OXIDATIVE ACTIVITY OF CYTOPLASMIC PARTICLES OF APPLES: ELECTRON TRANSFER CHAIN

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Some recent studies of apple respiration by Hackney (8) implied that the fruit may mediate its oxidations through a phenol oxidase system. In a later study Webster (21, 22) could find no evidence for a cytochrome oxidase system in apple tissue. However, more recently a number of reports have appeared which demonstrated cytochrome oxidase activity in apple tissue slices (14) and in cytoplasmic particles from apples (13, 16). There are also data showing Krebs cycle activity in cytoplasmic particles from apples (9, 18). Nevertheless, a number of workers are still experiencing considerable difficulty in isolating active cytoplasmic particles from apples (15). The difficulties might be traced to characteristics of the apple fruit, such as a highly acid cell sap (pH 3.5), an extremely low concentration of protein (approximately 0.3 %), and a high concentration of polyphenol substances that may injure cytoplasmic particles during homogenization. As a first step in isolating active cytoplasmic particles from such tissues it seemed advisable, as previously noted (13), to investigate the effect of homogenization in media of increasing pH.

This paper reports the results of such a study. Data are presented to show that alkaline homogenates yield the most active cytoplasmic particles. The properties of the particles from homogenates of different pH are described in some detail and spectra are presented demonstrating the presence of cytochrome b and cytochrome a_3 in the particles. These data and additional evidence from inhibitor studies lead to the conclusion that the apple particles isolated from alkaline homogenates contain the usual type of electron transfer chain.

MATERIALS AND METHODS

Malus sylvestris (Miller) Rome Beauty apples harvested during the first week of October, were used in these experiments. The fruit held in cold storage (0° C) and used during the storage season from October to May.

Three hundred grams of apple pulp were grated into a blendor cup containing 300 ml of cold 0.25 M sucrose, 0.2 M buffer (phosphate, or tris(hydroxymethyl) amino methane (tris), or sodium carbonate), 0.01 M ethylene-diamine-tetra-acetic acid (EDTA) and 0.01 M ascorbic acid. Homogenization was carried out for 2 minutes at reduced speed (approx 40 v) controlled with a variac. The homogenates were strained through four layers of cheesecloth and centrifuged for 10 minutes at approximately 1,000 × G followed by a high speed centrifugation of the supernatant at about 17,000 × G for 15 minutes. The particles obtained from the high speed centrifugation were washed in 0.25 M sucrose and resedimented at 17,000 × G. The particles were finally suspended in 6 ml of 0.25 M sucrose and 0.001 M adenosine triphosphate (ATP) adjusted to pH 7.0. These suspensions generally had a nitrogen (N) content (N of ATP subtracted) that varied from 250 to 900 μg/ml depending on the buffer of the homogenizing medium. All operations were carried out in a cold room held at 2 to 4° C.

ASSAY PROCEDURES: Spectrophotometric assays were conducted at room temperature (approx 20° C) with a Beckman DU spectrophotometer in cuvettes of 1 cm path length and 3 ml volume. Readings were taken every 30 seconds for 3 minutes after adding the enzyme, DPNH, or cytochrome c at zero time to start the reactions. All rates given are corrected for endogenous activity as determined from blanks.

Cytochrome oxidase activity was determined by following the decrease in optical density at 550 mμ after adding the apple particles to reducted cytochrome c (Sigma), according to the method of Cooperstein and Lazarow (4). The reaction mixture contained 2.98 ml of reduced cytochrome c (0.076 μ moles) in 0.03 M phosphate buffer pH 7.1, and 0.02 ml of the particles added at zero time. The oxidation of reduced cytochrome c was found to be linear for at least 1 minute and was proportional to enzyme concentration from 0.01 to 0.04 ml of the particulate suspension. This assay was run against a water blank.

The oxidation of reduced diphosphopyridine nucleotide (DPNH) (Pabst) was determined by following the rate of decrease in optical density at 340 mμ after adding 0.05 ml of the particulate suspension at zero time, to a cuvette containing 60 μ moles of tris buffer pH 7.4, 0.15 μ moles DPNH, 0.03 μ moles cytochrome c, and water to bring the volume to 3 ml. The readings were compared to a blank containing all the components except for the absence of DPNH.

