Control of adenine nucleotide metabolism in hepatic mitochondria from rats with ethanol-induced fatty liver

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Male rats developed fatty liver after being fed on an ethanol-containing diet for 31 days. Liver mitochondria from these animals catalysed ATP synthesis at a slower rate when compared with mitochondria from pair-fed control rats (control mitochondria), and demonstrated lowered respiratory control with succinate as substrate, owing to a decrease in the State-3 respiratory rate. Respiration in the presence of uncoupler was comparable in mitochondria from both groups of rats. Translocation of both ATP and ADP was decreased in mitochondria from ethanol-fed rats, with ADP uptake being lowered more dramatically by ethanol feeding. Parameters influencing adenine nucleotide translocation were investigated in mitochondria from ethanol-fed rats. Experiments performed suggested that lowered adenine nucleotide translocation in these mitochondria is not the result of inhibition of the translocase by either long-chain acyl-CoA derivatives or unesterified fatty acids. Analysis of endogenous adenine nucleotides in these mitochondria revealed lowered ATP concentrations, but no decrease in total adenine nucleotides. In experiments where the endogenous ATP in these mitochondria was shifted to higher concentrations by incubation with oxidizable substrates or defatted bovine serum albumin, the rate of ADP translocation was increased, with a linear correlation being observed between endogenous ATP concentrations and the rate of ADP translocation. The depressed ATP concentration in mitochondria from ethanol-fed rats suggests that the ATP synthetase complex is replenishing endogenous ATP at a slower rate. The lowered ATPase activity of the ATP synthetase observed in submitochondrial particles from ethanol-fed animals suggests a decrease in the function of the synthetase complex. A decrease in the rate of ATP synthesis in mitochondria from ethanol-fed rats is sufficient to explain the decreased ADP translocation and State-3 respiration.

Gordon (1977) has demonstrated that the content of ATP is decreased by approx. 50% in hepatic tissue of rats maintained on an ethanol-containing diet for 4 weeks. Although not eliminating the possibility of increased demand for ATP in hepatic tissue during ethanol consumption, the above study emphasized the probable link between lowered liver mitochondrial energy metabolism and decreased phosphate potential in hepatic tissue of animals that had developed ethanol-induced fatty liver. In studies where ATP synthesis has been determined by measuring the P/O ratio (Christiansen *et al.*, 1969) it has been observed that mitochondria from ethanol-fed animals have lowered capacity for oxidative phosphorylation. Cederbaum *et al.* (1974), utilizing

NADH-linked substrates, observed both a lowered P/O ratio and a depressed rate of ATP synthesis in mitochondria from ethanol-fed rats. With succinate as substrate a slower rate of ATP synthesis was observed, but the P/O ratio was not significantly affected. Thayer & Rubin (1979), using NADH and succinate, noted a slightly lowered P/O ratio (10–12%) and a substantial decline (35–41%) in the rate of ATP synthesis with submitochondrial particles prepared from ethanol-fed rats. In the present study we have also observed a lowered rate of ATP synthesis, but not a decline in P/O ratio, in mitochondria from ethanol-fed rats. The above observations document that the rate of ATP synthesis is affected to a greater degree than is energy coupling (P/O ratio) in mitochondria from ethanol-fed animals. This decreased rate of ATP

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synthesis may well affect cytosolic concentrations of ATP in hepatic tissue.

In this paper we report an investigation of those factors that influence the rate at which ATP is synthesized in liver mitochondria from ethanol-fed rats. This study, which focuses on ethanol-induced alterations in the control mechanisms for ADP transport into mitochondria, suggests that the ATP synthetase complex (Pedersen *et al.*, 1978) functions at a lower rate in mitochondria from rats that have developed ethanol-induced fatty liver. Furthermore, this study demonstrates for the first time the interrelationships between the ethanol-induced alterations in respiratory activity, ADP translocation, endogenous concentrations of adenine nucleotides, and the rate of ATP synthesis in liver mitochondria. A preliminary report of this work has appeared elsewhere (Cunningham *et al.*, 1979).

