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Effect of Pyrithioxine on Phospholipids Constituents of Tissue[1]

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Phospholipid extracted with $CHCl_3$–MeOH (2:1 v/v) from the brain and liver of pyrithioxine-treated animals, as compared to that from control animals, was greater in amount. The amount of phospholipid extracted with $CHCl_3$–MeOH (7:1 v/v) saturated with NH_4OH from tissue residue after $CHCl_3$–MeOH (2:1) extraction is found to be smaller in pyrithioxine-treated animals. This decrement of phospholipid is represented by phosphatidylserine and phosphatidylinositol, which both account for the increment of $CHCl_3$–MeOH (2:1)-extracted phospholipid.

We reported in our previous paper that pyrithioxine exerts its action on the blood-brain barrier by facilitating passive transport across capillary endothelial cells.[3]

Recent studies have shown that local anesthetics and narcotic analgesics bind to phospholipid in the excitable cell membrane and thereby cause Ca^{2+} displacement.[4] Metcalfe, *et al.*, measuring the nuclear magnetic proton relaxation of benzyl alcohol, revealed anesthetics to alter lipid-protein interaction in the cell membrane through their interaction with lipid.[5] Burt and Green also showed that *n*-butanol may give rise to a change in phospholipid-protein interaction in the cell membrane of red blood corpuscles.[6]

Based on the fact that pyrithioxine, like local anesthetics and narcotic analgesics, is a basic compound having membrane action, we investigated its action on phospholipid.

Experimental

Lipid Extraction——Male ddY strain mice, weighing 20 to 25 g, were used. Lipid extraction was carried out 20 min after pyrithioxine administration (200 mg/kg *i.p.*) by the method of Folch, *et al.*[7] One gram portion of the brain or liver was homogenized with 20 ml of $CHCl_3$–MeOH (C/M 2:1), the homogenate was centrifuged for 10 min at 2500 rpm, and the supernatant was transferred into a 50-ml glass-stoppered test tube. The tissue residue was reextracted twice with 10 ml $CHCl_3$–MeOH (2:1), and the extracts were combined. To the lipid extract was added 0.2 vol of 0.9% NaCl with subsequent 1-min shaking; the mixture was then centrifuged and the upper aqueous layer was decanted. The $CHCl_3$ layer was washed twice with

1) Part of this work was presented at the Third Symposium on Drug Metabolism and Action, Fukuoka, November 1971.
2) Location: *Aobayama, Aramaki, Sendai.*
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6) D.H. Burt and J.W. Green, *Biochim. Biophys. Acta*, **225**, 46 (1971).
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equal part of "Folch upper phase ($CHCl_3$: MeOH: 0.9% NaCl=3: 48: 47)." Pooled extracts were evaporated to dryness in a rotatory evaporator and the residue was then dried thoroughly over P_2O_5 overnight under reduced pressure.

Extraction of tightly Bound Lipid——The tissue residue following the initial lipid extraction with $CHCl_3$–MeOH (2:1) was subjected to further extraction with 20 ml of $CHCl_3$–MeOH (7:1) saturated with NH_4OH (C/M-NH_3) according to the method of Rouser, *et al.*[8] Non-lipid water-soluble material was eliminated in the same manner as in the preceding extraction with C/M (2:1). While the method of Rouser, *et al.* was shown by Wells and Dittmer not to permit total lipid extraction,[9] the use of such a solvent as $CHCl_3$–MeOH–HCl and the like, as pointed out by Rouser, *et al.*,[8] allows large quantities of impurities to be extracted concurrently, producing thus great technical difficulty. In order to check on how much lipid still remains in the tissue residue after extraction with C/M-NH_3 we carried out therefore further lipid extraction from the tissue residue following extraction with C/M-NH_3 using 20 ml of C/M (2:1) containing Triton X-100 (1% of original tissue wet weight).

