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LIPID COMPOSITION OF THE SYNAPTOSOME AND ERYTHROCYTE MEMBRANES DURING CHRONIC ETHANOL-TREATMENT AND WITHDRAWAL IN THE RAT

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Abstract—Male Sprague–Dawley rats were intoxicated by inhalation of ethanol vapor for 21 days. This allowed high tolerance to the hypnotic effect of ethanol and withdrawal syndrome to be developed. The chronic intoxication brought about modifications of the synaptosome and erythrocyte membrane lipid composition which were not due to the reduction in food intake that parallels intoxication. The fatty acid composition of the phospholipids was modified but the level of cholesterol and the level of phospholipid remained unchanged. The modifications concerned the levels of linoleic (18:2) and arachidonic (20:4) acids which decreased in the synaptosomes. In the red blood cell membranes, ethanol affected the levels of the octadecenoic acids (18:1) which rose, and linoleic acid (18:2) which fell. These disturbances were present when the withdrawal syndrome was at its highest and also 3 days after withdrawal when the signs of hyperexcitability were no longer visible in the animal.

Modifications in the brain membrane lipid composition parallel the behavioral tolerance to ethanol; however the present results show that the apparent readaptation of the central nervous system to withdrawal of alcohol occurs earlier than the return to normal of the membrane lipid modifications.

Numerous investigations suggest that the pharmacological effects of ethanol are not due to its binding to a specific receptor. In fact ethanol could act by dissolving in the membrane lipids: the use of spin markers [1, 2] and fluorescent probes [3] has shown that ethanol causes biomembranes to become disordered—the lipid matrix being more fluid; it has also been noted that the fluidifying effect of ethanol is less strong in membranes isolated from chronic ethanol-treated rats [4]. This membrane adaptation is at the origin of hypotheses explaining behavioral tolerance to and physical dependence on ethanol [2].

Two mechanisms of adaptation could explain how the membrane lipid matrix is rigidified in response to the fluidifying effect of the alcohol:

(i) an increase in the level of cholesterol as demonstrated by Chin *et al.* [5].

(ii) an increase in the saturation of the fatty acids of the membrane phospholipids as shown by Littleton and John [6].

However, the modifications of the membrane lipid composition reported by various researchers are not always in agreement. Using a crude synaptosome preparation, Johnson *et al.* [7] did not detect any significant modification in the level of cholesterol in mice tolerant to ethanol. Sun and Sun showed a significant increase in the polyunsaturated fatty acids of the phosphatidyl ethanolamines in the synaptic plasma membranes from the brains of ethanol-treated guinea-pigs [8]. In the mouse, Wing *et al.* did not find any significant variation in the level of cholesterol nor in the fatty acid composition of the phospholipids in synaptosome membranes isolated from mice intoxicated by inhalation; however, in the same animals three other types of plasma membrane showed different patterns of change in their phospholipid fatty acids [9].

Few works deal with the modifications in synaptosomal lipid composition and even fewer involve the simultaneous study of the levels of cholesterol and fatty acids of the membrane phospholipids [9, 10]. The purpose of the present study is to confirm the existence and investigate the nature of the action of ethanol on the synaptosomes of rats tolerant to and dependent on ethanol. Furthermore, the lipid composition of erythrocyte membranes was analysed in parallel. All the measurements were made during chronic intoxication and withdrawal in order to determine the possible interrelationships.

MATERIALS AND METHODS

Ethanol administration. During the different experiments, male Sprague–Dawley rats weighing 190–210 g were treated by continuous inhalation of ethanol vapor for 21 days as described previously [11]. The animals placed in the vapor chamber received food (A 103 solid chow, U.A.R., Villemoisson sur Orge, France) and tap-water *ad libitum*. Two groups of animals were kept in a normal atmosphere: one of the groups (controls) had free access to food and drink, and the other (pair-weight) was given a quantity of food such that the growth of the rats was identical to that of the ethanol-treated animals.

Measurement of blood ethanol and acetaldehyde. Blood ethanol and acetaldehyde were assayed by

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head-space gas chromatography using isopropanol as internal standard [12]. The test sample was composed of 100 μl blood taken from the retro-orbital sinus.

Quantification of tolerance to ethanol and withdrawal syndrome. Ethanol tolerance was estimated by the level of blood ethanol at the moment when the animal regains its righting reflex after intraperitoneal injection of 20% (w/v) ethanol in saline at 4 g ethanol/kg in non-treated rats and 3 g/kg in alcohol-treated rats (tolerance test at end of intoxication). The rats were considered to have regained their righting reflex if they returned to the normal supine position three successive times within 30 secs.

Quantification of withdrawal syndrome was carried out after the second tolerance test: ethanol treated and pair-weight animals were placed in a normal atmosphere without noise or bright light and the evaluation of withdrawal symptoms was performed double-blindly—every hour spontaneous signs of withdrawal (tremor, rigid posture etc.) were noted and quantified [13] then the intensity of the convulsions on handling were evaluated using Goldstein's scale [14].

