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Preparation of Avocado Mitochondria Using Self-Generated Percoll Density Gradients and Changes in Buoyant Density during Ripening

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ABSTRACT

Mitochondria from avocado (*Persea americana* Mill, var. Fuerte and Hass) can be rapidly prepared at every stage of ripening using differential centrifugation and self-generated Percoll gradients. The procedure results in improved oxidative and phosphorylative properties, especially for mitochondria isolated from preclimacteric fruits.

A gradual change in the buoyant density of avocado mitochondria takes place during ripening. Climacteric and postclimacteric avocado mitochondria have the same buoyant density as other plant mitochondria (potato, cauliflower), whereas mitochondria from preclimacteric fruit have a lower density. The transition in buoyant density occurs during the climacteric rise, and two populations of intact mitochondria ($\rho = 1.060$ and $\rho = 1.075$) can be separated at this stage. Evidence indicates that the difference in mitochondrial buoyant density between preclimacteric and postclimacteric mitochondria is likely due to interactions with soluble cytosolic components.

Mitochondria have been isolated from avocado at every stage of fruit ripeness by Hobson *et al.* (6) and these authors established that the mitochondria are active and coupled throughout the climacteric (6, 10). Subsequently, Ozelkok and Romani (15) observed that mitochondria from preclimacteric fruit have a much reduced capacity to maintain RC when incubated at 25°C. Because the RC of preclimacteric mitochondria appeared to be constrained by cytoplasmic contaminants (15), we have sought a rapid method to remove cytoplasmic contaminants, *i.e.* to 'clean' the organelles.

Relatively clean mitochondria have been obtained from etiolated tissues using either discontinuous or continuous sucrose gradients (16–18). Modified silica sol gradients have also been used and are superior to conventional sucrose gradients in being isosmotic and of low viscosity (5, 7). Recently, Jackson *et al.* (7) and Bergman *et al.* (1) used a three-step discontinuous Percoll gradient to prepare mitochondria from several plant tissues.

In this paper, we describe the use of Percoll, self-generated gradients with 22.5% Percoll as starting solution, and isosmotic conditions. Taking advantage of the 'S'-shaped gradient formed by this method, it has been possible not only to remove cytosolic contaminants but to demonstrate a change in the buoyant density of mitochondria during ripening. The biochemical behavior of these PGC[3] avocado mitochondria in relation to malate metabolism and cyanide-insensitive respiration is examined throughout the climacteric.

A preliminary report of this work has appeared (13).

MATERIALS AND METHODS

Fruits. 'Fuerte' and 'Hass' avocado fruits (*Persea americana*, Mill) were obtained from the University of California South Coast Field Station and placed at 5°C upon arrival.

Measurement of Respiratory Rates. Individual avocado fruit were placed in respiration jars at 15°C and supplied with a metered air flow, and their respiration was measured with the Claypool-Keefer method (3). Fruit were selected at points along the climacteric for the isolation of mitochondria.

Preparation of Mitochondria. Mitochondria were isolated from avocado fruits and once-washed by standard differential centrifugation as described by Ozelkok and Romani (15). The mitochondria were then combined with a solution of 22.5% Percoll[4] containing 5 mM Tris-HCl (pH 7.5) and 0.25 M sucrose. After centrifugation at 35,000*g* (average revolutions) for 30 min in a fixed angle rotor (Spinco, rotor 30), the banded mitochondria were collected with a pipette, diluted about 10 times with wash medium containing 0.25 M sucrose, 50 mM phosphate buffer (pH 7.2), 0.1% BSA, and 5 mM β-mercaptoethanol, and immediately centrifuged at 10,000*g* for 10 min. Generally, a second resuspension in wash medium and centrifugation was necessary to completely remove the Percoll and yield PGC mitochondria.

To determine gradient distribution profiles, the Percoll gradients were fractionated into 40 to 45 fractions and held at ice temperature, and the refractive index was measured using a Zeiss refractometer at 35°C.

