# Kinetics and control of oxidative phosphorylation in rat liver mitochondria after chronic ethanol feeding

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Changes in the kinetics and regulation of oxidative phosphorylation were characterized in isolated rat liver mitochondria after 2 months of ethanol consumption. Mitochondrial energy metabolism was conceptually divided into three groups of reactions, either producing protonmotive force ($\Delta p$) (the respiratory subsystem) or consuming it (the phosphorylation subsystem and the proton leak). Manifestation of ethanol-induced mitochondrial malfunctioning of the respiratory subsystem was observed with various substrates; the respiration rate in State 3 was inhibited by $27 \pm 4\%$ with succinate plus amytal, by $20 \pm 4\%$ with glutamate plus malate, and by $17 \pm 2\%$ with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine/ascorbate. The inhibition of the respiratory activity correlated with the lower activities of cytochrome *c* oxidase, the $bc_1$ complex, and the ATP synthase in mitochondria of ethanol-fed rats. The block of reactions consuming the $\Delta p$ to produce ATP (the phosphorylating subsystem) was suppressed after 2 months of ethanol feeding, whereas the mitochondrial proton leak was not affected. The contributions of $\Delta p$ supply (the respiratory subsystem) and $\Delta p$ demand (the phosphorylation and the proton leak) to the control of the respiratory flux were quantified as the control coefficients of these subsystems. In State 3, the distribution of control exerted by different reaction blocks over respiratory flux was not significantly affected by ethanol diet, despite the marked changes in the kinetics of individual functional units of mitochondrial oxidative phosphorylation. This suggests the operation of compensatory mechanisms, when control redistributes among the different components within the same subsystem.

Key words: control coefficient, kinetic response, top-down control analysis.

## INTRODUCTION

Chronic intake of large quantities of ethanol is associated with marked alterations in mitochondrial structure and function, both in humans and in experimental animals (reviewed in [1–4]). Abnormalities in mitochondrial morphology, e.g. deformed and enlarged mitochondria, often with paracrystalline inclusions, are evident in liver, muscle and other tissues. The most prominent site of ethanol-induced mitochondrial changes is the liver. Early studies [5,6] established that liver mitochondria isolated from rats fed an ethanol-containing diet for 3–5 weeks exhibited a decrease in NAD-linked respiration, both under conditions of active respiration (State 3, defined as the mitochondrial state in which ADP supply is saturating and excess substrate and $P_i$ are available) and in uncoupled mitochondria. Subsequent studies (reviewed in [2]) demonstrated that these effects had their origin in a decrease in the activity of select components of the oxidative phosphorylation machinery, notably NADH dehydrogenase, cytochrome *b*, cytochrome oxidase and ATP synthase. Since all of these protein complexes contain one or more subunits encoded by mitochondrial DNA, a defect in mitochondrial protein synthesis may account, at least in part, for these altered activities, and there is evidence that the effects of chronic ethanol treatment may be due to dysfunctional mitochondrial ribosomes [7,8]. However, other mechanisms may contribute to the changes in electron transport and ATP synthase activity, e.g. the accumulation of dysfunctional proteins in the form of acetaldehyde adducts or through damage by free radical species, or changes in membrane structure associated with altered properties of mitochondrial cardiolipin [4]. By contrast, isolated mitochondria from heart, skeletal muscle and brain show less evidence of defects in these pathways [1,3,9,10]. The progression of ultrastructural changes in the heart is slower than in the liver, and the onset of biochemical alterations is much later.

Despite many studies indicating effects of chronic alcoholism on various mitochondrial enzyme activities, there is as yet neither a clear picture of the relative importance of such changes for overall mitochondrial respiration, nor a quantitative assessment of alterations in the kinetic responses and regulation. In the present paper, we investigate the effect of chronic ethanol treatment using an analysis known as 'top-down' metabolic control analysis (reviewed in [11,12]). In this context, the oxidative phosphorylation system is conceptually divided into three subsystems, either producing or consuming the protonmotive force ($\Delta p$). $\Delta p$ is generated by the respiratory subsystem (*R*), which includes the substrate transport systems in the inner membrane, the tricarboxylic acid cycle enzymes and substrate dehydrogenases providing electrons to the respiratory chain, and the complexes of the electron transport chain. The remaining two blocks of reactions consume $\Delta p$: the phosphorylation subsystem (*P*) and the proton leak subsystem (*L*). The phosphorylation subsystem includes the phosphate transport systems, the ATP synthase, the adenine nucleotide translocator, and any reactions that may be present in the medium (or in the matrix) converting

Abbreviations used: $\Delta p$, protonmotive force; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; $TPP^+$, tetraphenylphosphonium.

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ATP into ADP. The proton leak unit represents uncoupled respiration (mainly the leak of protons across the mitochondrial membrane, but also any other non-phosphorylating ion fluxes that dissipate $\Delta p$).

The present paper analyses the dependencies of the fluxes through the respiratory subsystem ($J_R$), the phosphorylation subsystem ($J_P$) and the proton leak pathways ($J_L$) on $\Delta p$. Under conditions where changes in $\Delta$pH can be negligible, the mitochondrial membrane potential ($\Delta\Psi$) may be considered instead of $\Delta p$. This simplification is valid as long as $\Delta\Psi$ is the major contributor to $\Delta p$, or there is a reasonably constant relationship between $\Delta\Psi$ and $\Delta$pH [13]. In applying top-down analysis to mitochondria from the ethanol-fed and control rats, the present paper investigates how the functional units of the system are affected by chronic ethanol treatment.

