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The effect of temperature and chronic ethanol feeding on the proton electrochemical potential and phosphate potential in rat liver mitochondria

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The relationship between the proton electrochemical potential ($\Delta\tilde{\mu}_H$) and the maximal free energy of ATP hydrolysis (ΔG_P) in coupled respiring rat liver mitochondria was investigated as a function of temperature and chronic ethanol-feeding. The flow dialysis method was utilized to measure the temperature dependence of $\Delta\tilde{\mu}_H$ from the uptake of ^{86}Rb (in the presence of valinomycin) and [^{14}C]DMO. ΔG_P in state 4 was determined by a null-point titration of the reversible, H^+-coupled ATPase against the phosphate potential. $\Delta\tilde{\mu}_H$ increases with temperature from 196 mV at 10°C, to 217 mV at 40°C. The maximal ΔG_P at state 4 decreases as a function of temperature from 67.8 kJ/mol at 10°C, to 54.8 kJ/mol at 40°C. As a result, the ratio $\Delta G_P/\Delta\tilde{\mu}_H$ decreases with temperature from 3.56 at 10°C to 2.60 at 40°C. Similar studies with mitochondria from rats which were chronically fed with ethanol show that, while ΔG_P at state 4 decreases in these rats from 61.2 to 56.0 (25°C), the $\Delta\tilde{\mu}_H$ is essentially unchanged at 212 mV. Thus the ratio $\Delta G_P/\Delta\tilde{\mu}_H$ in ethanol-fed rats at 25°C is 2.77 as compared with 2.97 in control. Similar reduction of ΔG_P was observed in inverted inner membranes from ethanol-fed rats. Both the temperature dependence of $\Delta G_P/\Delta\tilde{\mu}_H$ and the effect of ethanol-feeding cannot be easily explained by the chemiosmotic hypothesis which postulates that $\Delta\tilde{\mu}_H$ is the only driving force for ATP synthesis. In contrast, a parallel coupling model, which postulates that intramembrane proton transfer from redox pumps to ATPase is mediated by the formation of dynamic aggregates of the mitochondrial innermembrane proteins, can easily accommodate these findings. Accordingly, the temperature effect is due to weakening of these fragile aggregates, while the ethanol-feeding effect is the result of reduced concentration of active pumps, which decrease the frequency of formation of functional aggregates.

Introduction

Oxidative phosphorylation in mitochondria is catalyzed by a sequence of proton pumping enzymes. First, substrate oxidation by the electron-transport chain drives electrogenic proton extrusion from the mitochondrial matrix; then, the extruded protons flow back to the matrix via the

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Abbreviations: $\Delta\tilde{\mu}_H$, the proton electrochemical potential difference across the mitochondrial inner membrane; ΔP, protonmotive force; ΔG_P, the free energy of ATP hydrolysis; Phosphate potential, the ratio [ATP]/[ADP] [P_i]; DMO, 5,5-dimethyloxazolidine-2,4-dione; di-S-C_3(5), 3,3'-dipropylthiocarbocyanine; P_i, inorganic phosphate; Pipes, 1,4-piperazinediethanesulphonic acid.

H^+-ATPase, driving the synthesis of ATP [1,2]. In the chemiosmotic hypothesis Mitchell postulated that the protons are first released into the medium surrounding the inner membrane, thus generating a transmembrane electrochemical potential difference between the matrix and the bulk phases, $\Delta\tilde{\mu}_H$ [3] *. Subsequently, $\Delta\tilde{\mu}_H$ drives the reversal of the ATPase to generate ATP. When mitochondria are allowed to phosphorylate ADP to the maximal extent, the system approaches a steady state (state 4 or 'static head'), in which a maximal $[ATP]/[ADP][P_i]$ ratio ('phosphate potential') is maintained without further net synthesis. The phosphate potential at state 4 depends on the thermodynamic degree of coupling of oxidative phosphorylation. Since, according to chemiosmotic theory, the direct driving force for ATP synthesis is $\Delta\mu_H$, at state 4 the reaction catalyzed by the pump is at equilibrium, and the free energy of the coupled reaction $ATP + nN_{in} \leftrightarrow ADP + P_i + nH_{out}$ should vanish. Hence, at state 4, $n = \Delta G_P/\Delta\tilde{\mu}_H$. In recent years, several methods have been developed for the measurement of $\Delta\tilde{\mu}_H$ in the mitochondria [4–6] and the relation between $\Delta\tilde{\mu}_H$ and ΔG_P, as well as $\Delta\tilde{\mu}_H$ and the rate of oxidation and phosphorylation have been investigated (for reviews, see Refs. 7–9). Although the results of these extensive studies cannot be described here in detail, it was suggested that $\Delta\tilde{\mu}_H$ is not the only intermediate in oxidative phosphorylation. In particular, it was noted that the ratio $\Delta G_P/\Delta\tilde{\mu}_H$, which is expected to remain constant when $\Delta\tilde{\mu}_H$ is reduced by ionophores, uncouplers or electron transport inhibitors, actually increases. These results suggest that there is a more direct pathway (not via the bulk phases) for proton transfer between the electron-transport chain and the ATPase. A similar conclusion was arrived at previously from a study of the relationship between respiratory control and $\Delta\tilde{\mu}_H$ [10] and the rate of phosphorylation and $\Delta\tilde{\mu}_H$ [11]. More recently, we have observed that the coupling efficiency of oxidative phosphorylation in rat liver mitochondria, as assessed by the acceptor respiratory control, is strongly dependent on temperature with a maximum at 25–30°C [12,13]. This suggested to us that direct interactions of intramembrane pump, plays a role in energy conversion. However, it is also possible that for one reason or another $\Delta\tilde{\mu}_H$ itself is maximized at this temperature range. In that case the results would be compatible with $\Delta\tilde{\mu}_H$ as sole intermediate. It was, therefore, necessary to measure accurately $\Delta\tilde{\mu}_H$ as a function of temperature, a task we set out to accomplish in this study. In addition, in order to assess more accurately the temperature dependence of the efficiency of energy conversion, we also measured ΔG_P as a function of temperature. If $\Delta\tilde{\mu}_H$ is the sole intermediate in energy conversion we would expect the ratio $\Delta G_P/\Delta\tilde{\mu}_H$ to be independent of temperature. In another related study [14] we have found that the efficiency of coupling, as measured by respiratory control in rats which were chronically fed ethanol, is reduced. This finding may also suggest that intramembrane coupling increases energy-conversion efficiency, since it is now well established that in mitochondria from chronically alcoholic rats the activity and/or content of the ATPase and selected electron transport complexes is reduced (cf. Refs. 15 and 16). However, it is possible that $\Delta\tilde{\mu}_H$ is also reduced in mitochondria from ethanol-fed rats a condition which would be sufficient to explain the reduced efficiency. Therefore, we decided to determine the magnitude and the temperature dependence of $\Delta\tilde{\mu}_H$ in rat liver mitochondria from ethanol-fed rats and their pair-fed controls, and also the magnitude of ΔG_P at state 4, which is maintained by these mitochondria.

