RESPIRATION AND PHOSPHORYLATION OF MITOCHONDRIA FROM NORMAL AND CROWN-GALL TISSUE CULTURES OF TOMATO[1, 2]

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Knowledge of the initiation mechanism of the crown-gall disease incited by *Agrobacterium tumefaciens* (Smith & Town.) and the subsequent growth of the abnormal tissue is fundamental to understanding diseased growth (2, 18, 24). The nutritional requirements for normal and gall tissues have been intensively studied (25), and the necessity for proper balance of amino acids and growth substances in the gall formation has been emphasized (2, 23). Braun (3) has suggested that conversion of a normal cell into a crown-gall cell is accompanied by the permanent activation of a series of growth-substance-synthesizing systems, which are precisely regulated in all normal cells. Similarly, Klein (17) has proposed stepwise activation of metabolic systems, such as respiration as well as protein and nucleic acid syntheses, during the period of transformation of a normal cell into a tumor cell.

Biochemical analyses are needed to clarify further the metabolic characteristics of the diseased growth. Experiments are especially interesting with isolated enzymes or enzyme systems from normal and diseased tissues. Recent results with plant tissue cultures in studies of cell cytology and physiology (16), and pathology (12, 36) have made attractive the use of such cultures for biochemical analyses.

Earlier respiratory studies of normal and crown-gall tissues were concerned largely with tissue slices or suspensions of intact cells from tissue cultures at various stages of growth. In general, reduced respiratory levels were observed with crown-gall tissue (24). The results, however, were not always consistent; they were dependent upon the basis for expressing the respiratory activity (2).

The purpose of the present paper is to report the oxidative and phosphorylative properties of mitochondria isolated, respectively, from normal and crown-gall tissue cultures of tomato. Portions of the work already have been summarized (31, 33).

MATERIALS AND METHODS

TISSUE CULTURES: The tissue cultures were originally isolated from normal stem or crown gall on stems of tomato plants (*Lycopersicum esculentum* Mill var. Bonny Best). They had been cultivated for 5 years on a White's agar medium (35) modified by Hildebrandt et al (13), and supplemented with 2,4-dichlorophenoxyacetic acid and coconut milk (29). Both normal and crown-gall tissue cultures grew at similar rates on the above medium. Normal tissue cultures were unable to grow in the absence of coconut milk, whereas crown-gall tissue cultures maintained growth at a reduced rate (30).

PREPARATION OF MITOCHONDRIA: Tissue cultures 15 to 20 days old were used as a source of mitochondria. During this period, tissues were actively growing and had a relatively constant respiratory level [avg Qo_2 (N), 80] and respiratory quotient (avg R.Q., 0.9) (30). About 50 g of tissue were washed in 1 liter of M/200 phosphate buffer, pH 7.4, for a few minutes to remove agar, salts and other components of the medium which might be on the surface of the cells. The suspension of cells was then filtered on four layers of cheesecloth. Any necrotic tissue was removed. The washed cells were ground in a glass homogenizer with a Teflon pestle in a solution containing 0.5 M mannitol, 0.005 M cysteine, 0.02 M KH_2PO_4 and sufficient tris-hydroxymethyl-aminomethane (tris) to bring the pH to 7.4. The homogenate was centrifuged at 500 G for 10 minutes to remove cell debris. The supernatant was centrifuged further at 5,000 G for 10 minutes, and the pellet was suspended in 0.4 M mannitol of pH 7.8 adjusted with tris. The suspension again was centrifuged first at low and then at high speeds. The final pellet from the high speed centrifugation was resuspended in a small amount of the 0.4 M mannitol, and this suspension was used as the mitochondrial preparation in the following experiments.

A high percentage of particles in the preparation was similar in appearance to mitochondrial particles seen with phase optics in living tomato cells in cul-

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ture. These particles were dark-brown, spherical bodies that ranged in size from 0.5 to 2 μ. No morphological differences were observed between mitochondrial particles from the normal and crown-gall tissue cultures. With a fraction spun down at 12,000 G for 10 minutes in 0.5 M sucrose solution, roughly half of the particles stained with Janus green B.

