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Malate Oxidation and Cyanide-Insensitive Respiration in Avocado Mitochondria during the Climacteric Cycle

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ABSTRACT

After preparation on self-generated Percoll gradients, avocado (*Persea americana* Mill, var. Fuerte and Hass) mitochondria retain a high proportion of cyanide-insensitive respiration, especially with α-ketoglutarate and malate as substrates. Whereas α-ketoglutarate oxidation remains unchanged, the rate of malate oxidation increases as ripening advances through the climacteric. An enhancement of mitochondrial malic enzyme activity, measured by the accumulation of pyruvate, closely parallels the increase of malate oxidation. The capacity for cyanide-insensitive respiration is also considerably enhanced while respiratory control decreases (from 3.3 to 1.7), leading to high state 4 rates.

Both malate dehydrogenase and malic enzyme are functional in state 3, but malic enzyme appears to predominate before the addition of ADP and after its depletion. In the presence of cyanide, a membrane potential is generated when the alterntive pathway is operating. Cyanide-insensitive malate oxidation can be either coupled to the first phosphorylation site, sensitive to rotenone, or by-pass this site. In the absence of phosphate acceptor, malate oxidation is mainly carried out via malic enzyme and the alternative pathway. Experimental modification of the external mitochondrial environment *in vitro* (pH, NAD^+, glutamade) results in changes in malate dehydrogenase and malic enzyme activities, which also modify cyanide resistance. It appears that a functional connection exists between malic enzyme and the alternative pathway via a rotenone-insensitive NADH dehydrogenase and that this pathway is responsible, in part, for nonphosphorylating respiratory activity during the climacteric.

It has been known for some time that mitochondria isolated from avocado fruit at various stages of ripeness exhibit unaltered oxidative and phosphorylative properties provided that all required cofactors are present (8–10). Nonetheless, some alteration in mitochondrial respiratory function *in vivo* is implied by the dramatic burst in respiratory activity that accompanies ripening of the avocado and other climacteric-type fruit (4). An observed increase in the capacity for cyanide-resistant respiration appeared as a plausible explanation for the respiratory climacteric (28, 29) but was subsequently discounted in light of further evidence (30). Another and still plausible explanation (23) infers intracellular homeostatic relationships but leaves unexplained the metabolic connections between the climacteric respiratory rise and mitochondrial activity.

It is interesting in this context that malate oxidation, which can be carried out by both MDH[3] and ME (6, 13, 14, 21), has recently been shown (25, 26) to exhibit a varying degree of cyanide-insensitivity, depending on the relative activity of either enzyme. It seemed promising, therefore, to re-examine malate oxidation by avocado mitochondria throughout the climacteric with special attention to the cyanide-insensitive alternative pathway.

In a previous paper (20), we described the preparation of avocado mitochondria on self-generated Percoll gradients. The present study concerns the biochemical behavior of PGC avocado mitochondria with special attention to malate oxidation and cyanide-insensitive respiration. A preliminary report of this work has appeared (19).

MATERIALS AND METHODS

Source of Mitochondria. 'Fuerte' and 'Hass' avocado fruits (*Persea americana*, Mill) were obtained from the University of California, South Coast Field Station. The conditions for ripening, measurement of fruit respiration, and preparation of mitochondria on self-generated Percoll gradients have been described (20). At intermediate ripening stages, the mitochondria separate into two bands on the gradients. In this work, the light and heavy mitochondria were combined for subsequent study.

Mitochondrial O_2 Uptake and Protein Determination. O_2 uptake was measured at 25°C with a Clark-type polarographic electrode using a medium containing 0.3 M mannitol, 10 mM phosphate buffer, 10 mM KCl, 5 mM $MgCl_2$, and 0.1% BSA. Depending on the experiment, pH was adjusted to 6.8, 7.2, or 7.5. Protein was measured by the Lowry procedure.

