THE ELECTRON TRANSFER AND OXIDATIVE PHOSPHORYLATION SYSTEM AND CYTOCHROME C REDUCTASE ACTIVITY OF MITOCHONDRIA ISOLATED FROM *SOLANUM TUBEROSUM L.*[1]

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Abstract: Mitochondria capable of oxidative phosphorylation and respiratory control have been isolated from *Solanum tuberosum* L. using polyvinylpyrrolidone as a complexing agent for oxidizing compounds formed during cellular disruption. An intermittent low speed centrifugation at 200×g combined with isolation medium containing mannitol, bovine serum albumin and cysteine proved satisfactory for the sendimentation of starch and separation of functional mitochondria having high protein content.

Isolated mitochondria were found to be actively oxidizing substrates such as succinate, malate and pyruvate. The efficiency of oxidative phosporylation approaches the expected maxima. As expressed by the P:O ratio and measured polarographically by oxygen electrode it is 1.6. A similar method of investigation evidenced tight coupling of respiratory activity with oxidative phosphorylation, the rate of which is regulated by adenosine diphosphate. This ratio of respiratory control is found to be 4.1.

Some information on the cytochrome components of potato mitochondria is obtained. The activities of DPNH and succinate cytochrome c reductases have been demonstrated. They are found to absolutely require lipids for their normal functioning.

Test on storage effect revealed that the best method of preservation for isolated mitochondria from potato is deep freezing in liquid nitrogen.

INTRODUCTION

Isolation of metabolically active mitochondria have been very successful for animal tissues (Chance and Williams, 1955) and for some higher plants (Ikuma, 1970; Lance and Bonner, 1968). But the extraction of active mitochondria from potato tuber (*Solanum tuberosum L.*) has met with some difficulties (Hackett, Haas, Griffiths and Niederpruem, 1960). Numerous attempts have since been made by adding alkaline buffers, various binding and reducing agents to the isolating medium to protect mitochondria during extraction (Bonner, 1967). The present paper reports the results of mitochondria isolation with added polyvinylpyrrolidone (PVP) in isolating medium. Protein yield is low but quality is high.

In recent years, plant mitochondria have been good materials for studying respiratory metabolism (Erecinska and Storey, 1970) since respiratory activity has been demonstrated and oxidative phosphorylation are coupled and regulated by the

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availablity of phosphate acceptor (ADP). That is, the mitochondria has a substrate oxidation which is coupled to phosphorylation, the rate of which is controlled by ADP (Verleur, 1960). The two criteria used in judging integrity of the mitochondria were the values of the ratio of orthophosphate atoms incorporated into ATP to atoms of oxygen consumed (P:O ratio) and the respiratory control (R.C.) ratio. As a measure of coupling, the respiratory control ratio indicates the degree of leaking of electron transfer without concomitant phoshorylation (Chance and Williams, 1955). These ratios are usually low in plant mitochondria. However, in the present paper, our findings on both P:O and R.C. ratios of isolated potato mitochondria as assayed polarographically with the Clark oxygen electrode (Hagihara, 1961) approach the maximum values.

Most of the cytochromes in the electron transfer chain are bound tightly to the mitochondrial membrane, but one of them, cytochrome c, can easily be solubilized in aqueous medium. The other compounds can be isolated as multi-enzyme complexes: b and c_1 as cytochrome reductase complex and a and a_3 as cytochrome oxidase. The reductase donates electron to cytochrome c and the oxidase accepts them again (Lieberman, 1961). In this paper we demonstrated the activities of DPNH and succinate cytochrome c reductases in our mitochondria preparation.

Later, experiments were designed to see if phospholipid is required for the normal functioning of these reductases. Lipids were "gently" removed from mitochondria (Lester and Fleischer, 1961). After lipid extraction the activities of cytochrome c reductases were tested in the presence and absence of phospholipids and coenzyme Q and in the presence of coenzyme Q only. The results showed that lipid is absolutely required for the normal functioning of this enzyme.

