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PREPARATION OF INTACT PLANT MITOCHONDRIA

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SUMMARY

Mitochondria from mung bean hypocotyls and potato tubers have been separated from contaminating organelles and membrane fragments on discontinuous sucrose gradients. After removal from the centrifuge tubes only intact mitochondria survive dilution to 0.3 M sucrose. The physiological integrity of these purified preparations is confirmed by three integrity tests. The influence of purification on oxidative capacities and carrier concentrations are detailed.

INTRODUCTION

Commonly used criteria for intactness of isolated mitochondria include good ADP:O ratios, strong control by ADP and reasonable appearance in electron micrographs. These criteria appear to be routinely achieved, yet it is clear that mitochondria prepared by the usual differential centrifugation techniques, while tightly coupled, are contaminated by various subcellular structures[1-3]. In addition the outer membranes of an unknown percentage of the isolated mitochondria may have suffered damage.

The purpose of this paper is to demonstrate that intact plant mitochondria, after separation from contaminants on sucrose gradients, survive dilution to iso-osmolar conditions. The remarkable homogenity and intactness of these purified mitochondrial preparations are described as are some of their oxidative capacities. A preliminary account of these results has already been presented[4].

METHODS

The plant material used in these experiments included potato tubers (*Solanum tuberosum*) and etiolated mung bean hypocotyls (*Phaseolus aureus*). Bean seedlings were grown in a dark room maintained at 28 °C and 60% relative humidity. Freshly harvested potatoes were obtained in Gilbertsville, Pa., from Mr R. L. Blimline. Mitochondria were prepared by methods that have been described previously[5], except that a Moulinex mixer 66 (Alençon, France) was used for tissue disruption. The washed mitochondria preparations were suspended in a small volume of wash medium (50 mg protein/ml).

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The method used for the purification of washed mitochondria consists of layering them on top of discontinuous sucrose gradients and centrifuging in a swinging bucket rotor for 45 min at 20000 rev./min (40692 × *g*) (Beckman Model L preparative centrifuge, SW 25:1 rotor). The gradients were prepared by layering sucrose solutions containing 0.1 % bovine serum albumin and 10 mM phosphate buffer into centrifuge tubes in the sequence of concentrations: 1.8 M (6 ml), 1.45 M (6 ml), 1.2 M (6 ml), 0.9 M (3 ml), and 0.6 M (3 ml). Following centrifugation the mitochondria were found at the boundary between 1.2 and 1.45 M sucrose. The mitochondria were removed by injecting 2.0 M sucrose solution into the base of the tube and collecting the appropriate fraction. These mitochondria, now in 1.35 M sucrose, were diluted slowly at 0 °C with 10 mM phosphate buffer containing 0.1 % bovine serum albumin until a sucrose concentration of 0.3 M was achieved. Regardless of the volume of the mitochondrial suspension, the time required for the dilution process remains about 20 min. The diluted mitochondria were centrifuged at 9000 × *g* for 15 min, and the purified mitochondria were collected and suspended in a small volume of 0.3 M sucrose, containing 10 mM phosphate buffer and 0.1 % bovine serum albumin (suspension medium). This final suspension contained around 100 mg mitochondrial protein per ml. All operations were carried out between 0 and 2 °C and at pH 7.2.

Oxygen uptake was measured at 25 °C in a 3-ml stirred cell using a Clark oxygen electrode (Yellow Springs Instrument Co.) as described by Estabrook[6]. The reaction medium (Medium A) contained 0.3 M mannitol (or sucrose), 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2) and 0.5–2 mg of mitochondrial protein. The oxygen concentration in air-saturated medium was taken as 250 μM[6].

Spectrophotometric measurements were performed with a sensitive scanning split beam spectrophotometer described by Chance[7]. The mitochondrial concentrations of cytochromes and flavoproteins were measured at room temperature from succinate anaerobic minus oxidized difference spectra; the pairs of wavelengths selected for measurement of individual components, as well as the extinction coefficients, were those given by Chance and Williams[8], and Lance and Bonner[9]. The extraction and enzymatic estimation of pyridine nucleotides are described by Williamson and Corkey[10], using the apparatus described by Mayer *et al.*[11]

The media and the conditions used in each assay for the determination of enzyme activities were as follows:

(1) Succinate:cytochrome *c* oxidoreductase (EC 1.3.99.1): 5 mM phosphate buffer (pH 7.2), 0.05 mM cytochrome *c*, 1 mM KCN, and 0.1–1 mg of mitochondrial protein. The reaction was initiated with 10 mM succinate. The final volume was 3 ml.

