

The Influence of Mitochondrial Concentration and Storage on the Respiratory Control of Isolated Plant Mitochondria¹

Received for publication August 13, 1969

JOHN K. RAISON AND JAMES M. LYONS

Plant Physiology Unit, Commonwealth Scientific and Industrial Research Organization Division of Food Preservation, Ryde, Australia; and School of Biological Sciences, University of Sydney, Sydney, Australia

ABSTRACT

The respiration of mitochondria isolated from various plant tissues was studied over a range of mitochondrial concentrations and at various times after isolation. Respiration at 25 C expressed as nanomoles of O₂ per minute per milligram of protein was constant for mitochondrial concentrations higher than some critical amount, usually 0.25 to 1.0 milligram of protein per reaction. Below this concentration the state 3 respiration rate declined and the mitochondria appeared to lose respiratory control. The respiration of isolated mitochondria stored in ice but measured at 25 C generally declined over long time periods although mitochondria from some tissues showed an initial increase. The results indicate that valid comparisons of the respiratory activity of mitochondria isolated from different tissues or from different parts of the same tissue cannot be made at least until the influence of the above factors has been evaluated.

A number of workers have described methods of isolating tightly coupled mitochondria which are applicable to a variety of plant tissues (*cf.* Reference 2). However, little attention has been given to the characteristics of the isolated mitochondria which may influence the interpretation of comparative studies (5, 11, 14). In a study of the effect of temperature on the oxidative activity and phosphorylative efficiency of mitochondria isolated from a variety of plant tissues (8) it became apparent that oxidative activity and the capacity to demonstrate respiratory control were greatly influenced by the quantity of mitochondrial protein in the assay system and by changes occurring during storage of the isolated mitochondria. A study was therefore undertaken to evaluate these conditions imposed by the assay system which can affect the respiration of isolated mitochondria but do not have any physiological implications.

The results presented here show that the respiration rate of plant mitochondria, expressed as nanomoles of O₂ per min per mg of protein, is not proportional to the amount of protein in the assay system and, below a certain critical mitochondrial concentration, there is an apparent loss of respiratory control. Furthermore, mitochondria isolated from some plant tissues and stored at 0 C increase in oxidative activity during the 1st hr after

isolation, when measured at 25 C; subsequently the activity declines. These results clearly show the importance of selecting reaction conditions such that valid evaluations can be made of the respiration and phosphorylative capacity of mitochondria isolated from a variety of tissues.

MATERIALS AND METHODS

Plant Material. Mitochondria were prepared from the following plants: mature green tomato fruit (*Lycopersicon esculentum* Mill. cv. Grosse Lisse); sweet potato roots (*Ipomoea batatas* L. cv. White Maltese); cucumber fruit (*Cucumis sativus* L.); potato tubers (*Solanum tuberosum* L. cv. Sebago); cauliflower buds (*Brassica oleraceae* L. var. botrytis); beet roots (*Beta vulgaris* L.); avocado fruit (*Persea gratissima*, cv. Fuerte) and pear fruit (*Pyrus communis* L. cv. Packham). The avocado and pear fruit were harvested at the hard green stage and tomato fruit at the mature green stage from commercial plantings. All other plant material was purchased from local markets and was of high visual quality and free of defects. The cauliflower, cucumber, and beet root were of unknown cultivars.

Preparation of Mitochondria. The pericarp tissue of tomato, cucumber, and pear fruit was separated from the seeds and placental material and cut into sections. Potato, sweet potato, beet root, cucumber, and pear sections were peeled and washed in distilled water. Avocado fruit was thinly peeled and the fruit tissue was divided into two fractions: the outer green layer and the pure yellow tissue near the seed. Only the outer 2 to 4 mm of cauliflower were used and with the other prepared tissues chilled in crushed ice. The tissue (usually 1 kg) was macerated in a commercial juice extractor (9) with continuous addition of isolating medium and filtered through a double layer of Miracloth (Chicopee Mills, Inc., New York, N.Y.) forming a lining in the juice extractor basket, or through several layers of muslin after discharge from the juice extractor. For potato and tomato tissue, the Miracloth lining was coated with a 2- to 4-mm layer (10-15 g) of Dicalite 4200 (Grefco, Inc., Dicalite Division, Torrance, Calif.). The use of Dicalite effectively removed large starch grains and cell debris and provided an easier separation of the mitochondrial fraction in the first stages of centrifugation. Usually the tissue to isolating medium ratio was 1:1 (w/v); with avocado and pears 300 g of prepared tissue were macerated with 1200 ml of medium. The pH of the macerated tissue was maintained at 7.2 by dropwise addition of 2 N KOH. Because of the high water content, 1 kg of cucumber tissue was macerated with 500 ml of isolating medium containing all components at three times the concentrations shown below. Pear mitochondria were prepared as described by Romani, Yu, and Fisher (12).

