Biochem. J. (1991) 274, 565–573 (Printed in Great Britain)

Maintenance of structural and functional characteristics of skeletal-muscle mitochondria and sarcoplasmic-reticular membranes after chronic ethanol treatment

Francesc CARDELLACH,* Theodore F. TARASCHI, John S. ELLINGSON, Christopher D. STUBBS, Emanuel RUBIN and Jan B. HOEK†

Department of Pathology and Cell Biology, Jefferson Medical College, Philadelphia, PA 19107, U.S.A.

The effect of long-term ethanol intake on the structural and functional characteristics of rat skeletal-muscle mitochondria and sarcoplasmic reticulum was investigated. Functionally, skeletal-muscle mitochondria were characterized by a high respiratory control index and ADP/O ratio and a high State-3 respiration rate with different substrates. These parameters were not significantly different in preparations from control and ethanol-fed rats, except for a small increase in the rate of oxidation of α-oxoglutarate/malate in the latter. In submitochondrial particles from the two groups of animals there was no significant difference in cytochrome content, ATPase activity or the activity of respiratory-chain complexes. Mitochondrial membranes from untreated and ethanol-fed rats showed no difference in the baseline e.s.r. order parameter, and both preparations were equally sensitive to disordering by ethanol *in vitro*. Similarly, sarcoplasmic-reticulum preparations were not significantly affected by long-term ethanol feeding with respect to Ca^{2+}-ATPase activity or in baseline order parameter and susceptibility to membrane disordering by ethanol *in vitro*. These membranes were also equally sensitive to degradation by exogenous phospholipase A_2. Ethanol feeding did not alter the class composition of mitochondrial or sarcoplasmic-reticulum membrane phospholipids, nor the acyl composition of individual phospholipid classes. Specifically, the changes in acyl composition that characteristically occur in liver microsomal phosphatidylinositol and liver mitochondrial cardiolipin were not observed in the corresponding phospholipids from skeletal-muscle membranes. In experiments where membrane preparations from liver and skeletal muscle from the same ethanol-fed animals were compared, the liver membranes developed membrane tolerance, with the muscle membranes retaining normal sensitivity to disordering effects by ethanol. It is concluded that: (*a*) different tissues from the same animals differ in their susceptibility to ethanol; (*b*) the tissue-specific lack of development of membrane tolerance correlates with a lack of chemical changes in the phospholipids and with a retention of normal function of mitochondria and sarcoplasmic reticulum; (*c*) effects of chronic ethanol intake on muscle function are not due to a defect in the mitochondrial energy supply.

INTRODUCTION

Chronic alcoholism is not infrequently associated with degenerative conditions of skeletal and cardiac muscle (alcoholic myopathy and cardiomyopathy respectively). The effects of ethanol are dose-related, and occur in patients who show no evidence of malnutrition or electrolyte imbalance [1]. It has been suggested [2–4] that these functional deficiencies of muscle may be due to structural and functional defects in the mitochondria and sarcoplasmic reticulum.

In liver, chronic ethanol feeding affects mitochondrial oxidative phosphorylation, causing a 30–50% decrease in functionally competent cytochrome oxidase and NADH dehydrogenase and also affecting the mitochondrial ATPase activity [5–7]. Morphologically, the mitochondria in the liver of ethanol-fed animals appear enlarged and swollen and have a distended matrix [8–11]. Dilation of the endoplasmic reticulum in rat liver has also been observed after chronic ethanol feeding [9,10], accompanied by changes in the activity of Ca^{2+} transport in microsomal preparations isolated from chronically ethanol-fed rats [12]. These functional defects are accompanied by alterations in the chemical and physical characteristics of intracellular membranes of liver, which appear to be associated primarily with the acidic phospholipid fraction [13–15].

Morphological studies of skeletal muscle in human volunteers [16,17] and in experimental animals [3] indicate that long-term ethanol feeding may induce changes in intracellular membranes that are qualitatively similar to, but less pronounced than, those found in liver. However, functional and structural studies on isolated organelles from skeletal muscle are scarce and inconsistent. Ohnishi and co-workers [18] reported that skeletal-muscle sarcoplasmic reticulum from ethanol-fed rats had a lower order parameter (was more 'fluid') and was less sensitive to the membrane-disordering effects of ethanol than were control preparations. These changes were accompanied by an increase in the passive permeability of these membranes to Ca^{2+} [19]. The results of studies on the functional characteristics of isolated skeletal-muscle mitochondria from ethanol-fed animals are variable. One study found that long-term ethanol feeding significantly decreased the respiratory control index of isolated mitochondria from sedentary rats, but not of mitochondria from animals that were exercised [20]; another study reported a small decrease in the State-3 rate of respiration of rat and baboon skeletal-muscle mitochondria, but no change in the respiratory control index or

Abbreviations used: DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; DCPIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulphate; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine; SA*n*, *n*-[β-(4',4'-dimethyloxazolidinyl-*N*-oxyl)]stearic acid, where $n = 5, 7, 12$; NBD, *N*-(4-nitrobenzo-2-oxa-1,3,-diazole); C6-NBD-PC, 1-palmitoyl-2-*N*-(4-nitrobenzo-2-oxa-1,3-diazole)aminohexanoylphosphatidylcholine.

* Permanent address: Department of Internal Medicine, Hospital Clinic i Provincial, University of Barcelona, Barcelona, Spain.

† To whom reprint requests should be addressed.

the ADP/O ratio [3]. Studies on the physical or chemical characteristics of skeletal-muscle mitochondria of ethanol-fed animals have not been reported. We therefore investigated the effect of long-term ethanol feeding on the functional and structural characteristics of rat skeletal-muscle mitochondria and sarcoplasmic reticulum, and compared these data with those of liver microsomes, mitochondria and submitochondrial particles. The results demonstrate that skeletal-muscle mitochondria are not susceptible to the deleterious functional effects of long-term ethanol feeding, whereas liver mitochondria from the same animals are damaged. The physical and chemical characteristics of the skeletal-muscle mitochondrial and sarcoplasmic-reticular membranes are unchanged, even when the mitochondrial and microsomal membranes from the liver of the same animals show adaptive changes characteristic of membrane tolerance.

EXPERIMENTAL

Experimental animals

Male Sprague–Dawley rats, initially weighing 120–130 g, were given a nutritionally adequate liquid diet (Bioserv, Frenchtown, NJ, U.S.A.) for 35–70 days, which contained 36% of total energy as ethanol [21]. Littermate control rats of similar body weight were pair-fed on a control liquid diet, in which carbohydrates were isoenergetically substituted for ethanol.