(2) NADH:cytochrome *c* oxidoreductase (EC 1.6.99.3): 5 mM phosphate buffer (pH 7.2), 0.05 mM cytochrome *c*, 1 mM KCN, and 0.1–1 mg of mitochondrial protein. The reaction was initiated with 0.3 mM NADH. The cytochrome *c* reduction was measured in a Zeiss spectrophotometer using the millimolar absorbance coefficient at 550 nm of 21.0 $mM^{-1} \cdot cm^{-1}$ (ref. 12).

(3) Substrate: $K_3Fe(CN)_6$ oxidoreductase: Medium A, 170 μM ATP, 1 mM KCN, substrate (see Results) and 0.5–2 mg of mitochondrial protein. The reaction is initiated with 1 mM $K_3Fe(CN)_6$, and its reduction was measured in a Zeiss spectrophotometer using the millimolar absorbance coefficient at 420 nm of 1.03 $mM^{-1} \cdot cm^{-1}$

(final volume, 3 ml). The contribution of the nonenzymatic reduction of $K_3Fe(CN)_6$ was subtracted from the reaction in each assay.

Mitochondrial protein content was determined by a modified Lowry method[13] with crystallized bovine serum albumin (Miles Laboratories, Inc.) as the standard.

For electron microscopy, mitochondria in suspending medium were fixed for 1 h in 2 % glutaraldehyde. After centrifugation the pellet was washed briefly with suspending medium and was postfixed with 1 % OsO_4 dissolved in suspending medium. The pellet was then washed with distilled water, dehydrated in acetone and embedded in araldite. Ultra thin sections were cut with an LKB ultramicrotome and floated on to copper grids. The specimens were examined and photographed in a Philips 200 operating at 60 kV.

RESULTS

Purification of washed mitochondria on a discontinuous sucrose gradient

Fig. 1 shows the position of mitochondria from various preparations after centrifugation on discontinuous sucrose gradients. Whatever their origin, the mitochondria always aggregate at the interface between the 1.45 M and the 1.2 M sucrose.

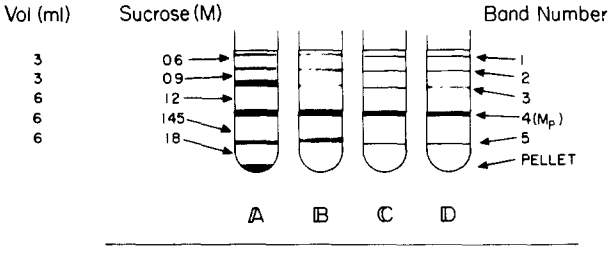

Fig. 1. Mitochondrial preparations centrifuged on discontinuous sucrose gradients. (A) Mung bean mitochondria. (B) Potato mitochondria. (C) Pigeon heart mitochondria. (D) Rat liver mitochondria. Potato mitochondria M_w protein/M_p protein = 1.9, average of 38 experiments. Mung bean mitochondria: M_w protein/ M_p protein = 3.7, average of 26 experiments. Abbreviations for all figures. M_w, washed mitochondria; M_p, purified mitochondria.

Interestingly, mitochondria isolated from potatoes (B) are less dense than those isolated from mung beans (A); therefore, the concentration of the sucrose layers between which the mitochondria accumulate is very important. Thus an interface of 1.35 M sucrose will stop potato mitochondria, but will allow mung bean mitochondria to pass through. Fig. 1 also shows that the kind and amount of contamination varies in washed mitochondria according to their source. It is clearly seen from the ratio of the total protein added to the top of the gradient to the total protein collected in Fraction 4 (purified mitochondria), that the contamination is very great in mung bean washed mitochondria. This contamination is for the main part due to an unknown subcellular structure which passes through the 1.8 M sucrose layer and forms a pellet at the bottom of the tube.

Electron micrographs of purified mung bean mitochondria (Fig. 2) show a homogeneous population of mitochondria with the inner and outer membranes clearly distinguishable. Similar pictures have been obtained from purified potato mitochondria.

