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Laboratory Exercises

Teaching About Citric Acid Cycle Using Plant Mitochondrial Preparations

SOME ASSAYS FOR USE IN LABORATORY COURSES*

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Potato tubers and turnip roots were used to prepare purified mitochondria for laboratory practical work in the teaching of the citric acid cycle (TCA cycle). Plant mitochondria are particularly advantageous over the animal fractions to demonstrate the TCA cycle enzymatic steps, by using simple techniques to measure O_2 consumption and transmembrane potential ($\Delta\Psi$). The several TCA cycle intermediates induce specific enzyme activities, which can be identified by respiratory parameters. Such a strategy is also used to evidence properties of the TCA cycle enzymes: ADP stimulation of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase; activation by citrate of downstream oxidation steps, *e.g.* succinate dehydrogenase; and regulation of the activity of isocitrate dehydrogenase by citrate action on the citrate/isocitrate carrier. Furthermore, it has been demonstrated that, in the absence of exogenous Mg^{2+}, isocitrate-dependent respiration favors the alternative oxidase pathway, as judged by changes of the ADP/O elicited by the inhibitor *n*-propyl galate. These are some examples of assays related with TCA cycle intermediates we can use in laboratory courses.

Keywords: Plant mitochondria, O_2 consumption and $\Delta\psi$ determinations, TCA cycle features.

Owing to the simplicity of the isolation process, most studies of bioenergetics are carried out with isolated mitochondrial fractions [1]. Plant mitochondrial fractions, easily obtained with high yield of purity, offer advantages over animal fractions [2, 3]. Among about 30 different plant tissues already tested [4], potato tubers and turnip roots were selected for isolation of mitochondrial fractions owing to the high purity of fractions and the stable functional activities [3]. These mitochondria last longer and exhibit superior performance of respiration activities with several substrates, as compared with animal preparations [3]. Therefore, the use of plant mitochondrial fractions is advantageous in biochemical studies, *e.g.* bioenergetics and metabolism, for practical works in undergraduated and graduated curricula.

In potato tuber and turnip root mitochondria, the activities dependent on the following citric acid (TCA)[1] cycle enzymes were assayed: malate dehydrogenase, NAD^+-dependent malic enzyme, aconitase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. The appropriate substrates for these activities, yielding ubiquinol (succinate dehydrogenase) or NADH (the remaining enzyme steps), activate Complex II and Complex I, respectively, triggering respiration (oxygen consumption) and the coupled transmembrane potential [5]. Respiratory indices report the bioenergetic competence of tested mitochondria and also characterize each intermediate activity. Enzyme properties of TCA cycle intermediate steps were also evaluated with the methodology proposed in this work.

The present project is directed to teachers and instructors of laboratory practicals, but is not immediately suitable for use by the students. For instance, after presentation of the TCA cycle scheme (Fig. 1), students are motivated to experimentally demonstrate activities of each TCA cycle intermediate by using mitocondrial preparations to access respiration parameters. Properties of the TCA cycle enzymatic activities can be previously given to the students knowledge and then demonstrated in assays performed with plant mitochondria, as those expressed in this work. Or, alternatively, records and parameters can be produced and then considered for discussion and interpretation.

EXPERIMENTAL PROCEDURES

Young potato tubers *(Solanum tuberosum* L.) and turnip roots (*Brassica napus* L.) were peeled to expose a clean tissue kept in distilled water. All operations are performed at 0–4 °C. The tissue was homogenized in a juicer (Moulinex) at 2 g of fresh weigh per milliliter of homogenization medium (A) containing 250 mM sucrose, 2 mM EDTA, 40 mM HEPES (pH 8.1) (or 20 mM for turnip), 0.1% bovine serum albumin (BSA), and 4 mM cysteine (added just before homogenization) [6, 7]. After filtration through four to six

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[1] The abbreviations used are: TCA, citric acid; BSA, bovine serum albumin; TPP, tetraphenylphosphonium; RC, respiratory control.

cheesecloth layers (for potato, a previous centrifugation at 400 × *g* for 5 min is required to remove large amounts of starch), the homogenate was centrifuged at 3,500 × *g* for 15 min and the supernatant was centrifuged again at 10,000 × *g* for 20 min (crude mitochondria). The final pellet was resuspended and applied to a 22% Percoll gradient (containing 300 mM mannitol, 10 mM HEPES, 0.1 mM EDTA, at pH 7.2) and centrifuged for purification of the crude mitochondria [6]. The mitochondrial fraction (a dense brownish layer close to the bottom) was collected from the Percoll gradient with a Pasteur pipette and washed twice, by centrifugation, at 28,000 × *g* for 5 min (or 10,000 × *g* for 15 min) in washing medium (B) containing 250 mM sucrose, 0.1% BSA, and 10 mM HEPES (pH 7.2). The pellet was gently resuspended in medium B at a protein concentration of 20–40 mg/ml. Protein was determined by the procedure of Bradford [8] calibrated with BSA standards.

