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EFFECT OF ACUTE AND CHRONIC ETHANOL TREATMENT ON RAT BRAIN PHOSPHOLIPID TURNOVER*

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Abstract—Phospholipid turnover was studied in the rat brain after treatment *in vivo* with (1) an acute dose of ethanol, (2) after the development of tolerance to ethanol, (3) of physical dependence on ethanol, and (4) of withdrawal from dependence. It was found that the turnover of phosphatidylcholine, of phosphatidylinosotol/phosphatidylserine and of phosphatidylethanolamine was increased in tolerant animals and that tolerance developed to these increases in dependent animals. A drastic decrease in ^{32}P-labeled phosphatidylinositol/phosphatidylserine of microsomal fractions was observed in animals withdrawing from dependence.

Ethanol is known to exert an effect on biological membranes [1]. Chin and Goldstein [2, 3], studying the fluidity of spin-labeled membranes in the presence of ethanol, have shown that ethanol may increase membrane fluidity *in vitro*; tolerance is developed to that effect after chronic ethanol treatment. Supporting evidence for this fluidization has been provided by Curran and Seeman [4]. We have also reported that chronic ethanol treatment results in more rigid lipid bilayers extracted from synaptosomal membranes of tolerant mice [5]; the increase in rigidity is not due to the change in cholesterol content [6]. We have also provided evidence [7] that the change in brain membrane lipid composition or organization responsible for the attenuation of ethanol-induced fluidization of 'tolerant' membranes is related to tolerance to the anesthetic actions of ethanol, because there is cross tolerance between membrane fluidizing properties of ethanol and pentobarbital but not of morphine. Further experiments revealed that with the absence of ethanol from the body for a period of time, the fluidizability of the lipid bilayer returned to normal [7]; these lipid changes may be generalized to all strains and species of rodents [8].

Since the ability of ethanol to induce fluidity may be related to the phospholipid composition of the membrane, we studied phospholipid turnover in brain membranes isolated from acute, tolerant, dependent and withdrawn rats.

METHODS

Male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA), weighing 300–350 g, were implanted with a guide cannula over the lateral ventricle while under pentobarbital anesthesia. Before beginning the experiment, 4–6 days were allowed for recovery from surgery. We studied the effects of (1) an acute dose of ethanol, (2) tolerance to ethanol, (3) physical dependence on ethanol, and (4) withdrawal from dependence.

To produce acute effects, animals were injected i.p. with either 3.5 g/kg of ethanol (20%, w/v) or with an equivalent volume of isotonic saline. Thirty minutes later the animals were given intraventricularly 200 μCi [^{32}P]phosphoric acid and 50 μCi [^{3}H]glycerol dissolved in 20 μl of a modified Krebs–Ringer bicarbonate buffer from which the sodium diphosphate was omitted.

Each animal to be made tolerant was tested for basal sleep time prior to implantation of the guide cannula. They were injected i.p. with 3.5 g/kg of ethanol, and upon onset of narcosis, were placed on their backs. The time for an animal to right itself twice within 60 sec was recorded as the sleep time. Groups could thereby be balanced for basal sleep times. The animals were thereafter made tolerant, while under light CO_2 anesthesia, by intubation with 3.0 g/kg ethanol [20% (w/v) solution in Slender, Carnation Co., Los Angeles, CA.] twice daily for 3 days. Control animals were similarly intubated with equivalent amounts of isocaloric sucrose (35%, w/v) in Slender. On the day following the treatment period, tolerance was assessed in both groups by again determining sleep times [5]. Radioisotopes were administered on the next day 1 hr after the treatment of both groups with 3.5 g/kg of ethanol, i.p.

