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Inhibition of the State 3 Respiration of Isolated Mitochondria and its Implications in Comparative Studies*

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Abstract

The initial state 3 rates of respiration of mitochondria from plant tissue and from rat liver are usually less than the state 3 rates obtained after the mitochondria experience several state 3-state 4 cycles.

Reduction of the absolute rate of respiration by decreasing the temperature or increasing the osmolarity of the reaction medium increased both the magnitude of the inhibition and its persistence in subsequent cycles. Under particularly adverse reaction conditions the initial state 3 was depressed to the extent that no distinction was observed in the state 3 and state 4 rates and the mitochondria appeared to have lost respiratory control. Thus, for comparative studies, invalid conclusions regarding the effect of these factors on respiration could be drawn unless the inhibition were removed.

The inhibition was observed with substrates other than succinate and was therefore not attributed to oxaloacetate inhibition of succinate dehydrogenase.

Increasing the amount of ADP added at each cycle did not appreciably reduce the inhibition.

The inhibition of initial state 3 rates was satisfactorily relieved by incubating the mitochondria with substrate, and several cycles of ADP under conditions which increased the absolute rate of respiration. Once relieved of the inhibition, mitochondria could be stored at 0° for several hours without the inhibition being apparent again.

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Introduction

In polarographic studies of the oxidation of succinate by tightly coupled plant mitochondria, the initial state 3 rates of respiration usually exhibit some inhibition compared to the state 3 rates observed after sequential additions of ADP [1, 2, 3]. Also, there is often a time delay before a steady rate of state 3 respiration is reached in response to the first addition of ADP [2, 4]. Both these effects have been attributed to inhibition of succinate dehydrogenase by oxaloacetate [1, 2] and various methods have been tried to decrease or eliminate this inhibition [1, 3].

Inhibition of the initial state 3 rates of oxidation is also apparent in the polarographic traces of mitochondria oxidizing α-ketoglutarate [5] and malate plus pyruvate [6]. Furthermore the inhibition can extend through several cycles of ADP addition (state 3-state 4 transitions), although the degree of inhibition is usually less after each successive addition.

This type of inhibition was noted in a study of the effect of temperature on the rate of respiration of mitochondria from a variety of plant tissues [7]. The degree of inhibition of the intial and subsequent state 3 rates was observed to increase when the absolute rate of respiration was reduced in response to a decrease in the temperature of the reaction. Thus for a comparative study it was essential to differentiate the effect of temperature on the inhibition of state 3 respiration from the effect of temperature on the kinetics of respiratory enzymes.

This paper describes in detail the inhibition of the initial state 3 rate of respiration of mitochondria from rat liver and various plant tissues under a variety of reaction conditions and demonstrates the type of invalid conclusions which can be drawn in comparative studies using preparations of mitochondria which show this type of inhibition.

When the absolute rate of respiration was depressed by conditions imposed by the experimental conditions it was necessary to pretreat the mitochondria with substrate and three successive additions of ADP under conditions which stimulate the absolute rate of respiration. The results indicate that stimulation of the state 3 rate to a value in excess of the ultimate state 4 rate, which allowed the mitochondria to undergo several cycles of state 3-state 4 transition, consistently abolished the inhibition.

Materials and Methods

Preparation of Mitochondria

Mitochondria were isolated from the following plants: mature green tomato fruit (*Lycopersicon esculentum* Mill. c.v. Grosse Lisse), cucumber fruit (*Cucumus sativus* L.), cauliflower buds (*Brassica*

oleraceae L. var. *botrytis*), and potato tubers (*Solanum tuberosum* L. c.v. Sebago), by methods described previously [8]. After isolation, mitochondria were suspended in a medium consisting of 0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 10 mM tris, 10 mM KH_2PO_4, and 0.5 mg/ml of bovine serum albumin (BSA) adjusted to pH 7.2 with HCl. The BSA (fraction V, Sigma Chemical Co.) was extracted three times with diethyl ether before use. The mitochondrial suspension usually contained 7-15 mg of protein per ml. Mitochondria were also isolated from rat liver (Wistar and Carsworth Farm strain) by the method described previously [9] and stored in a medium consisting of 250 mM sucrose, 10 mM tris, and 0.5 mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.6 with HCl. The mixture usually contained 20-25 mg of mitochondrial protein per ml.