Isolation of mitochondria and sarcoplasmic reticulum

Mitochondria were isolated essentially as described by Ashour & Hansford [22], with some modifications. Animals were killed by decapitation, and the muscles of both hindlimbs were removed and immediately placed into ice-cold Chappel–Perry isolation medium (KCl 0.1 M, Tris/HCl 50 mM, $MgCl_2$ 5 mM, ATP 1 mM, EGTA 1 mM, pH 7.5). The tissue was cleaned of fat and connective tissue, weighed, finely minced with scissors, and incubated with subtilisin (1 mg/g of muscle) for 25 min in 5 ml of medium/g. The tissue was homogenized with a Potter–Elvehjem homogenizer, filtered through gauze and centrifuged for 10 min at 900 *g*. The supernatant was decanted and centrifuged at 9000 *g* for 10 min. The supernatant was used for the isolation of sarcoplasmic-reticulum vesicles (see below), and the pellet was diluted with SHE medium (sucrose 0.25 M, Hepes 10 mM, EGTA 1 mM, pH 7.2) and centrifuged again at 9000 *g* for 10 min. This procedure was repeated once, and the final pellet was suspended and homogenized by hand with a minimal amount of SHE. The protein concentration was then adjusted to 27 mg/ml. Protein concentration was determined by the biuret method. For the preparation of sarcoplasmic reticulum, the supernatant of the first 9000 *g* spin was centrifuged at 100000 *g* for 45 min. The pellet was resuspended in SHE and the protein concentration was adjusted to 10 mg/ml.

Preparation of submitochondrial particles

The mitochondria were incubated with digitonin (0.09 mg/mg of mitochondrial protein) for 10 min on ice, rapidly diluted with SHE and centrifuged at 21000 *g* for 10 min. The pellet was resuspended in SHE and centrifuged twice at 21000 *g* for 10 min. This pellet was resuspended with sonication medium (10 mM-Na-Hepes, 1 mM-$MgCl_2$, 5 mM-succinate, 1 mg of BSA/ml, pH 7.4) and centrifuged at 21000 *g* for 10 min. The pellet was hand-homogenized and, after adding 10 mM-$MgCl_2$, was sonicated for 2×15 s period at 0 °C. The sonicated mitochondria were spun at 25000 *g* for 10 min. The supernatant was centrifuged at 140000 *g* for 45 min, and the pellet was washed once with SHE. The final pellet was resuspended in a small amount of SHE to adjust the protein content to 25 mg/ml.

Extraction, separation and analysis of lipids

Lipids were extracted from sarcoplasmic reticulum and mitochondrial membranes by the Bligh & Dyer [23] procedure, with extraction solvents that contained 0.01% butylated hydroxytoluene, and were flushed with N_2 to prevent oxidation of the fatty acids. Phospholipids were separated from neutral lipids by silicic acid chromatography and stored under N_2 in $CHCl_3$ at −20 °C. Phospholipid phosphorus was determined by the Bartlett [24] procedure.

The composition of the phospholipids extracted from the sarcoplasmic reticulum or mitochondria was determined by quantitative two-dimensional t.l.c., as previously described [25]. Phospholipids were identified by comparison of their R_F values with those of standards and by the use of spray reagents [25].

Fatty acid analysis

Phospholipids separated by two-dimensional t.l.c. were detected by using a dichlorofluorescein spray reagent. The areas containing the phospholipids were scraped off the plate, and fatty acid methyl esters were prepared with 12% BF_3 in methanol [26]. Methyl esters were separated by capillary g.l.c. on a Hewlett–Packard 5890A gas–liquid chromatograph with a fused-silica highly polar stabilized, bis-cyanopropylphenyl polysiloxane phase SP-2380 column (30 m × 0.32 mm internal diam., 0.2 μm film thickness) (Supelco, Bellefonte, PA, U.S.A.). The column temperature was linearly programmed from 170 °C to 195 °C at 3 °C/min, with initial and final times of 3 and 20 min respectively. The injector and detector temperatures were 250 °C. Methyl esters were analysed by using a Hewlett–Packard 3393A computing integrator to calculate the area percentage of each fatty acid. The integrator response was checked daily with methyl ester standards (Supelco, and Applied Science, State College, PA, U.S.A.). Fatty acid methyl esters were identified by comparison of retention times of standards and/or equivalent chain lengths.

Mitochondrial assays

Oxygen utilization by mitochondria. This was measured polarographically with a Clark oxygen electrode (Yellow Springs Co.) in a 1.9 ml water-jacketed cell at 27 °C. The medium consisted of 0.1 M-sucrose, 50 mM-KCl, 20 mM-Hepes, 5 mM-sodium phosphate, 2 mM-$MgCl_2$, pH 7.2. The following substrate conditions were used: succinate (5 mM)/rotenone (0.15 μM), pyruvate (2.5 mM)/malate (5 mM), glutamate (10 mM)/malate (1 mM), *NNN'N'*-tetramethylene-*p*-phenylenediamine (TMPD) (120 μM)/ascorbate (2 mM), acetyl-L-carnitine (5 mM)/malate (1 mM), palmitoyl-L-carnitine (20 μM)/malate (1 mM), α-oxoglutarate (0.5 mM)/malate (1 mM), and acetoacetate (1 mM)/α-oxoglutarate (0.5 mM)/malate (1 mM). State-3 respiration was achieved by addition of 300 nmol of ADP. The respiratory control index was obtained as the ratio of State 3 (in the presence of ADP)/State 4 respiration (after consumption of ADP). Other additions were made as indicated. Respiratory activity of submitochondrial particles was determined with NADH (0.2 mM), TMPD (120 μM)/ascorbate (2 mM) or succinate (5 mM) as substrates. Respiratory control ratios were determined as the ratio of the uncoupled rate to the oligomycin-inhibited rate.

ATPase activity in submitochondrial particles. This was determined spectrophotometrically at 340 nm in an Aminco DW2 spectrophotometer in the split-beam mode, in a medium containing 2.5 ml of respiration buffer containing rotenone (0.15 μM) and antimycin (1 mg/ml), with 25 mM-ATP and an ATP-regenerating system consisting of NADH (0.1 mM), phos-

phoenolpyruvate (2 mM), lactate dehydrogenase (5 units/ml) and pyruvate kinase (2.5 units/ml). NADH oxidation was corrected for the rate in the absence of rotenone (0.1 μg/mg of protein).

Cytochrome content of submitochondrial particles. This was analysed in an Aminco DW2A spectrophotometer (dual-wavelength mode) in a medium containing mitochondrial respiration buffer (2.5 ml) and 1 mg of protein [8]. Cytochromes were fully oxidized by addition of carbonyl cyanide *m*-chlorophenylhydrazone ('CCCP'; 0.15 μM) and fully reduced by addition of sodium dithionite. Cytochrome contents were calculated from the oxidized-minus-reduced spectra as described previously [8], by using the wavelength pairs 605–630 nm (cytochrome aa_3, absorption coefficient 24.0 $mM^{-1} \cdot cm^{-1}$), 562–575 nm (cytochrome *b*, absorption coefficient 20.0 $mM^{-1} \cdot cm^{-1}$) and 550–540 nm (cytochrome $c+c_1$, absorption coefficient 19.1 $mM^{-1} \cdot cm^{-1}$).

Preparation and spin labelling of membranes

Intact membranes were labelled with the fatty acid spin probe 12-[β-(4',4'-dimethyloxazolidinyl-*N*-oxyl)]stearic acid (SA12), by adding the probe in a minimal volume of ethanol (2–4 mM final concn. of ethanol). This amount of ethanol had no effect on the molecular order of the respective membranes as determined by e.s.r. The samples were flushed with N_2 and shaken for 15–30 min at 25 °C. The membranes were pelleted by centrifugation at 100000 *g* for 30 min, and washed several times to remove unincorporated spin label. Final lipid to spin-label ratios were 120:1–150:1.