Measurement of Products of Malate Oxidation. The products of malate oxidation (OAA and pyruvate) were determined spectrophotometrically. Mitochondria (about 1 mg protein/ml) were incubated at 25°C in 7 ml of the same medium as used for O_2 uptake determinations plus 1 mM arsenite and 25 mM malate. This suspension was maintained aerobic by gentle stirring. After starting the reaction by the addition of 20 mM malate, 1-ml aliquots were taken every 2 min, mixed rapidly with 0.1 ml of cold 20% $HClO_4$ containing 1 mM EDTA, immediately neutralized with a few drops of 10 N KOH in the presence of 20 μl methyl orange (0.05%), and then centrifuged at 2000*g* for 5 min. Aliquots of the supernatant fraction were added to 0.1 M phosphate buffer (pH 7.2) and 0.12 mM NADH in a final volume of 3 ml. The oxidation of NADH was determined at 30 nm after addition of 2 μl of MDH (Calbiochem, porcine heart, 11,370 IU/ml) or lactate dehydrogenase (Sigma Chemical Co., rabbit muscle, type 1, 3900 units/ml)

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[3] Abbreviations: MDH, malate dehydrogenase; ME, malic enzyme; OAA, oxaloacetate; TPP, thiamine pyrophosphate; SHAM, salicylhydroxamic acid. Substrate state, rate of O_2 uptake in the presence of substrate alone. State 3 and state 4, rates of O_2 uptake in the presence and after the depletion of ADP, respectively; PGC, Percoll gradient-cleaned; RC, respiratory control (state 3/state 4); CN-R: cyanide resistance.

to measure OAA and pyruvate contents, respectively.

Measurement of Membrane Potential. Membrane potential was measured using safranine as an optical probe (1), as described in greater detail elsewhere (24). Mitochondria (0.5–1 mg protein/ml) were equilibrated in the presence of 5 μM safranine at room temperature and change in fluorescence at 575 nm following excitation at 525 nm was recorded using a Farrand model 801 spectrofluorimeter. No changes were discernible at 575 nm in the absence of safranine, thereby ruling out artifacts due to mitochondria shrinking and swelling.

RESULTS

Cyanide-Insensitive Respiration by Postclimacteric Mitochondria. PGC postclimacteric mitochondria exhibited considerable cyanide-insensitive respiration, especially with α-ketoglutarate (40%) and malate (60%) as compared with exogenous NADH (15%) and succinate (25%) as substrates (Fig. 1). This observation supports the concept that the alternative pathway is firmly bound to mitochondrial membranes and cannot be removed by centrifugation through Percoll gradients. Moreover, it is clear that the participation of this pathway depends on the substrates being oxidized. On the other hand, there was apparently no relationship between the maximal oxidation rates observed in state 3 and the level of cyanide resistance.

Although the cyanide-insensitive oxidation rates are high with both α-ketoglutarate and malate as substrates, malate oxidation has some unique features in postclimacteric avocado mitochondria (Fig. 1). First, malate oxidation was rapid in the substrate state, *i.e.* in the presence of substrate only. Second, as observed by Lance *et al.* (11), state 4 was usually biphasic. After a 'pseudo-state 4,' which corresponds to an inhibition of malate dehydrogenase by OAA, the subsequent real state 4 was appreciably higher and

