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EFFECTS OF CHLORPROMAZINE AND OTHER PHENOTHIAZINE DERIVATIVES ON THE PERMEABILITY OF LIPOSOMES

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Abstract—Chlorpromazine and other phenothiazine derivatives increased the permeability of D-glucose or D-amino acids through the membrane of liposomes. The effect of chlorpromazine was observed at its concentration of more than 50 μM. The presence of halogen atom at C-2 position of phenothiazine nucleus increased the effect. The lipid composition of liposomes influenced the effects of phenothiazine derivatives; the incorporation of cholesterol into the membrane reduced the effects.

On the other hand, macromolecular marker, D-glucose oxidase [β-D-glucose: O_2 1-oxidoreductase, EC 1.1.3.4] or D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3], trapped into the liposomes, was not leaked from the liposomes upon incubating with penothiazines.

Phenothiazine derivatives have been known to affect the biological membranes; erythrocytes were lysed by phenothiazines at their high concentrations, while stabilized at their low concentrations [1–3]. However, the mechanism of the interaction of the drugs with the membrane has not been well elucidated. In this paper, the effects of phenothiazines on membrane permeability have been studied using liposomes as membrane model.

MATERIALS AND METHODS

Materials. The following firms graciously donated drug samples: Shionogi Pharmaceutical Co., Osaka: chlorpromazine HCl, levomepromazine HCl, thioproperazine, promethazine HCl, trimeproprimine dimaleate (trimepramine dimaleate), prochlorperazine dimethanesulfonate, amitriptyline HCl and medazepam. Squibb Institute for Medical Research, Princeton: triflupromazine HCl. Yoshitomi Pharmaceutical Co., Osaka: perazine dimaleate, trifluoperazine dimaleate, perphenazine dimaleate, carpipramine diHCl and clocapramine diHCl. Banyu Pharmaceutical Co., Tokyo: promazine HCl. Upjohn International Co., Kalamazoo: filipin.

Phosphatidylcholine was purified from egg yolk [4]. Sodium diethylbarbiturate, stearoylamine and D-amino acids were purchased from Nakarai Chemicals, Kyoto, and dicetyl phosphate from Phaltz and Bauer, Inc., Stanford. Cholesterol and D-glucose oxidase [β-D-glucose: O_2 1-oxidoreductase, EC 1.1.3.4] were purchased from Sigma Chemical Co., St. Louis. D-Amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] was prepared by the method of Yagi *et al.* [5]. All other chemicals used were of reagent grade.

Preparation of liposomes. Phosphatidylcholine (50 μmol) was dissolved in chloroform and mixed with other lipids in a 100 ml round-bottomed flask. After evaporated on a rotary evaporator and dried in a desiccator, the lipids were suspended with M D-glucose or 0.05 M D-phenylalanine in 5 ml of 10 mM Tris–HCl buffer, pH 8.5, containing 50 mM NaCl. The solution was stirred for 15 min under argon gas. After equilibrated at 5° overnight, the liposomes were dialyzed against 10 mM Tris–HCl buffer, pH 8.5, containing 50 mM NaCl at 5°, changing the dialyzing solution several times. Thus the liposomes trapping D-glucose or D-phenylalanine were obtained. The liposomes trapping D-glucose oxidase or D-amino acid oxidase were prepared as described previously [6]. The amount of the enzyme was calculated from the protein content measured by the method of Lowry *et al.* [7]. The trapped enzyme was mixed with deoxycholate (0.1 % in final concentration) before protein measurement.

Assay for permeability through liposomes. The leakage of D-glucose or D-amino acid was assayed using an oxygen analyzer, Bioxygraph (Kyusui Kagaku Kenkyusho Co., Tokyo), with D-glucose oxidase or D-amino acid oxidase. The reaction was carried out in a closed vessel in 2.8 ml of 0.1 M sodium pyrophosphate buffer, pH 8.3, containing liposomes trapping the substrate, and the rate of oxygen consumption was followed after the addition of the oxidase. The permeability of glucose or amino acid into the liposomes was followed by measuring oxygen consumption of the liposomes trapping D-glucose oxidase or D-amino acid oxidase after the addition of the substrate (0.05 M in final concentration). The initial oxidation rates in these experiments were dependent on the amount of the permeant substrate. The rates of oxidation in the absence and presence of Triton X-100 (0.1 % in final concentration) were measured. The amount of Triton X-100 was sufficient to dissolve liposomes [8]. The rate of the permeability was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100.

