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A MECHANISM FOR ETHANOL-INDUCED DAMAGE TO LIVER MITOCHONDRIAL STRUCTURE AND FUNCTION *

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Summary

Mitochondria isolated from rats chronically fed ethanol demonstrated a marked inability to produce energy. The respiratory control ratio, the ADP/O ratio and state 3 respiration rates were all decreased. Coupled with other data, a progression of ethanol-induced changes is proposed with site I being altered prior to site II. Quantitation of mitochondrial cytochromes revealed decreases in cytochromes *b* and aa_3 and an increase in c_1. Evaluation of respiration activity in relation to temperature showed ethanol-induced changes in the transition temperature (T_f) which may have been related to changes in the lipid composition of the inner membrane. Mitochondrial membranes were separated, and analysis of fatty acids and phospholipids was performed. Various fatty acids were altered in both membranes; however, the outer membrane was altered more severely. A decrease in the arachidonate : linoleate ratio was observed only in the outer membrane; however, there was no ethanol-induced change in degree of unsaturation in either membrane. Phospholipid quantitation showed a reduction of total lipid phosphorous/mg protein in both membrane fractions; however, the inner membrane was most affected. Cardiolipin was the only phospholipid in this membrane which remained unaltered. The evidence indicates that the mechanism for ethanol-induced damage to the liver mitochondrion involves lipid compositional changes as well as changes in cytochromes and possibly other proteins.

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Introduction

That chronic ethanol ingestion induces changes in liver mitochondrial morphology has been well established [1—3], and several laboratories have shown that the morphological changes may be related to functional changes [4—8]. Recently, our laboratory published a study [9] which showed that the effects of ethanol on liver mitochondrial structure and function could be altered by dietary fat. In this study, we observed chronic ethanol ingestion to alter markedly the fatty acid and phospholipid composition of isolated whole mitochondria. The decrease in phospholipid resulted from a decrease in both cardiolipin and phosphatidylcholine. The major change in fatty acid composition was observed as a decrease in the arachidonate-to-linoleate (20 : 4/18 : 2) ratio. This was first observed in mitochondria by French and Morin [4]. French et al. [10] fractionated the mitochondria into inner and outer membranes and measured the fatty acid composition of each membrane. They observed decreases in the 20 : 4/18 : 2 ratio in both membranes.

In addition to changes in the lipid composition, changes in the mitochondrial cytochrome content have been reported [8]. These observers reported a decrease in cytochrome aa_3 and cytochrome b, but no change in cytochrome cc_1. Bernstein and Penniall [11] have shown that chronic ethanol ingestion produced a marked decrease in the synthesis of three subunits of cytochrome c oxidase.

Altered respiratory functions also have been reported. Decreased respiratory control has been observed by several investigators [12—15], oxidative phosphorylation, as measured by the ADP/O ratio, was decreased [13,16], and ATPase activities have been reported to be altered [16,17].

The studies reported in this paper attempt to correlate changes in phospholipids, fatty acids, cytochromes and respiration into a mechanism whereby ethanol alters the functioning of liver mitochondria.

Materials and Methods

Animals and mitochondria. Long-Evans rats were obtained from Simonsen Labs, Gilroy, CA. The animals were isocalorically pair-fed a low-fat (4.6% of calories) liquid diet for 35—45 days [9]. Ethanol amounted to 36% of the calories. Rats were killed by decapitation, their livers were removed immediately and placed in ice-cold 0.25 M sucrose containing 1 mM EGTA, 3.4 mM Tris-HCl (pH 7.4) and 1% defatted bovine serum albumin. The albumin was defatted according to the method of Goodman [18]. Mitochondria were isolated according to the procedure of Chappell and Hansford [19] as modified by Thompson and Reitz [14]. Succinate and β-hydroxybutyrate oxidations were followed polarographically using a Clark oxygen electrode. Oxygen content was determined from reported values [20]. β-Hydroxybutyrate (5 mM) and succinate (10 mM) were added to a final reaction volume of 1.5 ml. Each concentration value represents the final concentration in the reaction vessel. The reaction medium consisted of 0.25 M sucrose, 0.015 M Tris-HCl (pH 7.4), 0.015 M potassium phosphate (pH 7.4), 7.5 mM $MgCl_2$ and 1.5 mM EDTA. The reaction was initiated by the addition of 1.5 mg protein. The

