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Effect of Pyrithioxine on the Histo-Hematic Barriers[1]

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Pyrithioxine (pyridoxine 5-disulfide) increased the pentobarbital-induced sleeping time in mice. This potentiation was divided into two phases; one appeared immediately after the administration of pyrithioxine and reached a maximum within 20 min. The other appeared 8—10 hr later and corresponded to the inhibition of hepatic drug-metabolizing enzymes.

When the potentiation without relation to the inhibition of hepatic drug-metabolizing enzymes occurred, the pentobarbital concentration increased in the brain and liver, but was unchanged in the serum. Pyrithioxine stimulated the transport of ^{14}C-labeled fatty acids, glucose, amino acids, acetic acid, and inulin into the brain and liver, same as pentobarbital. Since $[Na^+ + K^+]$-ATPase, K^+-phosphatase, and $[Mg^{2+} + Ca^{2+}]$-ATPase activities were not influenced by pyrithioxine, this stimulation was not dependent on the increase of active transport, but depended on the increase of passive transport. However, the uptake of radioiodinated human serum albumin into the brain and liver of mice and the water content of these tissues were not affected by pyrithioxine treatment. The brain was not stained by Trypan Blue in pyrithioxine-treated and in control mice, but the liver, kidneys, intestines, skin, abdominal wall, and tail were stained deeper in pyrithioxine-treated mice.

Increase in radioactivity from octanoate-1-^{14}C was detected only in the free fatty acid fraction in the brain, but the radioactivity in the liver increased in the free fatty acid and the triglyceride fractions. This shows that pyrithioxine affects not only the histo-hematic barrier but also the fatty acid metabolism in the liver.

Brain pentobarbital concentration at the time of awakening was lower in pyrithioxine-treated mice than the control. This result suggests that pyrithioxine increases the susceptibility of central nervous system to barbiturates. In addition, increased uptake of ^{45}Ca in the brain indicates the disturbance of ion transport regulation which is necessary to preserve the central nervous excitability.

These results suggest that pyrithioxine increases the pentobarbital sleeping time by three mechanisms of (1) inhibition of hepatic drug-metabolizing enzymes, (2) increase in brain pentobarbital concentration, and (3) decrease of excitability of the central nervous system.

It has previously been reported that pyrithioxine inhibited the hepatic drug-metabolizing enzymes.[3] Since data presented in the preceding paper have demonstrated that the barbiturate-induced sleeping time was already prolonged by pyrithioxine at the time when the enzyme activity was not inhibited yet, it was assumed that other factors besides inhibition of the enzyme might be present for barbiturates potentiation by pyrithioxine.

The purpose of the present investigation was to determine these unknown factors.

Experimental

Chemicals——All radioactive compounds were obtained from Daiichi Chemical Co., Tokyo, and their specific activities were as follows: Sodium acetate-1-^{14}C (20.0 mCi/mmole), sodium butyrate-1-^{14}C (10.0 mCi/mmole), stearic acid-1-^{14}C (25.0 mCi/mmole), D-glucose-U-^{14}C (5.2 mCi/mmole), amino acid-U-^{14}C (protein

1) Part of this work was presented at the 91th Annual Meeing of Pharmaceutical Society of Japan, Fukuoka, April 1971.
2) Location: *Aobayama, Aramaki, Sendai.*
3) Y. Endo and M. Uchiyama, *Biochem. Pharmacol.*, **19**, 2839 (1970).

hydrolysate, 19.9 mCi/mmole), $^{45}CaCl_2$ (7.55 mCi/mg Ca), human serum albumin-^{131}I (RISA, 1 mCi/10 mg albumin), inulin [carboxyl-^{14}C] (1.83 mCi/g).

Cab-*O*-Sil, 2,5-diphenyloxazole (PPO) and 2,2′-*p*-phenylene-bis(5-phenyloxazole) (POPOP) (Packard Co.), Silicic acid (Mallinckrodt, 100 mesh), disodium *p*-nitrophenylphosphate (Wako Pure Chemicals Co., Tokyo), and disodium adenosine triphosphate (ATP) (Sigma Co.) were used.

Determination of Pentobarbital Sleeping Time——Male ddy strain mice, weighing 20—25 g, were used. Pyrithioxine was administered intraperitoneally in a dose of 200 mg/kg, and control animals were treated as in the preceding experiment.[1] All the experiments reported below were carried out under the same condition. Barbiturate sleeping time was designated as the time interval, in minutes, from the loss to the regaining of the righting reflex after sodium pentobarbital administration (30 mg/kg, *i.p.*).

Quantitative Determination of Pentobarbital——Sodium pentobarbital (60 mg/kg) was injected intraperitoneally. Pentobarbital concentration in brain, liver, and plasma was determined by the method of Kalser, *et al.* at the time of the loss and regaining of the righting reflex.[4] Blood was collected by heart puncture.

