Plant Physiol. (1983) 71, 627–634
0032-0889/83/71/0627/08/$00.50/0

Properties of Substantially Chlorophyll-Free Pea Leaf Mitochondria Prepared by Sucrose Density Gradient Separation[1]

Received for publication July 27, 1982 and in revised form November 5, 1982

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ABSTRACT

Mitochondria isolated from pea leaves (*Pisum sativum* L. var Massey Gem) and purified on a linear sucrose density gradient were substantially free of contamination by Chl and peroxisomes. They showed high respiratory rates and good respiratory control and ADP/O ratios. Malate, glutamate, succinate, glycine, pyruvate, α-ketoglutarate, NADH, and NADPH were oxidized but little or no oxidation of citrate, isocitrate, or proline was detected. The oxidation of NADPH by the purified mitochondria did not occur via a transhydrogenase or phosphatase converting it to NADH. NADPH oxidation had an absolute requirement for added Ca^{2+}, whereas NADH oxidation proceeded in its absence. In addition, oxidation of the two substrates showed different sensitivities to chelators and sulfhydryl reagents, and faster rates of O_2 uptake were observed with both substrates than with either alone. This indicates that the NADPH dehydrogenase is distinct from the exogenous NADH dehydrogenase.

In recent years, a number of techniques have been described for the isolation of mitochondria, completely or partially free of Chl, from leaf tissue. Bergman *et al.* (6) have developed a system for isolating Chl-free mitochondria from spinach leaves using a three-step procedure involving differential centrifugation, partition in an aqueous dextran-polyethylene glycol two-phase system, and Percoll gradient centrifugation. This method yields intact mitochondria with good respiratory rates, respiratory control, and ADP/O ratios. Its major disadvantage is that different dextran batches have different mol wt distributions (6) which necessitates considerable testing of each separate batch to determine the critical point for partitioning of materials in the phase system. Differential centrifugation and discontinuous Percoll density gradient centrifugation have been utilized by Jackson *et al.* (16) to yield spinach leaf mitochondria substantially free of Chl contamination and with good respiratory rates, respiratory control, and ADP/O ratios. Fuchs *et al.* (15) have isolated Chl-free mitochondria from oat mesophyll cells by protoplast isolation and subsequent linear sucrose density gradient centrifugation. However, this method is quite lengthy (8 h) and resulted in mitochondria with poor respiratory control.

Sucrose density gradient centrifugation has generally yielded poor leaf mitochondria with inadequate separation of the mitochondria from contaminating material (17). However, Arron *et al.* (4) have prepared intact *Sedum praealtum* leaf mitochondria on a linear sucrose density gradient, but the mitochondria lost some respiratory control during preparation and contained more Chl than washed mitochondria.

In this report, we describe the isolation of pea leaf mitochondria, substantially free of Chl, by linear sucrose density gradient centrifugation. The mitochondria have high respiratory rates and good respiratory control and ADP/O ratios. Some oxidative properties of the mitochondria, including malate and NADPH oxidation, are described in more detail and we present evidence that the NADPH dehydrogenase of pea leaf mitochondria is distinct from the exogenous NADH dehydrogenase.

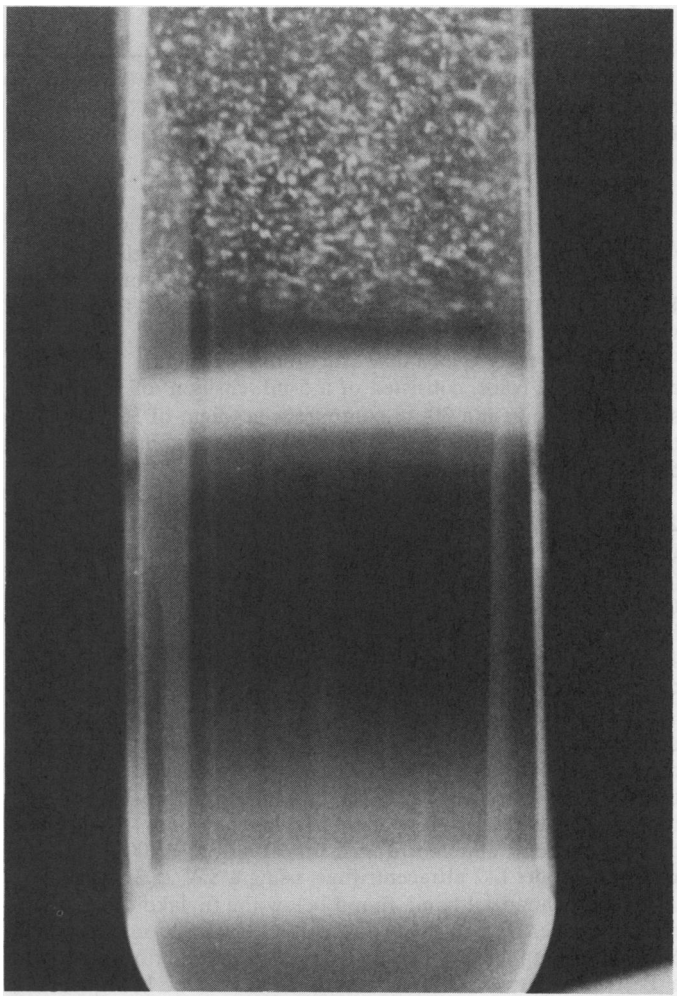

FIG. 1. Separation of pea leaf homogenate on a gradient of 30.6 to 41.5% (w/w) sucrose prepared as described in "Materials and Methods." The globules in the supernatant fraction were normally located on the top of the supernatant but were disturbed during photography.

[1] Financial support from the Australian Research Grants Committee is gratefully acknowledged.

[2] Recipient of a Commonwealth Postgraduate Research Award.