Spin-labelled liposomes of sarcoplasmic-reticulum phospholipids

Multilamellar vesicles of sarcoplasmic-reticulum phospholipids were prepared by drying of a chloroform solution containing the phospholipid mixture under N_2, high-vacuum desiccation of the dried lipid film for 30 min, and hydration with phosphate-buffered saline (120 mM-NaCl/2.75 mM-KCl/10 mM-sodium phosphate), pH 7.2. The fatty acid spin probe SA12 was incorporated into the reconstituted membranes as described previously [27]. The molar ratio of membrane phospholipid/spin probe was 120:1.

E.s.r. spectroscopy

The spin-labelled sample, after addition of the appropriate amount of ethanol, was vortex-mixed for 2 min at room temperature, transferred to a 400 μl quartz flat-cell (Wilmad Glass, Buena, NJ, U.S.A.) and placed in an IBM Instruments ER 200D e.s.r. spectrometer cavity in a quartz dewar which contained silicone oil to maintain thermal stability. All samples were equilibrated for 7 min in the cavity at 37 °C, before recording the spectrum. Temperatures were accurate to 0.5 °C. Spectra were accumulated with an IBM 9000 computer interfaced to the spectrometer. The molecular order parameter, *S*, was calculated as described previously [27]. Typical spectrometer settings were: spectral scan 100 G, modulation amplitude 1.0 G and microwave power 5 mW. All measurements were made at 37 °C.

Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was determined spectrophotometrically by a coupled enzyme assay [28]. The assay mixture (1 ml) consisted of 50 mM-Hepes buffer, pH 7.4, 5 mM-$MgSO_4$, 1 mM-$CaCl_2$, 1 mM-phosphoenolpyruvate, 0.15 mM-NADH, 2.5 mM-ATP, pyruvate kinase (9 units/ml) and lactate dehydrogenase (13 units/ml). The Ca^{2+}-ATPase activity was calculated from the rate of NADH oxidation in the presence and absence of 5 μM ionophore to uncouple Ca^{2+} transport from the Ca^{2+}-ATPase activity.

Phospholipase A_2

Exogenous phospholipase A_2 (*Crotalus durissus terrificus*; Sigma) activity against sarcoplasmic-reticulum membranes was determined by measuring the release of NBD-hexanoic acid from C6-NBD-PC as previously described [29]. C6-NBD-PC (5 μM) was added by injection of a 20 μl aqueous dispersion into a suspension of sarcoplasmic-reticulum membranes (1 mg/assay) in 0.4 ml of 50 mM-Hepes, pH 7.4, containing 1 mM-Ca^{2+}. Phospholipase A_2 (0.1 μg) was added and the mixture was incubated for 2 min; in this period not more than 5% of the phospholipids were hydrolysed. The reaction was stopped by the addition of chloroform/methanol (1:2, v/v), and the mixture was separated into a lower chloroform and an upper aqueous phase [23]. A sample of the upper aqueous phase containing NBD-hexanoic acid released from the C6-NBD-PC was removed, and the fluorescence intensity at 530 nm determined after excitation at 470 nm in a spectrofluorimeter. A standard curve was obtained for NBD-hexanoic acid, which was also subjected to the chloroform/methanol extraction.

Statistical analysis

Unless otherwise indicated, all experiments were carried out on four or more independent preparations from pair-fed alcoholic and control animals. Results are expressed as means $\pm$ S.E.M. Statistical significance was estimated by Student's *t* test.

RESULTS

Effect of ethanol feeding on mitochondrial function

Intact mitochondria. Previous studies from this and other laboratories [5–7] have demonstrated that mitochondria isolated from the liver of ethanol-fed rats have a decreased respiratory activity and a diminished capacity for ATP synthesis compared with mitochondria from control animals. These changes were associated with deficiencies in mitochondrial ATPase and in the electron-transport chain at the level of NADH dehydrogenase and cytochromes *b* and aa_3.

As shown in Table 1, mitochondria isolated from skeletal muscle did not exhibit any of the functional deficiencies found in liver mitochondria. Rates of oxidation in State 3 and State 4 with a variety of different substrates were very similar in mitochondria of skeletal muscle from ethanol-fed and control rats, and were comparable with those reported by Ashour & Hansford [22] and others [30–32]. They were markedly higher than the activities observed by Lieber and co-workers [3] or Farrar *et al.* [20]. Respiratory control ratios and ADP/O ratios were uniformly high, and were not affected by the addition of cytochrome *c* or $MgCl_2$ (results not shown). A significantly higher State 3 oxidation rate was found with α-oxoglutarate/malate in the mitochondria from ethanol-fed animals than in control preparations. The reason for this difference has not been investigated. The rate of oxidation of pyruvate/malate was somewhat lower than that reported by Ashour & Hansford [22], but the ratio of palmitoylcarnitine oxidation to pyruvate oxidation in the control preparation (0.70 $\pm$ 0.05) was similar to the value obtained by those authors, consistent with the mitochondria being derived from both white and red muscle [33]. This ratio was somewhat elevated in mitochondria from ethanol-fed animals (0.83 $\pm$ 0.08; see the Discussion section), although the change did not reach statistical significance in these experiments. This shift did not reflect a difference in the recovery of mitochondria from these tissues. The amount of muscle tissue used (6.7 $\pm$ 0.2 and 7.0 $\pm$ 0.2 g) and the total yield of mitochondria (1.48 $\pm$ 0.11 and 1.30 $\pm$ 0.07 mg of protein/g of tissue) recovered from ethanol-fed and control animals respectively were not significantly different.

Table 1. Effect of chronic ethanol administration on respiratory activity of intact mitochondria from rat skeletal muscle

Abbreviations: A, ethanol-fed; C, control preparations; RCR, respiratory control ratio. The values given are means ± S.E.M. for 4–6 pairs of rats: *$P < 0.01$ comparing ethanol-fed and control rats.

	Oxygen uptake (ng-atoms/min per mg)									
	State 3		State 4		Uncoupled		ADP/O		RCR	
Substrate	C	A	C	A	C	A	C	A	C	A
Glutamate/ malate	410±21	439±26	41±3	39±4	325±83	321±89	2.62±0.06	2.63±0.07	10.0	11.5
Pyruvate/ malate	268±15	268±14	29±1	26±1	172±14	174±13	2.85±0.04	2.88±0.04	9.2	10.3
Succinate	440±12	437±13	95±3	89±5	392±22	385±29	1.70±0.04	1.72±0.03	4.6	4.9
Ascorbate/TMPD	340±8	341±7	199±4	191±4	350±11	340±9	1.24±0.02	1.31±0.04	1.7	1.8
Acetylcarnitine/ malate	153±24	167±16	30±2	26±2	151±10	158±7	2.77±0.07	2.84±0.08	5.1	6.4
Palmitoylcarnitine/ malate	188±32	221±26	40±2	36±2	178±11	196±7	2.50±0.08	2.46±0.01	4.8	6.1
α-Oxoglutarate/ malate	41±7	63±6*	18±1	17±1	11±5	18±4	2.70±0.20	3.08±0.11	2.3	3.7
Acetoacetate/ α-oxoglutarate/ malate	93±15	105±9	23±1	21±1	50±6	48±4	2.87±0.10	3.00±0.09	4.0	5.0

Under most substrate conditions, the rate of uncoupled oxidation was the same as, or lower than, the State 3 rate of oxidation, indicating that the control of the respiration rate was exerted primarily at the level of substrate transport and/or dehydrogenation, rather than in the electron-transport or phosphorylation reactions. The mitochondria from control and ethanol-fed rats did not behave differently in that respect. It is conceivable that small differences in the electron-transport activity and ATP synthase activity in the two mitochondrial preparations would not be manifested in the respiration rate in a system which is controlled primarily at the level of the substrate supply and dehydrogenation. We therefore investigated the electron-transport activity in an isolated mitochondrial-inner-membrane preparation, in which the electron-transport components are more readily accessible.