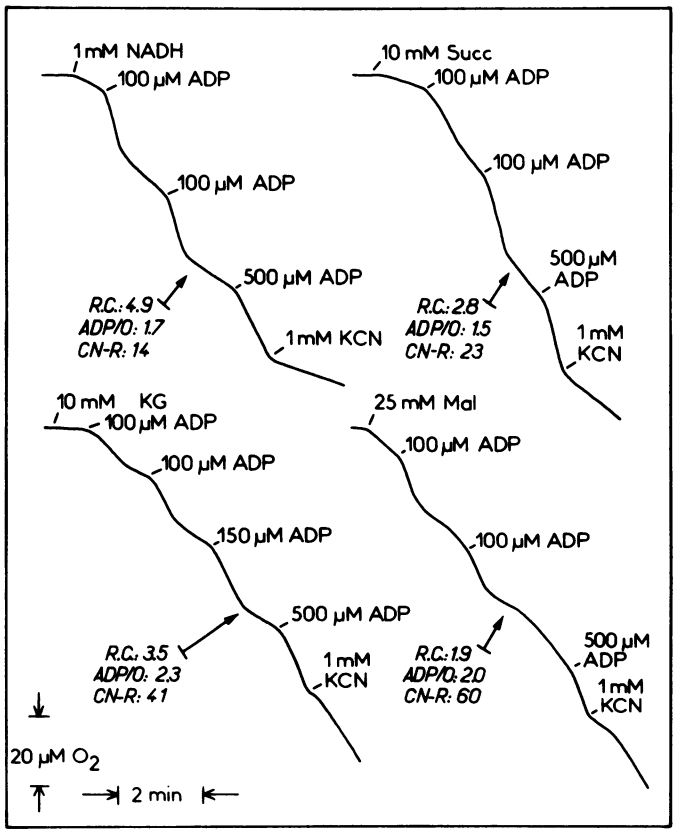

FIG. 1. Oxidative properties and cyanide-resistant respiration by Percoll gradient-cleaned postclimacteric avocado mitochondria. CN-R is expressed as a percentage of state 3 rates. Succ, succinate; Mal, malate.

Table I. *NADH and Malate Oxidation throughout the Climacteric by Percoll Gradient-Cleaned Avocado Mitochondria*

Substrate	Stage of Ripening				
	Early preclimacteric	Late preclimacteric	Climacteric rise	Climacteric peak	Postclimacteric
NADH					
State 3[a]	135	160	140	140	145
State 4[a]	40	43	33	30	32
RC	3.3	3.7	4.2	4.5	4.5
ADP/O	1.6	1.6	1.8	1.7	1.4
CN-R	20[b]	17	20	15	10
Malate					
State 3[a]	55	43	70	130	130
State 4[a]	16	17	29	56	76
RC	3.3	2.4	2.4	2.3	1.7
ADP/O	2.4	2.2	2.0	2.0	1.8
CN-R	44[b]	26	60	75	70

[a] nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$.
[b] Cyanide resistance as percentage of state 3 rates.

Table II. *Malate Oxidation in the Substrate State by Percoll Gradient-Cleaned Avocado Mitochondria Isolated at Different Stages of the Climacteric*

	Stage of Ripening				
	Preclimacteric	Climacteric minimum	Climacteric rise	Climacteric peak	Postclimacteric
	nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$				
−1 mM KCN	15	16	13	92	121
+1 mM KCN	12	9	2	63	90

loosely coupled to phosphorylation (RC < 2). In contrast, with α-ketoglutarate as substrate, little or no oxidation occurred in the substrate state, there was very little biphasic transition in state 4, and the rate of oxidation was strongly controlled by phosphorylation, presumably at the substrate level, resulting in a high (>4) RC.

Changes in Malate Oxidation throughout the Climacteric. Data comparing NADH and malate oxidation by PGC avocado mitochondria isolated at four different stages of ripening (preclimacteric, climacteric minimum, climacteric rise, and climacteric peak), are given in Table I. Various properties of NADH oxidation, including rate of O_2 uptake, RC, ADP/O, and CN-R, changed very little throughout the climacteric. In contrast, qualitative and quantitative changes occurred with malate as substrate. The rate of malate oxidation more than doubled with ripening while RC decreased and ADP/O remained constant or decreased slightly. Concomitantly, the capacity for cyanide-insensitive malate oxidation was appreciably enhanced during the climacteric and at the climacteric peak, it was equal to about 70% of the normal state 3 rate which had itself increased over 2-fold.

An especially dramatic change associated with the climacteric was the 8-fold increase in rate of malate oxidation in the substrate state (Table II). At the climacteric peak, this oxidation represented almost 90% of the maximal oxidation rate in state 3. Moreover, the substrate state malate oxidation was largely cyanide-insensitive.