Experimental

Materials

Male Sprague-Dawley rats (150–200g) and many of the reagents were obtained from sources listed previously (Spach *et al.*, 1979; Parce *et al.*, 1980). Myokinase, lactate dehydrogenase, NAD⁺, NADH and NADPH were purchased from Boehringer Mannheim Corp. (Munich, Germany). Glucose, atractyloside, DL-carnitine, cytochrome *c* (type III), β -glycerophosphate, AMP, dithiothreitol, α -oxoglutarate and CoA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fatty acid-free bovine serum albumin (Fracton V) was purchased from Miles Research Laboratories (Elkhart, IN, U.S.A.). Palmitoyl-CoA was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.).

Methods

Male Sprague-Dawley rats weighing 150–250g 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. Preparation of tightly coupled mitochondria, measurement of respiratory rates and protein determinations were performed as described previously (Spach *et al.*, 1979). Respiratory rates in the presence of uncoupler were measured in an assay mixture containing 0.56 μ M-carbonyl cyanide *p*-trifluorophenylhydrazone. The rate of ATP synthesis was determined by the procedure for P/O-ratio measurements described previously (Parce *et al.*, 1980). Succinate (20mM) was the oxidizable substrate. ATP synthesis was allowed to proceed for 6.5 min, at which time a sample of the assay mixture was removed for measurement of its ATP content (Parce *et al.*, 1980). The cytochrome oxidase,

NADPH-cytochrome *c* reductase and acid phosphatase activities of the mitochondria preparations were determined as described previously (Morton *et al.*, 1976).

The endogenous adenine nucleotide (ATP, ADP and AMP) content of mitochondria was measured by the method of Williamson & Corkey (1969) in freshly prepared mitochondria and in mitochondria incubated with defatted albumin or oxidizable substrates. Samples of mitochondria incubated in the adenine nucleotide translocase assay buffer with defatted albumin (see procedures below) were centrifuged at 46 000 *g* for 5 min, rinsed with 0.5 ml of 0.25 M-sucrose, resuspended in 0.2 ml of 0.25 M-sucrose and then analysed for their adenine nucleotide content.

Translocation of ATP and ADP was measured in the forward direction at 0°C by the inhibitor-stop technique (Vignais, 1976), as described previously (Parce *et al.*, 1980). One modification was utilized in some of the experiments. Two techniques were used to wash the mitochondria free of unincorporated radiolabelled adenine nucleotide. The first was a washing procedure utilizing centrifugation, described by Parce *et al.* (1980). In the second the assay mixtures were quenched with atractyloside as previously (Parce *et al.*, 1980), and immediately filtered over Whatman GF/F glass-microfibre filters and washed free of unincorporated adenine nucleotide with 0.25 M-sucrose. The filters were blotted free of adhering 0.25 M-sucrose and measured for radioactivity in a solution containing 1 ml of 10% (w/v) deoxycholate and 10 ml of Ready-Solv VI (Beckman). Although both procedures gave comparable results, the filtration procedure was preferred because it decreased sample handling time. $V_{\max}$ and K_m values for ADP translocation were measured by the filtration assay, with the ADP concentration being varied from 2.5 to 20 μ M. ADP uptake was allowed to proceed for 30 s, the time period during which ADP translocation occurred more rapidly under the above assay conditions (see Fig. 1).

In those assays where the effects of defatted albumin on ADP translocation were measured, mitochondria were preincubated in the translocase assay medium with defatted albumin (1 mg/mg of mitochondrial protein) for 4 min at 0°C. Control samples without albumin were incubated identically. Samples were washed in preparation for adenine nucleotide analyses (see above). The assays were initiated by the addition of radiolabelled ADP immediately after the incubation period. In assays where ADP translocation was measured in the presence of carnitine, mitochondria (5–6 mg of protein) were incubated for 10 min at 22°C in the translocase assay medium, which also included either 11.0 μ mol of carnitine, pH 7.4, or 12.9 μ mol of

Tris/HCl, pH 7.4. The assay mixture was then cooled to 0°C in an ice slurry before the assay was initiated.

In one experiment, oxidizable substrate was added to shift the adenine nucleotide distribution in the mitochondrion toward ATP. Mitochondria (70 mg) were incubated for 1 min at 10°C in 125 mM-sucrose/60 mM-KCl/10 mM-Tris/HCl (pH 7.4)/2 mM-glutamate/2 mM-malate(0.5 mM-P_i in a total volume of 4 ml. Oligomycin (16 µg) was added, samples were quick-frozen in liquid N₂ for adenine nucleotide determinations, and the incubation suspension was cooled immediately to 1°C in a salt/ice slurry (-5°C). Portions (7 mg of mitochondrial protein) were then assayed for ADP-translocation activity at 0°C.