It is found that the activities of our mitochondria preparations decrease with the increase of time. Each experiment should start again from the very beginning of isolation. It would make life much easier if we could find either a better and more suitable way for the isolation and separation of mitochondria having the highest activity or a better procedure of storage so that we could concentrate our attention on respiratory activity (Raison and Lyons, 1970). In this respect, we decided to measure the effects of storage on our mitochondria preparation.

MATERIALS AND METHODS

For 1.5 kilograms of peeled potato tissues 5 liters of isolating medium which contained the following reagents was prepared: 0.6 M mannitol; 0.02 M HEPES, pH 7.55; 0.001 N EDTA-Na_2; 0.1% bovine serum albumin (BSA); 0.5% polyvinylpyrrolidone (PVP) and 0.002 M cysteine-HCl which was added immediately before use. Final pH was adjuted to 7.4 by 6 N KOH. 100 ml of washing solution I was prepared: 0.6 M mannitol; 0.01 M HEPES, pH 7.4; 0.1% BSA and 0.001 M cysteine-HCl (added immediately before use with final pH adjusted to 7.1 by 2 N KOH). Also, 100 ml of washing solution II with pH adjusted to 7.1 was also prepared: 0.6 M mannitol; 0.01 M HEPES, pH 7.4 and 0.1% BSA.

The media, utensils and healthy potato tubers bought from a retailer in the local market were prechilled overnight before use. All operations were performed at 0–4°C except for an occassional going in and out of the cold room.

After chilling, about 4 lbs of potato tubers were peeled and 1.5 kg tissues were grated with a salad grater. They were blended with a Waring Blender for two seconds at rheostat 45, after four seconds they were again blended for three seconds

at 75. The pH was maintained between 7.2 and 7.5 by dropwise addition of 2 N KOH. The brei was squeezed through one layer of muslin and the filtrate fed into 4 Mistral bottles (one liter in volume) and subjected to the following centrifugations: Spun at 200 xg in Mistral for ten minutes. The precipitates of cell debris, starch granules and nucleus were discarded. The supernatant was spun at 8,000 xg by Spinco (rotor 19) for ten minutes. Supernatant so formed was discarded and the pellet was washed and homogenized with about 30 ml of washing solution I. Spun at 200 xg for ten minutes, and the starch precipitated was discarded. The supernatant was spun at 8,000 xg for ten minutes. The pellet was washed with 6 ml of washing solution II and taken up in a labelled celluose nitrate tube. This contained the final preparation of mitochondria. It was used for testing the protein content and enzyme activities.

The Folin procedure for protein determination (Lowry, Rosenbrough, Farr and Randall, 1951) was used in the estimation of protein content. After adding Folin reagent B (phosphomolybdic acid reagent) into the diluted mitochondria preparation (about 0.1 ml to become 0.6 ml) in Folin reagent A (containing 1% $CuSO_4$, 2% Na-tartarate and 2% Na_2CO_3 in 0.1 N NaOH) it was read in the Beckman spectrophotometer at 750 mμ exactly 30 minutes after adding reagent B. Protein concentration was calculated against BSA standard curve.

For assay of mitochondrial activity (oxidation of substrate) polarographically by the Clark oxygen electrode (Wiskich, Young and Biale, 1964; Lessler and Brierley, 1969) the following medium was added to the electrode chamber and mixed with a magnetic stirrer: 0.7 M mannitol; 0.001 M HEPES, pH 7.4; 0.02 M K-PO_4, pH 7.4; 0.0005 M EDTA-Na_2 and 0.1% BSA. About 0.2 ml of the mitochondrial preparation of about 10 mg protein/ml was added to the solution mixture and the electrode was placed in the cell and run for several minutes to get background rate without substrate. The reaction was started with the addition of substrate. For oxidation of substrate, 0.02 ml of 1 M K-succinate or 0.02 ml of 0.1 M pyruvate and 0.1 M malate was used and the final volume made up to 2 ml with distilled water. For respiratory control and oxidative phosphorylation 0.02 ml of 0.5 μM ADP was added to start the reaction by injection with a syringe. For an old sample, 0.02 ml of 10 mg/ml cytochrome c (-cysteine) may be required. The temperature of the oxygen electrode chamber was regulated at 26°C by continuous circulation of water from a water bath. The polarizing voltage was set to 0.6 V and microvolt ammeter full scale was 0-10 mv. Chart speed was selected at one inch/minute. The rate of oxygen uptake as determined from oxygen electrode record was calculated by the following formula (in μatoms O_2 consumed/min. /mg protein):

