

# Mechanism of Chilling Injury in Sweet Potato

## X. CHANGE IN LIPID-PROTEIN INTERACTION IN MITOCHONDRIA FROM COLD-STORED TISSUE<sup>1</sup>

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### ABSTRACT

Seventy per cent of the phospholipid in mitochondria from sweet potato roots was removed by aqueous acetone treatment. The amount of phospholipid that could be rebound to these lipid-depleted mitochondria roughly corresponded to the amount of phospholipid in untreated mitochondria. The activities of NADH-cytochrome *c* oxidoreductase, succinate-cytochrome *c* oxidoreductase, cytochrome oxidase, and succinoxidase in lipid-depleted mitochondria were restored by addition of mitochondrial phospholipid to about 60, 50, 15, and 35%, respectively, in comparison to untreated mitochondria. The capacity of lipid-depleted mitochondria from 14-day cold-stored tissue to bind mitochondrial phospholipid from healthy tissue was lower than that from healthy tissue. However, there was no large difference in activities of NADH-cytochrome *c* oxidoreductase and succinate-cytochrome *c* oxidoreductase between both phospholipid rebound lipid-depleted mitochondria from healthy and 14-day cold-stored tissues. On the other hand, activity of succinoxidase in phospholipid rebound lipid-depleted mitochondria from 14-day cold-stored tissue was decreased by about 50% of that from healthy tissue. Furthermore, the capacity of lipid-depleted mitochondria from 2-day cold-stored tissue to bind mitochondrial phospholipid from healthy tissue was higher than that from healthy tissue.

Tropical and subtropical plants are often subjected to irreversible cytological change when stored at low temperature (0–10 C), and as a consequence they are readily infected by soft rot organisms. This phenomenon is being investigated to (a) obtain a better insight into the biochemical mechanism by which this hereditary character was acquired by plants during their phylogeny and (b) seek means of storing fruits and vegetables sensitive to chilling.

Respiratory activity of cold-stored sweet potato tissue (15) and of its mitochondria (9, 6) was reduced, with a concomitant decrease in the respiratory control ratio (10). Decreases in the levels or activities of the following mitochondrial components were also noted: (a) cytochrome *c* (16), (b) malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) (16), and (c) phospholipid (18). Furthermore, mitochondrial membranes in cold-stored tissue readily released phosphatidylethanolamine and in rather large amounts (19). Studies with the electron microscope revealed that mitochondria from cold-stored tissue

were swollen (20) and that vacuolar membranes in some regions of the tissue were degraded at some parts (21).

Those functional and structural changes in mitochondria suggest that during the chilling treatment an alteration in the lipid-protein complex of the mitochondrial membrane occurred. Thus, the capacity to rebound mitochondrial phospholipid by LDM<sup>2</sup> isolated from cold-stored tissue was also investigated.

Previous studies by Lyons and his co-workers (7, 11–14) on a temperature-induced phase change in the mitochondrial membrane showed that the lowered respiratory activity in mitochondria isolated from chilling injury-sensitive plants corresponded with a phase transition of lipid. These findings also reported that some membrane-bound enzymes are dependent on the physical state of membrane lipids.

### MATERIALS AND METHODS

Each sweet potato root (*Ipomoea batatas* L., cv. Okinawa 100) was cut perpendicularly into halves. The cut surfaces were covered with petroleum jelly and two sheets of paraffin paper to exclude air. Halves were divided into two groups, each of which was stored for 2 or 14 days at 0 to 1 C as cold-stored sweet potato or at 10 to 14 C as healthy sweet potato. Corresponding blocks of the same root were used in each analytical experiment.

**Preparation of Mitochondrial Fraction.** Tissue was homogenized in a homogenizing medium, 50 mM tris-HCl buffer, pH 7.3, containing 0.6 M sucrose, 10 mM EDTA, and 1% potassium ascorbate. That fraction between 700 and 8000g was collected, purified by linear density gradient centrifugation ranging between 32.5 and 65% sucrose (w/v), and referred to as the mitochondrial fraction (18).