Oxygen consumption was monitored with a Clark oxygen electrode [9], at 25 °C with stirring. The polarographic measurements were performed in 1.5 ml of reaction medium (C) containing 250 mM sucrose, 10 mM HEPES (pH indicated in Table I for each TCA cycle enzyme), 20 mM KCl, 2 mM K_2HPO_4, 4 mM $MgCl_2$, and 0.1% BSA. Respiration rates were calculated taking an oxygen concentration of about 250 nmol O_2/ml in the experimental medium at 25 °C [10]. The mitochondrial transmembrane electric potential ($\Delta\psi$) was monitored with a TPP^+-electrode prepared according to Kamo *et al.* [11]. Tetraphenylphosphonium cation (TPP^+) distributes across the mitocondrial membrane, according to the transmembrane potential, *i.e.* the distribution follows a Nernstian behavior [1]. Therefore, from the distribution followed with a specific electrode (TPP^+-electrode), it has been possible to estimate the $\Delta\psi$ by means of the Nernst equation. All experiments were carried out in an open vessel at 25 °C, with efficient magnetic stirring in 1.5 ml of medium C, supplemented with 3 μM TPP^+. About 0.3 mg/ml mitochondrial protein were used in reaction medium supplemented with the additions indicated in the legends of figures. Membrane potentials were calculated using the equation developed by Kamo *et al.* [11] [$\Delta\psi = 59\log (v/V) - 59\log (10^{(\Delta E)/59} - 1)$, where v = mitochondrial internal volume, and V = external volume of the assay medium]. The deflection corresponding to the TPP^+-electrode potential (ΔE) was determined according to the size of the trace displaced as a consequence of valinomycin-induced K^+ diffusion, by using 30 ng/ml valinomycin at the end of each assay to collapse the transmembrane potential.

Tetraphenylphosphonium chloride (TPP^+) was purchased from Merck (Whitehouse Station, NJ), and substrates, cofactors, and other reagents were purchased from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

TCA Cycle Enzymatic Steps Reflected in Mitochondrial Respiratory Activity—Shown results were obtained with potato and turnip mitochondria, after purification in a Percoll gradient. However, similar data were obtained with crude mitochondria washed with medium B by centrifugation at 8,500 × *g* for 15 min. Washed mitochondria, saving about 2 h of the isolation time, are preferred for the use in experimental work during the day of isolation. For assays to be carried out in several consecutive days, Percoll-purified mitochondrial fractions are required. The purified fractions, kept at ice-cold temperature, sustain good respiratory activities for several days [3].

The TCA cycle is represented in Fig. 1, with indication of the examined steps. The enzymatic activities of TCA cycle were elicited by adding pyruvate plus malate (malate and pyruvate dehydrogenases), malate (NAD^+-dependent malic enzyme), citrate (aconitase plus isocitrate dehydrogenase), isocitrate (isocitrate dehydrogenase), and α-ketoglutarate (α-ketoglutarate dehydrogenase). These substrates yield NADH, which is readily oxidized by Complex I, triggering O_2 consumption. Succinate dehydrogenase activity is also detected by O_2 consumption due to oxidation of succinate to fumarate by Complex II. The main TCA cycle enzymatic steps are conveniently followed on the basis of the O_2 consumption with a Clark oxygen electrode.

