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CONTROL OF OXIDATIVE PHOSPHORYLATION BY THE EXTRAMITOCHONDRIAL ATP/ADP RATIO*

U. KÜSTER, R. BOHNENSACK and W. KUNZ**

Institut für Physiologische Chemie, Medizinische Akademie, 301 Magdeburg (G.D.R.)

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SUMMARY

The control of mitochondrial ATP synthesis by the extramitochondrial adenine nucleotide pattern was investigated with rat liver mitochondria. It is demonstrated that any stationary state between the two limit states of maximum activity (state 3) and of resting activity (state 4) can be obtained by a hexokinase-glucose trap as an ADP-regenerating system. These intermediate states are characterized by stationary respiratory rates, stationary redox levels of the cytochromes *b* and *c* and stationary levels of extramitochondrial ATP and ADP between the rates and levels of the limit states. At a constant concentration of inorganic phosphate the activity of mitochondria between the limit states is controlled by the extramitochondrial ATP/ADP ratio independent of the total concentration of adenine nucleotides present. The control range was found to be between ratios of about 5 and 100 at 10 mM phosphate. At lower ratios the mitochondria are in their maximum phosphorylating state. With succinate+rotenone and glutamate+malate the same control range was observed, indicating that it is independent of the nature of substrate oxidized.

The results suggest that in the control range the mitochondrial activity is limited by the competition of ADP and ATP for the adenine nucleotide translocator.

INTRODUCTION

Respiring eukaryotic cells meet their energy requirement mainly by mitochondrial oxidative phosphorylation. The velocity of this process is well adapted to the demand of the cell. In his pioneer work, Chance [1–3] postulated that ADP is the most favourable candidate of the respiratory control [4]. This conception is reflected in the widely used "state nomenclature" proposed by Chance and Williams [1, 2] implicating a kinetic transition only between state 3 of active phosphorylation and the resting state 4. Shortly afterwards the reversal of the electron transport through

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** To whom correspondence should be addressed.

the respiratory chain was demonstrated in the presence of ATP [5–7] and a thermodynamic control of the respiration rate in a broad concentration range was postulated by Klingenberg [8]. According to this concept the respiration rate is controlled by the "phosphate potential", i.e. the mass action ratio of the reaction

$$ADP + P_i \rightleftharpoons ATP \qquad (1)$$

Meanwhile, there have been many reports on the extramitochondrial phosphate potential and its relations to respiring mitochondria [9–12]. But the results published mainly refer to the controlled state or were not obtained under stationary conditions of the extramitochondrial adenine nucleotides, as far as the active phosphorylating state is concerned. Therefore, they are of limited value for the interpretation of the relations between the extramitochondrial energy consumption and the mitochondrial ATP synthesis.

The first results under conditions of stationary extramitochondrial ATP/ADP ratios were obtained by Davis and Lumeng [13, 14]. They differ considerably in some points from results obtained by us. In the present paper it is shown that under stationary conditions, in the presence of a constant amount of Mg^{2+} and a nearly constant amount of inorganic phosphate:

(i) each kind of steady state between the fully active state 3 and the resting state 4 is possible and stable;

(ii) in these intermediate states the respiration activity is controlled by the ATP/ADP ratio independently of the total amount of adenine nucleotides present in the incubation medium;

(iii) the redox ratios of the cytochromes *b* and $c+c_1$ change parallel to the rates of respiration and phosphorylation; and,

(iv) the control range of the ATP/ADP ratio is nearly independent of the nature of the oxidized substrate.

MATERIALS AND METHODS

Chemicals and enzymes

Yeast hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), lactate dehydrogenase (EC 1.1.1.27), adenylate kinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), phosphoenolpyruvate, carboxyatractyloside, ATP, ADP, succinate, Tris, NADP, and NADH were purchased from Boehringer, Mannheim. L-glutamate and oligomycin were obtained from Serva, Heidelberg, and L-malate from Ferak, Berlin.