Synaptosome preparation. Brain synaptosomes of ethanol-treated rat were prepared at 3 different stages: on removal from the alcohol atmosphere and 10 and 72 hr after. The animals were decapitated, the brains removed rapidly and homogenised in 10 vol of an ice cold 0.32 M sucrose, 5 mM HEPES solution (pH 7.4). The homogenate was centrifuged at 1000 *g* for 10 min and washed again. The combined supernatants were centrifuged for 15 min at 14500 *g* and the pellet was resuspended and centrifuged at 15900 *g* for 20 min. The synaptosome pellet was purified on a 4-step sucrose density gradient [15]. The two interfaces corresponding to the light and heavy synaptosomes were removed and pooled. The quality of the preparation was checked by electron microscopy.

Part of this preparation was used to assay the proteins using Lowry's technique [16].

Preparation of erythrocyte membranes. Erythrocyte membranes were prepared according to the method of Hanahan and Ekholm [17] at 5 different stages in the same animals: after 1 week of alcohol inhalation, at the end of ethanol-treatment and 10, 72 hr and 7 days after recovery of the righting reflex of the final tolerance test. Erythrocyte membranes were also prepared from the control and pair-weight animals. The blood samples (200 μl) were taken from the retro-orbital sinus.

Lipid extractions and assays. The lipids were extracted from the subcellular fractions by Bligh and Dyer's technique [18]. Aliquots of the synaptosome lipid extract were used to assay the total cholesterol [19] and the phosphorus of the total phospholipids [20]. The remainder of the synaptosome lipid extract and all the erythrocyte membrane lipid extract were concentrated and spotted on silica gel plates (60 F 254, Merck). The solvent used—petroleum ether: diethyl ether: acetic acid (85:15:2, v/v)—allowed separation of the neutral lipids from the phospholipids which remain at the origin. After checking the migration of the neutral lipids under u.v. light, the phospholipid spots were scraped off and washed from the silica several times with a chloroform: methanol: water mixture (43:43:14, v/v). They were then transesterified by refluxing in methanol: sulfuric acid (10:1, v/v) for 90 min. The methylated fatty acids were extracted with petroleum ether and assayed by gas chromatography (Intersmat IGc 120 DFL) on a capillary column (WCOT fused silica

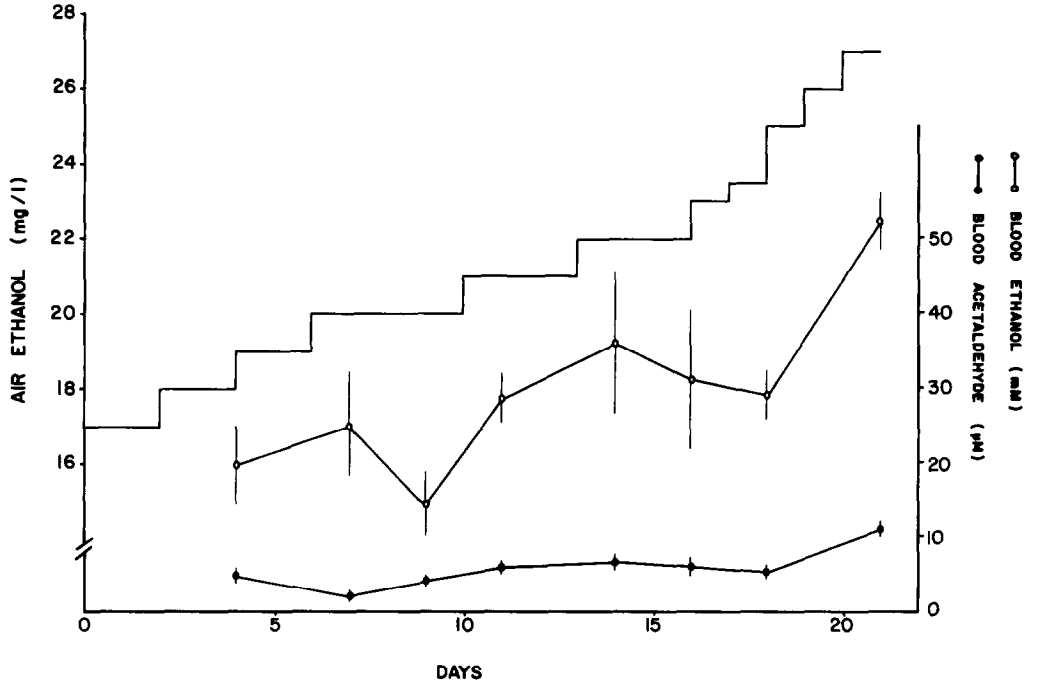

Fig. 1. Ethanol concentrations in the air of the inhalational chamber and levels of blood acetaldehyde and ethanol during the experiment. Means ± S.E. (N = 10).

column; 25 m; liquid phase: CP Wax 51; Intersmat Instruments) with temperature programmation (160–210°; 1°/min). The retention times and the areas under the peaks were obtained with a programmable integrator-calculator (Shimadzu C-R I B). Peak identification was by comparison of retention times with those of methyl-esterified standard fatty acids (Sigma).