Products of Malate Oxidation during the Climacteric. In the experiment represented in Figure 2, the products of malate oxidation were measured during the substrate state at three stages of ripening (preclimacteric, climacteric rise, and climacteric peak). Under these conditions, pyruvate formation increased dramati-

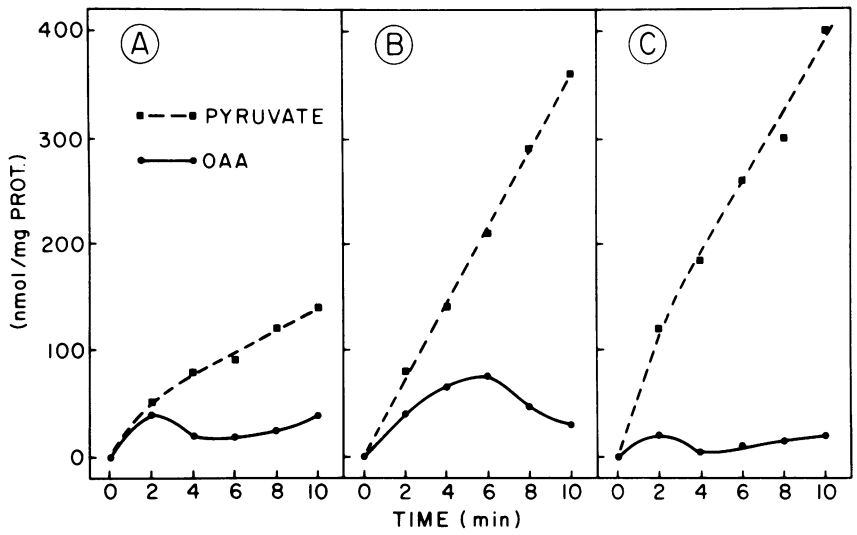

FIG. 2. Products of malate oxidation in substrate state by Percoll gradient-cleaned mitochondria isolated at different stages during the ripening of avocado. A, Preclimacteric; B, climacteric rise; C, climacteric peak. Pyruvate (■- - -■) and OAA (●——●) were determined as described in "Materials and Methods." PROT., protein.

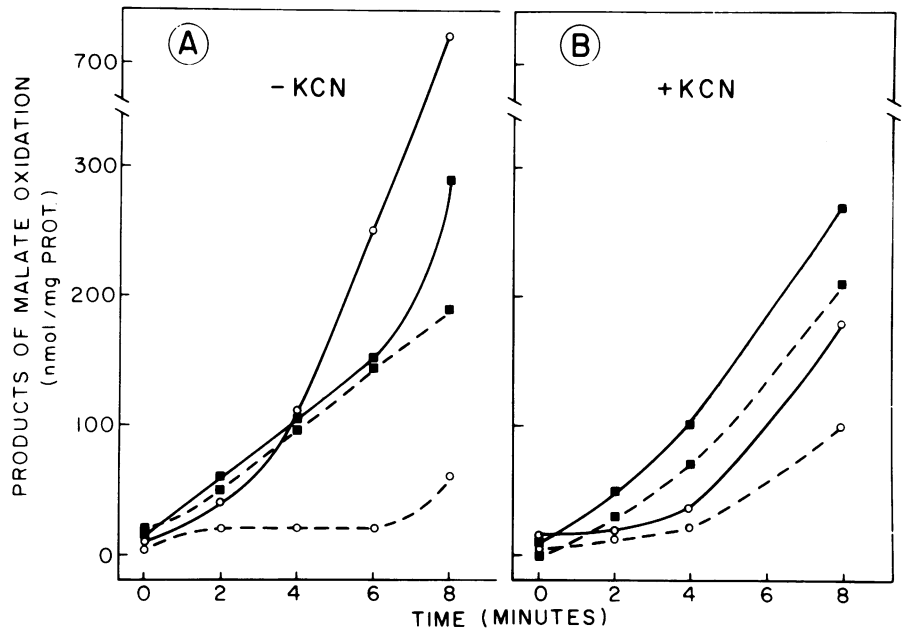

FIG. 3. Products of malate oxidation by Percoll gradient-cleaned climacteric avocado mitochondria in state 3 and state 4 in the presence (A) or in the absence (B) of KCN. State 3 (——), state 4 (- - -), pyruvate (■), and OAA (○). PROT., protein.