Radioactivity Measurement——Radioactive compound was injected intravenously *via* the tail in a dose of 2 μCi/20 g of body weight each of acetate-1-^{14}C, butyrate-1-^{14}C, octanoate-1-^{14}C, stearate-1-^{14}C, glucose-U-^{14}C, and amino acid-U-^{14}C, 5 μCi/20 g each of $^{45}CaCl_2$ and inulin [carboxyl-^{14}C], and 10 μCi/20 g of RISA. Radioactivity was measured 10 min after injection of these compounds by the method of Aronson and Davidson.[5]

Brain and liver were homogenized with cold 0.25M sucrose so that 100 mg of tissue might be contained in 0.5 ml of the homogenate. An aliquot of 0.5 ml of the homogenate was suspended in 9.5 ml of dioxane scintillator with 10 ml of Cab-*O*-Sil. In the case of serum, 0.2 ml of the serum was suspended directly in the scinitillator with Cab-*O*-Sil. Radioactivity was measured with Aloka liquid scintillation counter, Model LSC-601. Dioxane scintillator consisted of 750 ml of dioxane, 125 ml of anisole, 125 ml of dimethoxyethane, 12 g of PPO, and 0.4 g of POPOP.

Tissue Water Content——Water content of the brain and liver was determined by the method of Waddell and Butler.[6]

Incorporation of Octanoate-1-^{14}C into Lipids——Ten minutes after intravenous administration of 5 μCi/20 g of octanoate-1-^{14}C, lipid was extracted and purified in the manner described by Folch, *et al.*[7] The tissue (1 g) was homogenized with 20 ml of $CHCl_3$–MeOH (2:1, v/v), centrifuged and the organic solvent layer was collected. The tissue residue was reextracted with 10 ml of $CHCl_3$–MeOH twice. Extracts were combined, washed with 0.2 vol. of 0.9% NaCl solution, centrifuged, and the upper aqueous layer was decanted. $CHCl_3$ layer was washed twice with 'Folch upper phase ($CHCl_3$–MeOH–0.9% NaCl, 3:48:47 by volume)'. Pooled extracts were evaporated to dryness in a rotary evaporator at 30° and the residue was dried throughly over P_2O_5 for 24 hr *in vacuo*.

In order to separate the neutral lipid and phospholipid, the lipid extract was chromatographed over silicic acid by the method of Börgstrom.[8] After free fatty acids were extracted with 4% Na_2CO_3 solution, neutral lipid fraction was rechromatographed over silicic acid and divided into cholesterol ester, triglyceride, cholesterol, and di- and mono-glyceride fractions.[9] Each of the lipid fractions was evaporated to dryness and dissolved in 5 ml of ether. Radioactivity and lipid content were measured using a portion of these lipid solutions. Liquid scintillator consisted of 1000 ml of toluene, 4 g of PPO, and 0.1 g of POPOP. Lipids were quantitatively determined as follows: Cholesterol and cholesterol ester by the Lieberman–Burchard reaction,[10] free fatty acid according to Itaya and Ui,[11] glycerides by the method of Antonis,[12] and phospholipid phosphorous by the method of Bartlett.[13]

Vital Staining with Trypan Blue——Trypan Blue (200 mg/kg) was injected intravenously. Thirty minutes later, the whole body was perfused with 0.9% NaCl solution injected through the aorta and photographed.

Urinary Excretion of Human Serum Albumin-^{131}I——Urine was collected for 2 hr after intravenous administration of RISA (10 μCi/20 g) and protein was sedimented by the addition of 25% trichloroacetic acid (TCA) solution, to a final concentration of 5%. Protein suspension was centrifuged and the supernatant was decanted. Acid-insoluble protein fraction was washed with 2 ml of 5% TCA twice, washed with 2 ml

4) S.C. Kalser, E. Forbes, and R. Kunig, *J. Pharm. Pharmacol.*, **21**, 109 (1969).
5) N.N. Aronson and E.A. Davidson, *J. Biol. Chem.*, **243**, 4494 (1968).
6) W.J. Waddell and T.C. Butler, *J. Clin. Invest.*, **38**, 720 (1959).
7) J. Folch, M. Lee, and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).
8) B. Börgstrom, *Acta Physiol. Scand.*, **25**, 101 (1952).
9) J.C. Dittmer and M.A. Wells, *Methods in Enzymol.*, **16**, 510 (1969).
10) R.P. Moore and C.A. Baumann, *J. Biol. Chem.*, **195**, 615 (1952).
11) K. Itaya and M. Ui, *J. Lipid Res.*, **6**, 16 (1965).
12) A. Antonis, *J. Lipid Res.*, **1**, 485 (1960).
13) G.R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).

of EtOH–ether (3:1, v/v), dissolved in formic acid, transferred to a planchette, dried with an infrared lamp, weighed, and radioactivity was measured with Aloka 2π gas flow counter.