In this work we quantified the changes in the kinetic responses of the functional units of the oxidative phosphorylation machinery (the respiratory subsystem, proton leak and phosphorylation subsystem) in rat liver mitochondria after 2 months of ethanol feeding, using different respiratory substrates: succinate, glutamate plus malate, and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate. The oxidation of these substrates involves different mitochondrial enzymes, and the supply of reducing equivalents occurs at different levels of the electron transport chain. Using the plots of $J_R$, $J_P$ and $J_L$ versus $\Delta\Psi$, we determine the control exerted by the suppliers and consumers of the $\Delta p$ over mitochondrial respiration in ethanol-fed and control rats. Our results demonstrate that in rat liver mitochondria, a 2-month treatment with ethanol diet suppresses both the respiratory and phosphorylation subsystems, but to a different degree depending on the substrate used; proton leak was not affected. Remarkably, despite the changes in the activity of respiratory complexes in the mitochondria from ethanol-fed and control animals, there were no significant changes in the control distribution.

We applied kinetic modelling in order to understand why the control distribution does not change, despite marked alterations in the kinetics of individual functional units of oxidative phosphorylation. Theoretical analysis demonstrated that simultaneous depression of the supply and demand blocks, as found in ethanol-affected mitochondria, can occur without a significant change in the control distribution. Moreover, the depression of the activity of the individual components within the same block can cause the control redistribution among these components, even though the net control exerted by the block does not change.

## EXPERIMENTAL

We used male Sprague–Dawley rats (275–300 g) which were pair-fed according to the protocol of DeCarli and Lieber [14] for 5–7 weeks, with ethanol-fed animals receiving a diet containing 36% ethanol, and carbohydrate substituting for ethanol in the control diet. This dietary treatment results in a distinct pattern of changes in activity of respiratory chain components and ATP synthase, without causing overt liver disease [2].

Liver mitochondria were isolated in 5 mM Hepes (pH 7.4)-buffered sucrose (0.25 M), containing 3 mM EGTA and 0.2% BSA (HMS medium), using standard differential centrifugation procedures. The pellet of the first low-speed centrifugation step was resuspended in HMS medium and recentrifuged to recover mitochondria retained in the low-speed pellet. The final wash of the mitochondrial pellet was in EGTA-free HMS medium. The mitochondrial pellet was resuspended in the same medium, protein was adjusted to 50 mg/ml, and the preparation was stored on ice until use. Protein concentrations were determined by the biuret or Lowry methods. Previous studies have documented exhaustively that the mitochondrial content of the liver, and the relative purity of mitochondria, are comparable in ethanol-fed and control rats following 5–8 weeks of feeding acording to the Lieber–DeCarli protocol [15–18].

Mitochondria were incubated at 30 °C in a closed, stirred and thermostated 1.5 ml glass vessel, fitted with both a Clark oxygen electrode (Rank Brothers, Cambridge, U.K.) and a tetraphenylphosphonium ($TPP^+$)-selective electrode (A. Zimkus, Vilnius, Lithuania). The incubation medium contained Hepes (20 mM, pH 7.4), KCl (50 mM), sucrose (200 mM), $KH_2PO_4$ (3 mM), EGTA (0.1 mM), $MgCl_2$ (1 mM) and dithiothreitol (1 mM). $TPP^+$ (133–266 nM) was addded for membrane potential measurements. Incubations were carried out under the following substrate conditions: 5 mM glutamate plus 5 mM malate, 5 mM succinate (plus 1 mM amytal, used for technical reasons in preference to rotenone, since we had difficulty washing out the relatively hydrophobic rotenone from the $TPP^+$-selective electrode, whereas the more polar amytal did not cause this problem.), or 10 mM ascorbate plus 350 $\mu$M TMPD. Mitochondrial protein was 0.5 mg/ml. The creatine kinase ADP-regenerating system (50 mM creatine, 1 mM ATP and 30 units/ml creatine kinase to obtain State 3 conditions) was used to clamp the ATP/ADP ratio and to maintain stationary respiration rates. The creatine kinase system does not itself control respiration [19], but enables the ATP/ADP ratio to be clamped at different respiratory states throughout the range from State 4 (the mitochondrial state in which oxidative phosphorylation is limited by lack of external ADP, but in the presence of ATP) to State 3. Control experiments demonstrated that the respiration rate was not limited by the amount of creatine kinase, therefore all control over respiration rate was exerted by mitochondrial enzymes. The rate of mitochondrial respiration corresponding to State 3 was registered after addition of 1 mM ATP to the incubation medium. The following inhibitor concentrations were used in titrations to establish the relationship of respiratory activities to membrane potential: 0.03–1 mM malonate, 1.3–10 nM rotenone, 5–75 $\mu$M KCN, and 15–150 nM carboxyatractyloside.

The membrane potential of mitochondria was calculated from the distribution of $TPP^+$ using a binding correction factor of 0.16. This correction was based on a series of experiments with isolated mitochondria in which the ratio of accumulation of $Rb^+$ and $TPP^+$ was compared over a range of membrane potentials, titrated by addition of KCl in the presence of valinomycin [20]. Control experiments established that the $TPP^+$-binding correction in liver mitochondria was not significantly affected by chronic ethanol consumption (results not shown).