DETERMINATION OF OXYGEN CONSUMPTION: Oxygen consumption was measured with an oxygen electrode apparatus (9) which consisted of a rotating, polarizing, platinum electrode and a recording millivoltmeter. The change in concentration of oxygen in the reaction solution was detected as the change in the cathode reaction current which was converted into voltage and recorded automatically. The reaction cells used (9) were of 2.0 to 2.5 ml capacity with two narrow necks, one for inserting the electrode and the other for adding reaction components and subsequent stirring with a glass rod. Normally the reaction took place with the following amounts of materials per ml of the reaction mixture: 5.0 μmoles of inorganic orthophosphate, 0.5 μmoles of ethylenediamine tetraacetic acid, 5.0 μmoles of $MgCl_2$, 10.0 μmoles of KCl, 0.25 μmoles of mannitol, 2.5 μmoles of substrate and a mitochondrial suspension that contained 130 to 200 μg protein nitrogen. The concentration of oxygen in the air-saturated, isotonic medium was regarded as 240 mμ M at 26° C (6).

DETERMINATION OF PHOSPHORYLATION: The reaction mixture contained, in addition to the basal components mentioned above, radioactive inorganic orthophosphate (60,000 to 100,000 cpm/ml), adenosine triphosphate (ATP, 1 μmole/ml) and sufficient hexokinase and glucose. An aliquot of 0.1 ml of the reaction mixture was withdrawn soon after adding the mixture of ATP, glucose and hexokinase. The second and third 0.1 ml aliquots were taken at the end of the reaction after all oxygen in the reaction mixture had been consumed. Esterified P^{32} in each aliquot was analyzed according to the chromatographic method of Hagihara and Lardy (10). The oxygen consumption between the first and second (or third) samplings was usually 300 to 400 mμatoms, and the time elapsed was 3 to 6 minutes. From a few reaction mixtures with a low rate of respiration, the second and third aliquots were taken before oxygen consumption was completed to avoid introducing an error from air diffusion.

PREPARATION OF ASCORBIC ACID OXIDASE: Ascorbic acid oxidase was extracted from the cell debris fraction of crown-gall tissue cultures by treatment with 0.8 M $(NH_4)_2SO_4$ (pH 7.0 with NH_4OH) for 12 hours at 5° C. After extraction the cell debris was removed by centrifugation. Ammonium sulfate was added to the supernatant until the concentration was 1.6 M. The precipitate was discarded. The soluble fraction was dialyzed against M/15 phosphate buffer, pH 7.0, and then used as the ascorbic acid oxidase preparation.

RESULTS

Typical respiratory responses of mitochondria from normal and crown-gall tissue cultures to various substrates, to cofactors and to inhibitors as traced by the oxygen electrode apparatus are illustrated in figure 1 with the same right to left direction as the record sheet. Starting at the upper right edge, the oxygen consumption by the endogenous respiration of the preparations was 0.8 mμatoms of oxygen/minute/ml of the reaction mixture. Upon addition of succinate, the rate was increased to 13.6 and 11.7 mμatoms, respectively, for normal and crown-gall particles. These rates were accelerated 2.4- and 1.4-fold, respectively, by adding adenosine diphosphate (ADP). Malonate inhibited the succinate oxidation completely. Oxidation of citrate occurred in the presence of malonate and was enhanced by adding diphosphopyridine nucleotide (DPN). Reduced diphosphopyridine nucleotide (DPNH) was oxidized rapidly, as indicated by a sharp change in slope of the curves after its addition. Antimycin A strongly inhibited DPNH oxidation, whereas ascorbate was oxidized rapidly in the presence of Antimycin A.

Figure 1 shows that the mitochondrial preparations from normal tissue cultures oxidized succinate, citrate and DPNH more rapidly, but ascorbate more slowly, than the mitochondrial preparations from crown-gall tissue cultures, as discussed later.

