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OXIDATIVE PHOSPHORYLATION AS A FUNCTION OF TEMPERATURE

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SUMMARY

1. The activation energy of succinate oxidation by rat-liver mitochondria changes at a temperature of about 17° in State 3 as well as in the uncoupled state.

2. Over the whole temperature range investigated (0–23°) the rate of phosphorylation of intramitochondrial ADP during succinate oxidation exceeds that of added ADP.

3. The activation energy of the ADP–ATP and P_i–ATP exchange reactions and of the 2,4-dinitrophenol-induced ATPase also changes at about 17°.

4. The temperature coefficients of the State-3 oxidation and of the P_i–ATP and ADP–ATP exchange reactions are similar and, at temperatures below 17°, are high in comparison with that of the phosphorylation of intramitochondrial ADP.

5. The translocation of ADP and ATP through the inner membrane is rate-limiting for the process of oxidative phosphorylation in rat-liver mitochondria.

INTRODUCTION

The conversion of added ADP into extramitochondrial ATP by oxidative phosphorylation in the inner space of the mitochondrion requires the transport of ADP and ATP through the mitochondrial membranes. Whereas the outer membrane is readily permeable to nucleotides, passage through the inner membrane requires a specific translocator[1,2] that is sensitive to atractyloside[3–5]. HELDT[6,7] found that the translocation of adenine nucleotides is rate-limiting for oxidative phosphorylation at temperatures below about 10°, but not at higher temperatures. HELDT AND KLINGENBERG[8] suggested that this might be the reason for the discontinuous Arrhenius plot found for the rate of O_2 uptake during succinate oxidation in the presence of ADP.

We have confirmed this discontinuous Arrhenius plot[9,10] but from the experiments described in this paper conclude that the translocation is rate-limiting at all temperatures between 0° and 23°.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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RESULTS

Effect of temperature on rate of succinate oxidation

In order to test the proposal of HELDT[7] that low temperature has an effect similar to that of low concentrations of atractyloside in making the overall process of oxidative phosphorylation dependent upon the activity of the adenine nucleotide translocator, the experiment summarized in Fig. 1 was carried out. It can be seen that varying the temperature between 6° and 30° has practically no effect on the P/O

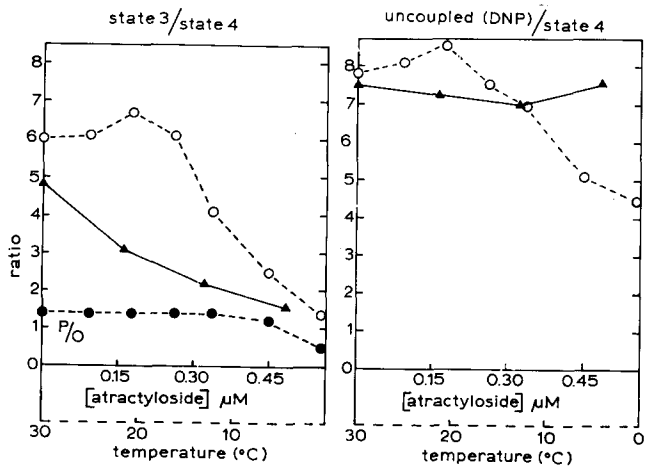


Fig. 1. The influence of atractyloside (▲—▲) and of reaction temperature (○---○) on the rate of succinate oxidation in State 3 and in the uncoupled state in comparison with State-4 oxidation rate. Experimental conditions as described in METHODS. 20 mM succinate, 0.2 μg rotenone per mg protein, 0.1 mM 2,4-dinitrophenol (DNP). In the experiment with atractyloside, 1.16 mg mitochondrial protein was used and a temperature of 25°. In the experiment with different temperatures, 0.84–8.4 mg mitochondrial protein were used, depending on the temperature. ●---●, P/O ratio.

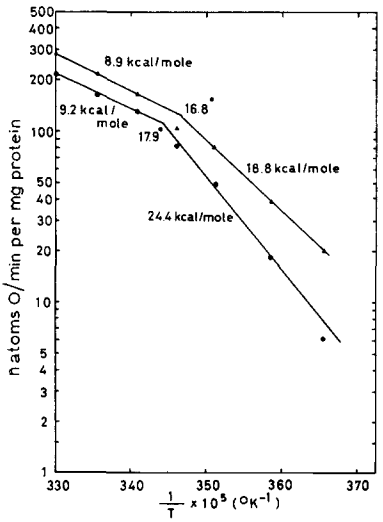
ratio with succinate as substrate. This was found also to be the case with other substrates tested (β-hydroxybutyrate, pyruvate *plus* malate, α-oxoglutarate). As was to be expected, atractyloside lowered the respiratory-control index measured with ADP but not with 2,4-dinitrophenol. In the absence of atractyloside, both indices were constant between 30° and 15°, but declined sharply between 15° and 0°. It is apparent from these curves that differences exist between the effects of adding atractyloside and lowering the temperature. Furthermore, it is clear that both the ADP- and uncoupler-induced respiration are more sensitive to low temperature than the controlled (State 4) respiration.

