Quantitative Analysis of Effects of Substituted Phenols on Membrane Characteristics of Lecithin Liposomes

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The partition coefficient of a series of substituted phenols in a lecithin liposome/water system, P(L/W), and the effects of these compounds on the perturbation of liposomal membranes were quantitatively investigated using physicochemical molecular parameters. Partition behavior is primarily governed by hydrophobicity, represented in terms of the 1-octanol/water partition coefficient. Steric and electronic characters also were of significance. Potency in perturbation of liposomal membranes was governed by the degree of partitioning to the membrane and the steric bulkiness of the phenol. This kind of quantitative procedure was very helpful in investigating the effects of organic molecules on membrane characteristics.

A variety of medicinal and agricultural chemicals interact with cell membranes as a part of their pharmacological or physiological effects. Thus, characterization of such interactions is important for understanding their overall mechanisms of action. Substituted phenols have fungicidal activity due to their causing an abnormal release of intracellular substances during their incorporation into the cell membranes.[1-3] The relationship between their fungicidal activity index and a hydrophobicity parameter derived from their partition coefficient in organic solvent/water systems is linear.[4-6] These studies suggest that the hydrophobicity of substituted phenols is most important, but the effect of other factors such as steric bulk and electronic property on the interaction with cell membranes should not be overlooked.

Here, we selected the lecithin liposome as a model of the cell membrane, since the effect of solutes is easily analyzed physicochemically with this system, the components of which are chemically uniform. We measured the partition coefficient of a number of substituted phenols between the lecithin liposome and an external aqueous phase. The substituents were chosen so that their steric and electronic characters would be as different as possible under experimental conditions. We also evaluated the degree of perturbation of the liposomal membrane by phenols, in terms of the change in the membrane permeability to glucose. Using physicochemical parameters of substituents and regression analysis, we first examined factors influencing their partitioning into the lecithin liposome. Then we analyzed the degree of liposomal membrane perturbation in terms of the liposome–aqueous phase partition coefficient, along with other parameters.

Experimental

Materials. Lecithin was prepared from fresh egg yolk and purified by the method of Singleton.[7] Phenols were purchased from the Nakarai Chemical Co. and purified before use. Hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and ADP were obtained from the Oriental Yeast Co. *N*-Dansylhexadecylamine (DSHA) was bought from the Sigma Chemical Co.

Preparation of Bilayered Liposome. Lecithin, 100 mg, was dissolved in 1 ml of $CHCl_3$ and placed in a 30 ml flask under Ar gas. The solvent was removed in vacuo. The lipid film was suspended in 20 ml of pH 7.2 Tris buffer (10 mM Tris-HCl, 50 mM NaCl (1 M=1 mol dm^{-3}), 0.2 mM EDTA) and sonicated with a Type 4280 sonicator (Kaijodenki Co.) in an ice-cooled bath under Ar gas to avoid oxidation of lecithin. Bilayered liposome was separated from the undispersed lecithin by centrifugation of the suspension at 15000 rpm for 30 min, and from multilayered liposome by gel filtration on Sepharose 4B at 4 °C.[8] The amount of lecithin was calculated from that of phosphorus by a modification of the Bartlett method.[9] The resultant solution of liposome contained about 1 mg lecithin/ml. Electron microscopy (JME-100C) was used to study the liposome negatively stained with 2% sodium tungstophosphate.

Measurement of Partition Coefficient. The partition coefficient, P(L/W), of substituted phenols between the liposome and the external aqueous phase was measured by equilibrium dialysis.[10] The solution of phenols was prepared with the same pH 7.2 Tris-HCl buffer as for the liposome solution. For phenols other than those that were alkyl-substituted, the pH of the buffer was adjusted by addition of dilute HCl so that they would exist as the nonionized form, except for 2,4-$(NO_2)_2$ and 2-*s*-Bu-4,6-$(NO_2)_2$ derivatives, which ionize by 3—10% even at pH 3.0. The concentration of the phenol in the buffer was 10^{-5} to 10^{-4} M. The pH of the liposome solution was adjusted to that of the phenol solution. Using an equilibrium dialysis cell (10×1 ml chambers, Sanko Plastic Co.), we equilibrated the liposome suspension (1 ml) with the phenol solution (1 ml) through a cellophane dialysis membrane at 25 °C for 12 h. The partition coefficient, P(L/W), was calculated by Eq. 1,

$$P(\mathrm{L/W}) = \frac{2(C_o - C)}{C \times C_L}\left[\frac{\text{mol phenol/kg lecithin}}{\text{mol phenol/l external solution}}\right], \quad (1)$$

where C is the concentration of phenol in a liposome-free chamber at equilibrium, C_o is that of phenol in a control experiment in which the phenol solution was equilibrated with the Tris buffer without liposome, and C_L is the concentration of lecithin (kg l^{-1}) in the liposome suspension. The total amount of lecithin in 1 ml of the liposome suspension was about 1 mg. The concentration of the phenol was measured spectrophotometrically on a Shimadzu UV-360

spectrophotometer. The P(L/W) value was calculated at least three times with different initial phenol concentrations, and the log P(L/W) values were averaged. For 2,4-dinitro- and 2-*s*-butyl-4,6-dinitrophenols, the partition coefficient is an apparent value for a mixture of ionized and nonionized species in the aqueous phase. For these two derivatives, the effect of variation in pH was examined.