Phospholipid Analysis——C/M (2:1) extract derived from one gram of tissue was chromatographed over silicic acid by the method of Börgstrom after being dissolved in a small quantity of $CHCl_3$.[10] Into a glass column, 8 mm in inner diameter, which was preliminarily filled with a suspension of 2 g silicic acid (Mallinckrodt, 100 mesh) in $CHCl_3$ with subsequent washing with 10 ml $CHCl_3$, the lipid extract was poured and the neutral lipid and then phospholipid were eluted with 20 ml $CHCl_3$ and 20 ml MeOH, respectively. The phospholipid fraction thus obtained was subjected to DEAE-cellulose column chromatography according to the method of Rouser, *et al.* to permit separation of two fractions: neutral phospholipid and acidic phospholipid.[8] DEAE-cellulose (Brown, 0.83 meq/g), which was made into the acetate form by the method described by Rouser, *et al.* and then suspended in glacial acetic acid, was poured into a glass column, 11 mm in inner diameter, up to a height of 20 cm. No pressure was exercised during this operation so as to provide as homogeneous a column as possible. At the top of the DEAE-cellulose column sea sand was placed to a thickness of 1 cm. The column was rinsed with 50 ml each of MeOH, C/M (7: 3) and $CHCl_3$ and 50 ml each of C/M (7: 3), $CHCl_3$–CH_3COOH (C/HAc, 3:1) and C/M (2:1) saturated with NH_4OH in that order and finally with 50 ml of C/M (7: 3). The phospholipid fraction, dissolved in C/M (7: 3), was allowed to flow down the column and the eluate with 50 ml each of C/M (7: 3) and C/HAc (3:1) was taken for the neutral phospholipid fraction and that with 50 ml of C/M (2:1) saturated with NH_4OH, for the acidic phospholipid fraction. Each of these phospholipid fractions, after evaporating out of the solvents, was run on thin-layer chromatography to separate and determine quantitatively constituent phospholipids. The thin-layer chromatography was performed by the method of Parker and Peterson.[11]

Both on C/M-NH_3 and C/M-Triton X-100 extracts silicic acid column chromatography followed by thin-layer chromatography was done instead of DEAE-cellulose column chromatography.

Phospholipid-facilitated Calcium Transport——This was determined on the phospholipid fraction separated from C/M (2:1) extract of brain tissues by silicic acid column chromatography according to the method of Mulé.[4d] Determination conditions were the same as described by Mulé except that the dose of ^{45}Ca was reduced to 0.01 μCi.

Phosphorous Determination——This was carried out by the method of Bartlett.[12]

Result

Inhibition of Phospholipid-facilitated Calcium Transport by Pyrithioxine

The amount of ^{45}Ca transported from methanol–water layer to $CHCl_3$ layer increases in proportion to the phospholipid content of up to 200 μg P (Fig. 1), but at levels of above 100μg P much fluff is produced, rendering it awkward and difficult to carry out experimental procedures. For this reason a phospholipid content of 75 μg P was used in our present study.

As shown in Table I, pyrithioxine inhibits the phospholipid-facilitated calcium transport. The capacity of phospholipid to transport Ca^{2+} between $CHCl_3$– and MeOH–water layers increases with rising pH. Inhibition by pyrithioxine (a tertiary amine) of phospholipid-facilitated calcium transport, on the contrary, occurs to a lesser degree with increasing pH (Fig. 2).

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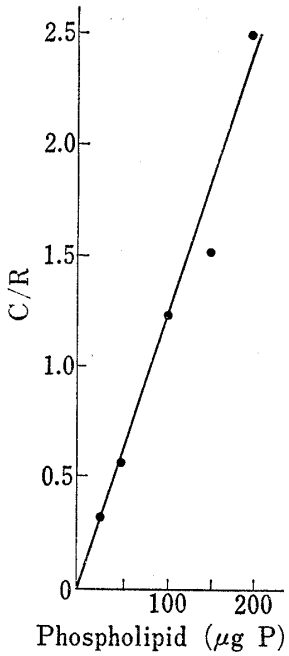

Fig. 1. Phospholipid-facilitated Calcium Transport between Chloroform and Methanol–Water

Lipid was extracted from brain with $CHCl_3$–MeOH (2:1). Phospholipid was isolated as described in Experimental. Two ml aliquots of $CHCl_3$–MeOH (2:1) containing phospholipid (25—200 μg of phosphorus) were shaken for 1 min in 10 ml glass-stoppered tubes with 1 ml of Ringers solution containing 116 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, and 0.01 μCi/ml of ^{45}Ca in 2 mM Tris-HCl (pH 7.4). $CHCl_3$ and Ringers phase were transferred to planchettes, dried with an infrared lamp, and radioactivities were measured with Aloka 2π gas flow counter. C/R represents the ratio of radioactivity of $CHCl_3$ layer to that of Ringers phase.

