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Effect of Chronic Ethanol Administration on Energy Metabolism and Phospholipase A_2 Activity in Rat Liver

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1. For a period of 31 days male rats were given a liquid diet containing 36% of its energy as ethanol. Liver mitochondria from these animals demonstrated lowered respiratory control with succinate as substrate, a diminished energy-linked anilinonaphthalene-sulphonic acid fluorescence response, and lowered endogenous ATP concentrations. The phospholipid/protein ratio in mitochondria from these animals was unchanged; only minor alterations in the phospholipid fatty acid composition were observed. 2. In experiments where mitochondria were incubated at 18°C in iso-osmotic sucrose (aging experiments), the above energy-linked properties were lost at an earlier time in organelles from ethanol-fed animals. Phospholipase A_2 activity was depressed in mitochondria from control animals until respiratory control was lost and ATP was depleted. In contrast, no lag in the expression of phospholipase activity was observed in mitochondria from ethanol-fed rats. This loss of control of the phospholipase resulted in an earlier degradation of membrane phospholipids under the conditions of the aging experiments. 3. The ATPase (adenosine triphosphatase) activities, measured in freshly prepared tightly coupled mitochondria and in organelles uncoupled with carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone, were not significantly different in ethanol-fed and liquid-diet control animals. When the mitochondria were aged at 18°C, the activity increased with time of incubation in organelles from both groups of animals. A lag was observed, however, as the ATPase activity increased in control preparations. This lag was not present as ATPase activity increased in mitochondria from ethanol-fed animals. 4. The significantly lowered values observed for energy-linked functions with succinate as an energy source demonstrate that ethanol elicits an alteration in liver mitochondria that affects the site II–site III regions of the oxidative-phosphorylation system. The apparent lack of control of the phospholipase A_2 and ATPase activities in mitochondria from ethanol-fed animals suggests that the membrane microenvironment of these enzymes has been altered such that they can exert their catabolic effects more readily under conditions of mild perturbation. The fatty acid analyses demonstrate that the observed alterations both in the energy-linked functions and in control of the phospholipase and ATPase are not mediated through changes in the acyl chain composition of bulk-phase phospholipids.

Chronic ethanol intake has been shown to produce fatty liver in rats (Lieber *et al.*, 1965; Porta *et al.*, 1965; DeCarli & Lieber, 1967), baboons (Lieber *et al.*, 1975) and man (Lieber *et al.*, 1965). Liver mitochondria prepared from rats demonstrating fatty liver exhibit ultrastructural changes (Porta *et al.*, 1965), increased fragility (French, 1968; French & Todoroff, 1970), and alterations in normal functions. The latter include decreased mitochondrial energy metabolism, which accompanies the development of fatty liver. Respiratory control is diminished in organelles from ethanol-fed animals (Banks *et al.*, 1969; Cederbaum *et al.*, 1974; Thompson & Reitz, 1976), and oxidative phosphorylation, as measured by P/O ratios (Cederbaum *et al.*, 1974), is also significantly decreased. In addition the micro-environment of the mitochondrial ATPase appears to be altered as a result of chronic ethanol feeding (Hosein *et al.*, 1977).

In addition to diminished energy metabolism, the fatty acid distribution in mitochondrial phospholipids appears to be slightly altered (French & Morin, 1969; Thompson & Reitz, 1976), the most notable change being a lowered arachidonate/linoleate ratio. This

Abbreviation used: ANS, 8-anilinonaphthalene-1-sulphonate.

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latter observation demonstrates that phospholipid metabolism is altered in animals maintained on an ethanol diet; such alterations could be initiated at the level of the endoplasmic reticulum or the mitochondrion. Changes in microsomal phospholipid metabolism as a result of chronic ethanol feeding have been noted previously (Mendenhall *et al.*, 1969). Moreover, alterations could occur in mitochondrial lipid-metabolizing enzymes that would influence the fatty acid composition of phospholipids (Miceli & Ferrell, 1973), with the mitochondrial phospholipase A_2 participating in such alterations (van den Bosch, 1974). Changes in fatty acid composition of phospholipids elicited by diet manipulation (Houtsmuller *et al.*, 1970; Clandinin, 1976) have resulted in alterations in mitochondrial functions. Notably, those activities related to the oxidative-phosphorylation system are influenced considerably by the fatty acyl composition of membrane phospholipids (Janki *et al.*, 1974).

In the present study we have investigated the interrelationships between mitochondrial energy-linked functions, the endogenous phospholipase A_2 activity and the fatty acid composition of the phospholipids in organelles isolated from ethanol-fed animals. To assess more accurately the interrelationships between the energy-linked properties of mitochondria and the activity of the phospholipase A_2 on membrane phospholipids we have carried out aging experiments *in vitro* with isolated organelles and have followed simultaneously the changes that occur in several mitochondrial properties with time of aging. The aging procedure has provided a mechanism for demonstrating more clearly those alterations in mitochondrial properties related to energy and phospholipid metabolism. We have also determined the fatty acid composition of the phospholipids and measured the phospholipid/protein ratio in mitochondria from ethanol-fed animals. These composition studies have demonstrated that ethanol feeding can initiate alterations in mitochondrial energy-linked properties without dramatically affecting phospholipid composition.

A preliminary report of this investigation has appeared elsewhere (Cunningham & Spach, 1977).