Supernatant fractions from preclimacteric and postclimacteric fruits were prepared by centrifugation of postmitochondrial supernatant at 80,000*g* for 60 min to remove microsomes and other small particles, and then frozen.

To assess the effects of the supernatant fraction, PGC mitochondria were resuspended in 60 ml of either preclimacteric or postclimacteric postmitochondrial supernatant and held at ice temperature for 30 min. The mitochondria were then pelleted, resuspended, and assayed for density distribution using the self-generating Percoll gradients.

Assays. O_2 uptake by mitochondria was measured at 25°C with

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[3] Abbreviation: PGC, Percoll gradient-cleaned.

[4] Percoll was purchased from Pharmacia, and gradients were prepared as described in *Percoll, Methodology and Applications*, Pharmacia Fine Chemicals, AB Box 175 S-75104, Uppsala, Sweden.

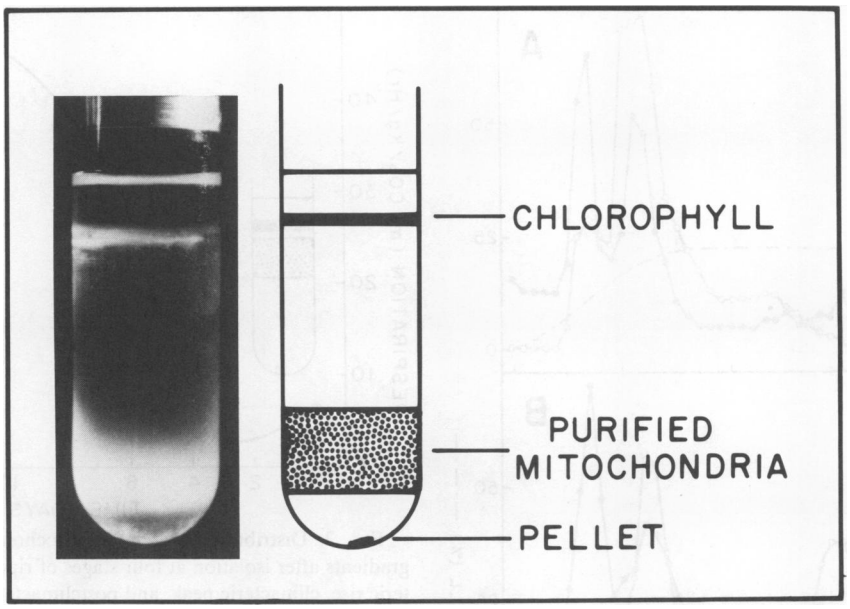

FIG. 1. Postclimacteric avocado mitochondria after centrifugation with a Percoll self-generated gradient. A 1-ml aliquot of washed mitochondria in 22.5% Percoll, 0.25 M sucrose, and 5 mM Tris-HCl (pH 7.5) was mixed with 27 ml similar solution and centrifuged at 35,000*g* for 30 min.

a Clark-type polarographic electrode using a medium containing 0.3 M mannitol, 10 mM phosphate buffer (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, and 0.1% BSA.

Succinate and NADPH-Cyt *c* reductase were determined spectrophotometrically at 25°C following the reduction of Cyt *c* at 550 nm. Aliquots of each fraction were diluted in a medium containing 10 mM phosphate buffer (pH 7.2), 40 μM Cyt *c*, and 1 mM KCN. Reaction was started by addition of 10 mM succinate or 1 mM NADPH.

Protein was determined by the Lowry procedure as modified by Miller (12).

RESULTS

Preparation of Mitochondria on Self-Generated Percoll Gradients. Effective use of the self-generating gradients required some trial and error. After starting solutions of 45% and 30% Percoll proved too dense, 22.5% Percoll (ρ = 1.06) was chosen with centrifugation at 35,000*g* for 30 min. At 20,000*g* the gradient was not generated and the mitochondria were pelleted. At 40,000*g* the gradient distribution was too steep and the resolution was unsatisfactory.