Table I. Inhibition of Phospholipid-facilitated Calcium Transport by Pyrithioxine *in Vitro*

Pyrithioxine concentration (M)	C/R	Inhibition (%)
—	1.37	—
1×10^{-3}	0.24	82.5
5×10^{-4}	0.92	32.8
1×10^{-4}	1.25	8.8
1×10^{-5}	1.27	7.2

Details were described in Fig. 1.

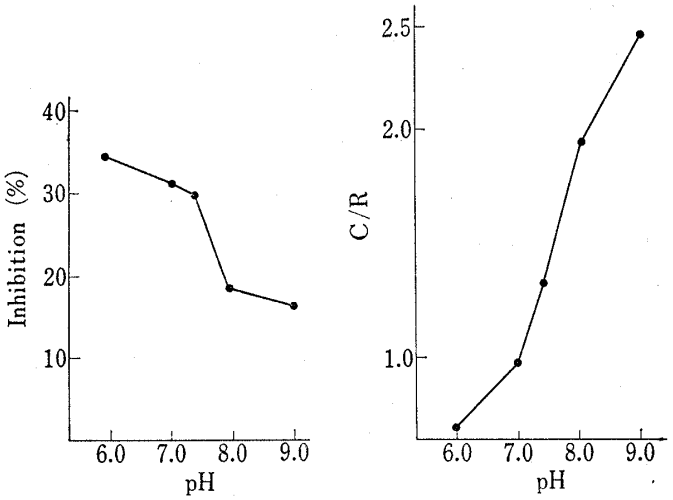

Fig. 2. Effect of pH on the Phospholipid-facilitated Calcium Transport (right) and its Inhibition by Pyrithioxine (left)

Phospholipid-facilitated calcium transport was measured as described in Fig. 1. Amount of phospholipid added in each tube was 75 μg of phosphorus. Pyrithioxine was made up in Ringers solution to provide a final concentration of 5×10^{-4} M.

These results are in good agreement with those with narcotic analgesics reported previously by Mulé.

Effect of Pyrithioxine on Phospholipids Constituents *in Vivo*

The amount of phospholipid extracted with C/M (2:1) from the brain and liver of pyrithioxine-treated animals, as can be seen in Table II, showed a tendency to increase as compared to non-treated controls.

The tissue residue that remains after lipid extraction with C/M (2:1) is known to still contain a minute quantity of lipid. This fraction of lipid (*i.e.* tightly bound lipid) can be extracted only with organic solvents added with acid or alkali but its biochemical identity and physiological function are not as yet cleary understood. It has recently been reported by Metcalfe, *et al.*[5] and also by other workers[6,13] that anesthetics can alter protein-lipid interaction in cell membranes and this warrants us to expect that the extractability of the tightly bound lipid with organic solvents might vary. An attempt was made therefore to extract tightly bound phospholipid from the brain and liver of pyrithioxine-treated animals. As can be seen in Table II, the amount of tightly bound phospholipid (phospholipid-B+phospholipid-C) extracted both from brain and liver was apparently reduced by the administra-

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Table II. Total Phospholipid Content of Control and Pyrithioxine-treated Mouse Tissue

Brain	Exptl. I		Exptl. II		Exptl. III		Exptl. IV	
	Cont.	Pyri.	Cont.	Pyri.	Cont.	Pyri.	Cont.	Pyri.
PL-A	1.390	1.505	1.260	1.592	1.760	1.940	1.685	1.887
PL-B	0.080	0.006	0.113	—	0.055	0.021	0.022	0.006
PL-C	0.010	0.003	0.095	—	0.010	0.003	0.007	0.002
Total	1.480	1.513	1.469	1.592	1.825	1.964	1.714	1.895
Liver	Cont.	Pyri.	Cont.	Pyri.	Cont.	Pyri.	Cont.	Pyri.
PL-A	0.980	1.150	1.029	1.172	1.110	1.240	1.113	1.245
PL-B	0.250	0.110	0.048	—	0.065	0.025	0.026	0.010
PL-C	0.035	0.026	0.041	—	0.027	0.015	0.005	—
Total	1.265	1.286	1.118	1.172	1.252	1.280	1.144	1.255