The isolating medium consisted of 0.5 M mannitol, 0.01 M KCl, 0.005 M EDTA, 0.025 M tris, 0.002 M MgCl₂, 0.004 M cysteine

¹ This work was supported in part by a Commonwealth Scientific and Industrial Research Organization, Australia, study grant awarded to J. M. Lyons while on sabbatical leave from the Department of Vegetable Crops, University of California, Riverside, California 92502.

hydrochloride, and 0.5 mg/ml BSA² (Fraction V; Sigma Chemical Co., St. Louis, Mo.) all adjusted to pH 7.6. The wash medium consisted of 0.5 M mannitol, 0.01 M KCl, 0.001 M MgCl₂, 0.01 M tris, 0.01 M KH₂PO₄, and 0.5 mg/ml BSA, adjusted to pH 7.2. The BSA was extracted three times with A.R. grade diethyl ether before use.

The mitochondria were isolated by centrifuging the tissue brei at 2,500g for 15 min, discarding the pellet, and centrifuging the supernatant at 11,700g for 20 min. The resulting pellet was resuspended in approximately 240 ml of wash medium and centrifuged at 1,900g for 10 min. The resulting pellet was again discarded and the supernatant was centrifuged at 7,700g for 15 min. The final pellet was suspended in wash medium to give a protein concentration of 10 to 15 mg/ml.

Oxidative Activity. Oxygen uptake was measured polarographically with a Clark O₂ electrode in a reaction vessel of 4.2 ml total volume. The electrode was calibrated with air-saturated water (4). The reaction medium contained 0.25 M sucrose, 0.01 M tris, 0.01 M K₂HPO₄, 0.005 M MgCl₂, 0.005 M EDTA, 0.5 mg/ml BSA, adjusted to pH 7.2 with HCl. Succinate was used as the substrate for oxidation at a concentration of 3.3 mM. Additions of ADP, 150 to 370 nmoles, were made to the reaction vessels in 2- to 5- μ l amounts. ADP:O ratios and respiratory control (RC) ratios (*i.e.*, the ratio of state 3 to state 4 respiration rates) were calculated as shown by Estabrook (3). ADP (Sigma Chemical Co.) was prepared as an approximately 70 mM solution in 0.1 M tris buffer, pH 7.2; the actual concentration was estimated by the spectrophotometric determination of the oxidation of NADH with a coupled lactate dehydrogenase (EC 1.1.1.27)-pyruvate kinase (EC 2.7.1.40) system as described by Atkinson, Burton, and Morton (1). Protein was estimated by the method of Lowry *et al.* (7).

RESULTS AND DISCUSSION

Effect of Mitochondrial Protein Concentration on Respiratory Activity. During preliminary investigations, it was noted that the state 3 respiration rate of isolated mitochondria was not directly proportional to the quantity of mitochondrial protein used in the reaction mixture. At low protein concentrations (0.25 mg per reaction) mitochondria from each of the tissues exhibited a low rate of succinate oxidation and showed no increase in respiration in response to the addition of ADP, and thus they appeared to lack respiratory control. However, when the concentration of these same mitochondria was increased to approximately 1.0 mg of protein per reaction, there was an increase in the rate of respiration and a response to the addition of ADP, and the mitochondria now exhibited respiratory control. Since the variation in the rate of respiration, which appeared to depend on the concentration of mitochondrial protein in the reaction mixture, would greatly confound comparisons of other factors influencing the respiration rate, and there was no information available on this point, it was considered important to investigate this parameter of mitochondrial respiration.