Physical dependence was produced as described previously [8–10] using a maximally tolerable dosage regimen. Briefly, animals were intubated while under

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Table 1. Effect of acute ethanol on [^{3}H]glycerol or [^{32}P]phosphate incorporation into membrane lipid fractions*

		Synaptosomal fractions			Microsomal fractions		
		Control cpm/μmole	%	Ethanol % of control	Control cpm/μmole	%	Ethanol % of control
PC	[^{3}H]	1850 ± 848	100 ± 6.5	172.6 ± 19.2†	4950 ± 942	100 ± 11.9	193.9 ± 6.5‡
	[^{32}P]	3070 ± 493	100 ± 13.8	133.1 ± 18.9	6100 ± 599	100 ± 7.9	197.9 ± 14.7
PI + PS	[^{3}H]	10,030 ± 833	100 ± 6.1	167.9 ± 15.8‡	9900 ± 756	100 ± 7.2	164.6 ± 20.3‡
	[^{32}P]	28,800 ± 686	100 ± 7.0	91.1 ± 9.6	48,300 ± 1153	100 ± 12.7	105.0 ± 14.0
PE	[^{3}H]	4700 ± 386	100 ± 10.7	105.6 ± 15.6	7330 ± 646	100 ± 6.5	126.4 ± 16.9
	[^{32}P]	8610 ± 244	100 ± 4.8	87.8 ± 8.0	14,480 ± 4080	100 ± 8.1	104.9 ± 11.3

* Data are means ± S.E.; N. = 9–11.
† $P < 0.025$.
‡ $P < 0.005$.

light CO_2 anesthesia three times daily for 3 days. The dose was adjusted according to degree of intoxication (determined largely by righting ability) prior to intubation with possible doses of 6.0, 4.0, 3.0, 2.0 and 0.0 g/kg of ethanol; the ethanol solution was 20% (w/v) in Slender. To maintain body weight, the total volume of each intubation was made up to 14 ml with additional Slender and a fourth daily 14-ml intubation of Slender was given. Each control animal was intubated with an amount of the isocaloric sucrose–Slender diet proportional to the mean amount given the experimental group; additional Slender was similarly given. Radioisotopes were administered to dependent animals 1 hr after the first missed dose, a time at which blood alcohol begins to decline but at which withdrawal has not yet commenced, and to withdrawn animals 9 hr after the missed dose, a time at which various withdrawal symptoms are found [10].

For all treatment conditions, animals were killed 1 hr after radioisotope administration. Brains were removed and homogenized in 30 ml of 0.32 M sucrose with 5 mM Hepes,* pH 7.8 at 4°. Crude synaptosomal and microsomal fractions were prepared by standard centrifugation techniques [11]. A portion of the homogenate was mixed with 2 vol. of 10% trichloroacetic acid and an aliquot of the supernatant fraction was counted for total nonprecipitable ^{32}P and ^{3}H in order to normalize the lipid incorporation results. Phospholipids were extracted from synaptosomal and microsomal fractions with chloroform–methanol (2:1, v/v) [11, 12]. The lipid extract was partitioned with 0.1 M KCl, washed several times with theoretical upper phase until no radioactivity appeared in the upper phase, and then evaporated to dryness at 30°; the residue was dissolved in 100 μl of chloroform–methanol (2:1). Twenty-five microlitres was spotted on silica gel G thin-layer chromatography plates (Merck), and the plates were developed in a solvent system of chloroform–methanol–acetic acid–water (25:15:4:2, v/v). Spots were identified with iodine vapor and scraped, and the phospholipids were eluted into chloroform–methanol (2:1), aliquots of which were then used for scintillation counting and for lipid phosphorus analysis [13]. The results are reported as percentage of control for simplicity. In Table 1, we also listed cpm/μmole P_i so that readers may calculate back to that unit from percentage if they wish. Statistical analysis was done using Student's *t*-test.

* Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

RESULTS AND DISCUSSION

Table 1 presents the aggregate results of two experiments. An acute dose of ethanol had no effect on the incorporation of $^{32}P_i$ into phosphatidylcholine (PC), phosphatidylinositol + phosphatidylserine (PI + PS) or phosphatidylethanolamine (PE) in either synaptosomal or microsomal fractions. There is evidence, however, of increased incorporation of

Table 2. Effect of chronic ethanol treatment on [^{3}H]glycerol or [^{32}P]phosphate incorporation into membrane lipid fractions of tolerant and nontolerant rats*

