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The Role of State 4 Electron Transport in the Activation of State 3 Respiration in Potato Mitochondria

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Abstract

The initial state 3 respiration rate of potato mitochondria is markedly depressed, or attenuated. With several consecutive state 3/state 4 cycles the state 3 rate rises to a maximum, while the state 4 rate remains essentially unchanged. The development of state 3 respiration has been termed conditioning. An analysis of the process has indicated that state 4 is a better conditioner than state 3 *per se*. Conditioning is also attained by preincubation in state 2, or under conditions designated pseudo-state 2, wherein ADP is present, with or without oligomycin, and inorganic phosphate is absent. ADP implements the conditioning process in the absence of oxidative phosphorylation. The action of ADP in its secondary or modulator role appears to be positively cooperative, the kinetics of ADP involvement being second-order. $S_{0.5}$ for ADP as a modulator of the conditioning process is approximately 62 μM, a value in excess of the K_s for ADP in oxidative phosphorylation. Electron transport is indispensable for conditioning, and it is suggested that conditioning and ATP synthesis represent alternative uses of respiratory energy. It is further suggested that to some extent state 4 underlies state 3.

Introduction

The initial state 3 respiration rate of isolated plant mitochondria which exhibit good respiratory control is more often than not lower than

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successive state 3 rates. Furthermore, following the first addition of ADP, the attainment of the initial constant state 3 rate follows a noticeable lag period, or "roll-off". The state 3 rate is enhanced during active respiration, or better still by a series of successive state 3/state 4 cycles. Raison *et al.* [1] have described the phenomenon in both plant and animal mitochondria—elucidating the influence of experimental conditions, the magnitude of attenuation, and the implication of the attenuation phenomenon with respect to comparative studies of mitochondrial respiration.

Succinate oxidation by isolated mitochondria has long been associated with depressed early state 3 rates. The putative explanation has been in terms of inhibition of succinate dehydrogenase by a product of succinate oxidation, e.g. oxalacetate [2, 3, 4]. ATP as well as successive cycles of ADP addition were shown to enhance state 3, and it was presumed that ATP facilitated oxalacetate removal. For this reason the diminished initial state 3 was considered an inhibited state 3, and the attainment of maximal state 3 rate was spoken of as deinhibition. However, Tyler [5] recognized that relief of succinate dehydrogenase "inhibition" did not require the removal of oxalacetate, and Drury *et al.* [4] discovered that ADP served as a modulator of succinate dehydrogenase, favoring succinate binding over oxalacetate. More to the point, Raison *et al.* [1] as well as Papa *et al.* [6] and McDaniel and Sarkissian [7], observed a depressed initial state 3 rate with substrates other than succinate. For all these reasons we choose to refer to the initial diminished state 3 as attenuated, or deconditioned, and to the attainment of maximal respiratory activity in state 3 as conditioning.

As mentioned, Raison *et al.* [1] discovered that alternating cycles of state 3/state 4 were more effective than a continuous period of state 3 in conditioning plant mitochondria. Thus while ADP seemed a requirement for conditioning, the nature of its involvement has remained obscure. The *sine qua non* for conditioning is vigorous electron transport [1]. In what follows we demonstrate that mitochondria can be experimentally deconditioned by impairing electron transport, and that attenuation can be overcome by appropriate stimulation of electron transport. While ADP enhances electron transport by dint of its conventional role in oxidative phosphorylation, our observations herein implicate ADP in a second, modulator, role, directly related to the conditioning process.

Materials and Methods

Mitochondria were prepared from untreated potato tubers (variety Russet Burbank). Washed tubers are cut in half along the short axis and 1 in. cores removed from within the vascular ring with a stainless steel borer. The tissue is chilled in ice, and 100 g comminuted in a commercial

juice extractor (Oster Mfg. Co., Milwaukee) with 200 ml extraction medium consisting of 0.35 M mannitol, 0.25 M sucrose, 0.025 M TES buffer, pH 7.8, 1 mg/ml of fat free bovine serum albumin (BSA) and 0.1 mg/ml Na mercaptobenzothiazole (MERCAP, Hopkin and Williams, U.K.). The juicer comprises a toothed plate fixed in the base of an aluminium basket centrifuge (3000 rev/min, 750 x g), all contained in a plastic housing. The basket is lined with a double layer of Miracloth (Chicopee Mills, Inc., N.Y.). The filtered homogenate is centrifuged at 800-1000 x g for 10 min to remove starch and debris. The ensuing supernatant is thereupon centrifuged at 12,000-14,000 x g for 20 min. The pellet is resuspended in extraction medium and the suspension centrifuged once again at 12,000-14,000 x g for 20 min. The pellet is finally suspended in extraction medium lacking MERCAP, mitochondria from 100 g tissue being brought to a final volume of 2.0 ml (*circa* 1 mg protein N/ml). Respiration rates are reported in terms of activity per ml of this final mitochondrial suspension.