Material and Methods

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories) were used for this study. Chow-fed animals were sacrificed when weighing 250–300 g after ad-lib feeding. For the ethanol feeding experiments, littermates weighing 150–160 g were pair-fed a totally liquid diet, in which ethanol provided 36% of total calories, protein 16%, fat 35% and carbohydrate the remainder [17]. Pair-fed controls received the same diet except that carbohydrate isocalorically replaced ethanol.

* In Mitchell terminology, the 'protonmotive force', P, is related to μ_H^+ by the following relation: $P = -\Delta\tilde{\mu}_H/F$, where F is the Farady constant.

Rats were maintained on this diet for 5 weeks. Ethanol consumption averaged 14.3 ± 0.8 g per kg of body weight per day.

Preparations

Liver mitochondria were isolated by conventional differential centrifugation in a medium of 0.25 M sucrose and 1.0 mM EDTA (pH 7.0) as described previously [14]. Submitochondrial particles were prepared by passing mitoplasts through a Yeda-press as described previously [18].

Assays

Protein was determined by a cyanide biuret procedure [19]. Phosphorylation rates and ATP hydrolysis rates were measured by the rate of disappearance or appearance of protons using a pH electrode as described previously [20]. The proton electrochemical potential ($\Delta\tilde{\mu}_H$) was calculated from the estimated membrane potential ($\Delta\psi$) and pH gradient (ΔpH), using the flow dialysis method [21]. The dialysis cell was modified as described [30]. ^{86}Rb in the presence of valinomycin was used to estimate $\Delta\psi$, and [^{14}C]DMO was used to estimate ΔpH. The internal water volume was measured from the distribution of [^{14}C]sucrose and ^3H_2O between pellet and supernatant [5]. Oxygen consumption was measured with an oxygen electrode.

Results

Temperature dependence of $\Delta\tilde{\mu}_H$

The preferred method for estimation of $\Delta\tilde{\mu}_H$ in mitochondria is from the determination of the pellet to supernatant ratio of ^{86}Rb (in the presence of valinomycin) and [^{14}C]DMO after centrifugation in a microfuge [5]. However, it was difficult to adapt this method for the study of the temperature dependence of $\Delta\tilde{\mu}_H$, because it was impossible to control the temperature in the microfuge. We have, therefore, adapted the flow dialysis method [21] in which the dialysis chamber is thermostated with a water jacket, allowing a precise control of temperature [30]. This method has been used extensively for $\Delta\tilde{\mu}_H$ determination with both right-side out and inside-out bacterial vesicle preparations and with submitochondrial particles [21,7]. It can be easily adapted to determination of $\Delta\tilde{\mu}_H$ in mitochondria by utilizing ^{86}Rb (for $\Delta\psi$) and [^{14}C]DMO (for pH). The only necessary precaution is to choose a medium in which ΔpH is relatively large (> 40 mV), since it is difficult to accurately measure small values of ΔpH with this method. When checked against the centrifugation method the two methods give comparable values of both $\Delta\psi$ and ΔpH.

