Biochemical Pharmacology, Vol. 33, No. 10, pp. 1625–1632, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

CHANGES IN MEMBRANE LIPID CONTENT AFTER CHRONIC ETHANOL ADMINISTRATION WITH RESPECT TO FATTY ACYL COMPOSITIONS AND PHOSPHOLIPID TYPE

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(*Received* 9 *September* 1983)

Abstract—Changes in the relative proportions of the phospholipid fatty acids of erythrocyte membranes in mice after chronic ethanol treatment (4.5 g/kg, i.p. twice daily for one week) were shown to vary with the differing control profiles observed. It is suggested that certain changes in membrane lipid composition after ethanol administration may not be interpreted simply in terms of an adaptation to a disordering effect of the drug. The fatty acid changes were, in addition, distributed asymmetrically within the individual phospholipid classes. Depending on the control profile, the effects varied from being mainly in phosphatidylethanolamine (PE; 80%) and phosphatidylserine–inositol (PS + PI; 10%), phospholipids primarily located on the inner half of the membrane bilayer, to being more evenly distributed between PE and phosphatidylcholine (PC) and probably, therefore, between the two halves of the bilayer. Changes in the monounsaturated acid remained primarily with PE, suggesting a specific functional role for this species. The remaining results are discussed in the light of possible effects on cell morphology and their potentially similar consequences of increasing cell volume.

In recent years evidence has accumulated showing that functional effects of both acute and chronic ethanol administration may be exerted at the level of cell membranes. It was proposed by Hill and Bangham [1] that tolerance to general depressants such as ethanol could result from alterations in membrane lipids in response to the disordering influence of the drugs. Using an electron paramagnetic resonance technique, Chin and Goldstein [2] showed that ethanol did, in fact, disorder brain and erythrocyte membranes. When such membranes were isolated from mice that had been chronically treated with ethanol, they showed resistance to the disordering effects of the drug *in vitro* [3]. The hypothesis that chronic ethanol exposure can induce adaptive changes in membranes towards an increased rigidity was further supported by studies with rat-liver-mitochondrial membranes [4].

It has been suggested that these physicochemical effects can, indeed, be explained by changes in membrane lipid composition. Thus with synaptosomal membranes from ethanol-tolerant mice, the effects were located in the lipid extracts [5]. In addition, Chin *et al.* [6] reported an increased cholesterol content of synaptosomal and erythrocyte membranes from mice chronically treated with ethanol. Littleton and John [7] have shown an increase in the relative saturation of fatty acids in synaptosomal membranes after chronic exposure of mice to the drug. Both these types of change are consistent in direction with an adaptation to a more ordered membrane.

However, not all the reported changes in membrane lipid composition *in vivo* after chronic ethanol exposure are consistent with this simple hypothesis. Thus Sun and Sun [8] reported a proportionate increase in polyunsaturated fatty acids in synaptosomal membranes from guinea-pig brain. Wing *et al.* [9] found that changes in membrane fatty acyl composition after chronic ethanol treatment in mice varied with membrane type. Moreover, in mice selectively bred for differences in ethanol sensitivity, no differences in synaptic membrane phospholipid, fatty acid or cholesterol composition were observed [10].

Using the erythrocyte membrane as a model, the present study indicates the importance of the prevailing fatty acyl composition of the membrane phospholipid in relation to the resulting changes induced by ethanol, whether they may appear to be towards a more fluid or a more rigid structure. The distribution of the changes within individual groups of phospholipids has also been studied, and the implications for common functional effects are discussed.

MATERIALS AND METHODS

Mice, Charles River CD-1, males 24–29 g, were maintained on Charles River diet no. 22RF with free access to water. Lighting was provided from 07.30 hr to 19.30 hr and the animal room temp was 22°.

Ethanol was administered by i.p. injection twice daily at 09.00 hr and 18.00 hr with 20% (w/v) ethanol in saline (0.85% w/v aq. NaCl) at 4.5 g ethanol/kg body wt for 7 days [11]. Controls were injected with saline containing glucose equicaloric to the ethanol dose and were 'pair-fed' so as to parallel the food intake of the ethanol-treated mice.

