

Metabolically Driven Self-Restoration of Energy-Linked Functions by Avocado Mitochondria

General Characteristics and Phosphorylative Aspects

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ABSTRACT

To assess the restorative capacity of isolated avocado (*Persea americana*) fruit mitochondria, the organelles were first aged in the absence of an energy source at 25°C for several hours until respiratory control and oxidative phosphorylation were greatly diminished or totally lost. Energy-linked functions were then gradually restored over a period of several hours after the addition of substrate. Restoration of respiratory control resulted from both an increase in state 3 and a decrease in state 4 respiratory rates. Either α -ketoglutarate or succinate served as restorants, each with distinctive rates of recovery in state 3 and state 4 respiration. ATP also served as a restorative agent but not as effectively as metabolizable substrate. ATP synthase activity was modulated by stress and restoration but neither the extent nor the rate of change was sufficient to constrain state 3 rates. Orthophosphate was released from the mitochondria during substrate-deprived stress. Restoration of phosphorylation preceded that of RC with phosphate uptake and phosphorylation being evident immediately upon the addition of substrate. During restoration [³²P]orthophosphate was incorporated into several organic fractions: phospholipid, ATP, a trichloroacetic acid-precipitable mitochondrial fraction, and an organophosphate that accumulated in the medium in relatively large amounts. The organophosphate was tentatively identified as a hexosephosphate. Incorporation into ATP and the putative hexosephosphate continued unabated beyond the point of maximum restoration. Phosphate metabolism thus appears to be a necessary but not sufficient precondition for mitochondrial restoration and maintenance. Based on the recovery kinetics of the various phosphorylated components, the mitochondrial-bound fraction appears to be most directly linked with restoration. Results are discussed with reference to specific characteristics and components of self-restoration and to possible underlying mechanisms. We suggest that a degree of self-restoration is consistent with the quasi-autonomous nature of mitochondria and that this intrinsic capacity may be pivotal to the respiratory climacteric in senescent fruit cells and to cellular homeostasis in general.

of mitochondria in the climacteric respiratory rise that accompanies the senescence of many fruit and other plant organs. Decades later it is still generally held that the respiratory climacteric has mitochondrial origins but causal relationships remain unresolved. Early postulates were premised on the notion that mitochondrial respiration increased during the climacteric in compensation for declining mitochondrial efficiency, with the latter attributed to either phosphorylative uncoupling (20) or the interposition of the energy-inefficient alternative electron transport pathway (37). These postulates gave way to subsequent evidence that mitochondria remain energy-efficient throughout the respiratory climacteric (16), and that the alternative pathway, though present, remains unengaged (39).

Based on the growing perception that mitochondria are quasi-autonomous and on evidence (34, 35) of mitochondrial repair *in situ*, it has been proposed (29) that the respiratory climacteric may represent the mitochondria's own maintenance metabolism. The postulate calls for mitochondria that have a capacity for self-maintenance and self-restoration. In this regard, a degree of self-maintenance was implicit in a series of studies culminating with evidence that isolated fruit mitochondria can maintain energy-linked functions for as long as 4 d at 25°C (33). Moreover, it should be noted that maintenance at physiologic temperatures is distinctly different and in some ways the obverse of the more commonly reported 'aging' of mitochondria at 0°C. The latter, which relies largely on the removal of harmful substances and the use of protective agents, has passive connotations. Maintenance, on the other hand, is dynamic and energy-dependent (32). Nonetheless, confirmation of mitochondrial self-restoration calls for direct evidence along the lines first explored in a preliminary study by Romani and Howard (30). The purpose of the work reported in the present paper is to confirm the existence of mitochondrial self-restoration by describing its characteristics and principle components in greater detail.

MATERIALS AND METHODS

Isolation of Mitochondria

Avocado fruit (*Persea americana* var. 'Fuerte' or 'Hass') were used because they are available year-round and are a reliable source of long-lived mitochondria (24). The fruit were ripened at 20°C and then chilled at 0°C before use. Mitochon-

The pioneering study of Millerd, Bonner, and Biale published in 1953 (20) gave impetus to much research on the role

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dria were isolated as previously described (32) with the exception that PVP was increased from 0.2% to as high as 0.8% for late season fruit high in lipids. Where indicated, further purification of the organelles was achieved via the self-forming Percoll gradient centrifugation procedure of Moreau and Romani (22). Mitochondrial protein was initially determined by a modified Lowry procedure (19) and in later studies with the Bradford method (4). The former proved more sensitive by about a factor of 2 for mitochondrial protein relative to a serum albumin standard thus accounting for some variance in Q_{O_2} (protein) among experiments. Because inter-experiment comparisons of respiratory rates were not pertinent to the objectives of this study, we have chosen to indicate the protein assay employed in each experiment rather than attempt retroactive normalization.

Monitoring the Loss and Restoration of RC³ and ADP/O

Mitochondria were suspended at a concentration of 0.5 to 1.5 mg mitochondrial protein per mL in medium (0.25 M sucrose, 50 mM phosphate [pH 7.2], 1 mM MgCl₂, 10 μM CoA, 100 μM TPP, 100 μM NAD, and 100 μg/mL chloramphenicol) lacking substrate. This combination of osmoticum, buffer, and cofactors—but with added substrate and BSA—had been found optimal for the maintenance of RC under energized conditions (31, 32). Chloramphenicol, added to suppress bacterial contamination, had been shown to be effective for at least 24 h (33).

The mitochondrial suspension was placed in beakers approximately five times the volume of the fluid contained and incubated at 25°C in a reciprocating waterbath shaker set at about 50 oscillations/min. After a several hour period of energy-deprivation and accompanying loss of energy-linked functions, restorative activity was initiated by adding 10 mM substrate and 50 μM ADP.

At frequent (1–2 h), designated intervals during substrate-deprived stress and subsequent substrate-dependent restoration, an aliquot of the incubating mitochondrial suspension was transferred to a 1 mL chamber equipped with a polarographic O₂ electrode (Yellow Springs Instrument Co.) and substrate (10 mM) added, except during the restoration phase when substrate was already present. In the latter instance, it was often necessary to increase O₂ tension in the assay aliquot by gently bubbling in air for 5 to 10 s just prior to closing the electrode chamber. State 3 and state 4 rates of oxidation were assessed following the second or third addition of stoichiometric amounts of ADP. RC and ADP/O were calculated according to conventional methods (6, 9).