cally with the climacteric, whereas OAA formation remained nearly unchanged. Taking into account the data of Figure 2 and Table II, it appears that the enhancement of malate oxidation is accompanied by an increase in ME activity. It has already been reported by Lance *et al.* (11) that pyruvate and OAA accumulate in avocado mitochondria oxidizing malate in state 3. The same was true with the PGC avocado mitochondria used in this study (Fig. 3A). However, the data in Figure 3 also reveal a dramatic difference between OAA production in state 3 and in state 4. During state 3, OAA was the main product being formed whereas its accumulation was very low in state 4. In contrast, the accumulation of pyruvate was almost unchanged, indicating that the state 3/state 4 transition does not significantly modify ME activity. In the presence of 1 mM cyanide (Fig. 3B), pyruvate is the main product of malate oxidation in both state 3 and state 4. Some OAA is also accumulated under these circumstances, but to a lower extent.

Phosphorylation during Cyanide-Insensitive Malate Oxidation. Trace A in Figure 4 shows that postclimacteric mitochondria oxidizing malate respond to ADP but that after release of pseudo-state 4 the real RC was low and the cyanide-insensitive, SHAM-sensitive oxidation was very high. In the presence of cyanide (trace B), malate oxidation was slowly inhibited but did respond to stoichiometric additions of ADP. Oxidation in the presence of cyanide (Fig. 4, trace C) could be partly inhibited by oligomycin and released by the uncoupler carbonylcyanide *m*-chlorophenylhydrazone. Self-inhibition of the cyanide-insensitive malate oxidation can be related to the slow accumulation of OAA (*cf.* Fig. 3B). Moreover, the delay in this inhibition depends on the mitochondrial concentration which presumably modulates the rise in OAA concentration in the medium, *e.g.* mitochondrial concentration represented in Figure 4, trace B, is roughly twice that in trace

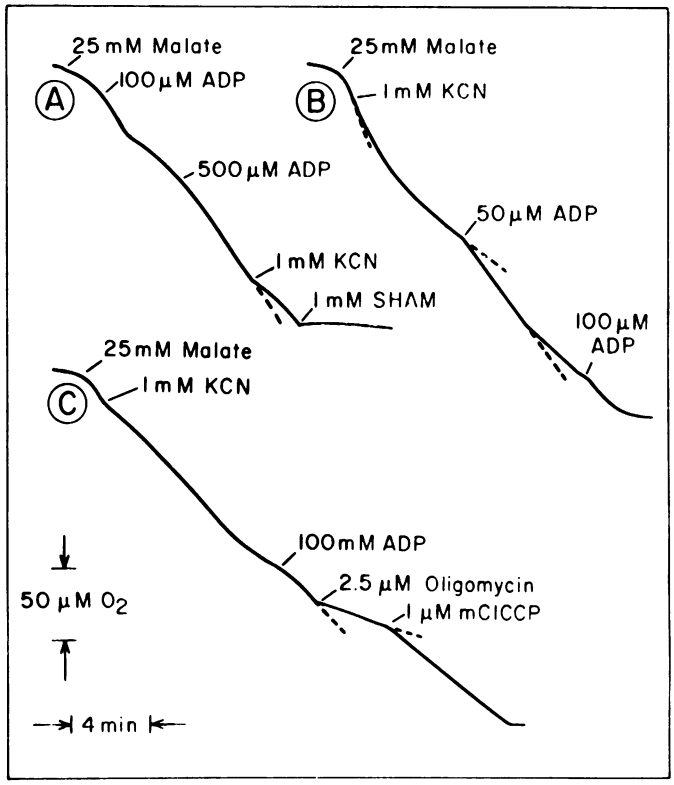

FIG. 4. Oxidative phosphorylation linked to malate oxidation in the absence (A) or in the presence (B) of 1 mM cyanide and the presence of 1 mM KCN plus oligomycin and carbonylcyanide *m*-chlorophenylhydrazone (mCLCCP) (C).