Preparation of membranes. On the eighth morning, without further injection, mice were killed by cervical dislocation and bled from the neck. Blood was collected in heparinized tubes at 4°. The method

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of Hanahan and Ekholm [12] was used for isolation of erythrocyte membranes as described earlier [9] and their purity was checked by electron microscopy and acetylcholinesterase activity. A portion of the membrane preparation was kept for assay of protein content by the method of Lowry *et al.* [13]. The remainder, usually 80% of the total preparation, was extracted for lipid analysis.

Lipid extractions and estimations. After initial uptake in methanol and two 40 sec periods of sonication in a standard Sonor device, membrane lipids were extracted into 2:1 (v:v) chloroform:methanol [14]. Lipids were then separated by silicic acid column chromatography [15] into neutral lipid (chloroform eluate), glycolipid (acetone eluate) and phospholipid (methanol eluate) fractions. Phospholipid was assayed by measurement of the phosphorus content according to the method of Morrison [16]. After alkaline hydrolysis of one portion of the phospholipid, the constituent fatty acids were methylated and assayed by gas chromatography using a 2 m × 2 mm glass column of 10% SP-2330 on 100/120 Chromosorb WAW (Supelco Inc., PA, U.S.A.) as described earlier [9]. Cholesterol from the neutral lipid fraction was also assayed by gas chromatography using a 2 m × 2 mm glass column of 3% SE-30 on 100/120 Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA, U.S.A.) as described earlier [9]. Peak areas in both assays were measured using a 3390A Hewlett-Packard Recording Integrator.

The remaining portion of phospholipid was subjected to TLC. In this procedure, plates were coated with a 0.5 mm layer of 4:1 (w:w) silica gel HF_{254} (av. 15 μm): silica gel G (10–40 μm) (E. Merck, Darmstadt, F.R.G.). Plates, pre-washed in the solvent mixture (see below), were stored in a desiccator. After activation at 100°, approximately 100 μg phospholipid were applied as a streak in 60 μl 2:1 (v:v) methanol:chloroform. Plates were then returned to the desiccator for 15 min before being developed in a solvent mixture of chloroform: methanol:glacial acetic acid:water in the ratio 25:15:4:2 (v:v:v:v) [17]. Standard phospholipids and additional sample spots, which were run in parallel with the main sample, were visualised independently using iodine vapour contained within a perspex restriction box. Because of similar *R*f values for phosphatidylserine (PS) and phosphatidylinositol (PI), and the relatively small amounts of each present in the membrane samples, these phospholipids were combined. The regions occupied by phosphatidylethanolamine (PE), PS + PI, phosphatidylcholine (PC) and sphingomyelin (SM) were scraped clean after identification of their positions from the *R*f values of the visualised reference and sample spots. Phospholipids were eluted from the gel with the solvent mixture by shaking at 40° for 20 min. This was repeated once. Eicosadienoic acid was added as an internal standard at this stage. The phospholipid samples were then hydrolysed and the fatty acids obtained were methylated for assay by gas chromatography as described earlier [9] and above.

Chemicals. All reagents were of 'AR' grade and obtained from B.D.H. Ltd. (Poole, U.K.) and Sigma London Chemical Co. (London, U.K.). Organic solvents were doubly distilled before use.