$[Na^+ + K^+]$-ATPase, K^+-Phosphatase, and $[Mg^{2+} + Ca^{2+}]$-ATPase activity——The brain and liver were homogenized with 9 vol. of cold 0.32M sucrose, using a Teflon-glass homogenizer. This homogenate was used as the enzyme preparation. Sodium *p*-nitrophenyl phosphate and ATP were converted to tris (hydroxymethyl)aminoethane (Tris) form by passing through a column of Amberlite IR-120 and neutralized with Tris. $[Na^+ + K^+]$-ATPase, K^+-phosphatase, and $[Mg^{2+} + Ca^{2+}]$-ATPase activities were measured by the method of Ohashi, *et al.*[14] Protein was determined by the method of Lowry, *et al.*[15]

Result

Increase of Pentobarbital Sleeping Time by Pyrithioxine

Pentobarbital sleeping time was measured at various periods after *i.p.* administration of pyrithioxine. As shown in Fig. 1, pentobarbital sleeping time was prolonged immediately after administration of pyrithioxine and a peak appeared after 20 min.

Data presented in the previous paper have demonstrated that the inhibition of hepatic drug-metabolizing enzymes appeared about 6 hr after administration of pyrithioxine and reached maximum after 10 hr (Fig. 2 in Reference 3).

These two results show that barbiturates potentiation induced by pyrithioxine consists of two different parts; the one appearing rapidly after the administration of pyrithioxine, unrelated to the inhibition of hepatic drug-metabolizing enzymes by pyrithioxine, and the other appearing about 6 hr later and corresponding to the inhibition of hepatic drug-metabolizing enzymes.

The experiments described below deals with this potentiation of barbiturate action by pyrithioxine, which appeared in an early stage.

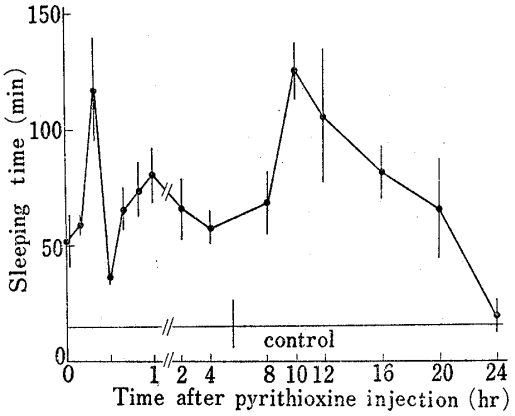

Fig. 1. Increase of Pentobaribital Sleeping Time by Pyrithioxine

Sodium pentobarbital (30 mg/kg, *i.p.*) was administered at various time intervals after pyrithioxine injection. Each value was represented as the mean for five determinations. Vertical lines show ± S.D. population.

Effect of Pyrithioxine on Brain, Liver, and Plasma Pentobarbital Level

Brain and liver pentobarbital concentration increased significantly in pyrithioxine-treated mice when the righting reflex was lost and, on the contrary, decreased significantly at the time of awakening after narcosis. Plasma pentobarbital concentration did not show

Table I. Pentobarbital Concentration of Control and Pyrithioxine-Treated Mice

Treatment	Brain	Difference (%)	Liver	Difference (%)	Plasma	Difference (%)
Control	61.9±11.4[a]		148.9±12.1		107.4±17.1	
pyrithioxine	104.0±11.7	+66.5[b]	194.6±20.0	+30.7[c]	106.9±10.5	—
Control	43.8± 6.7		61.6± 3.3		31.0± 2.6	
pyrithioxine	30.0± 2.3	−30.8[c]	49.4± 5.1	−19.1[d]	28.0± 4.7	—

Ten male mice were used in each experiment. Pyrithioxine (200 mg/kg) or 0.9% NaCl solution was injected intraperitoneally. After 20 min, sodium pentobarbital (60 mg/kg, *i.p.*) was administered. When righting reflex was lost (upper cloumn) and regained (lower column), pentobaribital concentration was determined.
a) mean ± S.D. of pentobarbital in μg/g tissue of ml plasma; *b*) $P<0.001$; *c*) $P<0.01$; *d*) $P<0.05$

14) T. Ohashi, S. Uchida, K. Nagai, and H. Yoshida, *J. Biochem.*, **67**, 635 (1970).
15) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

any significant change in both cases (Table I). This indicates that pyrithioxine stimulates the transport of pentobarbital into the tissue and decreases the effective concentration of pentobarbital in the central nervous system (CNS).

Permeability Change of Histo-hematic Barriers for Brain and Liver by Pyrithioxine

As shown in Table II, the transport of tracer substances (^{14}C-labeled fatty acids, amino acids, acetic acid, and glucose) into the brain and liver increased significantly by the administration of pyrithioxine. Since both substances incorporated by the active transport (glucose and amino acids) and the substance penetrated by passive diffusion (fatty acids) were stimulated, it seemed reasonable to assume that pyrithioxine stimulates the substrate transport by increasing the passive permeability nonspecifically.