### Determination of mitochondrial volume and $\Delta$pH

Mitochondrial volume was determined from the distribution of $^3H_2O$ and [$^{14}$C]sucrose, and $\Delta$pH from the distribution of [$^3$H]acetate and [$^{14}$C]sucrose [20], under the same incubation conditions as mitochondrial respiration. The results show that in State 3 the matrix volume and $\Delta$pH of isolated liver mitochondria oxidizing succinate/amytal were not significantly affected by 5–7 weeks of ethanol feeding. Matrix volume and $\Delta$pH in the preparations from the ethanol-fed animals changed, compared with controls, from $0.89 \pm 0.02$ $\mu$l/mg to $0.84 \pm 0.03$ $\mu$l/mg, and from $31.3 \pm 0.4$ mV to $28.1 \pm 1.8$ mV respectively. Also, under the conditions used here (i.e. in the presence of excess $P_i$), $\Delta$pH made only a modest contribution to the total $\Delta p$, and this contribution was not significantly affected by the inhibitor

titrations (results not shown). Thus the conditions were appropriate for using membrane potential measurements as quantitative indicators of changes in $\Delta p$.

### Measurements of ATPase, cytochrome oxidase and ubiquinol:cytochrome *c* (*bc*$_1$ complex) activities

ATPase and cytochrome oxidase activities were measured in fractured mitochondria from rat liver. Fractured mitochondria were made by rapidly freeze–thawing liver mitochondria (repeated three times). ATPase activity was measured at 30 °C by recording the pH changes in the incubation chamber during ATP hydrolysis reaction in the presence of 250 mM sucrose, 5 mM $MgCl_2$, 2.5 mM Tris (pH 8) and 1 mM ATP [21]. The buffering capacity of the reaction mixture was determined experimentally by adding a known amount of HCl (5 $\mu$l of 50 mM HCl $\equiv$ 250 nmol ions $H^+$) to the reaction mixture after each measurement, and recording the pH change induced by this addition. The ATPase activity was estimated from the initial rate of increase in $H^+$ concentration during ATP hydrolysis. Control experiments showed that $H^+$ formation was $> 95\,\%$ inhibited by oligomycin (1.5 $\mu$g/ml).

Cytochrome oxidase activity was measured at 30 °C by monitoring the rate of oxygen consumtion by fractured mitochondria (0.4 mg protein/ml) in the presence of 0.35 mM TMPD, 10 mM ascorbate and 0.015 mM cytochrome *c*, under the same incubation conditions as for mitochondrial respiration.

Activity of the $bc_1$ complex was measured spectrophotometrically by following the kinetics of cytochrome c reduction. Measurements were carried out at 30 °C in the presence of $KH_2PO_4$ (35 mM pH 7.2), $MgCl_2$ (5mM), BSA (fatty acid-free, 2.5 mg/ml), KCN (0.2 mM), oxidized cytochrome *c* (20 $\mu$M), rotenone (4 $\mu$g/ml), fractured mitochondria (10 $\mu$g mitochondrial protein) and antimycine (0 or 10 $\mu$g/ml). Reactions were started by adding 20 $\mu$M decylubiquinol. Enzymic activity was measured at 550 nM for 2 min, with an absorption coefficient of 18.5 $mM^{-1} \cdot cm^{-1}$.

### Statistical analysis

Data are expressed as means $\pm$ S.E.M. of 3–7 experiments carried out on different mitochondrial preparations. Each point for every individual preparation is averaged from two or three repetitive runs.

## RESULTS

### Activities and kinetic responses of the components of oxidative phosphorylation in chronic alcoholism

We measured the activities of ubiquinol:cytochrome *c* reductase ($bc_1$ complex), cytochrome oxidase, and the hydrolytic activity of ATPase in submitochondrial particles, from the livers of control and ethanol-fed animals. The results presented in Table 1 demonstrate that 2 months of chronic alcohol treatment diminished the activities of the $bc_1$ complex, cytochrome oxidase and ATPase in rat liver mitochondria by $41 \pm 5\,\%$, $40 \pm 6\,\%$ and $36 \pm 4\,\%$ respectively (Table 1). These data are consistent with previous studies [5,10,22–26]. The alterations in the component activities bring about the differential responses of each block of reactions *R*, *L* and *P* to changes in $\Delta\Psi$. These responses were determined by a series of inhibitor titrations as follows: first, the dependence of the flux through the respiratory subsystem, $J_R$, on $\Delta\Psi$ was determined by titrating mitochondria in State 3 with an inhibitor of the phosphorylation subsystem (carboxyatractyloside or oligomycin). Inhibition of phosphorylation caused an increase in $\Delta\Psi$ (similar to that occuring during the State 3/State 4 transition), which was accompanied by a decreased rate of $O_2$ uptake. At steady state, $J_R$ is equal to the sum of the rates of $O_2$ consumption needed to drive the phosphorylation subsystem ($J_P$) and the proton leak ($J_L$): $J_R = J_P + J_L$. Secondly, the dependence of $J_L$ on $\Delta\Psi$ was determined under conditions of complete inhibition of $J_P$ by excess (1 $\mu$M) oligomycin, so that $J_R = J_L$ [27]. Mitochondria oxidizing succinate were titrated with malonate (an inhibitor of succinate dehydrogenase). Titrations with rotenone or KCN were used to inhibit oxidation of glutamate plus malate, and TMPD plus ascorbate respectively. Inhibition of the respiratory subsystem caused $\Delta\Psi$ to decrease, and $J_L$ was detemined as the rate of $O_2$ consumption at different values of $\Delta\Psi$. Thirdly, a similar titration protocol with inhibitors of the respiratory subsystem (malonate and rotenone or KCN in the absence of oligomycin) was used to determine the dependence of $J_P$ on $\Delta\Psi$. The phosphorylation flux at each $\Delta\Psi$ was calculated by substracting the proton leak $J_L$ from $J_R$ at corresponding $\Delta\Psi$ values ($J_P = J_R - J_L$).