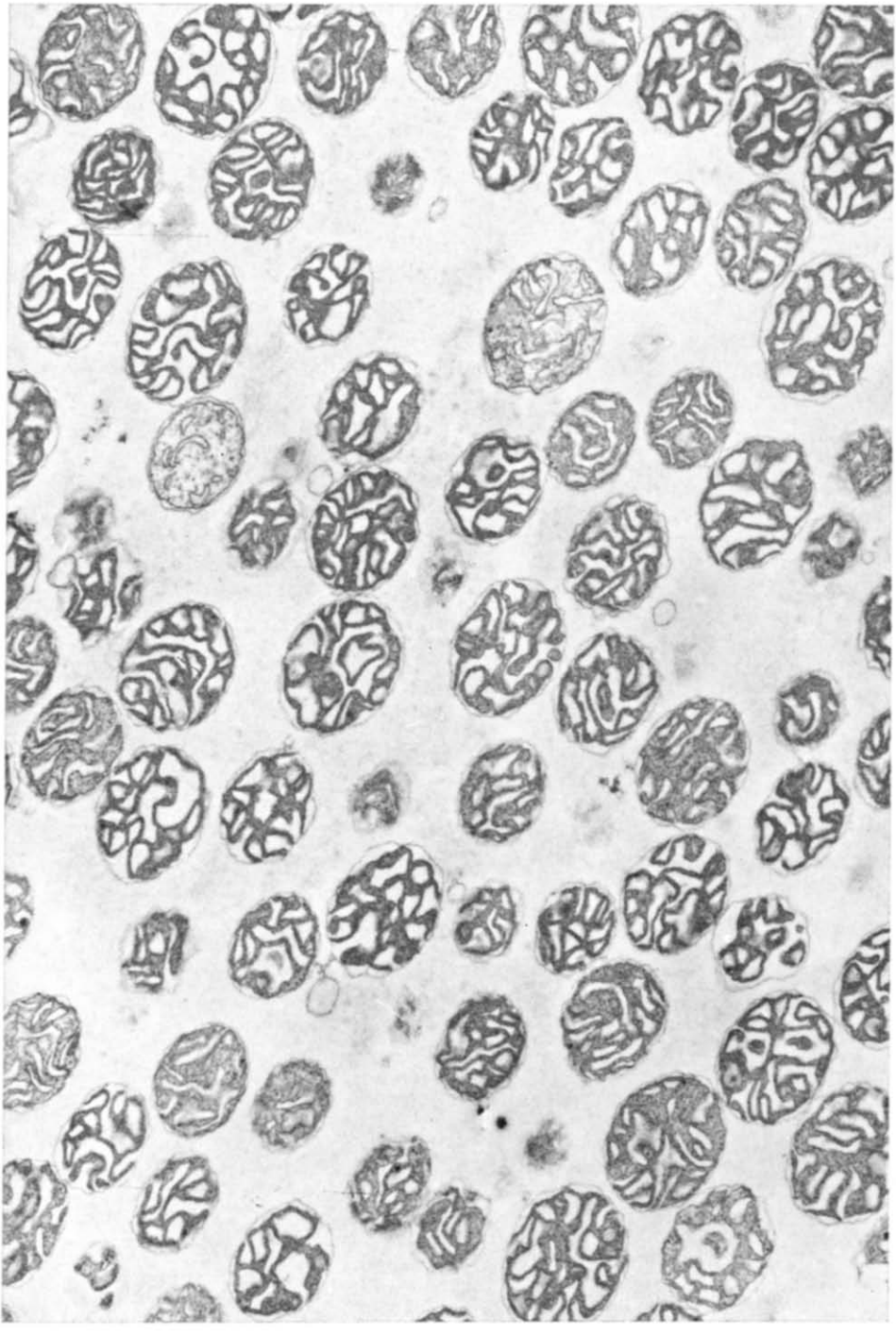

Fig. 2. Electron micrograph of purified mung bean mitochondria. Fixation methods described in text. Magnification = 19600.

The activity of washed and purified mitochondrial preparations

The quality of the purified mitochondrial preparation can be appraised rapidly by measurements of certain characteristic parameters. Respiratory activity is shown in Fig. 3 which illustrates several oxygen electrode traces obtained from mung bean preparations (washed and purified mitochondria). These traces show that on a protein basis, the rate of oxygen uptake in State 3 is much faster in purified mitochondria than in the washed mitochondria. Tables I and II give the average values for rates of oxidation, ADP:O ratios and respiratory control indices with succinate, malate and NADH as substrates. These values are averages from a great number of preparations; the maximal rates usually obtained with the second addition of ADP have been used. Inspection of Tables I and II shows that the quality of the purified mitochondrial preparations is far superior to those prepared in the conventional way[5]. The State 3 rates for all the substrates are somewhat faster than those of the washed mitochondria; approximately 3 times in mung bean and 1.7 times in potato. In addition, there is very strong control by ADP, and the ADP:O ratios are improved over those of the washed mitochondria. In fact, when carefully prepared, purified plant mitochondria are more active than mitochondria derived from animal tissues[15].