PL-A: Phospholipid obtained with $CHCl_3$–MeOH (2:1) extraction.
PL-B: Phospholipid extracted with $CHCl_3$–MeOH (7:1) saturated with NH_4OH from tissue residue after $CHCl_3$–MeOH (2:1) extraction.
PL-C: Phospholipid obtained with $CHCl_3$–MeOH (2:1) containing Triton X-100 (1% of fresh tissue weight) extraction from tissue residue after PL-A and PL-B were extracted.
Cont.: control; Pyri.: pyrithioxine-treated Result was represented as mg of phospholipid phosphorus per g of tissue.

tion of pyrithioxine. A study of the composition of the extracted phospholipid (Table III) showed that the tightly bound phospholipid fraction contained phosphatidylserine and phosphatidylinositol, both of which were found to be decreased in yield after pyrithioxine administration. In phospholipid-A fraction there was noted an increase sufficient to compensate for these decrements.

Table III. Phospholipid Contents of Control and Pyrithioxine-treated Mouse Tissue

		Exptl. I				Exptl. II			
		Brain		Liver		Brain		Liver	
		Cont.	Pyri.	Cont.	Pyri.	Cont.	Pyri.	Cont.	Pyri.
PL-A	PC	427	520	452	484	351	388	395	447
	PE	630	693	268	328	517	584	216	238
	PS	281	419	39	55	225	300	33	52
	PI	16	72	11	45	14	43	15	44
	Sph	96	92	52	56	64	72	50	50
PL-B	PC	—a)	—	—	—	—	—	—	—
	PE	—	—	—	—	—	—	—	—
	PS	29	7	14	—	35	18	20	5
	PI	21	5	27	7	20	13	25	4
	Sph	—	—	—	—	—	—	—	—
PL-C	PC	—	—	—	—	—	—	—	—
	PE	—	—	—	—	—	—	—	—
	PS	—	—	—	—	—	—	—	—
	PI	9	4	5	3	16	—	10	—
	Sph	—	—	—	—	—	—	—	—

Results were represented as mean for duplicate determinations in μg of phosphorus. PC: phosphatidylchloline; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: poosphatidylinositol; Sph: sphingomyeline
a) nondetectable

The results shown in Tables II and III indicate that pyrithioxine produces a change in preexisting (tight) phospholipid-protein interaction in cell membranes, in other words, a looselification of membrane structure.

Discussion

It is well known that calcium ions and phospholipids play an important role in the maintenace of excitability of nerve cells.[14] Present results showed that pyrithioxine inhibited the phospholipid-facilitated calcium transport. Since local anesthetics and narcotic analgesics inhibit the phospholipid-facilitated calcium transport, it has been considered that the reaction mechanism of these drugs is the displacement of calcium ions within the cell membrane (reference 4), except 4*i*)). But Hauser and Dawson opposed such assumption on the ground that not only local anesthetics but also some stimulant drugs as brucine, strychnine and amphetamine as well as bases without distinct pharmacologic action are capable of binding Ca^{2+} and thereby causing displacement of these ions.[4i]

It is considered, therefore, that inhibition of the phospholipid-facilitated calcium transport by pyrithioxine determined *in vitro* does not always relate to its *in vivo* effects. Furthermore, the inhibition of the phospholipid-facilitated calcium transport is suitable to explain the decrease of membrane permeability postulated by Feinstein,[4a] but pyrithioxine increases the blood-brain barrier permeability reported previously.[3]

Since it has been demonstrated that anesthetics alter the membrane structure by the interaction with lipid[5,6,13,15] and increase the membrane permeability,[16] the effect of pyrithioxine on the lipid-protein interaction *in vivo* was investigated.

The lipid-protein interaction in the membrane is known to be divided roughly into two types: hydrophobic and hydrophilic.[17] Although the details of the lipid-protein interaction in the membrane are not obvious, a few reports indicate that the hydrophilic phospholipid-protein interaction is quite susceptible to pH and ionic strength,[18] and the reports referred to above,[5,6,13,15,16] in common, indicate that hydrophilic lipid-protein interaction in the membrane is affected as well as hydrophobic interaction by the action of anesthetics.