The hexokinase was deionized by a small column of Sephadex G 25 prior to use.

Isolation of mitochondria

Rat liver mitochondria were prepared as previously described [15] in 0.25 M deionized sucrose adjusted to pH 7.4 with Tris · HCl. The respiratory control ratio of all preparations was better than 4 in the presence of 10 mM succinate plus rotenone.

Incubation conditions

The standard incubation medium contains 110 mM sucrose, 60 mM Tris, 60 mM KCl, 15 mM glucose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, and 0.5 mM EDTA.

The pH was adjusted to 7.4 with HCl. Further additions are given in the legends of the figures. Usually an amount of mitochondria equivalent to 1–2.5 mg protein per ml were incubated at 22 °C in a 5-ml cuvette, both for polarographic and spectrophotometric determinations. In each case, the incubation mixture was covered by a paraffin layer, reducing the back diffusion of oxygen from the air. The sample compartment of the photometer was equipped with two tubules of stainless steel, permitting additions or withdrawal of samples without interruption of the measurements. Rapid mixing was achieved using a magnetic stirrer. Samples for the determination of metabolites were stopped in two different ways.

Method a: 0.6-ml samples were added to the same volume of 1.9 M $HClO_4$ and centrifuged within 60 s with centrifuge TH 11, VEB Zentrifugenbau, Leipzig. The supernatant was neutralized with a solution of K_2CO_3 and immediately frozen in liquid nitrogen. The time between the withdrawing and the freezing was 2.5–5 min.

Method b: 0.6 samples were added to 2 ml of a phenol/chloroform/isoamylalcohol mixture (38 : 24 : 1, w/w) and vigorously shaken [10]. After centrifugation, the upper aqueous layer was removed and frozen in liquid nitrogen. Because the extraction of metabolites was incomplete by this method, a correction of the determined values was necessary. The percentage of extraction was the same for ATP, ADP, AMP and glucose 6-phosphate. Therefore, the sum of adenine nucleotides was determined in a second sample according to method A and used for the correction.

Determination of the rates of respiration and of phosphorylation and of the degree of reduction of the cytochromes

The respiration rate was registrated by a Clark electrode. The redox state of the cytochromes *b* and $c+c_1$ was measured according to Chance and Williams [16] with a Phoenix dual-wavelength spectrophotometer at 564–575 nm and 550–540 nm, respectively. The oxidized reference state was obtained after 2 min incubation without substrates in the presence of adenine nucleotides. The fully reduced state was achieved after consumption of oxygen in the incubation mixture.

The rate of mitochondrial ATP production was calculated from the measured concentrations of adenine nucleotides and glucose 6-phosphate according to Eqn. 2:

$$V_{ATP} = \left[\frac{(\Sigma P)_2}{(\Sigma AdN)_2} - \frac{(\Sigma P)_1}{(\Sigma AdN)_1}\right] \cdot \frac{(\Sigma AdN)_\phi}{C_p \cdot (t_2 - t_1)} \tag{2}$$

Here

$$\Sigma P = 3[ATP]+2[ADP]+[AMP]+[G6P]$$
$$\Sigma AdN = [ATP]+[ADP]+[AMP]$$

C_p, concentration of mitochondrial protein. The indices 1 and 2 refer to two samples at different times *t*, the index ϕ to the mean of all samples of an experiment. The normalisation through the total content of adenine nucleotides was necessary for the reduction of experimental errors. The whole amount of adenine nucleotides in the extract was taken as extramitochondrial because the intramitochondrial contribution is negligible.

Assays

The protein content was measured by the biuret method [17]. The activity of hexokinase and the metabolites were determined by standard enzymic procedures

[18]. ATP and glucose 6-phosphate were measured with hexokinase and glucose-6-phosphate dehydrogenase, ADP and AMP with pyruvate kinase, lactate dehydrogenase and adenylate kinase.