In order to calculate ΔpH from flow-dialysis, it is necessary to measure the matrix volume of the mitochondria. We measured, in separate experiments, under identical incubation conditions, the matrix volume using the [^{14}C]sucrose and ^3H_2O as described elsewhere [5]. The matrix volume under the experimental conditions of the experiment described below varied little. An average value of 1.03 ± 0.09 was obtained with a large number of preparations under state 4 conditions and was used routinely in $\Delta\tilde{\mu}_H$ calculation. There was no significant difference in matrix volume between control mitochondria and mitochondria from ethanol-fed rats. The possible temperature dependence of the volume is somewhat more difficult to assess because it is difficult to control the temperature in the microfuge precisely. However, in a separate study in which $\Delta\tilde{\mu}_H$ and matrix volume were determined for each point by the centrifugation method after incubation at 37°C and 25°C, we found no significant difference in the volume between the two temperatures (unpublished data collected during the course of study described in Ref. 27). Fig. 1 shows a flow dialysis experiment with rat liver mitochondria under conditions of large $\Delta\psi$ (165 mV) and small ΔpH (40 mV). The addition of mitochondria and valinomycin induced uptake of both ^{14}C[DMO] and ^{86}Rb indicating a small but significant residual $\Delta\tilde{\mu}_H$. The addition of substrate greatly enhanced Rb uptake, but only slightly that of DMO. State 4 $\Delta\tilde{\mu}_H$ was estimated to be 205 mV. The addition of ADP lead to a transient reduction of $\Delta\psi$ (state 3) which returned to its state 4 value after complete phosphorylation of ADP. Addition of the uncoupler CCCP completely abolished $\Delta\tilde{\mu}_H$. In this experiment, state 4 $\Delta\tilde{\mu}_H$ was estimated in the presence of a relatively high phosphate concentration which reduced the magnitude of ΔpH.

In subsequent experiments we increased the protein concentration and reduced the phosphate

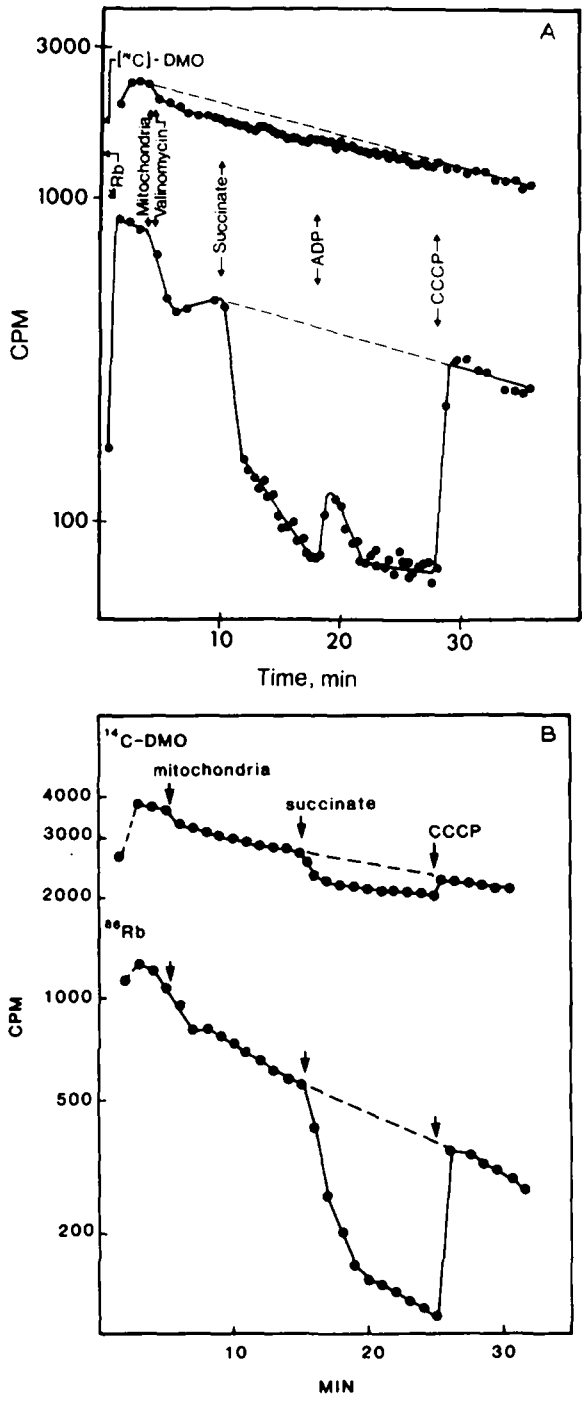

Fig. 1. (A) $\Delta\tilde{\mu}_H$ determination by flow-dialysis: The upper chamber contained 300 μl of 0.2 M sucrose/50 mM NaCl/10 mM Pipes/4 mM $MgCl_2$/5 mM Na_2HPO_4 (pH 7.0). The identical buffer, equilibrated at 25°C, was pumped through the lower chamber at a rate of 6 ml/min. Fractions of 1.4 ml were collected continuously. The upper trace shows an experiment in which [^{14}C]DMO (15 μM) was added at time zero. The lower trace shows a parallel experiment in which ^{86}Rb (3 μM) was added at time zero. Further additions were made where indicated: rotenone (1 μM) and mitochondria (2.8 mg/ml), valinomycin ($1 \cdot 10^{-7}$ M), succinate (10 mM), ADP (0.3 mM) and finally, CCCP (2 μM). Calculation of ΔpH and $\Delta\psi$ as in Ref. 21. (B). Conditions are as in (A), except that the phosphate concentration was reduced to 1.0 mM, the protein concentration was increased to 4.9 mg/ml, and ADP was omitted.

concentration which resulted in a substantial decrease in the error of ΔpH. This is shown in Fig. 1B where the course of a typical determination of state 4 $\Delta\tilde{\mu}_H$ is under the same conditions that were employed in subsequent experiments (Figs. 2 and 3).