ADP/O ratio, and the state 3 and state 4 respiration rates were determined as described by Estabrook [21]. Preparations having a respiratory control ratio of less than 4.0 were discarded because their stability was insufficient to complete a temperature-rate experiment or to allow good separation of the mitochondrial membranes. The protein concentration was determined by using the procedure of Lowry et al. [22].

Outer and inner mitochondrial membranes were prepared from isolated mitochondria as described by Greenawalt [23]. The purity of the mitochondrial membrane preparation was determined by measuring the activity of marker enzymes. Glutamate dehydrogenase activity was estimated by using the method of Raijam [24]. Cytochrome oxidase was assayed by monitoring the oxidation of reduced cytochrome *c* according to the method of Smith [25]. Monoamine oxidase was analyzed according to the method of Tabor et al. [26]. Adenylate kinase was assayed according to the method of Schnaitman and Greenawalt [27].

Arrhenius plot analysis. Each curve was obtained by computer analysis. Initially, all points were evaluated by least-squares linear regression to determine the slope of the line. Then, beginning with the data points at either end of the curve, linear regression analyses were performed on a point-by-point basis to determine if a second line could be drawn which had a standard error of the slope less than that obtained for all of the points. These analyses were repeated starting with the data points from the opposite end of the curve. In order for a break to be considered real, the point-by-point analysis must have produced one line which contained four or more points and of which the standard error of the slope was less than that for all points. The actual break-point between regressions was determined by varying the break-point until the pooled variance was minimized.

Lipid analysis. Mitochondrial membrane lipids were extracted by using the procedures of Bligh and Dyer [28]. The phospholipids were separated by thin-layer chromatography (TLC) using a solvent consisting of $CHCl_3/CH_3OH/CH_3CO_2H/H_2O$ (100 : 55 : 16 : 6, v/v). The various phospholipids were located with 2,6-dichlorofluorescein, eluted from the TLC scrapings according to the method of Arvidson [29], and quantitated by using the total phosphate procedure of Bartlett [30]. An internal standard fatty acid, heptadecanoic acid, was added to the TLC scraping prior to elution by the procedure of Arvidson [29]. Methyl esters were prepared [31], and membrane fatty acids were quantitated by gas chromatography as described previously [32].

Cytochrome analysis. Mitochondrial cytochromes were estimated according to the procedure of Williams [33]. The four simultaneous equations were solved by a standard computer program [34]. Extinction coefficients used in the calculations were as follows. (a) Cytochrome *c*, $E = 25.1\ mM^{-1} \cdot cm^{-1}$ [35]; (b) cytochrome c_1, $E = 18.8\ mM^{-1} \cdot cm^{-1}$ [33]; (c) cytochrome *b*, $E = 14.3\ mM^{-1} \cdot cm^{-1}$ [33]; and (d) cytochrome aa_3, $E = 24\ mM^{-1} \cdot cm^{-1}$ [36]. Difference spectra were obtained using an Aminco DW-2 spectrophotometer.

Results and Discussion

Table I presents the results of mitochondrial respiration studies. Chronic ethanol ingestion reduced state 3 by approx. 30% using either succinate or

TABLE I

OXIDATION RATES IN LIVER MITOCHONDRIA FROM RATS MAINTAINED FOR 35—45 DAYS ON AN ETHANOL DIET

Each value represents the mean ± S.D. from five animals. Respiration measurements were performed at 33°C, and the incubation medium is described in Materials and Methods. Oxidation rates are expressed as nmol O_2/min per mg. RCR, respiratory control ratio. n.s., not significantly different from controls.