Experimental

Methods

After being adapted to the diet over a period of 6 days, male Sprague–Dawley rats weighing 150–200g were fed for 31 days on a nutritionally adequate liquid diet (DeCarli & Lieber, 1967) in which ethanol provided 36% of the total energy. Pair-fed control rats received the same diet, but with maltose/dextrin isoenergetically substituted for ethanol. A second set of controls received Purina chow and water *ad libitum* throughout the feeding period. The average energy intake of the animals on the liquid diets was 234kJ/day, which corresponded to a value of 96.3 and 99.2kJ/day per 100g body wt. for liquid-diet control and ethanol-fed animals respectively over the entire feeding period. The weight gains were 5.1, 3.1 and 3.0g/day for chow-fed, control liquid-diet-fed and ethanol-fed animals respectively. The above nutritional parameters of our animals on the liquid diet were comparable with those observed by other investigators (Porta *et al.*, 1965; DeCarli & Lieber, 1967).

At 40min before death, rats were injected intraperitoneally with 40μCi of 2-amino[1-^{3}H]ethanol hydrochloride (sp. radioactivity 3.8Ci/mmol). The livers were homogenized in ice-cold 0.25M-sucrose adjusted to pH7.4 with NaOH, and mitochondria were prepared by the method of Schneider (1948). With this procedure freshly prepared mitochondria generally have 92% of the total mitochondrial radioactivity in phosphatidylethanolamine, 6% in phosphatidylcholine, and 2% in lysophosphatidylethanolamine. The mitochondria were suspended in ice-cold 0.25M-sucrose, pH7.4, at a protein concentration of 30mg/ml. Portions were frozen either in liquid N_2 or at −20°C for later analyses. The remaining portion of the mitochondrial preparation was placed in an 18°C water bath and allowed to age for 12h. During the aging period the mitochondria were continually monitored for respiratory control, membrane-associated energy-linked ANS fluorescence, ATP concentrations, phospholipase A_2 activity and ATPase activity. Phospholipid/protein ratios and fatty acid analyses of phospholipids were carried out on samples that had been frozen. In addition, the free fatty acid content of freshly prepared mitochondria was determined.

Respiratory-control ratios were measured by the method of Estabrook (1967) by using a Clark oxygen electrode. The assays were carried out at 30°C in an assay mixture containing 15mM-Tris/HCl, pH7.5, 15mM-potassium phosphate, 0.25M-sucrose, 7.5mM-$MgCl_2$, 1.5mM-EDTA, 2μM-rotenone, 11mM-succinate and mitochondria (0.3mg of mitochondrial protein), with State-3 respiration being measured in the presence of 0.27μmol of ADP. Mitochondrial ATP concentrations were measured by using the hexokinase–glucose 6-phosphate dehydrogenase couple and following $NADP^+$ reduction fluorimetrically (Williamson & Corkey, 1969).

The energy-linked ANS fluorescence response (Azzi, 1969; Datta & Penefsky, 1970) was measured as follows: 10μl of mitochondria (30mg of protein/ml) was added to a fluorescence cuvette containing 2ml of a fluorescence assay mixture composed of 20mM-Tris/HCl, pH7.4, 0.3M-sucrose, 10μM-rotenone and 0.25mM-ANS. The fluorescence was measured at

room temperature (23°C) in a Farrand mark I spectrofluorimeter with 90° optics and 5nm slits. The excitation and emission wavelengths were 395 nm and 485 nm respectively. The low protein concentration (0.15 mg/ml) allowed use of the 90° optics system; preliminary experiments to establish the above conditions demonstrated that the energy-linked fluorescence response was unaffected by light-scattering. After measuring the initial fluorescence of the ANS–mitochondria complex, succinate was added to give a final concentration of 10mM and the resulting decrease in fluorescence was recorded. After the relative emission reached a minimum, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to give a final concentration of 0.4 μM and the relative emission was recorded until it reached a maximum. The percentage change in relative emission was calculated as the highest relative emission minus the lowest relative emission divided by the average of the highest and lowest relative emissions. The frequent additions and accompanying mixing procedures precluded any settling of the mitochondria during the fluorescence assay.

In carrying out mitochondrial ATPase-activity measurements it was necessary to ensure that an increase in ATPase activity did not occur during the assay period owing to disruption of mitochondria; such disruption does occur in hypo-osmotic assay mixtures. Therefore an assay mixture was devised that had the same osmolarity as that of the respiratory-control-ratio assay mixture. It was composed of 2.5mM-$MgSO_4$, 2.5mM-ATP, 2.5mM-phosphoenolpyruvate, 45mM-Tris/sulphate and 0.23M-sucrose; the pH of the mixture was adjusted to 7.5 and the osmolarity was 0.32. Tubes containing 1.0ml of this mixture and 16 μg of pyruvate kinase were temperature-equilibrated in a 30°C shaking water bath. To those assays in which uncoupled ATPase activity was measured, 1 nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added. The assay was initiated by the addition of 0.05ml of mitochondrial protein (30mg/ml). After incubation for 10min the assay was stopped by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid. The tubes were centrifuged for 5min at 2000g and 0.5ml portions of the supernatants were taken for phosphate analysis. These samples were maintained on ice until all assays were completed. Phosphate was determined by the method of Lohmann & Jendrassik (1926). The assays were performed at 40min intervals during the period of respiratory control and at 1 h intervals thereafter. All assays were carried out in duplicate and a control quenched with trichloroacetic acid before addition of substrate was included at each time interval. Preliminary experiments established that in tightly coupled mitochondria, and in mitochondria with low respiratory control, ATP hydrolysis was linear with time from 1 to 10min with this assay procedure.