Statistics. Either the significance of the difference

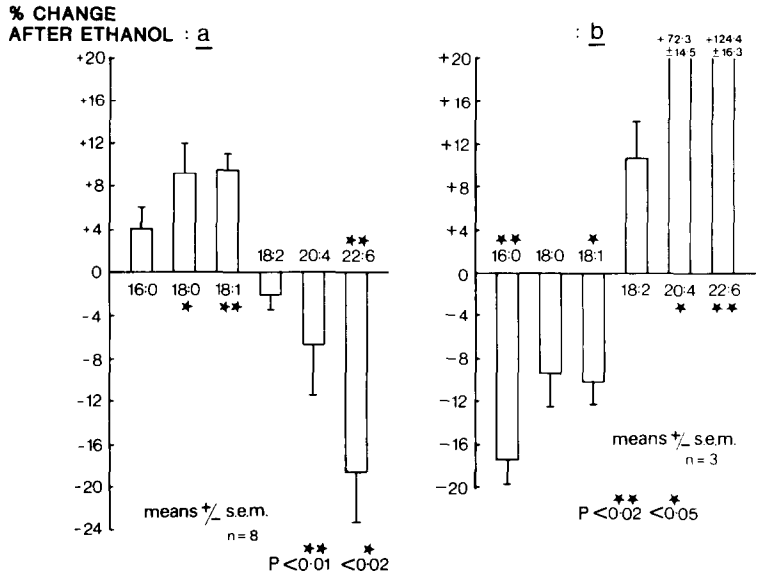

Fig. 1. Different patterns, 'a' and 'b', of percent change in total fatty acid content of erythrocyte membrane phospholipid after chronic ethanol treatment. Pattern 'a' depicts significant increase(s) among 16:0, 18:0 and 18:1 acids and significant decrease(s) among 18:2, 20:4 and 22:6. Pattern 'b' depicts significant reversals of the changes in 'a'. Mean values ± S.E.M. are given for the percent changes (expressed as a percentage of each control fatty acid value taken as 100%). 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid. P refers to the probability of the change not being significantly different from zero. n = 8 groups of 5–8 mice for 'a'; 3 groups of 8 mice for 'b'.

between two means was tested by Student's *t*-test or the probability of a change being significantly different from zero was tested according to the Null Hypothesis. For Fig. 2 a variance ratio test was performed. A value for $P < 0.05$ was taken as significant.

RESULTS

Total phospholipid. Changes in the major fatty acids of the erythrocyte membrane phospholipids after chronic ethanol administration are shown in Fig. 1. The changes are expressed as a percentage of each control fatty acid value, taken as 100%. The results obtained in early studies shown in 'a' (Fig. 1) indicate significant increases in stearic and oleic acids, and a significant decrease in docosahexaenoic acid. Results of this type have also been reported in an earlier publication [9]. In studies more than a year later, using the same strain of mice and identical experimental conditions, a reversal of this effect was observed and is shown in 'b' (Fig. 1). These results show a significant decrease in both palmitic and oleic acids and a significant increase in both arachidonic and docosahexaenoic acids.

Analysis of these discrepant results showed that there were distinguishing patterns in the phospholipid fatty acid composition of the control membranes between types 'a' and 'b'. These control profiles are shown as percentage compositions in Table 1 and were significantly different from each other. Mean quantitative values are also given. The differences in % composition between 'a' and 'b' controls represent larger effects (e.g. if calculated as in Fig. 1 for comparison) than those produced by ethanol itself. It can be seen, therefore, in Fig. 2 that a qualitatively smaller standard deviation of the values is obtained with each fatty acid from ethanol-treated mice. This represents a significant effect overall. It was observed, in addition, that when no significant changes in fatty acid composition were seen after ethanol treatment, the control membranes showed profiles that were intermediate in nature to those of 'a' and 'b'. In these experiments a range of % changes (relative to 100% for the control fatty acids) from -3.9 ± 2.2 (4) for palmitic acid to $+4.6 \pm 1.8$ (4) for stearic acid (means ± S.E.M.; *n* groups) were observed, and the compositional profiles are also included in Fig. 2.

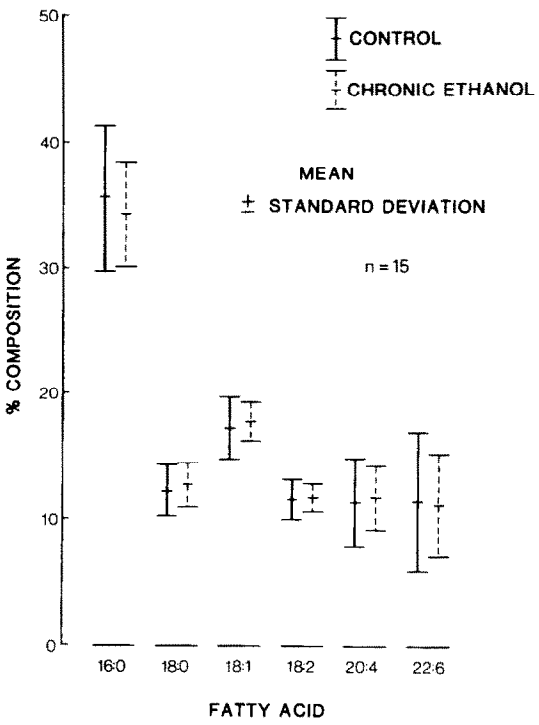

Fig. 2. Mean values and S.D. of percent composition of total fatty acid content of erythrocyte membrane phospholipid from all types of control mice and all types of mice chronically treated with ethanol. Assuming an equal chance for a larger or smaller S.D., the repetition of a qualitatively smaller standard deviation with ethanol-treated mice for all six fatty acids represents a significant effect. Comparison of the variance between ethanol and control was not significantly different for each individual fatty acid. $n = 15$ groups of 5–8 mice for both test and control.