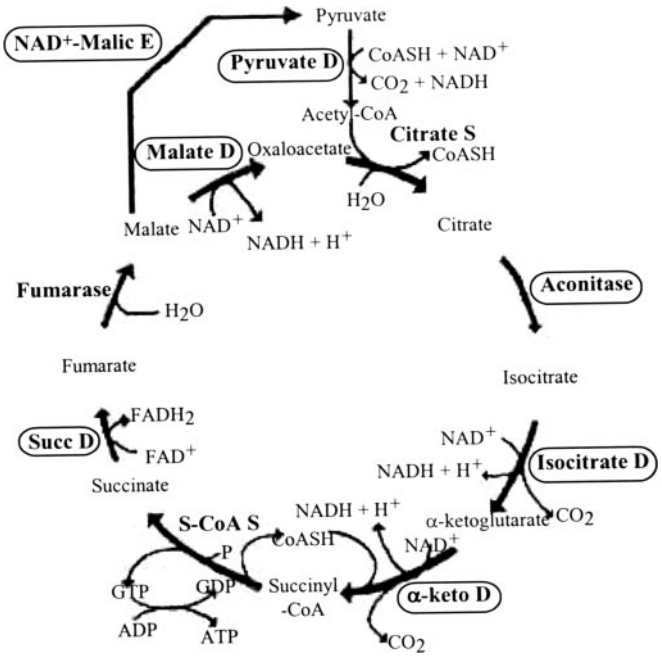

FIG. 1. **Citric acid cycle.** Enzymes tested in this work are boxed, namely: *NAD^+ malic E*, NAD^+ malic enzyme; *Pyruvate D*, pyruvate dehydrogenase; *isocitrate D*, isocitrate dehydrogenase; *α-keto D*, α-ketoglutarate dehydrogenase; *Succ D*, succinate dehydrogenase; *Malate D*, malate dehydrogenase. (Adapted from Dennis and Turpin [11].)

In Table I, optimal concentrations of substrates (TCA cycle intermediates), cofactors, optimal pH, and mitochondrial protein amounts are provided for studying each step of the TCA cycle. The cofactors thiamine pyrophosphate (for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase) and NAD^+ have specific transporters in plant mitochondria [12], at variance with animal fractions. Therefore, the cofactors are released during the isolation procedure, requiring further addition during the assays. Activities of all NAD^+-dependent enzymes often increase with thiamine pyrophosphate and NAD^+, evidencing the existence of transporters for the cofactors [13].

Activities related with the TCA cycle intermediates can be followed in two consecutive days, except for citrate (aconitase plus isocitrate dehydrogenase), because aconitase is easily inactivated by the release of a Fe^{2+} from its Fe-S cluster in less than 24 h [14]. Isocitrate dehydrogenase sustains its activity at the second day after isolation of mitochondria, as shown in Fig. 4, where the only intermediate of the TCA cycle used is exogenous isocitrate.

In Fig. 2, the oxidations of succinate and NADH from several TCA cycle steps are processed with different performances, reflected in values of several parameters: RC (respiratory control), the respiratory rate attained during maximal ATP synthesis or in the presence of a proton translocater, divided by the rate in the absence of ATP

TABLE I

Optimal conditions for assaying O_2 consumption or corresponding elicited $\Delta\psi$ induced by the respiratory substrate produced at each TCA cycle step

These conditions were defined using potato or turnip Percoll-purified or washed mitochondria. For good results, pyruvate and NAD^+ solutions should be freshly prepared. Tpp, thiamine pyrophosphate.

Step of TCA cycle	Substrates	Cofactors	pH	mg protein/ml
Succinate dehydrogenase	Succinate (5 mM)	ATP (0.2 mM)	7.2	0.2–0.3
Malate dehydrogenase	Pyruvate (20 mM) + malate (2 mM)	NAD^+ (0.5 mM) + Tpp (0.5 mM) + ATP (0.2 mM)	7.5	0.3–0.5
NAD^+-malic enzyme	Malate (20 mM)	NAD^+ (0.5 mM) + Tpp (0.5 mM) + ATP (0.2 mM)	6.5	0.3–0.5
α-Ketoglutarate dehydrogenase	α-Ketoglutarate (2 mM)	NAD^+ (0.5 mM) + Tpp (0.5 mM) + ATP (2 mM) + ADP 100 μM)	7.2	0.3–0.5
Aconitase plus isocitrate dehydrogenase	Citrate (2 mM)	NAD^+ (0.5 mM) + Tpp (0.5 mM) + ATP (2 mM)	7.2	0.3–0.5
Isocitrate dehydrogenase	Isocitrate (4 mM)	NAD^+ (0.5 mM) + Tpp (0.5 mM) + ATP (2 mM)	7.2	0.3–0.5