RESULTS

Alcohol intoxication, body weight, food intake. Increasing the level of ethanol in the vapor chamber allows high blood ethanol concentrations to be obtained throughout experimentation. After 21 days of intoxication the blood was 50 mM for alcohol and 10 μM for acetaldehyde (Fig. 1). The chronic ethanol-treatment is paralleled by a drop in weight and decreased food intake with respect to the controls which had free access to food and drink (Fig. 2). To obtain an equivalent decrease in growth in the animals not exposed to ethanol (pair-weight group) their solid food intake was reduced but still remained 30% greater than the quantity freely ingested by the ethanol-treated animals (Fig. 2).

Development of tolerance and withdrawal syndrome. When the behavioural response was tested with the first injection of ethanol the animals regained their righting reflex at lower blood alcohol levels (72.9 ± 2.07 mM) than when tolerance was evaluated after three weeks of ethanol treatment (108.0 ± 9.66, $P < 0.01$); the level of blood acetaldehyde however was not significantly modifed (7.9 ± 0.69 and 9.1 ± 0.62 μM). The method of alcohol administration used therefore allows a high tolerance to the hypnotic effect of ethanol to be developed; it also leads to a strong withdrawal syndrome. Figure 3 presents the development of this syndrome during 48 hr. The highest scores were obtained between 10 and 20 hr. The animals presented decreased spon-

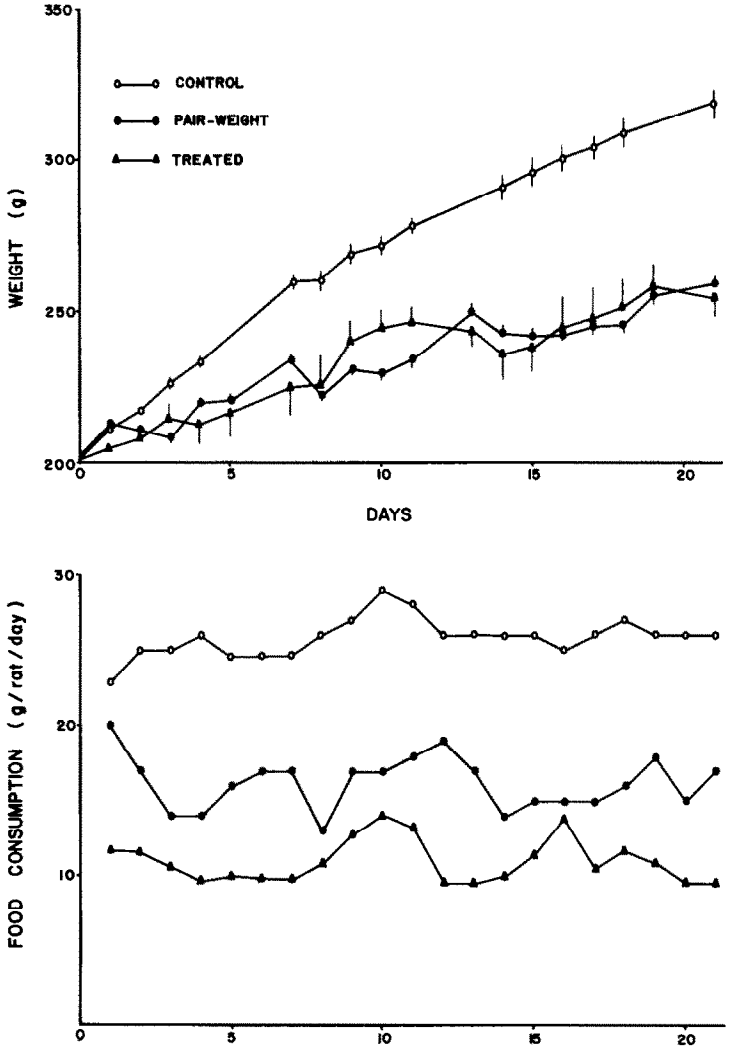

Fig. 2. Body weight and food intake of the three groups of animals during the period of experimentation (N = 10). The control and ethanol-treated animals received food and drink *ad libitum*. The pair-weight animals received a quantity of food each day which allowed their growth to follow that of the alcoholic rats.

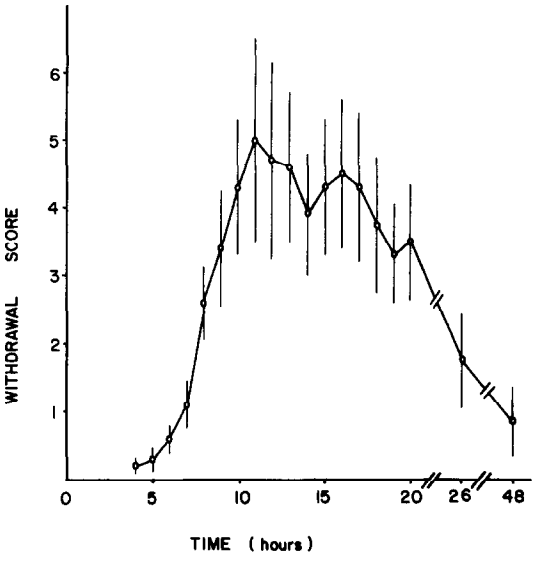

Fig. 3. Quantification of withdrawal syndrome. For each rat, the spontaneous signs of withdrawal and the convulsions on handling were scored. Each point represents the mean total score and its standard error (N = 6). This evaluation was carried out after the tolerance test. Time zero corresponds to the moment when the animals regained their righting reflex.