Submitochondrial particles. The electron-transport activity in skeletal-muscle submitochondrial particles from control and ethanol-fed rats was measured with NADH, succinate and TMPD/ascorbate as substrates, with oxygen as the electron acceptor. The results are shown in Table 2. Although the respiratory control index (determined as the ratio of the uncoupled rate to the oligomycin-inhibited rate) was lower in the skeletal-muscle preparations than in liver submitochondrial particles, the rates were not significantly different between the control and ethanol-fed rats. The activity of Complex I and III of the respiratory chain was determined in two separate preparations of submitochondrial particles, by using ferricyanide or 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB) (1 μM) as electron acceptor for NADH oxidation and dichlorophenol-indophenol/phenazine methosulphate (DCPIP/PMS) as electron acceptor for succinate oxidation. Both the NADH–ferricyanide reductase activity (rotenone-insensitive) and the NADH–DB reductase activity (> 95% inhibited by rotenone) were unaffected by ethanol treatment, and the succinate–PMS reductase activity was less than 10% different (results not shown). The cytochrome content of the submitochondrial particles was also measured spectroscopically. Liver mitochondria lose almost 50% of their cytochromes *b* and aa_3 after chronic ethanol feeding. By contrast, the content of cytochromes $c+c_1$ is not affected in liver [7,8]. Farrar *et al.* [20] reported a loss of up to 30% of all cytochromes in mitochondria from the gastrocnemius–plantaris muscle. By contrast, our data (Table 2) show that the cytochrome composition of the skeletal-muscle submitochondrial particles was unaffected by ethanol feeding, a finding consistent with the electron-transport activities. Similarly, ATPase activity, measured in both the absence and the presence of uncoupler, was identical for the two preparations.

Effect of ethanol feeding on ethanol-induced membrane disordering

Mitochondrial membranes. The development of functional deficiencies in liver mitochondria is accompanied by an adaptive change in the chemical and physical characteristics of the mitochondrial inner membrane, referred to as membrane tolerance [34]. This can be detected as an increased resistance to the membrane-disordering effects of ethanol and a marked change in the acyl composition of cardiolipin, which contains a smaller proportion of linoleic acid ($C_{18:2}$). The relationship between the physicochemical changes and the functional defects is not clear. We therefore also investigated the effect of chronic ethanol feeding on the structural properties of the skeletal-muscle mitochondrial membrane preparations, as detected by e.s.r., using SA12 as a spin probe. As shown in Figs. 1(*a*) and 1(*b*), both in intact mitochondria and in submitochondrial particles, the molecular order parameter *S*, derived from the motionally sensitive spectral hyperfine splittings, showed a dose-dependent decrease upon addition of ethanol (50–100 mM) *in vitro*, indicative of significant molecular disordering induced by the incorporation of ethanol into the membrane. The baseline order parameters (i.e. in the absence of ethanol) determined for mitochondrial membrane preparations from ethanol-fed animals were not significantly different from the control.

Sarcoplasmic-reticulum membrane preparations. Since the mitochondrial preparations showed little evidence of a structural or functional responsiveness to ethanol feeding, it was important to assess whether other membrane preparations from the same tissue were susceptible to ethanol-induced alterations. We there-

Table 2. Functional characteristics of skeletal-muscle submitochondrial particles from control and ethanol-fed rats

The values given are means ± S.E.M. from 11 pairs. Abbreviation: RCR, respiratory control ratio.

	Control	Ethanol-fed
Electron-transport activity (ng-atoms of O/min per mg)		
NADH/O_2	1555±127	1477±134
+Oligomycin	1158±43	1115±39
RCR	1.34	1.32
Succinate/O_2	220±16	206±14
+Oligomycin	159±11	151±10
RCR	1.39	1.36
TMPD/ascorbate/O_2	226±7	233±7
+Oligomycin	217±6	223±5
RCR	1.04	1.04
ATPase activity (μmol/min per mg)		
Uncoupled	3.72±0.37	3.63±0.35
+Oligomycin	1.87±0.23	1.75±0.22
Cytochrome content (nmol/mg)		
aa_3	0.62±0.02	0.60±0.01
b	0.57±0.01	0.54±0.02
$c+c_1$	0.79±0.01	0.80±0.01

fore assayed for membrane tolerance in skeletal-muscle sarcoplasmic reticulum and combined these studies with the determination of Ca^{2+}-ATPase activities. Contrary to earlier studies [18,19], in our sarcoplasmic-reticulum preparations we were unable to identify any significant differences in the Ca^{2+}-ATPase activity between ethanol-fed and control animals (9.08±0.27 and 9.01±0.86 nmol/min per mg of protein in preparations from ethanol-fed and control animals respectively; means ± S.E.M. for four preparations). The intact sarcoplasmic-reticulum membranes did not readily lend themselves to an accurate estimation of the order parameter, owing to a low partitioning of SA12 into this highly ordered membrane. We therefore analysed the order parameter of the reconstituted phospholipid fraction of the sarcoplasmic reticulum, since our previous studies had demonstrated that membrane tolerance arises from alterations in the membrane phospholipids, particularly acidic phospholipids [14,15,34]. As shown in Fig. 1(*c*), the reconstituted sarcoplasmic-reticulum phospholipids from control and ethanol-fed rats were disordered to the same extent by the addition of ethanol (50 or 100 mM). Moreover, in disagreement with a previous report [18], we found no significant difference in the baseline order parameter of the sarcoplasmic-reticulum membranes between the two preparations.

Comparison of liver and skeletal-muscle preparations from the same animals. The lack of effect of ethanol feeding on the baseline order parameter of sarcoplasmic-reticulum membrane preparations, and the retention of sensitivity to membrane disordering by ethanol, raised the question of whether the animals used in these studies were, for unknown reasons, less responsive to chronic ethanol administration than animals previously investigated. We therefore compared preparations from the skeletal muscle and liver in the same animals. The results of two of these experiments are illustrated in Fig. 2. Submitochondrial particles from skeletal muscle and liver from the same rats are compared in Figs. 2(*a*) and 2(*b*), and liposomes of muscle sarcoplasmic-reticulum phospholipids are compared with liver microsomal preparations from the same animals in Figs. 2(*c*) and 2(*d*). The results demonstrate that the liver membranes had developed tolerance to the disordering effects of ethanol (100 mM), whereas the skeletal-muscle preparation from the same animal retained its sensitivity to ethanol-induced disordering. Thus the lack of membrane adaptation to ethanol in skeletal muscle is a tissue-specific phenomenon, and is not related to individual characteristics of the animals.

