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Separation of Mitochondria from Contaminating Subcellular Structures Utilizing Silica Sol Gradient Centrifugation[1]

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ABSTRACT

Discontinuous Percoll density gradients have been developed for the purification of mitochondria, permitting rapid separation under isosmotic and low viscosity conditions. Mitochondria from several etiolated tissues have been successfully separated from contaminating subcellular structures by this method. For potato tuber the ratio of washed to purified mitochondrial protein was 2.6, similar to the increase in specific activity of cytochrome *c* oxidase following separation. The purification of mitochondria from green leaf tissues on Percoll gradients has reduced chlorophyll contamination of spinach mitochondria from about 70 micrograms chlorophyll per milligram protein to approximately 8 micrograms chlorophyll per milligram protein.

The ratio of protein content of the washed mitochondria compared to that in the purified preparation was 7 for spinach and respiratory activity was retained. The physiological integrity and oxidative properties of washed and gradient mitochondria are compared.

Mitochondrial preparations from diverse sources such as rat liver (13) and higher (3, 6) and lower plants (14) are to some extent contaminated with other subcellular structures such as peroxisomes, cell debris, and in the case of mitochondria from the leaves of higher plants (8), broken thylakoid membranes. In order to undertake enzyme localization and other studies, it is desirable to obtain mitochondria as free as possible from such contamination, while retaining integrity and respiratory activity. Such a result can be achieved by discontinuous and continuous sucrose gradient centrifugation (6, 7, 20) or by centrifuging through a 0.6 M sucrose cushion (6). However, the former methods have the disadvantage of being time consuming and require careful dilution of the mitochondrial fraction to isosmotic conditions, while the latter method fails to remove much of the contaminating subcellular material. In addition, some mitochondrial preparations do not successfully undergo the isosmotic dilution (5, 7) and in the case of higher plant leaf preparations, these techniques fail to remove the Chl contamination and retain respiratory activity (11). Evidently, a rapid procedure allowing the removal of contaminating material under isosmotic and low viscosity conditions would have many advantages over the more conventional sucrose techniques (6, 7, 20). Although colloidal silica such as Ludox has been used in the past, success has been limited because of its toxicity. The present work describes a technique which has the advantage of being rapid and separates mitochondria from contaminating materials under isosmotic conditions. It has been successfully applied to the purification of mitochondria isolated from etiolated tissues and subsequently adapted to remove most of the Chl contamination from green leaf preparations.

The density medium used is Percoll, a silica sol combining low viscosity, low osmolarity, and nontoxicity. The latter is achieved by coating the silica particles with PVP. Although both continuous and discontinuous gradients can be formed with this material, a three-step discontinuous system has been found most useful.

MATERIALS AND METHODS

Plant Material and Reagents. Mung bean (*Phaseolus aureus*) and french bean (*Phaseous vulgaris*) hypocotyls were dark-grown at 25 C for 5 to 7 days. Potato tubers, spinach, and cos lettuce (*Lactuca sativa*) leaves were obtained from commercial sources and *Chenopodium album* leaves from our own gardens.

Percoll was purchased from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden (18). All other reagents were of the highest quality commercially available.

Preparation of Mitochondria. Washed mitochondria were prepared from etiolated tissues by the general method of Bonner (3) whereas mitochondria from green leaves were isolated according to Douce *et al.* (8) with the following modifications. The initial low speed centrifugation was 3,000*g* for 5 min. Following centrifugation at 12,000*g* for 20 min, the crude mitochondrial fraction was resuspended and centrifuged at 1,500*g* for 10 min followed by final pelleting at 11,000*g* for 15 min. The washed mitochondria were resuspended in approximately 4 ml of wash medium containing 0.3 M mannitol, 1 mM EDTA, BSA (fraction V, 0.2%, w/v) and 20 mM MOPS[3] (pH 7.2). The protein concentration was 10 to 20 mg/ml for etiolated tissues and the Chl concentration not more than 0.8 mg/ml for leaf tissue.