Table II. Stimulation of Fatty Acids, Glucose, Amino Acids, and Acetic Acid Incorporation into Brain and Liver by Pyrithioxine

	Treatment	Brain (dpm/100 mg)	Difference (%)	Liver (dpm/100 mg)	Difference (%)
Stearic acid-1-^{14}C	control	304± 25[a]		24602±3088[a]	
	pyrithioxine	415± 49	+36.5[b]	34782±3225	+41.4[b]
Octanoic acid-1-^{14}C	control	1032± 169		9832± 716	
	pyrithioxine	1742± 260	+68.9[b]	12707±1887	+29.3[b]
Butyric acid-1-^{14}C	control	1372± 244		12400±1508	
	pyrithioxine	1807± 159	+31.7[c]	16987±1974	+37.0[b]
Glucose-U-^{14}C	control	12410±1022		33888±2759	
	pyrithioxine	16514±1090	+33.1[b]	40050±3911	+18.1[d]
Amino acids-U-^{14}C	control	1226± 211		17901±2639	
	pyrithioxine	1685± 156	+37.4[c]	20923±1252	+16.9[d]
Acetic adid-1-^{14}C	control	731± 68		33804±2491	
	pyrithioxine	1037± 119	+41.9[b]	43019±4508	+27.2[d]

Radioactive compounds were injected intravenously 10 min after pyrithioxine administration. Ten minutes thereafter, radioactivity was measured. Details were described in Experimetal.
a) Each value represents the mean ±S.D. for 10 determinations; *b*) $P<0.01$; *c*) $P<0.02$, *d*) $P<0.05$

Table III. Effects of Pyrithioxine on Inulin and Radiodinated Human Serum Albumin (RISA) Influx to Brain and Liver, and on the Water Contents of Brain and Liver

	Treatment	Brain dpm/50 mg	Brain Diff. (%)	Liver dpm/50 mg	Liver Diff. (%)	Serum dpm/0.2 ml	Serum Diff. (%)
Inulin	control	155± 20		1345± 215		68615± 10296	
	pyrithioxine	367± 83	+136.8	2495± 377	+85.8	53010± 8253	−22.7[a]
RISA	control	3027±1005		42503±8105		987253±113829	
	pyrithioxine	2971±1107	—	41185±6278	—	882962±100771	−10.5[a]

Water content	Brain (g water/100 g tissue)	Liver (g water/100 g tissue)
Control	78.1±15.3	67.5±21.1
Pyrithioxine	80.1±12.5	65.4±18.3

Readioactivity was measured as described in Table II. A portion of tissue was cutted off 20 min after pyrithioxine administration and wet weight was measured. This small piece of tissue was then dried throughly in oven at 110° for 24 hr, cooled in desicator and immediately weighted. The difference between the wet and the dry weight was made the water content of tissue. Results were represented as the mean±S.D. for 5 determinations.
a) nonsignificant

Data given in Table III show that the incorporation of inulin[carboxyl-^{14}C] into the brain and liver increased significantly, that in the serum inclined to decrease, and tissue water content did not change by pyrithioxine. These results suggest that the increased transport by pyrithioxine was due to the increase in capillary permeability.

Since the tissue RISA concentration of pyrithioxine-treated mice was no different from that of the control, pyrithioxine did not induce a change which would allow macromolecules such as albumin to pass through.

Vital Staining with Trypan Blue

Trypan Blue and other acidic dyes have been used as a tracer for measuring the brain capillary permeability.[16] Since the transport of inulin into the brain tissue was increased by pyrithioxine administration (Table III), it was expected that pyrithioxine would increase the staining of brain tissue with Trypan Blue. However, as shown in Fig. 2, there was no visible staining of the brain tissue with Trypan Blue by pyrithioxine treatment, as in the control. Furthermore, any visible staining was not found in various cross sections of the brain.