C. Finally, in the presence of cyanide, ADP/O was about 0.5 ± 0.1 and RC was close to 2.

It was also observed that a transmembrane potential ($\Delta\psi$), as measured by safranine fluorescence, was generated in avocado mitochondria and that a transient response to ADP occurred with malate as substrate (Fig. 5A). Addition of 1 mM KCN partly dissipated $\Delta\psi$, whereas subsequent addition of uncoupler completely collapsed it. It is interesting that the addition of ADP in the presence of cyanide (Fig. 5B) led to a small but significant transition in $\Delta\psi$, implying that the alternative pathway can support some phosphorylation.

Rotenone inhibited malate oxidation by about 25% in both state 3 and state 4 (Table III). In the presence of KCN, malate oxidation was also inhibited by about 35% in both state 3 and in state 4 and in the presence of both cyanide and rotenone, malate oxidation did not respond to the addition of ADP. Consequently, it is clear that postclimacteric mitochondria are also able to oxidize malate by a pathway which by-passes the first, rotenone-sensitive site, and which is not phosphorylating.

Effect of External Environment. It is well established that high pH (7.5) or addition of glutamate stimulates MDH activity, whereas low pH (6.8) or addition of exogenous NAD^+ stimulates ME activity (7, 8, 12–14).

As shown in Table IV, pH was the most efficient effector of cyanide-insensitive malate oxidation in preclimacteric mitochondria. Low pH increased O_2 uptake, decreased RC without significant changes in ADP/O, and enhanced cyanide-insensitive malate oxidation. High pH had the opposite effect. Exogenous NAD^+ had a questionable effect on cyanide resistance except when used in conjunction with low pH. RC was then minimal and cyanide-resistant respiration at its maximum. In the presence of glutamate, cyanide-insensitive malate oxidation was lowered, the total O_2 consumption remaining unchanged while the effect of low pH could also be observed but to a lower extent.

Generally similar responses were obtained with postclimacteric mitochondria (Table V). The effects of pH were even more dramatic. The combined NAD^+ and low pH further increased O_2 consumption with a very low RC (<2) and a remarkably high cyanide resistance, *i.e.* near 90% of state 3 rates. In contrast,

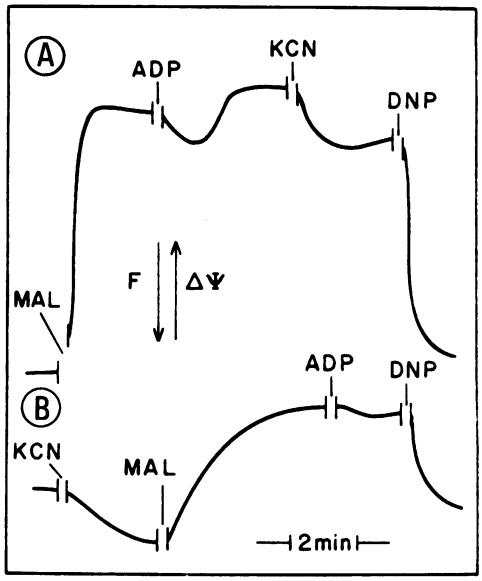

FIG. 5. Membrane potential (ψ), as measured by fluorescence of safranine in Percoll gradient-cleaned postclimacteric avocado mitochondria oxidizing malate in the absence (A) or in the presence (B) of cyanide. In A, approximately 0.50 mg/ml mitochondria protein was incubated in 2.5 ml medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2), and 5 μM safranine. Respiration was initiated by the addition of 10 mM malate. Further additions consisted of 100 μM ADP, 0.5 mM KCN, and 80 μM dinitrophenal (DNP). The same conditions pertained in B except for the initial presence of 0.5 mM KCN. MAL, malate.