The combined periods of stress and restoration often exceeded 10 to 12 h. For experimental convenience mitochondria were, on occasion, isolated late in the day, incubated at 25°C in medium lacking substrate until partial loss of energy-linked functions, and then stored on ice. Overnight storage resulted in some additional loss of RC but did not significantly impair subsequent restoration. The amounts of mitochondria and time required for multiple assays over the many hours of

a complete experiment generally precluded assay replicates. Confidence in the results derives from the regular pattern of progressive change in a given function during stress and restoration and in replicates of the entire experiment—at least three replicates for all except the chromatographic separation (see “Results”).

P/O Ratios

Mitochondria were incubated in medium altered to contain 5 to 10 mM (as indicated) phosphate and 45 mM Hepes buffer (pH 7.2). At designated intervals a 1.2 mL aliquot of the mitochondria was transferred to the O₂ electrode chamber and supplied with 20 mM glucose, 0.2 mg/mL hexokinase, 0.25 mM ADP, and, if being tested during substrate-deprived stress, 10 mM substrate. Duplicate 0.1 mL aliquots were taken at the beginning and end of the polarographic assay for determination of inorganic phosphate by the method of Ames (1). P/O ratios were based on the consumption of oxygen and the corresponding uptake of inorganic phosphate.

ATP Synthase

ATP synthase activity was measured by esterification of ³²Pi as described by Grubmeyer *et al.* (11). The assay medium contained 0.25 M sucrose, 4 mM MgCl₂, 20 mM glucose, 4 mM K₂HPO₄, 50 mM Hepes, 10 mM α-KG, 0.5 mg/mL hexokinase, 1 mM ADP, and 2 μCi/mL ³²Pi (New England Nuclear), brought to pH 7.2 at 25°C with Tris base. The reaction was started by the addition of a 0.2 mL aliquot of incubating mitochondria to 0.2 mL assay medium and allowed to proceed for 20 min at 25°C. To stop the reaction 0.4 mL of chilled quench solution (0.3 M HCl, 0.12 M glycine, and 1.8 M NaClO₄) was added and the tubes placed on ice for 5 min. After centrifugation for 2 min at 10,000g, 0.2 mL of the supernatant was added to molybdate reagent and extracted with isobutanol:benzene (1:1) to remove the unesterified ³²Pi. A portion (0.1 mL) of the aqueous phase was combined with 10 mL Aquasol for scintillation counting.

Extramitochondrial Inorganic Phosphate

At designated intervals during stress or restoration, a 0.1 mL aliquot of the incubating mitochondria was pipetted into 1.5 mL Eppendorf centrifuge tubes containing 0.9 mL of 5.5% TCA. After centrifugation for 2 min at 10,000g, aliquots of the supernatant were assayed for inorganic phosphate by the method of Ames (1). Similar results were obtained when the phosphate transport blocking reagents, *N*-ethylmaleimide or mersalyl were substituted for TCA (18), confirming that the latter did not burst the mitochondria and thereby obscure the distinction between extra- and intramitochondrial Pi.

Extramitochondrial Esterified Phosphate(s)

Esterified (organically bound) phosphates in the extramitochondrial incubation medium were measured by the method of Avron (2) employing the ‘inhibitor stop’ technique of Palmieri *et al.* (25). One microcurie per milliliter of [³²P] orthophosphate (carrier free, New England Nuclear) was added to the mitochondrial reaction mixture just before the

³ Abbreviations: RC, respiratory control; α-KG, α-ketoglutarate; RCR, respiratory control ratio; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

initiation of stress at 25°C. At indicated intervals aliquots of the stressing mitochondria were pipetted into 1.5 mL centrifuge tubes containing 1 mM mersalyl and pelleted. An appropriate aliquot of the resultant supernatant was transferred to a test tube, 0.2 mL acetone added, mixed thoroughly and allowed to stand for 10 min. before adding 0.8 mL isobutanol:benzene (1:1) and mixing well. After phase separation, 0.2 mL of molybdate/H₂SO₄ reagent was allowed to trickle down the wall of the tube and react with the unesterified phosphorous. The aqueous phase was mixed gently and then set aside for 5 min allowing the molybdate complex to form. After a second mixing and phase separation the upper layer containing the phosphomolybdate complex was aspirated off and 20 μ L of 20 mM K₂HPO₄ followed by 0.8 mL of the isobutanol:benzene were added to the remaining aqueous phase and mixed again for 30 s. After phase separation a 100 μ L aliquot of the aqueous phase was placed in 10 mL water and radioactivity measured in a scintillation counter.

The several radiolabeled phosphate metabolites were separated via the chromatographic method of Heldt and Klingenberg (14). At the point of maximum recovery of RC, 0.5 mL of the reaction mixture was centrifuged in the presence of 1 mM mersalyl to prevent phosphorous efflux from the mitochondria. Then 0.2 mL of the supernatant plus reference standards (50 nmol each of ADP, NAD, and UTP plus 40 nmol ATP) were loaded onto a Dowex 1-X8 (<400 mesh) column (diameter 1.5 mm, length 200 cm) and eluted at 40°C with a continuous gradient, H₂O \rightarrow 9 M ammonium formate at 1.2 mL/h. UV absorbance was recorded continuously at 265 nm. Fractions (0.5 mL) were collected in scintillation vials to which 10 mL Aquasol were added for measurement of radioactivity.

Phosphate Incorporation into TCA-Precipitable Mitochondrial Constituents

³²P incorporated and retained in mitochondria was determined by a procedure based on the method of Kuhn and Wilden (15). At designated times, 0.5 mL aliquots of the incubating mitochondria were pipetted into 1.5 mL Eppendorf centrifuge tubes containing 0.5 mL ice-cold 10% TCA and 10 mM Pi. After several minutes at 0°C, the tubes were centrifuged for 2 min at 10,000g. The pellets were washed twice with 1.5 mL of 10% TCA plus 10 mM Pi with the tubes oriented in the rotor such that the pellets were driven through the TCA solution onto the opposite tube wall. Final pellets were dissolved by shaking for 2 h in 1.5 mL of a toluene-based scintillation cocktail containing 10% tissue solubilizer (NCS, Amersham Searle). The capped Eppendorf tubes were placed in scintillation vials for measurement of radioactivity.