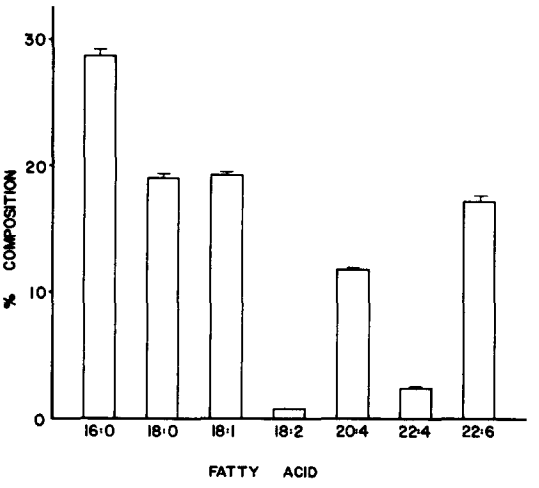

Fig. 4. Fatty acid content of synaptosome phospholipids. Per cent composition of major fatty acids in pair-weight rat are given. Means ± S.E. (N = 10).

taneous activity but their reaction to sensorial stimulation was abnormally strong (squeals, tremor, convulsion etc.). After 72 hr there were no longer any visible signs of hyperexcitability of the central nervous system.

Effect of ethanol-treatment on the lipid composition of the synaptosomes and changes occurring during the withdrawal period. The levels of cholesterol and phospholipid expressed per mg protein and the cholesterol : phospholipid ratio were not modified in the chronic ethanol-treated animals nor in the animals with a restricted diet. No variations of these values were observed during the withdrawal period (Table 1).

The relative levels of the main fatty acids of the synaptosome phospholipids are presented in Fig. 4, these reference values were obtained from the pair-weight animals whose growth was adjusted to that of the alcoholic animals. Figure 5 presents the changes (%) of these levels in rats at the end of the ethanol-treatment and during withdrawal. The significant modifications occurring due to ethanol (Fig. 5a) concern linoleic acid (18:2) and arachidonic acid (20:4) the levels of which decrease. Ten hours after the start of withdrawal (Fig. 5b) the decrease in arachidonic acid was still significant whereas the octadecenoic acids (18:1) increase. After 72 hr of withdrawal (Fig. 5c) only a decrease in 20:4 was observed. The composition of the synaptosome fatty acids of ethanol-treated, and withdrawn animals (10 or 72 hr) showed no significant differences when compared between each other. There is therefore no observable change in the composition during the first 72 hr of withdrawal.

Fatty acid composition of erythrocyte membrane phospholipids during intoxication and withdrawal. Table 2 reports the effect of alcoholic intoxication and food restriction on the fatty acid composition of erythrocyte membrane phospholipids. Variance analysis decomposing the effects shows that only intoxication has an effect on the composition—food restriction does not cause any change. Ethanol affects the levels of the octadecenoic acids (18:1), which increase, and of linoleic acid (18:2) and docosatetraenoic acid (22:4) which decrease.

Figure 6 presents the modification kinetics of erythrocyte membrane fatty acid composition obtained by means of series of blood samples from the same animals. The animals were also used to evaluate the acquisition of tolerance and the intensity of the withdrawal syndrome (Fig. 3). After a week of intoxication by inhalation the phospholipids of the erythro-

Table 1. Levels of cholesterol and phospholipids of the synaptosomes of ethanol-treated, withdrawn, pair-weight and control animals

	Phospholipid (nmol/mg protein)	Cholesterol (nmol/mg protein)	Cholesterol/phospholipid molar ratio
Control	572.1 ± 13.30	223.9 ± 13.60	0.39 ± 0.019
Pair-weight	594.0 ± 12.57	241.0 ± 9.55	0.41 ± 0.012
Ethanol	568.2 ± 6.35	227.4 ± 5.21	0.40 ± 0.007
Withdrawn (10 hr)	581.4 ± 8.23	232.6 ± 7.54	0.40 ± 0.009
Withdrawn (72 hr)	561.0 ± 12.03	223.1 ± 8.67	0.40 ± 0.011

No significant difference was found on comparing the data by means of Student's *t*-test. Means ± S.E. (N = 10).