Gradient Centrifugation. Etiolated tissue preparations were fractionated on a gradient prepared as described in the Pharmacia Percoll manual (18) with final concentrations of 13.5% (v/v), 21% (v/v), and 45% (v/v) Percoll, each containing 0.25 M sucrose, 0.2% (w/v) BSA, and 10 mM MOPS. The final pH was adjusted to 7.2 with KOH. Each gradient comprised 6 ml of 45%, 12 ml of 21%, and 12 ml of 13.5% Percoll mixtures in a 50-ml tube. Centrifugation conditions were 30 min at 7,500*g*, utilizing an angle rotor. All operations were carried out at 0 to 4 C.

Mitochondria from green leaves were separated under similar conditions except that the final concentrations of components in the 13.5% layer were 0.239 M sucrose, 50 mM propane −1,2-diol, BSA (0.2%, w/v), and 10 mM MOPS (pH 7.2). After separation and collection of the mitochondrial fraction it was resuspended in

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[3] Abbreviations: MOPS: morpholinopropanesulphonic acid.

wash medium (about 60 ml) and pelleted at 11,000*g* for 15 min.

Mitochondrial Respiration and Enzyme Assays. Mitochondrial respiration was measured polarographically using a Hansatech O_2 electrode (Kings Lynn, Norfolk, U.K.) (16). Cyt *c* oxidase was determined by the method of Tolbert (20) in the medium used for mitochondrial respiration (16). Catalase was assayed using an O_2 electrode according to the procedure of Rich *et al.* (19), and glycolate oxidase was measured as described by Feierabend and Beevers (9). Glycine decarboxylase was estimated by following release of $^{14}CO_2$ from [1-^{14}C]glycine according to Moore *et al.* (16). NADP-glyceraldehyde-3-P dehydrogenase was assayed in a 3-ml

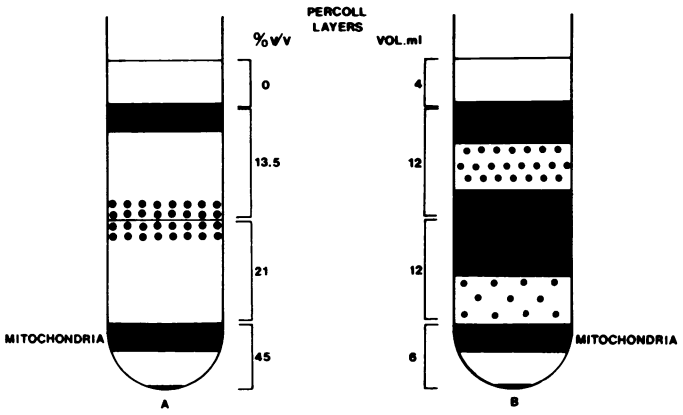

FIG. 1. Purification of washed mitochondria on discontinuous Percoll density gradients. Separation of mitochondria from etiolated tissues (mung bean hypocotyl and potato tuber) is represented diagrammatically in A; distribution of mitochondrial fractions from green leaves (spinach and cos lettuce) is indicated in B. Density of shading, other than that for the mitochondrial bands, represents amount of contaminating material. Nature of contaminants can be deduced from Figure 2.

Table I. *Membrane Integrity of Washed and Gradient Mitochondria Measured by Release of Cyt c Oxidase*

Gradient mitochondria were prepared from washed fractions utilizing the Percoll gradient for etiolated tissues in the case of mung beans and a similar gradient containing propane-1,2-diol for spinach. All procedures were as described under "Materials and Methods." Mitochondria were disrupted by 0.02% (v/v) Triton X-100 (final concentration). Rates are expressed as nmol Cyt *c* oxidized/min·mg protein. Similar results have been observed for mitochondria isolated from potato tuber, french bean hypocotyls, and cos lettuce leaves.

	Washed Mitochondria			Gradient Mitochondria		
	Control	Detergent treated	% Intact	Control	Detergent treated	% Intact
Mung bean	11.9	190	94	6.5	285	98
Spinach	36.9	213	83	52.6	330	84

reaction volume containing 67 mM Tris-HCl, (pH 7.2) 3.3 mM ATP, 10 mM $MgCl_2$, 4 mM EDTA, 130 μM NADPH, 3.3 μg/ml phosphoglycerate kinase, 1 mM DTT. From 50 to 100 μl of sample was incubated in this medium for 5 min to allow activation (12). The reaction was initiated by the addition of 3-P-glycerate to a final concentration of 1 mM. Mitochondrial membrane integrity was assessed by release of Cyt *c* oxidase from Triton X-100-treated mitochondria, as described by Tolbert (20). Protein was estimated by a modification of the Lowry procedure (21) and Chl was measured according to the procedure of Arnon (2). Assuming a protein to Chl ratio of 7, the amount of mitochondrial protein can be corrected for the contribution of broken thylakoids, as described by Douce *et al.* (7).