Respiratory measurements. Oxygen uptake was measured polarographically with a Clark type O_2 electrode, calibrated with air saturated water. The reaction medium was as follows: 0.4 M mannitol, 0.025 M TES, pH 7.4, 5 mM K phosphate, pH 7.4, 5 mM $MgSO_4$, and 1 mg/ml BSA. Final volume, 3.3 ml. The substrate was citrate, at a final concentration of 5 mM, and 1 mM NAD was added in all cases. Additions of ADP (100-150 nmoles/ml) were made in 2-3 μl volumes. Carbonyl cyanide *m*-chlorophenyl-hydrazone (*m*-Cl-CCP), and oligomycin final concentrations 10^{-5} M, and 1 μg/3.3 ml respectively.

Results

Experimental attenuation of isolated mitochondria. Plant mitochondria show considerable variability in the extent of attenuation on isolation, presumably attributable in varying degree to the state of the intact tissue and to the isolation procedure. In potato mitochondria the first steady rate following ADP addition is frequently 30-40% lower than the maximal state 3 rate achieved after several cycles of state 3-state 4 alternation. Both the time required for the attainment of the first steady state 3 rate, and the number of cycles required for full conditioning are directly related to the degree of initial attenuation [1]. The duration of the lag period following the first addition of ADP is independent of the amount of ADP added. Conditioning is reflected in an increasing rate of state 3 respiration. State 4 rates remain essentially constant. Consequently the extent and progress of conditioning is best perceived when the state 4 rate is first subtracted from a given state 3 rate, and the difference compared with the comparable difference relating to the ultimate maximal state 3 rate.

An insight into the basis of attenuation is attained by experimentally deconditioning mitochondria by preincubation with an inhibitor of electron transport. Table I sets out the effect of preincubation in 10 mM sodium amytal for 1 min at 27°C. Amytal pretreatment increases the

TABLE I. Deconditioning of mitochondria by preincubation with amytal

ADP 150 nmoles/ml	No pretreatment Attenuation		Amytal pretreatment Attenuation	
	State 3	RC	State 3	RC
Cyles of addition	%		%	
1	53	1.7	76	1.9
2	28	2.3	42	2.4
3	21	2.5	26	2.5
4	15	2.6	20	2.5
5	0	2.7	14	2.6
6	0	2.8	0	2.8
7	0	2.8	0	2.8

Pretreatment: 0.5 ml final mitochondrial suspension (see Methods) incubated for 1 min with 10 mM amytal at 27° C. Mitochondria pelleted, and resuspended in 0.5 ml extraction medium lacking MERCAP. Oxygen uptake measured polarographically as described in Methods with 5 mM K citrate as substrate. 1.0 mM NAD in all cases. Attenuation expressed as per Fig. 1. RC, the respiratory control ratio, represents ratio of given state 3 rate divided by following state 4 rate.

degree of attenuation from 53% to 76%, increases the lag time, or roll-off, from 72 to 138 sec, and increases the number of state 3-state 4 cycles required for full conditioning. Amytal pretreatment has but a small effect on the ultimate maximal state 3 rate. The increasing respiratory control (RC) values with successive state 3-state 4 cycles reflect increasing state 3 rates. Attenuation is effected by an impairment of electron transport, and conditioning follows upon subsequent re-establishment of electron flow. Can one define more explicitly which facet of the mitochondrial respiratory process is most directly linked to conditioning? The observation that alternating cycles of state 3-state 4 are more effective than continuous state 3 in conditioning mitochondria [1] has caused us to examine the possible role of non-phosphorylating electron transport in the conditioning process, and in this connection we have studied the effect of state 2, of state 4 and of pseudo-state 2 (substrate and ADP in the absence of inorganic phosphate, with or without oligomycin) on the conditioning phenomenon.