DPNH—Cytochrome c reductase activity was assayed by determining the increase in optical density at 550 mμ in the above system except that 0.06 μ moles of cytochrome c was used and the oxidation of reduced cytochrome c was blocked with 8 μ moles of sodium

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azide. The assay was run against a blank containing all the components except DPNH.

Diaphorase activity was determined by following the reduction of 2,6,dichlorophenol-indophenol (DCPIP) as a decrease in optical density at 600 mμ, in a system containing the same components as the DPNH—cytochrome c reductase assay except that 0.15 μ moles of DCPIP was used instead of cytochrome c. The assay was run against a blank containing all the above additions except DPNH.

Succinic—cytochrome c reductase activity was assayed by following the increase in optical density at 550 mμ after incubating the following mixture for 10 minutes at 25° C: 60 μ moles phosphate pH 7.0, 6 μ moles succinate, 8 μ moles sodium azide, 3 μ moles $MgCl_2$, 0.05 ml of the particulate suspension and water to a volume of 2.9 ml. The reaction was started by adding 0.06 μ moles of cytochrome c to the incubated mixtures. The blanks were treated exactly the same except that they contained no succinate.

Succinoxidase was determined by standard Warburg manometric methods (20). The vessels contained in addition to 30 μ moles succinate, 1 μ mole adenosine triphosphate (ATP), 12 μ moles of magnesium, 20 μ moles phosphate, 0.1 ml of 1 % yeast concentrate (Sigma), 0.3 ml 2 % bovine serum albumin, 0.001 μ moles cytochrome c, 1.0 ml of the particulate suspension, and 0.25 M sucrose to a volume of 3 ml. All the additions were buffered to pH 7.1.

Manometric techniques were also used to determine the carbon monoxide inhibition of cytochrome oxidase and its reversal by light. The vessels contained 0.06 μ moles cytochrome c (Sigma), 40 μ moles phosphate buffer pH 7.1, 2 μ moles ATP, 12 μ moles Mg, 0.5 ml of the particulate suspension, 30 μ moles ascorbic acid, freshly prepared and adjusted to pH 7.1, and 0.25 M sucrose to a volume of 3.0 ml. The ascorbic acid, used to reduce cytochrome c, was placed in the side arms and tipped into the main compartment of the vessel at zero time, after 10 minutes of equilibration to temperature (25° C). Darkness was achieved by completely covering the bath and vessels with a double layer of black cloth. Light was provided by two 150 w G.E. photoflood lamps (PAR 38) hung about eight inches from the surface of the water bath. These lamps emit about 3.5 per cent of their energy in the 350 to 700 mμ region of the spectrum.

Nitrogen determinations were made by the Thompson and Morrison method (19).

The data reported are the averages of at least three experiments.

Additional experimental details are described in the text and legends of the tables and figures.

RESULTS

Most of the difficulties encountered in isolating active cytoplasmic particles from apple fruit appear to be related to the presence in the cells of high concentrations of potentially inhibitory substances, notably polyphenols (10, 15), and to the acidity of the cell sap (17). The effects of polyphenols were considerably reduced by eliminating the peel, which contains the bulk of these substances, and by introducing 0.01 M ascorbic acid into the homogenizing medium, thus maintaining the polyphenols in the reduced (non-inhibitory) state (12). Having established what appeared to be a satisfactory operating procedure with respect to avoiding inhibition by phenolics, the effects of polyphenols were not further considered in these studies. However, a detailed analysis was made of the effects of homogenizing media of increasing alkalinity on the activity of cytoplasmic particles isolated from them.