Oxidative Activity

Oxygen uptake was measured polarographically with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) or a Rank oxygen electrode (Rank Bros., Cambridge, U.K.) in a reaction volume of 3.0 ml. The electrodes were calibrated with air-saturated water. The concentration of oxygen in air at various temperatures was obtained from published tables [10]. Unless otherwise stated, the reaction mixture contained 250 mM sucrose, 10 mM tris/HCl pH 7.2, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM EDTA and 0.5 mg/ml of BSA. Additions of ADP, 180-500 nmoles, were made in 2-5 μl amounts. Protein was estimated by the method of Lowry *et al.* [11].

Results and Discussion

Examples of the Variation in the Degree of Inhibition of Initial State 3 Rates

During comparative studies of the effects of temperature [7] and osmolarity [12] on the respiration rate of mitochondria isolated from both plant tissue and rat liver, large variations were noted in the degree of inhibition of the initial state 3 rate of respiration compared with subsequent state 3 rates. Differences in the degree of inhibition were apparent in mitochondria from different plants as well as in mitochondria from the same tissue prepared at different times, or even prepared at the same time by different workers. Examples of this variation in the degree and duration (i.e., persistence in subsequent cycles) of the inhibition of mitochondria isolated from the same batch of potato tubers by different workers are shown in Fig. 1. Both preparations contained approximately the same amount of protein. Since the state 3 rates ultimately reached values reproducible within the limits of ±5%, the

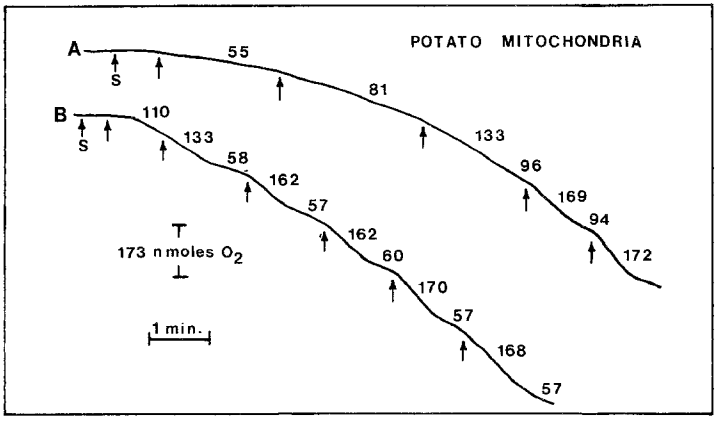


Figure 1. The inhibition of the initial state 3 rates of respiration of two preparations of mitochondria from potato tuber. The preparations were isolated at the same time by different workers from the same batch of tubers and were assayed within 15 min of isolation. The numbers beside each trace show the rate of oxygen uptake in nmoles O_2/min/mg of protein. Succinate (5 mM) was added as indicated by "S" and ADP (180 nmoles) was added as indicated by the arrows. The reactions were carried out at 25° with 0.86 mg of protein, A, and 0.97 mg of protein, B, in a volume of 3.0 ml.