RESULTS

The existence of stationary intermediate states

In the usual polarographic or spectrophotometric experiment with addition of ADP up to 300 μM, there is a quick transition between the active state and the controlled [2, 11] one. But according to the concept of the thermodynamic control [8], any condition between these two limit states should correspond to a stationary intermediate state, providing that the mitochondrial phosphorylation of ADP is coupled to an appropriate ADP regenerating system. For this purpose, we used limiting amounts of yeast hexokinase in the presence of glucose. It offers the possibility of computing the phosphorylation rate from the glucose 6-phosphate formed and the changes in the adenine nucleotide pool.

Fig. 1A shows that any stationary rate of respiration between the two limit states can be obtained by the addition of appropriate amounts of hexokinase. These states will be called intermediate states. The respiration rate depends on the concentration of the hexokinase up to 0.1 units/mg mitochondrial protein (Fig. 1B), further enzyme additions being without influence.

The stationary respiration rates occurring after the addition of hexokinase correspond to a constant rate of glucose 6-phosphate formation and nearly constant

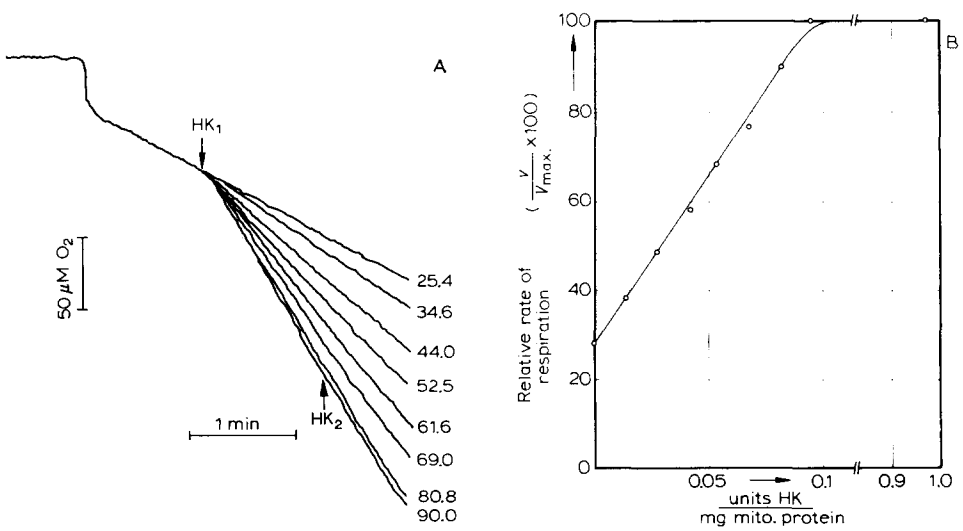

Fig. 1. Stationary respiration rates produced by additions of hexokinase. A. Incubation of rat liver mitochondria (2.92 mg protein/ml) in standard medium with 2 mM succinate, 1 μM rotenone and 1 mM ATP. HK_1: addition of 0–95 m I.U. hexokinase/mg mitochondrial protein. HK_2: further addition of 880 m I.U. hexokinase/mg mitochondrial protein. The respiratory rates are given at the polarographic traces in ngatoms 0/min per mg protein. B) The dependence of the resulting respiratory rate on the addition of hexokinase. The points are taken from the experiment shown in A.