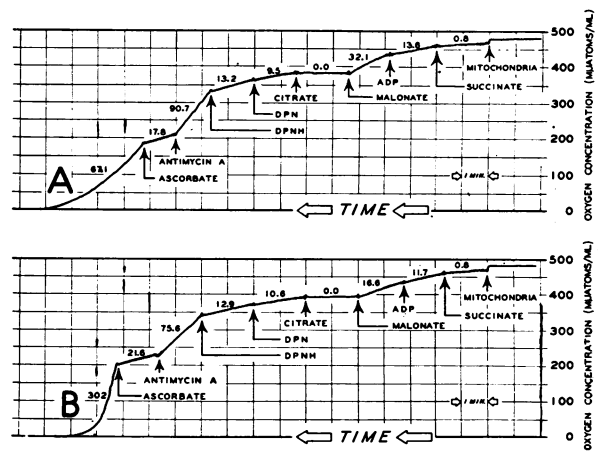

FIG. 1. Respiration curves at 26° C of mitochondria from normal and crown-gall tissue cultures of tomato. Recordings with Varian recorder. Figures in mμatoms/min/ml. Total reaction mixture 2.2 ml. Recording of the reactions was from right to left as traced from the original records.

A: Respiration curve of normal tissue mitochondria. Mitochondria, 134 μg N in 0.28 ml; succinate, 2.8 μmoles/ml; ADP: 0.2 μmole/ml; malonate, 14 μmoles/ml; citrate, 2.8 μmoles/ml; DPN; 0.5 μmole/ml; DPNH: 0.7 μmole/ml; Antimycin A, 0.4 μg/ml; ascorbate, 2.8 μmoles/ml. B: Respiration curve of crown-gall mitochondria. Mitochondria, 146 μg N in 0.28 ml. All others same as above (A).

OXIDATION OF KREBS CYCLE ACIDS AND DPNH: Table I shows rates of oxidation of DPNH, ascorbic acid, and the principal acids of the Krebs cycle by mitochondria from normal and crown-gall tissue cultures. These figures are based on several measurements for which a single substrate was tested to avoid the effects of other substrates, inhibitors, or change in oxygen tension which might be encountered in an experiment employing multiple compounds as in figure 1. Mitochondria from normal cultures oxidized the Krebs cycle acids tested more rapidly than mitochondria from crown-gall tissue cultures. With both normal and crown-gall particles the rates of oxidation of citrate, α-ketoglutarate, malate, and glutamate varied from preparation to preparation, but usually fell in the range of 0.4 to 0.8, when the rate of succinate oxidation was set to 1.0. DPNH was oxidized rapidly (about 7 times faster than succinate) both by normal and crown-gall mitochondria.

Mitochondria from both normal and crown-gall tissue cultures responded similarly to the cofactors and inhibitors tested (figs 1 & 2). Adding DPN enhanced oxidation of citrate, malate, α-ketoglutarate and glutamate 1.4- to 2-fold depending upon the preparation. The oxidation of DPNH was not affected by adding DPN or cytochrome c (fig 2, B), although the latter enhanced the oxidation rate about twofold when mannitol was omitted from the reaction mixture. Cytochrome c added to sucrose preparations of normal and crown-gall mitochondria increased the rate of oxidation of all the Krebs cycle acids tested by about 10 %. The oxidations of DPNH and of the Krebs cycle acids tested were inhibited about 90 % by Antimycin A (0.4 μg/ml) and cyanide (0.1 μmole/ml).

TABLE I

OXIDATION OF VARIOUS SUBSTRATES BY MITOCHONDRIA FROM NORMAL AND CROWN-GALL TISSUE CULTURES OF TOMATO

SUBSTRATE	NORMAL TISSUE		CROWN-GALL	
	O_2 UPTAKE Mμ ATOMS/ MIN/MG N	RELATIVE ACTIVITY*	O_2 UPTAKE Mμ ATOMS/ MIN/MG N	RELATIVE ACTIVITY*
Succinate	180	1.0	154	1.0
Citrate**	142	0.79	125	0.81
α-Ketoglutarate**	110	0.61	94	0.61
Malate**	85	0.47	75	0.49
Glutamate**	125	0.69	117	0.76
DPNH	1,250	6.9	1,090	7.1
Ascorbate	1,560	8.7	6,940	45.0

* Relative activity = Oxidation rate of substrate/ Oxidation rate of succinate.