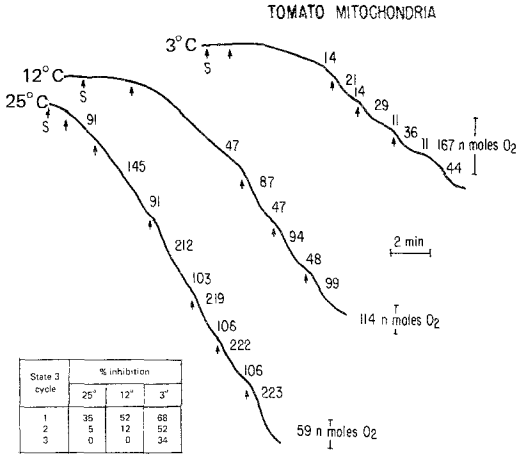
average of these rates is considered to represent the uninhibited rate. The difference between the initial state 3 rates and the subsequent uninhibited rate, expressed as a percentage of the uninhibited rate, represented the degree of inhibition. With preparation A of Fig. 1, the first and second cycles were inhibited 68% and 53% whereas in preparation B they were inhibited only 32% and 21%, respectively.

As shown in Fig. 1, trace B, the inhibition was abolished after three additions of ADP, i.e. after three cycles or two state 3-state 4 transitions. With mitochondria from preparation A (Fig. 1, trace A) there was a small increase in the rate of oxidation after each successive addition of ADP, but there was no evidence of a state 3-state 4 transition until the third addition of ADP.

The two examples of the inhibition of the initial state 3 rates of isolated potato mitochondria shown in Fig. 1 represent the range of the inhibition which has been observed with mitochondria isolated from roots of sweet potato, beet and maize, fruit of tomato, avocado and cucumber and buds of cauliflower [7] and reacted under optimal conditions for respiration. However, the degree of inhibition exhibited by a particular preparation of mitochondria varied depending on the reaction conditions. Factors which reduced the absolute rate of respiration, for example decreasing the temperature [7] or increasing osmolarity [14], increased both the percentage inhibition in the initial

state 3 rates and the persistence of the inhibition through subsequent additions of ADP.

The influence of the temperature on the rate of respiration of mitochondria from tomato fruit is shown in Fig. 2. The degree of inhibition in the initial cycle increased from 35% for mitochondria reacted at 25° to 52% and 68% for the same mitochondria reacted at 12° and 3°, respectively. As the absolute rate of respiration was depressed by lowering the temperature of the reaction, the number of state 3-state 4 transitions required to overcome the inhibition also increased. The insert of Fig. 2 shows that the inhibition was almost completely relieved after one addition of ADP at 25°, but even after three additions of ADP to the reaction at 3°, the state 3 rate was still inhibited by 34%.



State 3 cycle	% inhibition		
	25°	12°	3°
1	35	52	68
2	5	12	52
3	0	0	34

Figure 2. The influence of temperature on the inhibition of the initial state 3 rates of respiration of mitochondria obtained from tomato fruit. The mitochondria were reacted at the temperatures indicated at a concentration of 0.5 mg of mitochondria protein per ml. Succinate was added as indicated by "S" and ADP as indicated by the arrows. The table shows the percentage inhibition for the first, second and third cycles for each reaction.

The apparent activation energy of mitochondria from chilling sensitive plants has been shown to increase from approximately 8 kcal/mole in the temperature range of 12-24° to 10-30 kcal/mole for the range 1-12° [7]. This change is associated with a temperature-induced phase change in the lipids of the mitochondrial membranes [12, 13]. Because of the large decrease in the absolute rate of respiratory activity for mitochondria from chilling sensitive tissue as the temperature of reaction was reduced below approximately 10°, there was an increase in both the degree and persistence of the inhibition of the initial state 3 rates. This effect is

clearly illustrated in Fig. 3 showing the respiratory activity of mitochondria from cucumber fruit at 12° and 9°. Mitochondria reacted at 12° (Fig. 3, trace B) indicated that the inhibition in the initial state 3 rates was overcome after three additions of ADP, thereafter the rate remained relatively constant, and the mitochondria exhibited a respiratory control ratio of 1.5. When reacted at 9°, the same