Measurement of Glucose Permeability. The permeability of the liposomal membrane to glucose was calculated as the rate of release of glucose from the internal aqueous phase to the external aqueous phase at 25°C. Liposome enclosing glucose internally (glucose-liposome) was prepared by sonication of lecithin in pH 7.2 Tris buffer (10 mM Tris-HCl, 0.2 mM EDTA) containing 0.15 M glucose, and then separated with Sepharose 4B gel filtration. Glucose, which remained in the outer phase, was quickly removed with further gel filtration using Sephadex G25. An enzyme mixture for the glucose-assay was prepared in a quartz cell for fluorescence measurement.[11] The mixture consisted of 2.5 ml of pH 7.2 buffer (10 mM Tris-HCl, 0.2 mM EDTA, and 0.15 M NaCl), 0.2 ml of 0.02 M magnesium acetate, 0.1 ml of 0.02 M ATP, 0.1 ml of 0.01 M $NADP^+$, and 20 μl of aqueous solution containing 100 IU of hexokinase and 250 IU of glucose 6-phosphate dehydrogenase. After the addition of 80—130 μl of a stock solution in which various amounts of a phenol was dissolved in pH 7.2 Tris buffer, 0.5 ml (about 0.1 mg of lecithin) of the glucose-liposome suspension was quickly added to the cell. The final pH of the reaction mixture was 5.3. The amount of the phenol stock solution to be added was decided so as to cover the lower concentration region for highly effective compounds and the higher region for less effective compounds, in a range of final concentrations between 0.08 and 17 mM. The concentration of glucose in the outer phase was assayed at intervals with gentle stirring from the fluorescence intensity at 460 nm (ex. at 340 nm) arising from the NADPH produced from $NADP^+$ with glucose 6-phosphate dehydrogenase in the presence of the glucose 6-phosphate that is in turn derived from glucose with hexokinase and ATP. Fluorescence was recorded on a Shimadzu RF-503A spectrofluorophotometer. At 30 min after the start of the reaction, 10% Triton-X100 aqueous solution (300 μl) was added to the reaction mixture, and the glucose-liposome was completely disrupted to release the enclosed glucose. The fluorescence intensity after the disruption corresponded to the total amount of glucose. The course over time for the 4-*t*-Bu derivative is shown in Fig. 1 as an example. The total volume of the internal aqueous phase of liposome was calculated from the total amount of glucose in the entire reaction mixture and from the initial concentration of glucose (0.15 M) enclosed in the glucose-liposome. The pH of the external solution where the enzymatic reaction proceeds did not vary in this experimental period. Phenols used here are mostly not ionized at the final pH of 5.3 except for the 2-*s*-Bu-4,6-$(NO_2)_2$ derivative.

Estimation of Polarity of the Liposomal Membrane Surface. The variations in the polarity of membrane surface were estimated as the change of the λ_{max} of the emission spectrum of DSHA at 25°C. A chloroform solution of DSHA was added to lecithin at the molar ratio of 1:50 for DSHA to lecithin.[12] Chloroform was removed in vacuo and liposome (DSHA-liposome) was prepared in a similar way. The λ_{max} of the DSHA-liposome was recorded with excitation at 330 nm.

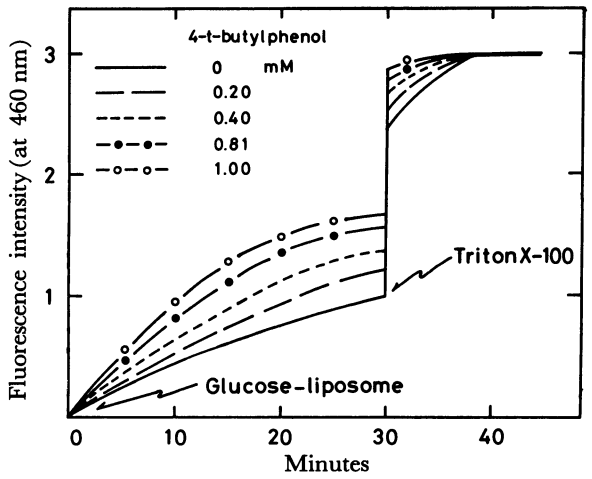

Fig. 1. Spectrofluorophotometric assay for glucose released from liposome in the presence of 4-*t*-butylphenol. Glucose-liposomes were added to experimental (+$NADP^+$) and control (−$NADP^+$) cuvettes at time zero. Triton X-100 (300 μl) to give the final concentration of 1% was added to the cuvettes at 30 min. Fluorescence intensity values were corrected for dilution following the addition of Triton X-100.

Table 1. log P(L/W) of 2,4-$(NO_2)_2$ Derivatives at Different pH

pH	2,4-$(NO_2)_2$ [pK_A=4.09]		2-*s*-Bu-4,6-$(NO_2)_2$ [pK_A=4.40]	
	% ionized	log P(L/W)	% ionized	log P(L/W)
7.2	99.9	1.90	99.8	2.95
5.3	94.2	2.00	88.8	3.16
3.0	7.5	2.42	3.8	3.33
Neutral		2.46[a]		3.35[a]

a) Estimated by correcting log P(L/W) at pH 3.0 according to the pH-partitioning model by adding $\log\{(K_A+[H^+])/[H^+]\}$, where K_A is the dissociation constant and $[H^+]$ is the hydrogen ion concentration.