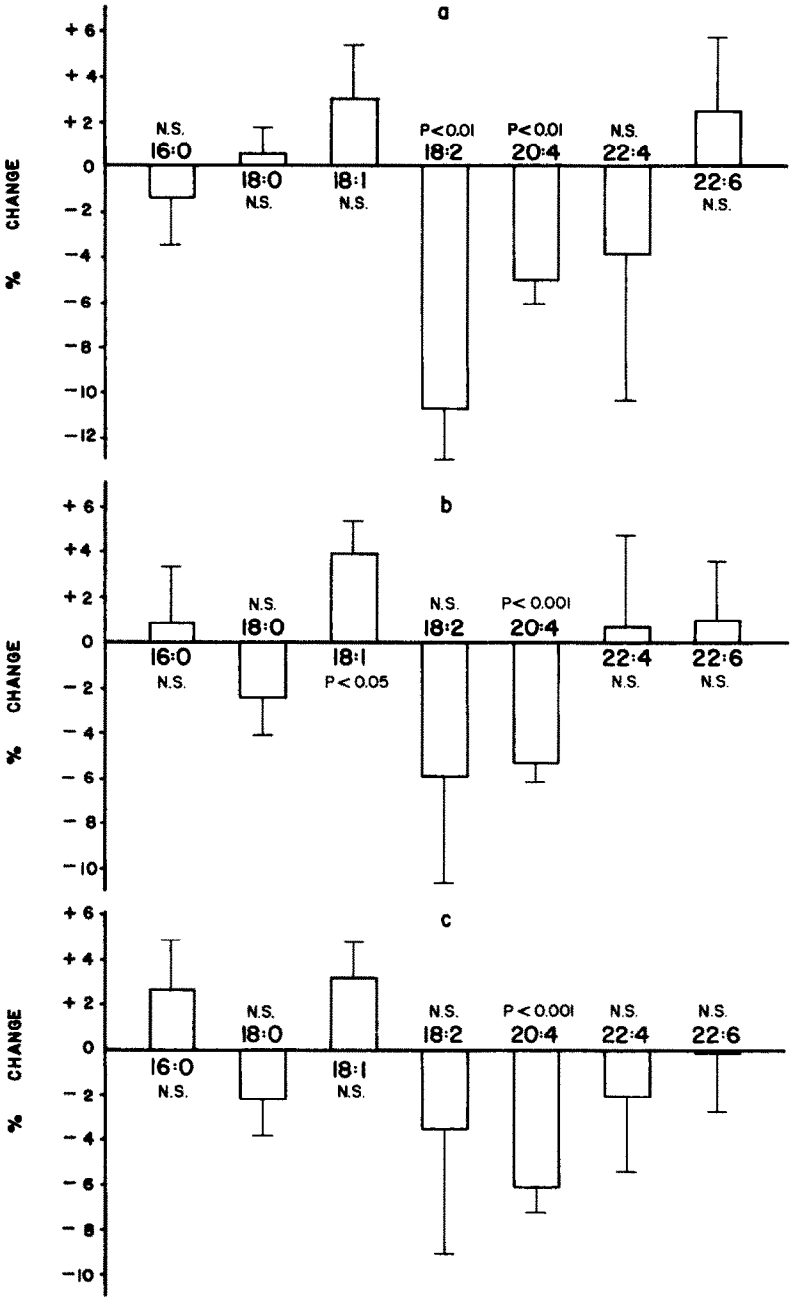

Fig. 5. Per cent changes in fatty acid content of synaptosome phospholipids at the end of the ethanol treatment (a) and 10 hr (b) and 72 hr (c) after withdrawal. The percentage change from the mean experimental value of the pair-weight animals taken as 100% was calculated and the probability of the change being significantly different from zero was tested [9]. Means ± S.E. (N = 10).

cyte membranes already showed modifications in their fatty acid compostion—the level of 18:1 increased and that of 18:2 decreased (Fig. 6a). At the end of the intoxication period this effect was even stronger and in addition the level of 22:4 was greatly decreased (Fig. 6b). When this determination had been carried out, tolerance was evaluated after injection of ethanol at 3 g/kg (see Materials and Methods). The values given in Fig. 6(c) were therefore measured on blood samples taken 10 hr after the animals had woken up: the increased variation in the levels of 18:1 and 18:2 observed could therefore be the result of the recent alcohol overload. From 10 hr withdrawal to 72 hr then 7 days the percentage modification in the levels of 18:1 and 18:2 were seen to decrease: +36.35 ± 8.08 (10 hr)