Susceptibility to degradation by phospholipase A_2. In earlier studies on liver microsomes, we reported that the structural alterations induced by chronic ethanol intake are associated with a decreased susceptibility to degradation by exogenous phospholipase A_2 [29]. We measured the susceptibility of the sarcoplasmic-reticulum membranes to phospholipase A_2-mediated degradation by prelabelling with C6-NBD-PC and determining the release of NBD-hexanoic acid in response to phospholipase A_2, as described elsewhere [29]. In preparations from three pairs of animals, the activity in control animals was 1.79±0.31 nmol of C6-NBD-PC hydrolysed/min per μg of

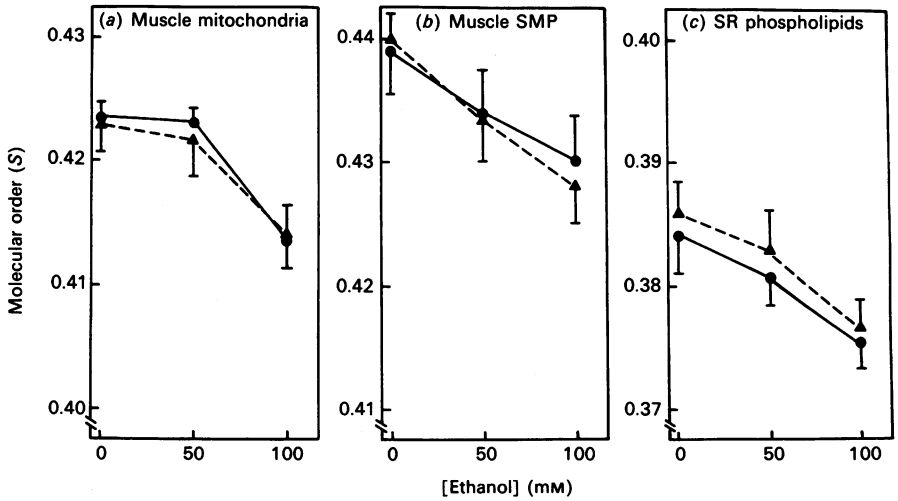

Fig. 1. Effect of ethanol *in vitro* on the molecular order parameter, *S*, obtained for SA12-labelled (*a*) muscle mitochondria ($n = 4$), (*b*) muscle submitochondrial particles (SMP) ($n = 4$) and (*c*) liposomes of sarcoplasmic-reticulum (SR) phospholipids ($n = 13$) from pair-fed untreated (●) and 35-day-ethanol-fed (▲) rats at 37 °C

Error bars represent S.D. No significant differences in baseline order parameters were observed for membranes from untreated and ethanol-fed rats.

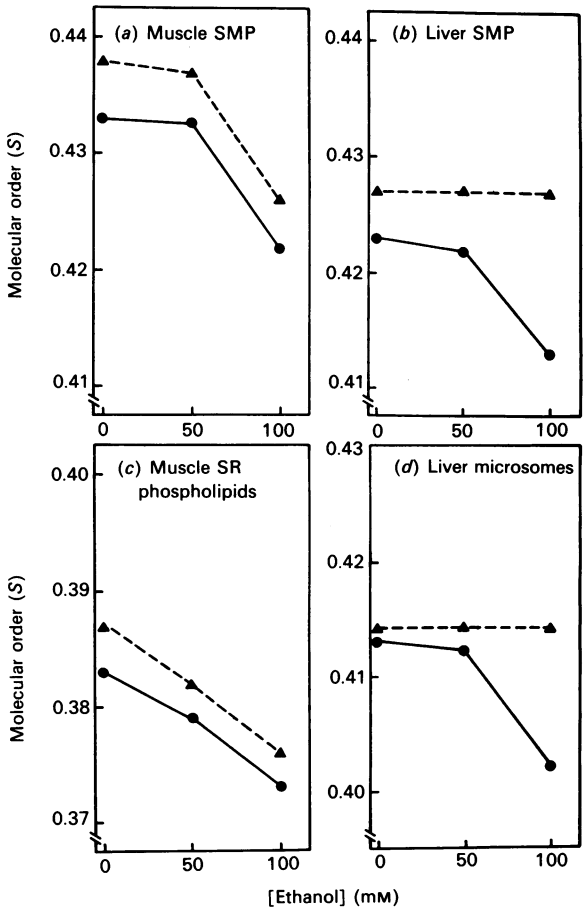

Fig. 2. Effect of ethanol *in vitro* on SA-12-labelled (*a*) muscle and (*b*) liver submitochondrial particles (SMP) and (*c*) muscle sarcoplasmic reticulum and (*d*) liver microsomes from the same ethanol-fed rat (▲) and its pair-fed control (●) at 37 °C

The liver membranes exhibit membrane tolerance, whereas the muscle membranes retain sensitivity to ethanol-induced disordering. Similar results were obtained from one other pair of rats. Membrane tolerance fails to appear in the muscle sarcoplasmic reticulum (SR) from an ethanol-fed rat (*c*), whereas liver microsomes from the same animal develop membrane tolerance (*d*). Similar results were obtained from two other pairs of rats.

phospholipase A_2, whereas in preparations from ethanol-fed animals the rate was 1.80±0.27. By contrast, in C6-NBD-PC-labelled liver microsomes from ethanol-fed rats the rate of C6-NBD-PC hydrolysis was only 52% of that in control preparations [29]. Thus these data support the conclusion that sarcoplasmic-reticulum membranes from ethanol-fed rats do not exhibit the structural alterations which are evident in liver microsomes.

Phospholipid composition of skeletal-muscle mitochondria and sarcoplasmic reticulum from control and ethanol-fed rats

In subcellular membranes from liver, the development of tolerance to the disordering effect of ethanol is accompanied by subtle, but consistent, changes in the acyl compositions of specific phospholipid classes, notably a significant decrease in the linoleic acid content of cardiolipin in mitochondrial membranes and a decrease in the arachidonate content of phosphatidylinositol in microsomal membranes [13–15]. We therefore investigated whether long-term ethanol feeding was associated with similar changes in the phospholipids of skeletal-muscle membrane fractions. The phospholipid compositions of the mitochondrial and sarcoplasmic-reticulum membrane preparations from the two groups of animals are shown in Table 3. In agreement with the e.s.r. studies, which revealed normal sensitivities to membrane disordering by ethanol, there were no significant differences in the phospholipid composition of either of these membrane preparations between the ethanol-fed and control animals.

Table 3. Phospholipid composition of skeletal-muscle sarcoplasmic reticulum and mitochondria in control and ethanol-fed rats

Membrane preparations were made from the skeletal muscle obtained from two pairs of control and ethanol-fed rats. The preparations were pooled and phospholipids extracted. Five separate analyses were conducted on the phospholipids from each pooled sample as described in the Experimental section. Two separate sets of these preparations were analysed, and the results are means ± S.E.M. of 10 analyses.