Table III. *Effect of Rotenone on Malate Oxidation by Postclimacteric Mitochondria in the Presence or in the Absence of Cyanide*

	Control	+1 μM rotenone
	nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$	
Absence of KCN		
State 3	100	75
State 4	63	50
Presence of KCN		
State 3	60	37
State 4	55	37

Table IV. *Effects of pH and Various Effectors on Malate Oxidation by Percoll Gradient-Cleaned Preclimacteric Avocado Mitochondria*

Substrate	pH	V_{O_2}[a]	RC	ADP/O	CN-R[b]
					%
Malate	7.5	33	3.0	2.1	36
	7.2	41	3.5	2.0	46
	6.8	72	1.9	1.9	58
Malate/NAD^+	7.5	39	3.1	2.0	25
	7.2	48	3.0	1.9	46
	6.8	64	1.6	2.1	63
Malate/glutamate	7.5	30	2.3	1.8	14
	7.2	45	2.3	1.8	22
	6.8	72	2.3	1.7	39

[a] nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$.

[b] Cyanide-resistant respiration as a percentage of the state 3 rate.

Table V. *Effects of pH and Various Effectors on Malate Oxidation by Percoll Gradient-Cleaned Postclimacteric Avocado Mitochondria*

Substrate	pH	Vo_2[a]	RC	ADP/O	CN-R[b]
					%
Malate	7.5	60	4.9	1.6	48
	7.2	73	1.7	1.6	72
	6.8	133	1.3	1.7	72
Malate/NAD^+	7.5	68	4.2	1.5	58
	7.2	77	1.5	1.6	77
	6.8	153	1.2	1.5	89
Malate/glutamate	7.5	73	2.5	2.0	51
	7.2	99	2.4	2.1	76
	6.8	149	1.8	2.1	73

[a] nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$.

[b] Cyanide resistance as percentage of state 3 rate.

glutamate had less effect on cyanide-insensitive malate oxidation, ADP:0 ratios were higher and the pseudo-state 4 (not shown) almost completely disappeared.

DISCUSSION

Several conclusions can be drawn from the results reported above. They deal with the peculiarities of malate oxidation in plant mitochondria and also with the climacteric scheme which pertains to many ripening fruits.

After a 'cleaning' with self-generated Percoll gradients, avocado mitochondria oxidize many substrates with an increased specific activity and retain a high cyanide-insensitive respiration. Malate oxidation takes place at all stages of ripening without a need for TPP. This contrasts with the performance of the usual, once-washed avocado mitochondria that require TPP for effective malate oxidation, especially when the organelles are obtained from preclimacteric fruit (11). The data above imply that TPP is not limiting, a notion supported by the finding that TPP levels in avocado undergo little change with ripening (5). We have also observed that Percoll centrifugation removes extramitochondrial MDH (unpublished results) which would contribute to high levels of OAA in the incubation medium with resultant product inhibition. Such an effect has been observed with washed spinach mitochondria (25).

PGC avocado mitochondria do demonstrate a climacteric-related increase in rate of malate oxidation, especially in the substrate state and in state 4, resulting in a progressive decrease in RC. In contrast, and as previously shown (11), the rate and phosphorylative properties of α-ketoglutarate oxidation do not change with the climacteric. Moreover, based on ADP/O and RC values, the cyanide-insensitive pathway is probably not functional with α-ketoglutarate, presumably because its oxidation is directly controlled by phosphorylation at the substrate level.

Several observations lead to the conclusion that a mitochondrial NAD^+-malic enzyme is involved in the climacteric-related increase in malate oxidation. First, the formation of pyruvate by mitochondria oxidizing malate parallels the increase in mitochondrial O_2 consumption during the climacteric. Second, at all climacteric stages, changes in ME activity, via modification of external environment, modulate O_2 uptake, rate of state 4, and, consequently, RC. Third, cyanide-insensitive malate oxidation is also modulated, without significant modification of ADP/O ratios.