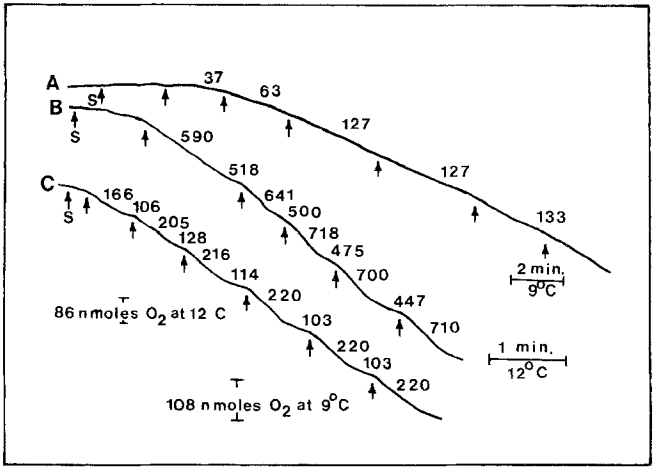


Figure 3. The relief of the inhibition of initial state 3 rates of respiration of mitochondria from cucumber fruit. The mitochondria were reacted at 9° (trace A) and 12° (trace B) at a concentration of 0.62 mg of mitochondrial protein per ml. For trace C mitochondria were pre-incubated at 15° for 3 min with 5 mM succinate and three successive additions of 230 nmoles of ADP. The mitochondria were immediately transferred to a reaction vessel at 9° and the rate of respiration determined at this temperature.

mitochondria (Fig. 3, trace A) showed a marked decrease in the absolute rate of respiration to the extent that the respiratory control, evident in the mitochondria reacted at 12° was not apparent when reacted at 9°. The small increase in the rate of respiration after each successive addition of ADP indicates that the respiration of these mitochondria was severely inhibited and the rates exhibited were not indicative of the maximum rates for these mitochondria at this temperature. When these mitochondria were incubated at 15° for 3 min with 5 mM succinate and three successive additions of 230 nmoles of ADP, and then transferred to a reaction vessel at 9°, the initial state 3 rate was 166 nmoles O_2/min/mg of protein (Fig. 3, trace C) and after two further additions of ADP increased to 220 nmoles O_2/min/mg protein and remained relatively constant through three further additions of ADP, showing a respiratory control ratio of 2.2. Thus after the inhibition, apparent in the reaction at 9°, was relieved by incubation at a higher temperature the mitochondria

showed an almost two-fold increase in respiratory rate and exhibited respiratory control.

The importance of recognizing this phenomenon in relation to the interpretation of results in comparative studies is clearly illustrated in Fig. 4 showing the respiratory activity of cucumber mitochondria at various temperatures presented as an Arrhenius plot (log of the

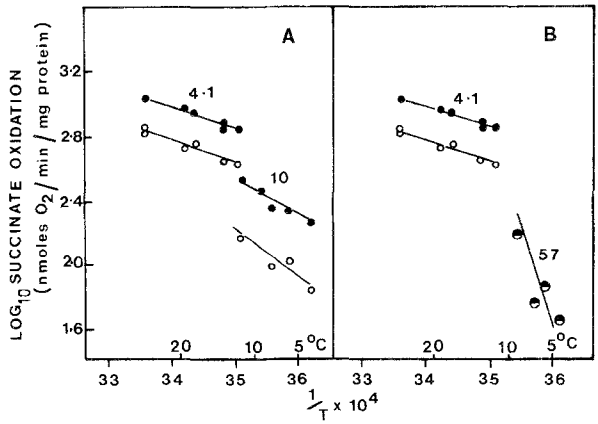


Figure 4. The influence of inhibited state 3 rates of respiration on the Arrhenius activation energy and respiratory control ratio of mitochondria from cucumber fruit. The state 3 (●) and state 4 rates (○) of respiration at the various temperatures were obtained from polarograph traces as shown in Fig. 3. In "A" the respiration rates at temperatures below 12° were obtained after the inhibition of the initial state 3 rates were relieved by pre-incubating the mitochondria at higher temperatures as described in Fig 3. In "B" the mitochondria were not pre-incubated, the respiration rates were inhibited and the mitochondria failed to exhibit respiratory control (◓) at temperatures below 12°. The numbers beside each line are the Arrhenius activation energy (kcal/mole) for the respective temperature range.