The temperature dependence of ΔpH, $\Delta\psi$ and $\Delta\tilde{\mu}_H$ in state 4, as determined from flow dialysis experiments, is shown in Fig. 2. It was observed that $\Delta\tilde{\mu}_H$ increases monotonically with temperature. Both ΔpH and $\Delta\psi$ increase, although ΔpH increases to a larger extent. The respiratory control ratio of these preparations was 4.8 ± 1.4 at 10°C, 5.8 ± 0.7 at 25°C and 3.3 ± 0.5 at 40°C. Hence, the decreased coupling efficiency at high temperatures cannot be attributed to a reduction of $\Delta\tilde{\mu}_H$. Fig. 3 shows a similar experiment with mitochondria from ethanol-fed rats and their pair-fed controls. Basically the same pattern of temperature dependence was observed for both preparations. Though it appears that ΔpH is somewhat higher in mitochondria from ethanol-fed rats, and $\Delta\psi$ is somewhat lower, these differences are not statistically significant. Moreover, these apparent differences compensate for each other, such that $\Delta\tilde{\mu}_H$ values for treated and control mitochondria, at all temperatures, are practically identical. The differences is respiratory control of these preparations are shown in Table I. Clearly the difference in efficiency between mitochondria from ethanol-fed and control animals cannot be a result of difference in $\Delta\tilde{\mu}_H$.

The determination of ΔG_P

ΔG_P is usually determined after a long incubation of respiring mitochondria in the presence of ADP, ATP and P_i to attain a steady-state, followed by quenching of the reaction and the subsequent determination of the steady-state concentra-

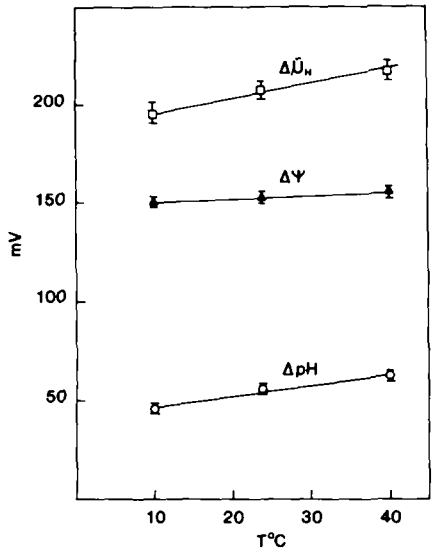

Fig. 2. $\Delta\tilde{\mu}_H$ as a function of temperature. Assays were performed essentially as in Fig. 1B at three temperatures: 10°C, 25°C and 40°C. A thermostatically controlled bath contained the buffer solution which was pumped into the lower chambers. Additionally, water from the bath was circulated through the water jacket surrounding the chamber. Protein concentration was increased to 4.9 mg/ml and phosphate reduced to 1.0 mM (pH 8.0). The medium and buffer were saturated with 100% O_2, and a stream of O_2 was flushed above the vigorously stirred upper chamber. Results are an average of three separate determinations.

tions of ADP and ATP. Because incubation of mitochondria at high temperatures for long periods of time (20–30 min) may result in deterioration of coupling, and because of persistent doubts concerning the accuracy of this method, particularly due to interference from myokinase, we have

TABLE I

RESPIRATORY CONTROL AS A FUNCTION OF TEMPERATURE IN ETHANOL-FED AND CONTROL RATS

Conditions for assays are as in Fig. 3, except for the protein concentration, which was 2.0 mg/ml. Results are from five pairs. Statistical analysis of significance is by the paired *t*-test.

Respiratory control			*P*
T (°C)	ethanol-fed	control	
10	4.2±0.9	5.8±0.8	> 0.001
25	5.4±1.3	6.9±0.9	> 0.001
37	3.1±0.9	4.5±0.8	> 0.001
40	2.9±0.5	3.6±0.4	> 0.001

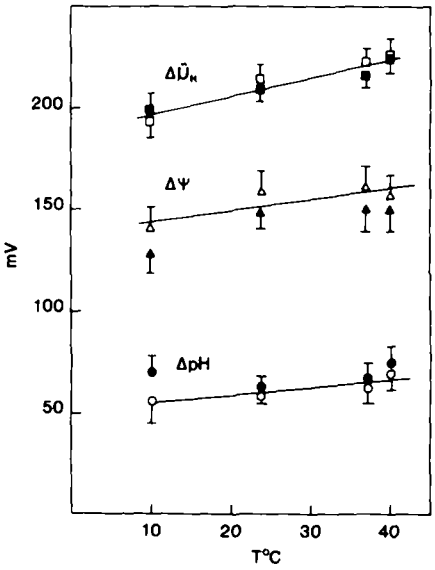

Fig. 3. $\Delta\tilde{\mu}_H$ determinations as a function of temperature in mitochondria from ethanol-fed rats (full symbols) and their pair-fed controls (empty symbols): Conditions and procedures are as in Fig. 2. Results are from five pairs.