Results

Characterization of Liposome. Electron microscopy showed that liposomes prepared in this way did indeed have an internal space surrounded by a spherical bilayered membrane. The diameter of the liposomes was between 150 and 300 Å, which agrees with that reported in the literature.[13] The volume of the internal cavity of the liposomes estimated from the total volume for the glucose-liposomes was $2.6(\pm0.3)\times10^{-4}$ cm^3 μmol^{-1} lecithin, which is close to the 3.1×10^{-4} cm^3 μmol^{-1} lecithin obtained by Brunner.[14]

Partition Coefficient. The partition coefficient, P(L/W), of some representative phenols was measured with different initial concentrations in the external aqueous phase from 5×10^{-6} to 10^{-3} M under conditions where they exist as the nonionized form. For the 4-Cl and 4-*t*-Bu derivatives, the log P(L/W) values were

Table 2. log P(1-octanol/water) and log P(liposome/water) of Substituted Phenols

Substituent	log P(O/W)[a]	Δlog K_A[b]	ΔV_w(m, p)[c]	ΔV_w(di-ortho)[c]	log P(L/W)	
			$\times 10\,cm^3\,mol^{-1}$	$\times 10\,cm^3\,mol^{-1}$	Obsd[d]	Calcd[e]
H	1.46	0	0	0	1.81(0.05)	1.90
2-Me	1.97	−0.30	0	0	2.25(0.04)	2.32
3-Me	2.02	−0.10	1.12	0	2.10(0.04)	2.30
4-Me	1.94	−0.16	1.12	0	2.17(0.03)	2.22
4-Et	2.58	−0.23[f]	2.14	0	2.68(0.06)	2.70
4-*n*-Pr	3.06[g]	−0.23[h]	3.07	0	3.09(0.02)	3.05
2-*t*-Bu	3.31	−1.18[f]	0	0	3.44(0.03)	3.43
3-*t*-Bu	3.31[g]	−0.14[f]	4.09	0	3.18(0.06)	3.20
4-*t*-Bu	3.31	−0.25[f]	4.09	0	3.21(0.05)	3.19
2-*s*-Bu	3.27	−0.36[f,i]	0	0	3.40(0.03)	3.45
2-Ph	3.09	0.01	0	0	3.50(0.02)	3.32
4-Ph	3.20	0.43	4.33	0	3.16(0.03)	3.12
4-*t*-Pent	3.87[g]	−0.25[j]	5.20	0	3.53(0.04)	3.59
2,6-$(Me)_2$	2.30[k]	−0.64[l]	0	2.24	2.52(0.02)	2.34
2,6-$(Et)_2$	3.03[k]	−0.59[l]	0	4.28	2.83(0.01)	2.75
2-Cl	2.15	1.50	0	0	2.47(0.04)	2.60
3-Cl	2.50	0.96	0.95	0	2.81(0.06)	2.80
4-Cl	2.39	0.60	0.95	0	2.80(0.03)	2.68
2,4-Cl_2	3.06	2.09[f]	0.95	0	3.42(0.01)	3.36
2,6-Cl_2	2.75[k]	3.19[l]	0	1.90	2.80(0.02)	3.02
3-Me-4-Cl	3.10	0.43[f]	2.07	0	3.15(0.01)	3.20
3-NO_2	2.00	1.58	1.43	0	2.52(0.02)	2.36
4-SO_2Me	0.58[m]	2.15	3.80	0	0.95(0.02)	0.99
2,4-$(NO_2)_2$	1.54	5.89[f]	1.43	0	2.42(0.03)	2.24
2,4,6-Cl_3	3.69	3.99[f]	0.95	1.90	3.75(0.01)	3.82
2-*s*-Bu-4,6-$(NO_2)_2$	3.56[k]	5.58[k]	1.43	5.52	3.33(0.02)	3.37

a) Unless otherwise noted, from T. Fujita, *Prog. Phys. Org. Chem.*, **14**, 75 (1983) and log P data bank compiled by Pomona College Medicinal Project, Claremont, California, U. S. A., 1983. b) Unless otherwise noted, from A. Albert and E. P. Serjeant, "Ionization Constants of Acid and Base," Methuen, London (1962), p. 130 c) From A. Bondi, *J. Phys. Chem.*, **68**, 441 (1964). d) For the nonionized form, see text. The figures in parentheses are the standard deviation. e) By Eq. 3. f) From "Tables of Rate and Equilibrium Constants of Heterolytic Organic Reactions," ed by V. A. Palm, Tartu State University, 1975, Vol. 1. g) Estimated from the value of closely related compounds. h) Taken as the same as that of 4-ethylphenol. i) Taken as the same as that of 2-isopropylphenol. j) Taken as the same as that of 4-*t*-butylphenol. k) Newly found. l) From G. J. Bijiloo and R. F. Rekker, *Quant. Struc.-Act. Relat.*, **3**, 91 (1984) or estimated according to a procedure presented there. m) Estimated according to a procedure published in T. Fujita, *Prog. Phys. Org. Chem.*, **14**, 75 (1983), assuming that $\rho_{SO_2Me}=\rho_{NO_2}$ (=−0.14).

almost constant regardless of the initial concentration in the aqueous phase, varying only slightly from 2.75 to 2.81 for the 4-Cl and from 3.19 to 3.28 for the 4-*t*-Bu compounds. For the 2,4-dinitrophenols, the P(L/W) value was measured at pH 7.2, 5.3, and 3.0. As shown in Table 1, these phenols were partitioned into the liposome membrane even from the aqueous phase where they exist almost always in the ionized form. Judging from the difference in log P(L/W) value between higher pH and a pH of 3.0, the partition of the phenolate ions is lower than that of the neutral phenols, but the difference does not seem to be very large. Although the partitioning system with liposomes seems to work according to a simple partition model as far as the nonionized species are concerned, it does not obey the pH-partition model.[15] The log P(L/W) value of 2,4-dinitro- and 2-*s*-butyl-4,6-dinitrophenols at pH 3.0 was "corrected" for the ionization according to the pH-partition model, since the "corrected" valus is thought to be the highest estimate for the valus for the nonionized neutral molecule. The log P(L/W) valus found at pH 3.0 did not differ much from the corrected value shown in Table 1 and was used without correction along with those for other compounds for further analyses, as an approximate value for the neutral form.