respiration rate versus the reciprocal of the absolute temperature). This shows an abrupt discontinuity at 12° with an increase in activation energy of respiration as the temperature is reduced below 12°. For mitochondria pre-incubated as described in Fig. 3, the activation energy increased from 4.1 to 10.1 kcal/mole (Fig. 4A), but there was little change in the respiratory control ratio, showing that decreasing the temperature did not effect the phosphorylative efficiency of the mitochondria [7]. In contrast, if mitochondria were not pre-incubated to overcome the inhibition, reduction in the reaction temperature below 12° appeared to reduce the respiration rate by approximately half, increase the activation energy to 57 kcal/mole, and abolish the respiratory control (Fig. 4B). If only the data of Fig. 4B existed, then the conclusion probably would have been made that the phosphorylative

capacity of these mitochondria respiring at temperatures below 12° was impaired. This, however, was clearly not the case, as shown in Fig. 4A. The decrease in the temperature of the reaction did not effect the phosphorylative efficiency of the mitochondria when the inhibition was abolished by pre-incubation at a higher temperature. This illustrates the necessity of overcoming the inhibition of the initial state 3 rates before imposing further restrictions on the respiration rate by lowering the temperature of the reaction.

The marked increase in the persistence of the inhibition, to the extent that the mitochondria failed to exhibit respiratory control when the absolute rate of respiration was reduced, was not confined to mitochondria from chilling sensitive tissue-like cucumber which exhibit a marked increase in activation energy when the temperature is reduced below about 10° [7]. A similar effect of lowering the reaction temperature was observed with mitochondria from potato tuber, a chilling-resistant plant which shows a constant activation energy for respiration between 0° and 25° [7]. Nor was the apparent increase in the inhibition of the initial state 3 rates restricted to effects of temperature. Other factors which reduced the absolute rate of respiration such as increasing osmolarity [14] also increased the apparent inhibition. This is shown for mitochondria from potato tuber in Fig. 5.

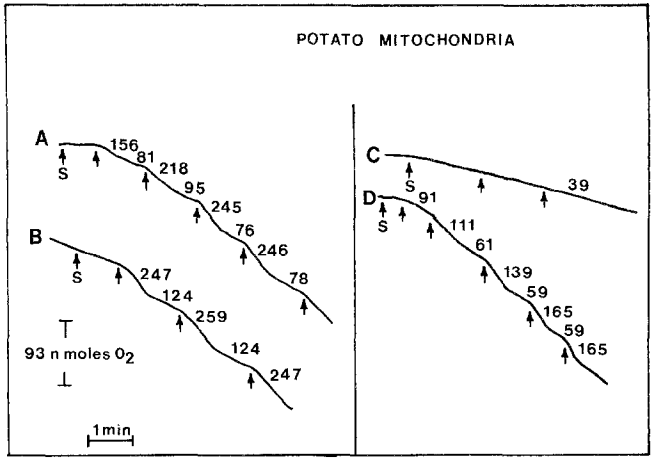


Figure 5. The effect of pre-incubation of mitochondria on the inhibition of state 3 respiration induced by increased osmolarity. Polarographic traces of mitochondria from potato tuber reacting at 25° in media containing 0.25 M sucrose (A and B) and 0.75 M sucrose (C and D). Traces A and C are mitochondria reacted immediately after isolation. In trace B and D the mitochondria were pre-incubated at 25° with 10 mM succinate and three successive additions of 400 nmoles of ADP for 5 min and stored at 0° until required. Succinate (5 mM) added as indicated by "S" and ADP as indicated by the arrows.