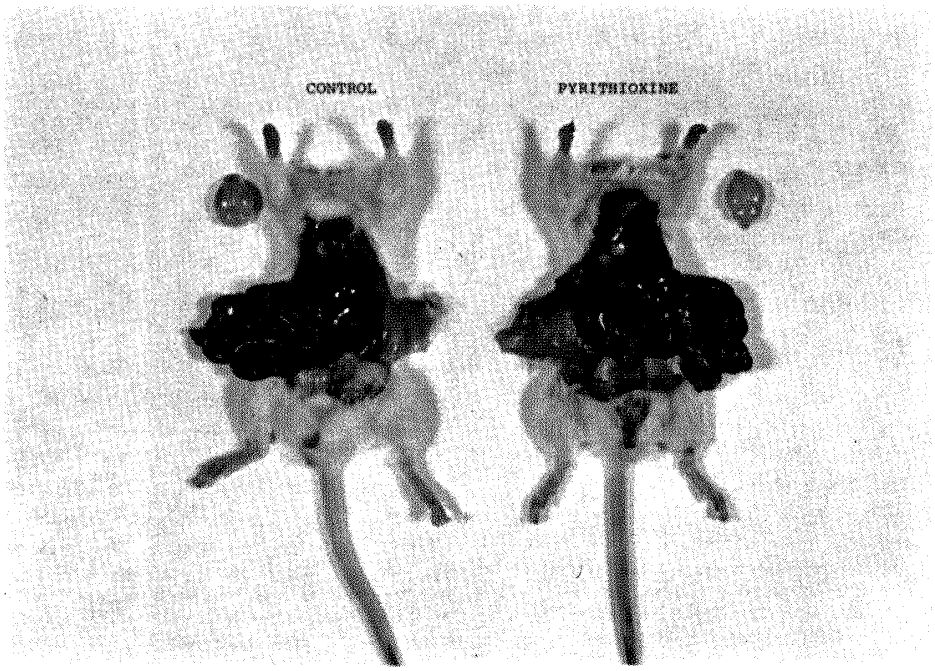

Fig. 2. Whole Body Photograph Stained with Trypan Blue

Pyrithioxine was administered 10 min after Trypan Blue injection. Twenty minutes thereafter, mouse was killed and treated as described in Experimental.

On the other hand, liver, kidneys, intestines, skin, abdominal wall, and tail were stained deeper in pyrithioxine-treated mice. This indicates that pyrithioxine provokes the increase of substrate transport extending to the whole body.

It was found during the course of an investigation on Trypan Blue staining that urine of pyrithioxine-treated mouse was deeper blue than that of the control. Tschirgi stated that Trypan Blue bound with albumin in blood and passed through the capillary wall as albumin complex.[17] It was expected from this theory and from our observation that urinary protein excretion would increase by pyrithioxine. Therefore, the counts of RISA excreted in urine was measured, but the radioactivity in TCA-insoluble protein fraction did not increase by pyrithioxine administration (Table IV).

16) *a*) R.A. Classen and S. Pandolfi, *J. Neuropath. Exptl. Neurol.*, **29**, 266 (1970); *b*) L. Bakay, *Progr. Brain Res.*, **29**, 315 (1968); *c*) L.S. King, *Arch. Neurol. Psychiat.*, **41**, 51 (1939).

17) R.D. Tschirgi, *Am. J. Physiol.*, **163**, 756 (1950).

Table IV. Urinary Excretion of Radioiodinated Human Serum Albumin (RISA)

	Protein in urine (mg)	Radioactivity of RISA	
		Total (dmp)	Specific activity
Control	1.25±0.44	1510±117	1350±393[a]
Pyrithioxine	1.35±0.64	1533±228	1374±492

Mice were tied up pronely and injected with RISA. Urine was collected with pushing occasionally over the bladder during 2 hr. Pyrithioxine was injected intraperitoneally 20 min before the end of collection. Values were represented as the mean ±S.D. for three determinations.
a) dpm/mg protein

Effect of Pyrithioxine on [Na^++K^+]-ATPase, K^+-Phosphatase, and [Mg^{2+}+Ca^{2+}]-ATPase of Brain

Quadbeck reported that the influx of ^{24}Na into the brain increased by pyrithioxine[18] and it is established that the active transport is correlated with cation-dependent ATPase activity. The experiment below refers to the effect of pyrithioxine on [Na^++K^+]-ATPase, K^+-phosphatase, and [Mg^{2+}+Ca^{2+}]-ATPase.

Table V shows that there is not very much change in these enzyme activities by pyrithioxine administration. These enzyme activities in the liver were very low (hardly detectable) and any alteration did not take place. It is concluded from this result that the increase of substrate transport induced by pyrithioxine is not accompanied by the stimulation of ATPase activity.

Table V. Effect of Pyrithioxine on [Na^++K^+]-ATPase, K^+-Phosphatase, and [Mg^{2+}+Ca^{2+}]-ATPase Activities of Brain

	Control	Pyrithioxine
[Na^++K^+]-ATPase	4.51 ±0.55	4.35 ±0.47
K^+-Phosphatase	1.014±0.361	1.035±0.343
[Mg^{2+}+Ca^{2+}]-ATPase	3.87 ±0.68	3.90 ±0.71

Five male mice were injected *i.p.* with pyrithioxine, sacrificed 20 min atter th drug administration and the enzyme activities were measured. Three enzyme activities were measured in same brain. Reaction mixtures were incubated at 37° for 10 min. Results were expressed as the mean ±S.D. of phosphorous liberated in μmoles per mg protein per hour for K^+-phosphatase, and of *p*-nitrophenol produced in the case of [Na^++K^+]-ATPase and [Mg^{2+}+Ca^{2+}]-ATPase.