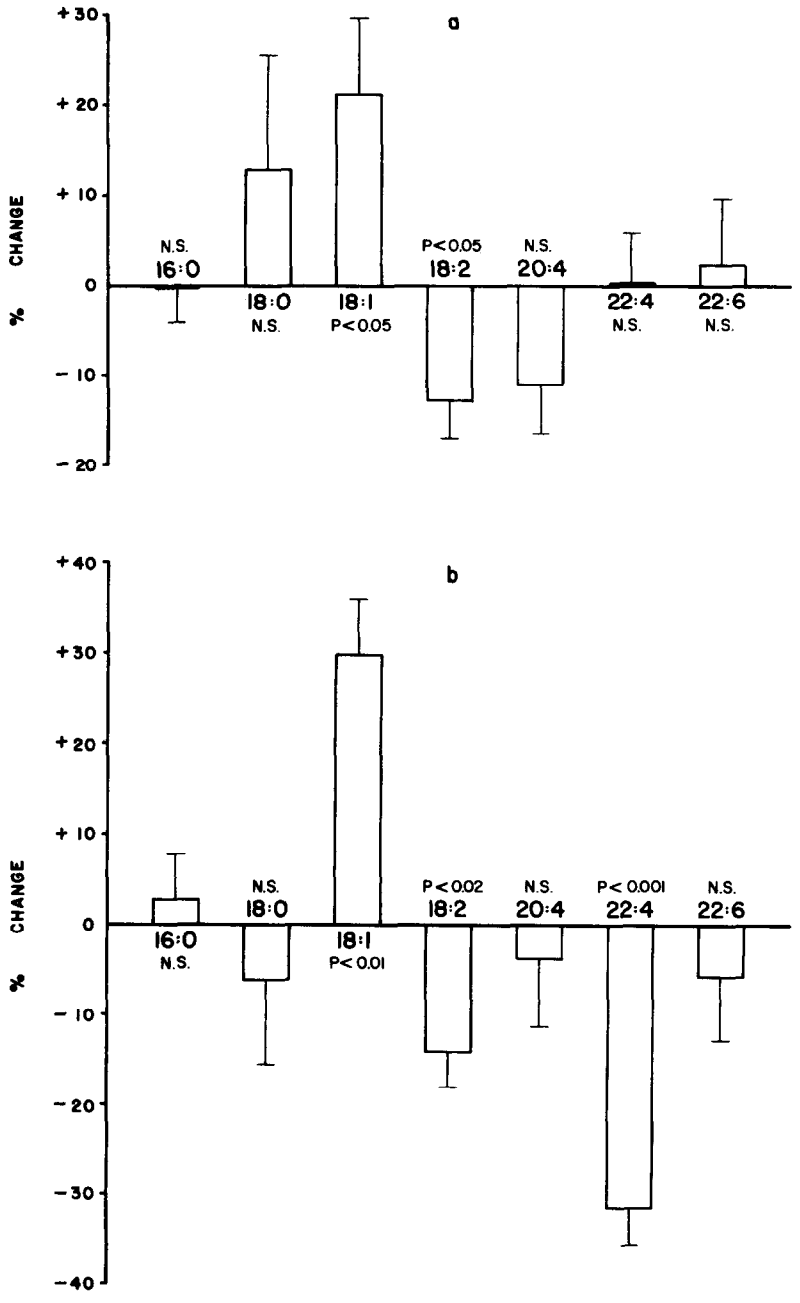

Fig. 6a and b (for caption see p. 622).

to +19.01 ± 6.63 (7 days) for 18:1 (non-significant difference) and −23.02 ± 3.19 (10 hr) to −13.79 ± 1.92 (7 days) for 18:2 ($P < 0.05$).

DISCUSSION

The mechanisms by which the central nervous system becomes resistant to the effects of ethanol administered repetitively or continuously are as yet unclear. The effects of various alcohols on the brain are considered to arise from a direct biophysical effect on the lipid bilayer of the neurone membranes [21]. The biophysical reaction can be compared to the effect of heat, increasing the membrane fluidity. In 1975 Hill and Bangham [22] already suggested that brain neurones could respond to the perturbation induced by ethanol in the same way that microorganisms become adapted to an increase in temperature: the lipid composition of the cell membrane becomes altered in such a way that it becomes intrinsically less fluid and therefore less sensitive to the effect of temperature.

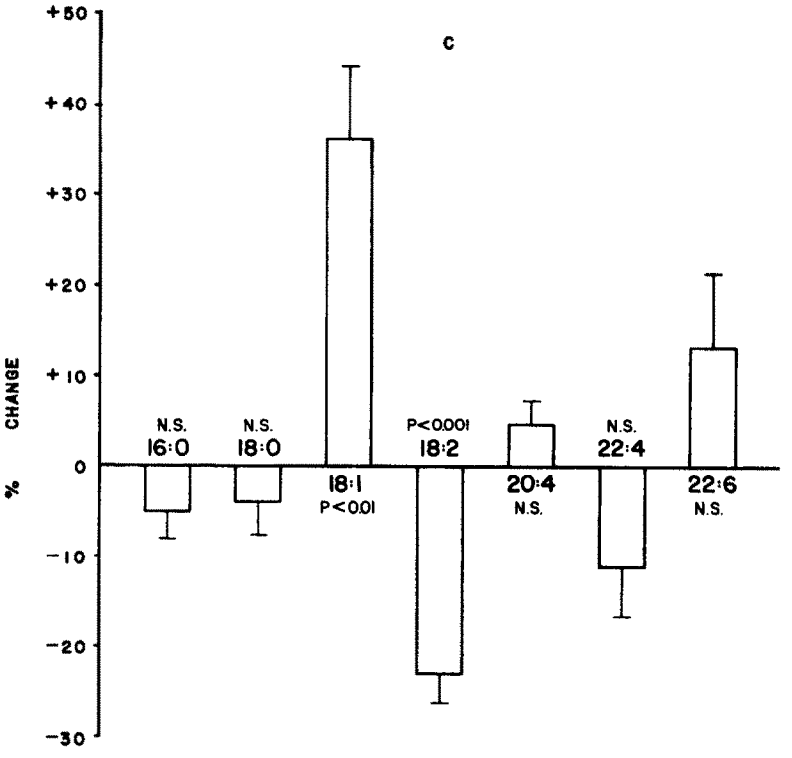

Fig. 6c (for caption see p. 622).