Substrate	Control	Ethanol	% Change	*P* value
Succinate				
State 3	96.9 ± 7.7	66.4 ± 12.0	−31.5	<0.001
State 4	19.3 ± 0.4	15.6 ± 2.5	−19.2	<0.001
RCR	5.02 ± 0.5	4.4 ± 0.3	−14.0	<0.02
ADP/O	1.50 ± 0.05	1.27 ± 0.02	−15.0	<0.001
β-Hydroxybutyrate				
State 3	43.2 ± 1.7	30.8 ± 3.2	−28.7	<0.001
State 4	4.6 ± 0.8	6.1 ± 1.9	+32.0	n.s.
RCR	9.7 ± 1.6	5.4 ± 1.2	−44.6	<0.001
ADP/O	2.11 ± 0.09	1.90 ± 0.06	−10.0	<0.001

β-hydroxybutyrate as substrates. State 4 was decreased when succinate was employed as substrate and increased when β-hydroxybutyrate was used. The respiratory control ratio was decreased 14% with succinate and 45% with β-hydroxybutyrate. The ADP/O ratio also was reduced using either substrate. The values for the ADP/O ratio in controls are consistent with values reported by Hinkle and Yu [37].

Previous investigations of aberrations in mitochondrial respiration have yielded reports suggesting different sites at which ethanol induces changes. Earlier reports indicated decreases in state 3 and 4 respiration with succinate as substrate as well as decreases in succinate dehydrogenase activity [8,38]. Cederbaum et al. [13] showed state 3 to be depressed to a greater extent with NAD^+-dependent substrates than with FAD^+-dependent substrates. This evidence along with data showing decreases in the respiratory control ratio and the ADP/O ratio with only NAD^+-dependent substrates was used as an indication of ethanol damage to site I (NADH-ubiquinone coupling site) of the oxidative phosphorylation system. Recently, Spach et al. [15] demonstrated a decrease in state 3 using succinate and suggested variations at both site I and site II (ubiquinone-cytochrome *c* coupling site).

Previous reports from our laboratory have shown a consistent decrease in state 3 respiration, regardless of whether a flavin- or pyridine-linked substrate was used [14]. Table I verifies the decrease in state 3 respiration as well as a decrease in the respiratory control ratio using either succinate or β-hydroxybutyrate. Animals used in studies prior to this were fed a low-fat diet (4.6% of the calories) for 77—99 days [9,14,16]. Mitochondrial respiration data from these earlier studies showed a 32% decrease in the respiratory control ratio using succinate as substrate and a 27% decrease using β-hydroxybutyrate as substrate. In the present study, animals were fed the same liquid diet [9] for 35—45 days. As shown in Table I, there was only a 14% decrease in the respiratory control ratio using succinate and a 44.6% decrease in the same parameter when β-hydroxybutyrate was the substrate. These data suggest a time-depen-

dent phenomenon in which ethanol appears to affect site I earlier than other sites, reaching a maximum effect before 45 days of ingestion. This concept is in agreement with the results of Spach et al. [15] who showed diminished mitochondrial energy properties using succinate. They fed rats the DeCarli-Lieber [39] high-fat (35% of calories) diet for 31 days. Prior to this time, Cederbaum et al. [13] demonstrated a preferential effect on site I after feeding rats the same diet for 24 days. These data were used to suggest a progression of chronic ethanol-induced alterations in mitochondria [15]. Chronic ethanol ingestion, then, exerts a progressive deterioration of the mitochondrial coupling system with alterations occurring first at site I and later at site II. These series of events seem to be accelerated by increasing dietary fat.