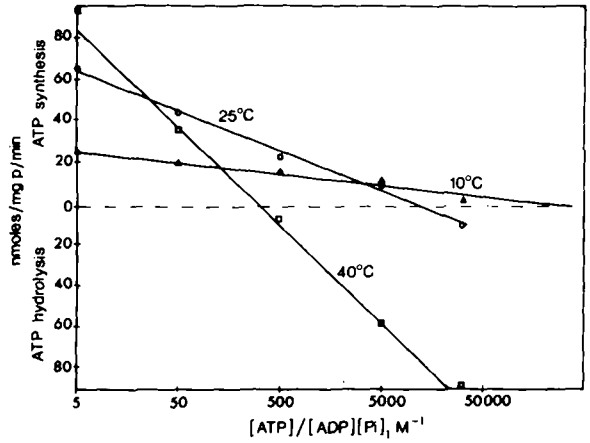

Fig. 4. Determination of the temperature dependence of ΔG_P at state 4 from a null-point titration of the reaction ATP ⇆ ADP + P_i + H^+: The rate of phosphorylation and ATP hydrolysis was measured using a pH electrode [20] at 10°C, 25°C and 40°C. The medium contained 0.2 M sucrose, 50 mM NaCl, 1 mM Na_2HPO_4 (pH 8.0), succinate (10 mM), rotenone (1 μM), valinomycin (0.1 μM) and mitochondria (2 mg protein/ml). The total nucleotide concentration (ADP + ATP) was 1.25 mM. The ratio of ADP to ATP was varied for each point. G_P was calculated for each temperature using values of ΔG^0_P extrapolated from Rossing and Slater [31] as follows: at 10°C, ΔG_P = 37.16 + 1.30 log([ATP]/[ADP] [P_i]) = 67.80 kJ/mol; at 25°C, ΔG_P = 38.17 + 1.36 log([ADP]/[ATP] [P_i]) = 61.10 kJ/mol; at 40°C, G_P = 39.13 + 1.43 log([ATP]/[ADP] [P_i]) = 54.82 kJ/mol.

developed a null-point titration which allows an instantaneous, in situ, determination of ΔG_p. To respiring mitochondria we added various mixtures of ADP, ATP and P_i to cover an extended range of ΔG_P. At a value of ΔG_P, which exceed the state 4 value, the mitochondria will hydrolyze ATP, while at values which are below the state 4 value, the mitochondria will synthesize ATP. The mixture which results in no net reaction is equivalent to ΔG_P at state 4. The initial rates of both phosphorylation and hydrolysis are conveniently measured with the aid of a pH electrode [20]. This method is insensitive to the myokinase reaction, and hence indicates only the direction of the ATPase reaction. The determination of ΔG_P by this method is illustrated in Fig. 4, where the value of ΔG_P is estimated at 10°, 25° and 40°C.

To enable comparison with $\Delta\tilde{\mu}_H$ measurement, the time-course of the experiment was identical to that of $\Delta\tilde{\mu}_H$ measurement (Fig. 1B). ATP hydrolysis is very slow at 10°C and could only be detected in the absence of added ADP. Because phosphate potential value is indeterminate under this condition, this point was not included in the figure, therefore the value of ΔG_P estimated at 10°C is a minimal value, which could be even higher if the ATPase data were taken into account. It is immediately apparent that ΔG_P is reduced as the temperature increases. The values obtained are 67.8 kJ/mol at 10°C, 61.1 kJ/mol at 25°C and 54.8 kJ/mol at 40°C. Hence, the reduction of efficiency at high temperature, deduced from respiratory control experiments [12–14], is also manifested by the values of ΔG_P at state 4.

Although the use of this method requires slightly different conditions from those utilized for the

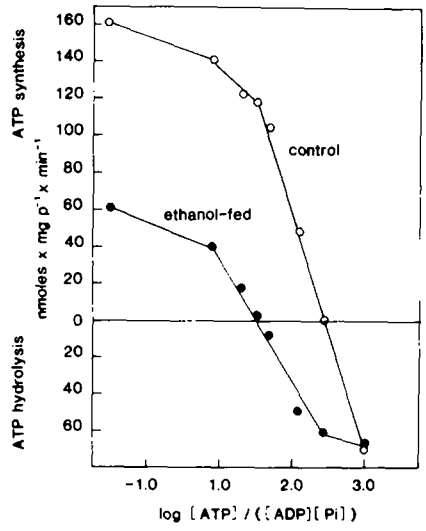

Fig. 6. Determination of ΔG_P at state 4 in mitochondria from an ethanol-fed rat and its pair-fed control. Temperature is 37°C; other conditions as in Fig. 4. The calculated ΔG_P for the control is 54.0 and for the ethanol-fed 49.0.

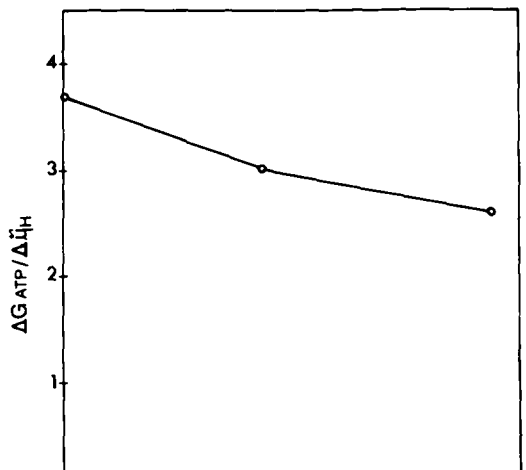

Fig. 5. The ratio $G_P/\Delta\tilde{\mu}_H$ as a function of temperature. Data are taken from Figs. 2 and 4.