Two types of variation in lipid make-up could explain the cellular adaptation to ethanol. The present work shows that in the Sprague–Dawley rat chronically intoxicated by alcohol inhalation, the phospholipid fatty acid composition of the synaptosome and erythrocyte membranes is disturbed whereas the cholesterol:phospholipid ratio is not modified in the synaptosomes. These results are not in agreement with data found in the literature where the level of cholesterol is increased in mouse erythrocyte and synaptosome membranes [5, 10] and in rat brain microsomes [23]. However, they confirm the modifications in phospholipid fatty acid composition observed in the synaptosomes [6] and other plasma membranes [9] in the mouse. The differences in the results could be explained by the fact that none of the authors mentioned used the same strain or the same species of animal. Another source of variation could arise from the differences in the intensity of intoxication. Wing *et al.* reported the absence of any effect of intoxication by inhalation on the lipid composition of mouse synaptosomes whereas other plasma membranes showed modifications of their phospholipid fatty acid composition [9]. This result can be explained by the weak intoxication of the animals (blood alcohol <1 g/l) and by the difference in sensitivity of the various membranes towards the effect of alcohol. Indeed, in our study the significant percentage changes reached 30 and 15% in the erythrocytes but were only 5 and 10% in the synaptosomes.

The question therefore arises as to whether these modifications were caused directly by the ethanol or were the result of the nutritional state following chronic ethanol administration. Many studies involving chronic alcohol administration to animals have involved adjustment of the caloric intake of controls with carbohydrate to match that of the ethanol-treated group [5, 10]. One disadvantage of administering ethanol by vapor inhalation is that the alcoholic caloric intake of the treated animals cannot be measured conveniently. In our feeding model the diet of the pair-weight rats is not distorted but the food intake is lower in the ethanol group than in the pair-weight group (Fig. 2), the reason for this is the intake of additional ethanol-calories in the treated group. So it is conceivable that changes in fatty acid composition may be the result of food restriction that parallels intoxication. However, we found no influence on the fatty acid composition of the erythrocyte membrane even when food intake had been severely restricted (Table 2). In another study, Wing *et al.* also showed that changes in the relative proportions of the phospholipid fatty acids of erythrocyte membrane in mice were not related to effects of the drug on nutrition [9]. After mice had been exposed to ethanol for two hours, the membrane phospholipids of their brain synaptosomes were shown to contain a decreased content of unsaturated acids compared with control mice [6]. This effect cannot be explained by nutritional changes. It seems, therefore, that the effects of ethanol on membrane com-

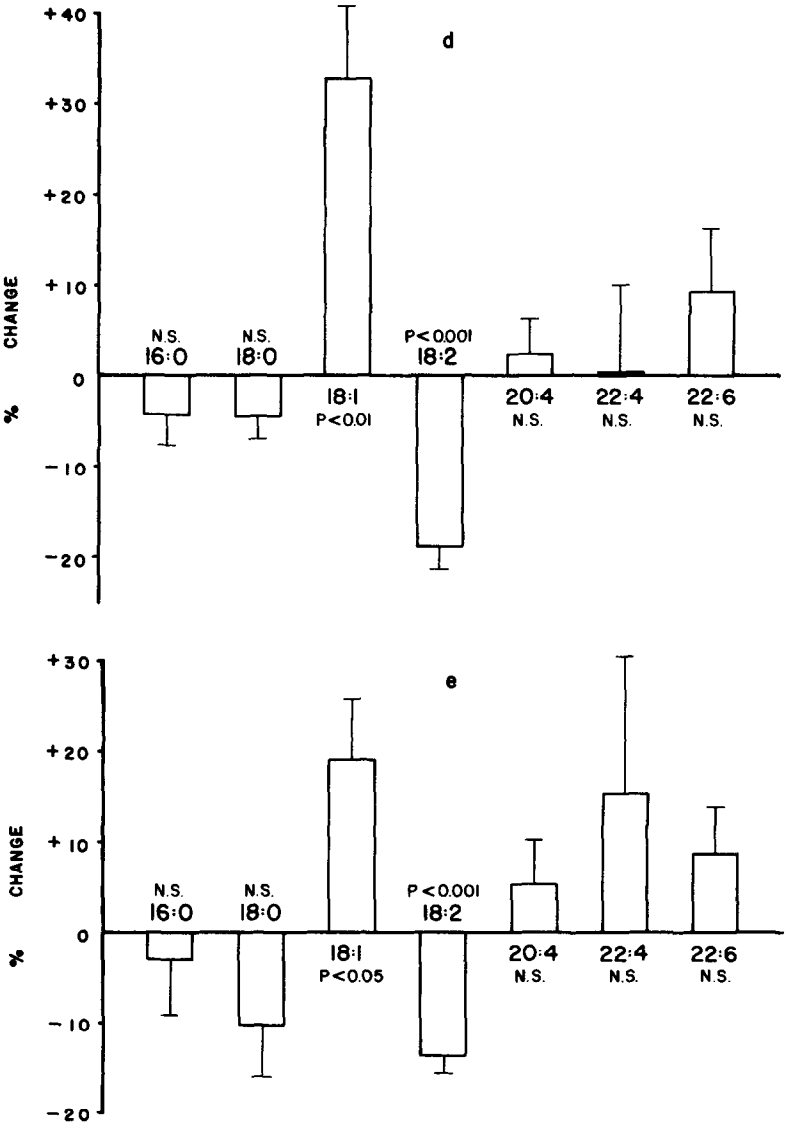

Fig. 6. Per cent changes in fatty acid content of erythrocyte membrane phospholipids after a week (a) and at the end of (b) the ethanol treatment and 10 hr (c), 72 hr (d) and 10 days (e) after withdrawal. Means ± S.E. are given for the per cent changes expressed as in Fig. 5 (N = 6).

position is a result of a direct action of ethanol on the membrane.