$$\text{Oxygen uptake} = \frac{\text{Number of boxes/min}}{100} \times \frac{1.07\ \mu\text{atoms } O_2 \text{ in } 2.0 \text{ ml solution}}{\text{mg protein in } 2.0 \text{ ml solution}}$$

The rate of oxygen uptake is equal to the oxygen consumed within the time elapsed. When oxygen is consumed 1.5 ml of the reaction mixture is added into a test tube containing 0.5 ml of 1.5 M perchloric acid (PCA). This was placed in ice. Then spun for three minutes in a clinical centrifuge at top speed. The mixture must be partitioned immediately for best results. 1.0 ml of supernatant is analysed for phosphate by the method of Berenblem and Chain (1938) with reagents containing $Na_2SiO_3 \cdot 8\,H_2O + Na_2WO_4 \cdot H_2O + H_2SO_4$; benzene-isobutanol; ammonium molybdate and $SnCl_2 \cdot H_2O$. The optical density is read at 720 mμ. The P:O ratio equals the concentration of ADP added to the total amout of oxygen uptake, and respiratory control ratio is the quotient of the rate of oxygen uptake during state 3 to state 4.

The activities of succinate- and DPNH- cytochrome c reductases were assayed

by the Gilford automatic spectrophotometer with the recorder running at speed of one inch/min and full scale from O to 100 (Lieberman, 1961). Four cuvettes were used each time, with one of them containing distilled water as a blank, the others contain, respectively, phospholipids and coenzyme Q, only coenzyme Q and the fourth with only reagent mixture and mitochondria. The reagent mixture contains: 1 M K-PO_4 buffer, pH 7.4; H_2O (32°C); cytochrome c (110 mg/ml); Tris-EDTA, pH 8 (0.02–0.001 M). Concentration of mitochondrial phospholipids and coenzyme Q_{10} is approximately 100 μg P/ml and 2 μg/ml EtOH, respectively. Mitochondrial enzymes were diluted to approximately 0.4 mg protein/ml. The reaction mixture was incubated at 32°C for ten minutes and then 0.02 ml of succinate-CN mixture, containing 1 ml 1 M succinate, 0.1 ml 1 M KCN and 0.9 ml H_2O was added and read at 550.5 mμ for the activity of succinate cytochrome c reductase for about 5 minutes. For assay of the activity of DPNH-cytochrome c reductase, after incubation at 32°C for ten minutes, 0.02 ml of 0.1 M sodium azide[1] was added followed by 0.10 ml 12% DPNH dissolved with cold 0.01 M Tris (pH 7.4). It was read at 550.5 mμ for about 5 minutes. Activity of cytochrome c reductase (in μmoles cytochrome c reduced/min/mg protein) was calculated by the following formula:

$$\text{Activity} = \frac{\text{o.D./min}}{\text{mg protein}} \times \frac{1}{18.5}$$

A typical procedure for the acetone extraction of lipids was done as follows (Lester and Fleischer, 1961): (1) Prepare 150 ml cold dry acetone with 15 ml water added to it. (2) 1 ml of mitochondria preparation was extracted for lipids in cold dry acetone with 10% water, and (3) homogenize for 5 minutes. (4) It was then spun in a clinical centrifuge for one minute at high speed, and then resupended in 120 ml washing medium II and spun at 6,000 xg for ten minutes. (5) The supernatant was discarded and the pellet washed with 11 ml washing medium II and spun again at 6,000 xg for ten minutes. (6) The pellet was collected in 1 ml of washing medium II. This preparation was used as mitochondrial enzymes for testing on the activities of succinate- and DPNH- cytochrome c reductases, and the analysis of the protein content, using procedures outlined as above.