No differences in amounts of cholesterol and phospholipid in the erythrocyte membranes of this study have been observed. A period of approximately 30 months elapsed from the initiation of experiments of type 'a' to the reported accumulation of results of type 'b'. Reasons for the shift in the fatty acid profile of the control membranes during this period are unknown at present. Checks on the strain, age and sex of the mice, fatty acid content of the diet, environmental conditions and seasonal

Table 1. Comparison of compositions of total fatty acid content of erythrocyte membrane phospholipid from control mice in experiments of type 'a' and of type 'b' (Fig. 1)

Fatty acid	% Composition Type 'a'	% Composition Type 'b'	P 'a' vs 'b'	Mean content as μg fatty acid/mg membrane protein Type 'a'	Mean content as μg fatty acid/mg membrane protein Type 'b'
16:0	35.5 ± 1.6	44.4 ± 2.5	<0.05	80.1	106.6
18:0	12.1 ± 0.6	15.4 ± 0.8	<0.02	27.6	37.1
18:1	16.4 ± 0.9	20.8 ± 0.9	<0.02	37.0	50.0
18:2	12.2 ± 0.6	9.5 ± 0.4	<0.02	26.6	23.1
20:4	13.1 ± 1.3	5.9 ± 1.6	<0.02	28.1	14.8
22:6	10.7 ± 1.4	3.7 ± 2.0	<0.05	21.6	9.2

Mean values ± S.E.M. are shown for percentage composition; $n = 5$ groups of 5–8 mice in 'a' and 3 groups of 8 mice in 'b'.

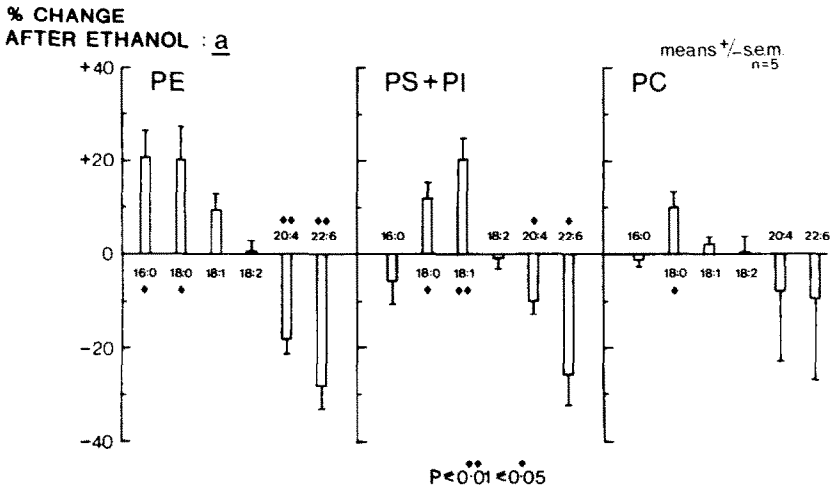

Fig. 3. Percent changes in fatty acid content of the major phospholipids from erythrocyte membranes after chronic ethanol treatment: type a. Mean values ± S.E.M. are given for the percent changes (expressed as a percentage of each control fatty acid value taken as 100%). PE = phosphatidylethanolamine, PS + PI = combined phosphatidylserine and phosphatidylinositol, PC = phosphatidylcholine. $n = 5$ groups of 8 mice. P refers to the probability of the change not being significantly different from zero.

variations have eliminated these factors as possibilities. Furthermore, no interference from methodological artifacts was apparent over the period of study.

Phospholipid classes. Figure 3 shows the percentage changes (relative to 100% for each control fatty acid) found after the ethanol treatment in membranes of type 'a' for PE, PS + PI and PC. It can be seen that most of the significant changes occurred in the PE and PS + PI fractions, and that they accounted for the effect in total phospholipid seen in Fig. 1a. Figure 4 shows the corresponding results for the phospholipids from membranes of type 'b'. Despite the reversal of direction, the most significant changes in fatty acid composition were again found in PE, but, with few exceptions, the direction and