Figure 1 illustrates the position of avocado mitochondria isolated from postclimacteric fruits following centrifugation on a self-generated Percoll gradient. Mitochondria were located near the bottom, whereas Chl and broken thylakoids aggregated near the top below a small lipid layer. For comparative purposes, the same Percoll procedure was tried with cauliflower and fresh potato mitochondria isolated by differential centrifugation using established methods (9). The density gradient position of these mitochondria (not shown) was analogous to that obtained with postclimacteric avocado mitochondria. Due to the S-shaped curve of the Percoll gradient, microsomes were banded near the top of the gradient.

Change in Buoyant Density and Ripening. The distribution profiles of the mitochondrial protein and succinate-Cyt *c* reductase activity for avocado mitochondria isolated at three ripening stages are shown in Figure 2. Mitochondria isolated from preclimacteric fruits (Fig. 2A) appeared as a single light density band (ρ = 1.060) as denoted by the distribution of succinate-Cyt *c* reductase activity. From 50% to 70% of the activity initially layered on the gradient was recovered in the mitochondrial fraction and the specific activity was roughly doubled. A large portion of the contaminant, nonmitochondrial protein was located near the top of the gradient (Fig. 2A) and presumably contained Chl, broken thylakoids, and microsomes. In studies (not shown) with nongreen tissues (potato and cauliflower), microsomal contaminants were identified near the top ($\rho \leq 1.051$) of the Percoll gradient.

The distribution of succinate-Cyt *c* reductase activity along the Percoll gradient used to prepare mitochondria from fruit on the climacteric rise (Fig. 2B) indicates the presence of two distinct populations (ρ = 1.060 and ρ = 1.075). However, some activity was distributed along the flat, middle region of the gradient, indicating that a gradual change in the buoyant density takes place during the climacteric. A significant amount of nonmitochondrial protein is again seen near the top of the gradient.

Only one population of more dense mitochondria (ρ = 1.075) was obtained from climacteric peak and postclimacteric fruits (Fig. 2C). The upper band of nonmitochondrial protein was absent at this stage of ripening and washed mitochondria were already 'cleaner' than preclimacteric mitochondria. Under these conditions, more than 80% of the initial protein was recovered in the PGC mitochondrial fraction.

Changes in mitochondrial population with ripening are shown schematically in Figure 3. A gradual change in buoyant density occurs coincident with the beginning of the respiratory climacteric. The final buoyant density is reached at the climacteric peak and does not change during the postclimacteric phase.

Effect of Cytosolic Supernatant Fraction on the Buoyant Density of Mitochondria. In order to determine if the change in buoyant density was an intrinsic property of mitochondria or the consequence of an interaction between mitochondria and cytosolic components, postclimacteric PGC mitochondria (ρ = 1.075) were incubated for 30 min at 0°C in preclimacteric supernatant (*cf.* "Materials and Methods"). After pelleting (10,000*g* for 10 min), resuspension, and a second centrifugation on a Percoll gradient, a shift in mitochondrial density was observed (Fig. 4). The final gradient distribution became bimodal, mimicking the profile of mitochondria isolated from fruit on the climacteric rise (compare Figs. 2B and 4). As a control (not shown), in which postclimacteric PGC mitochondria were treated in the same way with postclimacteric supernatant, no change in density distribution was observed. It is apparent that the supernatant fraction prepared from preclimacteric fruits affects the distribution of postclimacteric mito-