**Preparation of Lipid-depleted Mitochondria.** Extraction of lipid from mitochondria was performed by the method of Fleischer and Fleischer (3) with some modifications. Thirty-six milliliters of cold dry acetone were added to 4 ml of mitochondrial suspension (5–10 mg of protein per ml in 20 mM tris-HCl buffer, pH 7.3). The final concentration of water was 10% in acetone. The mixture was swirled slowly for 10 min and then centrifuged at 10,000g for 5 min. The precipitate was mixed with 20 ml of 25 mM tris-HCl buffer, pH 7.3, containing 0.3 M sucrose and centrifuged at 15,000g for 10 min. The resultant pellet was suspended in the same buffer as LDM. All operations were carried out at 0 C.

**Rebinding of Phospholipid to LDM.** Aqueous phospholipid micelles were prepared from sweet potato mitochondrial phospholipid by sonication in 25 mM tris-HCl buffer, pH 7.3, containing 0.3 M sucrose. Rebinding of the phospholipid to LDM

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<sup>2</sup> Abbreviations: LDM: lipid-depleted mitochondria; cyt. *c*: cytochrome *c*; CoQ<sub>10</sub>: coenzyme Q<sub>10</sub>.

was performed by the method of Fleischer and Fleischer (3) with some modifications. Three milliliters of LDM suspension (0.3–0.5 mg of protein per ml) in 25 mM tris-HCl buffer, pH 7.3, containing 0.3 M sucrose were incubated with 2 ml of phospholipid micelles for 15 min at 30 C. The binding reaction was stopped by addition of 10 volumes of the above cold (0 C) buffer (pH 7.3), and the reaction mixture was centrifuged at 15,000g for 10 min. The resultant precipitate was washed again with the same cold buffer. The pellet was homogenized in 1 ml of the above buffer.

**Enzyme Assay.** Activities of NADH-cyt. *c* oxidoreductase (EC 1.6.2.1) and succinate-cyt. *c* oxidoreductase (EC 1.3.99.1) were assayed according to the method of Fleischer and Fleischer (3) with some modifications. The assay medium was as follows: 90  $\mu$ moles of potassium phosphate, pH 7.4; 0.5  $\mu$ mole of NADH or 30  $\mu$ moles of succinate; 6  $\mu$ moles of sodium azide; 7.5 mg of bovine serum albumin; 0.4 mg of CoQ<sub>10</sub> (4 mg of CoQ<sub>10</sub> per ml of ethanol solution); enzyme preparation (5–30  $\mu$ g of protein); and sufficient water to give a final volume of 1.35 ml. The above mixture was incubated for 10 min at 30 C. For analysis of both enzyme activities, 0.15 ml of cyt. *c* solution (10 mg of cyt. *c* per ml of water) was added to the assay medium to initiate the reaction, and the activity was indicated as the increment change of absorbance at 550 nm.

Succinoxidase activity was analyzed with an oxygen electrode at 30 C according to the method of Mackler (8) with some modifications. The assay medium was as follows: 56  $\mu$ moles of potassium phosphate, pH 7.5; 200  $\mu$ g of cyt. *c*; 0.4 mg of CoQ<sub>10</sub> (4 mg of CoQ<sub>10</sub> per ml of ethanol); enzyme preparation (0.2–1.0 mg protein); and sufficient water to give a final volume of 2.8 ml for all experiments except where noted. The above mixture was incubated for 10 min at 30 C. Six micromoles of succinate were added to the assay medium to initiate the reaction, and the activity was indicated as oxygen uptake ( $\mu$ mole) per min.

Cytochrome oxidase (EC 1.9.3.1, cytochrome *c*:O<sub>2</sub> oxidoreductase) activity was analyzed according to the method described previously (18).

**Analysis of Mitochondrial Lipid and Protein.** Mitochondrial lipid and protein were separated by modification of Folch's method, as described previously (17). Phospholipid was determined as phosphorus (P) according to Allen's method (17). Protein was determined as nitrogen (N) according to Nessler's method (17).