RESULTS AND DISCUSSION

Figure 1A illustrates the position of mitochondria isolated from etiolated mung bean hypocotyls following centrifugation on a

Table II. *Oxidative and Phosphorylation Capacities of Washed and Gradient Mitochondria*

Gradient mitochondria were obtained from washed preparations using the Percoll gradient for etiolated tissues in the case of mung bean and a similar gradient containing propane-1,2-diol for green tissues in the case of spinach. All procedures were as described under "Materials and Methods." Rates of oxygen uptake, given in nmol O_2/min·mg protein, are the average of five experiments. Figures in parentheses are the highest and lowest values obtained. State 3 respiration refers to the rate of oxygen uptake in the presence of ADP, while state 4 refers to the rate on depletion of ADP. For terminology, see reference 4. *Mung bean mitochondria do not oxidize glycine (16). Similar results have been obtained with cos lettuce, potato tuber, and both etiolated and light-grown french bean hypocotyl mitochondria.

	Washed Mitochondria				Gradient Mitochondria			
	Glycine[1]	Malate[2]	Succinate[1]	NADH[3]	Glycine[1]	Malate[2]	Succinate[1]	NADH[3]
Mung bean								
State 3	*	79	123	118	*	145	147	159
		72–87	(118–130)	(100–138)		(132–163)	(130–162)	(118–198)
State 4		26	57	54		23	60	56
		(19–31)	(51–64)	(47–59)		(19–31)	(51–64)	(42–69)
R.C.R.[4]		3.4	2.2	2.2		6.7	2.3	2.4
		(2.5–4.3)	(1.8–2.6)	(1.7–2.6)		(4.5–8.4)	(2.0–2.6)	(2.1–2.9)
ADP:0		2.2	1.3	1.2		2.5	1.3	1.3
		(2.0–2.3)	(1.3)	(1.1–1.3)		(2.5)	(1.3)	(1.2–1.4)
Spinach								
State 3	56	47	38	71	96	99	48	121
	(41–61)	(41–56)	(37–38)	(64–80)	(80–107)	(72–123)	(40–55)	(86–148)
State 4	21	19	28	32	35	31	32	51
	(19–22)	(15–25)	(26–31)	(30–36)	(34–38)	(27–46)	(26–36)	(42–61)
R.C.R.[4]	2.7	3.0	1.3	2.3	2.8	3.8	1.6	2.6
	(2.4–2.9)	(2.6–3.1)	(1.3)	(2.1–2.5)	(2.6–3.0)	(3.5–4.6)	(1.5–1.7)	(2.4–3.0)
ADP:0	2.0	2.0	1.2	1.3	2.4	2.6	1.5	1.8
	(2.0)	(2.0)	(1.0–1.4)	(1.2–1.4)	(2.2–2.8)	(2.4–2.8)	(1.4–1.7)	(1.6–2.0)

[1] 10 mM.
[2] 25 mM.
[3] 2.5 mM.
[4] Respiratory control ratio, *i.e.* $\frac{\text{state 3}}{\text{state 4}}$.