The log P(L/W) value was listed in Table 2. The total volume of the internal cavity of vesicles is very small compared with that of the external aqueous phase. Even the partition into the cavity for the least hydrophobic phenols used here having the log P(L/W) value at about 1.00 can be neglected. The log P value measured with the 1-octanol/water system, log P(O/W), has been widely used as a standard for the hydrophobic parameter of the molecule.[16] We plotted the log P(L/W) value against the log P(O/W) for each phenol in Fig. 2, for which Eq. 2 was derived by regression analysis.

$$\log P(\mathrm{L/W}) = \underset{(\pm 0.102)}{0.767}\log P(\mathrm{O/W}) + \underset{(\pm 0.283)}{0.781} \quad (2)$$
$$(n = 26,\ s = 0.195,\ r = 0.953)$$

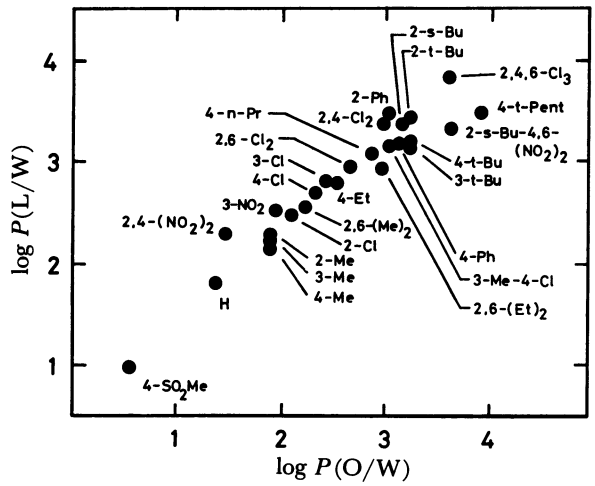

Fig. 2. Plot of log P(liposome/water) vs. log P(1-octanol/water) of substituted phenols.

In this and the following equations, n is the number of substituted phenols, s is the standard deviation, and r is the correlation coefficient. The figures in parentheses are the 95% confidence interval.

Although Eq. 2 seems to show reasonable correlation, slight negative deviations were observed for meta and para isomers. The deviations suggest contributions from factors other than the hydrophobicity of the molecule. Regression analysis was repeated using additional terms for steric and electronic factors of substituents. As the steric parameter, the van der Waals volume of substituents, ΔV_w (V_w relative to that of H and multiplied by 0.1 in order to make it equiscalar with other parameters), was found to delineate the effects best among steric parameters such as STERIMOL, E_s, and E_s^c.[17] The $\Delta\log K_A$ value ($\log K_A$ relative to that of the unsubstituted phenol) was used as the electronic parameter of substituents. Eq. 3 had the best correlation.

$$\log P(\mathrm{L/W}) = \underset{(\pm 0.070)}{0.870}\log P(\mathrm{O/W}) - \underset{(\pm 0.042)}{0.111}\,\Delta V_w(\text{di-ortho}) - \underset{(\pm 0.033)}{0.074}\,\Delta V_w(\mathrm{m,p}) + \underset{(\pm 0.029)}{0.064}\,\Delta\log K_A + \underset{(\pm 0.182)}{0.629} \quad (3)$$
$$(n = 26,\ s = 0.117,\ r = 0.986)$$

ΔV_w(di-ortho) is the sum of the parameters of both ortho substituents for di-ortho-substituted phenols. ΔV_w(m,p) is for the meta and para substituents. The addition of each of the ΔV_w and $\Delta\log K_A$ terms to Eq. 2 was justified at a level higher than 99.5%. The addition of the ΔV_w value for the mono-ortho-substituted compounds was not statistically significant. The coefficient of the log P(O/W) term in Eq. 3 is closer to 1 than that in Eq. 2. This is reasonable, since the degree of desolvation during partitioning from the aqueous to the organic phase is thought to be similar between the two partitioning systems. In the liposome/aqueous system, however, additional factors are significant. The ΔV_w terms suggest that the partitioning into liposomal membrane is slightly but significantly lowered by the bulkiness of substituents and that the effect is dependent upon the substitution patterns. From the regression coefficients, it appears that the effect of two ortho substituents is about double that of meta and para substituents. Interestingly enough, the single ortho substituent in monosubstituted compounds does not have this type of steric effect. The significance of the electronic term means that electron withdrawal from substituents favors partitioning. It should be noted that the electronic effect is not related to that on ionization, since the partition coefficient is that for the nonionized form. The electronic effect of substituents probably reflects a difference in the hydrogen bonding factor of the nonionized OH group with sites that can accept hydrogen between 1-octanol and lecithin.

Glucose Permeability. The rate of glucose release from liposome depends on the concentration of glucose in the internal aqueous phase. It follows first-order kinetics, as in Eq. 4.[14]

$$\text{Rate of glucose release} = k_p[\text{glucose}]_t^i, \quad (4)$$

where $[\text{glucose}]_t^i$ is the internal concentration of glucose at time t and k_p is the first-order rate constant. The integrated form of this equation cover the time t is shown as Eq. 5.

$$\ln\{[\text{glucose}]_{t=0}^i/[\text{glucose}]_t^i\} = k_p \cdot t. \quad (5)$$