Drug incubation experiments. The liposomes were suspended in the Tris–HCl buffer containing NaCl and phenothiazine was added to be 10 μM–1.0 mM in

final concentration. After incubation at 30°, an aliquot of the liposomal suspension was taken for the assay of the oxidation rate. The effect of incubation with phenothiazine on the permeability of the liposomes was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

RESULTS

Effect of chlorpromazine on the permeability of D-*glucose and* D-*phenylalanine.* After incubating the liposomes trapping D-glucose or D-phenylalanine with or without chlorpromazine at 30° for 15 min, D-glucose oxidase or D-amino acid oxidase was added and oxygen uptake was measured. Figure 1 shows the results on the liposomes trapping D-glucose. The incubation with chlorpromazine increased the oxygen uptake. When Triton X-100 was added, the rapid increase in oxygen uptake was observed (see Fig. 1, arrow III). Similar results were obtained with the liposomes trapping D-phenylalanine. Similar experiments were made on the liposomes trapping D-glucose oxidase or D-amino acid oxidase inside the membrane. After incubating the liposomes with or without chlorpromazine, D-glucose or D-phenylalanine was added and the oxygen uptake was measured. It was found that the incubation with chlorpromazine increased the oxygen uptake. The addition of Triton X-100 also induced the rapid oxygen uptake. After incubation with phenothiazine, the liposomes were washed by centrifugation (100,000 g × 60 min), and the enzymatic activity was measured, but no enzymatic activity was detected in the washing supernatant of the liposomes. Accordingly, it is clear that the trapped oxidase was not leaked from the liposomes and the effect of chlorpromazine can be ascribed to the change in the permeability of the liposomes to the substrates.

The effect of chlorpromazine required a definite incubation time to reach its maximum, as shown in Fig. 2. At the concentration of 0.5 mM of chlorpromazine, the effect increased with time of incubation and

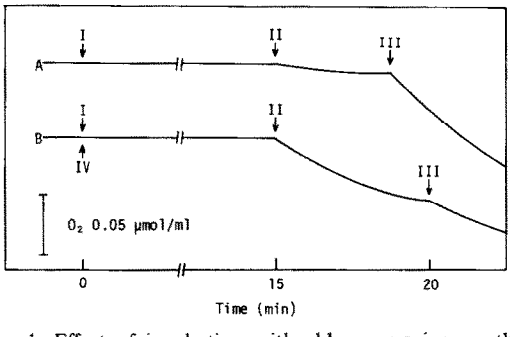


Fig. 1. Effect of incubation with chlorpromazine on the permeability of liposomes to D-glucose. Liposomes consisted of phosphatidylcholine–cholesterol–stearoylamine (7:2:1, molar ratio) and trapped D-glucose. Curves show the oxidation of D-glucose by D-glucose oxidase at 30°. To 2.7 ml of 0.1 M sodium pyrophosphate buffer, pH 8.3, were added 50 μl liposome suspension (0.3 μmol phospholipid containing 0.3 μmol glucose) at I, 4.7 nmole D-glucose oxidase at II and 30 μl Triton X-100 (10 %) at III. Curve A: liposomes without incubation with chlorpromazine; Curve B: liposomes incubated with chlorpromazine (added at IV, 1×10^{-4} M in final concentration).

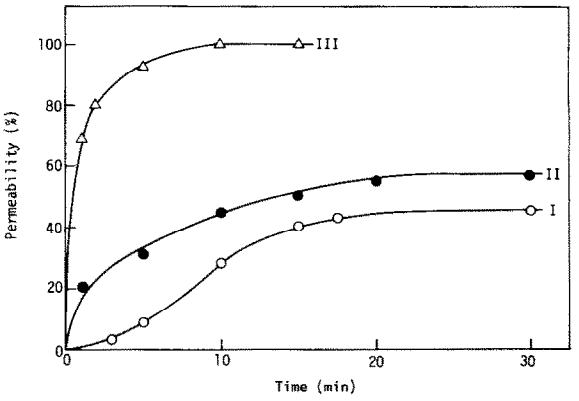


Fig. 2. Effect of incubation with chlorpromazine or filipin on the permeability of liposomes to D-glucose. The composition of liposomes and the reaction conditions were same as those in Fig. 1. Liposomes were incubated with 1×10^{-4} M chlorpromazine (Curve I), 5×10^{-4} M chlorpromazine (Curve II), and 1×10^{-4} M filipin (Curve III) at pH 8.3 and 30°. The changes in permeability were expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