The Effect of State 2 on Conditioning

As seen in Fig. 1A, the state 2 respiration rate rises with a sigmoidal time-course through some 30 min. While there is a lag between the

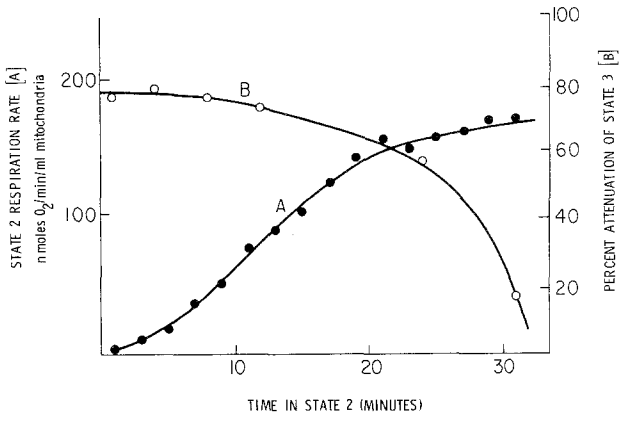


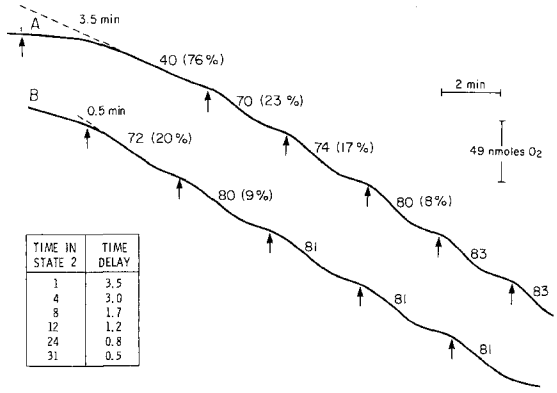
Figure 1. Conditioning of state 3 as a function of the development of state 2 respiration. Reaction mixture: 0.4 M mannitol, 0.025 M TES, 5 mM K phosphate, 5 mM $MgSO_4$, 1 mg/ml BSA, 5 mM K citrate, 1.0 mM NAD; pH 7.4. 0.1 ml mitochondrial suspension (ca. 100 μg protein N). Final volume, 3.4 ml. ADP, 0.15 mM each addition. A: State 2 respiration rate (nmoles O_2/min/ml mitochondrial suspension) as a function of time. B: Percent attenuation of state 3 as a function of state 2 duration. ADP (0.15 mM final concentration) added following designated intervals in state 2.

Percent attenuation: $\left[1 - \left(\frac{\text{Initial (state 3}-\text{state 4)}}{\text{Final (state 3}-\text{state 4)}}\right) \times 100\right]$

response of state 3 (Fig. 1B) and the development of state 2, the two events are clearly related, and the state 2 rise is part of the conditioning phenomenon. The course of respiration in response to successive additions of ADP to mitochondria preincubated in state 2 for 1 min and 31 min respectively is depicted in Fig. 2. Following 1 min in state 2, the initial state 3 is attenuated 76%, and four cycles are required for complete conditioning (Fig. 2A). By contrast, with 31 min preincubation in state 2, attenuation is initially 20%, and full conditioning is achieved after two cycles (Fig. 2B). Further, the roll-off time with 1 min preincubation is 3.5 min, while with 31 min preincubation the roll-off time is reduced to 0.5 min. Each cycle takes 3 min. Figure 2 demonstrates that the level of conditioning achieved in state 2 in 31 min (Initial state 3, Fig. 2B), is attained in approximately 6 min in the presence of ADP (second state 3, Fig. 2A). Thus ADP facilitates the conditioning process.

The Effect of State 4 Electron Transport on Conditioning

Figure 3 describes the course of conditioning of potato mitochondria as a function of time in state 4. Mitochondria were thrown into state 3



TIME IN STATE 2	TIME DELAY
1	3.5
4	3.0
8	1.7
12	1.2
24	0.8
31	0.5

Figure 2. Conditioning pattern of state 3 as a function of preincubation time in state 2. Reaction mixture as for Fig. 1. ADP, 0.15 mM for each state 3. A: 1 min preincubation in state 2. B: 31 min preincubation in state 2. Numbers beside traces represent respiration rates in nmoles O_2/min/mg mitochondrial protein. The numbers in parentheses indicate the percent attenuation of state 3 calculated as per the legend of Fig. 1. The insert table shows the lag, or roll-off, time between the initial addition of ADP and the attainment of the first steady state 3 rate, as a function of preincubation time in state 2.