As shown in Figure 1, when respiration was expressed as a function of the protein concentration (nanomoles of O₂ per min per mg of protein) and compared to the amount of mitochondrial protein in the reaction mixture, the state 3 respiration rate was relatively constant for a range of protein above some critical quantity which varied with mitochondria from different tissues. However, when the amount of mitochondrial protein was decreased below the critical quantity, the state 3 rate declined sharply to a value approximately equal to the state 4 rate. Under these conditions the mitochondria appeared to have lost respiratory control. The critical range of mitochondrial protein con-

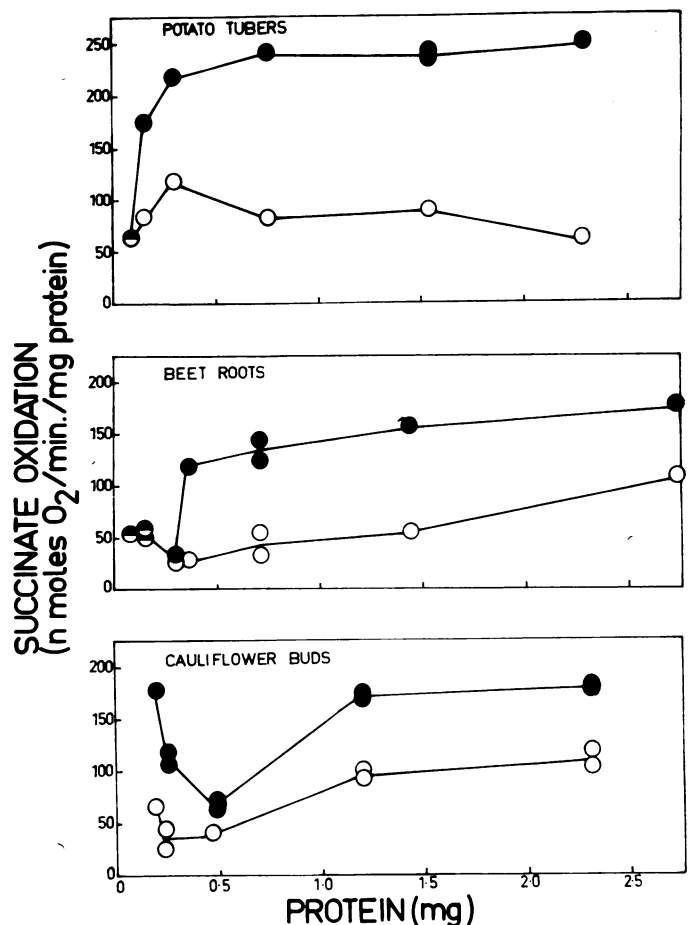


FIG. 1. The effect of protein concentration on the respiration of mitochondria at 25 C. Both the state 3 (●) and state 4 (○) rates of respiration were determined from an average of at least three cycles of ADP additions. Points shown as (●) indicate that there was no detectable difference between the state 3 and the state 4 respiration rates, *i.e.*, no respiratory control.

centration for the different tissues fell between 0.25 mg per reaction for mitochondria from potato (Fig. 1) and the green tissue of avocado to 1.0 mg per reaction for mitochondria from cauliflower (Fig. 1). The relationship between the respiration and protein concentration for mitochondria from the yellow tissue of avocado fruit, pear fruit, and sweet potato were similar to the relationship shown for beet root (Fig. 1). With mitochondria from beet root and potato tubers (Fig. 1) and from tomato fruit, variations of as little as 0.1 mg of protein per reaction in the region of the critical amount halved the state 3 rate. It was also noted with beet root and cauliflower mitochondria (Fig. 1) that in addition to the sharp decrease in state 3 respiration for reactions containing protein below the critical concentration, further reduction in the amount of protein resulted in an increase in the state 3 respiration rate and, in the case of mitochondria from cauliflower, enhanced respiratory control.

Although it was not the purpose of this study to make a detailed investigation of the factors associated with the observed decrease in respiration rate at low protein concentration, it was found that the addition of cytochrome *c* (6.0 μ g per reaction) partially overcame the reduction in state 3 respiration caused by low concentrations of protein but did not restore respiration to the level obtained with higher amounts of protein. Cytochrome *c* was not limiting state 3 respiration at protein concentrations in excess of the critical amount since no stimulation was observed on its

² Abbreviation: BSA: bovine serum albumin.

addition to these reactions. Thus, with a single preparation of mitochondria, if reactions are carried out at protein concentrations above the critical level, the mitochondria exhibit the essential criteria for "intactness" defined by Bonner (2). But, below this critical level of protein concentration, the mitochondria do not exhibit the same degree of intactness and the rate of respiration is dependent on the reaction conditions rather than on the physiological properties of the mitochondria.