**Table 1 Activities of respiratory chain components and ATPase after chronic alcohol treatment**

*Statistically significant effect of alcohol ($P < 0.05$)

| Component | Control | Ethanol | Inhibition (%) |
|---|---|---|---|
| Cytochrome *c* oxidase (n-atoms O/min per mg) | $504 \pm 35$ | $305 \pm 38$* | $40 \pm 6$ |
| ATPase (nmol $H^+$/min per mg) | $64.4 \pm 5.6$ | $39.6 \pm 1.9$* | $36 \pm 4$ |
| $bc_1$ complex ($\mu$M cytochrome *c*/min) | $2.16 \pm 0.17$ | $1.33 \pm 0.20$* | $41 \pm 5$ |

### Respiratory subsystem analysis

Generation of $\Delta p$ with different substrates involves different mitochondrial enzymes and encompasses different segments of the electron transport chain, resulting in different numbers of protons pumped per pair of electrons. As a consequence, plots of $J_R$ versus $\Delta\Psi$ vary with the oxidative substrate. Figure 1 shows the kinetic responses of the respiratory subsystems, $J_R$, in mitochondria from ethanol-fed and control rats oxidizing different substrates, which enter the respiratory chain at different levels.

The simplest substrate supply condition is when mitochondria oxidize TMPD plus ascorbate, where no substrate translocator system is involved and reducing equivalents enter the electron transport chain at the level of cytochrome *c*. At saturating TMPD/ascorbate concentrations, the flux through the respiratory subsystem is mainly ($> 80\,\%$) controlled by cytochrome *c* oxidase and no other respiratory complexes are involved. Figure 1(A) demonstrates an inhibition (15–19 %) of the respiratory subsystem over the entire membrane potential range in mitochondria from ethanol-fed animals compared with mitochondria from pair-fed control animals. This observation is in agreement with a decrease in cytochrome *c* oxidase activity following chronic ethanol consumption (Table 1) [2,6,7,24,26].

A somewhat different pattern was obtained with mitochondria respiring on succinate (Figure 1B). This substrate enters the mitochondrial matrix on the dicarboxylate translocator in exchange for malate. Reducing equivalents are derived from the substrate through succinate dehydrogenase, and enter the electron transport chain at the level of coenzyme Q. Excess amytal was added in these experiments in order to suppress the accumulation of oxaloacetate, a potent competitive inhibitor of succinate dehydrogenase. In State 3, mitochondria from ethanol-

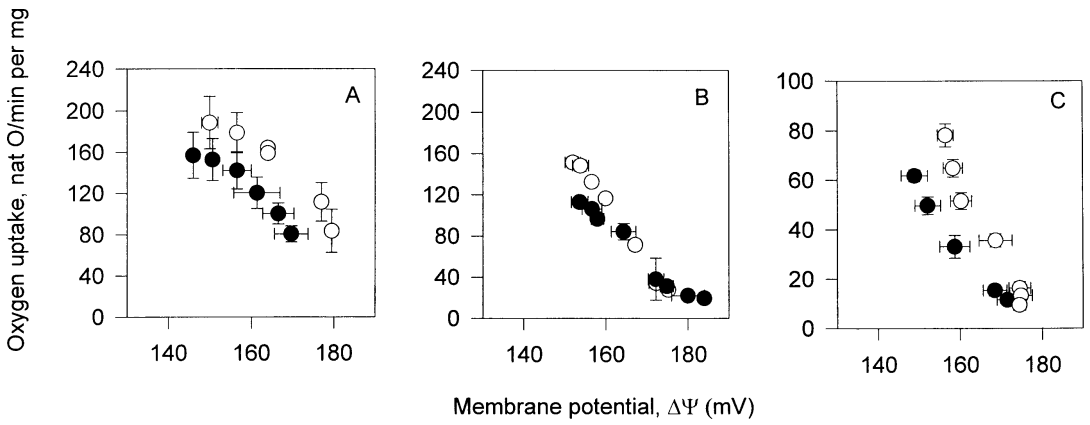


**Figure 1 Kinetic response of the respiratory subsystem to changes in the membrane potential**

Liver mitochondria oxidizing (**A**) TMPD (0.35 mM) plus ascorbate (10 mM), (**B**) succinate (10 mM) plus amytal (1 mM), (**C**) glutamate (5 mM) plus malate (5 mM). Control (○), ethanol-fed animals (●).