Incorporation of Octanoate-1-^{14}C into Brain and Liver Lipids

As shown in Table VI, radioactivity increases in the free fatty acid fraction of the brain and in both the free fatty acid and triglyceride fractions of the liver. Table VI also shows that the net amount of free fatty acid increases in the brain but does not change in the liver. These results indicate that pyrithioxine also stimulates fatty acid metabolism in the liver, different from the brain.

Since radioactivities incorporated into cholesterol ester and phospholipid do not change, pyrithioxine does not affect the acyl group turnover of these lipids.

^{45}Ca Influx into Brain and Liver

It is established that calcium ion plays an important role in the depolarization of the excitable cell membrane,[19] and it is necessary for the difference between internal and ex-

18) G. Quadbeck, *Progr. Brain Res.*, **29**, 349 (1968).
19) *a*) J.D. Judah and K. Ahmed, *Biol. Rev.*, **39**, 160 (1964); *b*) J.M. Tobias, *Nature*, **203**, 13 (1964); *c*) A.M. Shanes, *Pharmacol. Rev.*, **10**, 59 (1958); *d*) I. Tasaki, A. Watanabe, and L. Lerman, *Am. J. Physiol.*, **213**, 1465 (1967); *e*) B. Frankenhaeuser and A.L. Hodgkin, *J. Physiol.*, **137**, 218 (1957).

Table VI. Incorporation of Octanoic Acid-1-^{14}C into Brain and Liver Lipids

		Brain				Liver		
		Lipid[a] content	Radioactivity Total (dpm/g tissue)	Radioactivity Specific[b]		Lipid[a] content	Radioactivity Total (dpm/g tissue)	Radioactivity Specific[b]
Free Fatty Acid	C[c]	0.79	12910	16342	C[c]	0.68	56705	83389
	P	1.20	15040	8847	P	0.57	77525	136008
Cholesterol Ester	C	0.295	140	541	C	0.404	660	1633
	P	0.288	159	552	P	0.394	605	1535
Cholesterol	C	2.140	1347	629	C	0.715	1500	2097
	P	2.155	1416	657	P	0.785	1767	2250
Triglyceride	C	0.58	1461	2513	C	17.00	71925	4230
	P	0.62	1565	2524	P	17.70	102180	5772
Di- and Mono-glyceride	C	N.D.[d]	376		C	0.84	3105	3696
	P	N.D.	348		P	0.87	6520	7494
Phospholipid	C	1.429	585	409	C	1.078	635	589
	P	1.613	540	334	P	1.273	560	439

Three animals were used in one experiment. Twenty minutes after pyrithioxine administration, 3 brains (or livers) were combined and assayed as described in Experimental. Results were represented as the mean of duplicate determinations. Three experiments were perfomed and same results were obtained.

a) Units are μeq/g of wet weight for each of free fatty acid and glycerides, mg/g for each of cholesterol and cholesterol ester, and mgPi/g in the case of phospholipid.

b) Units are dpm/μeq for each of free fatty acid and glycerides, dmp/mg for each of cholesterol and cholesterol ester, and dpm/mg Pi for phospholipid.

c) C: control, P: pyrithioxine-treated

d) N.D.: non-detectable

ternal cellular concentration of calcium to be maintained strictly, especially for the CNS to be excited normally. It is also considered that calcium ion is essential for preserving rigidity of the membrane.[20] Interest in these functions of calcium ion has led to the suggestion that the effect of pyrithioxine on ^{45}Ca influx into tissues should be investigated. Pyrithioxine increased the incorporation of ^{45}Ca into the brain within 1 hr after its administration, as shown in Fig. 3. Similar effect was observed in the liver within 20 min after pyrithioxine administration.

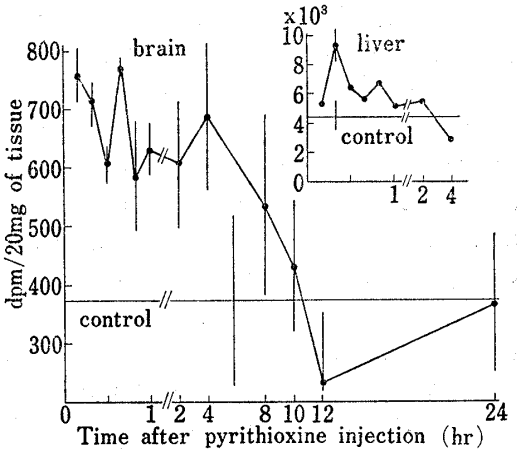

Fig. 3. Effect of Pyrithioxine on ^{45}Ca Incorporation

Radioactivity was measured at various time intervals after pyrithioxine adiministration. ^{45}Ca was injected 10 min before sacrifice. Each value was represented as the mean for 5 determinations. Vertical lines show ±S.D. popluation.