Long-chain acyl-CoA derivatives were measured as described by Tubbs & Garland (1969). Their procedure 7 was utilized for deacylation, with palmitoyl-CoA used as a standard. The α -oxoglutarate dehydrogenase reaction was used to measure CoA concentrations; α -oxoglutarate dehydrogenase was purified by the procedure of Sanadi (1969).

Phosphorylating submitochondrial particles were prepared from liver mitochondria by the method of Thayer & Rubin (1979). The uncoupler-stimulated ATPase activity of these particles was measured by including 10 µM-carbonyl cyanide *p*-trifluorophenyl-hydrazone in the assay described by Cunningham & George (1975).

The values reported in the Tables and Figures are

the averages $\pm$ S.E.M. for the numbers indicated. Statistical analyses were performed by using Student's *t* test for two means, employing a program from the Hewlett-Packard User's Library (Library No. 00115D). In this study differences between groups that gave *P* values of less than 0.05 were considered significant.

Results

Rate of ATP synthesis and adenine nucleotide translocation

As is shown in Table 1, the rate of ATP synthesis with succinate as oxidizable substrate is decreased by approx. 30% in mitochondria isolated from ethanol-fed animals. Respiratory control in such mitochondria is decreased by about the same amount. This fall in respiratory control ratio is due to a decrease in the rate of electron transport in the presence of ADP. The rate in the absence of ADP is also diminished, but not nearly to the degree observed during State-3 (Chance & Williams, 1956) respiration. Uncoupler-stimulated respiration was unchanged by ethanol feeding when measured in several pairs of animals. The data in Table 1 demonstrate a major decrease in respiration in the presence of ADP which is not observed when mitochondria from ethanol-fed rats are uncoupled. It suggests that the adenine nucleotide-transport mechanism (Klingenberg, 1976), rather than the electron-transport chain, is responsible for the decreased respiratory activity under conditions where

Table 1. *Effects of ethanol administration on ATP synthesis and electron-transport properties of rat liver mitochondria*
Abbreviation: N.S., not significant.

	Liquid-diet control	Ethanol-fed	Decrease (%)	<i>P</i> values
Expt. 1*				
ATP synthesized (µmol/min per mg of protein)	0.35 $\pm$ 0.03	0.25 $\pm$ 0.02	29	<0.01
P/O ratio	1.18 $\pm$ 0.12 $\parallel$	1.16 $\pm$ 0.08	2	N.S.
Expt. 2†				
Respiratory control ratio	4.85 $\pm$ 0.09	3.62 $\pm$ 0.12	25	<0.001
Rate of electron transport (µg-atoms of O/min per mg of protein)				
State 4 (-ADP)	0.058 $\pm$ 0.003	0.050 $\pm$ 0.003	14	<0.05
State 3 (+ADP)	0.27 $\pm$ 0.01	0.17 $\pm$ 0.01	36	<0.001
Expt. 3‡				
+Uncoupler	0.28 $\pm$ 0.01	0.29 $\pm$ 0.01	—	N.S.
Expt. 4§				
<i>V</i> _{max} for ADP translocation (nmol/min per mg)	1.66 $\pm$ 0.11	1.28 $\pm$ 0.07	23	<0.025
<i>K</i> _m for ADP translocation (µM)	7.15 $\pm$ 0.70	6.84 $\pm$ 0.65	4	N.S.

* Fifteen animals in each group.

† Forty-eight animals in each group.

‡ Six control animals and five ethanol-fed animals; one preparation of the pair-fed ethanol animals did not exhibit respiratory control and was omitted.

$\parallel$ Values obtained from mitochondria from chow-fed animals = 1.59 $\pm$ 0.02 (*n* = 4); see also data in Parce *et al.* (1980).

§ Six animals in each group.