Respiratory chain cytochromes were quantitated in order to assess variations in state 4 respiration. Table II shows the mitochondrial cytochrome quantitation. Cytochromes *b* and aa_3 were significantly reduced, 9.3 and 40%, respectively; however, there was a 41% increase in cytochrome c_1. The dramatic decrease in aa_3 was further emphasized by the aa_3/cc_1 and aa_3/c_1 ratios. These data corroborate the results of Rubin et al. [8] and Bernstein and Penniall [40] who demonstrated a consistent decrease in cytochrome aa_3 using either a high- or low-fat diet, respectively. Table II establishes a significant alteration in three of the four cytochromes measured. The change in the cytochromes was responsible, in part, for the decrease in energy efficiency of liver mitochondria after chronic ethanol ingestion.

One method of investigating the interaction between a group of proteins in a lipid matrix is evaluation of enzymatic activity with respect to different temperatures. Arrhenius plots were used to determine the extent of ethanol-induced damage to the protein-lipid interaction of the mitochondrial respiratory chain. Kemp et al. [41] have shown that the adenine nucleotide translocase is rate-limiting at all temperatures from 0 to 23°C; therefore, Arrhenius plots were used to analyze only state 4 respiration rates to avoid having to consider the translocase. The mean values from four to nine assays at each

TABLE II

MITOCHONDRIAL CYTOCHROME CONCENTRATIONS AFTER CHRONIC ETHANOL INGESTION

Each value represents the mean ± S.E. from six animals. The concentrations of each cytochrome were calculated by using the method of Williams [33]. n.s., not significantly different from control.

	Cytochrome concentration (nmol/mg protein)			
	b	c_1	*c*	aa_3
Control	0.312 ± 0.003	0.148 ± 0.003	0.161 ± 0.002	0.112 ± 0.002
Ethanol	0.283 ± 0.009 **	0.215 + 0.007 *	0.171 ± 0.003	0.067 ± 0.003 *
	−9.3%	+41%	n.s.	−40%
	aa_3/cc_1	aa_3/c_1		
Control	0.366 ± 0.010	0.790 ± 0.041		
Ethanol	0.172 ± 0.009 *	0.308 ± 0.016 *		
	−53%	−61%		

* Ethanol different from control at $P < 0.001$.

** Ethanol different from control at $P < 0.05$.

temperature and nine different temperatures were employed to obtain the data necessary for the Arrhenius plots. In order to avoid any bias in determining the shape of the Arrhenius plots, a new procedure was employed (see Materials and Methods). It is important to mention this procedure as it adds validity to the transition temperature differences which are discussed below.

Interpretation of Arrhenius plot breaks and activation energies (E_a) should be carried out with caution. Silvius et al. [42] studied the Mg^{2+}-ATPase of *Acholeplasma laidlawii* and showed that a variation in substrate binding affinity with temperature can strongly influence the behavior of Arrhenius plots. Also, there are specific membrane-bound enzymes which demonstrate the same K_m value and transition temperature regardless of the different lipid environments in which they are associated [43]. More common, however, are the correlations between membrane fatty acids and Arrhenius transition temperatures [44—47].

Fig. 1 demonstrates the effects of temperature on mitochondrial oxidations of succinate and β-hydroxybutyrate. The transition temperature was 29°C for the oxidation of succinate by control mitochondria. Succinate oxidation by mitochondria isolated from rats chronically fed ethanol, however, exhibited a break temperature at 25°C. The transition temperatures for oxidation of β-hydroxybutyrate were also different. Control mitochondria showed a break at 21°C, while in the ethanol-treated group, it appeared at 25.3°C. Thus,