**Electron Microscopy.** A mass of mitochondrial particles or LDM was fixed in 0.2 M potassium phosphate buffer, pH 7.2, containing 5% glutaraldehyde and 0.5 M sucrose for 1 hr at 0 C, then fixed in 0.2 M potassium phosphate buffer, pH 7.2, containing 1% OsO<sub>4</sub> and 4.6% sucrose for 1 hr at 0 C, as described previously (20). Thin sections were stained in lead citrate and observed with a Japan Electron Optics Laboratories EM-TS-7 microscope.

## RESULTS

**Removal of Phospholipid from Mitochondria.** The aqueous acetone treatment extracted about 70% of the mitochondrial phospholipid although the degree of extraction was not as much as reported with rat liver mitochondria (about 80%) (4). With rat liver mitochondria, an aqueous acetone treatment containing ammonium extracted larger amounts (95%) of the phospholipid than the aqueous acetone treatment (4); however, in sweet potato mitochondria, the treatment extracted only about 50% of the phospholipid.

Figure 1 shows untreated mitochondria and LDM from

healthy sweet potato observed by electron microscope. The untreated mitochondria show preserved reticulate cristae and outer membrane structure; however, LDM lost them and showed a slightly more expanded inner membrane than in the untreated mitochondria. It is considered that the expanded inner membrane occurred owing to degradation of the outer membrane by the aqueous acetone treatment. Therefore, the data in this paper seem to reflect mainly an influence on inner membrane.

**Effect of CoQ<sub>10</sub> on Respiratory Enzyme Activities in Phospholipid Rebound LDM.** Since coenzyme Q was extracted from mitochondria with the aqueous acetone treatment (2), CoQ<sub>10</sub> as coenzyme Q was added to the reaction mixture for analysis of respiratory enzyme activities. Figure 2 shows the extent of reactivation of NADH-cyt. *c* oxidoreductase, succinate-cyt. *c* oxidoreductase, and succinoxidase as a function of CoQ<sub>10</sub> concentration in phospholipid rebound LDM from healthy sweet potato. The amount of CoQ<sub>10</sub> per  $\mu$ g of enzyme protein-N required to obtain maximal activity was 10 to 100 times greater than in the case of rat liver mitochondria (2). This fact suggests that coenzyme Q contained in sweet potato mitochondria may differ from CoQ<sub>10</sub>. The decrease in three enzyme activities by the addition of more than 400  $\mu$ g of CoQ<sub>10</sub> was apparently induced by the ethanol in the CoQ<sub>10</sub> solution.

**Rebinding of Phospholipid to LDM from Cold-stored Sweet Potato.** Figure 3 shows the amount of mitochondrial phospholipid from healthy sweet potato rebound to LDM from healthy or 14-day cold-stored sweet potato. The amount of phospholipid rebound to LDM from cold-stored tissue was less than that from healthy tissue in various phospholipid concentrations (Fig. 3). This may indicate that some structural change in the protein moiety of the lipid-protein membrane complex was caused during the 14-day cold storage. The maximal values of lipid-P per protein-N rebound to both LDM from healthy and cold-stored tissues were roughly equal to those in untreated mitochondria. This fact suggests that the capacity of the mitochondrial protein moiety to bind phospholipid was not altered greatly by the aqueous acetone treatment.

To investigate whether changes in the lipid-protein interaction shown above are induced at an early stage of cold storage, rebinding of mitochondrial phospholipid from healthy sweet potato to LDM was tested using 2-day cold-stored sweet potato. As shown in Figure 4, larger amounts of phospholipid were rebound to LDM from 2-day cold-stored tissue than from healthy tissue in various phospholipid concentrations, while there was no difference in the amount of phospholipid per protein amount between untreated mitochondria from healthy and cold-stored tissues. It may be reasonable to assume that the increase in the capacity of LDM to rebound phospholipid was due to some change in the protein moiety of the lipid-protein complex by 2-day cold storage.