PH OF HOMOGENATES AND ACTIVITY OF PARTICLES: The acidity of the cell sap was counteracted by using strong alkaline buffers in the homogenizing media. Table I lists the types of buffers, the concentrations

TABLE I

PH OF HOMOGENATES AND PARTICULATE SUSPENSIONS DERIVED FROM HOMOGENIZING SOLUTIONS CONTAINING BUFFERS OF INCREASING ALKALINITY

BUFFER*	PH OF BUFFER	PH OF HOMOGENATE	PH OF PARTICULATE SUSPENSION**	MG N/ML PARTICULATE SUSPENSION***
0.1 M K_2HPO_4 0.1 M KH_2PO_4	6.7	6.0	6.3	0.90
0.2 M K_2HPO_4	8.1	6.9	6.6	0.90
0.2 M Tris	9.2	8.4	7.2	0.55
0.1 M Na_2CO_3	10.2	9.3	7.1	0.50
0.2 M Na_2CO_3	10.6	9.9	7.6	0.30
0.2 M Na_2CO_3†	12.0	10.7	8.5	0.25

* In addition to buffer all the homogenizing media contained 0.25 M sucrose, 0.01 M EDTA, and 0.01 M ascorbate. Three hundred grams of tissue were blended with 300 ml of homogenizing solution as described in the text. Distilled water homogenates of apples had a pH of 3.4.

** All particles were washed once in 0.25 M sucrose and finally suspended in 6 ml of 0.25 M sucrose and 0.001 M ATP at pH 7.0.

*** The N of the suspending medium was subtracted.

† This medium was made to pH 12.0 with solid KOH.

used, and the pH's of the resultant homogenates, under the conditions indicated in the legend. Cytoplasmic particles isolated from phosphate buffers which gave homogenates near neutrality showed little or no electron transport activity (table II). Thus, buffering the homogenate to yield a suspension near neutrality, a condition satisfactory for many plant tissues, was completely inadequate with apple fruit tissue. Particles isolated from alkaline buffers, however, yielding alkaline homogenates above pH 8 manifested significant electron transport chain activity. Maximum activity of DPNH oxidase, diaphorase, DPNH—cytochrome c reductase, and cytochrome oxidase was observed in cytoplasmic particles isolated from homogenates of pH 9.9 (table II). It should be borne in mind that the pH of the particulate suspension was 7.6 (table I).

CYTOCHROME OXIDASE ACTIVITY AND INHIBITION: The cytochrome oxidase activity of the particles isolated from 0.2 M sodium carbonate-sucrose medium (pH of homogenate 9.9 of table II), in a system in which cytochrome c was reduced by ascorbic acid (fig 1). The activity was stable for approximately two hours. The inclusion of an inhibitor of polyphenolase, as reported by Rubin and Ladygina (16) was not found necessary in these systems, even though the particles are known to contain polyphenolase. It is interesting to note, in addition, that the cytochrome oxidase system in these preparations was stable for at least 3 weeks at 5° C.

Carbon monoxide inhibition and reversal by light was demonstrated in these preparations (fig 1). It was not possible, however, to incorporate inorganic phosphorus into these systems even when supplied with hexokinose and glucose in addition to the other components of the system (ATP, Mg, phosphate). This indicates that the phosphorylation mechanism,

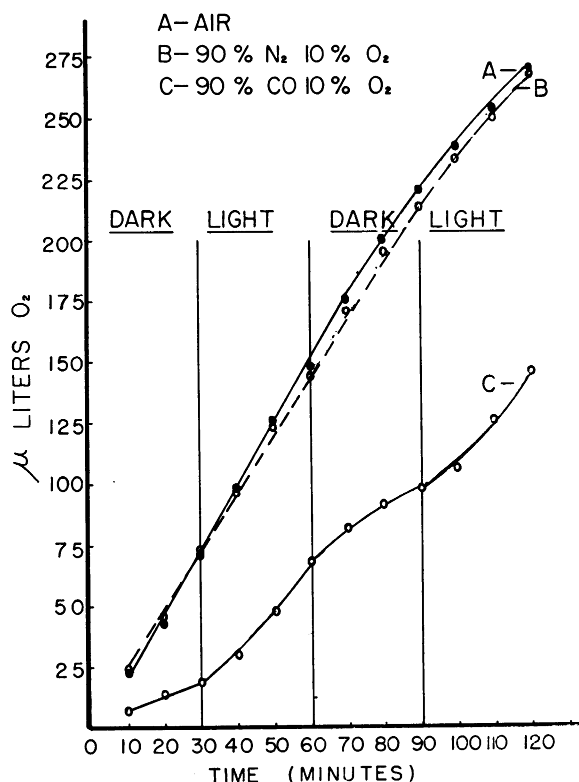

FIG. 1. Light reversal of carbon monoxide inhibition of cytochrome oxidase activity. The reactions were run at 25° C. in a Warburg apparatus. Each of the appropriate vessels was flushed with ten volumes of either N_2/O_2 mixtures in proportions of 9:1 or CO/O_2 in proportions of 9:1 before closing the system and equilibrating to temperature. Details of the reaction mixtures and other procedures are described in the text.