Glucose released to the outer aqueous phase is converted into glucose 6-phosphate, which produces NADPH. Since the conversion is also a rate process, and since the glucose concentration of the outer phase is also governed by both permeation and the enzymatic reaction, the concentration of NADPH that is found experimentally does not correspond with that of the glucose in the outer phase. If the overall enzymatic reaction of the glucose assay system is approximated to follow first order kinetics, the [NADPH] at time t is formulated as Eq. 6:

$$[\text{NADPH}]_t = [\text{glucose}]_t^o\{1 - \exp(-k_e \cdot t)\}, \quad (6)$$

where $[\text{glucose}]_t^o$ is the concentration of glucose in the external medium at time t and k_e is the rate constant of the enzymatic reactions. Since $[\text{glucose}]_t^i$ is related to $[\text{glucose}]_t^o$ by Eq. 7,

$$[\text{glucose}]_t^i = [\text{glucose}]_{t=0}^i - [\text{glucose}]_t^o \cdot (V_o/V_i). \quad (7)$$

Eq. 8 is derived by substituting Eq. 7 into Eq. 5, where V_o and V_i are the volume of the external and internal liposome, respectively.

$$\ln \frac{[\text{glucose}]_{t=0}^i}{[\text{glucose}]_{t=0}^i - [\text{NADPH}]_t\{1-\exp(-k_e \cdot t)\}^{-1}(V_o/V_i)} = k_p \cdot t \quad (8)$$

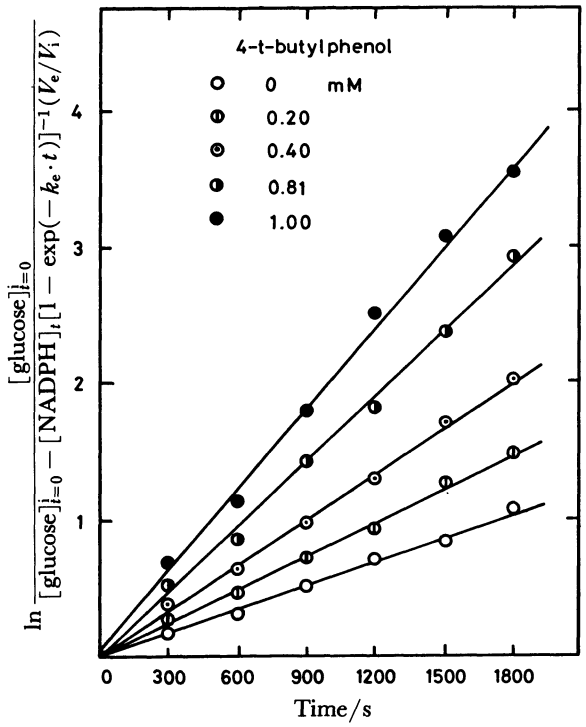

Fig. 3. Release kinetics of glucose. Data were plotted using Eq. 8.

The k_e value might be as a constant regardless of the phenols used. In fact, it varies depending upon the structure of the phenols, since the enzymatic reaction is more or less inhibited by individual phenols. The k_e value was determined under the same experimental conditions as the glucose release assay for different concentrations of each phenol in the presence of 1.5×10^{-5} M glucose without glucose-liposome. It ranged between $5\times10^{-4}\,s^{-1}$ and $3\times10^{-3}\,s^{-1}$ (data not shown). The plot of the left side of Eq. 8 against time t was linear, as shown in Fig. 3 for 4-*t*-butylphenol. When we neglected the effect of the enzymatic rate process by using $k_e \gg k_p$, the denominator of the ln term in Eq. 8 reduced to $[\text{glucose}]^{i}_{i=0}-[\text{NADPH}]_t\ (V_o/V_i)$. The plot with this modification against t was not linear. This means that the approximation made for Eq. 6 was in fact appropriate.

The permeability coefficient, P_m, is defined by Eq. 9, where A is the surface area of liposome and k_p is evaluated according to Eq. 8.

$$P_m = k_p \cdot V_i/A \tag{9}$$

The average surface area of liposome was calculated to be $2.0\times10^3\,cm^2\,\mu mol^{-1}$ lecithin from the average diameter of vesicles, estimated by electron microscopy. The mean value of the P_m of glucose, from 30 runs, was $6.6\pm1.3\times10^{-11}\,cm\,s^{-1}$. This value is close to the $3.0\pm2.0\times10^{-11}\,cm\,s^{-1}$ observed by Brunner.[15]

The P_m value of glucose increases with successive additions of phenols in the outer phase, as shown in Table 3. We defined a parameter, C_{200}, for each phenol

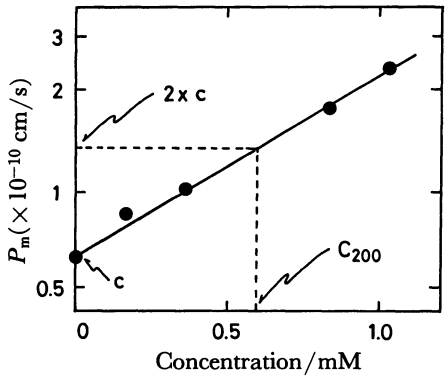

Fig. 4. Plot of the permeability coefficient of glucose vs. the concentration of 4-*t*-butylphenol. Data were analyzed by the least squares method using Eq. 10.

to represent potency of accelerating the release of glucose to twice that of the control in terms of the initial external concentration. For some phenols, the C_{200} concentration could not be found because of their limited solubility. In that case, we estimated this concentration by an empirical relationship such as that of Eq. 10 between P_m and the initial external concentrations of each phenol (C).

$$\log P_m = aC + c \tag{10}$$

Equation 10 holds for the 18 phenols used here with a correlation coefficient higher than 0.96 and a standard deviation smaller than 0.06. An example of the relationship is shown in Fig. 4. This relationship can be understood if we consider that the free energy of activation for the permeation process of glucose decreases linearly with the amount of each phenol partitioned into the liposomal membrane that is proportional to the external concentration. The a and c values in Eq. 10 were estimated by the least squares method and the quality of the correlation is shown in Table 4. The C_{200} value was calculated as $(\log 2)/a$ and listed in Table 4 as the logarithm.