Effect of Time of Storage on Activity of Isolated Mitochondria. Sarkissian and Srivastava (13) have shown that the changes in respiration rate which occur during storage at 0 C vary depending on the method used to isolate the mitochondria. Thus, in comparative studies, real differences in the activity of mitochondria from the same tissue can be easily masked by differential changes during storage. Since these differential changes would be further compounded in comparative studies involving mitochondria from a variety of tissues, the change in activity with time of storage of mitochondria from a number of different tissues was investigated.

As shown in Figure 2, marked differences in the changes which occurred with time after isolation were observed in the mitochondria from the different tissues studied. Mitochondria from avocado (Fig. 2) exhibited a decline in state 3 respiration over a period of 4 hr, as did those from tomato, pear fruit, beet root, and cauliflower buds. However, mitochondria from sweet potato (Fig. 2) and potato showed an initial rise in activity, within the 1st hr after isolation, after which time the activity declined. The magnitude of the rise in activity in the 1st hr of storage varied with different preparations of mitochondria from sweet potato and potato, but the increase was always observed. Little change was observed in the activity of mitochondria from cucumber fruit over a 5-hr period.

Because there was usually little change in the state 4 respiration during storage, when the state 3 respiration rate remained relatively constant (cucumber and sweet potato, Fig. 2), there was little change in the respiratory control ratio of the isolated mitochondria. However, where a marked decline in state 3 respiration

occurred, for example, avocado fruit (Fig. 2), there was an over-all decrease in the respiratory control ratio.

Differences in the rate of change occurring after isolation in both respiration rate and respiratory control of mitochondria from different tissues or even different portions of the same tissue (Fig. 2) probably reflect variations in the cellular content of compounds such as polyphenols which are released during maceration of the tissue and which are known to affect mitochondrial respiration (6). Deterioration of both respiration and respiratory control of mitochondria undoubtedly occurs during isolation. Palmer (10) and Sarkissian and Srivastava (13) have shown that the respiratory control of isolated mitochondria is diminished as the time involved in isolating the mitochondria increases. The decrease in both state 3 respiration and respiratory control shown by mitochondria isolated from avocado fruit, for example (Fig. 2), is probably a continuation of the deterioration process initiated during isolation. Hobson *et al.* (5) obtained mitochondria from the inner yellow portion of avocado fruit harvested at the hard green stage (early preclimacteric) which exhibited respiratory control, but they were unable to observe respiratory control with mitochondria isolated from the outer green tissue. The apparent disagreement between the results of Hobson *et al.* (5) and those shown in Figure 2 could easily reflect differences in the rate of deterioration of mitochondrial activity which occurs during isolation and storage.

CONCLUSION

Because of variability in both respiration rate and respiratory control of isolated mitochondria as a function of protein concentration and changes occurring during isolation and storage, it is apparent that extreme caution must be exercised in both the interpretation and the application of results obtained with isolated mitochondria. For example, the inability to demonstrate respiratory control in preparations of isolated mitochondria depends on the concentration of protein in the reaction mixture (Fig. 1) and the time of assay after isolation (Fig. 2) and does not necessarily reflect the true capacity of these mitochondria. Therefore, the absence of respiratory control and in some cases the magnitude of the respiratory control ratio of isolated mitochondria cannot be used as a criterion in relating the biochemical behavior of mitochondria to cellular metabolism. Similarly, the respiration rate of isolated mitochondria is influenced by these factors and, in making direct comparisons of mitochondrial respiration of preparations from different tissues, or even different preparations of the same tissue, these factors must be considered.