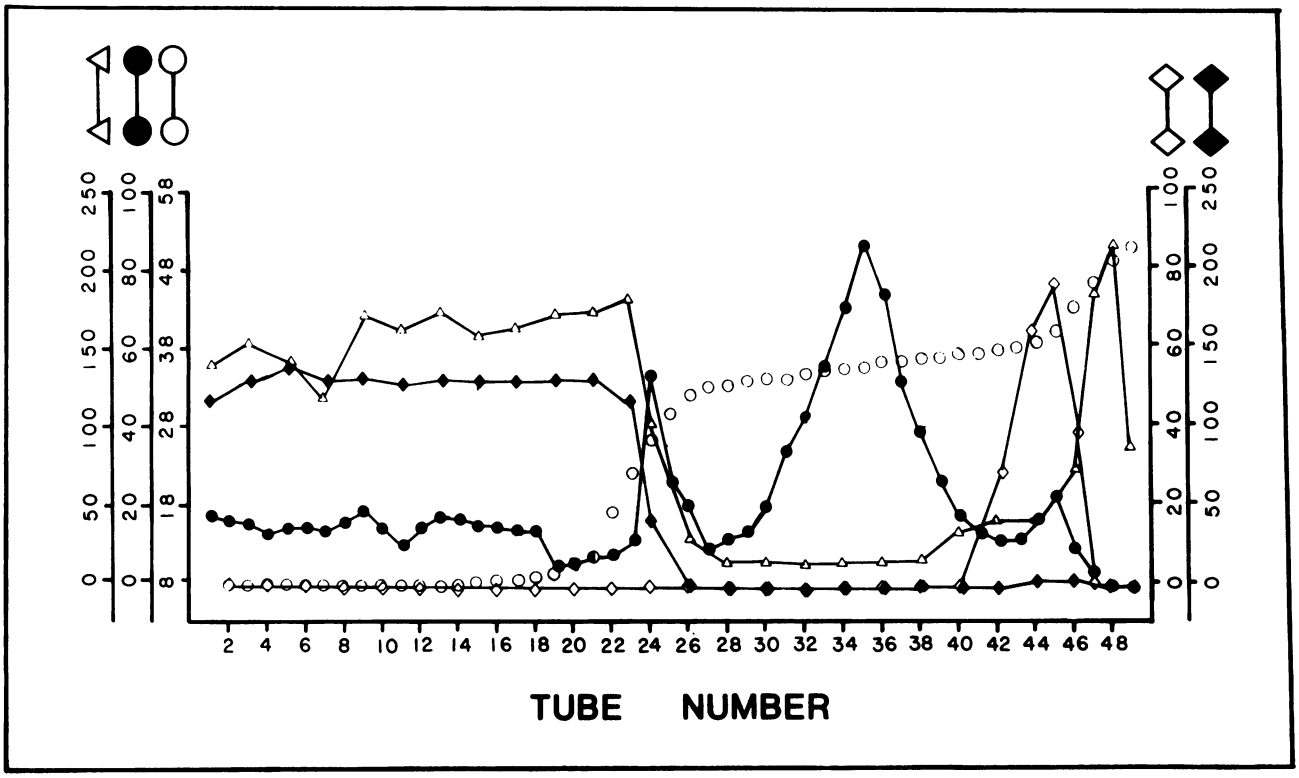

FIG. 2. Location of markers in a gradient of 30.6 to 41.5% (w/w) sucrose, fractionated into 1.2-ml volumes as described in "Materials and Methods." (△) Catalase activity (μmol H_2O_2 decomposed/min·tube); (●) μg Chl/tube; (○) % sucrose (w/w); (◇) NAD-isocitrate dehydrogenase activity (nmol isocitrate oxidized/min·tube); (◆) NADP-isocitrate dehydrogenase activity (nmol isocitrate oxidized/min·tube).

MATERIALS AND METHODS

Plant Material. Pea seedlings (*Pisum sativum* L. var Massey Gem) were grown for 10 to 12 d in vermiculite in a glasshouse.

Chemicals. Analytical reagent grade sucrose was obtained from B. D. H. Pty Ltd. All other chemicals were obtained from Sigma Chemical Co.

Sucrose Density Gradients. The gradients suitable for a Beckman SW 25.2 rotor, consisted of a 5-ml 'cushion' of 54% (w/w) sucrose underneath a 30-ml continuous gradient of 30.6% (w/w) to 41.5% (w/w) sucrose. All sucrose solutions contained 1 mM EDTA, 1 mM Tes, and 0.1% (w/v) BSA.

Isolation of Mitochondria. All isolations were carried out at 2°C. Mitochondria were isolated in the 'normal' manner (*i.e.* by differential centrifugation) by the method of Day and Wiskich (11) using a 5-s homogenization at speed 3 with a Polytron (PT 35 OD) homogenizer in an isolation medium containing 0.3 M sorbitol, 50 mM Tes, 1 mM EDTA, 1 mM $MgCl_2$, 1% (w/v) PVP-40, 10 mM isoascorbate, and 0.4% (w/v) BSA (pH 7.5). These mitochondria are termed washed mitochondria.

For density gradient isolation, 30 g prechilled leaves were disrupted in 150 ml of the above isolation medium, filtered through a double layer of Miracloth (Calbiochem) and centrifuged in a fixed angle rotor at 1500*g* for 10 min. Seventy to 75 ml of the resulting supernatant were carefully layered onto the sucrose density gradients and centrifuged for 90 min at 21,000 rpm in a Beckman model L-2 ultracentrifuge, using a SW 25.2 rotor.

The mitochondrial band, found below the thylakoid band, was removed either with an ISCO density gradient fractionator (model 640) by pushing the gradient up from the bottom, or by hand with a Pasteur pipette. The latter method was faster but generally resulted in greater contamination by thylakoid fragments. The mitochondria were slowly diluted at 0°C over a period of 30 to 40 min with 100 ml of 0.3 M sorbitol, 20 mM Tes, and 0.1% (w/v) BSA (pH 7.5). The suspension was centrifuged at 12,000*g* for 15 min in fixed angle rotor and the mitochondrial pellet was resuspended in 1 to 2 ml of the dilution medium. This yielded purified mitochondria.

Oxygen Consumption. O_2 uptake was measured polarographically in 2.0 to 2.5 ml of air-saturated standard reaction medium (250 mM sucrose, 10 mM K-phosphate buffer, 5 mM $MgCl_2$, 10 mM Tes, pH 7.4, unless otherwise specified) using a Rank O_2 electrode at 25°C. O_2 in air-saturated medium was assumed to be 240 μM. RC[3] and ADP/O values were calculated as described by Estabrook (14).