The assays for the mitochondrial phospholipase A_2 (Parce *et al.*, 1978) are described as follows. To measure Ca^{2+}-stimulated phospholipase A_2 activity, 4.5mg of radiolabelled mitochondria (batch B) was added to an assay mixture consisting of 50mM-Tris/HCl, pH7.5, 0.20M-sucrose and 2mM-$CaCl_2$. This mixture was incubated for 30min at 37°C. The reaction was terminated by addition of 3ml of chloroform/methanol (1:2, v/v). The lipids were extracted by the method of Bligh & Dyer (1959), evaporated to dryness under a stream of N_2 and redissolved in chloroform/methanol (9:1, v/v). For measurement of phosphatidylethanolamine hydrolysis occurring during the 18°C incubation, a portion of mitochondria (batch A) from the incubation suspension was added to the assay mixture and the lipids were immediately extracted by the method of Bligh & Dyer (1959) and concentrated as described above. The extracts from both batches A and B were fractionated by t.l.c. on silica-gel G plates and developed in chloroform/methanol/water/NH_3 (sp.gr. 0.88) (65:35:2:3, by vol.). The plates were exposed to I_2 vapour, and the spots corresponding to lysophosphatidylethanolamine and phosphatidylethanolamine were scraped into scintillation vials and counted for radioactivity in 10ml of a scintillation fluid composed of toluene, Triton X-100 and water in the proportions 10:5:1 (by vol.) containing 2.9g of Omnifluor/l. The percentage of phosphatidylethanolamine hydrolysis was calculated as [c.p.m. in lysophosphatidylethanolamine/(c.p.m. in lysophosphatidylethanolamine+c.p.m. in phosphatidylethanolamine)]×100. For batch A this was the percentage of phosphatidylethanolamine hydrolysed at 18°C. The percentage of phosphatidylethanolamine hydrolysed during incubation for 30min (Ca^{2+}-stimulated activity) was determined as the percentage of phosphatidylethanolamine hydrolysed in batch B minus the percentage of phosphatidylethanolamine hydrolysed in batch A.

Fatty acid analyses of the mitochondrial phospholipids were carried out on samples of freshly prepared mitochondria containing 5.0mg of protein. The lipids were extracted by the method of Bligh & Dyer (1959), evaporated to dryness under a stream of N_2, and redissolved in 0.2ml of chloroform/methanol (9:1, v/v). Phospholipids were separated from neutral lipids by t.l.c. by using silica-gel G plates and the solvent system light petroleum (boiling range 63–75°C)/diethyl ether/formic acid (30:20:1.0, by vol.). The phospholipids, which remain at the origin, were scraped into 40ml glass tubes fitted with Teflon stoppers. Butylated hydroxytoluene (0.3mg) and docosanoic acid (0.5mg) were added as an antioxidant and internal standard respectively. Transmethylation was carried out in methanolic 3% (w/w) HCl by the method of Litman (1975). The Litman (1975) procedure was modified by extending the

incubation time to 2h and by adjusting the final volume of the fatty acid methyl esters to 0.2ml of pentane. The fatty acid methyl ester mixture was analysed with a Barber–Colman gas chromatograph equipped with a column (1.8m×4mm) packed with 10% Silar 10C on 100–120 mesh Gas-Chrom Q. The temperature was programmed from 168 to 235°C with a rate of increase in temperature of 3°C/min. Quantification of the fatty acid methyl esters was based on the internal standard, docosanoic acid, and is reported in terms of mol percent.

To obtain the concentrations of non-esterified fatty acids in freshly prepared mitochondria, a sample containing 35mg of mitochondrial protein was extracted and separated from other lipids as described above. To locate the non-esterified fatty acids, the developed t.l.c. plates were sprayed lightly with a solution of 0.1% 2,7-dichlorofluorescein in 95% (v/v) ethanol and then illuminated with a u.v. lamp. Docosanoic acid (0.11mg) was applied directly to the non-esterified fatty acid band on the plate before the band was removed for methylation. Transmethylation and analysis by g.l.c. are described in the preceding paragraph.

Lipid phosphorus determinations were carried out by the method of Chalvardjian & Rudnicki (1970) on phospholipids extracted from mitochondria by the method of Bligh & Dyer (1959). Mitochondrial protein was determined by the procedure of Lowry *et al.* (1951) with the following modifications. Frozen suspensions of mitochondria were thawed and homogenized in a glass homogenizer with a glass pestle. Appropriate dilutions were carried out and the protein was solubilized in 5% (w/v) sodium deoxycholate/0.01 M-NaOH for 30min before addition of water and reagent C. The incubation time after the addition of reagent C was lengthened to 1.0h. Crystalline bovine albumin was used as a standard.

The values reported in the Tables and Figures are the averages ±S.E.M. for the numbers indicated. Statistical analyses were performed by using Student's *t* test for two means employing a progam from the Hewlett–Packard User's Library (Library No. 00115D). In this study differences between groups that gave *P* values less than 0.05 were considered significant.

Materials

Sprague–Dawley rats were obtained from Charles River Breeding Laboratories, North Wilmington, MA, U.S.A. Lieber/DeCarli liquid diets were obtained from Bio-Serv, Frenchtown, NJ, U.S.A. Special enzyme-grade sucrose used in the preparation of mitochondria was purchased from Schwartz/Mann, Orangeburg, NY, U.S.A. ANS was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and recrystallized twice from water as the magnesium salt. Rotenone, ADP and ATP were also obtained from Sigma. 2-Amino[1-^{3}H]ethanol hydrochloride was purchased from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. and Omnifluor from Fisher Scientific Co., Raleigh, NC, U.S.A. Sodium succinate, NADP^{+}, hexokinase and glucose 6-phosphate dehydrogenase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift of Dr. P. G. Heytler, E. I. duPont de Nemours and Co., Wilmington, DE, U.S.A. Methanolic HCl reagent, column packing for the gas chromatograph, docosanoic acid, and standard reference mixtures of fatty acid methyl esters were obtained from Applied Science Laboratories, State College, PA, U.S.A. Crystalline bovine albumin was purchased from Miles Laboratories, Elkhart, IN, U.S.A., and phospholipid standards for t.l.c. were provided by Dr. Moseley Waite of this department.