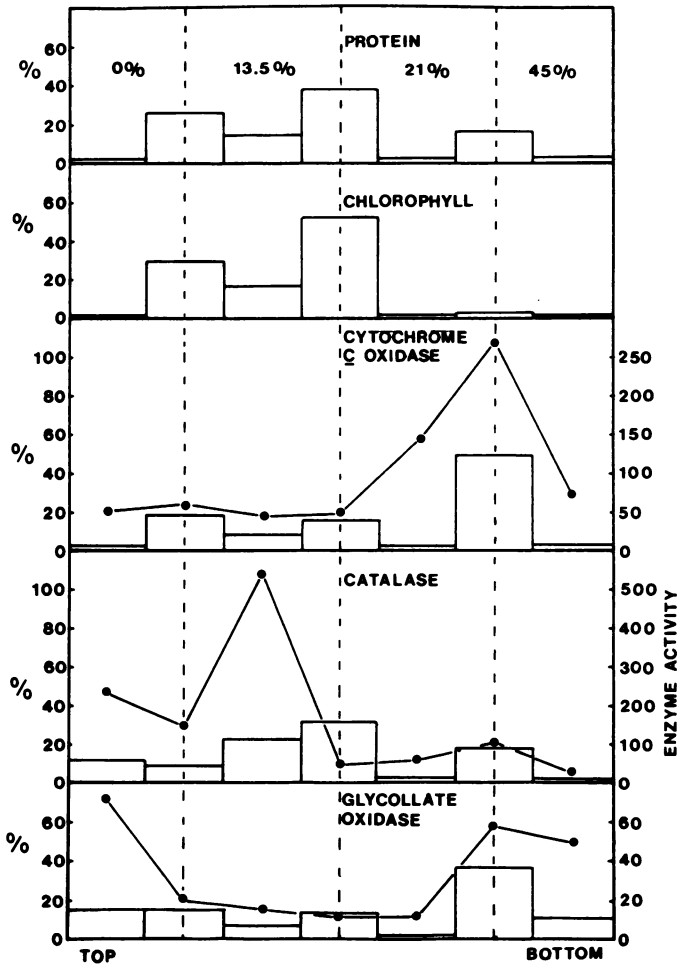

FIG. 2. Distribution of markers following separation on discontinuous Percoll gradients. A typical distribution of markers following separation of *C. album* mitochondria is shown. Similar results have been observed for spinach and cos lettuce. Glycolate oxidase activity is expressed as nmol O_2 consumed/min·mg protein and catalase activity as I.U./mg protein. Cyt *c* oxidase activity is expressed as nmol Cyt *c* oxidized/min·mg protein. Histogram denotes per cent of the original activity recovered in the fraction, and ●——● represents specific activity. Vertical dashed lines indicate interfaces between various layers and the figures in the protein distribution are per cent (v/v) Percoll in these layers.

discontinuous Percoll gradient. The mitochondria have aggregated at the interface of the 21 and 45% layers. Banding at this interface was observed for mitochondria from a variety of sources, *e.g.* potato tuber, french bean and mung bean hypocotyls.

With potato tuber preparations, catalase activity recovered in this fraction represented only 10% of the total activity layered onto the gradient and the specific activity was reduced from 100 I.U./mg protein (15) to 33 I.U./mg protein. From 50 to 70% of the Cyt *c* oxidase activity initially layered onto the gradient was recovered in the mitochondrial fraction and specific activity was increased from 1 μmol/min·mg protein to 2.4 μmol/min·mg protein. The ratio of protein content of the washed mitochondria to that in the preparation following gradient separation was 2.6, indicating removal of extraneous protein. Table I shows that membrane integrity, as measured by Cyt *c* oxidase, was retained and is normally at least 90% intact for both inner and outer membranes. Rates of state 3 oxidation (4), ADP:O and respiratory control ratios were maintained or increased following separation by this method (Table II). On the basis of these criteria, the mitochondria were largely intact, and this technique would therefore seem comparable to sucrose gradient procedures (6, 7).

Removal of Chl contamination, in addition to peroxisomes and cell debris from green leaf mitochondria, is only partially effective using sucrose procedures or the Percoll gradient for etiolated tissues. If it is assumed that the affinity of the mitochondria for the contaminants is due to a combination of density similarities, charge associations, and hydrophobic interactions, then changing the composition of part or all of the gradient to nullify or reduce these associations may make separation easier. The addition of low concentrations of species such as 10 or 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Na_2SO_4, or 2 mM EDTA tended to increase the aggregation between mitochondria and contaminants.