ATP is being synthesized at a maximal rate. Measurements of adenine nucleotide-translocation activity (Fig. 1) confirmed the above suggestion. ADP translocation into mitochondria is significantly lowered in organelles isolated from ethanol-fed animals. This observation was confirmed by measuring $V_{\max}$ from reciprocal plots of ADP uptake rate versus ADP concentration. As shown in Table 1, $V_{\max}$ is significantly decreased, but K_m for ADP is essentially unchanged in mitochondria from ethanol-

fed animals. Fig. 1 illustrates that ATP translocation is also decreased, but not to the extent observed for ADP.

Effect of ethanol feeding on organelle marker-enzyme activities

The possibility existed that adenine nucleotide transport was decreased in mitochondria from ethanol-fed rats owing to increased damage to the organelle by higher contamination by microsomal membranes and lysosomes in the mitochondrial preparations from ethanol-fed animals. To assess this possibility, marker-enzyme activities were measured on mitochondria from both control and ethanol-fed rats. The data in Table 2 demonstrate that the NADPH-cytochrome *c* reductase (microsomal marker) and acid phosphatase (lysosomal marker) specific activities are not significantly different in both kinds of mitochondria. Furthermore, Mezey *et al.* (1976) observed decreased content of lysosomal-associated catabolic enzymes in liver homogenates of ethanol-fed rats. The data in Table 2 and the work of Mezey *et al.* (1976) make it very unlikely that increased catabolic activity from either contaminating microsomal membranes of lysosomes is a factor in lowering adenine nucleotide-translocation activity in mitochondria from ethanol-fed rats. The specific activity of cytochrome oxidase, the mitochondrial marker, was decreased by 42% in preparations from ethanol-fed animals.

Fig. 1. Effects of ethanol feeding on ATP and ADP translocation in liver mitochondria

The centrifugation washing procedure was employed. The bars indicate S.E.M.; $n = 9$ for each group. ADP translocation in mitochondria from ethanol-fed rats was significantly lower at 60 and 120 s ($P < 0.05$, Student's *t* test). An analysis of variance (Jennerich & Sampson, 1977) indicated that the rates of ATP translocation were significantly different in mitochondria from ethanol-fed and control animals ($P = 0.025$). O, Control rats; Δ, ethanol-fed rats.

Lipophilic inhibitors and ADP-translocation activity

Unesterified fatty acids and long-chain acyl-CoA compounds are known to inhibit the activity of the mitochondrial adenine nucleotide-translocation system (Lerner *et al.*, 1972). If the translocase were being inhibited by either of these naturally occurring inhibitors, addition of defatted albumin to adsorb the lipophilic compounds should cause increased adenine nucleotide translocation. Mitochondria were incubated for 4 min with defatted albumin before being assayed for ADP-translocation activity. This preincubation resulted in ADP translocation being significantly increased in mitochondria from ethanol-fed animals (Fig. 2); no significant changes in ADP translocation were observed in control mitochondria. These observations supported the possibility of

Table 2. Marker-enzyme activities in mitochondria from ethanol-fed and control rats
There were six animals in each group. Abbreviation: N.S., not significant.

Animal group	Enzyme activity (nmol/min per mg of protein)		
	Cytochrome oxidase	NADPH-cytochrome <i>c</i> reductase	Acid phosphatase
Liquid-diet control	780 ± 86	5.9 ± 0.4	93 ± 8
Ethanol-fed	453 ± 42	4.9 ± 0.6	75 ± 6
Comparison of groups	$P < 0.005$	N.S.	N.S.

Fig. 2. Effect of defatted albumin on ADP translocation

The centrifugation washing procedure was employed. Seven control and eight ethanol-fed animals were used. One control preparation did not exhibit respiratory control and was not included in the study. Mitochondria from ethanol-fed rats preincubated with defatted albumin exhibited significantly higher translocase activity ($P < 0.025$ at 30 and 60 s; $P < 0.05$ at 120 s). Control mitochondria showed no significant differences. Δ , With defatted albumin; $\circ$, no albumin.

inhibition by naturally occurring lipophilic compounds. In a previous study we determined that the concentrations of unesterified fatty acids in mitochondria from ethanol-fed and control animals were not significantly different (see Table 3 of Spach *et al.*, 1979). Furthermore, the concentrations were not high enough to inhibit adenine nucleotide-translocation activity (Wojtczak & Zaluska, 1967). We have also measured the concentrations of long-chain acyl-CoA compounds in mitochondria from ethanol-fed and control rats, which were identical ($0.46 \pm 0.03 \mu\text{mol/mg}$ of mitochondrial protein).