**Respiratory Chain-Enzyme Activity in Phospholipid Rebound Mitochondria.** Table I shows the activity of NADH-cyt. *c* oxidoreductase, succinate-cyt. *c* oxidoreductase, or cytochrome oxidase in phospholipid rebound LDM from healthy or 14-day cold-stored tissue. The activity of NADH-cyt. *c* oxidoreductase in phospholipid rebound LDM from healthy and cold-stored tissues was restored by about 60% of that in untreated mitochondria. And there was no large difference in the activity of the enzyme between both phospholipid rebound LDM from healthy and cold-stored tissues, since no difference appeared between both untreated mitochondria, which was shown also in the previous paper (16). The activity of succinate-cyt. *c* oxidoreductase in LDM increased also to some extent by addition of phospholipid (Table I), as seen in NADH-

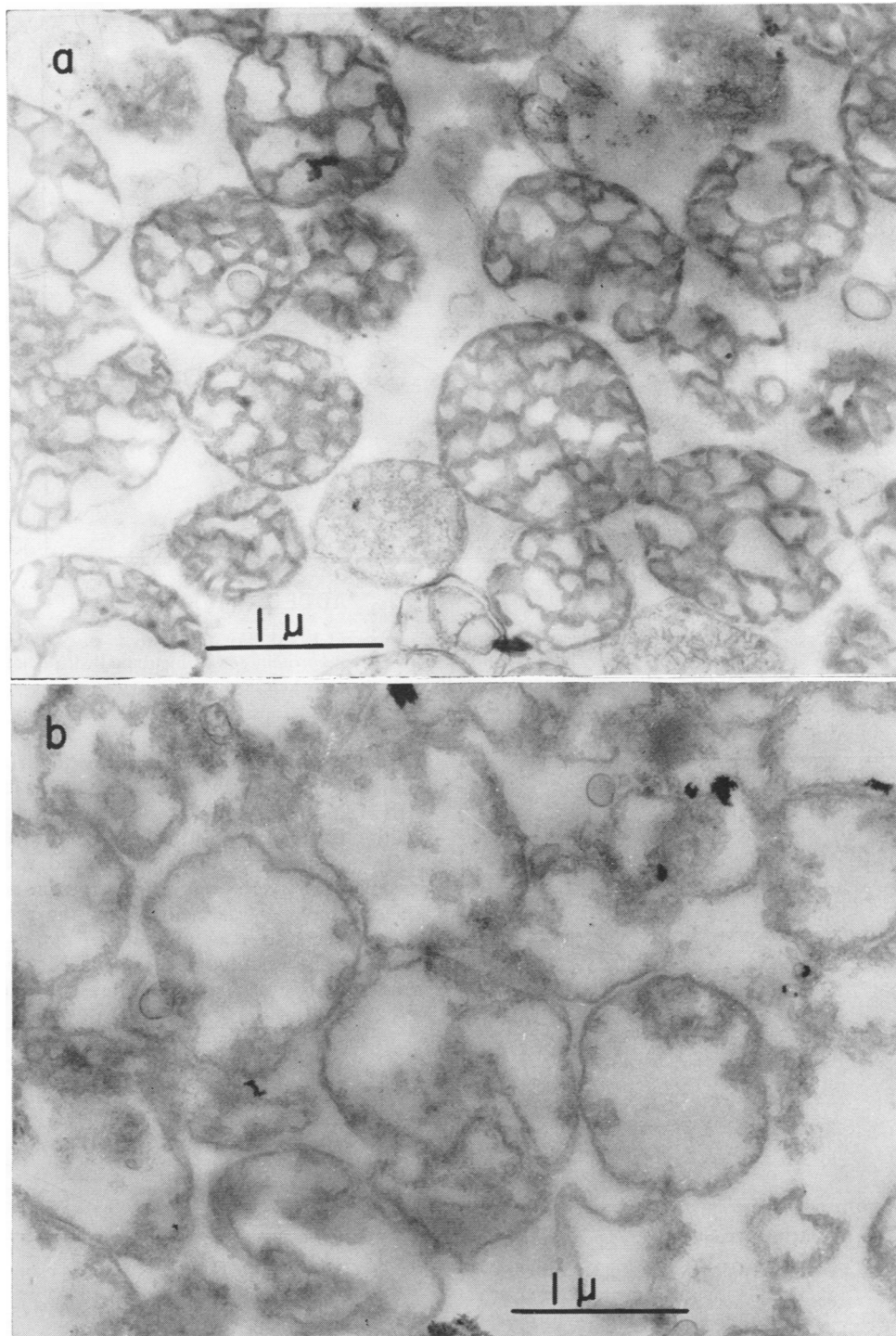


FIG. 1. Electron micrograph of untreated mitochondria or LDM from sweet potato. Both samples were fixed by 5% glutaraldehyde for 1 hr, then by 1% OsO<sub>4</sub> for 1 hr. a: Untreated mitochondria; b: lipid-depleted mitochondria.

cyt. *c* oxidoreductase. That is, the activity in phospholipid rebound LDM from healthy and cold-stored tissues was restored by about 50% of those in untreated mitochondria. There was no remarkable difference in the activity of the enzyme between both