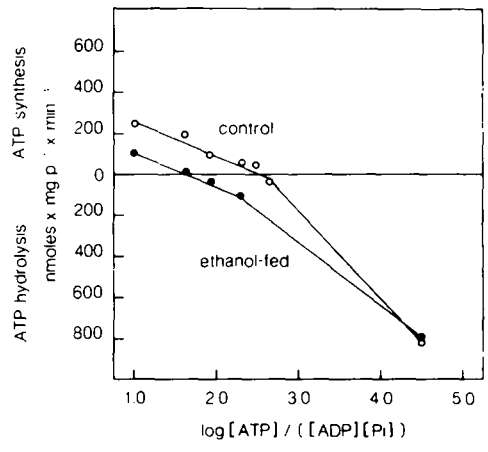

Fig. 7. Determination of ΔG_P at state 4 in submitochondrial particles. Medium was 0.2 M sucrose, 5 mM $MgCl_2$; temperature 25°C. Other conditions were as in Fig. 4. The calculation of ΔG_P for these conditions based on the extrapolated value of G_P^0 [31] is as follows: $\Delta G_P = 32.43 + 1.36$ log([ATP]/[ADP][P_i]). The value of control is 46.5 and for ethanol-fed 41.0.

determination of $\Delta\tilde{\mu}_H$, it was established that these changes have no effect on the value of state 4 $\Delta\tilde{\mu}_H$. Neither Pipes (or other buffer) nor Mg^{2+} has any significant effect on $\Delta\tilde{\mu}_H$. Adenine nucleotide also do not affect the value of state 4 $\Delta\tilde{\mu}_H$ (see for instance, Fig. 1A). Combining the data of Figs. 4 and 2, we have calculated the value of $\Delta G_P/\Delta\tilde{\mu}_H$ at each temperature. This is shown in Fig. 5. Since $\Delta\tilde{\mu}_H$ increases with temperature and ΔG_P decreases, the ratio obviously greatly decreases with increased temperature. Fig. 6 shows a similar experiment (at 37°C) with mitochondria from a pair of rats, one ethanol-fed and the other its pair-fed control. Clearly, the value of ΔG_P at state 4 is lower after ethanol feeding and is in agreement with the lower respiratory control. In nine similar experiments (at 25°C) the average difference was 5.19 kJ/mol (Table II). Since $\Delta\tilde{\mu}_H$ was not significantly different, this resulted in a significantly lower $\Delta G_P/\Delta\tilde{\mu}_H$ ratio in mitochondria from ethanol-fed rats (2.77 vs. 2.97). The high ΔG_P maintained in the mitochondrial suspension is partially dependent on the electrogenicity of the ADP/ATP translocator [22]. Since it is possible that the translocator function is impaired in mitochondria from ethanol-fed rat, it was necessary to find whether the difference in ΔG_P at state 4 between mitochondria from control and ethanol-fed animals is also observed in submitochondrial particles. Fig. 7 shows a typical titration of ΔG_P with submitochondrial particles from control and ethanol-fed rats. In these vesicles too the difference between preparations from control and ethanol-fed animals is maintained, and is practically identical to the difference in mitochondria (Table II). Hence the difference in the efficiency of oxidative phosphorylation is not due to a deficiency in the translocation of ATP and ADP across the membrane.

Because $\Delta\tilde{\mu}_H$ was not determined in submitochondrial particle, it was not certain that a difference of $\Delta\tilde{\mu}_H$ is not responsible in part for the reduction in ΔG_P. However, in view of the results with intact mitochondria we consider it unlikely that the difference in ΔG_P results from a difference in $\Delta\tilde{\mu}_H$.

Discussion

Temperature dependence of the coupling efficiency of rat liver mitochondria

Previous studies have indicated that the respiratory control and also the ATPase control in rat liver mitochondria is strongly dependent on temperature [12–14]. This finding suggested to us that coupling efficiency is affected by temperature-dependent structural parameters, which may control intramembranal proton pathways. However, two deficiencies in this preliminary observation precluded a definite conclusion. Firstly, it is possible that $\Delta\tilde{\mu}_H$ is also temperature-dependent and that the dependence of respiratory control simply reflects temperature effects on $\Delta\tilde{\mu}_H$. Secondly, it is not clear to what degree respiratory control is an accurate indicator of the coupling efficiency. The results of this study address these deficiencies and

TABLE II

THE FREE ENERGY OF ATP HYDROLYSIS AT STATE 4 AND $\Delta\tilde{\mu}_H$ IN MITOCHONDRIA FROM ETHANOL-FED RATS AND THEIR CONTROLS

$\Delta\tilde{\mu}_H$ measurements are from Fig. 3. ΔG_P in mitochondria was estimated as in Fig. 4. ΔG_P in submitochondrial particles was estimated as in Fig. 7. The statistical analysis of significance is by the paired *t*-test.

	n	Ethanol-fed	Control	Difference	*P*
(a) Mitochondria					
ΔG_P (kJ/mol)	9	56.04 ± 0.67	61.23 ± 0.33	5.19	> 0.0001
$\Delta\tilde{\mu}_H$ (kJ/mol)	5	20.26 ± 0.96	20.63 ± 0.75	0.37	not specified
$\Delta G_P/\Delta\tilde{\mu}_H$		2.77	2.97		
(b) Submitochondrial particles					
ΔG_P (kJ/mol)	4	40.93 ± 1.42	46.45 ± 0.67	5.52	> 0.001

firmly establish our suggestion that coupling efficiency decreases with temperature. Although the temperature dependence of the quenching of the potential indicator di-S-C_3(5), suggested that $\Delta\psi$ increases with temperature [13], the great uncertainty of potential measurements with this and other indicators, and the absence of ΔpH measurements precluded a definite conclusion regarding the temperature dependence of $\Delta\tilde{\mu}_H$. The determination of the temperature dependence of $\Delta\tilde{\mu}_H$ by the flow-dialysis method shows conclusively that $\Delta\tilde{\mu}_H$ increases with temperature. Most of the increase is due to an increased ΔpH, which may result from a difference in the activation energy between the redox proton pumps that generate ΔpH and the proton-coupled transport systems that lower ΔpH.