For the effects of storage on the activity of the mitochondria, the mitochondria were collected in cellulose nitrate tubes and stored in a cold room (about 0–4°C), ordinary freezing in a refrigerator (about –10°C) and deep freezing in liquid nitrogen (about –190°C). Activities of cytochrome c reductase were tested justed after isolation and after one, three and five days of storage by a Gilford automatic recording spectrophotometer with reagents and their respective concentration mentioned above.

RESULTS

As estimated by the Folin procedure of protein determination the protein content of a typical mitochondria preparation was about 14–18 mg/ml for isolation without PVP. The total protein obtained from 1.5 kg of potato tissue was therefore 84–108 mg. When PVP was added to the isolation medium the protein concentration dropped to about 5–7 mg/ml. These results are shown in Table I.

For succinate, the rate of oxidation was calculated to be 0.273 micro atoms oxygen consumed per minute per milligram of mitochondrial protein, or 16.38 micro atoms of oxygen per hour per mg mitochondrial protein.

(1) To block the oxidation of reduced cytochrome c. 0.01 ml of 0.05 M KCN has also been used with approximately the same results.

Table I. The activity of mitochondrial enzyme isolated from potato tuber with or without PVP and measured polarographically by the Clark oxygen electrode. 0.02 ml of 1 M succinate was the substrate used for oxidation.(1)

Mito. Prep.	Pr. Content(2)	Oxygen Uptake(3)	P:O Ratio	Respiratory Control
888 (−PVP)	17.21	0.096	0.87	2.0
890 (+PVP)	6.20	0.273	1.60	4.1

(1) For method of isolation and details of reagents used and their respective concentrations, see description under 'Materials and methods'.
(2) Protein concentration in mg/ml.
(3) Oxygen uptake in μ atom oxygen consumed/min/mg protein.

For the oxidation of succinate, the rate of uptake of oxygen increased immediately after adding ADP (state 3) and decreased rapidly to state 4 after all ADP was esterified to ATP. The P:O ratio of 1.60 is close to the theoretical maximun. The respiratory control ratio of 4.1 is considered to be quite high for plant mitochondria.

During the time of oxygen consumption (traced by oxygen electrode) addition of cytochrome c did not alter the respiration of the mitochondria. Addition of ADP increases the rate of substrate oxidation. When ADP is added for substrate oxidation there is no lag phase and the induction period of preincubation with ATP is not required. But when ADP is depleted, the rate of oxidation is also inhibited.

Results of the rate of oxygen uptake for mitochondria preparations with or without the addition of PVP in the isolation medium together with their respective protein content are given in Table I. P:O and R. C. ratios are also given in Table I.

Results of the rate of reduction of cytochrome c by succinate and DPNH cytochrome c reductases are calculated to be 610 and 660 mμ mole cytochrome c reduced per minute per mg protein respectively. These results are given in Table II.

Table II. Activities of succinate- and DPNH-cytochrome c reductases in potato tuber mitochondria.(1)

Mito. Prep.(2)	Pr. Content(3)	Rate of Reduction(4)		
		PL+CoQ	CoQ	None
894-I	17.35	610	615	585
894-II	12.11	660	710	699

(1) See 'Materials and Methods' for reagents used and details of assay with the Gilford automatic recording spectrophotometer.
(2) I is for succinate cytochrome c reductase and II for DPNH-cytochrome c reductase.
(3) Protein content in mg/ml.
(4) Rate of reduction in mμ mole of cytochrome c reduced/min/mg protein. PL: phospholipids; CoQ: coenzyme Q_{10}; None: without PL and CoQ.