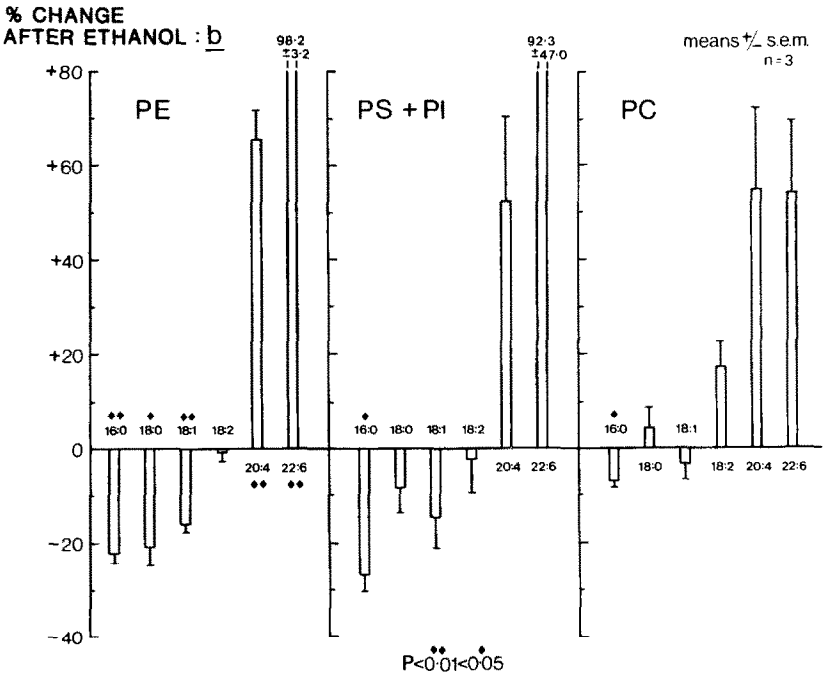

Fig. 4. Percent changes in fatty acid content of the major phospholipids from erythrocyte membranes after chronic ethanol treatment: type b. For details, see legend to Fig. 1. $n = 3$ groups of 8 mice.

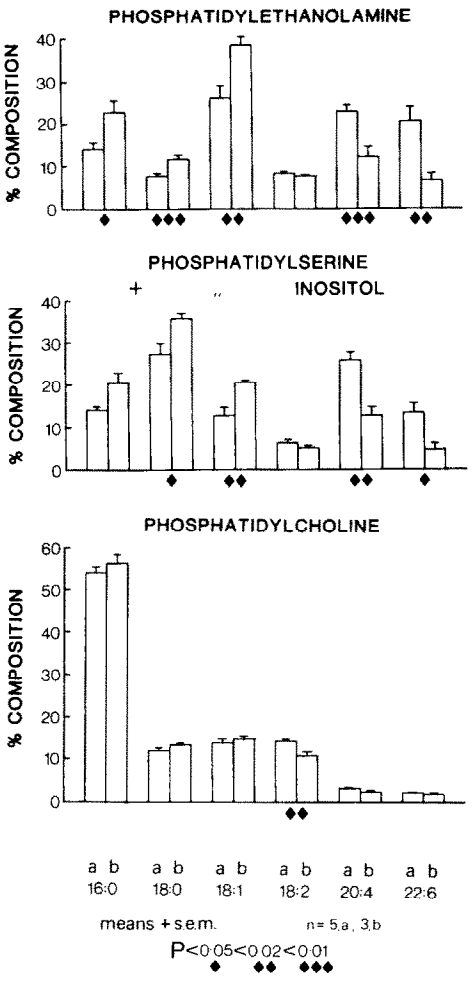

Fig. 5. Individual phospholipid classes: a comparison of their percentage fatty acid composition between erythrocyte membranes of types 'a' and 'b' from control mice. Mean values + S.E.M. are given; $n = 5$ groups of 8 mice in 'a' and 3 groups of 8 mice in 'b'. P refers to the significance of difference between 'a' and 'b'. Studies of relative percent composition are subject to smaller errors than ones of absolute quantitative measurement. Differences are therefore more highly significant in Fig. 5 than in Table 2.

pattern of the changes for all three phospholipid classes showed consistency with the pattern of change seen in the total phospholipid in Fig. 1b.