Even though the concentrations of long-chain acyl-CoA derivatives were identical in mitochondria from ethanol-fed and control animals, the possibility remained that equivalent concentrations of these lipophilic compounds could cause a greater inhibition of ADP translocation in the former mito-

chondria. To assess this possibility mitochondria were incubated with carnitine (see the Experimental section) to convert the acyl-CoA into acylcarnitine and free CoA via the fatty-acyl-CoA :carnitine fatty acid transferase reaction. Since acylcarnitine and CoA are not inhibitors of the adenine nucleotide translocase (Lerner *et al.*, 1972), conversion of long-chain acyl-CoA should result in increased ADP translocation if inhibition in mitochondria from ethanol-fed rats is due to the presence of long-chain acyl-CoA. Preincubation with carnitine did not result in increased translocase activity in mitochondria from either control or ethanol-fed rats. In a control experiment ADP translocation was measured after mitochondria were incubated in the presence of $6 \mu\text{M}$ -palmitoyl-CoA (5 min at 0°C) or $6 \mu\text{M}$ -palmitoyl-CoA followed by carnitine. Treatment with palmitoyl-CoA decreased ADP translocation to 64%, and carnitine restored this activity to 80% of the original activity of untreated mitochondria. These observations with carnitine suggest that equivalent concentrations of long-chain acyl-CoA do not cause greater inhibition in mitochondria from ethanol-fed rats. The effect of defatted albumin in increasing ADP translocation therefore does not appear to be related to its property of adsorbing lipophilic inhibitors of the adenine nucleotide translocase.

Endogenous adenine nucleotides and ADP-translocation activity

Since the adenine nucleotide translocase normally functions to exchange cytoplasmic ADP for mitochondrial-matrix ATP, the possibility existed that lowered ADP translocation in mitochondria from ethanol-fed rats was related to decreased concentrations of endogenous ATP. Measurements of mitochondrial concentrations of adenine nucleotides (Table 3) demonstrated a significant decrease in ATP and an increase in AMP in those from ethanol-fed rats. Total adenine nucleotide concentrations in mitochondria from ethanol-fed and control rats were not significantly different.

If endogenous ATP concentrations were limiting the rate of ADP translocation in mitochondria from ethanol-fed rats, then conditions that increase matrix ATP should increase ADP translocation. Such mitochondria were therefore preincubated with glutamate, malate and P_i to shift the endogenous adenine nucleotide distribution toward ATP. After preincubation they were assayed for ADP-translocation activity. Fig. 3 demonstrates that indeed ADP translocation is increased dramatically under conditions where ATP is increased in these mitochondria. Comparable increases were observed with control mitochondria, but with the initial activity and that after preincubation with glutamate + malate + P_i being higher than activities observed with mito-

Table 3. *Effect of chronic ethanol consumption on the adenine nucleotide content of mitochondria*

There were 12 animals in each group. The values reported are in nmol/mg of mitochondrial protein, $\pm$ S.E.M. Abbreviation: N.S., not significant.

Animal group	ATP	ADP	AMP	Total adenine nucleotides
Liquid-diet control	4.96 ± 0.35	2.23 ± 0.19	4.49 ± 0.40	11.06 ± 0.85
Ethanol-fed	2.94 ± 0.26 $P < 0.001$	2.66 ± 0.34 N.S.	6.54 ± 0.39 $0.005 > P > 0.001$	12.14 ± 0.67 N.S.

Fig. 3. Relationship between endogenous ATP content and ADP translocation in mitochondria from ethanol-fed rats

These mitochondria were incubated with 2 mM-glutamate/2 mM-malate/0.5 mM- P_i (O) as described in the Experimental section. The ATP content and ADP translocation activity of incubated samples were compared with those of the same mitochondria that were not incubated (Δ). The filtration washing procedure was employed, with five mitochondria preparations. Values in parentheses are nmol of mitochondrial-associated ATP/mg of mitochondrial protein.

chondria from ethanol-fed rats (results not shown). These results are in agreement with the studies of Klingenberg (1976), who also observed increased ADP translocation when endogenous ATP concentrations were elevated by incubation of mitochondria with oxidizable substrate and P_i .