The partitioning of organic solutes with a liposome/aqueous system reaches an equilibrium within a few minutes.[19] Since glucose release in this study obeys first-order kinetics very well, the effect of the conditions before equilibrium could be neglected. Release proceeds mostly under equilibrated conditions with respect to the partition of phenols. In fact, the C_{200} value (in log form) is almost identical to the value corrected for the partition equilibrium for each phenol. As an index for potency in perturbing the liposome membrane structure, it was suitable to use $\log(1/C_{200})$.

At pH 5.3, the phenols used here, except for 2-*s*-butyl-4,6-dinitrophenol, exist almost entirely as the neutral form. The $\log 1/C_{200}$ value of phenols other than this exception had a good linear relationship with log P(O/W) for the nonionized phenols, that of as Eq. 11.

$$\log 1/C_{200} = 0.912 \log P(\text{O/W}) + 0.162 \tag{11}$$
$$(\pm0.170) \qquad (\pm0.480)$$
$$(n = 17,\ s = 0.192,\ r = 0.947)$$

Table 3. Permeability Coefficient of Glucose for Substituted Phenols

Substituent	External Concd/mM	P_m $\times 10^{-11}$ cm/s	Substituent	External Concd/mM	P_m $\times 10^{-11}$ cm s^{-1}
2-Me	0	4.19	4-Ph	0	5.11
	3.57	5.36		0.098	6.77
	7.14	5.71		0.204	7.05
	14.30	9.66		0.253	8.06
	17.10	15.50		0.326	8.34
3-Me	0	6.15	4-*t*-Pent	0	8.08
	3.56	6.74		0.084	9.09
	6.91	8.59		0.166	11.10
	11.10	8.78		0.277	11.90
	13.80	10.80		0.356	18.10
4-Me	0	6.96	2-Cl	0	5.41
	3.17	8.05		1.73	5.95
	6.37	9.58		2.59	7.09
	7.91	10.50		4.17	8.61
	8.52	11.63		5.18	9.94
4-Et	0	9.15	3-Cl	0	7.23
	1.61	11.60		1.09	8.28
	2.07	11.50		1.77	8.84
	3.22	18.00		2.20	9.83
	6.23	21.70		2.83	10.90
4-*n*-Pr	0	5.88	4-Cl	0	7.31
	0.486	7.23		2.72	9.22
	0.770	8.73		3.45	10.00
	1.28	13.80		4.30	11.70
	1.59	17.10		6.88	18.80
2-*t*-Bu	0	5.43	2,4-Cl_2	0	6.04
	0.152	5.81		0.669	8.58
	0.228	7.51		1.62	16.10
	0.360	7.78		1.80	18.50
	0.746	15.79		2.01	23.00
3-*t*-Bu	0	5.80	3-Me-4-Cl	0	6.62
	0.220	6.57		0.560	7.48
	0.272	7.18		0.720	7.91
	0.454	8.14		1.12	10.30
	0.585	8.82		1.87	16.50
4-*t*-Bu	0	6.28	3-NO_2	0	2.94
	0.199	8.19		3.06	3.91
	0.399	9.32		6.12	7.21
	0.805	14.90		8.20	9.20
	0.999	16.70		12.20	11.30
2-Ph	0	5.05	2-*s*-Bu-4,6-(NO_2)$_2$	0	8.55
	0.118	6.26		0.166	9.77
	0.153	6.75		0.225	11.90
	0.354	10.10		0.268	12.90
	0.456	11.30		0.333	14.40

Table 4. log $1/C_{200}$ Value Estimated from Eq. 10 and Substituent Parameters[a)]

Substituent	a[b)]	c[b)]	s[c)]	r[d)]	log $1/C_{200}$[e)]	C_{200}^m[f)] mol/Kg lipids	V_w[g)] $\times 10$ cm^3 mol
2-Me	0.031[h)] (0.014)	−10.404 (0.148)	0.063	0.971	1.97	1.94	6.51
3-Me	0.017[h)] (0.008)	−10.214 (0.070)	0.028	0.968	1.75	1.67	6.51
4-Me	0.022 (0.004)	−10.160 (0.019)	0.005	0.999	1.88	1.67	6.51
4-Et	0.063[i)] (0.038)	−10.031 (0.127)	0.055	0.951	2.29	1.96	7.53
4-*n*-Pr	0.304 (0.079)	−10.262 (0.079)	0.031	0.990	2.99	1.19	8.46
2-*t*-Bu	0.637 (0.234)	−10.297 (0.091)	0.042	0.981	3.32	1.21	9.48
3-*t*-Bu	0.320 (0.068)	−10.239 (0.025)	0.010	0.993	3.02	1.34	9.48

Table 4. (Continued)