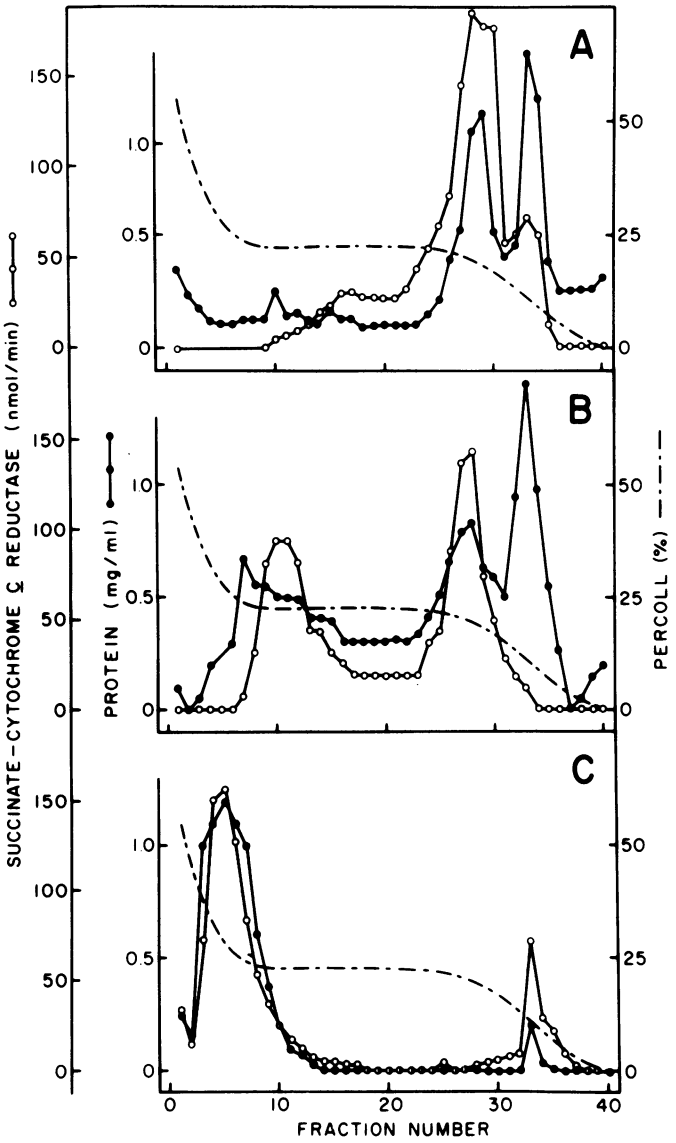

FIG. 2. Percoll density profile, protein distribution and succinate-Cyt *c* reductase activity of Percoll gradient-cleaned avocado mitochondria isolated at three stages of ripening: A, preclimacteric; B, climacteric rise; C, postclimacteric.

chondria by changing their apparent buoyant density.

Integrity and Oxidative Properties of Purified Avocado Mitochondria. Data in Table I show that membrane integrity as measured by succinate-Cyt *c* reductase (4) was retained in the light and heavy mitochondrial populations isolated on the climacteric rise. It is estimated that in both fractions about 90% of the organelles remain intact. Assuming that antimycin A-insensitive NADPH-Cyt *c* reductase serves as a marker enzyme for the microsomal fraction, a low level of microsomal contaminants can be detected in the PGC mitochondria (Table I). However, it has been observed (unpublished results) that avocado mitochondria can slowly oxidize NADPH (about 30 nmol/min·mg protein) with only partial inhibition by KCN or antimycin A. Consequently, the microsomal contamination cannot be accurately quantified. Moreover, the 'marker' enzyme activity is low and not much different in both the light and heavy mitochondrial population (Table II), increasing the likelihood that it is of mitochondrial origin and not a contaminant.

Oxidative and phosphorylative properties of washed and PGC postclimacteric mitochondria are compared in Table III. Rate of