Phosphate Incorporation into ATP

The method of Hanselman *et al.* (12) was employed to separate ATP from other phosphate metabolites. ³²Pi (1 μ Ci/mL) was added to the incubating mitochondria and at designated intervals 0.4 mL aliquots of the mitochondria were centrifuged in the presence of 1 mM mersalyl. A 0.2 mL aliquot of the resultant supernatant was added to 0.8 mL of 0.1 M Tris buffer (pH 8) and the mixture loaded on a DEAE-

Sephadex A-25 minicolumn packed in a Pasteur pipet. Specific phosphates were eluted with the following step gradient: (a) Pi, AMP, and glucose-6-phosphate with 10 mL 0.08 M NaCl in 0.1 M Tris-HCl (pH 8); (b) ADP with 9 mL 0.12 M NaCl in 0.1 M Tris-Cl (pH 8); (c) ATP with 5 mL 0.4 M NaCl in 0.1 M Tris-Cl (pH 8). The fractions containing ATP were collected directly into scintillation vials for measurements of radioactivity.

Phosphate Incorporation into Mitochondrial Lipids

Incorporation of ³²Pi into mitochondrial lipids was determined by adapting the procedure of Folch *et al.* (10). At designated intervals during stress or restoration 0.5 mL of the incubating mitochondria was centrifuged and the pellet homogenized in a 2:1 chloroform:methanol mixture. The organic extract was then washed first with 0.5 mL of 10 mM NaCl then again with a 1:1 methanol: 10 mM NaCl mixture. After phase separation an aliquot of the lower phase containing the lipids was placed in 10 mL Aquasol (New England Nuclear) for scintillation counting.

RESULTS

General Pattern of Stress and Restoration

In a typical experiment (Fig. 1A), avocado mitochondrial incubated at 25°C in reaction medium lacking substrate and ADP lost all RC and hence measurable ADP/O by the 5th h of substrate-deprived stress. Some phosphorylative capacity was retained (P/O of about 0.8 with α -KG as test substrate). At h 7, α -KG (10 mM) and sparker amounts of ADP (50 μ M) were added (arrow) to the incubating organelles. Four hours later energy-linked functions were restored, from 75% (RC) to 100% (ADP/O and P/O) of their original value.

Although it is a convenient and frequently used index of mitochondrial function, RC is not indicative of important underlying respiratory changes. As seen in Figure 1B, changes in both state 3 and state 4 respiration rates contributed to the loss and subsequent restoration of RC. The data points in Figure 1, A and B, were derived from recorder traces of O₂ consumption such as those obtained with aliquots of mitochondria taken at the four different incubation times (0, 7, 12, and 19 h) shown in Figure 1C.

Persistence and Variability of the Stress and Restoration Response

Data (Table I) from 22 comparable experiments performed over 1 year are illustrative of both the inter-experiment persistence of the restoration phenomenon and the variability within it. Mitochondria with low RCRs, *e.g.* <2.5 with α -KG, were found to be capable of limited, if any, restoration. Accordingly, mitochondrial preparations with low RC were discarded at the onset. As a consequence the mitochondria employed in these experiments were reasonably well coupled (Table I, function I). The RCRs of freshly isolated mitochondria were about 1 unit higher with α -KG as compared with succinate as substrate.

The duration of substrate-deprived stress at 25°C to the point of total loss of RC (function 2) varied widely (2–7 h)

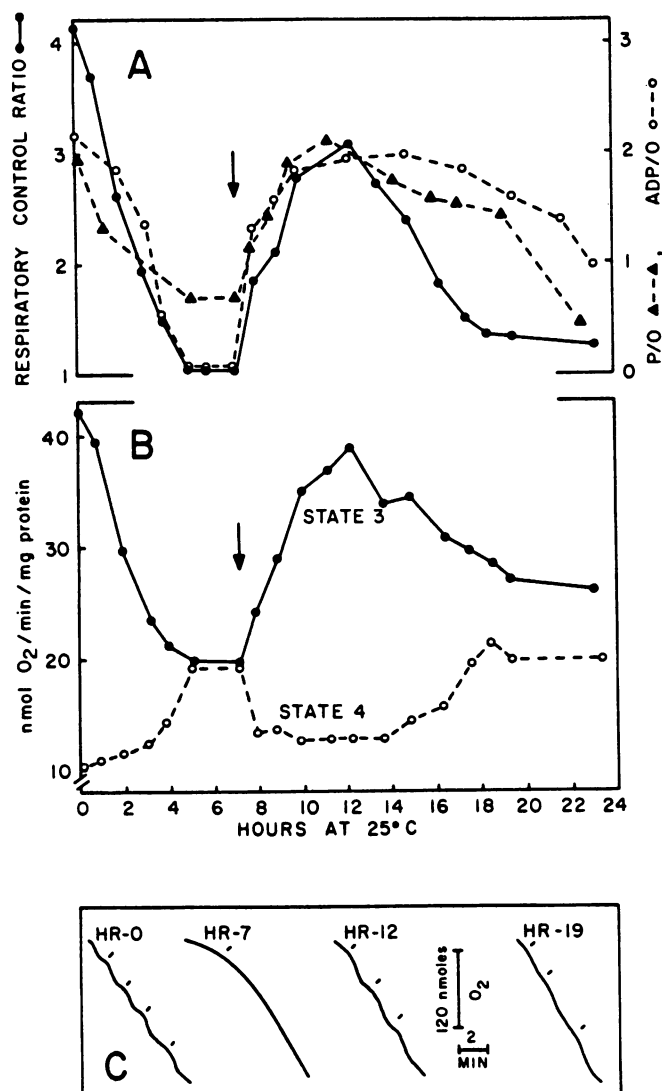


Figure 1. (A) Loss of respiratory control (●—●), P/O (▲---▲), and ADP/O (○---○) by mitochondria during their incubation in substrate-deprived medium for 7 h at 25°C and restoration of the same functions after the addition (↓) of α -KG (10 mM) and ADP (50 μ M). (B) Underlying changes in state 3 and state 4 respiratory rates. (C) Recorder traces of O₂ consumption by mitochondria sampled at time 0, and after 7, 12, and 19 h of incubation. Slashes denote the addition of 50 or 100 μ mol of ADP. Proteins were assayed with the Lowry method (19).

from experiment to experiment with no apparent correlation with the initial RCR. This seemed inconsistent with the fact that restoration was positively correlated to initial level of RC. However, the apparent discrepancy is readily explained by the realization that during substrate-deprived stress the mitochondria's energy generating capacity is inoperative and can not therefore contribute to maintenance.

The time required to achieve maximum restoration of RC after addition of substrate (Table I, function 4) ranged rather widely over a period of about 3.5 to 8 h. This function was affected, in part, by initial RC but more so, as elaborated below, by the length (depth) of the stress. On the average, RC

was restored to about 70% of its original value with α -KG as the energy source and near 100% with succinate (Table I, function 5).