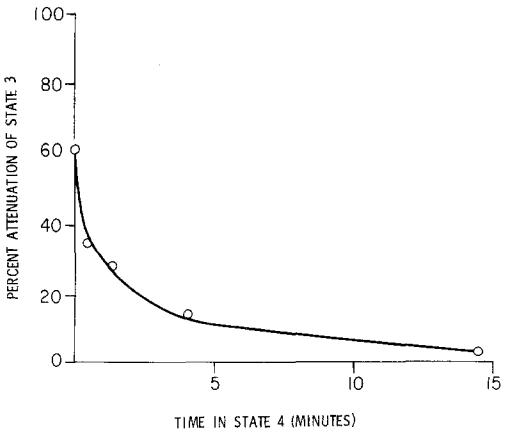


Figure 3. Conditioning of state 3 as a function of time in state 4. Reaction mixture as in Fig. 1. Abscissa: time in first state 4 before second ADP addition. Attenuation as per Fig. 1.

by addition of ADP after 1 min in state 2. ADP was added again either immediately following the end of the first state 3, or after varying intervals in state 4. About 90% of the intial attenuation was overcome in 6 or 7 min of incubation in state 4, while essentially full conditioning was achieved in 14 min (cf. Fig. 1). A comparison of conditioning effectiveness of state 2 with that of state 4 again points to the involvement of ADP in conditioning. While it was previously noted ([1] that prolonged incubation in the state 3 condition proved less effective in conditioning than several cycles of state 3/state 4, it remained to establish with certainty that ADP rather than ATP is the pertinent conditioning potentiating agent.

Mitochondria preincubated for 2 min in ADP in the absence of P_i show a markedly greater initial state 3 on subsequent P_i addition than do mitochondria preincubated in ATP (Table II). A detailed time-course of

TABLE II. Comparative effect of ADP and ATP on conditioning

Cycles ADP addition	ADP (0.1 mM)	ATP (0.12 mM)	ATP (0.71 mM)
1	46	70	65
2	8	30	23
3	0	11	5
4	0	0	0
5	0	0	0

Mitochondria were preincubated in 5 mM citrate and 1 mM NAD for 2 min in either ADP or ATP as indicated in the absence of inorganic phosphate. After 2 min P_i was added, together with 150 nmoles ADP/ml in all cases. Subsequently, as state 4 followed upon state 3, ADP was added again (150 nmoles/ml) to initiate another state 3. Percent attenuation expressed as follows:

$$1 - \left[\frac{\text{Initial (state 3}-\text{state 4)}}{\text{Final (state 3}-\text{state 4)}}\right] \times 100$$

conditioning in what is here termed pseudo-state 2, i.e. in the presence of substrate and ADP but in the absence of P_i, is depicted in Fig. 4. Conditioning under the specified conditions is seemingly as rapid as conditioning in state 4 (cf. Fig. 3). The course of conditioning in the presence of ADP is apparently hyperbolic with time (Figs. 4, 5), compared with the sigmoidal time-course in the absence of ADP (Fig. 1). It is uncertain whether the difference is intrinsic, or merely apparent—the consequence of a shortened time-scale. On the chance that endogenous P_i may yet have led to ATP formation, the effect of ADP *per se* in the absence of added P_i was assessed in the presence of oligomycin (Fig. 5). Conventional state 3 respiration is fully inhibited by oligomycin at the level used [8]. Consequently following the incubation

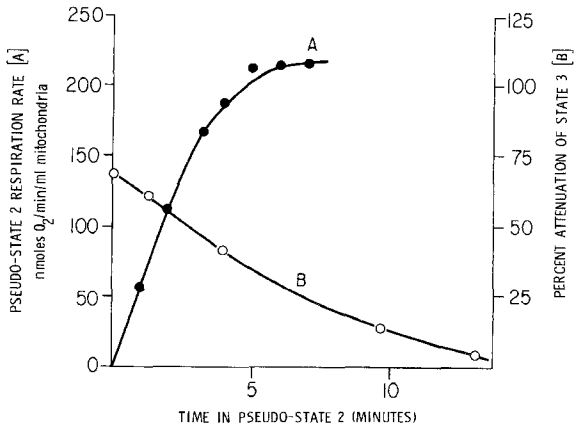


Figure 4. Conditioning of state 3 as a function of preincubation in pseudo-state 2. Reaction mixture as per Fig. 1 with the following exceptions: 0.15 mM ADP present in preincubation period; inorganic phosphate absent. State 3 initiated by addition of inorganic phosphate. Attenuation calculated as for Fig. 1. A: Respiration rate, pseudo-state 2. B: Percent attenuation of state 3.