Potato mitochondria reacted in a medium containing 0.25 M sucrose exhibited an inhibition in the initial state 3 rates of respiration which was evident through two additions of ADP (Fig. 5 trace A). When mitochondria from this same preparation were reacted in a medium containing 0.75 M sucrose (Fig. 5 trace C), the rate of respiration was reduced by approximately 85%, and the mitochondria did not exhibit respiratory control. To overcome the inhibition, a portion of the isolated mitochondria (0.5 ml containing 6 mg of mitochondrial protein) was incubated for 5 min at 25° with 10 mM succinate and three successive additions of 400 nmoles of ADP. The mixture was shaken vigorously during the incubation to maintain the mitochondria in an aerobic state. After this treatment, a sample was again reacted in medium containing 0.25 M or 0.75 M sucrose. This treatment completely relieved the inhibition when the mitochondria were reacted in 0.25 M sucrose (Fig. 5 trace B) and while not relieving the inhibition completely for those reacted in 0.75 M sucrose (Fig. 5 trace D) restored the state 3 state-4 transition in the second cycle (RC of 1.8) and relieved the inhibition after the third ADP addition. Thus in comparative studies of the effect of osmolarity on the activity of isolated mitochondria, it is essential to pre-incubate and thus insure that the mitochondria are immediately capable of attaining near maximal state 3 rates under optimal conditions.

These two examples clearly demonstrate ways in which invalid conclusions can be drawn regarding the effect of temperature (Fig. 4) and osmolarity (Fig. 5) on mitochondrial function if the confounding influence of the inhibition is not considered.

Additional Characteristics of the Inhibition

Inhibition of initial state 3 rates of respiration was not confined to mitochondria oxidizing succinate nor to mitochondria from plant tissue. The inhibition was evident and persisted through three additions of ADP (200 nmoles) for potato mitochondria oxidizing citrate (Fig. 6 trace A), see also [15]. Increasing the amount of ADP added to initiate each state 3 cycle from 500 to 800 nmoles did not appreciably alter the degree or persistence of the inhibition as shown in Fig. 6 trace B. Furthermore, incubation of the mitochondria with ATP (200 nmoles) and substrate for 5 min before the addition of ADP did not appreciably alter the degree of inhibition of the initial state 3 rates. Inhibition of respiration was also apparent with mitochondria from rat liver. Figure 7, trace A, shows that the initial state 3 rates of respiration were inhibited 18%, 12%, and 5% in the first, second and third cycles, respectively for rat liver mitochondria oxidizing succinate. When malate plus pyruvate or β-hydroxybutyrate was used as substrate the inhibition was even greater. Figure 7 also shows a comparison of the ultimate state 3 rate of respiration reached when ADP is added sequentially in small amounts

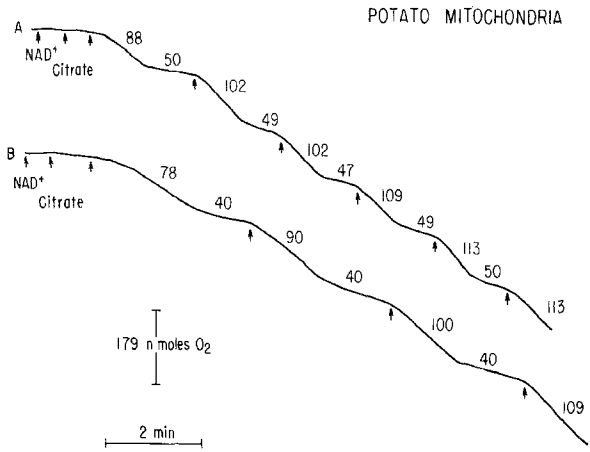


Figure 6. The inhibition of initial state 3 rates of respiration for mitochondria oxidizing citrate. Polarographic trace of oxygen consumption of mitochondria from potato tuber reacting at 25°. NAD and citrate were added as indicated. For trace "A", 500 nmoles of ADP, and for trace B, 800 nmoles of ADP were added as indicated by the arrows.