Restoration of State 3-State 4 Rates as Affected by Substrate

As seen in Figure 2 the patterns of RC loss and restoration were deceptively similar following a single addition (solid lines) or periodic replenishment of either α -KG or succinate as substrate. However, the underlying changes in state 3 and state 4 respiratory rates were distinctly different, especially when periodic additions of substrate (dashed lines) were made to compensate for that consumed. Periodic replenishment of α -KG had only a slight effect on the restorative changes in state 3 and state 4 suggesting that the initial 10 mM addition to initiate restoration was adequate to sustain metabolic activity for the 4 to 5 h restorative period. With succinate, however, the original 10 mM addition was clearly not adequate to sustain maximal respiratory rates beyond the first hour. This difference is consonant with the fact that respiration rates with succinate are nearly twofold those with α -KG while the latter, in effect, supplies two TCA intermediates. Quite unexpected, however, was the constancy in pattern of restoration and final decline of RC despite the marked effect of succinate replenishment on state 3 and state 4 rates. This, as can be seen (Fig. 2), resulted from proportionate increases in electron flux or "tracking" of both state 3-state 4 rates that, as discussed below, has implications for the nature of restoration and final loss of RC.

Requirement for Energy: Metabolizable Substrate versus ATP

With the addition of sufficient ATP to drive restoration, accompanying (contaminant) levels of ADP required the use

Table I. Summary of Data Obtained from a Series of Experiments in Which Isolated Mitochondria Were Stressed by Incubation at 25°C in the Absence of Substrate and then Permitted to Undergo Restoration by the Addition of Substrate (10 mM) and ADP (50 μ M)

Substrate (No. of Expts.)	Function ^a	Value Average (sd)
α -KG (n = 17)	(1) ^a Initial RCR	4.1 (\pm 0.8)
	(2) ^b Time to RCR = 1	4.7 (\pm 1.4) h
	(3) ^c Respiratory rate at RCR = 1	12.6 (\pm 7.9)
	(4) ^d Time to maximum restored RC	5.8 (\pm 2.3) h
	(5) ^e Restored RCR/RCR _i \times 100	69% (\pm 12%)
Succinate (n = 5)	(1) Initial RCR	3.0 (\pm 0.7)
	(2) Time to RCR = 1	4.9 (\pm 2.8) h
	(3) Respiratory rate at RCR = 1	17.9 (\pm 7.3)
	(4) Time to maximum restored RC	4.2 (\pm 1.2) h
	(5) Restored RCR/RCR _i \times 100	95% (\pm 25%)

^a (1) RCR of freshly isolated mitochondria. ^b (2) Stress hours at 25°C to total loss of RC, i.e. RCR = 1. ^c (3) Respiration rate [nmol O₂/min·mg protein] when RCR = 1 [protein assayed by the Lowry method (19)]. ^d (4) Restoration hours to maximum observed recovery of RC. ^e (5) Maximum restored RCR as a percent of initial RCR.

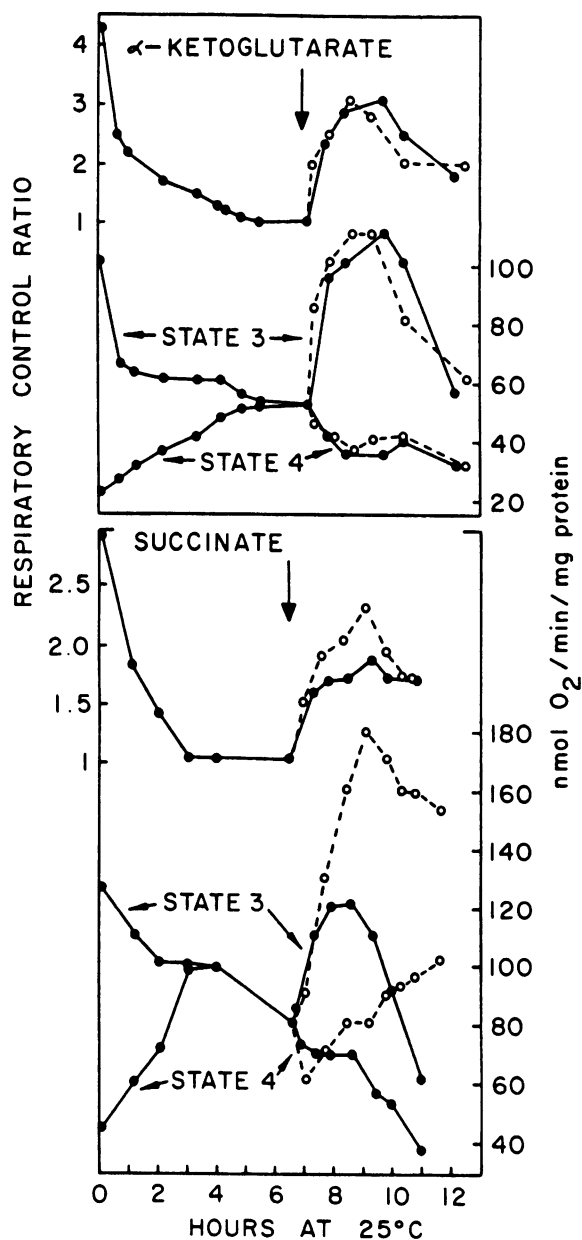


Figure 2. Loss and restoration of respiratory control and underlying changes in state 3 and state 4 respiratory rates with either α -KG (upper panel) or succinate (lower panel) as the test substrate during the 6.5 h stress and as the substrate added (\downarrow) to drive restoration. With no further addition of substrate, (●—●) or with more substrate added every hr (○ - - ○) to replenish the amount oxidized based on state 4 rates of O_2 consumption. Proteins assayed with the Bradford method (4).

Table II. Comparing the Restoration of State 3 and State 4 Respiration Rates and RC with Either ATP (5 mM) or α -KG (10 mM) + ADP (50 μ M) as Restorants

For the freshly isolated mitochondria (tested with α -KG as substrate) state 3 = 88, state 4 = 22 nmol O_2 /min·mg protein (assayed by the Bradford method [4]) and RCR = 4. After 5 h of substrate-deprived stress and at the beginning (0 h) of the restoration phase state 3 = state 4 = 45 nmol O_2 /min·mg protein and RCR = 1. Maximum restoration of RC occurred 4 h later.