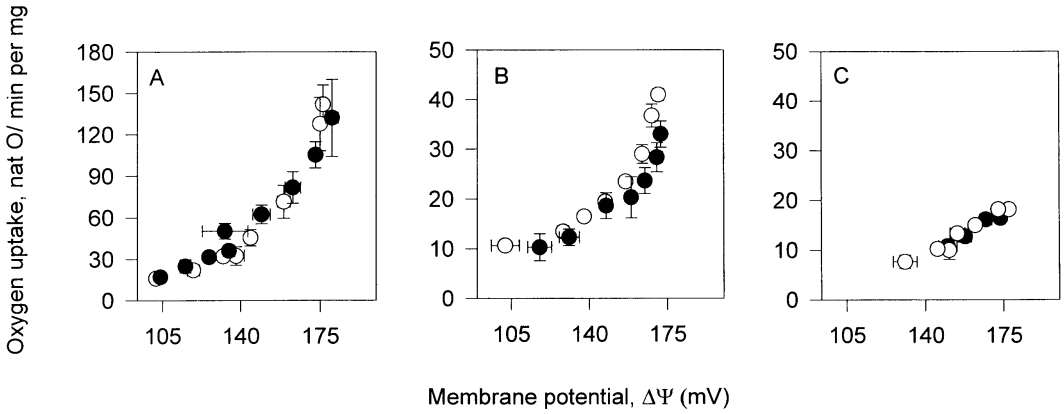


**Figure 2 Dependence of the proton leak on the membrane potential**

Liver mitochondria oxidizing (**A**) TMPD (0.35 mM) plus ascorbate (10 mM), (**B**) succinate (10 mM) plus amytal (1 mM), (**C**) glutamate (5 mM) plus malate (5 mM). Control (○), ethanol-fed animals (●).

fed rats exhibited lower succinate oxidation rates (by 26–33%) than mitochondria from pair-fed control animals, in agreement with previous studies (data from 4 pairs receiving ethanol for 5–6 weeks). When succinate oxidation rates were decreased by titration with carboxyatractyloside, the difference between mitochondria from control and ethanol-fed rats in the activity of the respiratory subsystem declined as a function of the membrane potential under the conditions approaching State 4 (Figure 1B). These observations are in agreement with earlier findings that the flux of succinate oxidation is controlled primarily by components other than the electron transport chain under conditions of submaximal respiratory activity [19].

Glutamate plus malate oxidation follows more complex pathways, which supply reducing equivalents to the respiratory chain mostly in the form of NADH. When glutamate is oxidized in the presence of malate, glutamate traps oxaloacetate derived from malate to form aspartate and $\alpha$-ketoglutarate. Aspartate is quantitatively exchanged for glutamate on the glutamate/aspartate translocator, whereas $\alpha$-ketoglutarate can be either exchanged for extramitochondrial malate, or oxidized through $\alpha$-ketoglutarate dehydrogenase. Hence the flux through malate dehydrogenase equals the sum of the fluxes through $\alpha$-ketoglutarate dehydrogenase and the malate/$\alpha$-ketoglutarate translocator. As shown in Figure 1(C), mitochondria from ethanol-fed animals oxidizing glutamate plus malate exhibited lower activities of the respiratory subsystem (by 11–35%) than mitochondria from control animals over most of the range of $\Delta\Psi$, except under maximally inhibited conditions.

## Proton leak subsystem analysis

Figure 2 shows the kinetic response of the proton leak ($J_L$) to $\Delta\Psi$ in mitochondria from ethanol-fed and control animals oxidizing different substrates. Mitochondria oxidizing TMPD plus ascorbate were titrated with KCN (Figure 2A), whereas malonate and rotenone were used to titrate succinate (Figure 2B) and glutamate plus malate (Figure 2C) oxidation respectively. Inhibition of the respiratory subsystem caused a decrease in $\Delta\Psi$. Proton leak is not directly affected by these inhibitors in the range of concentrations used [28].

Maximal rates of proton leak were obtained in State 4 where the membrane potential was maximal (no inhibitor added).

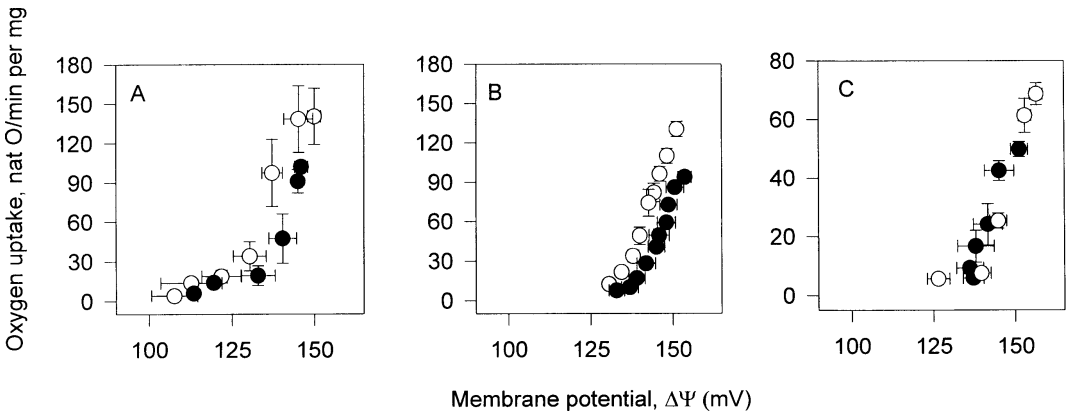


**Figure 3 Kinetic response of the phosphorylation subsystem to changes in the membrane potential**

Liver mitochondria oxidizing (**A**) TMPD (0.35 mM) plus ascorbate (10 mM), (**B**) succinate (10 mM) plus amytal (1 mM), (**C**) glutamate (5 mM) plus malate (5 mM). Control (○), ethanol-fed animals (●).