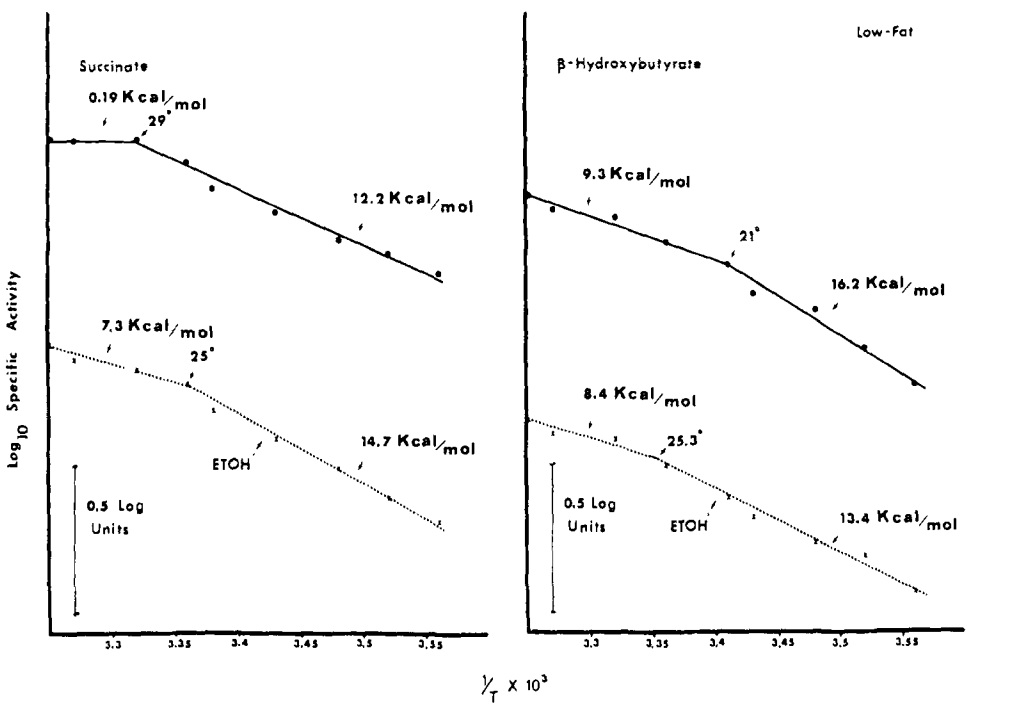

Fig. 1. Arrhenius plots of state 4 respiration supported by 5 mM β-hydroxybutyrate or 10 mM succinate. The respiration medium is described in Materials and Methods. Each point represents the mean value from four to nine assays at each temperature. Transition temperatures were determined according to the procedure described in Materials and Methods.

in control mitochondria, there was an 8°C difference in the transition temperature between β-hydroxybutyrate and succinate, and ethanol ingestion completely nullified this difference.

In a recent paper, Rottenberg et al. [48] have studied the temperature dependence of an NADH-linked substrate (glutamate-malate) in animals chronically treated with ethanol. They observed an increase in transition temperature of 7.3°C during state 4 respiration. This is consistent with the data in Fig. 1 in which we observed an increase of 4.3°C with β-hydroxybutyrate. These data support the idea that chronic ethanol ingestion has induced a decrease in membrane fluidity. The data in Tables IV and V (inner membrane fatty acids) also would support this concept. The fact that we observed a decrease in transition temperature of 4°C when succinate was used might argue against this interpretation; however, because site I has been shown to be the most lipophilic site in the electron transport chain [49], the inner membrane fatty acid composition may simply reflect the influence of the lipids from site I. Thus, these data seem to be supportive of the site I alteration reported by Cederbaum et al. [13] as well as suggestive of changes to other sites.

The activation energies (E_a) for the various Arrhenius plots were very similar with the exception of the E_a value for succinate oxidation at the higher temperatures. The control E_a value was 1.09 kcal/mol. This difference corroborated the altered state 4 respiration rates (see Table I). Using succinate, state 4 showed a statistical decrease (19%) in the ethanol-treated group. With β-hydroxybutyrate there was no statistical difference in state 4. These data, coupled with the variation in the transition temperature, indicate a possible difference in the physical composition of the inner mitochondrial membrane.