Restorant	At 1 h of Restoration			At 4 h of Restoration		
	State 3	State 4	RCR	State 3	State 4	RCR
α -KG	71	25	2.8	103	25	4.1
ATP ^a	72	52 ^b	1.4	142	39 ^b	3.6

^a α -KG used as test substrate to assay respiratory functions of mitochondria undergoing substrate-deprived stress or ATP-supported restoration. ^b State 4 in the presence of abundant ATP was obtained by the addition of oligomycin (20 μ g/mL).

of oligomycin to discern state 4 rates. Accepting that as a valid stratagem, ATP appeared to be nearly as effective a restorant of RC as α -KG. However, the underlying changes in state 3 and state 4 were distinctly different (Table II). ATP resulted, likely via activation of the dehydrogenase, in a state 3 rate that at the point of maximum restoration (4 h) was appreciably higher than that exhibited by the freshly isolated mitochondria. On the other hand, ATP was not nearly as effective as α -KG in reestablishing the restraints on state 4 that are indicative of tightly coupled mitochondria.

ATP Synthase

The decline in state 3 rates with increasing stress and the obvious need for energy to drive restoration implicate ATP synthase activity as a possible rate-limiting factor. In a typical experiment (Fig. 3), mitochondrial ATP synthase activity declined by only about 30% during stress and recovered rapidly and completely upon the addition of substrate. Meanwhile, state 3 rates (legend, Fig. 3) declined by 60% during stress and were restored to only 64% of the original. It seems unlikely, therefore, that ATP synthase activity was the causal factor in the decline of state 3 rates nor did it hinder the slower and less complete restoration of state 3 rate and RC. The relationship, if any, in the coincidence between the final decline in RC and ATP synthase activity was not explored.

Orthophosphate Flux during the Loss and Restoration of Energy-Linked Functions and Effects of Prolonged Stress

Phosphate flux—release of Pi from mitochondria into the medium during stress, rapid uptake during restoration, near equilibrium at the transition point in RC, and slow re-release of Pi during subsequent gradual uncoupling—is shown in Figure 4A. The concomitant loss and restoration of RC and P/O are shown in Figure 4B. Both the restoration of phosphorylation and its final decline appeared to precede analogous transitions in RC. Low but measurable levels of phosphorylative capacity persisted during the final 10 h even though intramitochondrial Pi was being released.

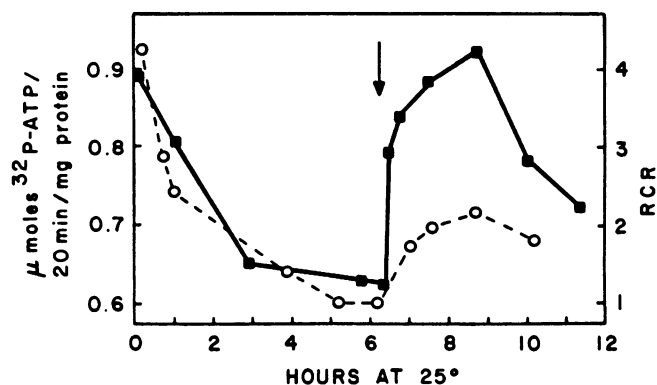


Figure 3. Changing levels of ATP synthase activity in avocado mitochondria undergoing stress and restoration at 25°C. Restorative phase was begun with the addition (↓) of 10 mM α -KG + 50 μ M ADP. ATP synthase (■—■); PCR (○ - - - ○). State 3 rates at time 0, at the end of the stress period (6.5 h), and at the point of maximum restoration (9 h) were 74, 27, and 47 nmol O_2 /min·mg protein, respectively. Protein was assayed with a Lowry procedure (19).

To amplify the temporal relationship between the flux of Pi and changes in RC, mitochondria were allowed to stress for increasing periods of time before restoration was initiated (Fig. 5, A and B). The addition of substrate after 2 or 4 h of stress resulted in Pi uptake (phosphorylation) and incipient return of RC that were nearly concomitant. However, when addition of substrate was delayed for yet another 4 h a distinct lag was evident between the immediate uptake of Pi and the first signs of RC over 1 h later.

That phosphorus metabolism is integral to restoration was especially evident when mitochondria were stressed at 25°C in medium containing adequate substrate but no Pi (Fig. 6). Pi-deprived organelles quickly lost demonstrable RC down to a minimal (1.3–1.5) level. Upon the addition of Pi to the incubating organelles their RC was gradually restored. Prolongation of stress for 7 or 10 h again resulted in an increasing time-lag between the introduction of Pi and the first signs of RC. Prolonged stress also diminished the extent of restoration.

Phosphate Incorporation

The fate of orthophosphate was assessed by adding $^{32}P_i$ at the onset of substrate-deprived incubation and noting the appearance of ^{32}P in four organic forms: (a) ATP, (b) phospholipid, (c) a TCA-precipitable mitochondrial fraction and, (d) a fraction released into the surrounding medium. Detectable levels of incorporation appeared only after the onset of restoration, *i.e.*, the addition of substrate. Quantitative distribution of ^{32}P among these various organic forms is shown in Table III. During the first hour of restoration roughly 95% of the radiolabeled organophosphate appeared in the medium. Of that about 9% was ATP. Only a small fraction remained with(in) the mitochondria; roughly 4% bound to a TCA-precipitable fraction and 0.2% as phospholipid. The amount of phosphate incorporated into the various fractions during the 4 h of restoration (about 300 nmol/mg mitoprotein) was roughly equivalent to that taken up by mitochondria as Pi during the restoration period.

The large amount of organic phosphate that appeared in the medium was analyzed via Dowex anion-exchange chromatography (Fig. 7). Except for the small amount of ATP and two unidentified minute fractions (No. 45 and 50), the bulk of organically bound ^{32}P in the medium appeared as a single peak (X) that was tentatively identified as a hexosephosphate based on cochromatography with glucose-6-phosphate, explorative TLC, and positive reaction to aniline phthalate (data not shown). Incorporation of ^{32}P into the putative hexosephosphate was clearly energy and transport dependent as evidenced by more than 94% inhibition by CCCP (0.8), oligomycin (5.2), or mersalyl (412), the number in parentheses referring to the concentrations of the inhibitors in nanomoles/mg mitochondrial protein.

Phosphate Incorporation: Kinetic Aspects

Incorporation of ^{32}P into the putative hexosephosphate (Fig. 8) was discernible immediately upon addition of substrate and ADP to stressed mitochondria and continued at a nearly linear rate (about 120 nmol/mg mitochondrial protein/h). Incorporation of ^{32}P into ATP (data not shown) occurred at less than 1/10 the rate (about 10 nmol [^{32}P]ATP formed/mg mitochondrial protein/h) but followed an analogous pattern—linear rates continuing past the maximum restoration of RC.