Results

The initial values for the respiratory control ratio were significantly lowered in mitochondria from ethanol-fed rats as compared with values from the two control groups (Table 1). The lowered respiratory control ratio was due to a decrease in the State-3 rate of O_2 utilization. The State-4 rate is comparable in the three groups (Table 1). Furthermore, when the mitochondria were aged at 18°C, respiratory control was lost earlier in preparations from ethanol-fed rats than in those organelles from pair-fed controls (Fig. 1*a*). The rates of loss of respiratory control were equivalent, however, as revealed by the first-order rate constants for decline in respiratory-control ratio. Moreover, the initial values of respiratory-control ratio (Table 1) and the rate of its decline were not significantly different in chow-fed and liquid-diet-fed rats (results not shown).

An analysis of the loss in respiratory control for mitochondria from ethanol-fed animals and liquid-diet controls revealed that the decline in respiratory-control ratio was due primarily to a loss of State-3 respiration. In mitochondria from both ethanol-fed and control animals, State-3 respiration declined more rapidly than State-4 respiration increased. Furthermore, the earlier loss of respiratory control in mitochondria from ethanol-fed animals (Fig. 1*a*) was due mostly to the lowered State-3 respiration, with the increase in State-4 respiration influencing respiratory control of ethanol-fed animals very little.

Measurement of endogenous ATP concentrations indicated a 57% decrease in mitochondrial ATP in freshly prepared mitochondria from alcoholic rats (Table 1). In mitochondria from both control and ethanol-fed animals ATP decreased at faster rates than did respiratory control (Fig. 1*b*). The rates of ATP loss were approximately equivalent in both

Table 1. *Effects of ethanol administration on energy-linked properties of rat liver mitochondria*
All assays and determinations are described in the Experimental section. The n values are 10 and 12 for chow-fed and liquid-diet animals respectively. The values for respiratory-control ratio, State-3 and State-4 respiration were obtained from a different group of animals than those used for Fig. 1. The ATP concentrations were measured in animal groups where $n = 9$. The maximum ANS fluorescence is the maximum decrease in fluorescence observed when succinate is added to freshly prepared tightly coupled mitochondria; calculation of the values is described in the Experimental section. Abbreviation: n.s., not significant.

Animal group	Respiratory-control ratio	State-3 rate (μg-atoms of O/min per mg of protein)	State-4 rate (μg-atoms of O/min per mg of protein)	Maximum ANS fluorescence	ATP concn. (nmol/mg of protein)
(A) Chow-fed	4.60±0.17	0.25±0.02	0.06±0.01	20.9±1.2	—
(B) Liquid-diet control	4.66±0.25	0.32±0.01	0.07±0.01	20.0±1.1	5.84±0.55
(C) Ethanol-fed	3.38±0.27	0.21±0.02	0.07±0.01	13.8±1.1	2.52±0.28
Comparison of (A) and (B)	n.s.	$P<0.03$	n.s.	n.s.	—
Comparison of (B) and (C)	$P<0.005$	$P<0.001$	n.s.	$P<0.001$	$P<0.001$

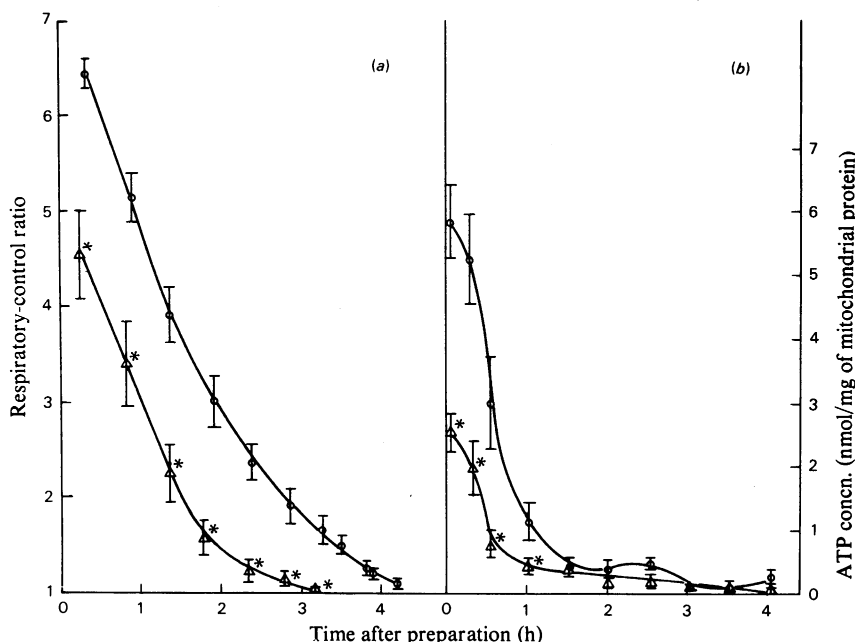

Fig. 1. *Loss of ATP with time of incubation at* 18°C
Mitochondria from liquid-diet control (○) and ethanol-fed (△) animals were assayed for (*a*) respiratory control with succinate as substrate and (*b*) ATP concentrations at the times indicated. *Statistically significantly different from liquid-diet controls ($P<0.05$, or lower). The bars represent ±S.E.M. for each point.

preparations, but the concentrations of ATP were significantly lower in mitochondria from ethanol-fed animals at all times during the early stages of incubation, owing to the lower initial concentrations.