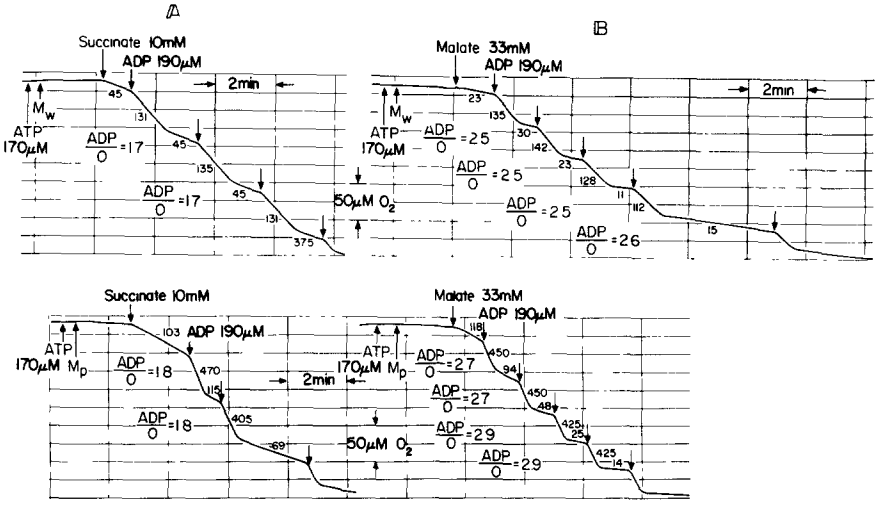

Fig. 3. Oxidation of succinate (A) and malate (B) by mung bean mitochondria The concentrations given are the final concentrations in the reaction medium The numbers on the traces refer to nmoles oxygen consumed per min per mg of mitochondrial protein.

Quantitative determination of electron carriers

The concentrations of the various electron carriers obtained by using the method of Chance and Williams[8] for two different kinds of mitochondria are given in Table III and illustrated in Fig. 4. Expressed in terms of nmoles per mg of mitochondrial protein, the concentration of the different carriers is greater in the purified mitochondria compared to washed mitochondria, 3 times in mung bean and 1.7 times in potato. It is interesting to note, just as the State 3 oxidation rates are more rapid in the purified mitochondria (Tables I and II), compared to washed

TABLE I

OXIDATION AND PHOSPHORYLATION CAPACITIES OF WASHED AND PURIFIED MUNG BEAN MITOCHONDRIA

10 mM succinate, 33 mM malate*, 1 mM NADH.

	Succinate		*Malate**		*NADH*	
	M_w	M_p	M_w	M_p	M_w	M_p
nmoles $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein (± S.D.)	170 ± 10	451 ± 25	162 ± 9	462 ±	182 ± 12	510 ± 30
ADP:O (± S.D.)	1.54 ± 0.10	1.68 ± 0.12	2.38 ± 0.21	2.56 ± 0.20	1.31 ± 0.11	1.55 ± 0.13
Respiratory control index (± S.D.)	2.7 ± 0.25	3.2 ± 0.26	4.8 ± 0.31	5.4 ± 0.33	3.9 ± 0.29	4.5 ± 0.30
Figures averaged from number of experiments	26	26	26	26	10	10

Abbreviations for all tables: M_w washed mitochondria; M_p, purified mitochondria.

* The dehydrogenase involved is the NAD linked malic enzyme[14].

TABLE II

OXIDATION AND PHOSPHORYLATION CAPACITIES OF WASHED AND PURIFIED POTATO MITOCHONDRIA

10 mM succinate, 33 mM malate*, 1 mM NADH.