Figure 5 indicates the % fatty acyl compositions of the individual phospholipids of control membranes of both type 'a' and 'b'. Quantities of fatty acid involved are given in Table 2. It can be seen that oleic acid was the most abundant fatty acid in PE, stearic (and arachidonic) acid was dominant in the PS + PI fraction and palmitic acid in PC. PE and PS + PI accommodated the changes in polyunsaturated fatty acid composition that were seen in total phospholipid between control membranes of types 'a' and 'b' in Table 1. Differences in the overall content of saturated and monounsaturated fatty acids between 'a' and 'b' controls seemed to be distributed throughout the individual phospholipid groups.

The mean changes in total amounts of fatty acid caused by ethanol administration are shown in Fig. 6 for membranes of types 'a' and 'b'. The mean % contribution to these changes by the separate phospholipids is also given. In membranes of type 'a' the increases in the amounts of saturated and monounsaturated fatty acids occurred mainly in PE and to a lesser extent in PS + PI and in PC. A similar contribution by these phospholipids was seen with the decrease in content of polyunsaturated acids. In contrast, the decrease in saturated fatty acid content observed in membranes of type 'b' was more evenly distributed between the two main phospholipid groups, PE and PC, with approx. 20% again attributable to PS + PI (Fig. 6). A similar pattern was observed with the distribution of the increase in polyunsaturated fatty acids in 'b', since these were also mainly in PE and PC. The change in the monounsaturated fatty acid content, however, despite being in the opposite direction to that in 'a', occurred mainly in PE as it did in membranes of type 'a'.

No significant quantities of fatty acid were obtained from any sphingomyelin fraction under the conditions of hydrolysis used in this study.

DISCUSSION

Total phospholipid

The experimental conditions used in this study were identical to those of an earlier report [9] in

Table 2. Quantitative fatty acid content of the major phospholipids from control erythrocyte membranes in experiments of type 'a' and of type 'b'.

Fatty acid	μg fatty acid/mg membrane protein					
	'a'			'b'		
	PE	PS + PI	PC	PE	PS + PI	PC
16:0	9.4 ± 2.7	4.0 ± 1.0	71.1 ± 18.5	14.4 ± 6.7	6.2 ± 1.7	100.7 ± 28.3
18:0	5.1 ± 1.4	7.9 ± 1.8	16.0 ± 4.8	7.6 ± 3.4	10.8 ± 2.5	23.7 ± 6.6
18:1	17.3 ± 4.1	3.7 ± 1.1	18.3 ± 5.4	24.1 ± 9.1	6.3 ± 1.3	26.7 ± 7.2
18:2	5.5 ± 0.8	1.9 ± 0.3	19.1 ± 3.0	4.8 ± 2.0	1.6 ± 0.3	19.5 ± 5.0
20:4	15.1 ± 2.2	7.5 ± 0.9†	4.2 ± 1.3	7.9 ± 3.0	4.0 ± 0.9	4.4 ± 1.5
22:6	13.7 ± 2.4*	3.8 ± 0.6†	3.0 ± 0.7	4.2 ± 1.8	1.5 ± 0.7	3.3 ± 1.6

* $P < 0.02$, † $P < 0.05$: significantly different from 'b'.
Mean values ± S.E.M. are given. $n = 5$ groups of 8 mice in 'a' and 3 groups of 8 mice in 'b'.