It is known that tissue residue left after lipid extraction with $CHCl_3$–MeOH (2:1 v/v) still contatins some phospholipid which can be extracted only with organic solvents containing acid or alkali.[8,9] These so called "tightly-bound phospholipid" was considered to bind to protein moiety in hydrophilic manner.

Our present study indicates that a tightly-bound phoshpolipid fraction which can be extracted only with $CHCl_3$–MeOH (7:1) saturated with NH_4OH in intact animals is extractable merely with $CHCl_3$–MeOH (2:1) in pyrithioxine-treated animals. Also known to us all is that the difference between the hydrophobic and the hydrophilic bound lipid as distinguished by extractability with organic solvents, *i.e.* loosely bound lipids (neutral lipids and part of phospholipid) and more strongly bound lipids (phospholipid), lies in the way in which they are bound to protein, namely hydrophobic binding in the case of the former and hydrophilic

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in the case of latter.[17,19] Furthermore, the extractability of gangliosides is known to vary with different Ca^{2+} concentrations.[20] From these facts it becomes clear that the ability of lipid to be extracted with organic solvents is determined by its state of being bound to protein. Conversely, one can gather the state of lipid-protein binding from the extractability of lipid.

It is considered, therefore, that pyrithioxine may alter the phospholipid-protein interaction in the biological system, and this may be observed as the increase of the extractability of tightly-bound phospholipid. But it does not clear whether the inhibition of the phospholipid-facilitated calcium transport by pyrithioxine detected *in vitro* relates to this *in vivo* results.

The tightly-bound phospholipid is thought to be accounted for by phosphatidylinositide (mainly di- and triphosphatidylinositide),[9] but whether it contains other phospholipids also or not is uncertain. While Manukyan postulated that phosphatidylserine of brain proteolipid is bound loosely to protein,[21] Uda and Nakazawa, since phsophatidylserine could not be extracted from brain proteolipid with $CHCl_3$–MeOH (2:1), inferred that phosphaditylserine might be tightly bound to protein.[22]

The tightly-bound phsopholipid we obtained was found to contain, in addition to phosphatidylinositide, phosphatidylserine as well. As mentioned earlier, Folch demonstrated that phosphatidylserine is bound to protein by ionic linkage, and the extractability of gangliosides, which likewise are protein-bound by ionic linkage (gangliosides-Ca-protein), reportedly is altered by ionic strength.

Combined consideration of these warrants us to surmise that the extractability of phosphatidylserine might also vary with the ionization status of the membrane. If phosphatidylserine is really bound to protein by an electrostatic bond, as claimed by Uda and Nakazawa, it should be natural for it to be found extracted in tightly-bound phospholipid. It may be said therefore that the failure of a part of phosphatidylserine to be extracted with $CHCl_3$–MeOH (2:1) is not due to mere technical faults but is a result arising necessarily from the existing physicochemical status of the substance itself within the membrane.

Seeman reported that local anesthetics, tranquilizers and lipid-soluble anesthetics exerted a lytic action on erythrocytes at high concentrations and that this effect was preceded by an increase in passive K^+ permeability of the cell membrane.[15] Similar effects were obtained also by Langslet using rat heart.[23] That such a membrane permeability change unquestionably is due to the drug-induced membrane structural change (dissolution of lipid-protein interaction) is very important for the explanation of pyrithioxine-induced damage to the histohematic barrier (increase in membrane permeability) we reported previously.[3]

Thus, it is considered that phospholipid-protein interactions in the membrane were weakened by *in vivo* pyrithioxine treatment and the membrane structure was lossened. Probably consequent on these effects is the observed increase in membrane permeablility.

Gahan and Maggi pointed out the pivotal importance of the tightly-bound lipid to the structure and function of cells.[24] Although it is true that the capillary endothelial cell plays a big role in the manifestation of specificity of the blood-brain barrier, there have been few reported biochemical studies on the mechanism thereof. It is likely that the tightly-bound lipid is of great significance in this regard, too.

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