Discussion

It has previously reported that pyrithioxine increases the influx of glucose into the brain and oxygen uptake of the brain.[21] In spite of these functions suggesting stimulation of the CNS metabolism, this drug prolongs barbiturate sleeping time.[3] Our earlier work[3] dealing with the inhibition of hepatic drug-metabolizing enzymes by pyrithioxine showed that barbiturate potentiation is already noticed before hepatic drug-metabolizing enzymes were inhibited by pyrithioxine. Results described in the present paper defined the above-mentioned finding. Barbiturate potentiation induced by pyrithioxine was divided into two, the early and later effects. The early effect was independent of the inhibition of hepatic

20) J.E. Manery, *Federation Proc.*, **25**, 1804 (1966).
21) G. Quadbeck, *Progr. Brain Res.*, **29**, 343 (1968).

drug-metabolizing enzymes and reached a maximum within 20 min after pyrithioxine administration. The later effect corresponded to the inhibition of the enzymes and reached a maximum 8—10 hr after pyrithioxine administration.

When the early effect appeared, the brain pentobarbital level at the time of the loss of righting reflex increased and that at the time of awakening after narcosis decreased in pyrithioxine-treated animals compared with the control. Blood pentobarbital concentration did not change. These results point out two facts, that is, the permeability increase induced by the injury of the blood-brain barrier (this was represented as the increase of the blood-brain barrier permeability for simplification) and the stimulation of susceptibility of the CNS to barbiturates.

It is known that a drug increasing the permeability of blood-brain barrier shows barbiturate potentiation in general.[22] Although it was postulated that each tracer had its own blood-brain barrier permeability[23] and that the effect of drugs on it varied with tracers,[18] the present result showed that pyrithioxine increased the transport of low-molecular tracers (ions, fatty acids, glucose, amino acids, and acetic acid) and inulin uniformly. The permeability increase induced by pyrithioxine was not due to the tissue swelling or to the increase of tracer concentration in blood, since the tissue water content did not change and the blood tracer level inclined to decrease.

There is a possibility that the transport seems to increase apparently by the increase in blood volume. Blood volume was not measured in the present experiment. Since radioactive albumin or Evans Blue, binding with serum albumin, was used to measure the blood volume,[24] the fact that RISA concentration in tissue and serum did not increase might be considered to indicate that blood volume did not change with pyrithioxine treatment. It is concluded, therefore, that the increased incorporation of tracers into the brain is not an apparent but a net increase.

If Trypan Blue would not enter into the brain because of binding with serum albumin, as postulated by Tschirgi,[17] it is difficult to explain the fact that tissues except the brain of pyrithioxine-treated mice were stained deeper with Trypan Blue than that of the control, although the incorporation of RISA did not change. It seems, therefore, that mechanisms other than binding with albumin must be considered for the failure of Trypan Blue to pass through the capillary wall.

Levin and Scicli mentioned that the brain barrier system has to be regarded as one of the histo-hematic barriers of the organism and not as an exclusive effect of the nervous system.[25] Tschirgi also suggested that blood-brain barrier is not a qualitative but a quantitative matter,[26] and Dobbing concluded that this barrier is a brain metabolic activity, since the development of blood-brain barrier is correlative with changes in brain metabolism with growth.[27] Nevertheless, blood-brain barrier is a concept on the substrate transport from blood into the brain and many reports on the injury of blood-brain barrier indicates structural and functional changes in the brain capillary and its surrounding architecture.[16b,28]

22) R.K. Richards and J.D. Taylor, *Anesthesiology*, **17**, 414 (1956); V.V. Cole, H.O. Hulpieu, and S.H. Hopper, *Proc. Soc. Exptl. Biol. Med.*, **73**, 554 (1950); M.E. Greig and T.C. Mayberry, *J. Pharmacol. Exptl. Therap.*, **102**, 1 (1951); M.E. Greig and W.C. Holland, *Arch. Biochem. Biophys.*, **52**, 175 (1954); J.M. Beiler and G.J. Martin, *J. Pharmacol. Exptl. Therap.*, **118**, 415 (1956).
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It may be reasonable to suppose, therefore, that there must be certain sites (or mechanisms) different from regulatory mechanisms which make the substrate transport into the brain markedly different from that into other organs, even if it were quantitative. In this respect, it is very interesting that blood-brain barrier exists principally in the capillary endothelial cell (especially in the cell membrane) regardless of molecular weight and charge of substance, as claimed by Hashimoto, *et al.*[29]

Ratio of the amount of tracer substance incorporated into the brain to that into liver, calculated from the values listed in Table II, was about 40% for glucose, below 10% for fatty acids, and about 1% for stearate. This indicates that there is a barrier for passive transport in the brain and that pyrithioxine decreases this barrier function.

It was concluded that the increase in blood-brain barrier permeability revealed at least within 20 min after pyrithioxine administration was not dependent on brain metabolism but is due to decrease of barrier function for the following reasons: (1) There is no marked difference in the increase between actively and passively transported substances, (2) $[Na^+ + K^+]$-ATPase activity was not affected, (3) incorporation of radioactive inulin increased, (4) radioactivity increase from ^{14}C-octanoate was restricted to the free fatty acid fraction, and (5) free fatty acid level in the brain increased.