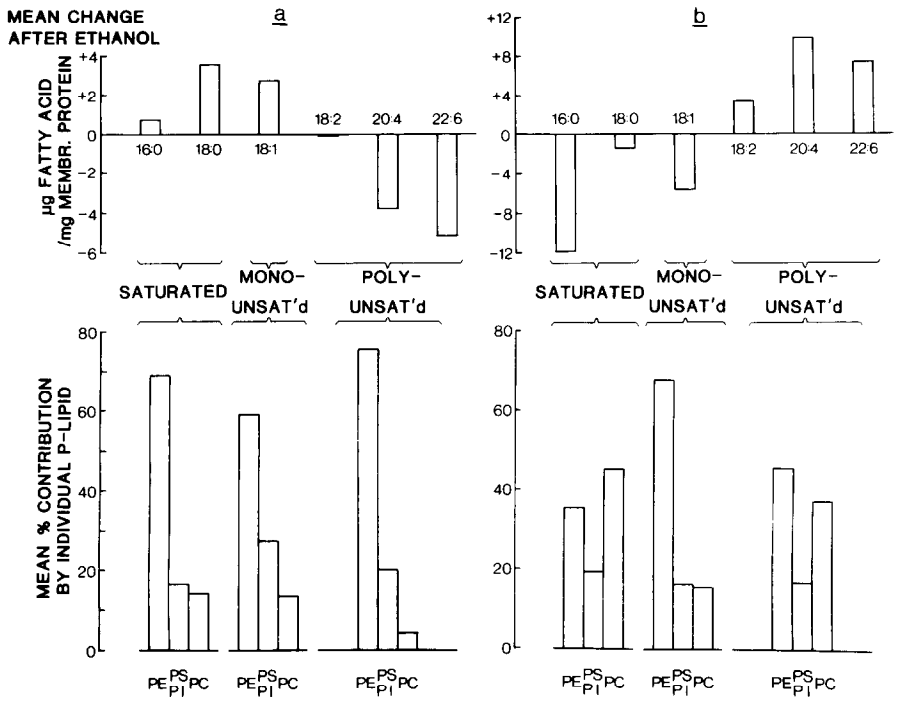

Fig. 6. Quantitative changes in fatty acid content of erythrocyte membranes of type 'a' and 'b' after chronic ethanol administration and their distribution within the major phospholipids. Mean values: n = 5 groups of 8 mice for 'a'; 3 groups of 8 mice for 'b'. Abbreviations as in Figs. 1 and 3.

which it was concluded that the observed changes in phospholipid fatty acid composition of the erythrocyte membranes were due to direct effects of ethanol. Thus effects of (a) stress of the injection routine, (b) equicaloric glucose substitution for ethanol and the diet restriction of pair-feeding in control mice, and (c) hypothermia caused by ethanol, were all eliminated as factors that could influence the significance of the results.

Under these experimental conditions the mice showed development of functional tolerance to ethanol [9]. The present results showed that, in this state, the relative concentration of the fatty acyl groups of the erythrocyte membrane phospholipids changed as a result of the drug treatment, but that the direction of the change appeared to relate to the control fatty acyl composition. The earlier observation [9] that different membrane types, varying in structure and function, responded differently to ethanol administration, has therefore now been extended to include a different response within the same type of membrane. The earlier results with the erythrocyte membrane reported in [9] are therefore related to the compositional profile (of type 'a') given at that time.

As shown in Fig. 2, the overall variability of composition of the fatty acids in the ethanol-treated membranes from all experiments was less than in the controls. Thus in the presence of ethanol, the fatty acyl groups of the membranes tended to change towards a similar composition irrespective of its variance in the absence of ethanol.

It is clear from the present results that total changes in the fatty acid composition of erythrocyte membrane phospholipids do not by themselves consistently support the hypothesis of membrane-lipid adaptation occurring to offset a disordering effect of ethanol *in vivo*. The nature of the fatty acid changes reported in a variety of membranes by other workers have also shown mixed responses to ethanol treatment [4, 8, 18, 19]. Varied responses were most common for palmitic and stearic acids and for oleic and linoleic acids, but from the data it is not possible to assess whether the changes related to initial fatty acid concentrations. It would clearly be of interest to determine whether ethanol would have similar effects on erythrocytes whose membrane fatty acid compositions had been deliberately manipulated *in vivo*. There is suggestive evidence that dietary fat, for example, can influence responses of mouse brain membranes to ethanol [20].

It may be that with such a large population of individual molecular species of phospholipids in membranes the overall level of saturation:unsaturation is not a sufficiently specific index. Where ethanol administration has generated membranes with altered cholesterol contents, however, increases have consistently been observed [6, 21, 22].

Phospholipid classes. The results of this study show that the changes in total fatty acyl composition of erythrocyte membrane phospholipid are not distributed proportionately throughout the main phospholipid groups. Furthermore, the distribution of the changes differs with the contrasting effects of ethanol administration.

In erythrocyte membranes, PC, together with SM,

are the dominant phospholipids of the outer half of the bilayer [23], while PE, PS and PI are mainly located on the inner half of the membrane. Mechanisms producing compositional changes differ on each side of the bilayer. Processes of whole phospholipid exchange are favoured for PC on the outside of the membrane [23] but fatty acid acylation mechanisms are favoured on the inner half of the bilayer [24]. Despite differences in mechanisms of fatty acyl modification and in location of the phospholipid, the total fatty acid pattern of its species is similar whether located on the outer or the inner half of the bilayer [24]. It may be assumed, then, that observed fatty acid compositional changes for a particular phospholipid will be distributed between the two halves of the bilayer in direct proportion to the bilayer distribution of the phospholipid itself.