Table 1. Effect of temperature on the action of chlorpromazine or filipin

Temp	Permeability (%) Chlorpromazine 1×10^{-4} M	Chlorpromazine 5×10^{-4} M	Filipin 1×10^{-4} M
20°	17	22	100
30°	28	43	100
40°	54	58	100

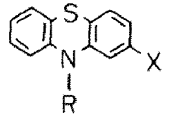
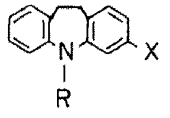
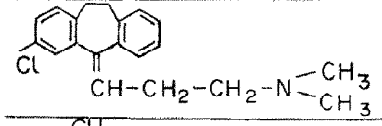
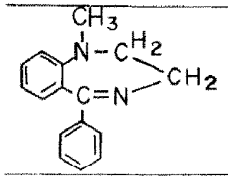
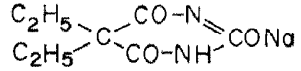
Liposomes consisted of phosphatidylcholine–cholesterol–stearoylamine (7:2:1, molar ratio) and trapped D-glucose as marker. Incubation with drugs was at pH 8.3 for 10 min. Permeability was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

Table 2. Effect of pH on the action of chlorpromazine or filipin

pH	Permeability (%) Chlorpromazine 1×10^{-4} M	Chlorpromazine 5×10^{-4} M	Filipin 1×10^{-4} M
5.0	17	30	100
7.0	17	36	100
8.3	28	43	100
9.0	60	80	100

Liposomes consisted of phosphatidylcholine–cholesterol–stearoylamine (7:2:1, molar ratio) and trapped D-glucose as marker. Incubation was at 30° for 10 min. Premeability was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

Table 3. Relation between chemical structure and effects of phenothiazines

R	X		Permeability (%) 1×10^{-4} M	5×10^{-4} M
 $-CH_2-CH_2-CH_2-N(CH_3)_2$	—H	Promazine	10	43
	—Cl	Chlorpromazine	39	48
$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	$-CF_3$	Triflupromazine	23	39
	$-OCH_3$	Levomepromazine	10	20
$-CH_2-CH_2-CH_2-N$ piperazine $-CH_3$	—H	Perazine	11	57
	—Cl	Prochlorperazine	30	42
	$-CF_3$	Trifluoperazine	39	62
	$-SO_2-N(CH_3)_2$	Thioproperazine	0	53
$-CH_2-CH_2-CH_2-N$ piperazine $-CH_2-CH_2-OH$	—Cl	Perphenazine	37	90
$-CH_2-CH(CH_3)-N(CH_3)_2$	—H	Promethazine	6	71
 $-CH_2-CH_2-CH_2-N$ piperidine $CONH_2$	—H	Carpipramine	4	21
	—Cl	Clocapramine	2	4
$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	—H	Trimepramine	15	33
		Amitriptyline	21	64
		Medazepam	16	45
		Sodium diethylbarbiturate	0	6

Liposomes consisted of phosphatidylcholine–cholesterol–stearoylamine (7:2:1, molar ratio) and trapped D-glucose as marker. Incubation was at pH 8.3 and 30° for 15 min. Permeability was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1% in final concentration).

Table 4. Effect of lipid composition of liposomes on the actions of phenothiazines

		Permeability (%) Liposomes composed of		
		Phosphatidylcholine	Phosphatidylcholine–cholesterol (1:1, molar ratio)	Phosphatidylcholine–cholesterol–stearoylamine (7:2:1, molar ratio)
Promazine	1×10^{-4} M	50	17	8
	5×10^{-4} M	100	43	32
Chlorpromazine	1×10^{-4} M	59	38	40
	5×10^{-4} M	83	49	50
Triflupromazine	1×10^{-4} M	72	37	41
	5×10^{-4} M	76	54	50
Levomepromazine	1×10^{-4} M	50	13	3
	5×10^{-4} M	74	29	6
Prochlorperazine	1×10^{-4} M	58	42	38
	5×10^{-4} M	81	43	79
Thioproperazine	1×10^{-4} M	35	8	14
	5×10^{-4} M	83	22	29

Liposomes trapping D-amino acid oxidase as marker were incubated with phenothiazine derivatives at pH 8.3 and 30° for 15 min. Permeability was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

reached its maximum after 20 min. At the concentration of 0.1 mM, however, it appears that a lag time is necessary for the appearance of the effect. On the other hand, filipin, one of polyene antibiotics affecting the biological membrane [9], increased the permeability within one min and reached its maximum in 10 min.

It was also found that the effect of chlorpromazine is dependent on the temperature of the incubation (Table 1). The effect of chlorpromazine increased with temperature, while the effect of filipin is not dependent on temperature.