The absolute lipid requirement for the normal functioning of cytochrome c reductase has been demonstrated unequivocally in our experiment. Results on the activity assay of succinate cytochrome c reductase in lipid depleted mitochondria with the addition of phospholipids and coenzyme Q_{10}, with coenzyme Q_{10} only, and with neither of them are given in Table III.

Table III. Demonstration of lipid requirement for the functioning of succinate cytochrome c reductase in potato mitochondria.(1)

Potato Mito.(2)	Pr. Content(3)	Rate of Reduction(4)		
		PL+CoQ	CoQ	None
903 Untreated	5.80	187.0	201.0	209.0
903 Lipid Depleted	3.24	50.2	0.0	0.0

(1) 0.02 ml of succinate-CN mixture is used. It contains 1 ml 1 M succinate, 0.1 ml 1 M KCN and 0.9 ml H_2O.
(2) For procedure of lipid extraction with acetone and assay with the Gilford automatic recording spectrophotometer, see 'Materials and Methods'.
(3) Protein content in mg/ml.
(4) Rate of reduction in mμ mole cytochrome c reduced/min/mg protein. PL: phospholipids; CoQ: coenzyme Q_{10}; None: without PL and CoQ.

The effects of storage on mitochondria activity are given in Table IV. It has been found that the best method of storage for our mitochondria preparation was freezing in liquid nitrogen. The activity of succinate cytochrome c reductase remains more than half as active as a freshly prepared enzyme even after five days of storage in liquid nitrogen. Ordinary freezing in a refrigerator was not satisfactory for after three days of storage its activity drops one half. For lipid depleted mitochondria storage in the cold room was not good enough for the maintenance of the activity of this enzyme.

Table IV. Effects of storage on the activity of succinate cytochrome c reductase in mitochondria isolated from potato tuber.(1)

Potato Mito.	Storage Cond.	Rate of cytochrome c reduction(6)								
		1st Day Storage			3rd Day Storage			5th Day Storage		
		PL+CoQ	CoQ	None	PL+CoQ	CoQ	None	PL+CoQ	CoQ	None
913-I Original	Fresh Mito.(2)	224	193	248	—	—	—	—	—	—
913-I Original	Cold Room(3)	—	—	—	75	70	102	51	33	0
913-I Original	Ord. Freezing(4)	—	—	—	130	112	112	61	37	19
913-I Original	N_2 Freezing(5)	—	—	—	209	135	84	130	70	33
913-II Lipid Depleted	Fresh Mito.(7)	50	0	0	—	—	—	—	—	—
913-II Lipid Depleted	Cold Room(8)	—	—	—	8	0	0	4	0	0

(1) Mitochondria prepared with added PVP in the isolation medium. Succinate concentration as in Table III.
(2) Protein content is 5.85 mg/ml.
(3) and (8) Temperature about 0-4°C.
(4) About -10°C.
(5) About -190°C.
(6) Rate of reduction in mμ mole cytochrome c reduced/min/mg protein. PL: phospholipids; CoQ: coenzyme Q_{10}; None: without PL and CoQ.
(7) Protein content is 3.24 mg/ml. For procedure of lipid extraction see 'Materials and Methods'.

DISCUSSION

Preparation of Mitochondria:

For good mitochondria preparation it is necessary to add appropriate alkaline buffers, binding and reducing agents into the medium for their isolation since acidity, tannin condensation and oxidation products such as quinones lead to the inactivation of mitochondria and the release of fatty acids which cause uncoupling of oxidative phosphorylation (Axelrod, 1955). Phosphate buffers should be avoided since it has been found that they extract cytochrome c and also lead to mitochondria rupture (Bonner, 1967). HEPES is used instead. BSA and EDTA bind a variety of substances and they are required for the isolation of good mitochondria. Cysteine (-HCl) is also added to the isolation and washing media since it will condense with quinones or reduce them to phenolics again, thus protecting mitochondria from damage. Mannitol is preferable to sucrose in isolation medium because it is easier to separate mitochondria from starch in mannitol (Ikuma and Bonner, 1967).