In addition to the lowered respiratory control and ATP concentrations, mitochondria from chronic alcoholic rats also showed a significantly decreased ability to conserve energy derived from electron transport as measured by the ANS-fluorescence response (Table 1). This response is purported to monitor the energization of the mitochondrial inner membrane, which occurs during substrate oxidation (Azzi, & Montecucco, 1976; Gains & Dawson, 1976; Williams *et al.*, 1977). In our experiment it can there-

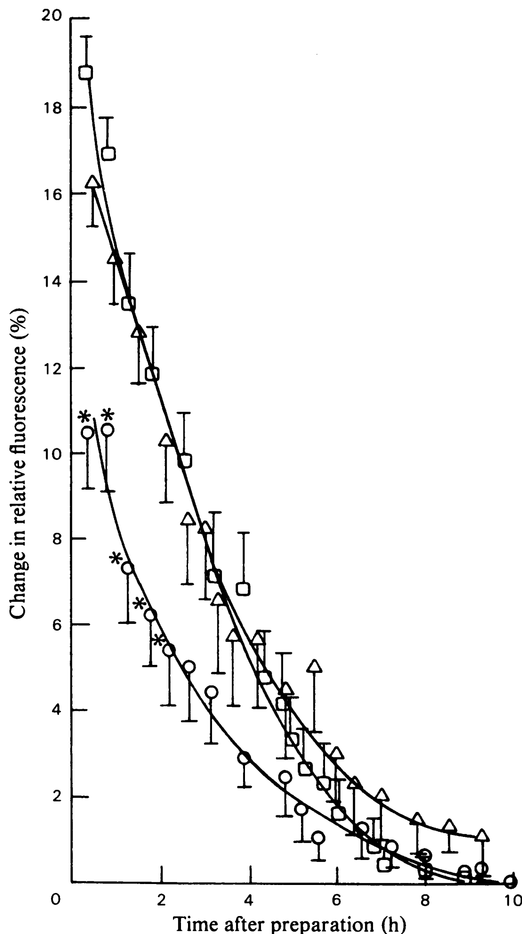

Fig. 2. *Loss in the energy-linked ANS fluorescence response with time of incubation at* 18°C

Mitochondria from chow-fed (□), control diet-fed (△) and ethanol-fed (○) animals were aged at 18°C and assayed for the energy-linked ANS fluorescence response at the times indicated. The calculation for percentage change in relative fluorescence is described in the Experimental section. *Statistically significantly different from liquid-diet controls ($P<0.05$, or lower).

fore be considered another indicator of energy conservation associated with succinate oxidation. The initial ANS fluorescence response of ethanol-fed animals was 44% lower than the response of their pair-fed controls, and remained significantly lower during the first 2h of mitochondrial aging (Fig. 2). The rates of loss of the fluorescence response in mitochondrial preparations from animals given the liquid diets were equivalent, however. Comparison of the curves for loss of respiratory control and loss of the ANS fluorescence response indicated that the mitochondria maintain the energy-linked fluorescence response for a longer time than they maintained respiratory control. Respiratory control was lost in control animals after 4–5h of aging, whereas the fluorescence response was observed for an additional 2–3h. Significant decreases in all three energy-linked parameters measured (Table 1) suggest a significantly lowered energy state in mitochondria from ethanol-fed rats.

The Mg^{2+}-stimulated ATPase activities of freshly prepared coupled mitochondria from both liquid-diet controls and ethanol-fed rats were low and approximately the same (Fig. 3). Furthermore, the uncoupler-stimulated activities in freshly prepared mitochondria were not significantly different, as also shown in Fig. 3. When the mitochondria were aged at 18°C, a dramatic difference was noted in the kinetics of increase of the ATPase activity. A distinct lag in the expression of maximal ATPase activity

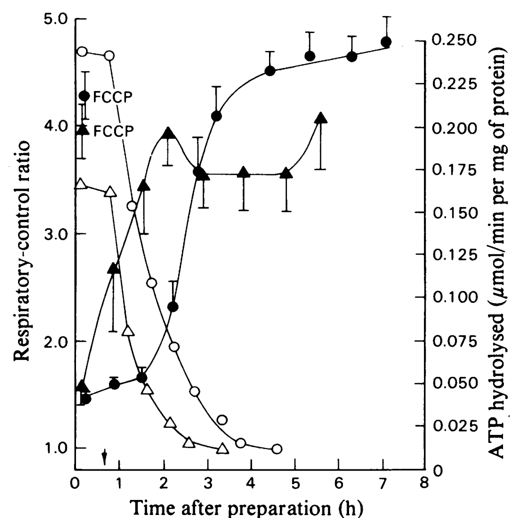

Fig. 3. *Loss of respiratory control and increase in ATPase activity with time of incubation at* 18°C

The arrow indicates the time at which the 18°C incubation was started. Mitochondria from control diet-fed animals are indicated by (○) and (●) and those from ethanol-fed animals by (△) and (▲). Open symbols (○, △) indicate respiratory-control ratio assays; solid symbols (●, ▲) indicate ATPase assays. ● FCCP and ▲ FCCP are the ATPase activities measured in mitochondria completely uncoupled with 1 nmol of carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone. The bars represent ±S.E.M. All points on the respiratory-control ratio decay curve for ethanol mitochondria are significantly lower than the corresponding points for control preparations ($P<0.05$, or lower). There were nine animals in each group.

(1.5h) was noted as mitochondria from control animals lost respiratory control. In contrast, the ATPase activity in mitochondria from ethanol-fed animals increased at a maximal rate from the onset of the 18°C incubation period, with maximal activity attained at the time respiratory control was lost. The maximal activity appeared to be somewhat higher in mitochondria from control animals, but statistical analyses did not verify that this was the case. The most obvious difference in mitochondria from the two groups was in the kinetics of the increase in ATPase activity during the time when the mitochondria lose their respiratory control.