phospholipid rebound LDM, as both untreated mitochondria showed no difference, which was the same as the previous data (16). The activity of cytochrome oxidase in phospholipid rebound LDM from healthy and cold-stored tissues was not restored when contrasted with the other two enzymes,

and the restoration was about 15% of those in untreated mitochondria (Table I). There was no large difference between the activity of NADH-cyt. *c* oxidoreductase in phospholipid rebound LDM from healthy and 2-day cold-stored tissues, as in the case of untreated mitochondria.

Table II shows how succinoxidase activity in untreated mitochondria or phospholipid rebound LDM was affected by cold storage. In untreated mitochondria, about 50% of the enzyme activity was lost by 14-day cold storage, as shown

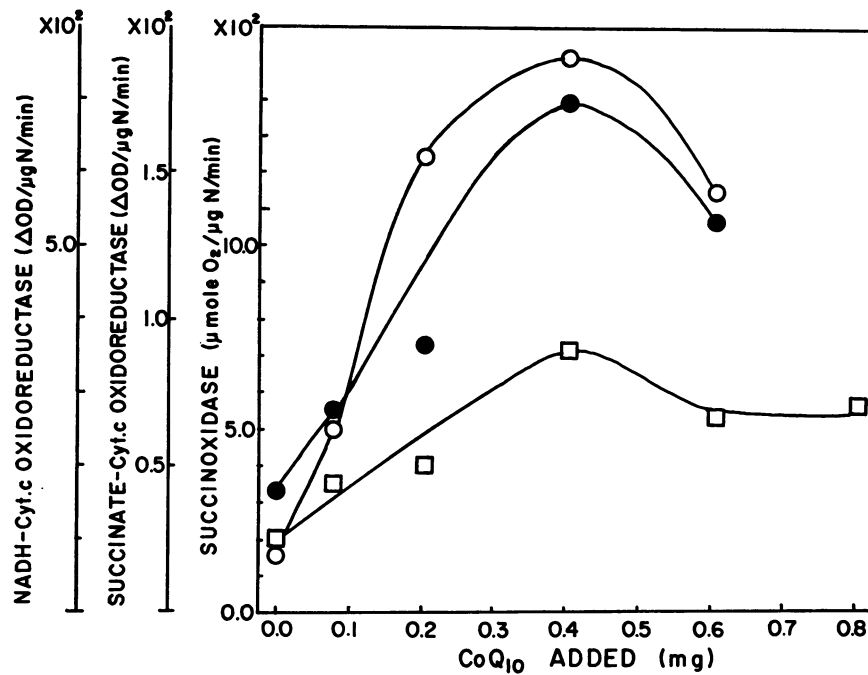


FIG. 2. Effect of  $\text{CoQ}_{10}$  on respiratory enzyme activities in phospholipid rebound LDM. For the analysis of NADH-cyt. *c* oxidoreductase, succinate-cyt. *c* oxidoreductase or succinoxidase activity, 4.7, 4.7, or 159  $\mu\text{g}$  of enzyme protein N was incubated with reaction medium. The horizontal axis shows the amount of  $\text{CoQ}_{10}$  added. The vertical axis shows the activity of NADH-cyt. *c* oxidoreductase, ( $\circ$ ), succinate-cyt. *c* oxidoreductase ( $\bullet$ ), and succinoxidase ( $\square$ ).