LITERATURE CITED

1. ATKINSON, M. R. R., M. BURTON, AND R. K. MORTON. 1961. Equilibrium constant of phosphoryl transfer from adenosine triphosphate to galactose in the presence of galactokinase. *Biochem. J.* 78: 813-820.
2. BONNER, W. D., JR. 1967. A general method for the preparation of plant mitochondria. In: R. W. Estabrook and M. E. Pullman, eds., *Methods in Enzymology*, Vol. 10. Academic Press, New York. pp. 126-133.
3. ESTABROOK, R. W. 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. In: R. W. Estabrook and M. E. Pullman, eds., *Methods in Enzymology*, Vol. 10. Academic Press, New York. pp. 41-47.
4. *Handbook of Chemistry and Physics*, 1960. C. D. Hodgman, ed. Chemical Rubber Publishing Co., Cleveland, Ohio. Ed. 42, p. 1706.
5. HOBSON, G. E., C. LANCE, R. E. YOUNG, AND J. B. BIALE. 1965. Metabolic processes in cytoplasmic particles of the avocado fruit. VIII. Particles from different regions of the fruit. *Biochim. Biophys. Acta* 133: 605-607.
6. HULME, A. C. AND J. D. JONES. 1963. Tannin inhibition of plant mitochondria. In: J. B. Pridham, ed., *Enzyme Chemistry of Phenolic Compounds*. Macmillan and Company, New York. pp. 97-120.
7. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
8. LYONS, J. M. AND J. K. RAISON. 1970. Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. *Plant Physiol.* 45: 386-389.
9. MILLARD, D. L., J. T. WISKICH, AND R. N. ROBERTSON. 1965. Ion uptake and

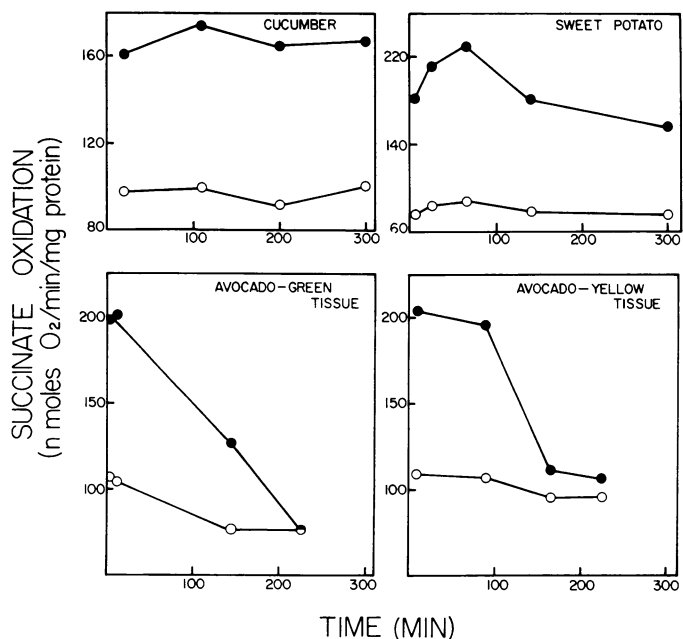


FIG. 2. Changes in the state 3 (●) and state 4 (○) rates of respiration of mitochondria with time of storage. The isolated mitochondria were stored as a suspension (6-50 mg of mitochondrial protein per ml) in ice for the times indicated and allowed to react at 25 C. The point shown as ● is as indicated in Figure 1.

- phosphorylation in mitochondria: Effect of monovalent ions. *Plant Physiol.* 40: 1129-1135.
10. PALMER, J. M. 1967. Rapid isolation of active mitochondria from plant tissue. *Nature* 216: 1208.
11. ROMANI, R. J., AND I. K. YU. 1967. Mitochondrial resistance to massive irradiation in vivo. III. Suppression and recovery of respiratory control. *Arch. Biochem. Biophys.* 117: 638-644.
12. ROMANI, R. J., I. K. YU, AND L. K. FISHER. 1969. Isolation of tightly coupled mitochondria from acidic plant tissues. *Plant Physiol.* 44: 311-312.
13. SARKISSIAN, I. V. AND H. K. SRIVASTAVA. 1968. On methods of isolation of active, tightly coupled mitochondria of wheat seedlings. *Plant Physiol.* 43: 1406-1410.
14. VERLEUR, J. D. AND I. URITANI. 1965. Respiratory activity of the mitochondrial fractions isolated from healthy potato tubers and from tissue incubated after cutting or infection with *Ceratocystis fimbriata*. *Plant Physiol.* 40: 1008-1012.