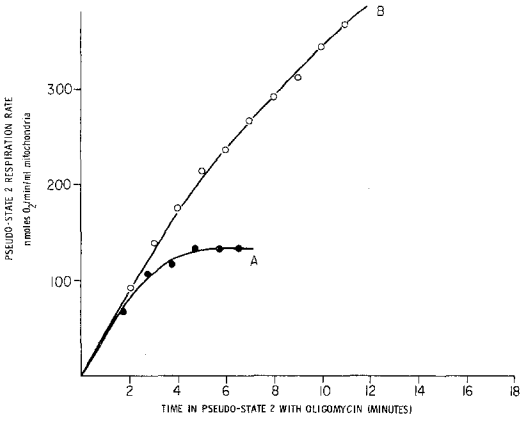


Figure 5. Effect of preincubation in pseudo-state 2 in the presence of oligomycin on mitochondrial Conditioning. Reaction mixture as per Fig. 1 with following exceptions: ADP (0.15 mM) and oligomycin, 1 μg total present in preincubation period; inorganic phosphate absent. Uncoupled respiration initiated by addition of *m*-ClCCP, 10^{-5} M final concentration. A: Respiration rate pseudo-state 2. B: Respiration rate in the presence of *m*-Cl-CCP.

of initially attenuated mitochondria in ADP and oligomycin for varying intervals, maximal respiratory capacity was subsequently estimated by addition of the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP). The course of pseudo-state 2 respiration is indicated in Fig. 5A. The uncoupled respiration rate as a function of preincubation time in pseudo-state 2 is shown in Fig. 5B. The pseudo-state 2 rate is maximal in about 5 min, while full conditioning takes something more than 12 min. As with state 2 implemented conditioning (cf. Fig. 1), conditioning lags behind the respiration rise attending preincubation. Nevertheless pseudo-state 2 approaches state 4 in conditioning effectiveness, being considerably more effective than state 2 *per se*.

Uncoupler added to deconditioned mitochondria in the absence of ADP has but a very limited effect (Fig. 6) [9]. When ADP is added subsequently, respiration is sharply stimulated, and the rise in respiration rate persists for some 10 min. Significantly, conditioning ostensibly proceeds in the presence of uncoupler, albeit more slowly.

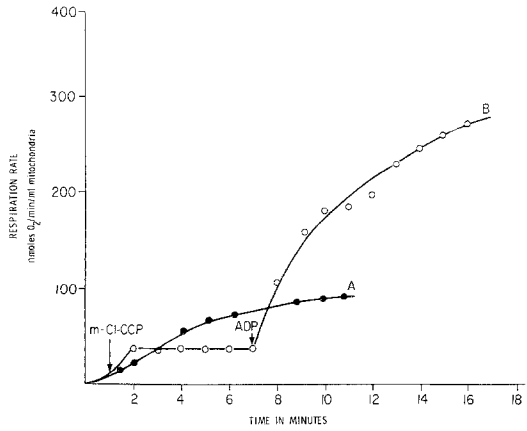


Figure 6. The effect of ADP on the respiration of uncoupled mitochondria. Reaction mixture as per Fig. 1 with oligomycin (1 μg total), *m*-Cl-CCP (10^{-5} M) added after 1 min (open circles), followed by ADP (0.15 mM) after 7 min. A: Control, mitochondria in state 2. B: Mitochondria in oligomycin and *m*-Cl-CCP, followed by ADP.