Titration with the inhibitor decreases respiratory rates and causes a decrease in ΔΨ. No significant differences were observed between mitochondria from ethanol-fed and control animals. Also, in State 4, the respiration rates were not significantly different between these preparations. Experiments in the presence of nigericin (1 $\mu$M), which equilibrates pH differences across the mitochondrial membrane in a $K^+$-containing medium, showed similar dependencies of $J_L$ on the $\Delta p$, confirming that under our experimental conditions the effects of ΔpH can be neglected (results not shown). Also, addition of BSA (2 mg/ml) did not affect proton leak rates in these mitochondria, indicating that non-esterified fatty acids did not contribute to the proton leak in either preparation (results not shown). It should be noted that the mitochondrial isolation medium contained BSA in order to suppress possible differences between ethanol-fed and control animals due to endogenous fatty acids.

### Phosphorylation subsystem analysis

Similar to the measurements of ΔΨ-dependence of the proton leak, inhibitors of the respiratory subsystem were used to determine the dependence of the phosphorylation flux on ΔΨ. In Figure 3, the flux through the phosphorylation subsystem was examined under State 3 conditions over a wide range of ΔΨ values. Results obtained with TMPD plus ascorbate (Figure 3A) and succinate (Figure 3B) as respiratory substrates were essentially similar, showing a significant inhibition of the phosphorylation subsystem in mitochondria from ethanol-fed animals over the entire range of ΔΨ values examined. In contrast, no inhibition was observed for mitochondria oxidizing glutamate plus malate at corresponding ΔΨ values (Figure 3C). The difference in sensitivity to chronic ethanol treatment is not unexpected and is related to different pathways of substrate oxidation. In mitochondria respiring on glutamate/malate, any substrate-linked phosphorylation associated with $\alpha$-ketoglutarate oxidation contributes to overall ATP production. When this step is inhibited with arsenite, the ΔΨ dependence of phosphorylation resembles the findings with other substrates, showing the suppression of the ATP-producing subsystem after chronic ethanol treatment (results not shown). This suggests that substrate-linked phosphorylation can compensate to some extent for the defect in ATP synthase-mediated ATP production in ethanol-fed rats.

**Table 2 Control coefficients of ΔΨ supply and ΔΨ demand processes over the respiration flux in State 3**

| | Control exerted by ΔΨ supply | | Control exerted by ΔΨ demand | |
|---|---|---|---|---|
| Substrate | Control | Ethanol | Control | Ethanol |
| TMPD/ascorbate | 0.68 ± 0.02 | 0.72 ± 0.09 | 0.32 ± 0.02 | 0.28 ± 0.09 |
| Succinate/amytal | 0.73 ± 0.08 | 0.67 ± 0.09 | 0.27 ± 0.08 | 0.33 ± 0.09 |
| Glutamate/malate | 0.48 ± 0.06 | 0.44 ± 0.03 | 0.52 ± 0.06 | 0.56 ± 0.03 |

### Analysis of control parameters

We conceptually grouped the reactions of the phosphorylation subsystem and the proton leak into a single block of reactions consuming $\Delta p$. In this manner, the oxidative phosphorylation machinery is divided into two parts only: ΔΨ supply and ΔΨ demand processes. The control exerted by either of these two parts on mitochondrial respiration is quantified as the control coefficient of ΔΨ supply and ΔΨ demand processes, $C_{supply}$ and $C_{demand}$ respectively. The control coefficients are calculated from the elasticities, $\epsilon^{supply}$ and $\epsilon^{demand}$, which are the normalized derivatives of the fluxes through the appropriate parts of the system with respect to ΔΨ [12]:

$$\epsilon^{supply} = \frac{\Delta\Psi}{J_R}\cdot\frac{\partial J_R}{\partial\Delta\Psi};\ \epsilon^{demand} = \frac{\Delta\Psi}{J_P+J_L}\cdot\frac{\partial(J_P+J_L)}{\partial\Delta\Psi}$$

These derivatives were determined from the kinetic dependencies shown in Figures 1–3 at ΔΨ values corresponding to State 3 respiration for each of the respiratory substrates examined. The control coefficients ($C_{supply}$ and $C_{demand}$) of ΔΨ supply and ΔΨ demand processes were calculated as:

$$C_{supply} = \frac{\epsilon^{demand}}{\epsilon^{demand}-\epsilon^{supply}};\ C_{demand} = \frac{-\epsilon^{supply}}{\epsilon^{demand}-\epsilon^{supply}}$$

Table 2 demonstrates how control of State 3 respiration rate is distributed among the different subsystems generating and consuming $\Delta p$. In mitochondria from both control and ethanol-fed rats, the respiratory subsystem makes a predominant con-

tribution to the flux control with TMPD/ascorbate (68 % and 72 % respectively) and with succinate/amytal (73 % and 67 % respectively), whereas it contributes only approx. 50 % when glutamate plus malate is used as substrate. Remarkably, the State 3 control coefficients were not significantly different between preparations from control and ethanol-fed rats for any of these substrate conditions, despite the substantial inhibition of both the electron transport and the phosphorylation reactions, as reflected in the State 3 oxidation rates with all substrates. This finding suggests that the mitochondrial oxidative phosphorylation system may compensate for the inhibition of the electron transport components and the phosphorylation reactions to maintain a substrate-specific pattern of control distribution, despite the alterations in the enzymatic components that occurred after chronic ethanol consumption.