The decrease in energy-linked properties observed in mitochondria isolated from ethanol-fed animals led us to begin an investigation to determine if there was an alteration in membrane composition that might influence energy conservation. Our analysis of mitochondrial phospholipids indicated no change in the phospholipid/protein ratio after 31 days of ethanol feeding (Table 2). Whereas total phospholipid content remained unchanged, some changes did occur in the fatty acid distribution within the mitochondrial phospholipids. Significant alterations in fatty acid distribution were seen between mitochondria preparations from the two control groups. Compared with mitochondria from chow-fed animals, those from liquid-diet-fed rats exhibited lower concentrations of palmitate and linoleate, and elevated oleate and arachidonate (Table 2). A comparison of mitochondria preparations from ethanol-fed animals with those from liquid-diet-control animals revealed that ethanol feeding initiates a further decrease of palmitate and an additional increase in oleate. The double-bond index, which increases in value as the proportion of unsaturated fatty acids increases, was unaltered by ethanol feeding, but a small difference was observed between the two control groups owing to the increase in arachidonate in liquid-diet animals. The value for total µmol of fatty acids present per mg of mitochondrial protein is twice the value for µmol of phospholipid P per mg of mitochondrial protein, and demonstrates a quantitative accuracy in recovery of the fatty acid methyl esters. These analyses do not preclude there being ethanol-induced alterations either in the concentrations or the fatty acid composition of individual phospholipids.

The measurements of phospholipase A_2 activity were carried out to determine if the changes in energy-linked functions induced in the mitochondria by chronic ethanol feeding rendered the organelles more susceptible to deacylation by this mitochondrial phospholipase. The radioactive ethanolamine injected intraperitoneally 40min before death is incorporated into tissue phospholipids. The mitochondria, from either ethanol-fed animals or control rats, are low in both non-esterified fatty acids and lysophosphatidyl-

Table 2. *Effects of ethanol feeding and diet on mitochondrial phospholipid composition*

The *n* values are 10 for the chow-fed animals and 12 for the liquid-diet controls and the ethanol-fed animals. Calculation of the double-bond index was done by multiplying the mol % value of each unsaturated fatty acid by the number of double bonds in that fatty acid, and then taking the sum of the values obtained. Abbreviation: n.s., not significant.

Animal group	Lipid P (µmol/mg of mitochondrial protein)	Fatty acid concn. (µmol/mg of mitochondrial protein)	Phospholipid fatty acid distribution (mol %)							Double-bond index
			$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	$C_{22:6}$	Other	
(A) Chow-fed	0.148±0.007	0.286±0.020	20.8±0.5	22.0±0.4	9.5±0.2	23.7±0.6	16.2±0.5	4.8±0.3	2.9±0.2	150.6±3.0
(B) Liquid-diet control	0.133±0.007	0.265±0.013	17.3±0.5	22.7±0.7	11.9±0.5	15.7±0.2	24.9±0.5	4.2±0.2	3.2±0.3	168.2±2.0
(C) Ethanol-fed	0.132±0.007	0.276±0.012	14.2±0.6	23.1±0.5	15.1±0.8	14.8±0.7	24.9±0.9	4.3±0.2	3.7±0.5	169.8±3.6
Comparison of (A) and (B)	n.s.	n.s.	$P<0.001$	n.s.	$P<0.001$	$P<0.001$	$P<0.001$	n.s.	n.s.	$P<0.001$
Comparison of (B) and (C)	n.s.	n.s.	$P<0.001$	n.s.	$P<0.005$	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3. *Effects of ethanol administration on mitochondrial phospholipase A_2 activity*
All assays and determinations are described in the Experimental section. The initial non-esterified fatty acid concentrations are expressed in nmol of total non-esterified fatty acids/mg of protein. The lysophosphatidylethanolamine concentrations are expressed in terms of the percentage of phosphatidylethanolamine converted into lysophosphatidylethanolamine. The maximum Ca^{2+}-stimulated phospholipase A_2 activities are the differences between the total phosphatidylethanolamine hydrolysed and hydrolysis occurring at 18°C; this activity is also expressed as the percentage of phosphatidylethanolamine converted into lysophosphatidylethanolamine.

Animal group	Initial non-esterified fatty acid concentrations	Initial lysophosphatidyl-ethanolamine concentrations	Lysophosphatidyl-ethanolamine concentrations after 12h incubation at 18°C	Maximum Ca^{2+}-stimulated phospholipase A_2 activity
(A) Chow-fed	—	2.9 ± 0.2 (10)	24.4 ± 5.8 (10)	14.4 ± 2.0 (10)
(B) Liquid-diet control	1.5 ± 0.3 (6)	3.3 ± 0.2 (12)	22.3 ± 3.7 (12)	17.2 ± 1.4 (12)
(C) Ethanol-fed	2.1 ± 0.3 (5)	2.9 ± 0.3 (12)	26.6 ± 4.0 (12)	18.3 ± 1.7 (12)