** In the presence of DPN.

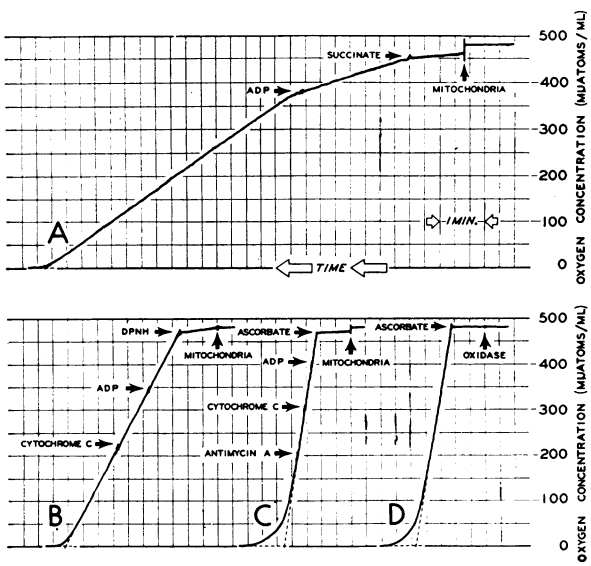

FIG. 2. Effect of ADP, cytochrome c, Antimycin A, and oxygen concentration on oxidation of succinate, DPNH, and ascorbate. Recordings with Brown recorder. Time scale for B, C, and D same as for A. Figures in mμatom/min/ml. Others same as in figure 1.

A: Oxidation of succinate by normal tissue mitochondria. B: Oxidation of DPNH by normal tissue mitochondria. C: Oxidation of ascorbate by crown-gall mitochondria. D: Oxidation of ascorbate by ascorbic acid oxidase extracted from cell debris fraction of crown-gall tissue cultures.

OXIDATION OF ASCORBIC ACID: Mitochondria from normal and crown-gall tomato tissue cultures oxidized ascorbate very rapidly; nine times faster than succinate by normal tissue mitochondria and 45 times faster by crown-gall mitochondria (table I). In contrast to the oxidation of Krebs cycle acids, ascorbic acid was oxidized by mitochondria from crown gall 4.5 times faster than by mitochondria from normal tissue. The mitochondrial oxidation of ascorbate was neither enhanced by DPN, ADP, or cytochrome c, nor inhibited by Antimycin A (fig 2,C). The addition of cyanide inhibited ascorbate oxidation. The minimum concentration of cyanide to cause maximum inhibition of ascorbate oxidation was 1.1 μmoles/ml, a concentration about ten times that required for maximum inhibition of DPNH oxidation (fig 3,A & B). Since the cell debris fraction of the tomato tissue cultures contained high ascorbic acid oxidase activity, ascorbic acid oxidase was extracted from this fraction. The sensitivity of mitochondrial oxidase to cyanide was similar to that of extracted ascorbic acid oxidase (fig 3,B & C).

The rate of ascorbic oxidation by the mitochondrial preparations decreased as the partial pressure of oxygen in the reaction mixture decreased (fig 2,C). Extracted ascorbic acid oxidase reacted in the same fashion (fig 2,D). This indicates a low affinity of the catalyst for oxygen. This contrasted with the oxidation of succinate or DPNH, in which the rate of oxidation was independent of the oxygen tension (fig 2,A & B).