## DISCUSSION

The findings reported in this study illustrate how mitochondria adapt to simultaneous changes in multiple components of the energy conservation system following chronic ethanol consumption. Ethanol treatment causes tissue-specific changes in mitochondrial morphology and membrane physical properties, including effects on mitochondrial inner membrane phospholipids and alterations in the activity and kinetic properties of several mitochondrial enzymes (reviewed in [2,4]). Mitochondrially encoded subunits of electron-transport-chain complexes and ATP synthase are specifically affected, resulting in a decrease by 30–50 % in the activity of NADH dehydrogenase, cytochrome $bc_1$ complex, cytochrome oxidase and ATP synthase [1,6,24,26]. These changes in mitochondrial characteristics occur in liver over a period of 3–5 weeks of ethanol feeding by the Lieber–DeCarli protocol [14] and may be more extensive with other more invasive ethanol-feeding protocols. Despite detailed studies of the molecular basis of these changes in oxidative phosphorylation, the consequences for cellular energy metabolism have not been characterized. We employed top-down regulatory analysis to answer the question of how these changes in the activity of multiple inner-membrane components affect the overall pathway rates and the integrative control of the oxidative phosphorylation machinery in isolated mitochondria. Top-down control analysis not only characterizes changes in key bioenergetic parameters, but can also identify alterations in the regulatory characteristics of mitochondrial oxidative phosphorylation. Specifically, top-down analysis can distinguish how the kinetic consequences of the ethanol-induced changes in enzymic components are distributed between the mitochondrial respiratory subsystem, the proton leak, and the phosphorylation subsystem.

In agreement with previous studies that reported a decline in electron transport components [5,10,22–26], the rate of generation of $\Delta p$ (the respiratory subsystem) with different substrates was inhibited after 2 months of ethanol consumption. With $NAD^+$-dependent substrates and TMPD/ascorbate a significant suppression of the respiratory subsystem was observed at all values of the mitochondrial membrane potential examined, with more pronounced inhibition in State 3 than State 4. Succinate oxidation was significantly suppressed in State 3 only, reflecting the predominant control of this pathway by succinate dehydrogenase, the activity of which is not affected by ethanol treatment [5]. Despite this decrease in the respiratory capacity, the response of the respiratory chain to changes in membrane potential (as reflected in the elasticity coefficient derived from the slope of the curves shown in Figures 1–3) was only modestly affected by ethanol treatment.

The $\Delta p$ dependence of the proton leak rate did not differ in mitochondria from control and ethanol-fed rats for any of the substrate conditions studied. This finding suggests that the changes in phospholipids and membrane proteins that occur after 2 months of ethanol feeding did not affect the permeability of the mitochondrial membrane to protons. This conclusion conflicts with some earlier studies, but is in agreement with the data reported by Murphy and Tipton [29]. It is interesting to note that in other studies we found that longer term ethanol feeding (up to 18 months) may be associated with an enhanced proton leak rate, possibly due to the expression of uncoupling protein (results not shown).

Ethanol effects on the phosphorylation subsystem can be reliably determined using succinate or TMPD/ascorbate, since inhibitor titrations of succinate dehydrogenase or cytochrome oxidase do not affect the enzymes of the phosphorylation subsystem directly. The data obtained with mitochondria oxidizing either of these substrates demonstrated a suppression of the responses of the phosphorylation subsystem to $\Delta\Psi$ changes. We were unable to detect any significant inhibition of the phosphorylation subsystem in mitochondria oxidizing glutamate plus malate. However, the application of top-down analysis is more complicated for substrates such as glutamate plus malate, which involve the substrate-linked phosphorylation step of $\alpha$-ketoglutarate dehydrogenase/succinate thiokinase, since an ATP-producing reaction appears both in the supply and demand blocks.

The distribution of control over the supply and demand processes is calculated from the elasticities of the phosphorylation and respiration subsystems. In State 3, mitochondria from ethanol-fed and pair-fed control rats did not show significant shifts in the distribution of control in favour of either the phosphorylation or respiration subsystem. (Table 2). The moderate changes that were observed, did not achieve statistical significance for any of the substrate conditions. This finding indicates that the control distribution in State 3 appeared relatively stable to substantial changes in the enzyme activities of both supply and demand blocks. Interestingly, this correlates with the findings of Brand and Kesseler [30], who showed that in potato tuber mitochondria, cadmium did not affect the control pattern at State 3 respiration, whereas it caused a progressive inhibition of the respiratory subsystem over the entire range of $\Delta p$ between States 3 and 4.

How does the mitochondrial system at State 3 compensate for marked changes in activity of the enzyme systems of oxidative phosphorylation? Some insight can be obtained from a kinetic model of mitochondrial respiration published elsewhere [31,32]. This model implements all available kinetic knowledge concerning the individual enzymes and non-enzymic steps of the mitochondrial oxidative phosphorylation machinery. The estimated $\Delta\Psi$ dependency of the respiratory subsystem and the phosphorylation subsystem generated by this model accounts reasonably well for the experimentally observed traces shown in Figures 1–3 (see Figure 4, solid lines). Figure 4 illustrates a theoretical analysis of how the oxidative phosphorylation system would be affected by a decrease in the content of electron-transport-chain components and ATP synthase observed in the range of 36–41 % (Table 1) during oxidation of succinate/amytal (Figure 4, broken lines). It is evident that the response patterns obtained in the computational analysis qualitatively match the patterns obtained experimentally, both in ‘normal’ mitochondria and in the partly inhibited state. The kinetic model illustrates that the simultaneous depression of the supply and demand blocks found in ethanol-affected mitochondria did not change the mitochondrial membrane potential in State 3. Indeed, in