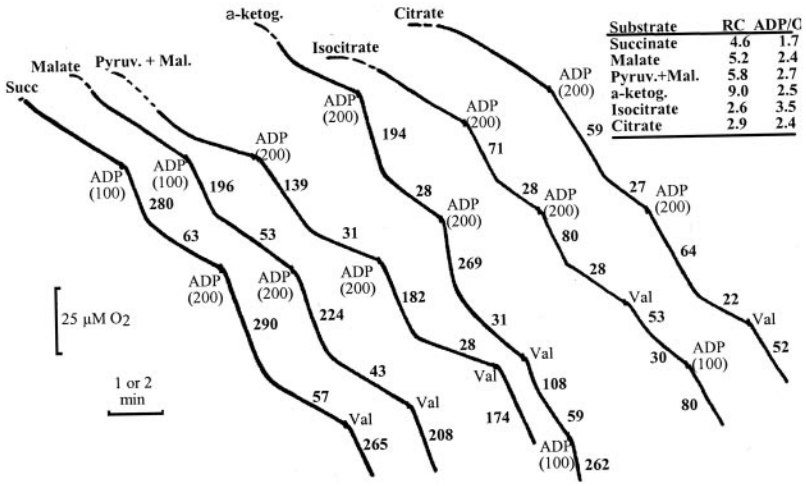

FIG. 2. **Respiratory activities of purified potato mitochondria.** Assays were performed in the conditions indicated in Table I for each step of TCA cycle. ADP was added where indicated, at the concentration expressed in parenthesis in μM. *Val.*, addition of 15 ng/ml valinomycin. The time scale corresponds to 1 min for succinate and 2 min for the other substrates. Numbers on the lines express activities in nmol O_2·mg protein^{-1}·min^{-1}. *Inset*, RC and ADP/O are indicated for the substrate of each step of TCA cycle. *Succ*, succinate; *Pyruv.+ Mal.*, pyruvate plus malate; *a-ketog.*, α-ketoglutarate.

synthesis (no ADP) or proton translocator [1]; ADP/O, ratio of ADP phosphorylated per oxygen consumed; V_{max}, maximal activities, etc. These parameters are closely related with the enzymatic activities at the level of the TCA cycle, according to the added substrates. The ADP/O ratio, indicating the phosphorylation efficiency of respiration dependent on each step of the TCA cycle, is similar for activities dependent on citrate, α-ketoglutarate, malate, and piruvate+malate (2.5 $\pm$ 0.2), because they all are dependent on the oxidation of a common product, NADH. However, different maximal respiration activity rates of state 3 are recorded for each TCA cycle intermediate. For instance, α-ketoglutarate-dependent activity (269 nmol O_2·mg^{-1}·min^{-1}) is significantly higher than citrate-dependent activity (64 nmol O_2·mg^{-1}·min^{-1}). Also, a very high RC value is recorded for α-ketoglutarate-dependent activity (9.0), at variance with a low value for citrate-dependent activity (2.9), and a middle range value for succinate-dependent activity (4.6). Usually, RC is an empirical parameter for assessing the integrity of mitochondrial membrane. However, in the present situation, the RC values also express differences in the TCA cycle enzyme activities, otherwise they should not be so significantly different for the different TCA cycle intermediate activities.

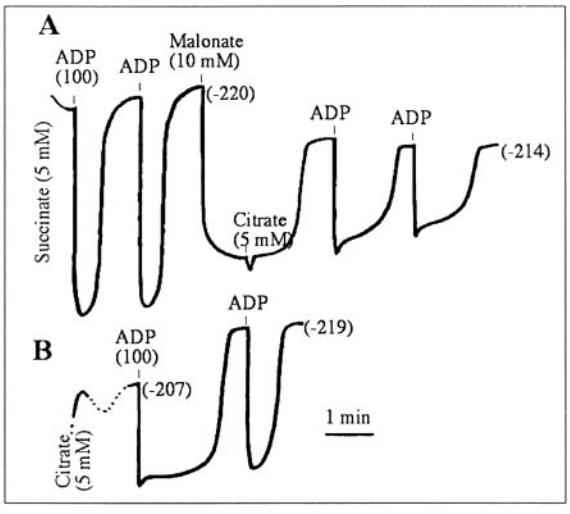

FIG. 3. **Detection of putative succinate production in potato mitochondria respiring citrate.** *A*, comparative phosphorylations before malonate addition (succinate-dependent) and after malonate addition (citrate-dependent). *B*, citrate-dependent phosphorylation paterns of two consecutive additions of 100 μM ADP. Numbers in parenthesis indicate $\Delta\psi$ in mV, at the corresponding positions.