	Composition (mol %)	
Phospholipid	Control	Ethanol-fed
Sarcoplasmic reticulum		
Phosphatidylcholine	63.1±0.4	62.2±0.4
Phosphatidylethanolamine	23.3±0.3	26.2±0.1
Phosphatidylinositol	4.7±0.2	3.4±0.2
Phosphatidylserine	3.0±0.2	2.9±0.2
Sphingomyelin	3.4±0.2	3.0±0.2
Cardiolipin	2.5±0.1	2.1±0.1
Mitochondria		
Cardiolipin	7.1±0.4	7.9±0.6
Phosphatidylethanolamine	39.2±0.3	40.6±0.6
Phosphatidylcholine	53.7±0.6	51.5±0.9

The fatty acid compositions of the skeletal-muscle mitochondria of control and ethanol-fed rats are shown in Table 4. The cardiolipin from the control mitochondria contained 84.6% linoleic acid and 8.5% oleic acid as the major fatty acids, whereas the corresponding amounts in the ethanol-fed animals were 82.1% and 8.2% respectively. This lack of change in the cardiolipin acyl composition is in marked contrast with the cardiolipin from liver mitochondria, which shows a drastic 30% decrease in linoleate content after ethanol feeding. Chronic ethanol consumption also did not alter the fatty acid composition of the phospholipids in the sarcoplasmic reticulum (Table 5). The phosphatidylinositol from controls had 48.9% stearic acid and 27.5% arachidonic acid, whereas in the ethanol-fed rats the amounts were 48.3% and 26.6%, respectively. Small but consistent changes have been reported in the arachidonate content of liver microsomal phosphatidylinositol after chronic ethanol feeding [14]. Although literature data on the acyl composition of rat skeletal-muscle phospholipids are limited, the values shown in Table 5 compare well with a previous report on normal rat sarcoplasmic reticulum [35].

The phospholipid acyl composition not only exhibits substantial species specificity [35], but it is also significantly different between corresponding preparations from muscle and liver. Markedly higher levels of the docosapolyenoic acids and lower levels of arachidonic acid are found in sarcoplasmic-reticulum phospholipids than we have observed in liver microsomes (J. S. Ellingson, N. Janes, T. F. Taraschi & E. Rubin, unpublished work). Similarly, mitochondrial phosphatidylethanolamine from skeletal muscle is highly enriched in docosapolyenoic acids (28.7%; see Table 4) compared with liver mitochondrial phosphatidylethanolamine (12.9%; J. S. Ellingson, E. Rubin & T. F. Taraschi, unpublished work), whereas arachidonate levels are 30–40% lower in mitochondrial phosphatidylcholine and phosphatidylethanolamine in skeletal muscle compared with

Table 4. Fatty acid composition of phospholipids in skeletal-muscle mitochondria of control and ethanol-fed rats

Phospholipids extracted from the pooled samples as described in Table 3 were analysed for fatty acid content as described in the Experimental section. Three fatty acid analyses were carried out on the phospholipids from each of the two pooled samples. The values are means ± S.E.M. of 6 analyses. Abbreviations: Ald, dimethylacetal of the aldehyde from the plasmalogens; n.d., not detected (detection limit 0.5%).

	Composition (% of total)					
	Cardiolipin		Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	Control	Ethanol-fed	Control	Ethanol-fed	Control	Ethanol-fed
$C_{16:0}$	1.7±0.3	1.6±0.3	26.2±1.0	26.3±0.9	3.7±0.2	3.3±0.4
$C_{16:0Ald}$	n.d.	n.d.	n.d.	n.d.	1.3±0.1	1.7±0.2
$C_{18:0}$	1.9±0.3	4.3±1.3	7.6±0.5	8.1±0.7	29.0±1.4	28.5±1.0
$C_{18:0Ald}$	n.d.	n.d.	n.d.	n.d.	0.8±0.1	< 0.5
$C_{18:1}$	8.5±0.2	8.2±0.4	12.4±0.2	13.1±0.8	3.0±0.2	4.5±0.2
$C_{18:1Ald}$	n.d.	n.d.	n.d.	n.d.	1.0±0.1	1.4±0.1
$C_{18:2}$	84.6±1.2	82.1±1.9	23.2±0.2	23.2±0.2	9.0±0.5	11.0±0.3
$C_{20:4}$	2.3±0.1	2.7±0.6	27.2±1.3	25.8±0.9	23.4±0.8	25.5±0.7
$C_{22:5}$	n.d.	n.d.	n.d.	n.d.	9.6±0.7	8.1±0.7
$C_{22:6}$	n.d.	n.d.	2.9±0.4	2.6±0.2	19.1±0.9	15.2±1.1

Table 5. Fatty acid compositions of phospholipids in skeletal-muscle sarcoplasmic reticulum of control and ethanol-fed rats

Conditions and abbreviations are as described in Table 4 legend.

	Composition (% of total)					
	Phosphatidylinositol		Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	Control	Ethanol-fed	Control	Ethanol-fed	Control	Ethanol-fed
$C_{16:0}$	3.3±0.4	2.7±0.2	34.5±0.6	34.4±1.4	8.7±0.6	7.8±0.4
$C_{16:0Ald}$	n.d.	n.d.	n.d.	n.d.	7.3±0.5	6.0±0.2
$C_{18:0}$	48.9±0.8	48.3±0.6	7.9±0.5	8.3±0.8	18.7±1.0	18.6±0.6
$C_{18:0Ald}$	n.d.	n.d.	n.d.	n.d.	4.9±0.3	3.8±0.2
$C_{18:1}$	3.6±0.1	3.2±0.4	9.2±0.6	9.8±0.8	5.3±0.2	5.9±0.2
$C_{18:1Ald}$	n.d.	n.d.	n.d.	n.d.	3.7±0.2	4.5±0.2
$C_{18:2}$	2.9±0.3	3.2±0.1	20.4±0.7	21.5±1.4	7.1±0.4	7.6±0.3
$C_{20:4}$	27.5±0.9	26.6±0.4	22.9±1.0	22.5±1.1	19.9±0.2	18.7±1.0
$C_{22:5}$	5.5±0.3	6.2±0.2	2.9±0.2	0.9±1.6	5.8±1.2	8.6±0.3
$C_{22:6}$	7.6±0.5	8.9±0.4	1.5±0.5	1.7±0.4	17.6±0.4	17.9±1.1

liver. Palmitate is the predominant saturated fatty acid in phosphatidylcholine in both skeletal-muscle mitochondria and sarcoplasmic reticulum, whereas liver microsomal and mitochondrial phosphatidylcholine contains about equal amounts of palmitate and stearate. In contrast with phosphatidylcholine, the saturated fatty acid content of phosphatidylethanolamine in muscle mitochondria is predominantly stearic acid, with very low amounts of palmitic acid. Liver mitochondrial phosphatidylethanolamine contains 3 times more palmitate than does phosphatidylethanolamine from skeletal-muscle mitochondria (J. S. Ellingson, E. Rubin & T. F. Taraschi, unpublished work). Thus the skeletal-muscle mitochondrial phosphatidylcholine must contain predominantly palmitate molecular species, whereas phosphatidylethanolamine contains principally stearate species. The functional implications of these differences between skeletal muscle and liver are not known, but they appear to be preserved after long-term ethanol feeding.