	Succinate		*Malate**		NADH	
	M_w	M_p	M_w	M_p	M_w	M_p
nmoles $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein (± S.D.)	180 ± 11	308 ± 18	122 ± 10	220 ± 12	70 ± 5	127 ± 9
ADP:O (± S.D.)	1.57 ± 0.12	1.64 ± 0.14	2.40 ± 0.22	2.59 ± 0.23	1.29 ± 0.10	1.48 ± 0.11
Respiratory control index (± S.D.)	3.20 ± 0.27	3.41 ± 0.29	4.20 ± 0.35	4.60 ± 0.36	2.1 ± 0.20	2.4 ± 0.21
Figures averaged from number of experiments	38	38	38	38	9	9

* The dehydrogenase involved is the NAD linked malic enzyme[14].

mitochondria, the carrier concentrations are similarly increased. When expressed on a relative basis taking the cytochrome aa_3 concentration as unity, there is virtually no difference between the purified and washed mitochondria. The stoichiometry of the different electron carriers found in plant mitochondria is similar to that reported for animal tissues[15]. From the measurements of the rates of succinate oxidation (Tables I and II) and from the data used to produce Table III, it is also possible to determine the turnover numbers of the different cytochromes[9]. Table IV shows that the turnover numbers of cytochromes $a + a_3$ and c are about the same for purified and washed mitochondria. These values are higher than those obtained with animal mitochondria[16–18] but compare favorably with those reported for yeast and plant mitochondria[16,19].

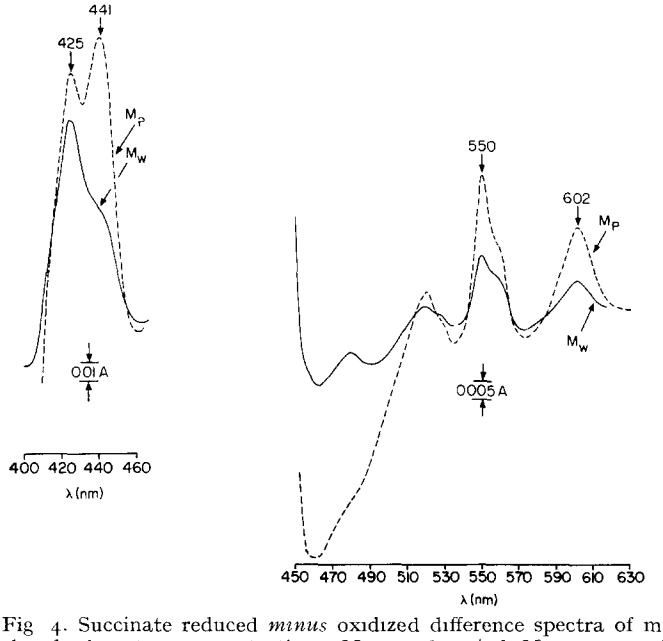

Fig. 4. Succinate reduced *minus* oxidized difference spectra of mung bean mitochondria. Mitochondrial protein concentrations: M_p = 3.6 mg/ml, M_w = 3.9 mg/ml; 25 °C, 10-mm light paths.

TABLE III

CONCENTRATIONS OF CARRIERS IN WASHED AND PURIFIED MUNG BEAN AND POTATO MITOCHONDRIA

All figures are mean values of 7 experiments for each type of mitochondria

Material		*Carrier concn (nmoles/mg protein)*				
		Cytochrome aa_3	*Cytochromes* b*	*Cytochromes* c**	*Flavoprotein*	*Pyridine nucleotide*
Mung bean	M_w	0.12	0.15	0.20	0.40	1.9
	M_p	0.38	0.33	0.60	1.4	6.5
Potato	M_w	0.16	0.18	0.27	0.56	2.1
	M_p	0.28	0.27	0.42	0.90	4.3

* Cytochromes b: $b_{553} + b_{557} + b_{563}$[8].
** Cytochromes c: $c_{547} + c_{549}$[8].

TABLE IV

ACTIVITY OF THE ELECTRON TRANSFER CHAIN IN MUNG BEAN AND POTATO MITOCHONDRIA

The data were determined from the highest succinate State 3 rate (Fig. 3) and the data in Tables I, II, and III.

Material	Q_{O_2} *of mitochondria* ($\mu l\ O_2 \cdot h^{-1} \cdot mg^{-1}\ N$)	*Turnover* (s^{-1})	
		Cytochrome aa_3	*Cytochrome c*
Mung bean	M_w 1430	94	56
	M_p 3800	79	50
Potato	M_w 1510	75	44
	M_p 2600	73	49

Assays on integrity of purified mitochondria

The quality of purified mitochondrial preparations can also be evaluated by the measurement of various enzyme activities. Fig. 5 shows that the activity of succinate:cytochrome *c* oxidoreductase in purified potato mitochondria is virtually negligible (Curve B), when the osmolarity of the medium is sufficient to prevent the bursting of the outer membranes. However, in a medium approximately 15 mosM such activity is considerable (Curve A). In both cases the introduction of antimycin A into the medium completely inhibits the reaction. The succinate dehydrogenase is located on the internal face of the inner mitochondrial membrane[20,21]. When the mitochondria have intact outer membranes, the exogenous cytochrome *c* is unable to pass through (R. Douce and W. D. Bonner, Jr. unpublished results), and is therefore not reduced. Under these conditions, any succinate:cytochrome *c* oxidoreductase activity would indicate that the mitochondrial outer membrane has been damaged. We have always found that the succinate:cytochrome *c* oxidoreductase activity of tightly coupled Mp from potato and mung bean is practically negligible.