Assays. NAD-isocitrate dehydrogenase was assayed at 22°C by the method of Cox (7) in the presence of 0.04% (v/v) Triton X-100, using 0.1 ml sample in a final volume of 3.1 ml. NADP-isocitrate dehydrogenase was assayed by the same method using 430 μM $NADP^+$ instead of NAD^+. Catalase was assayed by the method of Lück (20) using 0.1 ml sample in a final volume of 3.0 ml. Hydroxypyruvate reductase was measured as described by Tolbert (30) using 0.1 ml sample in a total volume of 3.0 ml and 1.0 mM hydroxypyruvate to start the reaction.

Protein content was measured by the method of Lowry *et al.* (19) using BSA (fraction V) as a standard. Chl was determined by the method of Arnon (1) using 100-μl aliquots for samples obtained by fractionation. The protein content of the washed mitochondrial suspension was corrected for any contamination by broken thylakoids by using a protein/Chl ratio of 7:1 (13).

Refraction was measured on an Abbe Refractometer model 10460 (American Optical Corp).

Electron Microscopy. After dilution and resuspension, purified mitochondria were fixed for 1 h at 4°C in resuspension medium

[3] Abbreviations: RC: respiratory control ratio; CCP: carbonyl cyanide *m*-chlorophenylhydrazone; EGTA: ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid: *p*CMB: *p*-chloromercuribenzoate; TPP: thiamine pyrophosphate.

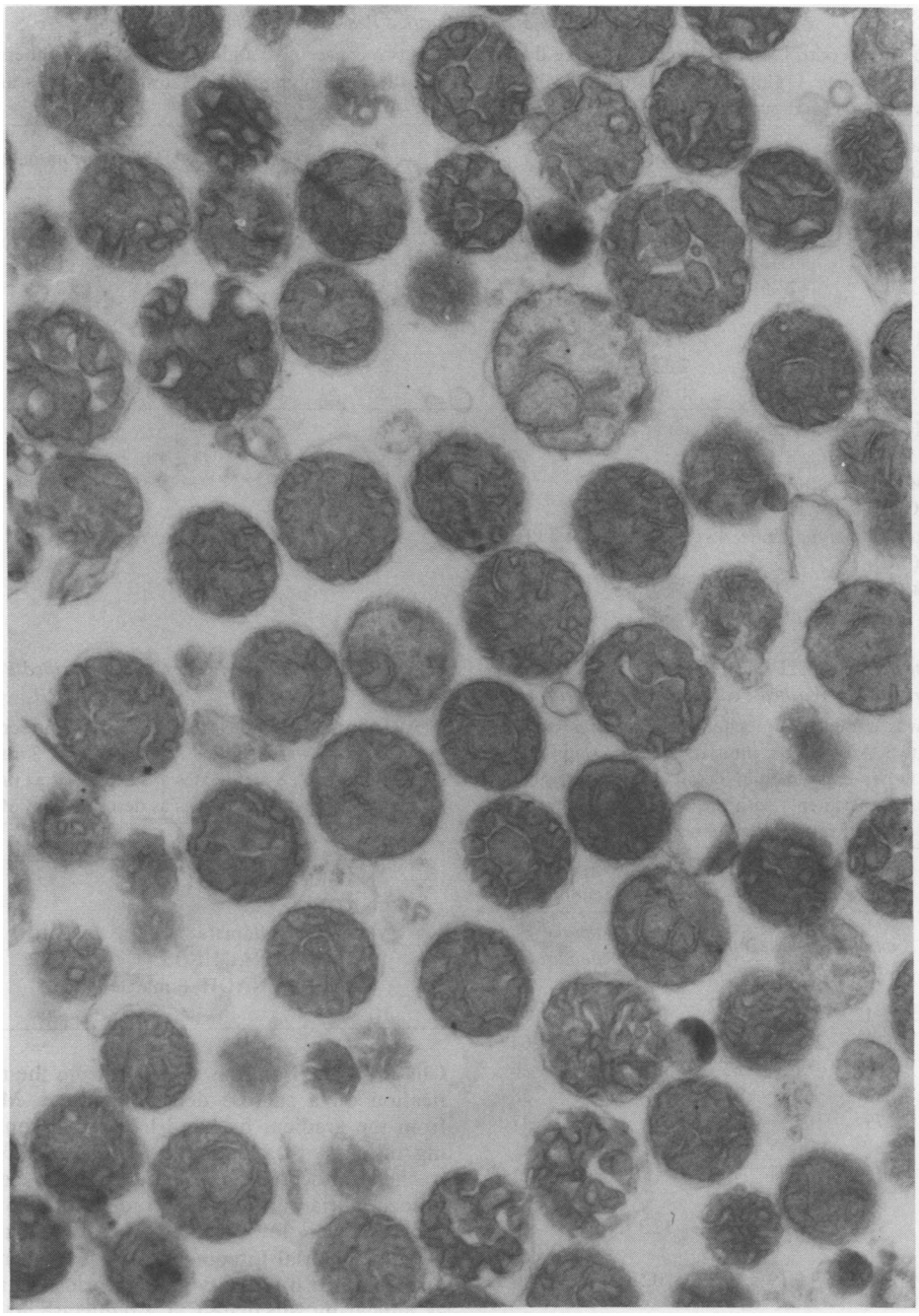

FIG. 3. Electron micrograph of mitochondria prepared by sucrose density gradient separation. Magnification × 8000.

containing glutaraldehyde at a final concentration of 2.5%. The suspension was then centrifuged at 40,000*g* for 10 min; the pellet was washed for 2 h in four changes of 0.1 M cacodylate buffer plus 3 mM $CaCl_2$ (pH 7.2), postfixed in 2% OsO_4 in buffer, dehydrated through a graded ethanol series, and embedded in Spurr's firm resin (29). Ultrathin sections were cut with glass knives on a Reichert Ultracut microtome, stained with aqueous uranyl acetate (1 h) and lead citrate (5 min), and examined in a Phillips 400 electron microscope.