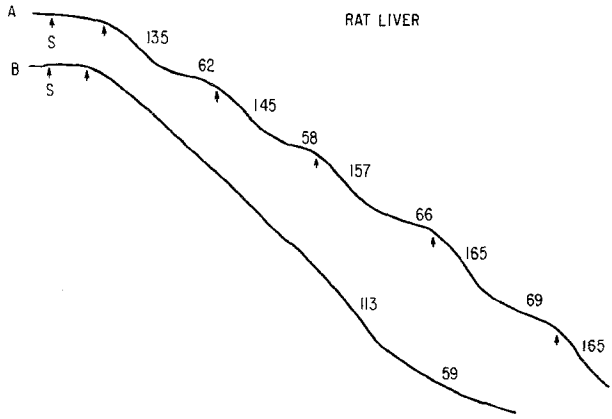


Figure 7. The inhibition of initial state 3 rates of respiration of mitochondria from rat liver. The mitochondria were reacted at 37°. Succinate (5 mM) was added as indicated by "S" and 270 nmoles of ADP (trace A) and 950 nmoles of ADP (trace B) as indicated by the arrows. The figures beside the trace show the respiration rate as nmoles O_2 /min/mg of mitochondrial protein.

(270 nmoles, trace A) or in one large amount (950 nmoles, trace B). After four cycles of ADP addition the state 3 rate was 165 nmoles O_2/min/mg protein whereas with one addition of ADP and one extended state 3 rate of respiration, the rate immediately before all the ADP was phosphorylated was only 113 nmoles O_2/min/mg protein. This apparent reduced rate was always observed when the mitochondria were permitted to continually respire in the presence of phosphate acceptor (state 3) in contrast to mitochondria undergoing alternate state 3 and state 4 respiration. This observation thus emphasizes the need to overcome the inhibition or allow the mitochondria to experience a number of 3-state 4 cycles before accepting the observed respiration rate as an uninhibited rate.

Conclusions

During the isolation of mitochondria, changes apparently occur which cause an inhibition of the initial state 3 rate of respiration. The magnitude of this inhibition varied considerably even with mitochondria from the same tissue isolated by different workers (Fig. 1) and depended on the reaction conditions and the absolute rate of respiration (Figs. 2, 3, 5, and 6). When the absolute rate of respiration was comparatively high, the inhibition was minimal and was alleviated in one or two cycles of ADP addition. However, when the reaction conditions impose a reduction in the absolute rate of respiration, the inhibition was maximal and persisted through a number of cycles of ADP addition or, in extreme cases, throughout the reaction. In the latter case the mitochondria usually failed to exhibit respiratory control, and the actual rate of respiration observed was less than half the rate shown by uninhibited mitochondria under the same conditions (Figs. 4 and 5).

The inhibition was probably not due to oxaloacetate inhibition of succinate dehydrogenase activity since it was evident with other oxidizable substrates (Fig. 6) and it was not relieved by pre-incubating the mitochondria with ATP as was observed by Wiskich and Bonner [1]. The most significant event in overcoming the inhibition appeared to be subjecting the mitochondria to a number of state 3-state 4 cycles. The addition of ADP in amounts which maintained the mitochondria in state 3 respiration does not appear to alleviate the inhibition (Fig. 7), as effectively as brief intervals of state 3 and state 4 respiration, and the respiration rate attained by this method was usually less than ultimate state 3 attained after sequential addition of smaller amounts of ADP. The role of ADP and the significance of the alternation between state 3 and state 4 respiration in overcoming the inhibition of state 3 respiration has been investigated and is described in the following paper [15].

The results described here clearly demonstrate that invalid conclusions can be drawn regarding the influences of factors such as temperature and osmolarity on mitochondrial respiration if the changes due to the

imposition of these factors are not differentiated from the changes in respiration due to alteration in the magnitude of the inhibition. Therefore, before meaningful comparative studies on the influence of factors such as temperature, osmolarity, pH and specific inhibitors on the respiration rate of mitochondria can be carried out, it is essential that the inhibition of the initial state 3 rate of respiration be removed.

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