Fig. 6 shows that the activity of NADH:cytochrome *c* oxidoreductase is

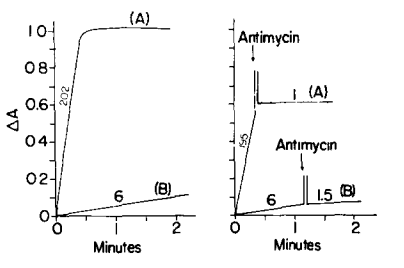

Fig. 5. Succinate:cytochrome *c* oxidoreductase activity of purified potato mitochondria. (A) Burst mitochondria in 5 mM phosphate buffer. (B) Intact mitochondria in 5 mM phosphate buffer containing 0.3 M sucrose. Antimycin A (0.5 μg/ml) was added as indicated. The numbers on the traces refer to nmoles cytochrome *c* reduced per min per mg mitochondrial protein.

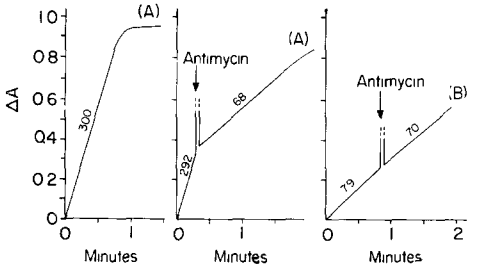

Fig. 6. NADH.cytochrome *c* oxidoreductase activities of purified potato mitochondria. (A) Burst mitochondria in 5 mM phosphate buffer. (B) Intact mitochondria in 5 mM phosphate buffer containing 0.3 M sucrose. Antimycin A (0.5 μg/ml) was added as indicated. The numbers on the traces refer to nmoles cytochrome *c* reduced per min per mg mitochondrial protein.

measurable in purified potato mitochondria. The addition of antimycin A to the incubation medium has virtually no effect on such activity (Curve B). On the other hand, when the mitochondrial outer membranes are burst osmotically, there is an increase in NADH:cytochrome *c* oxidoreductase activity (Curve A). Under these conditions, the addition of antimycin A results in a strong inhibition (75%). The rate of activity in the presence of this inhibitor is similar to that observed with intact mitochondria (Curve B). When the mitochondria are intact, the observed reductase activity is due solely to the antimycin A insensitive NADH:cytochrome *c* oxidoreductase which is located near the external face of the outer membrane (ref. 22 and R. Douce and W. D. Bonner, Jr, unpublished results). However, when the outer membranes are burst, there is a marked increase in the reduction of cytochrome *c*, the antimycin A insensitive NADH:cytochrome *c* oxidoreductase and the antimycin A sensitive NADH:cytochrome *c* oxidoreductase both contributing to the rate. In the latter case, the NADH dehydrogenase involved is located toward the outer surface of the inner membrane and is connected to the respiratory chain[1,21]; this dehydrogenase has been described in yeast[21] and plant mitochondria[1]. Inhibition of NADH:cytochrome *c* oxidoreductase by antimycin A in intact mitochondria is also indicative of damage to the mitochondrial outer membrane; we have always found that NADH:cytochrome *c* oxidoreductase activity of tightly coupled, purified mitochondria is virtually unaffected by the addition of antimycin A.

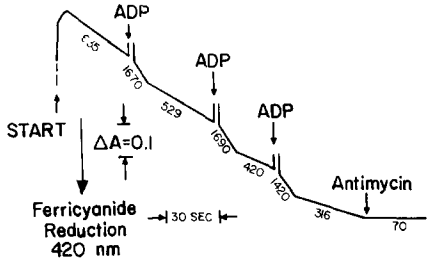

Fig. 7. Malate ferricyanide reductase activity of purified mung bean mitochondria. Mitochondria were incubated at 25°C in Medium A with 33 mM malate, 1 mM KCN and 170 μM ATP. The reaction was initiated with 1 mM $K_3Fe(CN)_6$. Where indicated, 50 μM ADP or antimycin A (0.5 μg/ml) were added. The numbers on the traces refer to nmoles $K_3Fe(CN)_6$ reduced per min per mg mitochondrial protein.