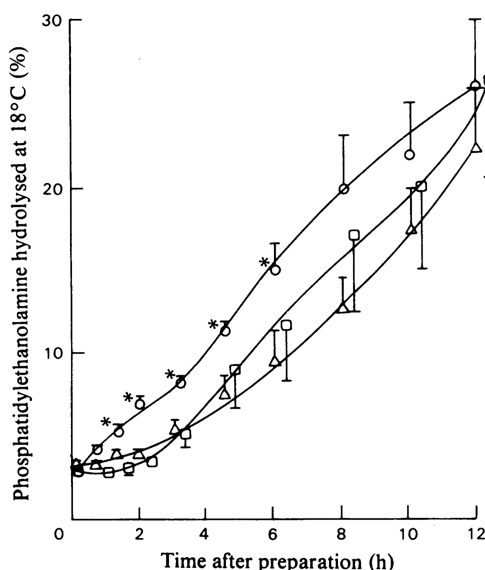

Fig. 4. *Hydrolysis of mitochondrial phosphatidylethanolamine by the endogenous phospholipase A_2 with time of incubation at* 18°C
Mitochondria from chow-fed (□), control diet-fed (△), and ethanol-fed (○) animals were aged at 18°C and analysed for concentrations of lysophosphatidylethanolamine and phosphatidylethanolamine at the times indicated. For details of assay, see the Experimental section. *Statistically significantly different from liquid-diet controls ($P < 0.05$, or lower).

ethanolamine (Table 3). No significant differences in the groups were observed with respect to non-esterified fatty acids or lysophosphatidylethanolamine concentrations, which indicates no appreciable phospholipase A activity initially or during the mitochondria preparation procedure. The values reported in Table 3 for non-esterified fatty acids represent approx. 1% of the total fatty acid complement from phospholipids. This value is lower than the amount of monoacyl phospholipid, as represented by lysophosphatidylethanolamine concentrations, and suggests that non-esterified fatty acids have been kept low by an active β-oxidation activity in the mitochondria preparations.

In the present study we have measured the activity of the phospholipase A under two separate conditions. As the mitochondria were aged at 18°C, the phospholipase hydrolysed phospholipids continuously. The percentage conversion of phosphatidylethanolamine into lysophosphatidylethanolamine after incubation of mitochondria at 18°C for 12h (Table 3) was not significantly different when mitochondria from ethanol-fed animals are compared with control preparations. To determine the maximal activity of the phospholipase on endogenous phospholipids, mitochondria previously aged at 18°C were incubated at 37°C in the presence of Ca^{2+}, an essential cofactor of the phospholipase. The maximal activity of the phospholipase towards endogenous phospholipids was obtained by the increase in lysophosphatidylethanolamine observed in the presence of Ca^{2+}. These measurements were carried out at several times during the 18°C incubation and the maximal additional hydrolysis in the presence of Ca^{2+} occurred at 8h after the 18°C incubation was begun. The maximum values for the additional hydrolytic activity were not significantly different when the three groups of mitochondria were compared. Although there were no significant differences in the amounts of phospholipids hydrolysed between mitochondria from ethanol-fed animals and preparations from the two control groups, there was a significant change in the time of onset of phospholipase A_2 activity during mitochondrial aging (Fig. 4). Mitochondria from control animals of both groups exhibited a lag period of 2–3h before lysophosphatidylethanolamine concentrations began to rise. This lag period was not evident in mitochondria from the ethanol-fed group. The earlier onset of phos-

pholipase A_2 activity resulted in significantly higher concentrations of lysophosphatidylethanolamine during the 1–6h portion of the aging period.

Discussion

We have observed a significantly lowered energy state in mitochondria isolated from chronic alcoholic rats. This lowered energy state is reflected in a 57% decrease in ATP in freshly prepared mitochondria, a lowered respiratory-control ratio with succinate as a substrate, and a decreased ability of the mitochondria to elicit the energy-linked ANS fluorescence response. Cederbaum *et al.* (1974), by using animals fed with the DeCarli & Lieber (1967) diet for 24 days, demonstrated that energy conservation was significantly decreased only when NAD^+-linked substrates were used as electron donors. Utilizing the same diet for 31 days, we have been able to establish that succinate-driven energy-linked properties of mitochondria are also diminished. We have determined that respiratory control with succinate as substrate is lowered in mitochondria from ethanol-fed animals, owing to decreased State-3 respiration. This observation has also been made in studies where animals were fed for even longer periods of time (Thompson & Reitz, 1976) or where slightly higher concentrations of ethanol were used (Banks *et al.*, 1969). Clearly both site I (NADH–ubiquinone coupling site) and site II (ubiquinone–cytochrome *c* coupling site) regions of the oxidative-phosphorylation system are affected by ethanol feeding, with site I appearing to be affected earlier during the feeding period. It is quite possible that there is a progression of alterations occurring in the mitochondrion, which ultimately affects all coupling sites of the oxidative-phosphorylation system when ethanol feeding is extended.

Further support for the idea that energy conservation is decreased in regions of the oxidative-phosphorylation system other than site I comes from the observation that the energy-linked ANS fluorescence response is lowered in mitochondria from ethanol-fed animals. Conditions under which these measurements were performed precluded electron flow through the NADH–ubiquinone segment of the electron-transport chain. This additional measure of energy conservation with succinate as oxidizable substrate provides further evidence that chronic ethanol consumption lowers energy conservation in the site II–site III segment, in addition to its effects on site I.

As mentioned above, the decrease in succinate-driven respiratory control is due to a lowered State-3 respiration, rather than an elevated State-4 rate. As suggested earlier (Gordon, 1973; Thompson & Reitz, 1976), this lowered State-3 respiration may be due to a decrease in adenine nucleotide translocation. Both Gordon (1973) and our laboratory (P. I. Spach & C. C. Cunningham, unpublished work) have observed decreased adenine nucleotide translocation in mitochondria from ethanol-fed animals. Furthermore, our observation that ATP concentrations are dramatically lowered in mitochondria from ethanol-fed animals suggests that under respiratory-control ratio assay conditions ADP translocation might be impeded, owing to lowered ATP amounts available for adenine nucleotide exchange.