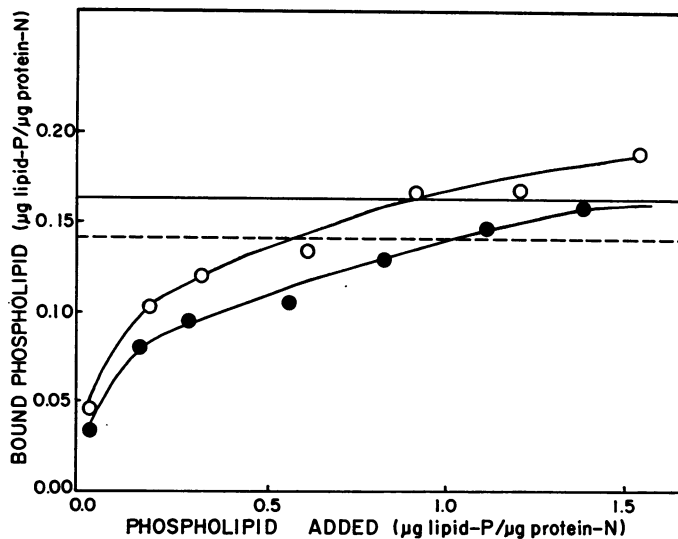


FIG. 3. Rebinding of mitochondrial phospholipid from healthy sweet potato to LDM from healthy or 14-day cold-stored sweet potato. Three milliliters of LDM suspension from healthy or 14-day cold-stored tissue (0.30 or 0.44 mg protein N) were incubated with 2 ml of mitochondrial phospholipid micelles from healthy tissue at 30 C for 15 min. Binding reaction was stopped by addition of 10 volume of cold buffer, and then reaction mixture was centrifuged at 15,000g for 10 min. The horizontal axis indicates the amount of added phospholipid. The vertical axis indicates the amount of rebound phospholipid ( $\mu\text{g}$  of lipid-P/ $\mu\text{g}$  protein-N).  $\circ$ ,  $\bullet$ : Phospholipid rebound LDM from healthy and cold-stored tissues, respectively. —, - - -: The amount of  $\mu\text{g}$  of lipid-P per  $\mu\text{g}$  protein-N in untreated mitochondria from healthy and cold-stored tissues, respectively.

previously (10, 16). However, the activity was restored to about 80% of that from healthy tissue by addition of cyt. *c*, which had no effect on that from fresh tissue. This suggests

that cyt. *c* was released from mitochondrial membrane by the chilling treatment, as reported previously (16).

In LDM rebound by mitochondrial phospholipid from healthy tissue, about 50% of the enzyme activity was lost after a 14-day cold storage, when assayed without cyt. *c*. It was possible that cyt. *c* was released from mitochondria by the aqueous acetone treatment. Thus, the activity of the enzyme was assayed also after addition of cyt. *c* to the reaction medium. In phospholipid rebound LDM, about 50% of the enzyme activity was lost by 14-day cold storage when assayed