Exemplifying Features of TCA Cycle—Several questions may be raised to understand the reason for the experimental results. It is possible that a particular TCA cycle activity may reflect the result of further activities of consecutive steps of TCA cycle. Starting with specific substrate(s) for a certain enzymatic step, can we demonstrate activities of

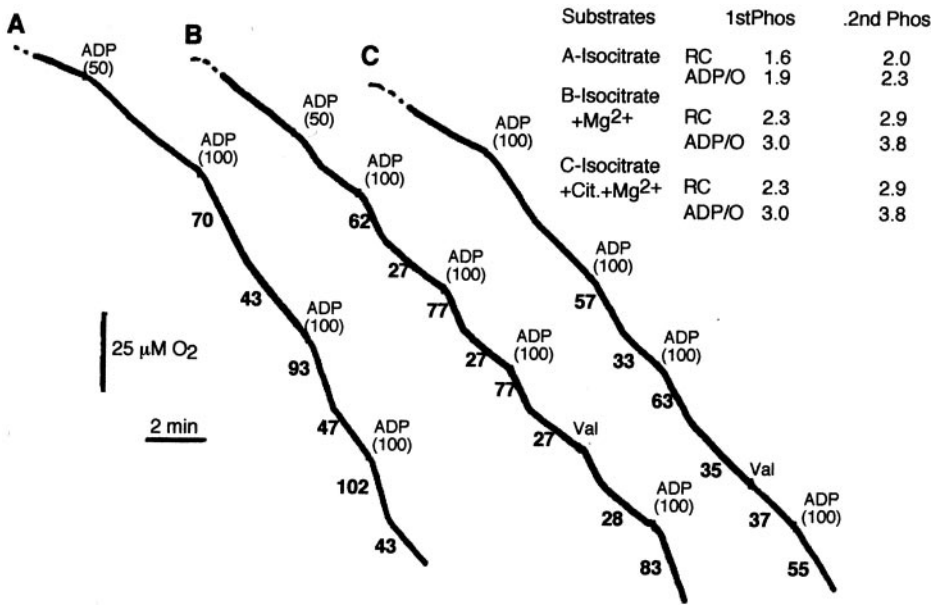

FIG. 4. **Isocitrate dehydrogenase activity detected by O_2 consumption of respiring potato mitochondria.** Assays were performed at the second day following isolation of purified mitochondria. *A*, isocitrate (4 mM) (without $MgCl_2$); *B*, isocitrate + $MgCl_2$ (4 mM); *C*, isocitrate + $MgCl_2$ + citrate (4 mM). ADP was added at the concentration indicated in parenthesis, in μM. *Val.*, addition of 15 ng/ml valinomycin. Numbers on the lines express activities in nmol O_2·mg protein^{-1}·min^{-1}. *Inset*, values of RC and ADP/O for two oxidative phosphorylations of ADP.

downstream steps? This question is approached in Fig. 3 reporting mitochondrial transmembrane potentials (TPP^+ electrode traces) dependent on the oxidative phosphorylation efficiency. Depolarization occurs after ADP addition followed by repolarization indicating the full phosphorylation of added ADP. This permits evaluation of the time required for the phosphorylation of 100 μM ADP and, therefore, determination of the phosphorylation rate. Thus, a consistent higher phosphorylation rate (Fig. 3*A*) was detected with succinate, as compared with citrate when succinate dehydrogenase is inhibited by malonate addition. However, in the absence of malonate (Fig. 3*B*), after a previous low rate citrate-dependent phosphorylation, a further elicited phosphorylation is as fast as the phosphorylation promoted by succinate oxidation. After one or more citrate-dependent events, succinate is produced, as demonstrated by others [15], and then used to fuel additional phosphorylations.

It is also apparent that citrate (4 mM), in conditions of active or inactive aconitase (at the second day, aconitase is not active, as referred before), inhibits isocitrate-dependent state 3 and state 4 activities and also RC and ADP/O (Fig. 4). Therefore, citrate, by itself, regulates the activity of isocitrate dehydrogenase, probably as a consequence of citrate/isocitrate competition, at the transporter level [16]. Finally, especially in Fig. 4*B*, we observe that, after valinomycin addition (phosphorylation inhibited), ADP stimulates isocitrate-dependent O_2 consumption with high activity. This agrees with the known allosteric regulation of isocitrate dehydrogenase by ADP [17].

The used methodology also allows assessment to other regulations of mitochondrial metabolic activity. Shown in Fig. 4, *A* and *B*, isocitrate-dependent ADP/O and RC are lower in the absence than in the presence of Mg^{2+}. Probably, in the absence of cytosolic Mg^{2+}, the alternative oxidase pathway is favored [18], because the addition of the alternative oxidase inhibitor *n*-propyl galate to isocitrate-respiring mitochondria increases ADP/O and RC (results not shown) to the levels obtained in the presence of Mg^{2+}.

The described methodology is quite appropriate for a fast and simple demonstration of the main steps of the TCA cycle activities and other related mitocondrial metabolic activities, particularly effective for the teaching of mitocondrial metabolism.

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