TABLE II

ACTIVITY OF ELECTRON TRANSPORT CHAIN COMPONENTS OF CYTOPLASMIC PARTICLES ISOLATED FROM HOMOGENATES OF DIFFERENT pH*

pH OF HOMOGENATE**	SPECIFIC ACTIVITY			
	DPNH OXIDASE***	DIAPHORASE†	DPNH-CYTOCHROME c REDUCTASE††	CYTOCHROME OXIDASE‡
6.0	0.005	0.006	0.000	0.000
6.9	0.011	0.012	0.003	0.000
8.4	0.025	0.041	0.022	0.100
9.3	0.081	0.067	0.072	0.630
9.9	0.153	0.079	0.204	1.120
10.7	0.047	0.035	0.026	0.150

* Details of assay procedures are described in the text. The rates are calculated from the absorption coefficients (5).
** Homogenates are those described in table I.
*** µM DPNH oxidized/min/mg N.
† µM 2,6-dichlorophenolindophenol reduced/min/mg N.
†† µM cytochrome c reduced/min/mg N.
‡ µM cytochrome c oxidized/min/mg N.

TABLE III

SUCCINATE OXIDATION BY CYTOPLASMIC PARTICLES ISOLATED FROM HOMOGENATES OF DIFFERENT pH*

pH OF HOMOGENATE**	SUCCINIC-*** CYTOCHROME C REDUCTASE (μM CYT. C REDUCED/MIN/ MG N)	SUCCINIC-† OXIDASE (μ MOLES SUCCINATE OXIDIZED/MIN/ MG N)
6.0	0.000	...
6.9	0.002	0.092
8.4	0.088	0.446
9.3	0.041	0.288
9.9	0.020	0.180
10.7	0.000	...

* Details of assay procedures are described in text.
** Homogenates are described in table I.
*** Calculated from spectrophotometric assay.
† Calculated from manometric assay.

which is usually assumed to be a more labile system in mitochondria, was damaged and that electron transfer was proceeding in an uncoupled manner.

OXIDATION OF SUCCINATE: In contrast to the oxidation of electron transport chain components, DPNH, and reduced cytochrome c, which showed maximal activity with particles isolated from homogenates of pH 9.9, the oxidation of succinate was maximal with cytoplasmic particles derived from homogenates of pH 8.4 (table III). This was true for both succinic-cytochrome c reductase and succinoxidase activity. As a matter of fact there was no demonstrable succinate activity in particles from homogenates of pH 6.0, and the activity of particles from pH 9.9 was much greater than that from pH 6.9.

These data offer evidence for the necessity of an alkaline homogenizing medium to isolate active cytoplasmic particles from apple fruit. It is further evident that a more alkaline homogenate (pH 9.9) yields particles with better DPNH cytochrome c reductase, DPNH oxidase, and cytochrome oxidase activity while homogenates of lower pH (8.4) furnish particles with better succinoxidase activity. In other data (unpubl), the oxidation of other Krebs cycle substrates was also favored by particles from homogenates of pH 8.4 compared to pH 9.9.

EFFECT OF INHIBITORS: The effects of antimycin A and amytal, reported to be inhibitors of specific sites in the electron transport chain (2), are shown in table IV. In these studies the particles were prepared in the alkaline homogenates that yielded the best activities as described above. More than 75 % of DPNH oxidase, DPNH-cytochrome c reductase and succinic-cytochrome c reductase were inhibited by antimycin A (0.5 μg per cuvette) (table IV). Inhibition of the succinoxidase system declined with time from 66 % to 21 % after 2 hours. This may indicate that electron transport from succinate to oxygen proceeds, under these conditions, via a pathway which circumvents the antimycin A block. Alternatively the decline in inhibition of the succinoxidase system may result from alteration or destruction of the antimycin A with time.