The above observations suggested the possibility that incubation with defatted albumin increased ADP translocation in mitochondria from ethanol-fed rats by eliciting elevated concentrations of endo-

Fig. 4. Effects of defatted albumin on ATP contents and ADP translocation in mitochondria

After incubation with defatted albumin, mitochondria were washed by centrifugation (see the Experimental section) before adenine nucleotides were measured. ADP translocation activities were measured on incubated, but unwashed, preparations. The filtration washing procedure was employed in the translocation assay. Atractyloside-sensitive ADP translocation was measured for 2 min. $n = 7$ for each group. Experimental protocol was as follows.

Symbol	Animal source	Incubation
O	Ethanol-fed	No additions
Δ	Ethanol-fed	Defatted albumin
$\square$	Liquid-diet control	No additions
∇	Liquid-diet control	Defatted albumin

genous ATP. To examine this possibility, ADP translocation and endogenous ATP concentrations were measured in mitochondria from both ethanol-fed and control rats, preincubated with defatted albumin, and also with the same mitochondria preparations incubated identically but without albumin. As shown in Fig. 4, there was a substantial increase in ATP content when mitochondria from ethanol-fed rats were incubated with defatted albumin. Coincident with the increase in endogenous ATP was a decrease in endogenous AMP content (results not shown), demonstrating a redistribution of the total mitochondrial adenine nucleotide to-

ward ATP as a result of preincubation with defatted albumin. There was also a smaller shift of endogenous ATP to higher concentrations in control mitochondria preincubated with the defatted albumin.

Fig. 4 also demonstrates that there is a direct relationship between the concentration of endogenous ATP and the amount of ADP translocated into the mitochondria in 2 min. This relationship appears to be applicable for mitochondria from both control and ethanol-fed animals. The experiment thus demonstrates that in the presence of defatted albumin both endogenous ATP concentrations and ADP translocation increase in mitochondria by a mechanism not yet established.

Endogenous ATP concentrations might be lower in mitochondria from ethanol-fed animals owing to decreased activity of the ATP synthetase complex. The uncoupler-stimulated ATPase activity of the ATP synthetase complex, when measured in phosphorylating submitochondrial particles, is decreased by 22% in preparations from ethanol-fed rats ($P < 0.025$). The ATPase activities were 4.55 ± 0.34 and $3.56 \pm 0.20 \mu\text{mol}/\text{min}$ per mg of protein for submitochondrial particles from eight control and nine ethanol-fed animals respectively.

Discussion

The observations in this paper demonstrate clearly that the rate of ATP synthesis is decreased in liver mitochondria isolated from ethanol-fed rats. The conditions utilized for measuring the rate of ATP synthesis (ADP added and excess succinate) were similar to those utilized in measuring State-3 respiration, which was also decreased by about the same amount in such mitochondria. This suggests a linkage between the decreased rate of ATP synthesis and lowered State-3 respiration in mitochondria from ethanol-fed animals. The possibility therefore exists that ATP synthesis is being controlled by the rate of respiration in such mitochondria; alternatively, the rate of State-3 respiration may be dictated by the rate at which ATP is synthesized. A third possibility is the rate of ATP synthesis is decreased because the mitochondria have lowered ability to conserve energy, i.e. they are less coupled.

The third possibility appears remote for several reasons. First, the P/O ratio measured with succinate as substrate was not significantly different in mitochondria from ethanol-fed and control animals (Table 1). Furthermore, the State-4 respiratory rate is lowered rather than elevated in the former mitochondria; an increase in State-4 respiration would be expected if the mitochondria were less coupled. In addition, the ATPase activity in tightly coupled mitochondria is comparable in organelles

from ethanol-fed and control animals (Spach *et al.*, 1979). Increased ATPase activity is another indicator of less-coupled mitochondria. Although these observations do not prove that mitochondria from ethanol-fed animals are comparable with control preparations in their ability to conserve energy, they are consistent with the supposition that there is no significant difference in energy coupling between organelles isolated from ethanol-fed and control animals maintained on the feeding regimen used in this study.