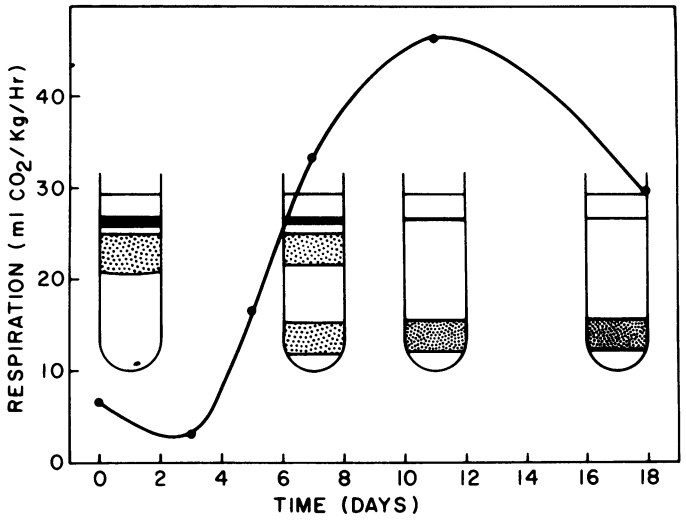

FIG. 3. Distribution of avocado mitochondria on self-generated Percoll gradients after isolation at four stages of ripening: preclimacteric, climacteric rise, climacteric peak, and postclimacteric.

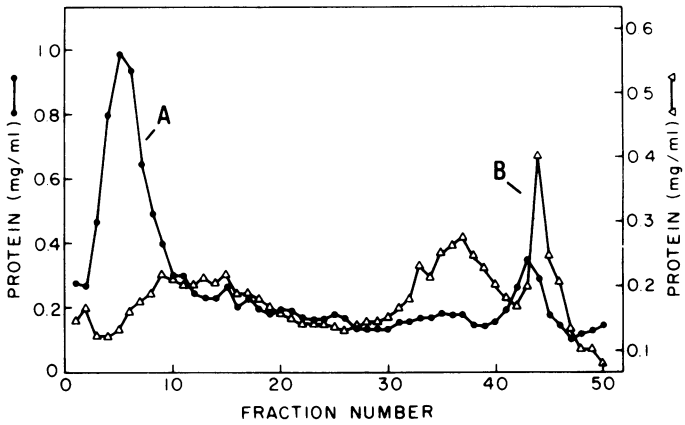

FIG. 4. Effect of the preclimacteric, postmitochondrial supernatant fraction on the distribution profile of postclimacteric mitochondria. A, Protein profile of control mitochondria; B, same mitochondria exposed for 30 min to the preclimacteric supernatant fraction (see "Materials and Methods").

Table I. *Mitochondrial Integrity and Microsomal-Like Activity in Light and Heavy Populations of Mitochondria from Avocado at the Climacteric Rise*

Activity	Mitochondrial Population	
	Light	Heavy
	nmol Cyt c reduced·mg^{-1} protein·min^{-1}	
Succinate-Cyt *c* reductase		
Hypotonic medium[a]	140 ± 10	110 ± 10
Hypertonic medium[b]	15 ± 5	11 ± 5
Antimycin A-insensitive NADPH-Cyt *c* reductase[b]	10 ± 5	14 ± 5

[a] Medium containing 10 mM phosphate buffer (pH 7.2).
[b] Same medium as Footnote a plus 0.3 M mannitol.

O_2 uptake, respiratory control, and ADP/O ratio were maintained or increased with the Percoll cleaning and cyanide resistance was retained. The rate of cyanide-insensitive oxidation was dependent on substrate. With substrates that are oxidized on the outer surface of the inner membrane, such as NADH or the artificial substrate

Table II. *Oxidative and Phosphorylative Properties of Light and Heavy Mitochondrial Populations Isolated from Avocado at the Climacteric Rise*

Substrate	Mitochondrial Population	
	Light	Heavy
NADH		
State 3[a]	114	97
RC	3.1	2.8
ADP/O	1.6	1.6
CN-R[b]	22	12
Succinate		
State 3[a]	72	85
RC	2.5	2.6
ADP/O	1.6	1.5
CN-R[b]	42	41
Malate		
State 3[a]	27	27
RC	2.7	2.3
ADP/O	2.1	1.8
CN-R[b]	47	52

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

Table III. *Oxidative and Phosphorylative Properties of Washed and Percoll Gradient-Cleaned Mitochondria from Postclimacteric Avocado*