In consideration of these results and the recent phase partition experiments of Albertsson (1) and Gardeström *et al.* (10) we attempted to change the hydrophobicity of the 13.5% layer by replacing part of the sucrose with 50 mM (0.38% w/v) propane-1,2-diol. With centrifugation conditions as described under "Materials and Methods," a banding pattern was obtained (Fig. 1B). Again, the mitochondria aggregated at the interface of the 21 and 45% layers and interestingly were a buff yellow in color. It is apparent from Figure 2 that the bulk of the Chl was removed by the 13.5% layer. This represents broken thylakoids only, since no glyceraldehyde-3-P dehydrogenase could be detected in any fraction. The mitochondrial fraction contained about 20% of the catalase activity originally present in the washed mitochondrial pellet and less than 5% of the original Chl. Although some glycolate oxidase activity was present which is indicative of peroxisomal contamination, the activity recovered in this fraction was lower than in the initial washed mitochondria. Additionally the protein to Chl ratio was increased from approximately 15 to 123, Cyt *c* oxidase specific activity was increased by up to 10-fold, and the ratio of protein content of the mitochondria layered onto the gradient compared to the amount recovered in the purified mitochondrial fraction averaged 7 for spinach (six experiments), again showing removal of contaminating protein. Table III demonstrates that Cyt *c* oxidase was enriched with respect to Chl after both differential and gradient centrifugation. Additionally, it can be seen from these results that the differential centrifugation steps also show an enrichment of Cyt *c* oxidase with respect to glycolate oxidase, a peroxisomal marker (about 45% of the Cyt *c* oxidase was recovered compared to only 10% of the glycolate oxidase). While the peroxisomes tend to co-sediment with the mitochondria during gradient separation (Fig. 2), the ratio of Cyt *c* oxidase to glycolate oxidase recovered in the purified mitochondria was slightly increased compared to that of the washed fraction. Rates of state 3 oxidation, ADP:O, and respiratory control ratios were improved as indicated in Table II. Membrane integrity was also maintained as shown in Table I, although due to the apparent fragility of green leaf mitochondria (16), this is somewhat lower (approximately 80% intact) than that obtained for etiolated tissue preparations.

Mitochondria from the leaves of higher plants are able to

Table III. *Recovery of Activities in Washed and Gradient Mitochondria from Spinach Leaves*

Gradient mitochondria were prepared from washed fractions via a Percoll gradient containing propane-1,2-diol as described under "Materials and Methods." Recovery is expressed as the average % of the total activity in the initial homogenate. Figures in parentheses show the maximum and minimum values obtained from different preparations. Similar results have been obtained with cos lettuce.

	Washed Mitochondria	Gradient Mitochondria
Chl	2.5 (1–3)	0.06 (0.02–0.1)
Cyt *c* oxidase	45 (40–50)	20 (12–23)
Glycollate oxidase	10 (5–15)	3.5 (1.5–6)
Cyt *c* oxidase / Chl	18 (13–50)	330 (120–1100)
Cyt *c* oxidase / Glycollate oxidase	4.5 (2.7–10)	5.7 (2–15)

catalyze the decarboxylation of glycine which is coupled to the electron transport chain (16). This decarboxylation is currently considered to be the major source of CO_2 released during photorespiration (16).

The rate of $^{14}CO_2$ release by the decarboxylase, which is dependent on mitochondrial membrane integrity (16), was increased from 24.6 nmol/min·mg total protein to 70 nmol/min·mg total protein following separation. These criteria indicate that green leaf mitochondria purified on such gradients are largely intact, as was found for the etiolated tissue preparations.

The use of four centrifugation steps has been found necessary to remove as much Chl contamination as possible from green leaf mitochondrial fractions before separation on a Percoll gradient. This procedure reduces the possibility of overloading such a gradient with Chl-containing material. However, for other tissues such as etiolated (no Chl) or light-grown (low levels of Chl compared to leaf tissue) mung bean hypocotyls, mitochondria from the first high speed centrifugation (3) can be applied to such a gradient without reducing the activity or integrity of the purified mitochondria. This procedure further reduces the preparation time of intact contaminant-reduced mitochondria from these sources.

The ability of propane-1,2-diol to act in the manner described is not entirely understood. The inclusion of this material introduces a separation based on surface properties (1), *i.e.* the countercurrent principle. It would seem possible that the combination of surface property and size/density separation techniques would be able to remove thylakoid contamination by a localized countercurrent partition at the interface of the 13.5% and 21% Percoll layers, while other material with surface properties similar to mitochondria are separated by conventional centrifugation. Materials such as dimethylsulfoxide, polyethylene glycols, or increased concentrations of propane-1,2-diol, which could be expected to increase hydrophobicity, did not significantly improve the separation of mitochondria from broken chloroplasts. The effects of these materials appear to be confined to the removal of chloroplast fragments, since their inclusion in gradients for purifying mitochondria from etiolated tissues does not result in any enhanced purification.