There are several factors that might affect State-3 respiration in mitochondria from ethanol-fed rats. It could be decreased as a result of the lowered cytochrome oxidase activity observed in such mitochondria. Cytochrome oxidase, however, does not appear to be rate-limiting for State-3 respiration in the preparations employed in this study. This is indicated by the observation that respiration in the presence of uncoupler is the same in mitochondria from ethanol-fed and control rats. Furthermore, the respiratory rate of uncoupled mitochondria is increased significantly over the State-3 respiratory rate in preparations from ethanol-fed animals. These observations suggest that the respiratory chain, including cytochrome oxidase, is not limiting the respiratory rate in the presence of added ADP in such mitochondria. A second possibility for the decreased State-3 respiratory rate in such mitochondria is the slower rate of ADP translocation observed in these organelles. Recognizing the important role of adenine nucleotide translocation in the ATP-synthesis process and its potential for controlling State-3 respiration (Davis & Davis-van Thienen, 1978; Lemasters & Sowers, 1979; Parce *et al.*, 1980), those factors were examined that could cause lowered ADP translocation in mitochondria from ethanol-fed rats.

Inhibition of the translocase by naturally occurring lipophilic compounds was eliminated as a possibility with the preparations utilized in this study. This conclusion is not in agreement with the earlier observations of Gordon (1977), which suggested that ethanol-related inhibition of the translocase was due to increased amounts of long-chain acyl-CoA in organelles from ethanol-fed rats. In the present study, however, no difference in long-chain acyl-CoA contents was observed and the increased translocation in the presence of defatted albumin appeared to be related to an increase in exchangeable ATP. These latter observations demonstrate that decreased adenine nucleotide translocation in mitochondria from ethanol-fed rats is not always related to inhibition by long-chain acyl-CoA. Clearly other factors, including the concentration of exchangeable endogenous ATP, are important in controlling the rate of ADP uptake into mitochondria isolated from ethanol-fed animals.

The experiments in which ATP concentrations were shifted to higher values by preincubation with substrate (Fig. 3) or albumin (Fig. 4) make it clear that the rate of ADP translocation does vary in mitochondria from ethanol-fed rats depending on the concentration of exchangeable ATP. These experiments further suggest that ADP translocation is decreased in such mitochondria because of lower endogenous ATP concentrations. The data in Table 3 indicate not only that ATP is lowered in such mitochondria, but also demonstrate that the total adenine nucleotide contents of mitochondria from ethanol-fed and control rats are essentially the same. It is the distribution of adenine nucleotide that is altered rather than the amount available as substrate for the ATP synthetase. This observation suggests that in mitochondria from ethanol-fed rats the rate of ADP translocation and the State-3 respiratory rate are controlled by the rate of ATP synthesis, rather than vice versa. The ATP synthetase complex may not be functioning at a rate rapid enough to maintain normal concentrations of ATP. The lowered content of exchangeable ATP depresses the rate at which ADP can be translocated into the mitochondrion. The slower rate of ATP synthesis by the ATP synthetase would also result in a depressed rate of utilization of the energy available from the electron-transport process, thereby controlling the rate of oxidation of substrate.

There is evidence that the ATP synthetase has lowered activity in liver mitochondria from ethanol-fed animals. Its ATPase activity is decreased both in mitochondria and in submitochondrial particles prepared from ethanol-fed rats. We previously observed decreased ATPase activity in aged (uncoupled) mitochondria from ethanol-fed rats (Spach *et al.*, 1979), which is statistically significant from that of aged control mitochondria, as indicated by the paired *t* test. Rottenburg *et al.* (1980) have also reported lowered ATPase activity in mitochondria from ethanol-fed rats. In addition, Hosein *et al.* (1977) and Thayer & Rubin (1979) have demonstrated decreased activity in submitochondrial particles from ethanol-fed rats, and their observations were confirmed in the present study (see the Results section, last paragraph). A more direct indicator of the ability of the ATP synthetase to function in ATP synthesis is its ATP- P_i exchange activity (Conover *et al.*, 1963). This energy-linked function of the synthetase complex is lowered in submitochondrial particles from ethanol-fed rats (Thayer & Rubin, 1979). We have also observed lower ATP- P_i exchange activity in both mitochondria and submitochondrial particles from ethanol-fed rats (R. E. Bottenus, P. I. Spach & C. C. Cunningham, unpublished work). The above studies support the supposition that the lower rate of ATP synthesis in mitochondria from ethanol-fed rats is

due to lowered activity of the ATP synthetase complex.

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