Substrate	Washed Mitochondria	PGC Mitochondria
Durohydroquinone		
State 3[a]	35	90
RC	4.0	4.7
ADP/O	1.5	1.5
CN-R[b]	0	0
NADH	50	130
State 3[a]	3.5	4.9
RC	1.7	1.7
ADP/O	15	18
CN-R[b]		
Succinate		
State 3[a]	120	200
RC	2.5	2.8
ADP/O	1.7	1.5
CN-R[b]	25	23
Malate		
State 3[a]	40	70
RC	3.9–1.7[c]	3.8–1.9
ADP/O	1.9	2.1
CN-R[b]	67	50
α-Ketoglutarate		
State 3[a]	60	150
RC	3.0	3.4
ADP/O	2.5	2.3
CN-R[b]	66	41

[a] nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$.
[b] Cyanide-resistant respiration as a percentage of state 3.
[c] Due to the biphasic shape of state 4 with malate as substrate, two RC can be calculated (14).

durohydroquinone, cyanide resistance was low or nil. This is consistent with the notion that durohydroquinone does not have access to the alternative pathway which is branched on the respiratory chain at coenzyme Q (17). In contrast, the rate of cyanide-insensitive oxidation is always higher with Krebs cycle substrates involving endogenous NAD^+-linked dehydrogenases, especially malate.

Table IV. *NADH and Malate Oxidation in Washed and Percoll Gradient-Cleaned Preclimacteric Mitochondria*

Substrate	Washed Mitochondria	PGC Mitochondria
NADH		
State 3[a]	31	107
RC	1.5–2.6	2.2–3.3
ADP/O	1.2–1.6	1.6–1.8
CN-R[b]	20	23
Malate		
State 3[a]	20	61
RC	1.0–2.0	2.0–4.4
ADP/O	2.4–2.6	2.5–2.8
CN-R[b]	40	38

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

With preclimacteric mitochondria, the beneficial effects of Percoll gradient centrifugation are clearly evident (Table IV). Rates of O_2 uptake and RC were markedly increased, although the ADP/O ratios and cyanide resistance remained roughly the same.

DISCUSSION

The data presented in this paper clearly show that self-generated Percoll gradients provide a convenient and effective method for the preparation of relatively clean avocado mitochondria. This method can easily separate preclimacteric mitochondria from plastid contaminants. These observations are consistent with data reported by Jackson *et al.* (7), who used discontinuous Percoll gradients to enrich several different plant mitochondria.

Taking advantage of the S shape and increased resolution of the self-generated Percoll gradient, it has been possible to demonstrate a change in the buoyant density of avocado mitochondria during ripening. Postclimacteric mitochondria have the same buoyant density (ρ = 1.075) as other plant mitochondria whereas preclimacteric mitochondria have an unusually low buoyant density (ρ = 1.060). However, it should be emphasized that both mitochondrial populations exhibit the same degree of integrity and similar biochemical properties, including the same level of cyanide-insensitive respiration.

Due to the isosmotic condition of the Percoll gradient, the mitochondrial structure is not stressed or altered during centrifugation as it is on sucrose density gradients. Consequently, plant (avocado, cauliflower, potato) mitochondria exhibit a lighter buoyant density on Percoll (ρ = 1.07) than on sucrose (ρ = 1.17) (18).

Luck (11) associated a change in buoyant density of mitochondria isolated from *Neurospora crassa* to a change in the phospholipid to protein ratio. A similar result has been recently obtained by Kotomin and Dontsova (8) with mitochondria isolated from *Planaria.* Chauveau and Tuquet (2) have also found changes in mitochondrial densities and activities during spadix development in *Arum maculatum.* In the latter case, changes in apparent densities can be related to deep structural alterations leading to a fragmentation of mitochondria.