Fig. 7 shows that intact purified mung bean mitochondria rapidly reduce ferricyanide in the presence of malate. The addition of a small quantity of ADP gives an increase in the rate of electron transport (State 3, Chance and Williams[23]). This fast rate declines after all the ADP has been phosphorylated (State 4, Chance and Williams[23]). This allows the ratio ADP:$2e^-$ (equivalent to the P:O ratio) to be calculated[24,25] and also the electron flow control index (equivalent to the respiratory control index[26]). This figure also shows that the reduction of ferricyanide is almost completely inhibited by the addition of antimycin A. The trace is very similar to that given in Fig. 3 for the oxidation of malate.

These results confirm the intactness of the purified mitochondria. The inner membrane represents an impenetrable barrier to the ferricyanide[24,27], and under these conditions it can react only with the cytochrome *c* which is localized on the

TABLE V

FERRICYANIDE REDUCTASE ACTIVITY AND OXYGEN UPTAKE OF PURIFIED MUNG BEAN MITOCHONDRIA

Rates of ferricyanide reduction were measured spectrophotometrically (see Fig 7) following addition of 192 μM ADP Rates of oxygen uptake were measured amperometrically following addition of 192 μM ADP (see Fig 3). 0.5 μg/ml antimycin A addition is when indicated.

Substrate	*Ferricyanide reduction* ($nmoles \cdot min^{-1} \cdot mg^{-1}$ *protein*)		*Oxygen uptake* ($natoms \cdot min^{-1} \cdot mg^{-1}$ *protein*)	
	—	*Antimycin*	—	*Antimycin*
Succinate	1830 (ADP: $2e^-$ = 0.9)	105	890 (ADP O = 1 8)	120
Malate*	1690 (ADP: $2e^-$ = 1.4)	100	910 (ADP:O = 2.6)	85

* The dehydrogenase involved is the NAD linked malic enzyme[14].

external face of the inner membrane[28,29]. Table V shows that for either electron acceptor, whether it be ferricyanide or oxygen, the rate of electron flow is the same and is inhibited by the addition of antimycin A. Conversely, when the mitochondria are damaged (osmotic shock, sonication), the rate of reduction of ferricyanide is no longer stimulated by ADP and shows little inhibition with antimycin A. In this case, the ferricyanide reacts directly with the dehydrogenase[21,24]. Therefore, we have always ascertained that in preparations of intact purified mitochondria the rate of reduction of ferricyanide is strongly sensitive to antimycin A. The ADP:$2e^-$ ratio is very close to the theoretical value (succinate, 0.9; malate, 1.4), assuming that Site III phosphorylation is not involved.

The effect of the osmolarity of the medium on the oxidative properties of purified mitochondria

The mitochondria are routinely suspended in a medium with an arbitrary osmotic concentration of 0.3 M sucrose. This concentration is hypertonic to that of the internal compartment of the mitochondria (R. Douce and W. D. Bonner, Jr, unpublished results). During the purification process the mitochondria are subjected to a fluctuating osmotic environment. It was therefore of interest to investigate the influence of the osmotic concentration of the suspending media on the integrity of purified mitochondria.

Fig. 8 gives the activity of succinate:cytochrome *c* oxidoreductase of purified potato mitochondria as a function of the sucrose concentration of the medium. Fig. 8 shows that at concentrations above 0.08 osM the mitochondria are preserved intact (see also Fig. 5). On the other hand, at concentrations below 0.08 osM the progressive increase in succinate:cytochrome *c* reductase activity reflects the bursting of the outer membrane of the mitochondria (see Fig. 5). The shape of the curve obtained clearly shows that the mitochondrial resistance to bursting of the outer membrane is quite variable; some begin to burst at 0.08 osM, and some do not burst until an osmolarity of 0.03 has been reached. This concentration corresponds to a very low osmotic pressure.

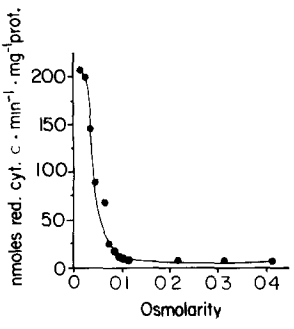

Fig 8. The effect of osmolarity on succinate cytochrome *c* oxidoreductase activity of purified potato mitochondria. The mitochondria were incubated at 25 °C in 5 mM phosphate buffer containing the indicated amount of sucrose.