RESULTS AND DISCUSSION

Gradient Purification. Figure 1 illustrates the separation, on a gradient of 30.6 to 41.5% sucrose, of a low speed supernatant fraction from pea leaves. The samples obtained from fractionation of the density gradients were assayed for mitochondria (NAD-isocitrate dehydrogenase activity), peroxisomes (catalase activity), and thylakoids (Chl) and are shown in Figure 2. Mitochondria banded at 40 to 43% sucrose (*i.e.* at the interface between the continuous gradient and the cushion where mixing of sucrose densities occurred). A small amount of Chl was associated with this band. Peroxisomes were located below the mitochondria. However, in some preparations, no separate peroxisomal band could be detected, presumably because harsher maceration of the tissue ruptured all the peroxisomes.

Isopycnic points for the mitochondria and peroxisomes were determined using a gradient of 30.6 to 45.2% sucrose. Mitochondrial density was 1.18 g cm^{-3} compared to 1.17 and 1.21 g cm^{-3} reported for *Sedum praealtum* (4) and spinach (27) leaf mitochondria, respectively; peroxisomal density was 1.23 g cm^{-3}, compared to 1.25 g cm^{-3} reported for spinach leaf peroxisomes (27). Figure 2 also shows the distribution of NADP-isocitrate dehydrogenase

Table I. *Substrate Oxidation by Purified Pea Leaf Mitochondria*

Respiratory rates, respiratory control, and ADP/O ratios were determined for two consecutive additions of ADP. Numbers in parentheses give the maximum (coupled rate) of malate + glutamate oxidation by mitochondria in the same preparation. Rates are given as nmol O_2 uptake/min·mg protein. Measurements were made as described in "Materials and Methods."

Substrate	Malate + Glutamate[a]	Succinate[b]	Glycine[a]	Pyruvate + Malate[c]	Malate[d]	α-Keto-glutarate[e]	NADH[f]	NADPH[g]	Glutamate[h]	Citrate[a]	Isocitrate[a]
First											
State 3 rate	168	115 (196)	58 (150)	132 (150)	38 (150)	153 (256)	183 (170)	153 (170)	40 (187)	30 (187)	48 (146)
RC	6.77	1.42	1.20	2.20		3.98	2.31	2.03	1.56		
ADP/O	2.20	1.25	2.12	2.14		3.08	1.28	1.34	2.83		
Second											
State 3 rate	187	138	123	127		163	185	146			
RC	6.19		2.32	2.52		2.12	2.17	1.87			
ADP/O	1.92		1.70	2.12		2.99	1.41	1.34			

[a] Malate, glutamate, glycine, citrate, and isocitrate at 21 mM.
[b] 21 mM succinate, 0.5 mM ATP.
[c] 9 mM pyruvate, 45 μM CoA, 45 μM TPP, 2 mM malate.
[d] 2 mM malate, 45 μM CoA, 45 μM TPP.
[e] 13 mM α-ketoglutarate, 1.3 mM malonate, 45 μM CoA, 45 μM TPP.
[f] 1 mM NADH, 1 mM $CaCl_2$.
[g] 2 mM NADPH, 1 mM $CaCl_2$.
[h] 21 mM glutamate, 45 μM CoA, 45 μM TPP.

Table II. *Effect of Rotenone and NAD⁺ on the Oxidation of Malate + Glutamate by Purified and Washed Pea Leaf Mitochondria*

Rotenone (21.7 μM) was added to mitochondria oxidizing malate + glutamate (20 mM) in state 3. After determining the rate of O_2 uptake, 260 μM NAD^+ was added. Other experimental detail was as described in "Materials and Methods." Experiments with the same number were performed on mitochondria isolated from the same batch of pea leaves.

	Purified Mitochondria			Washed Mitochondria		
	nmol O_2 uptake/min·mg protein			*nmol O_2 uptake/min·mg mitochondrial protein*		
Experiment number	1	2	3	1	2	3
Initial rate	128	259	183	91	73	64
+Rotenone	20	81	57	58	45	46
% Inhibition	84	69	69	36	38	28
+Rotenone + NAD^+	79	105	77	62	43	47
+Rotenone + NAD^+/rotenone	3.94	1.31	1.36	1.07	0.96	1.02
nmol Rotenone/mg total protein	95.6	73.6	71.0	12.3	14.8	18.2
nmol Rotenone/initial rate	0.39	0.19	0.27	0.55	0.69	0.95
nmol NAD^+/mg total protein	1150	877	838	148	177	219
nmol NAD^+/initial rate	4.69	2.32	3.28	6.58	8.21	11.40

activity. Most of the activity occurred in the supernatant, with a very small amount present in the mitochondrial fraction. This agrees with the work of Randall and Givan (25) who demonstrated that NADP-isocitrate dehydrogenase is located primarily in the cytoplasm and chloroplasts.

Removal of the mitochondrial fraction by hand generally resulted in greater contamination by Chl than did removal by fractionation, but had the advantage of speed. Some cross-contamination by Chl also occurred with the fractionation method, as a layer of green material could be seen adhering to the side of the polyallomer tubes used for gradients. The mitochondria were forced past this layer during fractionation and may have been contaminated with some of the Chl. Nevertheless, as can be seen from the data of Figure 2, a large proportion of the contaminating

Table III. *Addition of Substrates to Purified Pea Leaf Mitochondria Oxidizing NADPH*

All substrate additions were made in the presence of excess ADP. Final concentrations were: ADP, 0.7 mM; NADPH, 2 mM; NADH, 1 mM; malate, 8 mM; glutamate, 8 mM. Rates are expressed as nmol O_2/min·mg protein. Other experimental detail was as described in "Materials and Methods."

Addition	Rate
NADPH	166
NADH	183
Malate + glutamate	225
NADPH + NADH	257
NADPH + NADH + malate + glutamate	308

Chl and peroxisomes is removed from the mitochondria by purification on a sucrose density gradient. Mitochondria removed from the gradient by hand had a Chl content of 0.093 ± 0.023 mg/ml (n = 10), compared to 1.51 ± 0.25 mg/ml (n = 10) for washed mitochondria. Figure 3 is an electron micrograph of mitochondria purified on a gradient of 30.6 to 41.5% sucrose, and shows the very low level of thylakoid contamination.

Mitochondrial Intactness. The outer membranes of mitochondria obtained by density gradient centrifugation were 95% intact, as judged by succinate-Cyt *c* reductase activity in 0.3 M sucrose and hypotonic media. The inner membranes were also almost completely intact, as indicated by the lack of rotenone inhibition of exogenous NADH oxidation (data not shown).