The inhibitory action of antimycin A implicates cytochrome b as a component of the electron transfer chain in these particles. The fact that the DPNH-diaphorase reduction, a system that does not involve cytochrome b, is inhibited only 11 % (table IV), indicates that inhibition is specific to the cytochrome b site.

It is interesting that amytal, which inhibits electron transport between DPNH and the flavoprotein moiety in animal systems (2), was not effective in these particulates. Chance and Hackett (3) also noted the ineffectiveness of amytal in plant mitochondria.

SPECTRA OF APPLE PARTICLES: The suspensions of particles isolated from the alkaline homogenates

TABLE IV

INHIBITION OF ELECTRON TRANSFER BY CYTOPLASMIC PARTICLES*

INHIBITOR	DPNH OXIDASE**	DPNH-CYT. C** REDUCTASE	DIAPHORASE**	SUCCINIC-CYT. C** REDUCTASE
		*% Inhibition***		
Antimycin A***	76	77	11	78
Amytal†	4	2	2	...
		% Inhibition of succinoxidase††		
	10 MIN	1 HR	2 HR	
Antimycin A	66	40	21	

* The particles used for DPNH oxidase, DPNH cytochrome C reductase and diaphorase were from homogenates of pH 9.9. The particles used for the succinate assays were isolated from homogenates of pH 8.4.
** Assays were 3-minute spectrophotometric determinations as described in the text.
*** Antimycin A (0.5 μg) in 95 % ethanol was used in the cuvettes and Warburg vessels. An equivalent amount of 95 % ethanol was used in the blank.
† Amytal (3.6μ moles) dissolved in 95 % ethanol was used per cuvette. An equivalent amount of 95 % ethanol was used in the blank.
†† Assay was by Warburg manometric techniques. Assay system is described in text.

were very green and quite low in protein content (approx 2 mg/ml of suspension). It was therefore difficult to observe the spectra of the cytochromes in these particles. When the particles (derived from homogenates of pH 9.9) were concentrated from 3 kilograms of tissue, however, and the chlorophyll extracted by successive treatment with 25 volumes of cold (−15° C) acetone, until no green pigment was visible to the naked eye, it was possible to obtain difference spectra showing certain of the cytochrome bands.

The spectra shown in figure 2 are the difference spectra before and after adding dithionite to acetone-extracted concentrated particles. Absorption bands at 530, 560, and 605 mμ are clearly visible in these spectra. This corresponds to the β and α bands of cytochrome b and the α band of cytochrome a_3 (2). There is no obvious absorption band visible at 550 mμ to correspond to the α absorption peak of cytochrome c. Close scrutiny of this curve, however, reveals a hint of slight absorptions at 550 mμ and 520 mμ the α and β absorption peaks for cytochrome c. Since cytochrome c is the most soluble cytochrome it is possible that most of it has been extracted in the homogenization procedure. The residual cytochrome c bands may be masked by the much larger cytochrome b peaks. Skewing the cytochrome a_3 absorption band at 605 is due to the residual chlorophyll which tends to distort the band towards the higher wavelengths. In general, the curve is pitched sharply toward the higher wavelengths. This is due to the effect of dithionite on the overall absorption characteristics of the sample. For the most part, the absorption pattern observed in many plant cytoplasmic particles, demonstrating electron transport chain activity (7), was also seen in these apple particles.

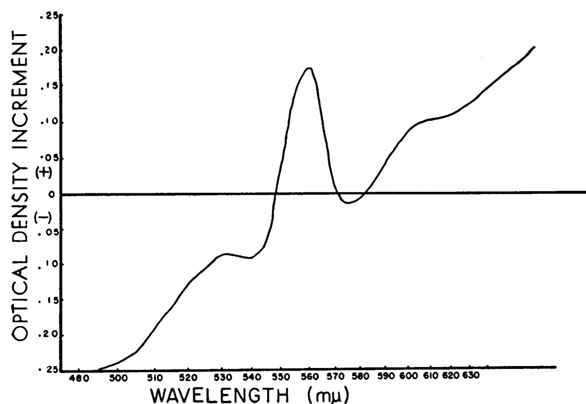

FIG. 2. Reduced minus oxidized difference spectra of apple particles obtained as outlined in text. The light path was 1.5 cm but was equivalent to an effective light path of about five centimeters resulting from a threefold intensification caused by endogenous scatter. The spectrophotometer used an end-window phototube and was specially designed to read through dense samples (1).