The Relationship of Conditioning to ADP Concentration

The influence of ADP concentration specifically on conditioning was deduced from the effect of ADP provided in a preincubation period without P_i—that is, in pseudo-state 2. Figure 7A depicts the pseudo-state 2 respiration rate after an 8 min preincubation period as a function of ADP concentration, while Fig. 7B describes the effect of ADP

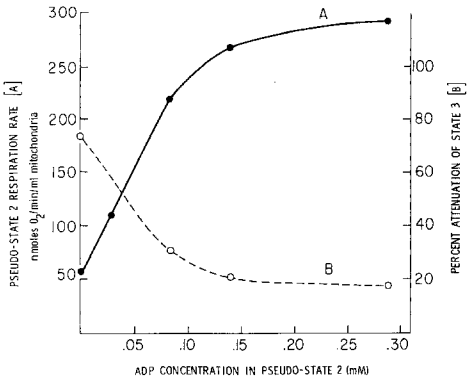


Figure 7. The influence of ADP concentration on the conditioning process. Reaction mixture as per Fig. 1, without inorganic phosphate. Mitochondria preincubated in pseudo-state 2 for 8 min in presence of designated ADP concentration. State 3 begun by addition of inorganic phosphate and additional ADP (0.15 mM). Attenuation calculated as per Fig. 1. A: Respiration rate after 8 min in pseudo-state 2 as a function of ADP concentration. B: Percent attenuation of state 3 as a function of ADP concentration in pseudo-state 2.

concentration on the conditioning process *per se*. When the reciprocal of the incremental increase in the rate of respiration due to conditioning is plotted against the reciprocal of the square of ADP concentration, the best fit indicates an apparent second-order involvement of ADP, with an $S_{0.5}$ of approximately 62 μM (Fig. 8A). A Hill plot of the data wherein log $(V/V_{max}-v)$ is plotted against log ADP concentration, yields a line of slope 2.3, suggesting a cooperativity number of 2 for ADP [10].

By contrast ADP involvement in oxidative phosphorylation is first-order. Further, the frequently observed theoretical P/O ratios in polarographic experiments point to a K_M for ADP well below the $S_{0.5}$ value noted above [11]. In any event the role of ADP in conditioning does not involve ATP formation since conditioning occurs in the presence of oligomycin (Fig. 5).

Discussion

In the past the phenomenon of attenuation has been focused primarily on state 3 of succinate oxidation and construed to represent an inhibition due to oxalacetate. Recently Douce and Bonner [12] have imputed inhibition by oxalacetate to NAD, formed in the process of oxalacetate reduction. However, Raison *et al.* [1] have shown attenuation to apply as well to the oxidation of malate and β-hydroxybutyrate,

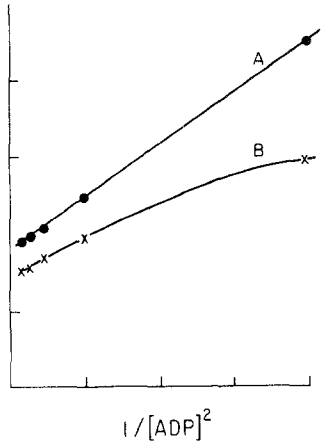


Figure 8. The kinetic order of ADP involvement in conditioning. Reciprocal plot of data of Fig. 7 with ADP involvement taken as a second-order. A: Respiratory increment due to conditioning plotted as a function of ADP concentration in pseudo-state 2. B: Total respiration rate plotted against ADP concentration in pseudo-state 2.

and McDaniel and Sarkissian [7] have demonstrated the phenomenon in connection with the oxidation of α-ketoglutarate. Furthermore, Drury *et al.* [4] found ADP to be a modulator of succinate dehydrogenase, enhancing a preference of the enzyme for its natural substrate, succinate, over the inhibitor, oxalacetate.

Herein we have shown conditioning to be a phenomenon elicited by mitochondrial electron flux, particularly in the absence of oxidative phosphorylation, but markedly favored by the presence of ADP. While the focus of attention with respect to conditioning remains on state 3, we have demonstrated that both state 2 and pseudo-state 2 (substrate and ADP in the absence of P_i) rise with time and that the rise is correlated with conditioning of state 3. Conditioning effectiveness of state 2 or pseudo-state 2 proceeds in time beyond the attainment of maximal state 2 or pseudo-state 2 rates, and consequently the rise in state 2 and pseudo-state 2 must be but one manifestation of the conditioning process.