As evidenced by the products of malate oxidation, MDH activity in mitochondria from climacteric fruit (Fig. 3A), as in other plant mitochondria (21, 22, 26, 31), is strictly regulated by the state 3/state 4 transition. In contrast, ME activity, *i.e.* pyruvate production, is relatively unaffected. Moreover, ME activity is mainly responsible for the cyanide-insensitive malate oxidation (Fig. 3B) which may or may not be coupled to the first site, depending on the presence of phosphate acceptor (15, 18, 22, 26).

These data emphasize that malate oxidation in climacteric avocado mitochondria can be phosphorylating or not, depending on metabolic state. In state 3, malate is oxidized by both MDH and ME, the Cyt pathway predominates, and the alternative pathway, if used, can be phosphorylating at the first site level. In the substrate state and state 4, malate is oxidized by a pathway involving ME, a rotenone-insensitive NADH dehydrogenase, which is presumably located on the inner face of the inner membrane (15, 22, 26), and the alternative oxidase (18, 26).

In this context, it seemed important to determine whether the state/3 state 4 transition in avocado mitochondria is accompanied by additional activation of the alternative path as proposed by Bahr and Bonner (2, 3). Unfortunately, using the methods of both Bahr and Bonner (2, 3) and Medenstev and Akimento (16), we have failed to demonstrate such regulation. In the first method, SHAM, an inhibitor of the alternative pathway (27), also partly inhibited the Cyt pathway in avocado mitochondria. In the second method, using ferricyanide as an artificial electron acceptor, it was not possible to maintain the cyanide-insensitive electron transport from malate to O_2, regardless of metabolic state.

Although the regulation of electron transport between the Cyt chain and the alternative path remains to be elucidated in avocado mitochondria, there is no definitive reason to rule out the participation of the alternative pathway in state 4. Moreover, the balance between MDH and ME activities during the state 3/state 4 transition can be more easily understood if the alternative path were activated. In plant mitochondria oxidizing malate, important changes occur in the redox level of endogenous pyridine nucleotides during the state 3/state 4 transition (31). Consequently, as a result of a high NADH/NAD^+ ratio and the accumulation of OAA, MDH is inhibited in state 4, whereas ME activity is almost unchanged (Fig. 3A). The NADH formed under this condition is probably partially diverted from the respiratory chain to progressively reverse MDH (21, 22), as evidenced by the disappearance of OAA (Fig. 2). Such an event could account for the pseudo-state 4 which occurs in avocado mitochondria. Lance *et al.* (11) have shown that the duration of pseudo-state 4 was dependent on the amount of OAA previously produced in state 3. Consequently, after elimination of OAA, NADH, which is continuously formed by ME, is reoxidized by the respiratory chain during the real state 4. In this situation, malate oxidation is carried out mainly by the cyanide-insensitive pathway (18, 26).

SUMMARY REMARKS

The foregoing observations lead to and support the general conclusion that the pathway involving ME and the alternative oxidase is likely to function under conditions of relatively low ATP demand and high energy charge (22, 26). These conditions exist in the later stages of the climacteric (28) and should therefore favor the metabolic pathway in question. On the other hand, Theologis and Laties (30) have demonstrated convincingly that the alternative path, although present and subject to stimulation by uncouplers, is normally not operative in tissue slices prepared from avocados at any stage of the climacteric. Remaining unclear is whether the energy and metabolic demands of freshly cut slices are analogous to those of the intact tissue.

The aim of this paper was to reappraise the role of malate oxidation in ripening, senescent tissue. Whereas the prevalence of the ME-alternative oxidase system in ripening tissue is still problematic, our findings demonstrate that it is not only operative in avocado mitochondria but that its magnitude increases with ripening.

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