After showing the existence of modifications in membrane lipid composition, the problem remaining is to find out the exact functional significance. The decrease of linoleic and arachidonic acid in the synaptosomes and of linoleic acid in the erythrocytes tends to rigidify the membrane and could therefore be responsible for the decrease in fluidity measured after chronic intoxication [1, 2] however, the increase in monounsaturated acids (18:1) would rather tend to fluidify the membrane. The overall effect of these modifications is difficult to evaluate, nevertheless, Waring *et al.* showed that there was a decrease in intrinsic membrane fluidity and an increase in 18:1 as well as other modifications in the fatty acid composition of the main phospholipids isolated from one mitochondria preparation of intoxicated rats [24]. The same team proposed the hypothesis that the decrease in intrinsic fluidity induced by chronic intoxication leads to a reduction in the membrane partitioning of ethanol to brain synaptosomes and hence to tolerance [2]. Our study demonstrates that animals with increased alcohol tolerance after chronic intoxication show modifications of the membrane lipid composition. These modifications are at the origin of functional alterations of the membrane with, for example modifications of the enzyme proteins [25] and receptor accessibility or affinity [26].

The physical dependence which brings about the withdrawal syndrome has been considered by several researchers to be the result of persisting tolerance of

Table 2. Effect of ethanol and food restriction on the fatty acid composition of erythrocyte membrane phospholipids

	Fatty acid					
	16:0	18:0	18:1	18:2	20:4	22:6
Control (C) (N = 7)	34.54 ± 1.073	13.37 ± 1.597	11.40 ± 0.576	11.83 ± 0.544	22.74 ± 0.982	2.22 ± 0.106	3.90 ± 0.190
Pair-weight (P) (N = 7)	36.47 ± 1.465	13.05 ± 0.518	10.40 ± 0.388	11.38 ± 0.416	22.72 ± 0.816	2.07 ± 0.124	3.91 ± 0.158
Ethanol (E) (N = 6)	37.48 ± 1.711	12.23 ± 1.220	13.51 ± 0.632	9.78 ± 0.460	21.88 ± 1.748	1.42 ± 0.087	3.69 ± 0.277
Global F	1.10^{NS}	0.23^{NS}	8.46^{**}	4.80^{*}	0.16^{NS}	14.55^{***}	0.33^{NS}
Ethanol effect							
C + P vs E	NS	NS	↗***	↘**	NS	↘***	NS
Food restriction effect							
C versus P	NS	NS	NS	NS	NS	NS	NS

The experimental data were treated with variance analysis. The table of results presents the means ± S.E., total F with its statistical significance and the orthogonal decomposition of the effects: the ethanol-treated group is compared with the control and the pair-weight group (effect of ethanol), then the latter 2 groups are compared with each other (effect of food restriction).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: not significantly different.

the nervous system in the absence of alcohol [2, 22]. Withdrawal syndrome would therefore be limited in time and intensity by the capacity of the central nervous system to readapt itself when the drug is no longer present: the fact that the lipid modifications remain on removal of the alcohol could explain the abnormal functioning of the membrane whereas in the presence of ethanol the functioning is close to that of a normal membrane [2]. According to this hypothesis the modifications in phospholipid fatty acid composition persist during withdrawal and are attenuated at the same time as the syndrome. Our results show that when the syndrome is at its highest—about 10 hr after the end of intoxication—the lipid modifications of the synaptosomes are present but they are still present after 72 hr of withdrawal when behavioural disturbances were no longer visible. These results are confirmed by the kinetic study of the fatty acid modifications of erythrocyte membrane phospholipids. A week after withdrawal the modifications brought about by 3 weeks of chronic intoxication were still present even though they were less intense than at the end of the intoxication period (Fig. 6). Readaptation of the central nervous system to withdrawal of alcohol therefore does not seem to be directly linked to the return to normal of the membrane lipids: they remain unchanged after the acute phase of withdrawal syndrome has become attenuated.

In conclusion this study demonstrates that chronic alcohol intoxication induces modifications of the phospholipid fatty acid composition in synaptosome and erythrocyte membranes. This suggests that the effect on membrane lipids is a general one, even though the two membranes chosen do not have the same composition nor the same alterations after intoxication.

It is assumed that the synaptosome membranes have an important functional role in the development of behavioural tolerance to ethanol and the results suggest that the membranes become adapted to ethanol through a modification of the relative proportions of fatty acids in the phospholipids thus becoming more tolerant to ethanol. The lipid modifications brought about by the alcohol are durable and the persistance of this effect can be compared with the persistance of tolerance to alcohol which is slow to disappear as shown by behavioural tests [27, 28]. If as suggested by various investigations [29, 30] membrane lipids play a part in the development of tolerance to ethanol, the readaptation of the nervous system on withdrawal of the alcohol occurs more rapidly than the return of the membrane lipids to the normal state.

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