Substrate Oxidation. Purified pea leaf mitochondria exhibited high rates of oxidation of various substrates with reasonable respiratory control and ADP/O ratios comparable to those reported for mitochondria isolated from other photosynthetic and nonphotosynthetic plant materials (Table I). Although low levels of thylakoid contamination occurred, no correction was made for this in determining oxidative rates of purified mitochondria. In every preparation, we determined the rate of oxidation of malate + glutamate as a standard, and this rate is shown in parentheses for each substrate. Respiratory control ratios up to 21 were observed for malate + glutamate oxidation, although generally they were in the region of 4 to 7. The rate of substrate oxidation, measured on a protein basis, was much greater with purified mitochondria than with washed mitochondria, presumably because of the removal of large amounts of nonmitochondrial protein

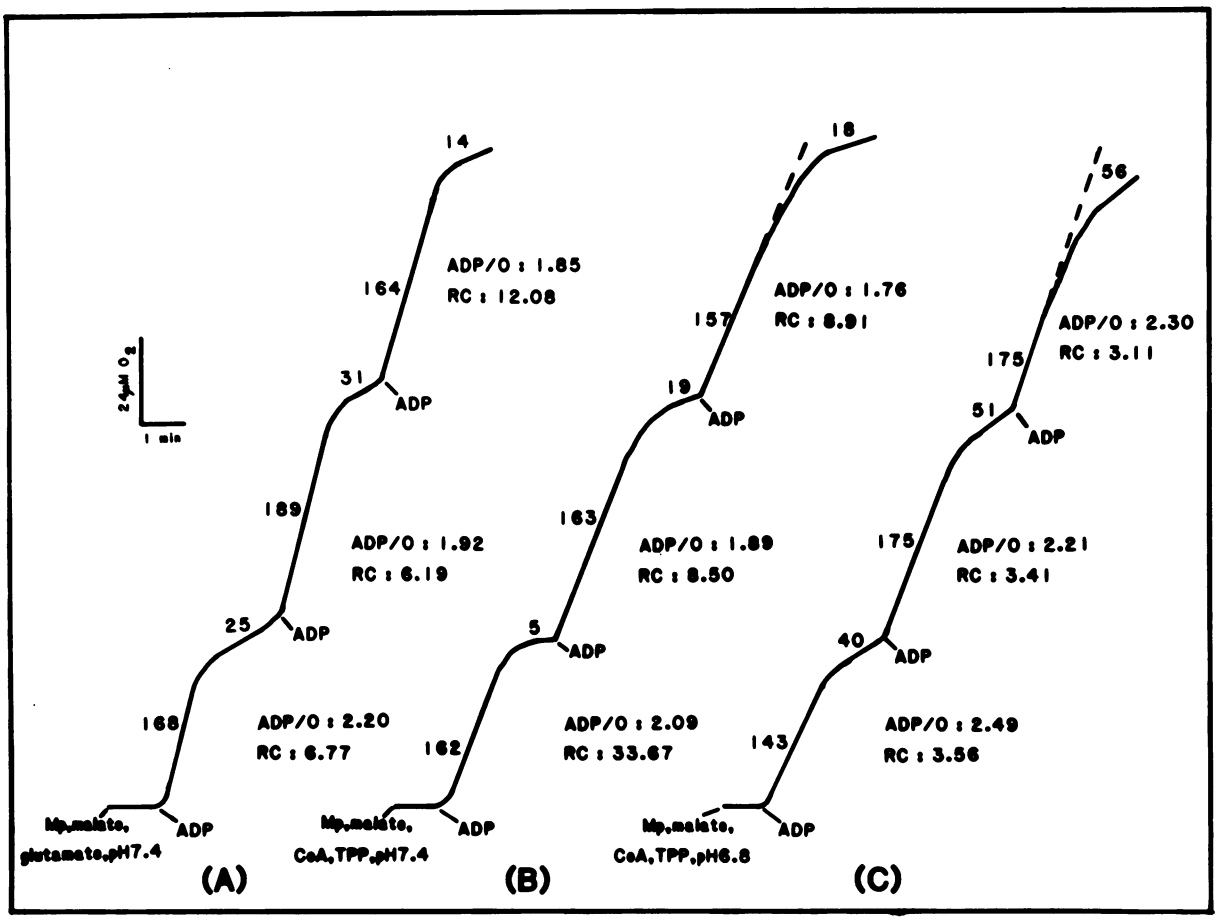

FIG. 4. Oxidation of malate by purified mitochondria (Mp) at varying pH. A, 21 mM malate, 21 mM glutamate at pH 7.4. B, 21 mM malate, 45 μM CoA, 45 μM TPP at pH 7.4. C, 21 mM malate, 45 μM CoA, 45 μM TPP at pH 6.8. The first addition of ADP in each trace was 177 μM, with subsequent additions being 221 μM.

by the purification procedure. This has also been reported with mitochondria isolated from *Sedum praealtum* leaves (4). Citrate and isocitrate were oxidized slowly compared to the rates for malate + glutamate oxidation, and no oxidation of proline could be detected.

Malate oxidation, measured in the presence of CoA and TPP, and the absence of glutamate, was almost as fast at pH 7.4 as it was at pH 6.8 (Fig. 4). However, at the higher pH, it had much greater respiratory control ratios, probably reflecting a buildup of oxalacetate producing a transient inhibition of the state 4 rate. In addition, the ADP/O ratios at pH 7.4 were 0.3 to 0.5 unit lower than at pH 6.8. The reason for this is unclear, but the lower external pH may help to maintain better the proton motive force.

This contrasts with the situation reported for mitochondria isolated from cauliflower buds (21), etiolated corn shoots (9), and *Kalanchoë daigremontiana* leaves (8), where malate oxidation was strongly inhibited by increasing the pH of the external medium. A possible explanation is that the cofactors CoA and TPP were included in the assay medium for malate oxidation by pea leaf mitochondria. These would help to remove oxalacetate by condensation with acetyl-CoA (from pyruvate produced by malic enzyme). Both pyruvate dehydrogenase (26) and citrate synthase (5) are inhibited by ATP, and this may explain why, in the absence of glutamate, the state 3 rate after the third addition of ADP began to decrease (Fig. 4, B and C). On the other hand, inhibition of corn mitochondrial malate oxidation by higher pH cannot be overcome by the addition of the cofactors CoA and TPP alone, but also requires added pyruvate (9). This could be explained by lower levels of malic enzyme activity in corn mitochondria compared to pea leaf mitochondria purified by our method.