The kinetics of loss of energy-linked parameters suggest that the lowered respiratory control, energy-linked ANS fluorescence response and endogenous ATP concentrations are an indication of the energy state of the mitochondrion within the cell. From Figs. 1 and 2, it is apparent that the rates of loss of the energy-linked parameters are the same in mitochondria from ethanol-fed and liquid-diet-control animals, whereas the initial values are lower in ethanol-fed animals in all three cases. If the lowered energy-linked properties observed in mitochondria from ethanol-fed animals are due to increased susceptibility to damage after tissue homogenization, rather than a different energy state within the intact cell, an increased rate of loss of the energy-linked properties would have been expected during the 18°C incubation period. The equivalent rates of loss of energy-linked properties suggest that within the cell the energy state of the mitochondrion is lowered owing to ethanol administration.

It has been suggested previously that alterations in phospholipid amounts or composition may be related to changes in the energy state of mitochondria from ethanol-fed animals (Banks *et al.*, 1969; French & Morin, 1969; French & Todoroff, 1970). Indeed, a number of studies have demonstrated in rats that chronic ethanol feeding will affect the phospholipid fatty acid composition of liver mitochondria (French *et al.*, 1971; Thompson & Reitz, 1976) in addition to its effect on energy metabolism within the same organelle. In our study, when comparing mitochondria from liquid-diet controls with preparations from ethanol-fed animals, we observed no change in the phospholipid/protein ratio and only minor changes in the fatty acid composition of these phospholipids; we noted dramatic differences in energy-linked properties, however. In contrast, when mitochondria from chow-fed animals were compared with those from liquid-diet controls, significant differences in fatty acid distribution of the phospholipids were observed with no differences observed in energy-linked properties between the two groups. These results demonstrate that conditions can be established for feeding ethanol that dissociate the loss of energy-linked functions from changes in phospholipid/protein ratios or major alterations in the distribution of fatty acids within phospholipids. When alterations in mitochondrial phospholipid composition do occur, they may well affect the energy

state of the organelle; our results indicate, however, that there are additional factors that influence energy-linked properties other than phospholipid/protein ratio and fatty acid distribution within phospholipids. Clearly, more extensive characterization of individual phospholipids is required, since there is a possibility that the phospholipid microenvironment of components of the oxidative-phosphorylation system might be altered by ethanol administration.

The observations with the membrane-associated ATPase and phospholipase A_2 activities (Figs. 3 and 4) indicate that the organization of the mitochondrial membrane is altered by ethanol consumption. Both the ATPase and the phospholipase A from control mitochondria demonstrate a lag period before their activities increase appreciably during the 18°C incubation period. In contrast, these activities increase without a lag from the onset of the incubation in mitochondria from ethanol-fed animals. These altered kinetics suggest that the organization of the membranes has been modified in mitochondria from ethanol-fed animals such that these particular catabolic enzyme activities are not subject to the control evidenced in mitochondria from control animals when the organelles are placed in a stressful environment. With both the ATPase and phospholipase A_2 the initial and final activities were not significantly different in mitochondria from ethanol-fed animals and control rats. The major alteration is in the ability of the mitochondria to control these activities under stressful conditions such as the 18°C incubation. Our observations with the ATPase and phospholipase are in basic agreement with those of Hosein *et al.* (1977), who have provided evidence that the microenvironment around the mitochondrial ATPase is altered in mitochondria from ethanol-fed animals.

In this study we measured the activity of the mitochondrial ATPase under iso-osmotic conditions. Although these conditions minimized disruption of the mitochondrion during assay they probably have not afforded us an accurate assessment of the ATPase activity of the ATP synthetase complex as it exists in the presence of endogenous pools of adenine nucleotides and oxidizable substrates. It must be noted that in these assays an exogenous source of ATP was added to the mitochondrion, which may have induced a higher ATPase activity than is present under conditions of no added substrate. If an activity existed in the mitochondrion equivalent to the initial activities reported in Fig. 3 the endogenous ATP would have been hydrolysed within 40s (this value was calculated by using the concentrations of ATP and ATPase in the mitochondrion, the volume of the mitochondrion, and the catalytic-centre activity of the mitochondrial associated ATPase). Therefore the ATPase activities reported in Fig. 3 cannot be used to explain the kinetics of ATP loss in Fig. 1. The loss in ATP probably reflects relative rates of synthesis of ATP-utilizing endogenous substrates, hydrolysis via ATPase activity, and utilization via mitochondrial activation reactions (Parce *et al.*, 1978).

Previous studies (Siliprandi *et al.*, 1973; Ozelkok & Romani, 1974) have demonstrated that mitochondrial energy-linked functions can be restored in isolated mitochondria that have been aged under conditions where the phospholipase A_2 activity is depressed. Previous work in this (Parce *et al.*, 1978) and other laboratories (Scarpa & Lindsay, 1972) concerning the sequence of events in mitochondrial aging has demonstrated that the endogenous phospholipase A_2 contributes to disruption of mitochondrial structure by hydrolysing membrane phospholipids. Once this degradation occurs, the mitochondrion loses the potential for restoration of energy-linked functions and is irreversibly damaged. In the present study we have presented evidence that demonstrates that chronic ethanol ingestion not only lowers the overall energy state of the mitochondria, but also elicits alterations in the microenvironments of enzymes that have the potential to lower the energy state further (ATPase) and disrupt mitochondrial structure (phospholipase A_2). The physiological relevance of these observations remains to be established, but this study does suggest that *in vivo* the mitochondria from ethanol-fed individuals may exist at a lowered energy state and may be lacking in the ability to control catabolic enzymes. This in turn may compromise the ability of the cell to deal with the stress of continued exposure to ethanol and acetaldehyde.

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