Consideration of the observed fatty acyl changes in the context of membrane bilayer fluidity revealed, in the present study, complementary and therefore dominating changes in the saturated and polyunsaturated fatty acids, the one increasing in amount while the other decreased. In the situation in which the content of the saturated acids increased and the polyunsaturated acids decreased, 90% of the changes were found in PE and PS + PI, the phospholipids that are mainly located on the inner half of the bilayer. This suggests that a more ordered inner half of the membrane might be anticipated with, in consequence, a reduced area. The morphological result of this could be a change in erythrocyte shape from biconcave disc to a more spherical form [25, 26] with an increase in cell volume. This is supported both quantitatively and qualitatively from *in vitro* studies in which the lipid composition of erythrocyte membranes has been manipulated and gross morphological changes observed [27–29].

Where a decrease in the amount of saturated fatty acids and an increase in polyunsaturated acids were observed in the present study, the changes were more evenly distributed between the main phospholipids, PE and PC. It may be assumed, therefore, that they were occurring in both halves of the bilayer. This could perhaps result in a more disordered membrane generally with increased potential for accommodating cells of increased volume.

In the context of membrane fluidity, the changes in the monounsaturated, oleic acid were opposite in direction to complementing the saturated–polyunsaturated acid changes. Moreover, irrespective of the direction of the change, the effects were mainly confined to PE. Several reports indicate that membrane metabolism of this phospholipid has distinguishing features. Thus it specifically showed a calcium-dependent stimulation of fatty acid incorporation in human erythrocytes [30] and recent evidence suggests some heterogeneity of its metabolism in the red cell membrane [31]. Furthermore, methylation of PE, a process that may be coupled to receptor activation and calcium transport [32], has been shown to be sensitive to ethanol in synaptosomal membranes [33].

The particular sensitivity of monounsaturated fatty acyl groups of PE to ethanol administration suggests that these species may have a pivotal role in the control of membrane function. It has been shown in NMR studies that unsaturated species of PE most readily adopt non-bilayer (hexagonal) phases in model membranes [34] and it was suggested that such phases may be important in transport processes across the membrane and in membrane fusion phenomena [34]. It may be that levels of monounsaturated species of PE are finely controlled in relation to polyunsaturated species, the changes being opposite in direction in this study, in order to maintain optimum activities of these important membrane functions. From the present data it is not possible to speculate in this context on the net functional consequences of the ethanol treatment. However, in brain tissue and isolated synaptosomal membranes from ethanol-tolerant rats, altered Ca^{2+}-sensitivities to a number of membrane-associated activities, including neurotransmitter release [35], have been reported. Since such a process involves membrane fusion, it is of interest that the formation of the non-bilayer phase by unsaturated species of PE has been shown to be sensitive to Ca^{2+} and ethanol [36]. Clearly, studies are required to investigate the possibility of such mechanisms occurring in the synaptic membrane and to delineate their functional significance in the erythrocyte membrane after ethanol tolerance development.

For membranes with total fatty acid profiles intermediate to the types discussed above, little, if any change in those profiles occurred as a result of the ethanol treatment. This did not, however, eliminate the possibility of changes also occurring within their individual phospholipid groups and of associated functional effects.

Summary. In conclusion, chronic ethanol administration has been shown to cause changes in the total fatty acyl composition of erythrocyte membrane phospholipids. The direction of the change varied with the control membrane profile towards a more consistent composition in the presence of ethanol. It is not known, however, whether the changes were directly dependent on the profile or on a related factor. The results may help to explain why some reported changes in membrane lipid composition after ethanol administration cannot be interpreted simply in terms of an adaptation to a disordering effect of the drug.

The changes have been found to be distributed asymmetrically within the individual phospholipid classes of the erythrocyte membrane. The varied asymmetry of distribution of divergent changes may result in similar functional effects, such as the suggested increases in cell size. An increase in mean corpuscular volume is, in fact, a common diagnostic test in human alcoholism [37], though it is appreciated that many other factors may contribute to this disorder in alcoholics. It is further suggested that monounsaturated species of PE may have specific functional roles that warrant more detailed studies with membranes from the CNS in investigations of tolerance development.

Acknowledgements—The authors are indebted to Miss Janet Hughes for expert technical assistance and to Mr. Rod Prior of the Dyson Perrins Laboratory, Oxford University for preparation of the TLC plates. The study was supported by a grant from the Medical Research Council.

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