The effect of temperature on both State-3 and uncoupler-induced respiration is shown in Fig. 2. Under both conditions, a break occurs in the Arrhenius plot at about 17°. Since adenine nucleotide translocation is not involved in uncoupled respiration, is seems unlikely that the break is connected with the action of the translocator.

Effect of temperature on rate of phosphorylation of endogenous and of added ADP was

The rate of phosphorylation of previously labelled endogenous ADP measured by determining the rate of ATP synthesis after injecting an anaerobic

suspension of mitochondria into an aerobic medium containing succinate and rotenone (see Fig. 3). Since the rates of phosphorylation calculated from 1-sec and 2-sec experiments were the same (Fig. 4) it seems that initial rates are measured by our procedure.



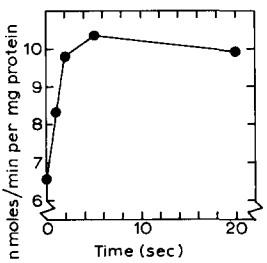


Fig. 2. Arrhenius plots of the rate of succinate oxidation in State 3 (●—●) and in the uncoupled state (▲—▲). Experimental conditions as in Fig. 1.

Fig. 3. Time-course of phosphorylation of intramitochondrial ADP. Experimental conditions as described in METHODS. The rate of phosphorylation of added ADP was 8.3 nmoles/min per mg protein in this experiment.

Fig. 4 shows that, in agreement with HELDT[7,8] we find a much lower temperature coefficient for the phosphorylation of endogenous than for added ADP. However, in contrast to HELDT's results, the rate of phosphorylation of endogenous ADP exceeded

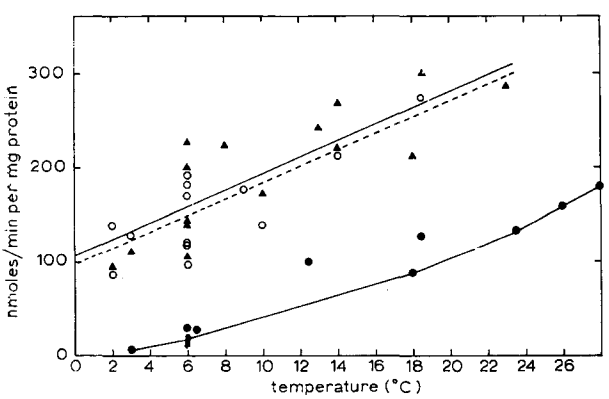


Fig. 4. Temperature dependence of phosphorylation rates of intramitochondrial ADP (▲—▲, O---O) and added ADP (●—●). The results in this figure were obtained in a number of different experiments. Experimental conditions as described in METHODS. 0.4 μg rotenone per mg protein. ▲—▲, rate measured over the first sec. O---O, rate measured over the first 2 sec. The straight lines are lines of best fit.

that of added ADP over the whole temperature range tested. We conclude that the translocation of adenine nucleotide is rate-limiting, not only at low temperature as proposed by HELDT[6-8], but at all temperatures normally used. This is in line with the conclusion of BYGRAVE AND LEHNINGER[11] that the translocator is responsible for the high affinity of mitochondrial oxidative phosphorylation for ADP.

The phosphorylation of endogenous ADP in the standard reaction mixture was not affected by the addition of either malonate or rotenone, but was strongly inhibited by the two inhibitors together (Table I). Apparently, with either endogenous NAD^+-linked substrates or added succinate the supply of reducing equivalents to the respiratory chain is not rate-limiting.

TABLE I

EFFECT OF ROTENONE AND MALONATE ON THE PHOSPHORYLATION RATE OF INTRAMITOCHONDRIAL ADP IN RAT-LIVER MITOCHONDRIA

Experimental conditions as described in METHODS. 1 or 2 sec at 6°.

Additions	*Phosphorylation rate (nmoles/min per mg protein)*		
	Mean value	*Range*	*Number of experiments*
None	158	97–227	8
Rotenone (0.4 μg/mg protein)	164	82–312	8
Malonate (5 mM)	223	212–235	2
Rotenone + malonate	8	0–20	4

The high rate of phosphorylation of endogenous ADP probably represents the maximum capacity of the respiratory chain. KRAAIJENHOF, TSOU AND VAN DAM[12] have measured an initial rate of succinate uptake of about 40 nmoles/min per mg protein at 0° with 1 mM succinate. Since the rate of succinate uptake is strongly temperature[12] and concentration[13] dependent, it seems unlikely that the rate of phosphorylation of added ADP at 28°, *viz.* 180 nmoles/min per mg protein, which would require a succinate uptake of 100–120 nmoles/min per mg protein, is limited by the uptake of succinate. It can be calculated from the data of VAN DAM AND TSOU[13] that the rate of uptake with 5 mM succinate, the concentration used in the experiment shown in Fig. 4, would be 77 nmoles/min per mg protein at 0°.