Mitochondrial membranes were fractionated in order to determine the extent of lipid compositional changes caused by chronic ethanol ingestion. Marker enzymes were assayed to detect the purity of each membrane fraction. Table III shows the percent total activity of each enzyme in the various mitochondrial fractions. These data indicate that the fractions used for subsequent lipid studies were relatively free from cross-contamination. It is important to note, however, that the phospholipid analysis (see Table VI) of the separate mitochondrial membranes indicates the possibility of more contamination than is evident from analysis of monoamine oxidase activity. Cardiolipin is thought to occur exclusively in the inner membrane fraction, while phosphatidylserine and phosphatidylinositol occur predominantly in the outer membrane fraction. The phospholipid data, then, indicate a small percentage of cross-contamination between the inner and outer membranes.

Membrane fatty acid composition has been reported to be primarily responsible for the transition temperature exhibited by Arrhenius plots. Because there were differences in transition temperatures (see Fig. 1), it was important to understanding this variation that the membrane fatty acid composition be determined. The effects of chronic ethanol ingestion on the fatty acid composition of the inner and outer mitochondrial membranes are compared in Table IV. The largest effect of ethanol ingestion on membrane fatty acids appeared in the outer membrane fraction. Palmitate was statistically decreased (19%), and oleate reduced (18%). Furthermore, the arachidonate-to-linoleate (20 : 4/18 : 2) ratio decreased 30%. The altered ratio was the result of a 40%

TABLE III

MITOCHONDRIAL ENZYME MARKERS

The numbers in parentheses are the reference numbers for the assay methods. Each value represents the mean ± S.E. from five preparations in which two animals were pooled for each preparation. Values are expressed as % of total activity.

	Cytochrome oxidase (25)	Monoamine oxidase (26)	Adenylate kinase (27)	Glutamate dehydrogenase (24)
Control				
Inner membrane	84.0 ± 2.0	0.0	3.2 ± 0.3	9.5 ± 3.3
Outer membrane	14.5 ± 2.6	100 ± 0.0	6.0 ± 0.7	4.8 ± 0.9
Intramembrane space	0.0	0.0	67.9 ± 3.5	0.0
Matrix	1.5 ± 1.5	0.0	22.9 ± 3.0	85.7 ± 2.8
Ethanol				
Inner membrane	82.4 ± 2.3	9.3 ± 4.3	3.8 ± 0.6	20.0 ± 2.7
Outer membrane	17.6 ± 2.3	90.7 ± 4.2	6.4 ± 4.5	6.2 ± 1.7
Intramembrane space	0.0	0.0	61.3 ± 2.2	0.0
Matrix	0.0	0.0	28.4 ± 3.0	73.9 ± 3.6

increase in linoleate with no change in arachidonate. Two other fatty acids were increased (20 : 3ω6, 50%; 22 : 6ω3, 100%).

In the inner membrane fraction, palmitate again was decreased 19%, and oleate was reduced 7% by ethanol. There was no significant change in the 20 : 4/18 : 2 ratio, stearate, 20 : 3ω6, or arachidonate.

TABLE IV

FATTY ACID COMPOSITION OF INNER AND OUTER MITOCHONDRIAL MEMBRANES

Each value represents the mean ± S.E. from five preparations, and each preparation came from pooled livers of two rats. Values are expressed as nmol%. n.s., not significantly different from control.