An increased value of $\Delta\tilde{\mu}_H$ with temperature was also observed by the conventional centrifugation method [27]. However, because the incubation temperature (37°C) could not be adequately controlled in the microfuge during centrifugation, there is uncertainty as to the exact temperature to be assigned to these measurements. The determination of the temperature dependence of ΔG_P at state 4 clearly shows a decreased efficiency with temperature. The value of ΔG_P at 25°C estimated by the null-point titration of the phosphorylation-ATPase reaction is in reasonable agreement with measurements by more conventional techniques, and agrees well with an estimation by ^{31}P-NMR (Rottenberg, H. and Taraschi, T., unpublished data).

The method of determination of $\Delta G_P/\Delta\tilde{\mu}_H$ from flow-dialysis measurements and parallel null-point titration is sufficiently different from more conventional methods to raise the question of confidence. We have adopted these methods because we believe them to be more reliable at high temperatures. Comparison (at room temperature) gave values which are similar to those obtained by conventional techniques. Both methods are not very accurate and may be subject to numerous artifact. However, because the results show not only quantitative but even qualitative differences in the effect of temperature and alcohol-feeding on ΔG_P and $\Delta\tilde{\mu}_H$, we believe that the changes indicated in $\Delta G_P/\Delta\tilde{\mu}_H$ values are real, regardless of their exact value. It should be noted that the results are not fully compatible with the respiratory control experiments (Table I), since respiratory control is maximal at 25°C, whereas ΔG_P is maximal at lower temperatures. We consider ΔG_P to be a more reliable estimate of the maximal efficiency of energy conversion, since it only depends on thermodynamic parameters and not on kinetic parameters. The opposing effect of temperature on coupling efficiency and $\Delta\tilde{\mu}_H$ strongly suggests that $\Delta\tilde{\mu}_H$ is not a sole intermediate. This is best expressed by the strong temperature dependence of the ratio $\Delta G_P/\Delta\tilde{\mu}_H$ (Fig. 5). This ratio is 3.56 at 10°C, falling to 3.05 at 25°C, and 2.60 at 40°C. This pattern is not compatible with the hypothesis that $\Delta\tilde{\mu}_H$ is the direct and only driving force for ATP synthesis. Since $\Delta\tilde{\mu}_H$ is higher at high temperatures it follows that $\Delta G_P/\Delta\tilde{\mu}_H$ increases with a reduction of $\Delta\tilde{\mu}_H$. This is quite similar to previous observations where $\Delta\tilde{\mu}_H$ was modulated by uncouplers and inhibitors (reviewed in Refs. 7–9). However, in all previous cases lowering $\Delta\tilde{\mu}_H$ also lowered ΔG_P although not in proportion to $\Delta\tilde{\mu}_H$, while here lowering $\Delta\tilde{\mu}_H$ (by lowering temperature) actually increased ΔG_P. There is a considerable amount of evidence that temperature changes in the 10°C–40°C range affect mitochondrial membrane structure. These include data derived from differential scanning calorimetry [23], studies on the energy of activation of membrane enzymes [24,14], and the temperature dependence of membrane spin-probe signals [25,26]. While it is not clear, as yet, what is the precise change that triggers these transitions, which show some characteristics of a broad phase-transition, it is possible that the weak forces holding protein aggregates in the membrane are overcome by thermal energy resulting in a breakdown of aggregates at elevated temperatures. If this is, indeed, the major temperature effect on mitochondrial membranes at this range, the decreased coupling efficiency at high temperature would mean that protein-aggregation enhances direct proton transfer between redox H^+-pumps and the H^+-ATPase. Similar conclusions were drawn recently from the effects of general anesthetics on oxidative phosphorylation [27].

Coupling efficiency in ethanol-fed rats

Chronic ethanol feeding produces both morphological and functional changes in rat liver