Malate oxidation by purified mitochondria was more sensitive to inhibition by rotenone than was that of washed mitochondria (Table II). Purified mitochondria also exhibited an increase in the rotenone-inhibited rate upon the addition of NAD^+, whereas washed mitochondria did not (Table II). In neither case did NAD^+ stimulate the uninhibited rate (data not shown), indicating that any increase in the rotenone-inhibited rate was not due to entry of NAD^+ into the matrix, and stimulation of the activity of malate dehydrogenase or malic enzyme.

The differences in rotenone sensitivity and response to NAD^+ may reflect either a change in the characteristics of the mitochondria after purification, which seems unlikely, or a difference in the effective concentrations of the two compounds. Table II shows that, expressed on a protein basis, the concentrations of rotenone and NAD^+ are approximately five times greater in the experiments with purified mitochondria than with washed mitochondria. However, as a large amount of nonmitochondrial protein is present in the washed mitochondria, a better comparison of the effective concentrations would be based on the activity (rate of O_2 uptake) in the controls. If this ratio is used, the effective concentration of rotenone and NAD^+ is two and a half times greater with the washed mitochondria than the purified mitochondria, which should have produced a greater inhibition of oxidation in the washed mitochondria. Hence, it must be concluded that there is some binding of rotenone and NAD^+ by nonmitochondrial protein in the washed mitochondrial suspensions. This demonstrates the precautions needed when working with unpurified mitochondria.

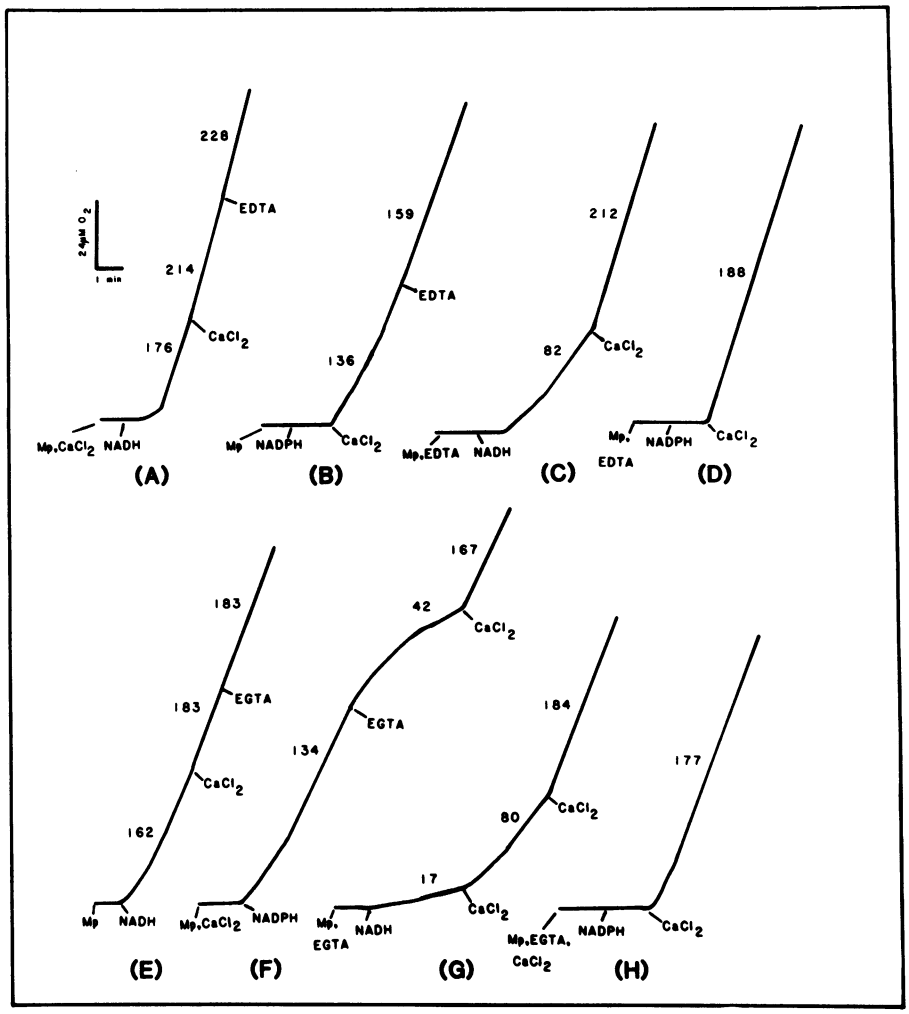

FIG. 5. Effect of $CaCl_2$ and chelating agents on oxidation of NADH and NADPH by purified mitochondria (Mp). Additions were: NADH, 1 mM; NADPH, 2 mM; $CaCl_2$, 1 mM; EDTA, 1 mM; EGTA, 1 mM. All experiments were carried out in the presence of 0.66 μM CCP.

Purified mitochondria also exhibited greater sensitivity to potassium cyanide, added at a concentration of 0.38 mM. Malate + glutamate oxidation by these mitochondria was inhibited by 80 ± 3% ($n = 4$) compared to 69 ± 3% ($n = 4$) for washed mitochondria. This may have been due to some nonmitochondrial binding of cyanide in the washed preparation and/or removal of lipoxygenase activity by gradient purification, as with mung bean mitochondria (28).