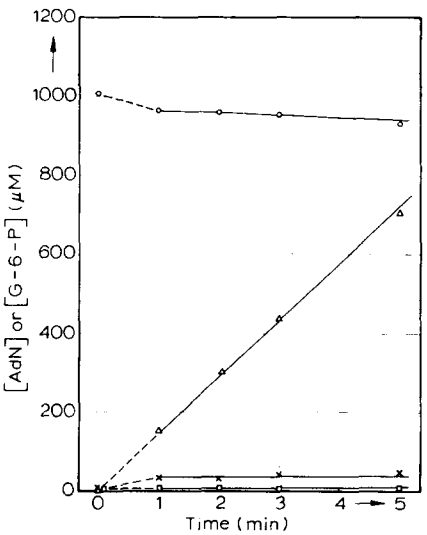

Fig. 2. Changes of the adenine nucleotide pattern after the addition of hexokinase. Incubation in standard medium with 1 mM ATP, 2 mM succinate and 1 μM rotenone (1.58 mg mitochondrial protein/ml). At zero time, 49 m I.U. hexokinase/mg mitochondrial protein were added. At the times indicated aliquots were taken, the reaction was stopped with perchloric acid and the mixture analyzed for ATP (○), ADP (×), AMP (□) and glucose 6-phosphate (△).

levels of ATP, ADP and AMP (Fig. 2). It is shown that the initial concentration of ATP decreases while the concentration of ADP increases until an equilibrium between the rates of mitochondrial ATP formation and ATP consumption by the hexokinase has been reached. Likewise, the level of AMP is slightly increased due to the adenylate kinase localized in the intermembrane space of the mitochondria. The transition into the new steady state is so fast that it cannot be monitored by the technique used in this experiment.

In Fig. 3, the response of the redox state of cytochrome *b* on the addition of hexokinase is illustrated. The oxidized state preceding the addition of the substrate and the reduced state after exhausting the oxygen are also recorded for the purpose of a quantitative evaluation of the degree of reduction. After addition of the substrate, the mitochondria shortly pass through the active state due to the small amount of ADP initially present in the incubation medium. It is shown that during the intermediate state (trace B) elicited by a limiting amount of hexokinase, the reduction degree is between that of the controlled state (trace A) and of the active state (trace C) produced by an excess of hexokinase. The degree of reduction is constant during the intermediate state; consequently, the mitochondria are in a true steady state comparable with those in the controlled state and in the active state. Furthermore, in these experiments, the very fast transition in the new steady state upon addition of hexokinase is demonstrated. In the following quantitative analysis the relationships between the intermediate states and the adenine nucleotide patterns are evaluated. For this purpose, the incubation mixtures were analyzed for the amounts of ATP, ADP, AMP and glucose 6-phosphate at points indicated by arrows in the figure.

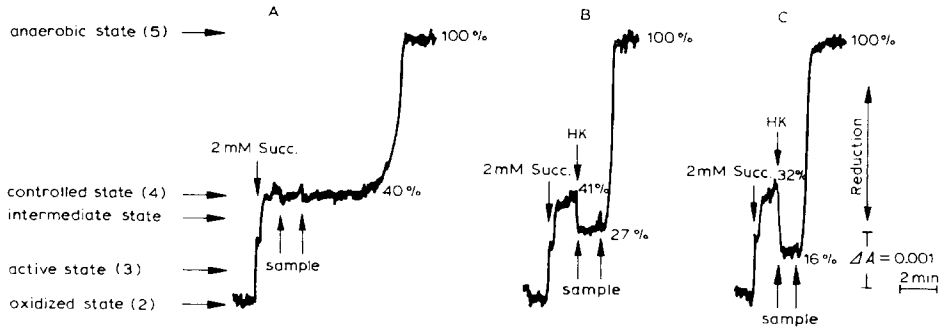

Fig. 3. Redox states of cytochromes *b* after different additions of hexokinase. Dual wavelength measurement at 564–575 nm. Mitochondria (1.76 mg protein/ml) were incubated in standard medium with 1 μM rotenone and 1 mM adenine nucleotides (98 % ATP, 1 % ADP, 1 % AMP) at 22 °C. 2 mM succinate was added after 2 min. When the mitochondria had reached the controlled state hexokinase (HK) was added in experiments B and C (50 m I.U./mg mitochondrial protein and 480 m I.U./mg protein, respectively). Arrows indicate when samples are taken for the assay of ATP, ADP, AMP and glucose 6-phosphate in the experiments illustrated in Figs. 4–7.