Effect of temperature on other ATP-requiring reactions related to oxidative phosphorylation

Fig. 5 shows that the P_i–ATP and ADP–ATP exchange reactions also show discontinuous Arrhenius plots, with breaks at 16° and 18°, respectively. The Q_{10} values both at high and low temperatures are very similar for the two reactions. The high Q_{10} values agree closely with those reported by KLINGENBERG AND PFAFF[14] for the adenine nucleotide translocator. This suggests that the rate of these exchange reactions, which require the translocator to work in an unfavourable direction *i.e.* to promote the entry of ATP into the mitochondria, is limited by the activity of the translocator.

In contrast to KLINGENBERG AND PFAFF[14] we also found a discontinuous Arrhenius plot for the 2,4-dinitrophenol-induced ATPase, with a break at 18° (Fig. 6). However, no break in the plot was found with the other uncouplers tested, *viz.*

2,6-dinitro-4-isooctylphenol, dicoumarol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The activation energy also varied with the uncoupler used. The differences in the uncoupler-induced ATPase at 0° is particularly striking. 2,4-Dinitrophenol, 2,6-dinitro-4-isooctylphenol and dicoumarol are particularly poor uncouplers at this temperature[8]. The action of uncouplers at different temperatures is under further investigation*. The Mg^{2+}-stimulated ATPase of sonicated rat-liver mitochondria gave a curved Arrhenius plot (Fig. 6).

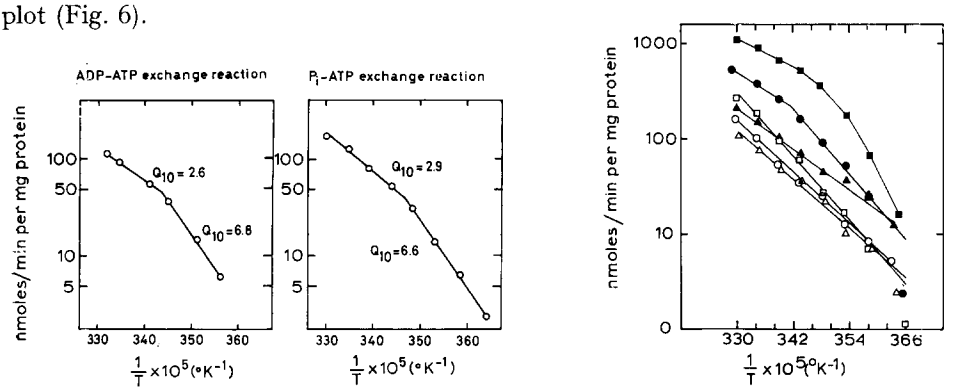


Fig. 5. ADP–ATP and P_i–ATP exchange reactions of rat-liver mitochondria as a function of temperature. The experiments were performed exactly as described by GROOT[19]. ADP–ATP exchange reaction, 1.0–3.0 mg protein depending on temperature. P_i—ATP exchange reaction, 0.5–4.0 mg protein depending on temperature.

Fig. 6. ATPase activity of rat-liver mitochondria as a function of temperature. Experimental conditions as described in METHODS. The amount of protein depended on temperature. ●—●, 0.1 mM 2,4-dinitrophenol, 0.41–3.30 mg protein; □—□, 0.005 mM 2,6-dinitro-4-isooctylphenol, 0.58–4.60 mg protein; ▲—▲, 0.005 mM FCCP, 0.38–3.06 mg protein; ○—○, 0.005 mM CCCP, 0.60–4.80 mg protein; △—△, 0.05 mM dicoumarol, 0.75–6.00 mg protein; ■—■, Mg^{2+}(5.0 mM)-induced ATPase of rat-liver mitochondria sonicated 4 times for 30 sec with a Mullard Ultrasonic power unit (MSE) at maximal output; 0.15–1.5 mg protein.