DISCUSSION

It seems that the dehydrogenases of the Krebs cycle, [as judged by succinate activity and oxidations of other Krebs cycle acids (data unpubl)], are better preserved by homogenates of pH 8.4, while the electron transport chain components are better retained in homogenates of pH 9.9. The characteristics of the pH 9.9 particles are similar to the ETP particles of Green and Crane (6) and therefore represent altered mitochondria. Since there is a basic difference in the localization and in the ease of extraction or solubilization of the Krebs cycle dehydrogenases and the electron transfer chain enzymes in the mitochondria, as noted by Green and Crane (6) and Lehninger (11), the action of alkali of pH 9.9 in these experiments, probably involves complete or partial removal by solubilization or otherwise of the substrate-level enzymes of the Krebs cycle. Even succinic dehydrogenase, a very insoluble system, is solubilized by a pH greater than 10 (6). The solubility effect may account for the lower nitrogen values of the particulate preparations from the more alkaline homogenates. On the other hand the much greater nitrogen content of the particulates from homogenates of low pH (6.0 and 6.9) is attributed to adsorptions of extraneous proteins that serve to inactivate the mitochondria. The greater activity of the electron transport chain in the pH 9.9 particles may be due to their more ready access to exogenous substrates in vitro, without interference of intervening layers of dehydrogenases which act as permeability barriers or regulatory controls.

Another way of looking at these data is simply to assume partial denaturation of the succinic dehydrogenase and other Krebs cycle substrate level enzymes, by the very alkaline homogenates. The more rugged electron transport enzymes encased as they are in phospholipid sheaths (11) are not injured and are in fact stimulated by the more alkaline buffers. One basis for this stimulation may be the purification of the enzymes since particles obtained from more alkaline homogenates have a lower specific nitrogen content. Another basis for stimulation may be related to an uncoupling effect caused by the high pH. Since the electron transport chain components are directly concerned with phosphorylation they may readily become uncoupled in very alkaline homogenates. As noted previously, oxidative phosphorylation was not observed with particles from pH 9.9 homogenates in the span from cytochrome c to oxygen, indicating that the system was uncoupled.

There is always the question whether or not the observed reductions of cytochrome c by DPNH and succinate are artificial extra-mitochondrial pathways. Inhibiting these reactions by antimycin A seems to support the view that the systems are intra-mitochondrial. Antimycin A inhibited both DPNH oxidase and DPNH-cytochrome c reductase about 80 %, which indicates that both these oxidations proceeded through cytochrome b. The antimycin A effect on succinoxidase seemed less conventional since there was a loss of inhibition with time from a value of 66 % to

21 %, after 2 hours. The length of time of the experiment may have somehow caused gradual degradation of antimycin A rendering it ineffective. However, it is also possible to explain the decline of inhibition by the gradual shunting of electrons around cytochrome b either by a secondary internal pathway or perhaps by means of some adsorbed microsomes. Shunting was not observed with the DPNH oxidase, DPNH-cytochrome c reductase, and succinic-cytochrome c reductase systems, because these assays were run for only 3 minutes.

Summary

A study of the isolation of cytoplasmic particles from apple homogenates of different pH (6.0–10.7), revealed that enzymatically active particles could be obtained only from alkaline homogenates. Particles isolated from homogenates of pH 9.9 gave the best electron transport chain activity while particles isolated from homogenates of pH 8.4 showed the best succinic-cytochrome c reductase and succinoxidase activity.

The cytochrome oxidase activity of these particles showed typical carbon monoxide inhibition and light reversibility. Antimycin A inhibited DPNH oxidase, DPNH-cytochrome c reductase and succinic-cytochrome c reductase, but did not inhibit diaphorase activity.

The difference spectra of the particles reduced with dithionite, after acetone extraction to remove chlorophyll, revealed absorption bands at 560, 530, and 605 mμ, which corresponds to the α and β bands of cytochrome b and the α band of cytochrome a_3. These data lead to the conclusion that the apple fruit has the usual type of electron transfer chain.

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