Control of the phosphorylation rate by the ATP/ADP ratio

If the overall reaction rate of the mitochondrial ATP formation depends only on the extramitochondrial ADP concentration [1–3], a shift in the total adenine nucleotide concentration at constant ADP concentration should be without effect. As shown in Fig. 4, this is not the case, in agreement with the results of other authors

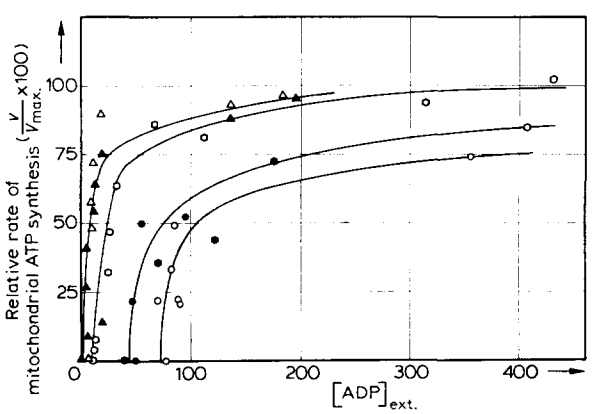

Fig. 4. Dependence on the ADP concentration of the rate of mitochondrial ATP synthesis. Mitochondria (1.7–2.0 mg protein/ml) were incubated as indicated for Fig. 3, with the exception of different concentrations of ATP. For each ATP concentration the amount of hexokinase was varied from 0 to 4.7 I.U./mg mitochondrial protein, resulting in different stationary states. The phosphorylation rate was calculated from the concentrations of ATP, AMP and glucose 6-phosphate determined in two samples before the addition of hexokinase and 1–3 min after that. Additions of ATP: 8.5 mM (○), 4.25 mM (●), 3.6 mM (◖), 1.08 mM (◇), 1.16 mM (△), 0.48 mM (▲). The curves are drawn for 8.5 mM, 4.25 mM, 1.08 mM and 0.48 mM ATP.

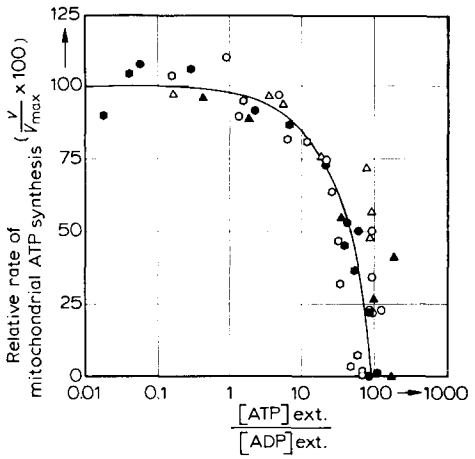

Fig. 5. Dependence on the extramitochondrial ATP/ADP ratio of the rate of mitochondrial ATP synthesis. The same experimental values and symbols are used as in Fig. 4.

[8–13, 19, 20]. Here the rate of the mitochondrial ATP formation is plotted against the concentration of ADP at different total concentrations of adenine nucleotides. The initial concentration of phosphate was equal, in all experiments, the amounts being high enough for the phosphate decrease during the experiment to be negligible. It results in a set of hyperbolic curves representing the saturation of oxidative phosphorylation by ADP. But the ADP concentration necessary for the saturation increases with increasing concentrations of adenine nucleotides, mainly caused by an increase in ATP. It must be pointed out that the curves do not cross the zero point of the abscissa as should be expected if the Michaelis-Menten relationship applied. Within the experimental error, however, all points can be represented by a common curve, if the ATP/ADP ratio is chosen as the independent variable for the mitochondrial ATP phosphorylation (Fig. 5). It must be concluded that for nearly constant phosphate concentrations the extramitochondrial ATP/ADP ratio controls the activity of the respiratory chain phosphorylation. Therefore, the constant phosphate potential in the resting state demonstrated by Slater et al. [10], as well as the constant phosphorylation potential at the half-maximum respiration stated by Wilson et al. [11], are particular properties of the general control characteristics. Furthermore, the results show that the control by the ATP/ADP ratio does not depend on the total concentration of adenine nucleotides in the range investigated between 0.5 and 8.5 mM.