NADPH Oxidation. Purified mitochondria oxidized NADPH, in the presence of 1 mM $CaCl_2$, at rates slightly lower (89 ± 5%, $n = 5$) than those of exogenous NADH, measured under the same conditions. No oxidation of NADPH was detected without the addition of Ca^{2+}. $MnCl_2$ (1 mM) or $MgCl_2$ (1 mM) could not replace $CaCl_2$. As reported for mitochondria from a variety of plants (2, 4, 8, 12), the oxidation of NADPH was coupled to two phosphorylation sites, insensitive to rotenone and completely inhibited by antimycin A, indicating that it is oxidized by the electron transport chain via a dehydrogenase located on the outside of the inner membrane and bypassing the first phosphorylation site, as occurs with exogenous NADH oxidation. Some reports (2, 18, 24) have shown that NADPH oxidation requires, or is stimulated by, Ca^{2+}. However, other studies of NADPH oxidation, including those of washed pea leaf mitochondria (12), have indicated no requirement for Ca^{2+}. With the exception of the mitochondria isolated by Arron and Edwards (2), the mitochondria were not purified and, consequently, the preparations may have contained higher levels of Ca^{2+} than ours. Alternatively, since the gradient preparation media in our experiments contained 1 mM EDTA, some Ca^{2+} may have been removed from the membranes during the purification procedure. NADH oxidation was stimulated by the addition of 1 mM $CaCl_2$ (22 ± 7%, $n = 4$) whereas malate + glutamate oxidation was not affected.

Four pathways of NADPH oxidation are possible: (a) via a transhydrogenase, NADPH + NAD^+ → $NADP^+$ + NADH; (b) via a phosphatase, NADPH → NADH + Pi; (c) via the same dehydrogenase as that responsible for exogenous NADH oxidation; (d) via a separate dehydrogenase located on the outside of the inner membrane.

In purified pea leaf mitochondria, oxidation of NADPH is not via a transhydrogenase with subsequent NADH oxidation since NAD^+, which would be expected to increase the rate of transhydrogenase activity, had no effect on NADPH oxidation either in the presence or absence of Ca^{2+} (data not shown). This is in contrast to the results obtained by Koeppe and Miller (18) with washed corn mitochondria, where the oxidation of NADPH in the presence of $CaCl_2$ was stimulated by NAD^+, and the results of Arron and Edwards (2) who obtained slight NAD^+ stimulation of NADPH oxidation by *S. praealtum*, corn, and potato tuber mitochondria. Møller and Palmer (23), however, showed inhibition by NAD^+ of NADPH oxidation by *Arum maculatum* mitochondria. We measured the activity of the NADH-specific enzyme, lactate dehydrogenase, in the presence of the same concentrations of mitochondria, NADPH, ADP, and $CaCl_2$ as we normally used in the oxygen electrode, with the addition of 19 mM pyruvate and

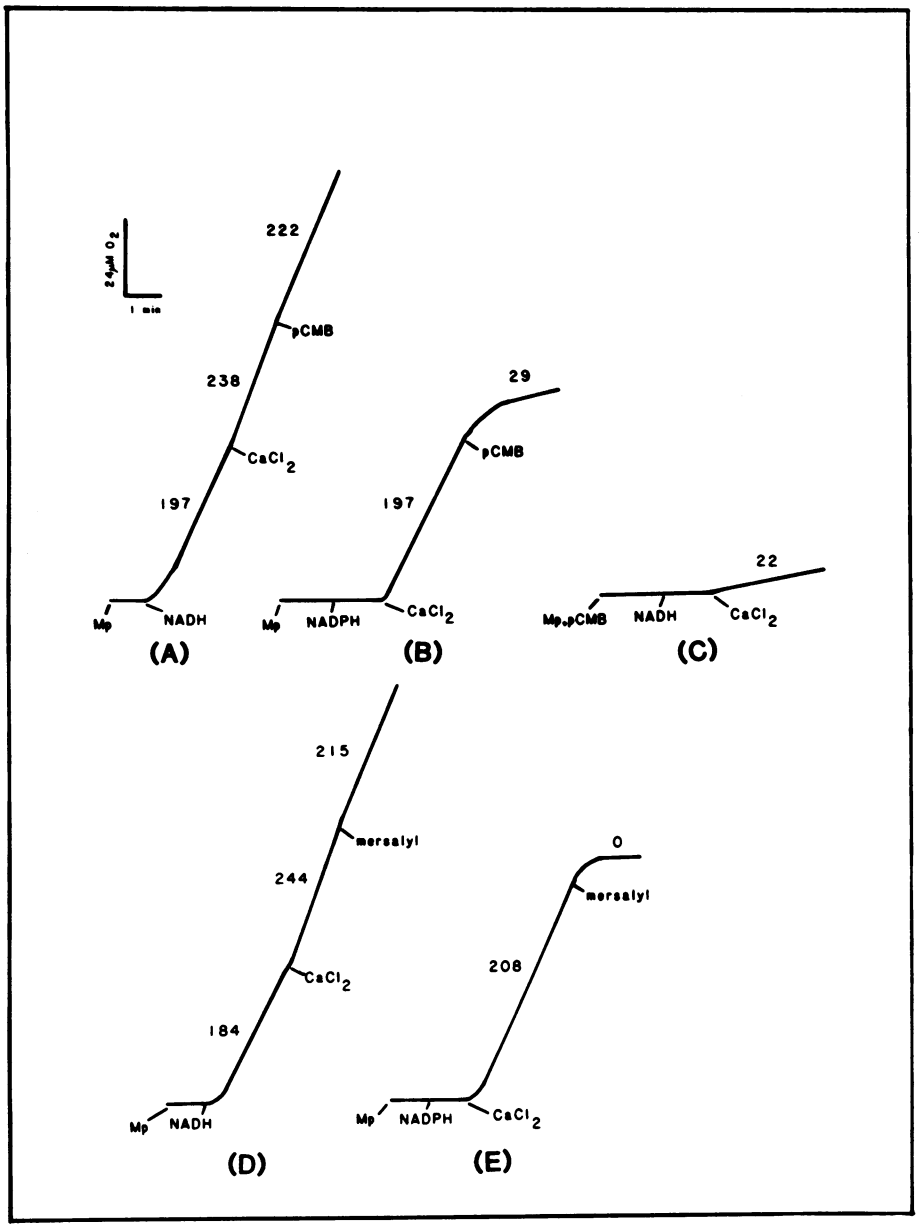

FIG. 6. Effect of $CaCl_2$, *p*CMB, and mersalyl on oxidation of NADH and NADPH by purified mitochondria (Mp). Additions were: NADH, 1 mM; NADPH, 2 mM; $CaCl_2$, 1 mM; *p*CMB, 50 μM; mersalyl, 6 μM. All experiments were carried out in the presence of 0.66 μM CCP.

sufficient antimycin A to inhibit mitochondrial NAD(P)H oxidation. If a phosphatase were present, it would convert NADPH to NADH which would subsequently be oxidized via lactate dehydrogenase. The net result would be pyridine nucleotide oxidation, at rates at least as fast as O_2 uptake measurements (244 nmol NADPH oxidized/min·mg protein). However, a value of only 4 nmol NAD(P)H oxidized/min·mg protein was measured spectrophotometrically. This indicates that neither a phosphatase nor a transhydrogenase (no effect of NAD^+) is involved in the oxidation of NADPH by pea leaf mitochondria and confirms the results of Møller and Palmer (23) for mitochondria from Jerusalem artichoke and *A. maculatum*.