		Synaptosomal fraction		Microsomal fraction	
		Acute	Tolerant	Acute	Tolerant
PC	[^{3}H]	100 ± 25.5	69.2 ± 22.5	100 ± 29.3	97.6 ± 22.1
	[^{32}P]	100 ± 30.6	84.3 ± 31.8	100 ± 3.8	213.6 ± 53.5†
PI + PS	[^{3}H]	100 ± 17.6	90.2 ± 6.3	100 ± 19.6	86.2 ± 12.1
	[^{32}P]	100 ± 17.0	167 ± 31.0‡	100 ± 22.4	187.5 ± 33.4‡
PE	[^{3}H]	100 ± 37.6	73.6 ± 9.9	100 ± 15.7	109.6 ± 8.6
	[^{32}P]	100 ± 16.9	125.7 ± 16.9	100 ± 15.7	165.0 ± 8.0†

* Values, means ± S.E., are percentage of control levels of cpm/pmole P_i; N = 4–5.
† $P < 0.005$.
‡ $P < 0.05$.

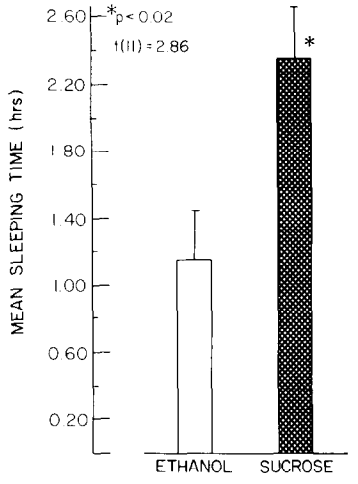

Fig. 1. Mean (± S.E.) sleeping time following 3.5 g/kg ethanol in chronic ethanol and sucrose control groups.

[^{3}H]glycerol into PC and PI + PS of the synaptosomal and microsomal fractions.

After the animals were made tolerant by intubation with 3.0 g/kg ethanol (see Methods), both control and tolerant animals were given ethanol prior to radioisotope treatment and killed so that tolerance in response to the drug, rather than any intrinsic state of tolerance, was being studied. Thus, the control animals were comparable to the acutely treated animals reported above. There was significant development of tolerance as indicated by the difference in sleep time between the tolerant and acute-control groups (Fig. 1).

Although the main effects of an acute dose were on [^{3}H]glycerol incorporation, there was no indication of tolerance developing to this effect, since the incorporation of the isotope was similar in all phospholipids in tolerant and acute groups (Table 2). On the other hand, the incorporation of $^{32}P_i$ appeared to be increased in all phospholipid components in microsomal membrane fractions in tolerant animals treated with ethanol; an increase was also observed in PI + PS fraction from synaptosomal origin. These increases were not seen in acute animals but only in tolerant ones, suggesting that it is unique to tolerant animals.

If the animals were treated with higher doses of ethanol, physical dependence developed as evidenced by Metrazol-induced withdrawal seizures [8]. Mean (± S.E.) blood alcohol levels in the dependent animals at the time of killing (132.6 ± 3.5 mg ethanol/100 ml blood) were at a level above that at which withdrawal might begin. Our results on phospholipid turnover in those animals showed that there was a significant increase of $^{32}P_i$ incorporation into microsomal PE; [^{3}H]glycerol incorporation was unchanged compared to sucrose control (Table 3). It should be pointed out that there was a significant increase in the ^{3}H-label in PC and PI + PS of synaptosomal fractions and PC of microsomal fractions after acute treatment (Table 1); no tolerance to these changes in tolerant animals was observed (Table 2), and yet in dependent animals (Table 3) almost all the changes returned to sucrose control levels. It is

Table 3. Incorporation of [^{3}H]glycerol or [^{32}P]phosphate into membrane lipid fractions of sucrose control, ethanol dependent and ethanol withdrawn rats*