Dependence of the redox quotients of the cytochromes b and cytochromes $c+c_1$ on the ATP/ADP ratio

As demonstrated, stationary redox states of the carriers of the respiratory chain can be obtained by appropriate additions of hexokinase. In Fig. 6, the resulting quotients of oxidized over reduced portions of the cytochromes *b* and cytochromes $c+c_1$ are plotted against the corresponding ATP/ADP ratios. One can see that in the

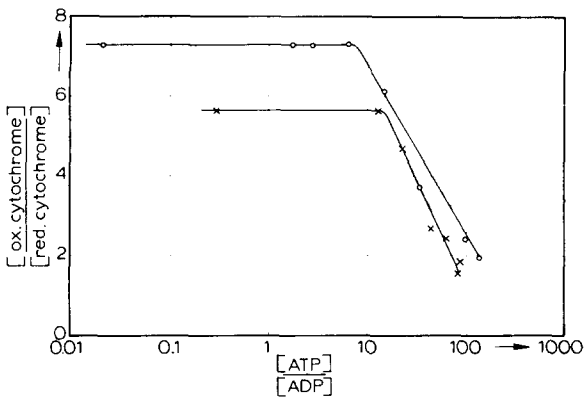

Fig. 6. Dependence on the extramitochondrial ATP/ADP ratio of the redox quotients of cytochromes *b* and *c*. Dual wavelength measurement at 564–575 nm and 550–540 nm for cytochromes *b* and $c+c_1$, respectively. Further conditions as indicated for Fig. 2 and 4. (×): cytochrome *b*, 4.25 mM ATP, 1.67 mg mitochondrial protein/ml; (○): cytochrome $c+c_1$, 4.8 mM ATP, 1.43 mg mitochondrial protein/ml.

range of small ATP/ADP ratios, i.e. in the range of maximum phosphorylation rate, the redox state of the carriers of the respiratory chain is also independent of the adenine nucleotide pattern. Furthermore, it is shown that with higher ATP/ADP ratios, the redox state is determined by the ATP/ADP ratio in the same range as the phosphorylation rate within the experimental errors. From further experiments (not shown), it follows that the influence of the ATP/ADP ratio on the redox quotients as well as on the phosphorylation rate is the same at different total amounts of adenine nucleotides.

The control range of the ATP/ADP ratio for oxidative phosphorylation

The experiments represented so far demonstrate that at constant concentrations of phosphate the activity of mitochondrial oxidative phosphorylation is controlled by the extramitochondrial ratio of ATP/ADP independent of the total amount of adenine nucleotides. The effective control range has been found to be between 5 and 100, under our experimental conditions. At lower ratios, the mitochondria operate at maximum activity. Here, the rate is limited by the availability of reducing equivalents to the respiratory chain*. The highest ratio attainable is determined by the activity of all the energy-consuming processes of the mitochondria preparation used in the experiment. The mitochondrial preparations used here contain some impurities, involving an active ATPase localized outside the intact mitochondria. This is demonstrated by the experiments presented in Table I. The respiration rate with succinate as substrate in the presence of 5 mM ATP is slightly higher (Expt. 2) than in absence of adenine nucleotides (Expt. 1). This stimulation of respiration can be prevented by carboxyatractylate-induced inhibition of the adenine nucleotide exchange across the mitochondrial membrane. The intramitochondrial splitting of ATP is comparably low, because the additional inhibition of the mitochondrial ATP

* Küster, U., Bohnensack, R. and Kung, W., unpublished.