On the basis of different sensitivities to chelators and the sulfhydryl reagents, *N*-ethylmaleimide, *p*CMB, *p*-chloromercuriphenylsulfonic acid, and mersalyl, Arron and Edwards (3) have suggested that the NADPH dehydrogenase is distinct from the NADH dehydrogenase. Purified pea leaf mitochondria also showed different sensitivities to chelators, *p*CMB and mersalyl (*p*-chloromercuriphenylsulfonic acid and *N*-ethylmaleimide were not tested). Since Ca^{2+} was essential for NADPH oxidation, measurements for both NADPH and NADH oxidation were made in the presence of 1 mM $CaCl_2$. In addition, an uncoupler (0.66 μM CCP) was added to prevent any rate limitation of the electron transport chain by decreased entry of phosphate due to inhibition of the phosphate translocator.

EDTA (1 mM) had no effect on the oxidation of either substrate if it was added after substrate oxidation had started (Fig. 5, A and B). If EDTA was added before the substrate, and in the absence of Ca^{2+}, NADH oxidation was inhibited 62% and no oxidation of NADPH occurred. However, both substrates were oxidized at normal rates upon the addition of 1 mM $CaCl_2$ (Fig. 5, C and D). EGTA (1 mM) did not inhibit NADH oxidation if it was added after the substrate (Fig. 5E) but NADPH oxidation was inhibited (Fig. 5F). This inhibition was reversed by the addition of a further 1 mM $CaCl_2$. If EGTA was added in the absence of $CaCl_2$ and prior to NADH, a 91% inhibition was observed (Fig. 5G). This was reduced to 56% by the addition of 1 mM $CaCl_2$ and fully overcome by the addition of a further 1 mM $CaCl_2$. Similar results

showing protection by NADH against ED(G)TA inhibition have been reported by Møller *et al.* (22). NADPH oxidation was completely inhibited if 1 mM EGTA and 1 mM $CaCl_2$ were added prior to the substrate (Fig. 5H). However, the addition of another 1 mM $CaCl_2$ restored full oxidative activity. Møller *et al.* (22) have shown that Jerusalem artichoke mitochondria have a specific requirement for Ca^{2+} for NADH oxidation. This requirement is in addition to the capacity of Ca^{2+} to screen electrostatically the fixed charges associated with the surface of the membrane. The results in Figure 5 indicate that pea leaf mitochondria also have a specific requirement for Ca^{2+} for both NADH and NADPH oxidation and that NADPH oxidation requires higher levels of Ca^{2+} than NADH oxidation.

NADH and NADPH oxidation exhibited marked differences in their sensitivity to the sulfhydryl reagents *p*CMB and mersalyl. NADH oxidation was only slightly inhibited (7%) by the addition of 50 μm *p*CMB whereas NADPH oxidation was inhibited 85% (Fig. 6, A and B). As reported for turnip mitochondria (10), we observed much greater inhibition of NADH oxidation (91%; Fig. 6C) if the mitochondria were preincubated with *p*CMB, indicating that NADH binds to its dehydrogenase via a sulfhydryl group or in close proximity to one. Mersalyl (6 μM) also inhibited NADH oxidation slightly (12%; Fig. 6D), but it completely abolished NADPH oxidation (Fig. 6E). As with *p*CMB, mersalyl inhibition of NADH oxidation was much greater (69%; data not shown) if the inhibitor was added prior to the substrate.

If separate dehydrogenases are involved in NADH and NADPH oxidation, and the rate of electron transfer to O_2 is not limiting, the addition of both substrates should result in faster rates of O_2 uptake than for either substrate alone. This was observed. The addition of 1 mM NADH to mitochondria oxidizing NADPH (2 mM) in state 3 increased the rate of O_2 uptake by 55% (Table III). A further increase (20%) occurred when malate and glutamate were added. This contrasts with the results obtained by Arron and Edwards (2), who reported that the rate of O_2 uptake of mitochondria oxidizing NADH could be increased by the addition of malate but not NADPH. It is not clear whether this represents an intrinsic difference between pea leaf mitochondria and the other mitochondria tested.

Since NADH and NADPH oxidation exhibit different requirements for calcium and different sensitivities to chelators and sulfhydryl reagents, and the addition of both substrates increases the rate of O_2 uptake compared to either substrate alone, it is evident that two separate dehydrogenases are involved in their oxidation. The rates obtained with purified pea leaf mitochondria show that their potential for NADPH oxidation *in vitro* is quite high (see Table I), and suggests that *in vivo* oxidation of cytoplasmic NADPH may occur at reasonably fast rates. At pH 7.4, NADPH oxidation by pea leaf mitochondria was only slightly slower than NADH oxidation. This contrasts with the results obtained using potato tuber mitochondria where NADPH oxidation was about 40% as fast as NADH oxidation (3) and with Jerusalem artichoke mitochondria, where the rate of NADPH oxidation at pH 7.2 was less than 5% of that of NADH oxidation (24).

This work demonstrates that leaf mitochondria of high integrity, and substantially free of Chl, may be obtained by purification on linear sucrose density gradients. Further refinement of the method, particularly the homogenization technique and removal of the mitochondria from the gradient, may make it possible to remove more of the contaminating Chl. However, it may be possible to remove all the Chl because during physical contact it may partition into the lipid of mitochondrial membranes.

Acknowledgments—The able technical assistance of S. Grubb, G. Napier, and A. L. Fox is gratefully acknowledged. We thank Dr. M. E. Lawrence for carrying out the electron microscopy work.

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