DISCUSSION

A striking finding in the present study is that whether or not the translocation of adenine nucleotide is rate-limiting, a discontinuous Arrhenius plot is obtained with

TABLE II

EFFECT OF TEMPERATURE ON REACTIONS RELATED TO OXIDATIVE PHOSPHORYLATION IN RAT-LIVER MITOCHONDRIA

Reaction	*Adenine nucleotide translocator rate-limiting*	*Break in Arrhenius plot*	Q_{10}	
			Low temp.	*High temp.*
Oxidation of succinate—State 3	Yes	18°	4.8	1.7
Oxidation of succinate—uncoupled	No	17°	3.0	1.6
Phosphorylation of endogenous ADP	No	Not measured	1.7	—
ADP–ATP exchange	Yes	18°	6.8	2.6
P_i–ATP exchange	Yes	16°	6.6	2.9
Dinitrophenol-induced ATPase	Not measured	18°	5.4	2.2

* See note added in proof on p. 34.

a break at about 17°. In all cases, the temperature coefficient is higher at lower than at higher temperatures. The data are summarized in Table II. ZEIJLEMAKER AND JANSEN[10] showed that the oxidation of succinate by the Keilin and Hartree heart-muscle preparation as well as by purified succinate dehydrogenase show a similar discontinuity in the Arrhenius plot at 17°. It is clear that such a phenomenon is not diagnostic for a particular enzyme or enzyme system. In particular, the break obtained with State-3 respiration is not due to the adenine nucleotide translocation becoming no longer rate-limiting above 17°.

METHODS

O_2 uptake was measured polarographically using a Clark-type electrode. The concentration of O_2 dissolved in the reaction mixture used, in equilibrium with air, at various temperatures between 1° and 30°, was determined by reacting with stoicheiometric amounts of NADH, with heart-muscle preparation as NADH oxidase. The values found are 7% less than the concentrations given in ref. 15 for pure water. The reaction medium (volume 1.60 ml) contained 15 mM KCl, 50 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA, 10 mM potassium phosphate buffer (pH 7.4), 25–75 mM sucrose, 5 mM $MgCl_2$, 0.16 μmole ADP or 0.10 mM 2,4-dinitrophenol and substrate as indicated in the legends of the figure.

The P/O ratio was calculated according to CHANCE AND WILLIAMS[16] by adding a known amount of ADP.

The ATPase was measured in a medium (volume 1.0 ml) containing 25 mM KCl, 0.5 mM EDTA, 25 mM Tris–HCl buffer (pH 7.4), 1 mM KCN and 100 mM sucrose. The reactions were started by adding a mixture of ATP (6.6 mM final concn.) and uncoupler as indicated in the legends. Reaction time, 4 min.

P_i was estimated according to FISKE AND SUBBAROW as modified by SUMNER[17].

The rate of phosphorylation of intramitochondrial ADP was measured in a medium containing 20 mM Tris–HCl buffer (pH 7.4), 2 mM EDTA, 5 mM succinate, 250 mM sucrose and 1 mM potassium phosphate buffer (pH 7.4). The standard reaction medium was gassed for 10 min with N_2. Carrier-free $^{32}P_i$ (about 10^6 counts per min per ml reaction mixture) and mitochondria (about 6 mg protein per ml reaction medium) were then added. 0.5 ml of this mixture was sucked into a glass syringe (without needle) which was then closed by a plastic cap. The syringe was placed in a bath at 25° for 5 min and then for a further 10 min in a bath at the reaction temperature. In this period the contents of the syringe became anaerobic and mitochondrial ADP and ATP became labelled by exchange. After this preincubation, the contents of the syringe were squirted at zero time into 0.5 ml of the standard reaction mixture gassed with O_2. The reaction was stopped after 1 or 2 sec by injection of 0.96 ml anaerobic $HClO_4$, immediately followed by 0.04 ml ATP (5 mM final concn.) as a carrier.

In the protein-free supernatant the radioactivity in the organic phosphate esters was measured as described by ERNSTER *et al.*[18]. The zero-time control was obtained by squirting the anaerobic contents of the syringe into the anaerobic $HClO_4$ directly. The rate of phosphorylation of added ADP was measured under the same conditions. 1 mM ADP was added 60 sec after adding the anaerobic labelled mitochondrial suspension to the oxygenated reaction mixture.

P_i–ATP and ADP–ATP exchange reactions were measured as described by GROOT[19].

Rat-liver mitochondria were isolated by the method of HOGEBOOM as described by MYERS AND SLATER[20].

Protein was determined with the biuret method as modified by CLELAND AND SLATER[21].

Atractyloside was kindly provided by Dr. V. Sprio, and FCCP as well as CCCP by Dr. P. G. Heytler. Rotenone was obtained from S.B. Penick, New York.

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Further investigations of the influence of temperature on the uncoupler-induced ATPase have now shown that in addition to 2,4-dinitrophenol all the uncouplers tested exhibit a discontinuous Arrhenius plot. The Q_{10} values both at low and at high temperatures are comparable with that of 2,4-dinitrophenol (see Table II).

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