Although Hulme and Jones (1963) reported that PVP did not protect mitochondria extracted from potato tuber tissue and Anderson (1968) stated in his review that PVP was unable to bind high concentrations of chlorogenic acids and other phenolics occuring in plant tissues, we have obtained good mitochondrial preparation by using PVP. Criteria for good mitochondria preparation includes, among others: a good respiratory control by ADP concentration, no influence of cytochrome c on any substrate oxidation rate, and the retention of respiratory control for some hours following isolation (Bonner, 1967). Investigators (e. g. Hulme, Rhodes and Wooltorton, 1967; Johnes, Hulme and Wooltorton, 1964, 1965) reported that coupled mitochondria with respiratory control have been obtained with isolation medium containing PVP. PVP has also been reported to form complexes with quinones and tannins which will inactivate or uncouple mitochondria (Loomis and Battaile, 1966; Walker and Hulme, 1965), thus protecting mitochondria during extraction.

Both the P:O and R.C. ratios are within the optimal range in our mitochondria preparation (Verleur and Uritani, 1965; Storey, 1970). In fact, these data indicate that our preparation is as good as one of the best preparations obtained by Stokes, Anderson and Rowan (1968), using sodium metabisulphite, a non-thiol reducing agent supposed to inhibit the activities of phenol oxidases completely.

As to the protein content of our mitochondria preparation we obtained about 100 mg of mitochondria protein using 1.5 kg of peeled potato tissues. For that amount of materials this is quite a high yield (Bonner, 1967). But, when PVP was added to the isolation medium protein content lowered and enzyme activity increased, thus obtaining a lower yield of protein but higher quality of mitochondria.

Since potato tubers contain a large amount of long and short starch molecules, one to two more extra centrifugations at 8,000 xg may be required for better and more homogenous mitochondria preparation. Storage overnight in a cold room before isolation will reduce the number of large starch granules. The third centrifugation at a lower speed of 200 xg is designed to get rid of the starch and it proved to be quite effective, since such prepartions contain less whitish starch granules compared with those obtained without intermittent low speed spinning. But starch molecules may be large or small and it is impossible to get rid of all of them. Thus, all preparations contain a certain amount of starch. This contamination certainly interferes with the purity of the mitochondria preparation but, fortunately, not with the enzyme activity. Spinning for ten minutes at 18,000 xg and 20,000 xg for the fourth

and fifth centrifugations gave essentially similiar results of protein content and enzyme activity, but not better. Since excellent results have been reported using lower speed of centrifugation (Bonner, 1967; Malhotra and Spencer, 1971), we therefore performed our isolation at the lower speed of 8,000 xg.

It is hoped that the isolation procedure can be improved in the years to come so that we may obtain a higher yield of high quality mitochondria.

Electron Transfer and Oxidative Phosphorylation:

According to Chance and Williams (1955b) there are four states when respiration of mitochondria is monitored by oxygen electrode. With only mitochondria added to the reaction mixture, it is state 1, and after subsequent addition of substrate it is state 2. State 3 starts with the addition of ADP and terminates when ADP has been consumed. State 4 is the period after depletion of the added ADP. If further ADP is added to the reaction mixture the reaction reverts to state 3 and again proceeds to state 4. The course of electron transfer and oxidative phosphorylation may be repeatedly reactivated by the addition of ADP until almost all the oxygen present in the cell has been used up.

The two criteria used in judging the integerity of mitochondria were the ratios of P:O and respiratory control. The P. O. ratio represents the amount of ADP added (μm) to the total oxygen uptake (μ atom oxygen) during state 3. The respiratory control ratio represents the quotient of maximum rate of oxygen uptake after ADP addition (state 3) to the rate when all ADP has been consumed (state 4).