Increased incorporation of radioactive inulin into the brain indicates either increased permeability of the blood vessels or the expansion of extracellular spaces. It was found during this investigation, however, that the brain water content did not change at all and that incorporation of radioactive inulin increased by labeling for 10 min, while it took 6 hr for equilibration between blood and brain tissue.[30] It was, therefore, decided that the permeability of blood vessels increased by pyrithioxine treatment.

Goldmann's theory that choroid plexus was the blood-brain barrier was denied by Walter[31] but, since it is well known that substances not entering into the brain by intravenous administration can penetrate rapidly into the brain by the intraventricular administration,[32] it may be possible that pyrithioxine acts through the choroid plexus. Since it has been elucidated that materials are exchanged directly between blood and brain tissue,[31] that the time required for blood and brain tissues to reach equilibrium is much faster than that for blood and cerebrospinal fluid,[33] that the choroid plexus must be considered the detoxication process because it has an active transport process, pumping out certain substance from the cerebrospinal fluid into the blood,[25 33 34] and that inulin seems not to enter directly from blood into the cerebrospinal fluid,[34] substances would rather flow out from brain than flow in if pyrithioxine were to act through the choroid plexus. It was concluded, therefore, that pyrithioxine did not act on the blood-brain barrier through the choroid plexus.

The area postrema and other paraventricular structures had once been thought the structures which capture the vital dyes, and therefore the blood-brain barrier, but this view has also been denied since not all substances enter into the brain through these structures,[31] and it was evidenced that these structures are regions devoid of blood-brain barrier.[29b] It may be concluded, therefore, that pyrithioxine may affect the capillary endothelial cell membrane and increase its permeability.

Although the action of pyrithioxine has been considered restrictively to capillary endothelial cells from the site of blood-brain barrier, there is no necessity to think that only this

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30) D.L. Woodward, D.J. Reed and D.M. Woodbury, *Am. J. Physiol.*, **212**, 367 (1967).
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32) M.W. Brightman, *Progr. Brain. Res.*, **29**, 19 (1968).
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34) T.Z. Csaky and B.M. Rigor, *Progr. Brain Res.*, **29**, 147 (1968).

type of cells are affected by pyrithioxine. It must be considered that pyrithioxine, once it enters the brain, may increase the membrane permeability of glial cells and neurons as it affects capillary cell membrane during its passage.

Substrate incorporation in the liver was also stimulated by pyrithioxine. The fact that incorporation of radioactive inulin increased but RISA uptake did not change indicates that the cell membrane permeability changed in the liver as well as in the brain. However, there is a difference in the effect of pyrithioxine on the liver and that on the brain, that is, the increase of radioactivity from ^{14}C-octanoate occurred in both the free fatty acid and triglyceride fractions and the amount of free fatty acid did not increase in the case of liver. This indicates that the fatty acid metabolism also increased in the liver. Such rapid incorporation of fatty acids into triglycerides has also been reported in the case of CCl_4-induced fatty liver.[35]

Recently, Jori, *et al.* reported that brain pentobarbital level at the time of awakening after narcosis in amphetamine (central nervous stimulant)-pretreated animals was higher than that of the control.[36] The result of present work showed that brain pentobarbital level at the time of regaining the righting reflex in pyrithioxine-treated mice was lower than that of the control. This suggests that pyrithioxine not only increases the blood-brain barrier permeability, but also suppresses the central nervous system.

It has been well known that calcium ions are necessary for nerve excitation and have a stabilizing effect on the nerve.[19] In addition, it has been considered that calcium ions enter into the cell preceding the influx of sodium ions, which generates the action potential, and acts as a trigger, increasing the cell membrane permeability to sodium ions.[19b] It is also considered that there is a barrier to calcium transport and a mechanism of pumping out the intracellular calcium ions in the cell membranes which maintain and regulate the concentration difference between extra- and intra-cellular calcium ions.[37] Increase of calcium influx by pyrithioxine indicates that such maintenance and regulation of difference between extra- and intra-cellular calcium concentration are lost, in other words, the decline of excitability.

In view of the above facts it was concluded that barbiturate potentiation induced by pyrithioxine was due to three mechanisms; (1) inhibition of hepatic drug-metabolizing enzymes, (2) increase of blood-brain barrier permeability, and (3) depression of the central nervous system excitability.

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35) B. Börgstrom and T. Olivecrona, *J. Lipid Res.*, **2**, 263 (1961).
36) A. Jori, A. Bianchetti, and P.E. Prestini, *Biochem. Pharmacol.*, **19**, 2687 (1970).
37) R. Whittam and K.P. Wheeler, *Ann. Rev. Physiol.*, **32**, 21 (1970).