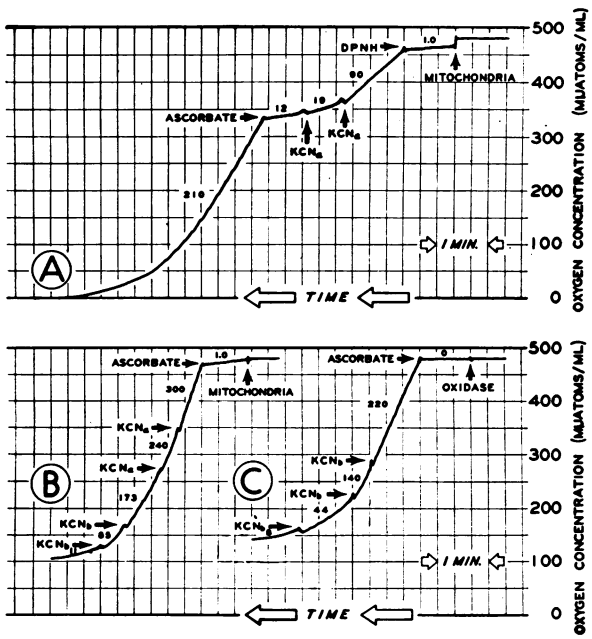

FIG. 3. Effect of cyanide on oxidation of DPNH and ascorbate by tomato tissue culture mitochondria and purified ascorbic acid oxidase. Recordings with Brown recorder. Figures in mμatoms/min/ml. KCN_a: KCN, 0.06 μmole/ml; KCN_b: KCN, 0.48 μmole/ml. Others same as in figure 1.

A: Oxidation of DPNH and ascorbate by crown-gall mitochondria. B: Oxidation of ascorbate by crown-gall mitochondria. C: Oxidation of ascorbate by ascorbic acid oxidase extracted from cell debris fraction of crown-gall tissue cultures.

EFFECT OF PHOSPHATE ACCEPTOR: Adding ADP or a mixture of ATP, glucose, and hexokinase increased the rate of oxidation of the substrates listed in table I except for DPNH (fig 2,B) and ascorbate (fig 2,C). With normal tissue particles, the increases were 1.6- to 2.5-fold (respiratory control ratios) for succinate (fig 1, fig 2,A), and 1.3- to 2-fold for the other substrates. In two experiments, 3.3- and 3.6-fold increases were observed for succinate oxidation. In comparison with the above, crown-gall particles gave lower respiratory control ratios; less than two for succinate oxidation.

The acceleration of respiration by ADP continued until all the oxygen in the reaction mixture was consumed, and there was no cessation of the enhanced respiration after the added ADP was supposedly exhausted by phosphorylation.

OXIDATIVE PHOSPHORYLATION: Table II shows phosphorylating efficiencies of mitochondrial preparations, respectively, from normal and crown-gall tissue cultures supplied succinate and citrate as substrates. With each substrate, particles from crown-gall gave considerably lower P/O ratios than did particles from normal tissue.

The P/O ratio in DPNH oxidation was less than half that obtained in succinate oxidation. No appreciable phosphorylation was observed to be coupled with oxidation of ascorbate.

DISCUSSION

In biochemical work on plant mitochondria, fast growing tissues from seedlings generally have been employed as the source of the particles (7). In the present study mitochondria from tomato cells cultivated in vitro were sufficiently active to examine respiratory and phosphorylative properties with the aid of an oxygen electrode apparatus.

A comparison of oxidation rates of Krebs cycle acids indicated that mitochondria from crown-gall tissue cultures had lower activity than mitochondria from normal tissue cultures. Crown-gall mitochondria also were lower in phosphorylating efficiency; the P/O ratio of crown-gall mitochondria was about 0.6 that of normal tissue particles. The lower oxidative and phosphorylative activities of crown-gall mitochondria contrast with the active growth of the tissue. This raises possibilities that A, crown-gall tissue cultures more efficiently utilize the energy produced than do normal tissue cultures, or B, crown-gall tissue cultures obtain energy other than through the energy-yielding system coupled with the oxidation of the Krebs cycle substrates.

The increase in respiration rate after ADP or phosphate acceptor (respiratory control) indicated tight coupling of the respiratory system to the phosphorylating system. This enhancing effect of a phosphate acceptor decreased with aging of mitochondria, and its decrease preceded a decrease in P/O ratio (11). Thus, the ability to exhibit respiratory control was a good indication of the quality of a mitochondrial preparation. The respiratory control has been shown with liver and kidney mitochondria (4- to 10-fold increase) (6, 11, 19), and to a lesser extent with particles isolated from heart muscle (4, 26, 27), ascites tumor cells (5), and certain plant species (7, 20). In succinate oxidation by mitochondria from normal tomato tissue cultures, the control ratio easily exceeded two; a value of 3.6 was obtained in one case. With crown-gall particles, on the other hand, the ratio rarely exceeded two.