It should be stressed that an effective separation of mitochondria and contaminants from green leaf tissues can only be successfully achieved if the washed mitochondrial fraction shows good ADP:O and respiratory control ratios and if the gradient is not overloaded with Chl (upper limit 3.5 mg Chl per gradient). Within these constraints this technique has proved successful for the separation of Chl and other contaminants from mitochondria in green leaf preparations from a variety of sources such as cos lettuce, spinach, and *Chenopodium album*. Hitherto, this has not proved possible utilizing other more standard techniques.

It is of particular importance to note the great flexibility of purification procedures involving silica sols. Not only can mitochondria be separated from other subcellular material as described in the present work, but similar techniques can be developed to isolate, for example, both intact and broken chloroplasts (17). Thus, methods of purification involving silica sols might be developed for the isolation of contaminant-free peroxisomes, glyoxysomes, and other subcellular organelles.

LITERATURE CITED

1. ALBERTSSON P-Å 1977 Separation of particles and macromolecules by phase partition. Endeavour 1: 69–74
2. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
3. BONNER WD JR 1967 A general method for the preparation of plant mitochondria. Methods Enzymol 10: 126–133
4. CHANCE B, GR WILLIAMS 1955 Respiratory enzymes in oxidative phosphorylation. III. The steady state. J Biol Chem 217: 409–417
5. COLLOT M, S WATTIAUX DE CONINCK, R WATTIAUX 1975 Deterioration of rat liver mitochondria during isopycnic centrifugation in an isoosmotic medium. Eur J Biochem 51: 603–608
6. DAY DA, JB HANSON 1977 On methods for the isolation of mitochondria from etiolated corn shoots. Plant Sci Lett 11: 99–104
7. DOUCE R, EL CHRISTENSEN, WD BONNER JR 1972 Preparation of intact plant mitochondria. Biochim Biophys Acta 275: 148–160
8. DOUCE R, AL MOORE, M NEUBURGER 1977 Isolation and oxidative properties of intact mitochondria isolated from spinach leaves. Plant Physiol 60: 625–628
9. FEIERABEND J, H BEEVERS 1972 Developmental studies in wheat leaves. I. Conditions influencing enzyme development. Plant Physiol 49: 28–32
10. GARDESTRÖM P, I ERICSON, C LARSSON 1978 Preparation of mitochondria from green leaves of spinach by differential centrifugation and phase partition. Plant Sci Lett 13: 231–239
11. HALLIWELL B 1974 Oxidation of formate by peroxisomes and mitochondria from spinach leaves. Biochem J 138: 77–85
12. HALLIWELL B 1978 The chloroplast at work. A review of modern developments in our understanding of chloroplast metabolism. Prog Biophys Mol Biol 33: 1–54
13. HOGEBOOM GH 1955 Fractionation of cell components of animal tissue. Methods Enzymol 1: 16–19
14. LAMBOWITZ AM, EW SMITH, CW SLAYMAN 1972 Electron transport in *Neurospora* mitochondria. J Biol Chem 247: 4850–4857
15. LÜCK H 1963 *In* HU Bergmeyer, ed. Methods of Enzymatic Analysis. Academic Press, New York, pp. 885–894
16. MOORE AL, C JACKSON, B HALLIWELL, JE DENCH, DO HALL 1977 Intramitochondrial localisation of glycine decarboxylase in spinach leaves. Biochem Biophys Res Commun 78: 483–491
17. MORGENTHALER J-J, CA PRICE, JM ROBINSON, M GIBBS 1974 Photosynthetic activity of spinach chloroplasts after isopycnic centrifugation on gradients of silica. Plant Physiol 54: 532–534
18. PHARMACIA FINE CHEMICALS 1977 (Percoll Manual) Box 175, S-751 04 Uppsala 1, Sweden
19. RICH PR, A BOVERIS, WD BONNER JR, AL MOORE 1976 Hydrogen peroxide generation by the alternate oxidase of higher plants. Biochem Biophys Res Commun 71: 695–703
20. TOLBERT NE 1974 Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. Methods Enzymol 31A: 734–746
21. WANG C-S, RL SMITH 1975 The Lowry determination of protein in the presence of Triton X-100. Anal Biochem 63: 414–417