TABLE I

INHIBITION BY CARBOXYATRACTYLOSIDE AND BY OLIGOMYCIN OF THE CONTROLLED RESPIRATION

Rat liver mitochondria were incubated in standard medium with 2.3 mM succinate and 1.1 μM rotenone, further additions as indicated. Carboxyatractyloside 0.9 nmol/mg protein, oligomycin 1.13 μg/mg protein, mitochondrial protein 2.26 mg/ml and 2.48 mg/ml in Expts. 1 and 2, respectively.

Expt.	Adenine nucleotide	Inhibitor	Respiration rate (ngatoms O/min per mg protein)
1	ϕ	ϕ	19.1
	200 μM ADP	ϕ	99.0
2	5 mM ATP	ϕ	29.0
	5 mM ATP	+carboxyatractyloside	18.7
	5 mM ATP	+carboxyatractyloside+oligomycin	17.3

synthesis by oligomycin has only a small effect. Therefore, the maximum value of 100 for the ATP/ADP ratio obtained in our experiments seems to be determined mainly by impurities of the preparation containing an ATPase activity [21]. Higher ratios may be expected if this ATPase does not operate, e.g. in magnesium-free media. Unfortunately, the hexokinase-glucose trap used in our experiments will not work either under these conditions.

The independence of the control range on the nature of the substrate oxidized by the mitochondria was proved with glutamate as substrate in the presence of a small amount of malate. In phosphorylating liver mitochondria at least 90 % of glutamate is oxidized via transamination [22–24]:

$$\text{Glutamate} + \tfrac{3}{2}\,O_2 + 9\,\text{ADP} + 9\,P_i \rightarrow \text{Aspartate} + CO_2 + 10\,H_2O + 9\,\text{ATP} \qquad (3)$$

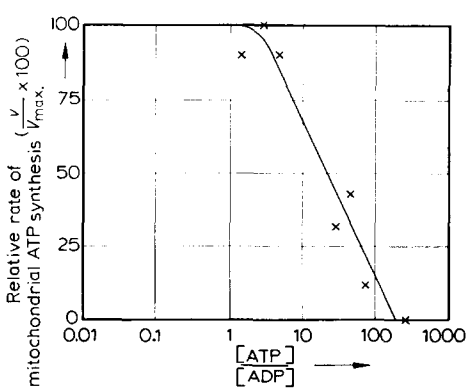

Fig. 7. Dependence on the extramitochondrial ATP/ADP ratio of the mitochondrial ATP synthesis with glutamate as substrate. The experiments were carried out according to those of Fig. 4. Mitochondria equivalent to 2.44 mg protein/ml were incubated with 9.7 mM glutamate and 0.48 mM malate instead of succinate and rotenone. The initial concentration of ATP was 2 mM and up to 2.4 I.U. hexokinase/mg mitochondrial protein were added. The reaction was stopped with a phenol/chloroform/isoamylalcohol mixture. A third sample was stopped with perchloric acid and used for the corrections of concentrations determined in the first two samples (see Methods and Materials).

Compared to the oxidation of succinate, the stoichiometric yield of ATP formed per mol substrate oxidized exhibits higher values in this case; a number of enzyme steps of intramitochondrial substrate conversion and a different transport system of the substrate uptake are involved. However, as shown in Fig. 7, the same range was obtained for the control of ATP formation by the ATP/ADP ratio.

DISCUSSION

The phosphorylation state of the adenine nucleotide system is particularly important for the coordination of cytosolic and mitochondrial processes. As shown by Veech et al. [25] the redox state of the cytosolic NAD system is controlled by the phosphorylation state of the adenine nucleotide system via the enzymes glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and 3-phosphoglycerate kinase (EC 2.7.2.3). The role of the competition for ADP of pyruvate kinase and of the respiratory chain phosphorylation was demonstrated by Gosalvez et al. [26] to be an important factor in the regulation of glycolysis. The present paper demonstrates the control of the mitochondrial ATP formation by the extramitochondrial energy requirement. Under conditions of nearly constant phosphate concentration this control is realized by the extramitochondrial ATP/ADP ratio.