Pseudo-state 2 and state 4 appear to be equally effective in implementing conditioning (compare Figs. 3 and 4), and considerably more effective than state 2. The significant element in conditioning is ADP, and its effectiveness in the absence of P_i and in the presence of oligomycin point to a modulator role unrelated to oxidative phosphorylation. Why is there no appreciable rise in the state 4 rate with time? There may well be, but by its very nature state 4 follows state 3, and in

our experiments attainment of state 4 followed a short time in state 2 and several minutes in state 3. There is reason to believe state 4 at least in part underlies state 3, and in consequence considerable conditioning has been achieved at the onset of the first state 4, and still more at its termination. Maximal pseudo-state 2 rates approach state 4 rates (as do maximal state 2 rates), and since the attainment of maximal pseudo-state 2 rates takes about 5 min, it is easy to see why a significant rise in state 4 rates is not often perceived. As with pseudo-state 2, maximal state 4 is achieved (Fig. 2) before full conditioning evokes maximal state 3 (Fig. 3). Respiratory control ratios approach 3 (Table I), and state 3 rates are therefore roughly three times greater than maximal state 2 or pseudo-state 2 rates.

Does state 4 underlie state 3, or is state 4 turned off upon addition of ADP in the presence of P_i, as asserted by Chance and Williams [13]? Alternating state 3/state 4 has been shown to condition more effectively than continuous state 3 [1]. Herein we have shown that it is state 4 rather than alternation which effects conditioning. Since state 3 *does* condition, albeit less effectively than state 4, there is a strong indication that state 4 to some extent underlies state 3. P/O ratios in plant mitochondria calculated by the convention of Chance and Williams [14] more often than not fall below the theoretical. If state 4 rates are subtracted from state 3 rates in plant mitochondria demonstrating good control, P/O ratios more nearly approach theoretical.

Conditioning is related to electron transport (and presumably to respiratory energy release), as indicated by attenuation induced by brief incubation of mitochondria in amytal in the absence of added substrate (Table I). In this connection it is significant that state 4 is amytal sensitive [15]. The view which emerges from the observations presented herein is that the potentially available energy arising from mitochondrial respiratory electron transport can be used *either* in oxidative phosphorylation or in the creation and maintenance of the conditioned state. On the assumption that there is a constant, although perhaps meager, tendency towards attenuation, some respiratory energy will be expended continuously in conditioning. That is, some state 4 will underlie state 3.

The concept of mutually exclusive utilization of respiratory energy proposed herein is akin to the alternative use of respiratory energy in oxidative phosphorylation and mitochondrial ion transport [16]. Does conditioning represent active transport—in this case predominantly substrate transport? While the possibility has not been ruled out, several considerations make such an explanation untenable. In mammalian mitochondria citrate uptake is dependent on exchange with malate, and malate absorption, in turn, is initially dependent on phosphate uptake [17, cf. 19]. Potato mitochondria demonstrate a comparable dependence on malate in the oxidation of citrate by NAD [18]. Nevertheless conditioning occurs in the absence of P_i and attenuation is unaffected by

preincubation in malate. Furthermore, conditioning is exhibited to the same extent with all TCA cycle intermediates, while transport of the latter varies with the substrate [17]. While conditioning takes from 5 min in state 4 to 30 min in pseudo-state 2, TCA cycle substrate transport is reported to be generally rapid [19], succinate uptake in rat liver mitochondria reaching an apparent equilibrium in 6-7 sec [20]. There has been no indication that mitochondrial substrate transport is ADP dependent, yet as seen herein conditioning is exceptionally responsive to ADP, even in the presence of uncoupler (Fig. 5). Finally, we have observed (unpublished) that the Arrhenius activation energy for citrate oxidation by unconditioned potato mitochondria is three to four times greater than that in the conditioned state, suggesting that conditioning may be related to a change in the conformation of the inner mitochondrial membrane favorable for the activity of one or more membrane-bound respiratory enzymes [21]. For all these reasons we reject substrate uptake as the basis of conditioning.

ADP has been shown to be a potentiator of uncoupler action in potato mitochondria with both 6C and 4C members of the tricarboxylic acid cycle. Thus the conditioning role of ADP cannot be attributed to its acceptor function in substrate level phosphorylation [9]. The ADP concentration relationships are much the same as for conditioning. It is uncertain whether the modulator effect of ADP is the same in both cases. The apparent persistence of conditioning in the presence of *m*-Cl-CCP (Fig. 6) at least leaves open the possibility.

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