		Synaptosomal fraction			Microsomal fraction		
		Sucrose (control)	Ethanol dependent	Ethanol withdrawal	Sucrose (control)	Ethanol dependent	Ethanol withdrawal
PC	[^{3}H]	100 ± 18.1	102.5 ± 19.7	72.5 ± 10.4	100 ± 7.2	100.5 ± 17.3	74.7 ± 13.3
	[^{32}P]	100 ± 28.5	93.9 ± 14.9	153.0 ± 51.0	100 ± 13.2	139.6 ± 29.7	75.9 ± 3
PI + PS	[^{3}H]	100 ± 53.7	158.4 ± 29.6	223.8 ± 33.4†	100 ± 27.9	114.5 ± 20.1	68.2 ± 18.3
	[^{32}P]	100 ± 30.4	109.2 ± 15.4	126.9 ± 17.5	100 ± 21.1	114.4 ± 33.3	30.4 ± 2.1‡
PE	[^{3}H]	100 ± 6.9	85.1 ± 8.6	85.2 ± 11.6	100 ± 22.8	103.3 ± 16.5	122.1 ± 16.1
	[^{32}P]	100 ± 19.5	105.1 ± 11.8	89.9 ± 12.1	100 ± 13.3	186.9 ± 3.6‡	111.0 ± 10.5

* Values, mean ± S.E., are percentage of control values of cpm/pmole P_i; N = 4–7.
† $P < 0.02$.
‡ $P < 0.001$.

apparent that tolerance develops to certain changes in phospholipid turnover in the dependent animals but not in the tolerant ones. In animals undergoing withdrawal, there was a significant decrease in the incorporation of $^{32}P_i$ into microsomal PI + PS and an increase in the incorporation of [^{3}H]glycerol into synaptosomal PI + PS; no other changes were significant.

The withdrawal state usually means a state after dependence has developed in which the drug is no longer given. Often, during withdrawal the behavioral symptoms that occur are opposite to those which occur in the (alcohol) acute or tolerant state. Although the underlying biochemical mechanism is not well understood, it is reasonable to assume that events induced by chronic ethanol exposure at the molecular level must undergo changes during withdrawal that will compensate for the absence of the drug. In examining the phospholipid turnover data, we observed that ^{32}P-incorporation by the PI + PS fraction of microsomes was significantly increased in tolerant animals. This increase diminished in the dependent state and decreased significantly in withdrawal animals, which is opposite to the change in tolerant animals. Therefore, it is possible that the increase of ^{32}P-incorporation by PI + PS fractions in tolerant animals represents a new state of biochemical events to which the animals adapted in the presence of chronic drug exposure; tolerance is eventually developed to it after longer exposure (presumably in the physical-dependent state).

A rebound seems to occur if ethanol is removed from the animal in withdrawal. ^{32}P-incorporation by PI + PS of the microsomal fraction, instead of increasing in the tolerant state, dramatically increased below control level in the withdrawal state. This may or may not be part of the reason for the severe withdrawal signs often seen in such animals. The fact that only ^{32}P, not ^{3}H, turnover is changed in this fraction indicates that the change may occur after 1,2-diglyceride production. Because PE is one of the major precursors of PS in the brain [14–16], and PE was increased in tolerant and dependent animals but returned to control levels in withdrawal, it is not likely that the decrease in PI + PS is primarily a change in PS resulting from a decrease in the precursor PE.

The increases in labeling of other phospholipids in either the acute or tolerant state all seemed to return to control level in dependent animals except ^{3}H-incorporation into the PI/PS fraction of the synaptosome; withdrawal did not precipitate any new change.

Chronic ethanol treatment is known to result in more rigid membranes [2–6], a factor which is related to the anesthetic action of ethanol [7, 8]. A change in phospholipid metabolism has been suggested for the change in membrane rigidity. Littleton and John [17] demonstrated that the level of saturated fatty acids was increased in membranes of ethanol tolerant mice, which may, in part, be responsible for the increased membrane rigidity. It is obvious that some phospholipid turnover is also altered by chronic ethanol treatment. Although both ICR and C57BL mouse strains exhibit decreased ethanol-induced fluidity in their lipid bilayers after chronic ethanol treatment [7], individual phospholipid levels were changed in ICR, but not in C57BL mice (D. A. Johnson and N. M. Lee, unpublished observation), indicating that the change in phospholipid level cannot be used to explain the change in membrane rigidity. The changes produced in phospholipid turnover by chronic ethanol treatment, however, may represent a novel shift in the membrane phospholipid metabolism. This signals membrane changes which may eventually be reflected as changes in membrane function.

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