The kinetic pattern of the small but readily distinguishable

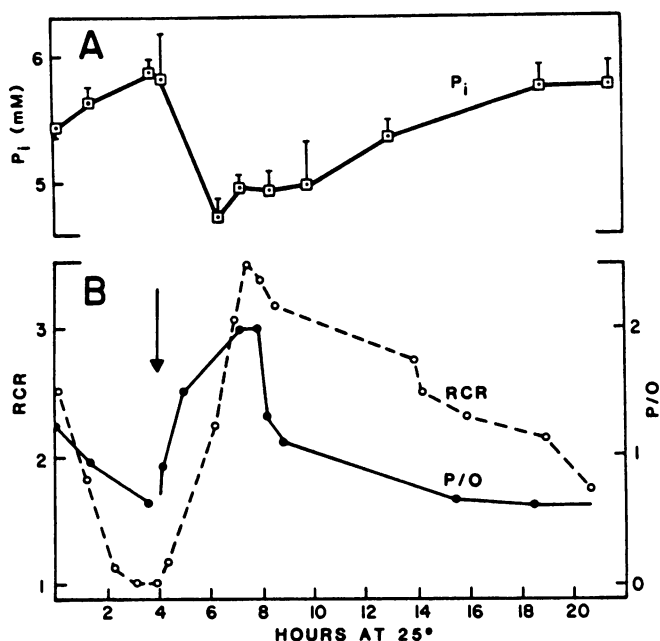


Figure 4. Changes in Pi levels of the incubation medium (A) and in mitochondrial RC and P/O (B) during substrate-deprived stress and subsequent substrate-driven restoration of energy-linked functions by avocado mitochondria held at 25°C. Pi (□—□), RCR (○ - - - ○), P/O (●—●). Mitochondria were isolated as described except that 50 mM phosphate in the incubation medium was replaced with 5 mM phosphate and 45 mM Hepes. Succinate (10 mM) and ADP (50 μ M) were added (↓) at hr 4. Each Pi datum represents the mean and so of four replicates.

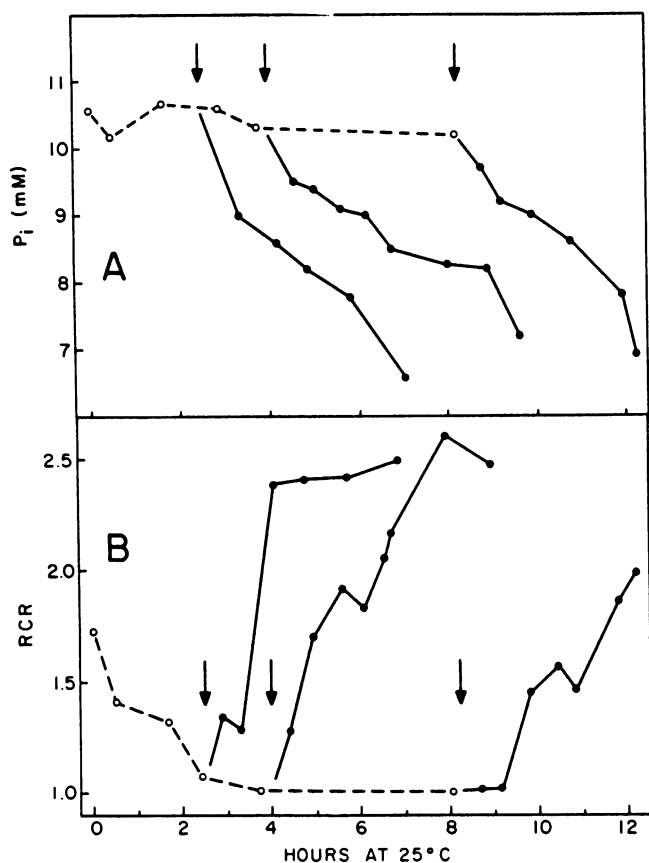


Figure 5. Uptake of Pi from the surrounding medium (A) and accompanying levels of RC (B) during stress and after the addition (\downarrow) of α -KG (10 mM) and ADP (50 μ M) to initiate mitochondrial restoration after progressively longer periods of substrate-deprived stress (\circ - - \circ). Mitochondria were isolated and treated as described except that the incubation medium contained 8 mM phosphate plus 40 mM Hepes, pH 7.2. The initial Pi levels (10–11 mM) is likely the result of Pi released by the organelles that had been exposed to 50 mM phosphate buffer during isolation. Relatively low mitochondrial RC at the beginning of 25°C stress was the result of overnight storage of the organelles in washing medium at 0°C (see "Materials and Methods").

amount of 32 P that was irreversibly bound to the mitochondria (Fig. 8) differed markedly from that for ATP or the hexosephosphate. Rapid at the onset of restoration, the incorporation rate was greatly attenuated as restoration of RC approached its maximum with little or no incorporation thereafter. These divergent kinetic patterns of incorporation into the four organophosphates were observed in three separate experiments.

DISCUSSION

The foregoing data are presented as evidence that isolated avocado mitochondria have an intrinsic capacity to restore energy-linked functions dissipated during a prolonged period of energy-deprived stress at 25°C. Self-restoration has far-reaching implications for mitochondrial functional autonomy and its relationship to cellular well-being. Accordingly, it

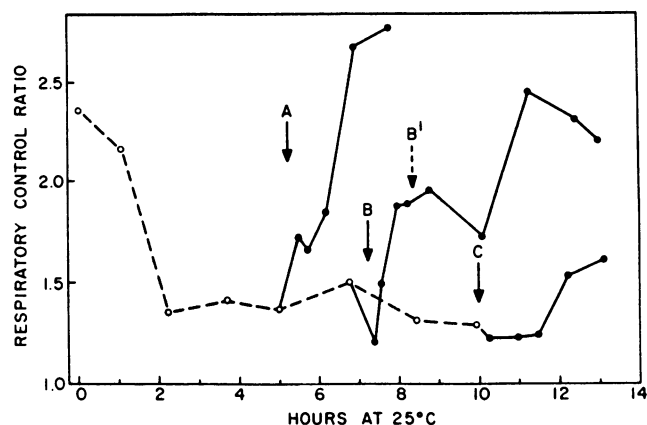


Figure 6. Loss (\circ - - \circ) and restoration (\bullet — \bullet) of respiratory control by mitochondria incubating at 25°C in medium complete with substrate (10 mM α -KG) but lacking Pi. Phosphate (8 mM) added after 5 (A), 7 (B) or 10 h (C) of phosphate-deprived stress. An additional 3 mM Pi added at B'.

seems advisable to examine and question the underlying premises and the limits and implications of the data.