For comparison of our results with intracellular conditions, the questions arise of the adenine nucleotide concentration in the liver cell cytosol and of the ATP/ADP ratio. For the cytosol, a value of 3–4 mM adenine nucleotides is estimated from the adenine nucleotide content determined after freezing stop of whole liver tissue [25, 27], considering the intracellular compartmentation of adenine nucleotides [28] and the volume of the cytosolic compartment [29]. These concentrations are covered by our experiments. Different values are available for the extramitochondrial ATP/ADP ratio in liver cells. Elbers et al. [28] obtained ratios up to 7.24 after cell fractionation in non-aqueous media. A value of 5.2 is reported by Siess and Wieland [30]. The highest values up to 19 were found by Zuurendonk and Tager [31] after digitonin lysis of hepatocytes. Considering the strong influence of hydrolytic splitting of ATP on ATP/ADP ratios greater than 1 and the high ATP turnover in liver cells, it seems that the last value determined is also rather too low.

The limitation of the control range from the ATP/ADP ratio of 5 to the ratio of 100 found under our conditions, corresponds to phosphate potentials of 10.9 and 12.7 kcal/mol [32], respectively. Therefore, a small change in the energy potential of the cell can rapidly transform the mitochondria from the state of minimum activity to its maximum by changes of the adenine nucleotide pattern.

Since the concentration of phosphate was nearly constant in the present experiments, we cannot decide whether the respiration is controlled by the ATP/ADP ratio per se, as it was concluded by Slater et al. [10], or it is the result of a thermodynamic control by the phosphorylation potential, i.e. by the mass action ratio $[ATP]/([ADP] \cdot [P_i])$. The latter assumption is supported by data of Wilson et al. [11]. According to the findings reported by Davis and Lumeng [13, 14], the respiration rate depends not only on the ATP/ADP ratio but can also be influenced by the total amount of adenine nucleotides. These workers found that the ATP/ADP ratio, necessary for a definite stimulation of respiration, rose with increasing amounts of adenine nucleotides. Accordingly, these findings differ from our results; such depen-

dence has not been observed under our experimental conditions. The reasons for this discrepancy are difficult to elucidate.

The mitochondria react with the extramitochondrial adenine nucleotides via the adenine nucleotide translocator localized in their inner membrane. The control characteristics found in our studies are consistent with the properties of the translocator. Due to the high affinity of the translocator for extramitochondrial ATP and ADP [33–35] the carrier is always saturated under the present experimental conditions. Therefore, as has been demonstrated, the results should not depend on the total amount of adenine nucleotides. The control by the ATP/ADP ratio seems to be the consequence of the competition between ATP and ADP for the carrier. The high ratio value necessary for an effective control corresponds to the different affinities of the carrier, found to be higher for ADP than for ATP in energized mitochondria [33–36]. However, it must be taken into consideration that the energetic state of mitochondria is not constant in the control range and, consequently, the kinetic preference of the ADP uptake may alter in this range. Studies using specific inhibitors (Küster, U., Bohnensack, R. and Kunz, W., unpublished) support the assumption that the actual capacity of the translocator for the ADP entry into mitochondria is the rate limiting step in the range where the respiration is controlled by the ATP/ADP ratio. The fact that the control characteristic does not depend on the nature of the substrate oxidized by mitochondria is in general agreement with this assumption.

However, it is questionable whether the maximum ATP/ADP ratio obtained during the resting state can be predicted by the apparent K_m values of the translocator for ATP and ADP, as was suggested by Davis and Lumeng [14]. In the resting state without net phosphorylation, the adenine nucleotide exchange reveals a thermodynamic equilibrium. The K_m values are related to the equilibrium constant by the Haldane relationship [37] including (depending on the kinetic mechanism) some additional constants [38] unknown in the present state.

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