DISCUSSION

The contamination in washed mitochondrial preparations is no surprise; what is surprising is the extent of the contamination in preparations from rapidly growing etiolated bean seedlings. The contamination in washed mitochondria from both mung bean and potato consists of microsomes, vacuolar membrane fragments and etioplasts; in addition those from mung beans contain the dense organelle already mentioned. Etioplasts contain cytochrome b_6; both the new dense organelle and microsomes contain *b* cytochromes. The removal of nonmitochondrial cytochromes *b* during purification is reflected in carrier concentrations (Tables III, Fig. 4) where amounts of *b* components in purified mitochondria from both potato and mung bean are hardly more than in the washed mitochondria, whereas the concentrations of all other carriers are markedly enhanced.

The purification procedure should be applicable to mitochondria from both higher and lower plants as well as to green plants. We have been successful in purifying mitochondria from cauliflower buds, jerusalem artichoke tubers, yeast, and light grown mung beans. In this latter case, where many fully developed green chloroplasts are present, buff-colored mitochondria were well separated from the green chloroplast fragments. Rat liver and pigeon heart mitochondria do not survive the purification procedure.

State 3 oxidation rates are much faster with purified mitochondria than with washed mitochondria. With potatoes, the ratio between the two rates (1.7) is very close to the value obtained for the ratio (1.9) of the mitochondrial protein added to the top of the sucrose gradient and the total mitochondrial protein recovered in Fraction 4. Such results clearly show that the physiological integrity of the mitochondria is maintained during the purification process. This interpretation confirms the fact that for all substrates used, respiratory control indices and ADP:O ratios are slightly higher in purified mitochondria compared to washed mitochondria. Further confirmation as to the intactness of the membranes of the purified mitochondria is shown by the fact that added soluble cytochrome *c* cannot pass through the outer membrane, and the inner membrane is an impenetrable barrier to the passage of ferricyanide.

The above conclusions are exactly the same with the mung bean preparations.

However, in this case the value for the ratio (3) of the State 3 oxidation rates between purified and washed mitochondria is not the same as the ratio (3.7) between the total mitochondrial protein placed on the top of the sucrose gradient and the mitochondrial protein recovered in Fraction 4. Nevertheless, this difference between the two ratios does not indicate mitochondrial damage during purification. With mung beans, approximately 25 % of the mitochondria pass through the 1.45 M sucrose and come to rest at the 1.8 M sucrose interface. Fraction 5 (Fig. 1) contains numerous etioplasts, and it is possible that the mitochondria found in this fraction are carried down with these organelles.

Two critical experimental conditions are required to maintain physiological integrity during the purification process. First, the mitochondria, after removal from the sucrose gradient, are under conditions of high osmolarity and are therefore extremely condensed. The volume of the matrix is greatly reduced, and the outer membrane is separated from the inner membrane by a greatly increased sucrose space. Consequently dilution to isoosmolar conditions must proceed very slowly to avoid damage resulting from too rapid inner membrane expansion. In the dilution procedure mung bean mitochondria are more easily damaged than are those from potato; some plant mitochondrial membranes are even more elastic than others. The second critical condition is the inclusion of bovine serum albumin in both the sucrose gradient and the dilution medium. Fatty acids, liberated by the destruction of phospholipid membranes, combine with bovine serum albumin; without this agent purified mitochondria show reduced ADP:O ratios and respiratory control compared to the initial washed mitochondrial preparation.

Recently Wattiaux *et al.*[30] have demonstrated that rat liver mitochondria show noticeable deterioration during centrifugation on discontinuous sucrose gradients. This deterioration was caused by high water pressures resulting from large centrifugal forces, and such pressures cause dissociation of inner membrane lipoproteins. The centrifugal force ($40\,700 \times g$) used on the plant mitochondria was much less than that used by Wattiaux *et al.*[31] ($200\,000 \times g$) and there was no such deterioration. We have found that plant mitochondria also fall apart and occasionally burst at centrifugal forces of $200\,000 \times g$.

The fact that plant mitochondria survive the purification procedure emphasizes the elasticity of their membranes. This elasticity is also shown by the fact that the mitochondria retain their integrity in a medium of very low osmotic pressure. Such a remarkable property is probably related to the fact that the water content of plant cells undergoes considerable fluctuation.

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