Stress as a Precondition for Restoration

Restorative capacity is both exacerbated and circumscribed by prior stress. In this study, the stress form was energy deprivation in the presence of potentially harmful extramitochondrial (cytoplasmic, vacuolar) inclusions and in the absence of exogenous protective agents. Additional cleansing of the mitochondria via Percoll gradient centrifugation or protection via additions of BSA to the incubation medium resulted in a two- to threefold prolongation of the time to total loss of RC (data not shown). While these observations suggest obvious experimental strategies in a search for stress factors, we chose not to include a Percoll wash or BSA

Table III. Amounts of 32 P Incorporated by Mitochondria into Various Organic Forms during Restoration of Energy-Linked Functions at 25°C

Freshly isolated avocado mitochondria with an RCR of 3.5 were stressed by incubation at 25°C (4 mg mitochondrial protein/mL) in reaction medium lacking substrate but to which 5 mM Pi and 1 μ Ci/mL of 32 P (2.6×10^5 cpm/ μ mol) were added. RC was lost (RCR = 1) after 4 h; 3 h later (7 h stress, 0 h restoration) α -KG (10 mM) and ADP (50 μ M) were added to initiate restoration. Maximum restoration (RCR = 2.5) was attained 4 h thereafter. Each datum is the average of duplicate assays.

Hours of Restoration (RCR Obtained)	Hexosephosphate ^a	ATP	Bound ^b	Lipid
	<i>nmoles Pi incorporated/mg mitochondrial protein</i>			
0 h (RCR = 1)	4.2	0.4	0.75	0.02
1 h (RCR = 1.6)	98.1	10.2	4.75	0.18
4 h (RCR = 2.5)	270.3	25.1	5.50	0.51

^a Tentative identification (see text). ^b TCA-precipitable mitochondrial fraction (see "Materials and Methods").

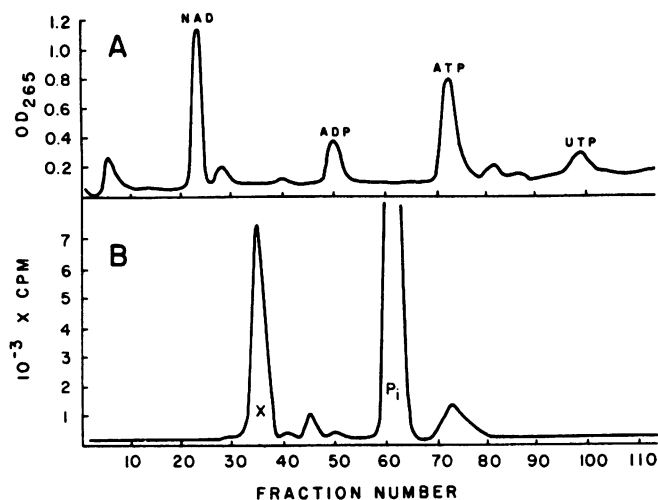


Figure 7. Dowex anion-exchange chromatography of organophosphates present in the surrounding medium after restoration of energy-linked functions by mitochondria. $A_{265\text{nm}}$ elution profile (A) with added markers NAD, ADP, ATP, and UTP. Distribution of radioactivity (B) with ^{32}P i and unknown (X) as the dominant fractions.

protection in this study so as to focus on the restorative activity under conditions possibly more closely akin to those *in situ*. Given the deterioration of plasmalemma and tonoplast of the ripening avocado cells (26), mitochondria therein are likely subjected to stressful substances and without the benefit of added BSA.

Energy Requirement

Energy is clearly necessary for restoration. ATP will support restoration, metabolically derived energy appears to be more effective. The latter observation is consistent with earlier studies on the requisites for long-term maintenance of energy-linked functions under energized conditions (32), and in the restoration of state 3 rates reported by Raison *et al.* (27). It is also in accord with Luzikov's (17) contention that various mitochondrial systems are kept functional more by underlying metabolic activity than by available energy *per se*. Although not studied in detail, NADH and malate also supported restoration.

Components of Restoration

RC is simply defined (6) as the respiration rate in the presence of ADP (state 3) divided by the respiration rate in its absence (state 4). Hence, RC can be affected by changes in either or both respiratory functions. It is clear (Figs. 1B and 2, Table II) that the loss and recovery of RC during energy-deprived stress and substrate- or ATP-driven restoration result from changes in both state 3 and state 4. These observations provide clues to underlying events. State 3 rates may be constrained by several factors such as substrate permeation, dehydrogenase inactivation, capacity of the electron transport chain, adenine translocase activity, or ATP synthase. Given the long, 3 to 8 h restorative periods (Table I, function 4), it seems very unlikely that substrate permeation should be a

limiting factor. If the decrease in state 3 were due to the inability of the phosphorylative system (ADP translocase, ATP synthase) to relieve proton back pressure, addition of a protonophore should result in a much faster state 3 respiratory rate. Addition of CCCP to mitochondria in state 3, in successive polarographic assays conducted during the periods of stress and restoration, did not result in a stimulated state 3 rate (data not shown). Moreover, the diminution of ATP synthase activity during loss of RC was found to be only about 30% with a very rapid recovery upon initiation of the restoration phase, thus it seems unlikely that ATP synthase activity was limiting the appreciably slower recovery in state 3 rates. Restraint due to leakage of NAD, as reported by Neuburger and Douce (23), was also unlikely since the medium was intentionally supplied with 100 μM NAD (see "Materials and Methods"). Dysfunctions at the dehydrogenase level or along the electron-transport chain seem more likely and merit further investigation.

State 4 respiration increased during stress and declined during restoration. According to chemiosmotic theory (21), the increase in state 4 during energy-deprived stress could be ascribed to either increasingly poor coupling (leaky F_0F_1 ATPase) or increased proton permeability of the mitochondrial membrane. The latter was unlikely in view of the rapid (few min) restoration of membrane potential observed upon the addition of substrate to uncoupled mitochondria (30). On the other hand, "tracking" of the rate changes in state 4 and state 3 (Fig. 2, succinate \pm replenishment) suggested a state 4 rate that was sensitive to proton back pressure and therefore indicative of a membrane "leak." The fact that α -KG, succinate, and ATP resulted in different state 3, state 4 restoration kinetics suggest complexity and involvement of various components of electron transport and ATP synthesis. Obviously, more information is needed before one can point to causal events.