TABLE II

COMPARISON OF PHOSPHORYLATING EFFICIENCIES OF MITOCHONDRIA FROM NORMAL AND CROWN-GALL TISSUE CULTURES OF TOMATO

EXP. NO.	SUBSTRATE	P/O RATIO	
		NORMAL TISSUE	CROWN-GALL
I	Succinate	1.0	0.66
	Citrate	1.1	0.67
II	Succinate	0.93	0.46
	Citrate	1.0	0.65

With rat liver mitochondria (6, 11) the ADP-enhanced respiration returned to the original rate when the added ADP was phosphorylated. Bonner and Ito (1) reported deceleration of respiratory rate with cauliflower mitochondria. This respiratory deceleration was not observed in the present experiments.

The rapid oxidation of ascorbate by mitochondria from normal and crown-gall tissue cultures deserves attention. In contrast to the Krebs cycle acids tested, ascorbate was oxidized more rapidly by mitochondria from crown-gall tissue cultures than by those from normal tissue cultures. The oxidation of ascorbate differed from the oxidation of the Krebs cycle acids or DPNH in A, insensitivity to Antimycin A, B, higher resistance to cyanide, and C, low affinity for oxygen (34). Thus, ascorbic acid oxidase seemed involved in this oxidation. In fact, purified ascorbic acid oxidase behaved similarly in a comparative experiment. Ascorbic acid oxidase has long been considered a soluble enzyme and has been highly purified from solution; however, recently at least some of its activity has been found to be associated with the cell surface (14, 22), and with mitochondria (21, 37). Since the cell debris fraction oxidized ascorbic acid rapidly, the mitochondrial oxidation of ascorbate from contaminating cell debris deserved consideration. However, the observations throughout the experiments supported the view that the mitochondria per se carried ascorbic acid oxidase.

Mitochondria from both normal and crown-gall tissue cultures oxidized DNPH rapidly (7 times as fast as succinate). Rapid oxidation of DPNH also has been reported with mitochondria from skunk cabbage (8), from lupine (15), and from many other plants (28). Spectrophotometric studies on DPNH oxidase, DPNH-cytochrome c reductase, cytochrome c oxidase, and diaphorase activities in mitochondria from normal and crown-gall tissue cultures are discussed in a forthcoming paper (32).

It may be concluded that the respiratory mechanisms of mitochondria from normal and crown-gall tissue cultures are quite similar. The only differences noted in their oxidative and phosphorylative activities were of a quantitative rather than a qualitative nature.

Summary

Oxidative and phosphorylative activities of mitochondria from normal and crown-gall tissue cultures of tomato were studied with an oxygen electrode. Mitochondria from crown-gall tissue cultures oxidized reduced diphosphopyridine nucleotide (DPNH), glutamate, succinate, citrate, malate, and α-ketoglutarate more slowly (about 0.9 times), but ascorbic acid more rapidly (4.5 times) than mitochondria from normal tissue cultures.

The phosphorylating efficiency of crown-gall mitochondria (P/O ratio, about 0.6 with succinate) was lower than that of normal tissue mitochondria (P/O ratio, about 1.0 with succinate). Adding a phosphate acceptor (adenosine diphosphate or glucose-hexokinase-adenosine triphosphate mixture) increased the rates of oxidation of the Krebs cycle acids tested, but not of DPNH and ascorbic acid. In succinate oxidation by normal tissue mitochondria, the enhancing effect by the phosphate acceptor was generally more than twofold (respiratory control ratio). With crown-gall mitochondria, on the other hand, the ratio rarely exceeded two.

The oxidation of ascorbic acid by the mitochondria from normal and crown-gall tissue cultures was compared to the oxidation by an ascorbic acid oxidase preparation; all the evidence obtained indicated that the mitochondria from both tissue cultures contained ascorbic acid oxidase.

Mitochondria from both normal and crown-gall tissue cultures responded similarly to cofactors (diphosphopyridine nucleotide, cytochrome c) and inhibitors (cyanide, Antimycin A).

In conclusion, the mitochondria from normal and crown-gall tissue cultures differed in their oxidative and phosphorylative activities in rather small quantitative rather than qualitative characteristics. Furthermore, mitochondria isolated from tomato tissue cultures, except for ascorbic acid oxidase activity, were similar in their respiratory properties to those from intact higher plants and animals.

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