Although a change in the lipid to protein ratio during the climacteric of avocado cannot be completely ruled out, the rapid change in buoyant density of postclimacteric mitochondria when exposed to preclimacteric, postmitochondrial supernatant for 30 min indicates that an interaction involving cytosolic components occurs during maceration of preclimacteric fruits, presumably modifying the conformation or the surface charges of these organelles without changing their functional integrity. The change in mitochondrial density which occurs during the climacteric (Fig. 2) may simply correspond to a decrease in effect of supernatant coincident with the climacteric rise. Because exposure of postcli-

macteric avocado mitochondria to a preclimacteric supernatant fraction also curtailed their long term maintenance of energy-linked functions at 25°C (15), it is possible that the same components are responsible for both the decrease in buoyant density and maintenance of functional integrity.

Using linear Percoll density gradients, Goldstein *et al.* (5) separated wheat mitochondria into two bands. Although only one of the two mitochondrial populations was free of contaminating lipoxygenase activity, the purification was much better than could be achieved with sucrose density gradients. The efficacy of Percoll gradients is also demonstrated by the positive effect on the oxidative and phosphorylative properties of preclimacteric avocado mitochondria. Indeed, Percoll purification reveals an interesting aspect of mitochondrial malate metabolism which is the subject of the companion paper (14).

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LITERATURE CITED

1. Bergman A, P Gardeström, I Ericson 1980 Method to obtain a chlorophyll-free preparation of intact mitochondria from spinach leaves. Plant Physiol 66: 442–445
2. Chauveau M, C Tuquet 1981 Changes in mitochondrial structure and activities during spadix development in *Arum maculatum*. Physiol Veg 19: 616
3. Claypool LL, RM Keefer 1942 A colorimetric method for CO_2 determination in respiration studies. Proc Am Soc Hort Sci 40: 177–186
4. Douce R, EL Christensen, WD Bonner Jr 1972 Preparation of intact plant mitochondria. Biochim Biophys Acta 275: 148–160
5. Goldstein AH, JO Anderson, RG McDaniel 1980 Cyanide-insensitive and cyanide-sensitive O_2 uptake in wheat I. Gradient-purified mitochondria. Plant Physiol 66: 488–493
6. Hobson GE, C Lance, RE Young, JB Biale 1966 The isolation of active subcellular particles from avocado fruit at various stages of ripeness. Nature (Lond) 209: 1242–1243
7. Jackson C, JE Dench, DO Hall, AL Moore 1979 Separation of mitochondria from contaminating subcellular structures utilizing silica sol gradient centrifugation. Plant Physiol 64: 150–153
8. Kotomin AV, GV Dontsova 1980 Change in the buoyant density of mitochondria during starvation and feeding of *Planaria*. Biochemistry (Eng Trans Biokhimiya) 45: 36–38
9. Lance C 1971 Les mitochondries de l'inflorescence de choufleur. I. proprietes oxydatives. Physiol Veg 9: 259–279
10. Lance C, GE Hobson, RE Young, JB Biale 1965 Metabolic processes in cytoplasmic particles of the avocado fruit. VII. Oxidative and phosphorylative activities throughout the climacteric cycle. Plant Physiol 40: 1116–1123
11. Luck DJL 1965 Formation of mitochondria in *Neurospora crassa*. A study based on mitochondrial density changes. J Cell Biol 24: 461–470
12. Miller GL 1959 Protein determination for large numbers of samples. Anal Chem 31: 964
13. Moreau F, RJ Romani 1981 Changes in the buoyant density, malate oxidation and cyanide resistance of avocado mitochondria with ripening. Plant Physiol 67: S-646
14. Moreau F, R Romani 1982 Malate oxidation and cyanide-insensitive respiration in avocado mitochondria during the climacteric cycle. Plant Physiol 70: 1385–1390
15. Ozelkok SI, R Romani 1975 Ripening and *in vitro* retention of respiratory control by avocado and pear mitochondria. Plant Physiol 56: 239–244
16. Quail PH 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
17. Storey BT 1980 Electron transport and energy coupling in plant mitochondria. *In* The Biochemistry of Plants, Vol 2. Academic Press, New York, pp 125–195
18. Tolbert NE 1974 Isolation of subcellular organelles on isopycnic sucrose gradients. Methods Enzymol 31A: 734–746