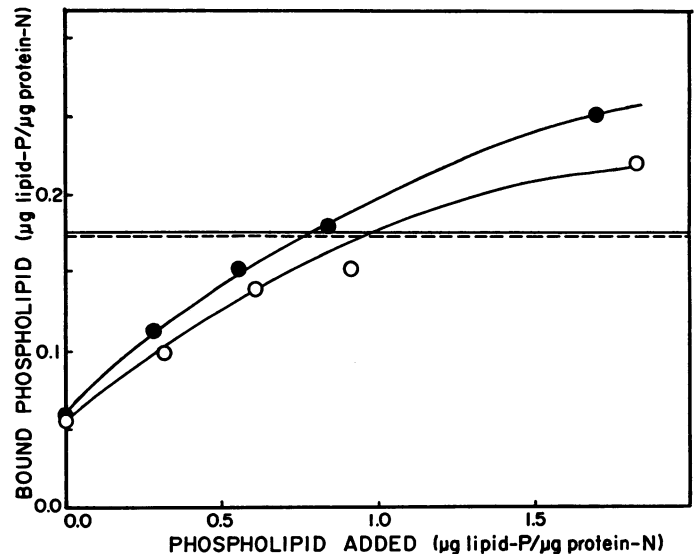


FIG. 4. Rebinding of mitochondrial phospholipid from healthy sweet potato to LDM from healthy or 2-day cold-stored sweet potato. Three milliliters of LDM suspension from healthy or 2-day cold-stored tissue (0.23 or 0.22 mg of protein-N) were incubated with 2 ml of mitochondrial phospholipid micelles from healthy tissue at 30 C for 15 min. Symbols are the same as in Figure 3.

Table I. *NADH-Cyt. c Oxidoreductase, Succinate-Cyt. c Oxidoreductase, or Cytochrome Oxidase Activity in Phospholipid Rebound LDM from Healthy or 14-Day Cold-stored Sweet Potato*

Phospholipid was added to LDM from healthy or 14-day cold-stored tissue in an amount almost sufficient to saturate the LDM. The activity of NADH-cyt. *c* oxidoreductase, succinate-cyt. *c* oxidoreductase, or cytochrome oxidase is reported as absorbance per  $\mu\text{g}$  of mitochondrial protein-N per min.

Sample	Lipid-P per Protein-N $\mu\text{g P}/\mu\text{g N}$	NADH-Cyt. <i>c</i> Oxidoreductase		Succinate-Cyt. <i>c</i> Oxidoreductase		Cytochrome Oxidase	
		$\Delta A/\mu\text{g N min} \times 10^{-2}$	relative value (%)	$\Delta A/\mu\text{g N min} \times 10^{-2}$	relative value (%)	$\Delta A/\mu\text{g N min} \times 10^{-2}$	relative value (%)
<b>Experiment 1</b>							
Untreated mitochondria (H) <sup>1</sup>	0.164	3.51	100				
Untreated mitochondria (I) <sup>2</sup>	0.124	3.84	100				
LDM (H)	0.045	0.49	14				
LDM (I)	0.034	0.40	10				
Phospholipid rebound LDM (H)	0.168	2.19	62				
	0.168	1.92	55				
Phospholipid rebound LDM (I)	0.129	2.35	61				
	0.147	1.89	49				
<b>Experiment 2</b>							
Untreated mitochondria (H)	0.155			9.63	100	7.69	100
Untreated mitochondria (I)	0.124			9.25	100	7.68	100
LDM (H)	0.047			1.85	19	0.64	8
LDM (I)	0.039			1.46	16	0.58	8
Phospholipid rebound LDM (H)	0.132			4.72	49	1.04	14
	0.164			4.54	47	1.09	14
Phospholipid rebound LDM (I)	0.110			4.00	43	1.02	13
	0.124			3.80	41	0.98	13

<sup>1</sup> (H): healthy sweet potato.

<sup>2</sup> (I): cold-stored sweet potato.

Table II. *Change of Succinoxidase Activity in Phospholipid Rebound LDM from Healthy or 14-Day Cold-stored Sweet Potato*

Three milliliters of LDM prepared from crude mitochondria (not subjected to sucrose density gradient centrifugation) from healthy or 14-day cold-stored sweet potato (1.77 or 2.08 mg of protein-N) were incubated with 2 ml of mitochondrial phospholipid micelles from healthy tissue (1.02 or 1.32 mg of lipid P) at 30 C for 15 min. The enzyme activity was indicated as  $\mu\text{mole}$  of  $\text{O}_2$  uptake per  $\mu\text{g}$  of mitochondrial protein-N per min.