mitochondria (cf. Refs. 15 and 16). In addition to reduced respiratory control, there is a significant reduction in the rate of oxidation and the rate of phosphorylation. These latter effects appear to be largely the result of a decrease in the membrane content and/or activity of several key components of the electron-transport chain and of ATPase [16]. This may be due either to inhibition of mitochondrial protein synthesis by ethanol or to impaired insertion of nascent proteins into the membrane. Lipid composition is also altered in these mitochondria [28], the most significant change being the increased saturation of cardiolipin acyl-chains. These changes in membrane composition lead to significant changes in the physical properties of the membrane. There is a slight decrease in membrane fluidity and an increase in the transition temperature of enzymatic activity [14] and membrane order [26]. These membranes are also more resistant to the fluidizing effect of ethanol [28], to the uncoupling effect of ethanol [14] and to the fluidizing effects of general anesthetics [29]. All the latter effects appear to be a result of reduced partition coefficients of alcohol and anesthetic in these membranes [29]. While the decrease in respiratory control suggests reduced coupling efficiency, this indicator is not sufficiently reliable for such a conclusion (see above). However, the finding that ΔG_P in state 4 is considerably reduced in mitochondria from ethanol-fed rats establishes that coupling is indeed impaired in these membranes. This difference persists both at 25°C and 37°C and also in submitochondrial particles prepared from ethanol-fed rats. The latter observation excludes the possibility that the impaired efficiency is due to impairment in potential-driven ADP/ATP translocation. Inspection of Table II shows that the difference in ΔG_P between control mitochondria and submitochondrial particles is 14.6 kJ/mol, while this difference in preparations from ethanol-fed rats is 15.1 kJ/mol. Since $\Delta\psi$ in mitochondria in both cases is 150 mV, which is equivalent to 14.9 kJ/mol, these data indicate that the electrogenic translocator in ethanol-fed mitochondria maintains the same gradient of ATP/ADP as control [22]. Hence, the reduction of ΔG_P in mitochondrial suspensions must be attributed to a lower efficiency of oxidative phosphorylation and not to the nucleotide transporter. The fact that $\Delta\tilde{\mu}_H$ is the same in mitochondria from control and ethanol-fed rats indicates that the reduced rate of oxidation and phosphorylation due to reduced enzyme content and/or activity could not, in itself, explain these data either. The rate of oxidation and/or phosphorylation would determine how fast state 4 would be reached, but not the eventual thermodynamic static head. Thus, the difference in $\Delta G_P/\Delta\tilde{\mu}_H$ between mitochondria from control and ethanol-fed rats indicates that the intramembranal coupling efficiency is reduced in mitochondria from ethanol-fed rats. Because the membrane concentration of active proton pumps is significantly reduced in this membrane, the frequency of aggregate formation, in which an active proton donor redox-pump and an active proton-acceptor H^+-ATPase co-exist will be greatly reduced. This should then reduce the efficiency of energy conversion.

In conclusion, the studies presented in this paper on the temperature dependence of $\Delta\tilde{\mu}_H$ and ΔG_P and on the effects of ethanol feeding on these parameters, reveal two cases in which increased or decreased coupling efficiency does not correlate with $\Delta\tilde{\mu}_H$. These data suggest that $\Delta\tilde{\mu}_H$ is not the sole intermediate in oxidative phosphorylation. On the other hand, both cases are compatible with and support the notion that efficient coupling is mediated by the formation of dynamic aggregates of redox H^+-pumps and H^+-ATPases [9,27].

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References

1 Mitchell, P. (1961) Nature 191, 144–148
2 Mitchell, P. (1979) Science 206, 1148–1159
3 Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative Phosphorylation and Photophosphorylation, Glynn Research, Bodmin, U.K.
4 Rottenberg, H. (1975) J. Bioenerg. Biomembranes 7, 63–76
5 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
6 Azzone, G.F., Pietrobon, D. and Zoratti, M. (1984) Curr. Top. Bioenerg. 13, 1–77

7 Ferguson, S.J. and Sorgato, M.C. (1982) Annu. Rev. Biochem. 51, 185–217
8 Westerhoff, H.V., Helgerson, S.L., Theg, S.M., Van Kooten, O., Wikström, M., Skulachev, V.P. and Dancshazy, Z.S. (1983) Acta Biochim. Biophys. Acad. Sci. Hung. 18, 125–149
9 Rottenberg, H. (1985) in Modern Cell Biology, Vol. 4, (Satir, B., ed.), Alan R. Liss, Inc, New York, in the press
10 Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437
11 Mandolino, C., DeSantis, A. and Melandri, B.A. (1983) Biochim. Biophys. Acta 723, 428–439
12 Rottenberg, H. (1978) FEBS Lett. 94, 295–297
13 Rottenberg, H. (1978) in Frontiers of Biological Energetics (Dutton, P.L., Leigh, J. and Scarpa, A., eds.), Vol. 1, 403–412, Academic Press, New York
14 Rottenberg, H., Robertson, D. and Rubin, E. (1980) Lab. Inv. 42, 318–326
15 Rubin, E. and Rottenberg, H. (1982) Fed. Proc. 41, 2465–2471
16 Thayer, W.S. and Rubin, E. (1981) J. Biol. Chem. 256, 6090–6097
17 DeCarli, L.M. and Lieber, C.S. (1967) J. Nutr. 91, 331–336
18 Hashimoto, K. and Rottenberg, H. (1983) Biochem. 22, 5738–5745
19 Szarkowska, L. and Klingenberg, M. (1963) Biochem. Z. 338, 674–697
20 Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182
21 Ramos, S., Schuldiner, S. and Kaback, H.R. (1979) Methods Enzymol. 55, 680–688
22 Klingenberg, M. and Rottenberg, H. (1977) Eur. J. Biochem. 73, 125–130
23 Blazk, J.F. and Newman, J.L. (1980) Biochim. Biophys. Acta 600, 1007–1011
24 McMurchie, E.J. and Raison, J.K. (1979) Biochim. Biophys. Acta 554, 364–374
25 Raison, J.K., Lyons, J.M., Melhorn, R.J. and Keith, A.D. (1971) J. Biol. Chem. 246, 4036–4040
26 Waring, A.J., Rottenberg, H., Ohnishi, T. and Rubin, E. (1982) Arch. Biochem. Biophys. 216, 51–61
27 Rottenberg, H. (1983) Proc. Natl. Acad. Sci. USA 80, 3313–3317
28 Waring, A.J., Rottenberg, H., Ohnishi, T. and Rubin, E. (1981) Proc. Natl. Acad. Sci. USA 78, 2582–2586
29 Rottenberg, H., Waring, A.J. and Rubin, E. (1981) Science 213, 583–585
30 Feldman, K. (1978) Anal. Biochem. 88, 225–235
31 Rossing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta 267, 275–290