Phosphorylation and P_i Flux

The observations regarding P_i flux and mitochondrial self-restoration may be summarized as follows:

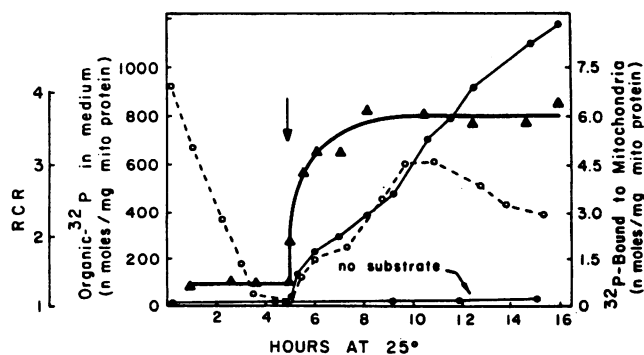


Figure 8. Incorporation of ^{32}P into organic fractions during restoration of mitochondrial RC. Organic phosphate appearing in the medium (\bullet — \bullet) ^{32}P bound to the mitochondria (\blacktriangle — \blacktriangle), RCR (\circ — \circ), α -KG (10 mM) and ADP (50 μM) were added (\downarrow) after 5 h of substrate-deprived stress at 25°C.

1. The loss, restoration, and final decline in RC is accompanied, respectively, by the efflux, uptake, and release of Pi by mitochondria.

2. The bulk of the Pi taken up becomes esterified via energy- and transport-dependent mechanisms as evidenced by the measured P/O, the distribution of ^{32}P , and the blocking of uptake by inhibitors of Pi transport and energy transduction.

3. Restoration is ordered with the recovery in phosphorylation preceding the incipient restoration of RC—this being most evident following prolonged energy-deprived stress.

4. Phosphorylation is necessary but not sufficient for restoration as is most evident during the final decline in RC when esterification of Pi continues but concomitant release of Pi into the medium suggests the dominance of degradative processes.

As elaborated by Tager *et al.* (38) in 1966 and in numerous studies since, phosphorylation is an integral part of chemiosmotic function. Our purpose was not to examine chemiosmotic mechanisms but rather aspects of phosphorylation as they may relate to mitochondrial restoration. For instance, a question arises with regard to the magnitude of phosphorylation by mitochondria supplied with only a minute amount of ADP. Based on the average observed state 4 rate (135 natoms/min for the mitochondria whose other functions are shown in Fig. 4) the amount of Pi taken up during the first hour of restoration would equate to a P/O of roughly 0.2. Even at such low levels of esterification the ADP added at the onset of restoration would have supported only a few minutes of state 3 respiration. Accordingly, Pi uptake, phosphorylation, and the restorative process(es) in general must have occurred during 'apparent' state 4 respiration.

Since it can be inferred from the foregoing that ATP synthesis is a requisite to restoration, it is puzzling why ATP turnover and hexosephosphate accumulation in the extramitochondrial medium (Fig. 8) proceed without reference to the pattern of restoration. Hexokinase and glucose were not added but the presence of contaminant or bound (28) hexokinase and of glucose (via invertase) can not be ruled out. It is not clear, therefore, whether the accumulation of hexosephosphate in the medium is the result of intra- or extramitochondrial phosphorylation. Regardless, the appearance of esterified phosphate in the medium represents wasteful metabolism with respect to the energy needs for restoration. It also suggests that the 'apparent' state 4 respiration during the restorative phase had a component of state 3 supported by ADP generated in the formation of hexosephosphate and in as yet undefined energy-demanding restorative processes.

With respect to *in vitro* restoration and the data at hand the least problematic portion of the phosphorous flux is the esterified phosphate that remains bound to the mitochondria. This synthesis proceeded immediately and rapidly upon the addition of substrate and ceased as the mitochondria achieved their maximum restored RC (Fig. 8). The mitochondrial bound ^{32}P fraction, thus far identified only by its TCA precipitability, could well represent phosphoproteins such as those recently found to be associated with the matrix or inner membrane surface of potato mitochondria (36). This probability is enhanced by the similarity in phosphorylation kinetics

for mitochondrial phosphoproteins (7, 36) and those observed during mitochondrial self-restoration.

The amount of phosphorus incorporated in phospholipids during restoration was small, so much so as to preclude meaningful kinetics with the analytical techniques employed. Nonetheless, given the importance of phospholipids to membrane structure and function this aspect also merits further investigation. Clearly, definitive interpretation(s) of the observed events in terms of sites and mechanisms of mitochondrial self-restoration will require much further research along several lines including those implicit in this study.

Cellular/Physiological Context

Restoration is neither a new term nor a new phenomenon with respect to mitochondria. In 1956 Ernster (8) reported on the restoration of phosphorylation by the addition of ATP and Mn^{++} to rat liver mitochondria uncoupled by preincubation for up to 20 min at 30°C. Some 10 years later Brierly *et al.* (5) examined the restoration of energy-dependent Ca^{2+} accumulation by beef heart mitochondria after aging at 38°C for 30 min. The improvement in state 3 rates often observed in the first few minutes of a polarographic assay, termed "conditioning" by Raison *et al.* (27), may also be seen as a form of restoration.

These and similar early studies of mitochondrial restoration had reference to relatively rapid (a few minutes) improvements or restoration of functions that were impaired during isolation of the organelles or by short-term (5–30 min) energy-deprived aging. The present experiments were conducted with initially well-coupled mitochondria known to be capable of maintaining energy-linked functions for days at 25°C. Loss of function was imposed by energy deprivation for 3 to 6 h and energy-driven restoration was equally protracted. Such temporal spans not only facilitate investigation of interdependent restorative events, *e.g.* ordered restoration of P/O and RC, but prompt one to view restoration as an intrinsic "life" function of the organelles. This view is consonant with the observations of Hartl and Neupert (13), who end their recent review of protein sorting to (and within) mitochondria by suggesting that, "mitochondria may be considered as miniature cells inside cells." Such an outlook is not meant to substitute vitalism for mechanism but to emphasize the complexity and integration inherent to such phenomenon as mitochondrial protein sorting and, we presume, to mitochondrial self-restoration.

SUMMARY

The preceding evidence for mitochondrial self-restoration *in vitro* supports the postulate (29) that mitochondrial self-maintenance or homeostasis is the root cause of the respiratory climacteric of avocados and other fruit undergoing the stress of senescence. There is, furthermore, an interesting analogy between the abundant phosphate esterification observed during mitochondrial self-restoration *in vitro* and the early findings by Young and Biale (40), recently confirmed by Bennett *et al.* (3), that cellular ATP content increases during the climacteric rise in avocados. Whether factual or coincidental, this analogy underscores the potential role of

quasi-autonomous mitochondria in meeting the energy needs of cells under stress.

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