Sample	Phospholipid per Protein-N $\mu\text{g P}/\mu\text{g N}$	CoQ <sub>10</sub> $\mu\text{g}$	Cyt. <i>c</i> $\mu\text{g}$	Succinoxidase	
				$\mu\text{mole O}_2/\mu\text{g N} \cdot \text{min}$	relative value (%)
Untreated mitochondria (H)	0.134	0	0	0.318	100
	0.134	0	200	0.318	100
Untreated mitochondria (I)	0.109	0	0	0.170	100
	0.109	0	200	0.250	147
LDM (H)	0.052	400	200	0.039	12
LDM (I)	0.045	400	200	0.037	22
Phospholipid rebound LDM (H)	0.113	400	0	0.072	23
	0.113	400	200	0.118	37
Phospholipid rebound LDM (I)	0.091	400	0	0.040	24
	0.091	400	200	0.058	34

with the addition of cyt. *c*. It should be noted that the activity was restored by about 35% of the enzyme activities without cyt. *c* addition in untreated mitochondria, in both healthy and cold-stored tissues (Table II).

LDM still reserved about 10 and 20% of the enzyme activi-

ties when assayed without cyt. *c* in untreated mitochondria in healthy and cold-stored tissues, respectively. Thus, there was no difference in the activity between LDM from healthy and cold-stored tissues.

## DISCUSSION

The ability of LDM to recombine with exogenously applied phospholipid to a value equivalent to that found in untreated mitochondria indicates that the protein moiety in the LDM was not severely denatured by the acetone treatment. Further evidence for this is the partial restoration of the activities of NADH-cyt. *c* oxidoreductase, succinate-cyt. *c* oxidoreductase, cytochrome oxidase, and succinoxidase by the addition of phospholipid to the LDM (Table I and II). Examination of the electron photomicrographs (Fig. 1) indicates that the outer membrane of the sweet potato mitochondria was degraded more than the inner membrane in which those enzymes reside. These findings seem to indicate that the protein moiety in the mitochondrial inner membrane was reconstituted in such a way that the complex could function similarly to the native inner membrane in untreated mitochondria, although the mitochondrial structure itself was broken down by the acetone treatment.

During a 14-day cold storage period the level of phospholipid in the mitochondria decreased in sweet potato tissue (Fig. 3). The amount of mitochondrial phospholipid (prepared from healthy tissue) which was rebound to the LDM prepared from cold-stored tissue was correspondingly less than the amount of phospholipid which recombined with LDM prepared from healthy tissue (Fig. 3). Thus, it appears that some structural changes occurred in the protein moiety of the lipid-protein complex during the chilling treatment. This alteration did not seem to affect the protein moiety in that complex which con-

trols NADH-cyt. *c* oxidoreductase and succinate-cyt. *c* oxidoreductase, as the activities of these enzymes upon addition of phospholipid were nearly equal to those of LDM obtained from healthy tissue.

The activity of the phospholipid-dependent succinoxidase in LDM obtained from 14-day cold-stored tissue was 50% of that obtained from healthy tissue (Table II). When cyt. *c* was added to the above reaction mixture, the activity of the enzyme increased proportionately in phospholipid rebound LDM obtained from both cold-stored and healthy tissue (Table II). The enhancement seems to occur by recompensation of cyt. *c* lost during the acetone treatment. When cyt. *c* was added to the intact mitochondria obtained from 14-day cold-stored tissue, succinoxidase activity was restored to near that of healthy tissue. Thus, it appears that the decrease in succinoxidase activity may partially be attributed to the loss in cyt. *c* during the chilling treatment and partially to the structural alteration in the protein moiety of the complex containing this enzyme.

In contrast to the 14-day cold-stored tissue, that stored for 2 days gave rise to LDM capable of binding more mitochondrial phospholipid (prepared from healthy tissue) than the control. Thus, chilling injury appears to involve a protein alteration which favors binding of phospholipid in the early stages and then a loss in this capacity. This loss in binding capacity is accompanied with a release of phospholipid from the membrane and lowering of respiratory activity.

The rapid alteration in protein structure which seemingly leads to an irreversible deterioration may account for the serious losses incurred by growers who inadvertently store their sweet potatoes at suboptimal temperature even for 1 day.

We speculate that the membrane protein of sweet potato is bound to the phospholipid, by way of hydrophobic bonds. These bonds, broken by the chilling treatment, result in "cold denaturation" which is reversible in the early stages but eventually becomes irreversible. Our results suggest that the hydrophobic protein moiety plays an important role in the initiation of irreversible chilling injury.

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