	Control	Ethanol	*P* value
Inner membrane			
16 : 0	25.4 ± 0.9	20.7 ± 1.0	<0.001
16 : 1	4.0 ± 0.2	4.1 ± 0.4	n.s.
18 : 0	28.8 ± 0.9	31.2 ± 1.8	n.s.
18 : 1	13.3 ± 0.3	12.4 ± 0.4	<0.005
18 : 2	13.0 ± 0.3	13.8 ± 0.9	n.s.
20 : 3ω6	0.9 ± 0.1	0.9 ± 0.1	n.s.
20 : 4ω6	9.5 ± 0.8	9.5 ± 1.0	n.s.
22 : 5ω6	0.9 ± 0.1	1.5 ± 0.1	<0.001
22 : 6ω3	0.5 ± 0.1	0.6 ± 0.1	n.s.
20 : 4/18 : 2	0.63 ± 0.1	0.64 ± 0.1	n.s.
Outer membrane			
16 : 0	31.1 ± 0.9	25.7 ± 0.8	<<0.001
16 : 1	2.3 ± 0.4	2.3 ± 0.2	n.s.
18 : 0	29.8 ± 0.9	32.0 ± 0.9	n.s.
18 : 1	13.6 ± 0.5	11.2 ± 0.5	<<0.001
18 : 2	6.5 ± 0.5	9.1 ± 0.3	<<0.001
20 : 3ω6	0.8 ± 0.1	1.2 ± 0.1	<<0.001
20 : 4ω6	16.6 ± 0.1	16.9 ± 0.1	n.s.
22 : 5ω6	0.9 ± 0.1	0.9 ± 0.1	n.s.
22 : 6ω3	0.7 ± 0.1	1.4 ± 0.2	<0.001
20 : 4/18 : 2	2.63 ± 0.2	1.86 ± 0.1	<<0.001

Previous investigations of the fatty acid composition of whole mitochondria have shown a consistent increase in linoleate (18 : 2ω6) and a decrease in the 20 : 4/18 : 2 ratio [4,9,10,14]. The present study verifies these aberrations but only in the outer membrane. This is in disagreement with French et al. [10] who demonstrated the same trends in both the inner and outer mitochondrial membranes. French et al. [10] used an inner membrane plus matrix fraction, and we have separated the matrix from the inner membrane. The use of detergent to break the inner membrane may have solubilized certain specific lipids which resulted in alterations in the 20 : 4/18 : 2 ratio. The accordant decrease in palmitate in both membranes is in agreement with prior reports [4,10].

It has been reported that a 5% change in the percent of unsaturated fatty acids of a membrane can alter the transition temperatures by nearly 15°C [46]. Table V is a comparison of the transition temperatures, percent unsaturation and the 20 : 4/18 : 2 ratio. Note that there was no difference between the two groups. Recently, McMurchie and Raison [50] reported feeding rats diets containing various unsaturated fatty acids and found no clear relationship between percent unsaturation and the temperature of transition. Table V verifies this idea.

Any small alteration of the specific lipids surrounding a membrane-bound enzyme system could definitely account for the changes in transition temperatures and for altered energy production in mitochondria from ethanol-treated rats. Because the structural organization of membanes is determined by phospholipid arrangement, the phospholipid composition of the different membrane fractions was determined. Table VI summarizes the change in membrane phospholipids. The total phospholipid/mg protein was significantly reduced in both membranes; however, the inner membrane contained only 17% less phospholipid compared to 46% less in the outer membrane. This was consistent with earlier reports which demonstrated that ethanol induced a reduction in the total lipid phosphate/mg protein in whole mitochondria [4,9,51].

TABLE V

RELATIONSHIPS BETWEEN UNSATURATION INDEX, 20 : 4/18 : 2 RATIO AND TRANSITION TEMPERATURE

	Control		Ethanol	
	% unsaturation	20 : 4/18 : 2 ratio	% unsaturation	20 : 4/18 : 2 ratio
Mitochondrial membrane				
Inner	42.10	0.63	43.30	0.64
Outer	41.40	2.63	43.0	1.86
	Transition temperature T_f(°C)			
Substrate				
Succinate	29		25	
β-Hydroxybutyrate	21		25.3	

TABLE VI

PHOSPHOLIPID ANALYSIS OF INNER AND OUTER MEMBRANES

CL, cardiolipin; PE, phosphatidylcholine; PS/PI, phosphatidylserine/phosphatidylinositol fraction; PC, phosphatidylcholine; Sph, sphingomyelin; LPC, lysophosphatidylcholine. Each value represents the mean ± S.E. from three to five preparations, and each preparation resulted from two rats. Values are expressed as % total phosphate. n.s., not significantly different from control.