DISCUSSION

The data reported here demonstrate that rats exposed to long-term ethanol treatment do not develop the structural and functional alterations in subcellular membranes of skeletal muscle that have been described for membranes of liver, brain, blood cells and pancreas. Specifically, the mitochondria from ethanol-fed animals had oxidation rates with a wide range of substrates that were comparable with those of control preparations and were not deficient in the activities of NADH oxidase, ATPase or cytochrome oxidase, which are decreased in liver mitochondria [5–7]. The lack of change in functional characteristics correlates with a lack of development of tolerance to the disordering effects of ethanol in the sarcoplasmic reticulum or mitochondrial membranes from ethanol-fed rats. Similarly, the changes in acyl composition that are characteristically observed in specific phospholipids in mitochondrial and microsomal membranes from liver [13–15] were absent from the corresponding preparations from skeletal muscle. We have reported previously [36] that the changes in the physical and chemical characteristics of mitochondrial membrane phospholipids correlate with the development of mitochondrial respiratory deficiencies, both during the onset of adaptation to ethanol and after withdrawal from the ethanol-containing diet. The present findings further substantiate this correlation, in that a tissue that appears to be resistant to the membrane structural alterations exhibits neither the chemical

changes in membrane phospholipids nor the deficiencies in mitochondrial and sarcoplasmic-reticulum function owing to long-term ethanol ingestion.

This lack of effect of ethanol feeding is not due to a lack of exposure to ethanol or to an unusual insensitivity of the animals used in this study. The animals had consumed an average of 12.3 ± 0.2 g of ethanol/kg body wt. per day for a period of 35–75 days, maintaining a circulating ethanol concentration of 25–50 mM. They sustained a mean growth rate of 5.4 ± 0.4 g/day. Furthermore, when directly comparing the characteristics of membrane preparations from liver and muscle in the same ethanol-fed animals, deficiencies in mitochondrial function and membrane tolerance were observed in both liver mitochondria and microsomes, whereas membrane preparations from the skeletal muscle of the same rats had retained their sensitivity to ethanol.

The lack of change in the structural and functional characteristics of skeletal-muscle membranes contrasts in some respects with reports in the literature. In the present study, neither skeletal-muscle mitochondria nor sarcoplasmic-reticular membranes from rats chronically fed on an ethanolic diet developed tolerance to disordering by ethanol *in vitro*. A previous investigation of sarcoplasmic-reticulum membranes from ethanol-fed rats reported a greater resistance to ethanol-induced disordering compared with control preparations [18]. Close examination of the data in [18] revealed that resistance was only observed in membranes labelled with SA7, whereas membranes labelled with SA5 or SA12 (which was used in the present study) were as sensitive to disordering by ethanol as membranes from control rats. It is difficult to compare directly the results in [18] with this investigation, since we examined the effects of much lower ethanol concentrations (< 100 mM in these studies, as compared with 300 mM and above in [18]). Differences in baseline order parameter (i.e. in the absence of ethanol) reported in [18] also showed probe dependence, with membranes labelled with SA7 showing the greatest difference. Liposomes of sarcoplasmic-reticulum phospholipids labelled with SA12 were not examined in [18], but, based on their studies with intact membranes, differences in baseline order would not be anticipated.

Our data on mitochondrial respiratory activity agree with the findings of Lieber and co-workers [3] that skeletal-muscle mitochondria maintain normal respiratory control ratios and ADP/O ratios; however, these authors observed a decreased rate of State 3 oxidation with glutamate as substrate. The site of action of this decrease was not identified. Glutamate oxidation, in the absence of a supply of oxaloacetate, is likely to be limited either by substrate transport or by the rate of regeneration of oxaloacetate through α-oxoglutarate oxidation. To what extent these factors might be affected by the altered metabolic state in the ethanol-fed animals is not clear. Farrar *et al.* [20], who studied mitochondrial function in rat gastrocnemius–plantaris muscle, found that, under sedentary conditions, ethanol feeding markedly decreased mitochondrial respiratory activity, ATPase activity and cytochrome content, whereas in animals that were exercised daily by a running regimen ethanol feeding had no effect on these parameters. However, the animals in the study of Farrar *et al.* [20] were not pair-fed, but received food *ad libitum*. The sedentary ethanol-fed rats, in contrast with the exercised ethanol-fed animals, had a significantly lower body weight and muscle weight than the corresponding control group [20]. The pair-fed animals used in our study are comparable with the control animals with respect to weight gain during the course of the treatment. It should also be pointed out that the rate of pyruvate/malate oxidation and the respiratory control ratio found by Farrar *et al.* [20] are markedly lower than those reported here (Table 1), for both ethanol-fed and control animals. In a recent study on rat brain mitochondria, which appeared while the present paper was in preparation, Farrar *et al.* [37] reported that respiratory rates and respiratory control characteristics were maintained after 10 weeks of ethanol feeding, with pyruvate/malate as substrate. The activity of electron-transport components was not reported in that study, but the brain mitochondria from ethanol-fed animals were found to have developed tolerance to an inhibition by ethanol of energy-driven Ca^{2+} uptake [37]. Brain mitochondria also develop tolerance to the disordering effects of ethanol as detected by e.s.r., as demonstrated by Chin & Goldstein [38], and membrane tolerance has also been observed in synaptosomal preparations from ethanol-fed rats ([38]; Y. Nie, J. S. Ellingson, E. Rubin & T. F. Taraschi, unpublished work). Thus, in contrast with the skeletal-muscle membranes studied here, membrane preparations from brain develop tolerance to ethanol in a similar way to membranes from liver, but the metabolic expression of this tolerance in the mitochondria may be different.

Several authors have noted that the mitochondria isolated from skeletal-muscle tissue are heterogeneous in their origin, a situation that may affect their performance characteristics. Krieger *et al.* [39] devised isolation procedures to separate mitochondria of subsarcolemmal and intermyofibrillar origin and found that the latter had a higher rate of State 3 respiration, but were less responsive to changes in aerobic demands induced by exercise or immobilization. Pande & Blanchaer [33] compared mitochondria from red and white muscle and reported differences in the rate of fatty acid oxidation relative to pyruvate oxidation. Our mitochondrial preparation did not differentiate between subsarcolemmal and intramyofibrillar sources, and is derived from both red and white muscle. However, we found substantially higher rates of respiration than in any of the preparations used by those authors [33,39]. A predominance of either of these types of mitochondria in our preparation could have obscured changes occurring only in a particular subfraction of the muscle. However, the yield of mitochondria was consistent with earlier studies and was not affected by ethanol feeding. Electron-microscopic studies showed no evidence of a selective deterioration of a particular subpopulation of mitochondria in the ethanol-fed animals (results not shown). A small shift in the ratio of palmitoylcarnitine oxidation to pyruvate oxidation was observed, from a value of 0.70 ± 0.05 in control mitochondria, intermediate between those observed for red and white muscle, to 0.83 ± 0.08 in the mitochondria from ethanol-fed animals, identical with the value of 0.84 reported for red muscle by Pande & Blanchaer [33]. Since the yield of mitochondria and the amount of muscle tissue recovered from both groups of animals was not affected by ethanol feeding, this may represent a shift in the metabolic characteristics of the mitochondria in response to ethanol feeding. Morphological studies have indicated that long-term ethanol feeding is associated with a preferential atrophy of Type II (fast) muscle fibres, in agreement with an increased reliance on red muscle [40]. Both this shift and the apparent enhancement of α-oxoglutarate oxidation (see Table 1) indicate that the skeletal-muscle mitochondria from ethanol-fed animals do show a slight change in their metabolic characteristics, even when the deleterious actions at the level of the mitochondrial membrane are not evident.