In Table 2, the effect of pH of the solution on the permeability of the liposomes incubated with chlorpromazine is shown. At alkaline side, the effect of chlorpromazine was greater than at acidic side. However, the effect of filipin was not dependent on pH.

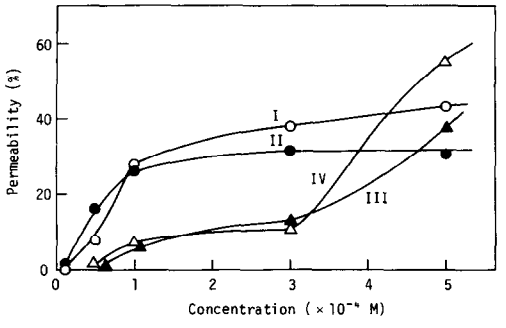


Fig. 3. Effects of concentrations of phenothiazine derivatives on the permeability of liposomes to D-glucose. The composition of liposomes and the reaction conditions were same as those in Fig. 1, and the incubation was for 10 min. Liposomes were incubated with chlorpromazine (Curve I), prochlorperazine (Curve II), promazine (Curve III) and perazine (Curve IV). The changes in permeability were expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

Chemical structure of phenothiazines and the effects of the permeability. Figure 3 shows that the chemical structure of phenothiazines influenced the increase in the permeability of the liposomes. Chlorpromazine and prochlorperazine, which have chlorine at C-2 position of phenothiazine nucleus, were effective at lower concentrations. On the other hand, promazine and perazine, which have hydrogen at C-2 position, were less effective for the increase in the permeability at their lower concentrations, but at their higher concentrations than critical micelle concentration (0.3 mM for promazine and perazine), a remarkable increase in the permeability was observed.

Table 3 summarizes the relation between the chemical structure of phenothiazines and the effects on the permeability of the liposomes to D-glucose. The halogen atom and halogen-containing residue at C-2 position of the phenothiazine nucleus increased the effects at the concentration of 0.1 mM. Other antipsychotic drugs examined were also found to have similar action, even though sodium diethylbarbiturate has a little or no action.

Effects of lipid compositions of the liposomes on the drug effects. Table 4 shows that the lipid composition of the liposomes affects the increase in the permeability of D-phenylalanine through the liposomes. The incorporation of cholesterol into the liposomes reduced the effects. The positively-charged liposomes composed of phosphatidylcholine–cholesterol–stearoylamine (7:2:1, molar ratio) and the negatively-charged liposomes composed of phosphatidylcholine–cholesterol–dicetyl phosphate (7:2:1, molar ratio) were less affected by chlorpromazine than the phosphatidylcholine liposomes (Table 5).

DISCUSSION

The present data clearly demonstrate that phenothiazine derivatives increase the permeability of the liposomes to low molecular substances such as D-glucose and D-phenylalanine. In this case, the

Table 5. Effect of lipid composition of liposomes in the actions of phenothiazines

Liposomes composed of	Permeability (%)			
	Chlorpromazine		Promazine	
	1×10^{-4} M	5×10^{-4} M	1×10^{-4} M	5×10^{-4} M
Phosphatidylcholine	70	100	60	100
Phosphatidylcholine –cholesterol–stearoylamine (7:2:1, molar ratio)	39	48	10	43
Phosphatidylcholine –cholesterol–dicetyl phosphate (7:2:1, molar ratio)	31	49	7	21

Liposomes trapping D-glucose as marker were incubated with phenothiazine derivatives at pH 8.3 and 30° for 15 min. Permeability was expressed as percent of the oxidation rate to the maximum oxidation observed in the presence of Triton X-100 (0.1 % in final concentration).

presence of cholesterol in the membrane reduced the effects of the drugs on the permeability. This is quite different from the case of polyene that requires the presence of cholesterol in the membrane in its effect to increase the permeability [9–11]. In addition, the effects of phenothiazine derivatives to increase the membrane permeability are dependent on both pH and temperature. This characteristic of the drugs is also quite different from that of polyene. Accordingly the mechanism of the effects of the present drugs to increase the membrane permeability is obviously different from that of polyene. In the case of polyene, it forms 8–10 adjacent adducts with sterols to form a channel on each side of the membrane [11].

Even though the mechanisms of the effects of polyene and phenothiazine derivatives are different, both effects equally result into the change in higher structure of the membrane. The well-known fact that higher concentrations of the drugs induce the lysis of cells such as erythrocyte would be resulted from the extreme change in the higher structure of the membrane mentioned above.

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