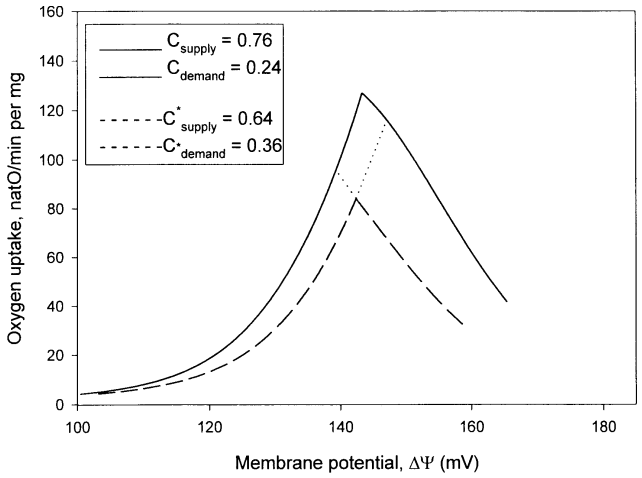


**Figure 4 Simulated responses of the supply and demand blocks of oxidative phosphorylation to $\Delta\Psi$ changes in mitochondria with 'normal' and suppressed activities of electron transport chain and ATP synthase**

Solid lines correspond to control mitochondria oxidizing succinate/amytal, and broken lines correspond to mitochondria with the ATP synthase activity inhibited by 36%, the $bc_1$ complex activity inhibited by 41% and the activity of cytochrome *c* oxidase inhibited by 40%. The dotted extensions of the dashed lines illustrate how the response pattern would change if either ATP synthase or the electron transport chain activity were suppressed separately. The control coefficients of the supply block (the respiration subsystem) were 0.89 and 0.72 in control ($C_{supply}$) and inhibited ($C^*_{supply}$) mitochondria respectively. The control coefficients of the demand block (which involves the proton leak and phosphorylation subsystem) are equal to one minus the control coefficients of the supply block.

Figure 4 the points of intersections of the two solid lines (State 3 of normal mitochondria) and the two broken lines (State 3 of affected mitochondria) correspond to the same $\Delta\Psi$ value of 141 mV. The dotted lines in Figure 4 illustrate the expected response patterns when only the ATP synthase or only the electron transport components are inhibited. The points of intersection of dotted and solid lines show that the State 3 membrane potential would change upwards if only the ATP synthase were inhibited by ethanol treatment, and downwards if only the respiratory chain were affected. As shown in Figure 4, the computed control coefficients appeared to be within the range of values observed experimentally, with a predominant fraction of the control being located in the respiratory subsystem. Inhibition of the activities of $bc_1$ complex, cytochrome oxidase and ATP synthase (Table 1) shifted the control away from the respiratory subsystem, towards the phosphorylation subsystem. However, the change in control distribution associated with the inhibited state was relatively modest, and would be difficult to identify experimentally.

How is it that the system requires so little compensation in control distribution for these marked changes in enzyme activity? We tried to answer this question using the model and calculating the control distribution, not only among the blocks of multiple reactions (as we did in our experiments), but also among the individual components within these blocks. In control mitochondria respiring on succinate/amytal at State 3, succinate dehydrogenase carries the major part (approx. 80%) of the control over respiration. The rest of the control is nearly equally distributed among the other components of the system: $bc_1$ complex, cytochrome oxidase, ATP synthase, adenine nucleotide translocator and proton leak. According to the data presented in Table 1, we calculated the control coefficients for each component in the system for mitochondria with suppressed cytochrome oxidase, $bc_1$ complex and ATP synthase activities. In this case, control shifted substantially from succinate dehydrogenase (now 30% of control) to $bc_1$ complex and cytochrome oxidase. However, the net control, exerted by the respiratory subsystem, remained almost the same, despite the substantial decrease in the activities of some of its components and the redistribution of the control within the subsystem. The conclusion from these theoretical observations is that, in chronic alcoholism, the suppressed capacities of some mitochondrial enzymes can cause a redistribution of the control among individual steps in the respiration pathway. However, ethanol-induced changes in control distriution are essentially undetectable in 'top-down' control analysis, where the number of reactions are conceptually grouped into complex blocks, and only the control distribution among these blocks is calculated. Thus compensation within each of the major blocks of oxidative phosophorylation can prevent the change in control distribution between these blocks.

Despite the lack of change in State 3 control distribution following chronic ethanol feeding, the compensations reflected in steady-state metabolite changes are likely to have important physiological ramifications, by making mitochondria much more susceptible to changes in energy demand. This may have significant implications for the performance of liver mitochondria from alcoholic animals under conditions of physiological stress, such as hypoxia or $Ca^{2+}$ overload. This may account for an enhanced mitochondrial capacity to generate reactive oxygen intermediates, which is largely a reflection of steady-state levels of semiquinone [33]. Also, matrix ATP/ADP ratio may be more susceptible to stress. Interestingly, some reports have demonstrated that liver cells from ethanol-fed rats have a decreased capacity to maintain adequate ATP/ADP ratios under conditions of modest hypoxia, even though ATP levels can be maintained adequately under normal oxygen supply conditions [34]. Under these conditions it would be more difficult for mitochondria from ethanol-fed animals to maintain the necessary rates of ATP production in the liver. The impact of these defects may be further strengthened by the increased exposure to hypoxic conditions that occurs particularly in the pericentral zones in the liver *in vivo*, which may contribute to the liver damage associated with chronic ethanol consumption [35].

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