Substituent	a [b]	c [b]	s [c]	r [d]	log $1/C_{200}$ [e]	C_{200}^{m} [f] mol/Kg lipids	V_w [g] ×10 cm³ mol
4-*t*-Bu	0.428 (0.075)	−10.191 (0.046)	0.020	0.995	3.15	1.06	9.48
2-Ph	0.791 (0.114)	−10.294 (0.031)	0.013	0.997	3.30	1.11	9.72
4-Ph	0.624 (0.364)	−10.267 (0.077)	0.029	0.953	3.42	0.655	9.72
4-*t*-Pent	0.897[i] (0.522)	−10.109 (0.114)	0.047	0.953	3.44	0.752	10.59
2-Cl	0.053 (0.017)	−10.287 (0.055)	0.021	0.986	2.20	1.83	6.34
3-Cl	0.062 (0.017)	−10.148 (0.032)	0.012	0.989	2.39	2.53	6.34
4-Cl	0.060 (0.025)	−10.173 (0.103)	0.039	0.975	2.30	2.48	6.34
2,4-Cl_2	0.287 (0.080)	−10.237 (0.107)	0.029	0.996	3.00	2.28	7.29
3-Me-4-Cl	0.217[h] (0.102)	−10.226 (0.108)	0.045	0.969	2.87	1.81	7.46
3-NO_2	0.050[i] (0.031)	−10.522 (0.215)	0.064	0.980	2.21	2.13	6.82
2-*s*-Bu-4,6-$(NO_2)_2$	0.700 (0.338)	−10.085 (0.077)	0.027	0.967	3.37	0.621	12.34

a) log P(L/W) and log P(O/W) values at pH 5.3 used in Eqs. 11 and 12 are equal to values for the neutral form listed in Table 2 except for 2-*s*-Bu-4,6-$(NO_2)_2$. log P(L/W) value at pH 5.3 of 2-*s*-Bu-4,6-$(NO_2)_2$ is 3.16. b) Slope and intercept in Eq. 10. Unless otherwise noted, the correlation was justified above the 99.5% level; figures in parentheses are the 95% confidence interval. c) Standard deviation of the correlation according to Eq. 10. d) Coefficient of correlation accoding to Eq. 10. e) The C_{200} value was calculated as (log 2)/a from Eq. 10. f) The concentration of phenols in the liposomal membrane equilibrated with C_{200}. g) The van der Waals volume of compounds estimated according to A. Bondi, *J. Phys. Chem*, **68**, 441 (1964). h) Justified at the 99% level. i) Justified at the 97.5% level.

This equation means that the potency of membrane perturbation is linearly dependent on the hydrophobicity of phenols in terms of log P(O/W). The addition of other parameters does not further improve the correlation. To incorporate 2-*s*-butyl-4,6-dinitrophenol into the analysis, the log P(L/W) value found at pH 5.3 was used as the independent variable, since the partitioning occurring during the glucose release is better simulated by the P(L/W) value. For 2-*s*-butyl-4,6-dinitrophenol, log P(L/W) is the value for the ionization equilibrium mixture, since the liposome can incorporate both species. Equation 12 was derived for perturbation potency.

$$\log 1/C_{200} = 0.868 \log P(\mathrm{L/W}) + 0.119\, V_w - 0.794 \quad (12)$$
$$(\pm 0.136) \qquad (\pm 0.035) \quad (\pm 0.280)$$
$$(n = 18,\ s = 0.086,\ r = 0.991)$$

In Eq. 12, the term of steric parameter V_w for the entire molecular volume is also significant, which indicates that perturbation potency is governed not only by partitioning into liposome but also by the steric bulkiness of the phenols. Although the collinearity between log P(L/W) and V_w is rather high (r^2=0.514) in this set of compounds, the addition of the V_w term is significant at a level higher than 99.5%.

Polarity of the Liposomal Membrane Surface. The fluorescent probe DSHA is thought to be buried in the lecithin bilayer in such a way that its polar dansyl group is directed toward the surface and the hydrophobic long alkyl chain is arranged along the alkyl chain of lecithin.[12] The emission maximum of DSHA has been used as an indicator of the surface polarity of liposomal membranes.[12] It was observed at 520 nm for the liposome suspension here. The maximum in methanol was at 518 nm and that in the 2:1 methanol–water mixture at 522 nm. Therefore, the surface region of liposomal membrane is probably more polar than methanol and less polar than 2:1 methanol–water. In this study, we examined the effect of the 2-*t*-butyl-, 4-methyl-, 4-propyl- and 4-*t*-butylphenols on the emission maximum. As shown in Fig. 5 for the 4-*t*-Bu derivative, the maximum was shifted to the polar side by the addition of substituted phenols. When the concentration increased, it approached to 528—529 nm, which corresponds to that of 1:1 methanol–water. The concentration range where the maximum shift was observed varied depending upon the structure of the phenol (data not shown). In terms of the concentration in the membrane phase, however, it was located in the range of 1.5—2.5 mol kg^{-1} lecithin.

Discussion

Several studies examined the partition behavior of

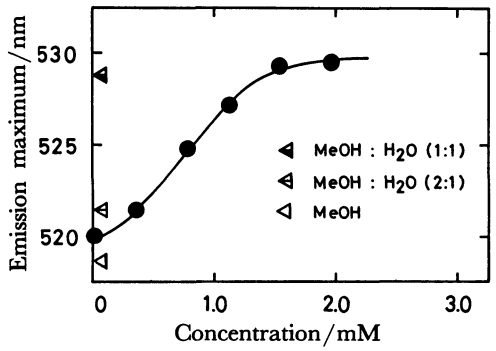

Fig. 5. Position of the fluorescence emission maximum of DSHA incorporated in liposomal membrane in the presence of 4-*t*-butylphenol. Emission maximum of DSHA in 1:1 MeOH–H_2O, in 2:1 MeOH–H_2O, and in MeOH are shown for reference.

various organic solutes in the liposome/water system as a model of biological membranes. Diamond and Katz[18] and Jain and his coworkers[19] found that the effects of the substituents and the substructural features such as chain-branching on the liposome/water partition coefficient are qualitatively similar to those observed for organic solvent/water partition systems. They also noticed that the effect of chain-branching in aliphatic alcohols in lowering the partition coefficient is greater in a liposome/water system than in systems with organic solvents such as olive oil and 1-octanol. They stated that this difference is due to steric repulsion on the more or less parallel arrangement of hydrocarbon tails in the interior of liposomal membranes. There seemed to be an extra steric effect when partition coefficients of liposome/water and organic solvent/water systems were compared.