	Control	Ethanol	*P* value
Inner membrane			
CL	21.55 ± 0.86	20.94 ± 0.65	n.s.
PE	35.49 ± 0.75	31.58 ± 1.06	<0.01
PS/PI	3.76 ± 0.27	5.28 ± 0.36	<0.001
PC	31.37 ± 0.64	35.11 ± 0.69	<0.001
Sph	2.27 ± 0.21	3.88 ± 0.49	<0.01
LPC	2.51 ± 0.12	3.77 ± 0.51	<0.05
% recovery	91.4	97.2	
Total (nmol P/mg protein)	320 ± 18	266 ± 17	<0.05
Outer membrane			
CL	4.59 ± 0.46	4.49 ± 0.20	n.s.
PE	22.05 ± 0.32	22.70 ± 0.53	n.s
PS/PI	13.87 ± 0.50	11.65 ± 0.70	<0.02
PC	49.04 ± 1.32	52.34 ± 1.17	n.s.
Sph	6.46 ± 0.32	4.62 ± 0.28	<0.001
LPC	1.34 ± 0.10	0.83 ± 0.21	<0.02
% recovery	88.1	90.4	
Total (nmol P/mg protein)	468 ± 32	301 ± 37	<0.01

These data do not provide anything concerning the relative composition of the individual phospholipids. The decreases in total phospholipids could represent 'across-the-board' alterations in all phospholipids, but they could also represent selective changes in certain phospholipids. Thus, the individual phospholipids were separated and the relative percentages were determined. Phospholipid compositional alterations were most evident in the inner membrane fraction. Cardiolipin was the only phospholipid not affected. Phosphatidylethanolamine was reduced 11%. Phosphatidylserine/phosphatidylinositol was increased 40%. Phosphatidylcholine was slightly elevated (12%), while sphingomyelin rose 71%. Lysophosphatidylcholine also increased (50%). Thus, the relative composition of 80% of all phospholipids in the inner membrane was altered by chronic exposure to ethanol.

The 50% increase in inner membrane lysophosphatidylcholine may have particular significance with respect to certain functions of this membrane. Pfeiffer et al. [52] related increases in inner membrane permeability to a phospholipase A_2-dependent increase in lysophospholipids. Spach et al. [15] showed that the hydrolysis of mitochondrial phosphatidylethanolamine by endogenous phospholipase A_2 was greater in mitochondria from chronic ethanol-treated animals. Thus, ethanol ingestion decreases the control over this enzyme which can alter mitochondrial structure. The importance of these observations lies in the fact that lysophosphatidylethanolamine and lysophosphatidylcholine are excellent detergents, and their increased production could contribute to altered membrane function, i.e., uncoupling via their detergent action on other membrane components.

The composition of the phospholipids of the outer mitochondrial membrane was affected less than the inner membrane. Only three phospholipid fractions were altered by ethanol feeding, and these were all decreased (phosphatidylserine/phosphatidylinositol, 16%; sphingomyelin, 29%; lysophosphatidylcholine, 38%). The altered phospholipids represent only 17% of the total phospholipids of the outer membrane. Ethanol feeding, then, altered the phospholipids of the inner mitochondrial membrane much more than those of the outer (80% compared to 17%).

Membrane phospholipids are responsible for providing the proper polar and spatial arrangement necessary for optimal activity of a membrane-bound enzyme, membrane fatty acids provide the required fluidity in the environment of these enzymes [53]. A small change in either one or both of the lipid components of a membrane system could cause a marked change in the activity and efficiency of the enzymes which comprise this membrane. It has been shown, in this paper, that both of the lipid components of the mitochondrial membranes were altered by chronic ethanol ingestion. These structural aberrations, i.e., lipid changes, coupled with changes in cytochrome content, could contribute to the observed differences in transition temperatures and to the reduction in the energy efficiency of mitochondria from ethanol-fed animals.

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