Using a Warburg respirometer, oxidative phosphorylation was first demontrated in potato tubers in 1960 (Hackett, Haas, Griffiths and Niederpruem). For succinate oxidation, the P:O ratio was reported to be 1.3. But this mitochondrial preparation did not exhibit any sign of respiratory control. Its first demonstration by Bonner and Voss was in 1961, using an oxygen electrode. But the degree of respiratory control was small. Using similar materials and modified methods, Wiskich and Bonner (1963) reported the values of P:O and R. C. ratios to be, repectively, 1.7 and 1.4 for the oxidation of malate and 1.8 and 1.3 for DPNH. Verleur (1965) reported the values of 1.4 to 1.7 for P:O and 4.0 for R. C. ratios. The best results reported has been those obtained by Stokes, Anderson and Rowan (1968) which was 1.61 for P:O and 4.7 for R. C. ratio.

Our results of 1.60 (P:O) and 4.1 (R. C.) showed that we have prepared mitochondrial suspensions in a reasonably intact condition.

The author tried standard Warburg manometry (Umbreit, Burris and Stauffer, 1964) to determine the oxygen uptake and oxidative phosphorylation with a hexokinase-glucose trapping system. Inorganic phosphate (Pi) was determined by the method of Berenblem and Chain (1938). But its inability to follow rapid changes and the length of time necessary to prepare and equilibrate samples convinced the author that the rapidly responding oxygen electrode is best suited for short term investigation of respiratory activity in isolated mitochondria (Hagihara, 1961). It provides a means of obtaining a reliable and continuous record of changes due to the activity of mitochondria in media of variable composition, gas content and pH (Lessler and Brierley, 1969; Lesser, 1972). All subsequent measurements were thus investigated by the oxygen electrode.

Cytochrome C Reductase:

The activities of succinate– and DPNH– cytochrome c reductases have been demonstrated in our present experiments. It has also been demonstrated unequivocally

that lipid is absolutely required for the normal functioning of cytochrome c reductases. Without phospholipids, the activities of reductases in lipid depleted mitochondria decreased to zero even in the presence of coenzyme Q_{10}. However, the activity of reductase maintained about one-fourth of its original activity after lipid was reinserted. We think that this might be due to several factors (Tzagoloff and Maclennan, 1965; Vernon, Mahler and Sarkar, 1952), among others, being (a) the acetone procedure of lipid extraction may be too rough and/or violent since after extraction the protein content was also lowered (down 3/5), and (b) the phospholipids we put in (about 300 μg/ml) were not extracted from plant mitochondria, but from an animal source (beef heart mitochondria). This may have caused difficulties in the reunion or restoration of membrane integrity and thus exhibited some anomalous behavior (Fleischer, Brierly, Klouwen and Slautterback, 1962; Lieberman, 1961). For future experiments, we are contemplating a smoother and better method of extraction and also using phospholipids from plant materials (Green and Fleischer, 1963).

Effects of Storage:

It appears to the present author that the best method for the storage of mitochondria is deep freezing in liquid nitrogen. The present results demonstrated that the activity of mitochondria remain more than half as active as freshly prepared mitochondria even after five days of storage. However, this practice certainly should not be construed as the normal method of storing active cell organelles, such as mitochondria, since bulk preparation of mitochondria from animal livers, hearts and lungs etc. are usually frozen in ordinary refrigerators and retain their full activity after thawing even after months of storage (Raison and Lyons, 1970; Ziegler and Doeg, 1959). Logically, the key to our problem of obtaining optimal results is to check, search and designed better or less violent modified method of isolation and preservation so that the usual method of freezing would allow full restoration of activity after storage (Tarjan and von Korff, 1967) and hopefully it will lessen the extra burden of preparing new mitochondria every time one wishes to do a new experiment.

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