The mechanism by which the animals maintain control over the functional integrity of the mitochondria in skeletal muscle under conditions of a sustained ethanol challenge remains a matter of conjecture, since the basis for the mitochondrial damage in liver and other tissues is not unequivocally established. It is unlikely that ethanol oxidation through alcohol dehydrogenase is responsible for the long-term damage to different tissues, since several organs that do not have this enzyme, such as brain or pancreas, show evidence of membrane tolerance [38,41].

Laposata & Lange [42] have suggested that tissues have a different capacity for non-oxidative ethanol metabolism, causing differential accumulation of fatty acid ethyl esters which could correlate with mitochondrial damage; they demonstrated that, after ethanol intake, relatively low amounts of these ethyl esters accumulate in skeletal muscle compared with liver, pancreas or brain. However, it is unknown to what extent these or other acute actions of ethanol relate to long-term alterations in membrane structure and function.

In summary, three main conclusions derive from this work. Firstly, the data demonstrate that the effects of long-term ethanol feeding on the structural and functional characteristics of subcellular organelles are tissue-specific, with skeletal-muscle mitochondria and sarcoplasmic reticulum being resistant to the alterations that are found in liver membranes of the same animals. Secondly, the lack of functional changes in skeletal-muscle mitochondria and sarcoplasmic reticulum from ethanol-fed animals correlates with a lack of membrane tolerance and with the maintenance of normal acyl composition of membrane phospholipids. These observations support a mechanism in which alterations in these parameters are mutually dependent. Thirdly, these studies indicate that any pathological effects of long-term ethanol feeding on muscle function are not due to a deficiency in the mitochondrial energy supply. Instead, the condition may reflect a deficiency in the excitation–contraction coupling mechanism, for instance owing to alterations in the control of Ca^{2+} release or in the contractile proteins. Further studies are needed to elucidate the effects of ethanol intake on these parameters.

This work was supported by Grants AA07186, AA07215 and AA00088 from the National Institutes of Alcoholism and Alcohol Abuse (NIAAA) and by grant CCA-8411/023 from the U.S.–Spain Joint Committee for Scientific and Technical Cooperation. T.F.T. is a recipient of a Research Scientist Development Award from NIAAA.

REFERENCES

1. Urbano-Marquez, A., Estruch, R., Navarro-Lopez, F., Grau, J. M., Mont, L. & Rubin, E. (1989) N. Engl. J. Med. **320**, 409–415
2. Segel, L. D., Rendig, S. V., Choquet, Y., Chacko, K., Amsterdam, E. A. & Mason, D. T. (1975) Cardiovasc. Res. **9**, 649–663
3. Shaw, S., Leo, M. A., Jayatilleke, E. & Lieber, C. S. (1982) Biochem. Med. **28**, 210–223
4. Del Villar Negro, A., Merino Angulo, J. & Rivera-Pomar, J. M. (1984) Acta Neurol. Scand. **70**, 185–196
5. Cederbaum, A. I., Kieber, C. S., Toth, A., Beattie, D. S. & Rubin, E. (1973) J. Biol. Chem. **248**, 4977–4986
6. Thayer, W. S. & Rubin, E. (1979) J. Biol. Chem. **254**, 7717–7723
7. Thayer, W. S. & Rubin, E. (1981) J. Biol. Chem. **256**, 6090–6097
8. Rubin, E. & Lieber, C. S. (1968) N. Engl. J. Med. **278**, 869–876
9. Rubin, E. & Lieber, C. S. (1973) Science **182**, 712–713
10. Rubin, E. & Lieber, C. S. (1974) N. Engl. J. Med. **290**, 128–135
11. Katz, A. M., Freston, J. W., Messineo, F. C. & Herbette, L. G. (1985) J. Mol. Cell. Cardiol. **17**, Suppl. **2**, 11–20
12. Ponnappa, B. C., Waring, A. J., Hoek, J. B., Rottenberg, H. & Rubin, E. (1982) J. Biol. Chem. **257**, 10141–10146
13. Rottenberg, H., Waring, A. J. & Rubin, E. (1981) Science **213**, 583–585
14. Ellingson, J. S., Taraschi, T. F., Wu, A., Zimmerman, R. & Rubin, E. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3353–3357
15. Taraschi, T. F., Ellingson, J. S., Wu, A., Zimmerman, R. & Rubin, E. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9398–9402
16. Song, S. K. & Rubin, E. (1971) Science **175**, 327–328
17. Rubin, E., Katz, A. M., Lieber, C. S., Stein, E. P. & Puszkin, S. (1976) Am. J. Pathol. **83**, 499–515
18. Ohnishi, S. T., Waring, A. J. & Fang, S.-R. G. (1985) Membr. Biochem. **6**, 49–63
19. Ohnishi, S. T. (1985) Membr. Biochem. **6**, 33–47
20. Farrar, R. P., Martin, T. P., Abraham, L. D. & Errickson, C. K. (1982) Life Sci. **30**, 67–75
21. DeCarli, L. M. & Lieber, C. S. (1967) J. Nutr. **91**, 331–336
22. Ashour, B. & Hansford, R. G. (1983) Biochem. J. **214**, 725–736
23. Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
24. Bartlett, G. R. (1959) J. Biol. Chem. **234**, 466–468
25. Ellingson, J. S. (1974) Biochim. Biophys. Acta **337**, 60–67
26. Morrison, W. R. & Smith, L. M. (1964) J. Lipid Res. **5**, 600–608
27. Taraschi, T. F., Wu, A. & Rubin, E. (1985) Biochemistry **24**, 7096–7101
28. Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 622–626
29. Stubbs, C. D., Williams, B. W., Pryor, C. L. & Rubin, E. (1988) Arch. Biochem. Biophys. **262**, 560–573
30. Davies, K. J. A., Packer, L. & Brooks, G. A. (1981) Arch. Biochem. Biophys. **209**, 539–554
31. Allshire, A. P. & Heffron, J. J. A. (1984) Arch. Biochem. Biophys. **228**, 353–363
32. Hutson, S. (1986) J. Biol. Chem. **261**, 4420–4425
33. Pande, S. V. & Blanchaer, M. C. (1971) Am. J. Physiol. **220**, 549–553
34. Taraschi, T. F. & Rubin, E. (1985) Lab. Invest. **52**, 120–131
35. Marai, L. & Kuksis, A. (1973) Can. J. Biochem. **51**, 1365–1379
36. Taraschi, T. F., Thayer, W. S., Ellingson, J. S. & Rubin, E. (1987) Ann. N.Y. Acad. Sci. **488**, 127–139
37. Farrar, R. P., Seibert, C., Gnau, K. & Leslie, S. W. (1989) Brain Res. **500**, 374–378
38. Chin, J. H. & Goldstein, D. B. (1977) Science **196**, 684–685
39. Krieger, D. A., Tate, C. A., McMillin-Wood, J. & Booth, F. W. (1980) J. Appl. Physiol. **48**, 23–28
40. Martin, F. C., Slavin, G. & Peters, T. J. (1984) J. Clin. Pathol. **37**, 448–454
41. Ponnappa, B. C., Hoek, J. B., Waring, A. J. & Rubin, E. (1986) Biochem. Pharmacol. **36**, 69–79
42. Laposata, E. A. & Lange, L. L. (1986) Science **231**, 497–499

Received 30 August 1990/19 October 1990; accepted 13 November 1990