The quantitative result formulated for substituted phenols in Eq. 3 seems to conform to these previous general findings. Phenol molecules are dehydrated in the lecithin membrane to a degree close to that occurring in the 1-octanol/water partitioning process. However, the steric bulkiness of substituents in terms of the group van der Waals volume lowers the partitioning into liposome more than into the octanol phase. The effect of ortho substituents is most important when they occupy both ortho positions, but not significant in mono-ortho-substituted derivatives, so that the bulkiest substituents, such 2-*t*-Bu, 2-*s*-Bu, and 2-Ph, alone have no significant steric effect. The effect of meta and para substituents is significant but less important than that of di-ortho substitution. From these positional specificities in the steric effect, the following model for the orientation of phenols in the liposomal membrane can be proposed. The substituted phenols are buried from the side of the substituted benzene ring holding the polar OH group toward the external aqueous phase. In this situation, meta and para substituents have an unfavorable effect on packing of the lecithin molecules, in proportion to their volume. For ortho-substituted derivatives, the OH and the single ortho substituent would be located together close to the outside of the membrane where the lecithin polar head groups are loosely packed, having no significant steric effect on the packing structure of the membrane. This kind of orientation would no longer be possible for di-ortho-substituted derivatives. In such compounds, interaction of the OH group with the polar head substituents of lecithin could be hindered. Since the di-ortho-substituted derivatives could behave as less polar molecules than the other substituted phenols, they tend to be buried deeper in the membrane structure. Thus, the steric effect of two ortho substituents acting as a wedge against the parallel arrangement of lecithin molecules is significant. As shown in Eq. 3, electron-withdrawing substituents are more favorable to the partitioning into liposome than into the 1-octanol phase. This could be rationalized by the fact that the basicity of the hydrogen-bond acceptor in liposome (perhaps phosphate anion) is higher than that of the oxygen of 1-octanol.

This orientation model is consistent with information from surface polarity measurements. The marked increase in the surface polarity of the membrane caused by substituted phenols shows that the interactions between polar head groups are weakened due to disorder of the membrane structure near the surface where phenols are buried, and the hydration around the head groups increases greatly. These results seems to be in accord with those obtained earlier,[20] where substituted phenols such as BHT (2,6-di-*t*-butyl-4-methylphenol) and its analogs disturbed molecular packing primarily near the polar head-group region of the lecithin bilayer.

Liposomal membrane perturbation caused by phenols, studied in terms of the increase in passive glucose permeability, was affected by partitioning into the membrane phase and steric bulkiness. The higher the partition coefficient and the steric bulkiness, the higher the degree of perturbation, leading to higher glucose permeability. Lipid-soluble drug molecules generally stabilize the membrane structure, to decrease permeability to permeants.[20–22] This stabilization is thought to be the origin of the antihemolytic activity on erythrocytes of various organic compounds, including anesthetics and tranquilizers. This kind of effects, however, was observed in concentration ranges that had a threshold, and high concentrations of organic molecules irreversibly disordered the membrane structure to increase permeability, as in haemolysis.[23] In this study, glucose permeability did not decrease in the lower range of phenol concentrations. The experimental system could inherently be such that no membrane stabilization was observable, requiring concentrations higher than the "threshold" to cause membrane perturbation.

Steric bulkiness increases the perturbation of the liposomal membrane, as shown in Eq. 12. We ex-

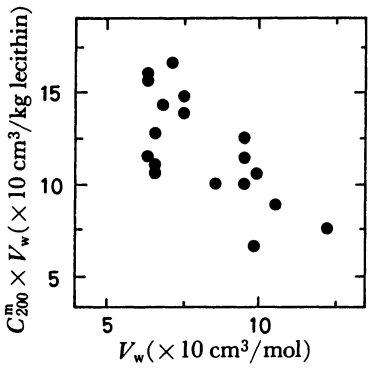

Fig. 6. Plot of the occupying volume in liposomal membrane vs. van der Waals volume of phenols. The scale of V_w was multiplied by 0.1.

amined the nature of this steric effect in terms of the occupying volume of substituted phenols in the membrane phase, and derived Eq. 13, where C^{m}_{200} is the concentration of phenols in the liposomal membrane (mol phenol/kg lecithin) equilibrated at the C_{200} concentration estimated from the log P(L/W) value, and where V_w is the molecular van der Waals volume of compounds.

$$C^{m}_{200} \cdot V_w = -1.133\, V_w + 21.141 \quad (13)$$
$$(\pm 0.626) \quad (\pm 5.229)$$
$$(n = 18, s = 2.193, r = 0.692)$$

Although the correlation coefficient is not very high, the V_w term is significant above the 99.5% confidence level. As shown in Fig. 6, there is a clear trend in which the larger the steric bulk of the molecule, the smaller is the occupying volume with the equivalent degree of membrane perturbation potency. This may mean that the effect of steric bulk is caused not by the bulk itself, but that the effect is enhanced proportionally to the bulk.

This study indicates that the separation of various effects is very helpful to understand the mechanisms of membrane phase partitioning and the membrane structure perturbation of organic compounds. Understanding can be achieved only by quantitative analysis using physicochemical molecular parameters. The effect of substituted phenols on these membrane characteristics can be separated in terms of the hydrophobicity and the steric bulk relative to these for appropriate reference systems. Work in progress in this laboratory indicates that a procedure of this sort can be used in analysis of the effect of the same series of compounds on the proton permeability across the lecithin bilayer, simulating the inner mitochondrial membrane.

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