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BIOCHEMICAL STUDIES OF CHILLING INJURY IN SWEETPOTATOES[1]

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Many plants indigenous to subtropical and tropical regions suffer physiological injury when subjected to low (1 to 10° C) but non-freezing temperatures (6, 14). The injury, manifested by discoloration, susceptibility to decay, and failure to ripen, is generally progressive with time indicating a gradual degeneration of physiological processes. There is also some evidence (7, 16) that low temperature has an immediate effect on cellular metabolism in sensitive tissues. Sachs (16) reported that some plant cells subjected to low temperatures (11° C) showed cessation of cytoplasmic streaming. Lewis (7) recently confirmed these observations with petiole trichomes of tomato (*Lycopersicon esculentum*) and of sweetpotato (*Ipomoea batatas*) and with other species sensitive to low temperatures. These cells showed cessation of protoplasmic streaming when subjected to 10° C for one or two minutes. In contrast, trichome cells of tissues not sensitive to low temperatures, such as those of radish (*Raphanus sativus*) and carrot (*Daucus carota*), showed protoplasmic streaming even at 0° C.

While the effects of chilling injury have been described adequately in many sensitive tissues (6, 14) there have been few metabolic and physiological studies of this phenomenon. It therefore seemed desirable to investigate biochemical aspects concerned with chilling injury. The emphasis in such a study should be to detect changes occurring at the cellular and subcellular level during exposure of sensitive tissues to low temperatures.

We recently studied cytoplasmic particles from sweetpotato roots (a tissue sensitive to low temperature) and had established a system for assaying their mitochondria (8, 9). It was therefore possible to investigate the effect of chilling on mitochondria, the organelles intimately concerned with energy metabolism in the cell. Our purpose was to compare mitochondrial activity from chilled and non-chilled tissues and also to study some metabolic differences between tissue slices from chilled and non-chilled tissues. It is hoped that this approach will yield clues to the mechanisms concerned in chilling injury.

MATERIALS AND METHODS

Porto Rico sweetpotato roots (*Ipomoea batatas* L. (Lam.)) grown on the Plant Industry Station farm at Beltsville, Maryland, were used in these experiments. The roots were harvested early to insure that no chilling occurred in the field. Immediately after harvest, the roots were held for 10 days at 29° C and approximately 95 % relative humidity to promote healing of surface injuries. After this curing period the sweetpotatoes were divided into two lots. One lot was transferred to a constant temperature room held at 15° C, a recommended storage temperature, and the second lot was transferred to a room held at 7.5° C, the chilling temperature used in these experiments. Both rooms were equipped with humidistats set to maintain a relative humidity of 80 to 85 %. The roots were sampled after curing and at predetermined intervals during storage at the two temperatures. The chilled and non-chilled roots were compared with respect to ion leakage from tissue slices and with respect to oxidation and phosphorylation exhibited by isolated mitochondria.

The leakage from tissue slices was determined by conductivity measurements of solutions surrounding slices which were approximately 2 mm thick and 1 cm in diameter. Duplicate 10-g samples from chilled and non-chilled roots, previously washed in distilled water for one hour, were placed in 120-ml flasks containing 15 ml of either distilled water or 0.5 M sucrose. The flasks were shaken in a constant temperature bath at 25° C for two hours at a shaking speed of 120 strokes per minute. Conductivity measurements were made with a Serfass Conductivity Bridge using a cell with a constant of 0.1. The tissue was sampled at harvest and at intervals as specified during a 10-week storage period.

In the 2nd year the solutions were frozen and held until the end of the experiment (10 weeks), after which time they were analyzed for inorganic ions. The analyses were made by flame photometry following the procedure of Myers, Dyal and Borland (13).

Preparation of mitochondria, assay of mitochondrial activity, and determination of phosphorus and nitrogen were all carried out as described in previous publications (8, 9) except for minor changes as indicated in the tables of this report.

Most of the experiments were carried out in duplicate in each of two years.

RESULTS

CONDUCTIVITY MEASUREMENTS AND ION LEAKAGE: Conductivity of the solutions (water and 0.5 M su-

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TABLE I

SPECIFIC CONDUCTIVITY (MICROMHOS/CM) OF MEDIA SURROUNDING SLICES OF SWEETPOTATO TISSUE AFTER TWO HOURS SHAKING IN A CONSTANT TEMPERATURE BATH AT 25° C *

PERIOD IN STORAGE	TISSUE IN WATER		TISSUE IN 0.5 M SUCROSE	
	NON-CHILLED	CHILLED	NON-CHILLED	CHILLED
None	2.4	...	1.0	...
3 weeks	2.2	6.5	1.0	1.9
4 weeks	2.8	7.8	1.0	2.9
7 weeks	3.2	10.4	1.3	4.5
9 weeks	2.8	10.2	1.2	6.5
10 weeks	2.8	9.6	1.0	5.0

* Conductance readings were made at 23° C with a Serfass conductivity apparatus using the 1,000 cycle per second bridge and a conductivity cell with a constant of 0.1. The data for the two years did not vary more than 10 % from the means.

crose) surrounding the tissue slices in the vessels was determined after two hours of shaking. Table I presents the average of the results obtained from two years of work. Approximately a 2-fold increase in conductivity was noted in the water around the chilled tissue. This difference was observed after 3 weeks storage of roots at 7.5° C and was more or less sustained throughout the experiments. Conductivity in the sucrose solutions around the chilled tissues showed approximately the same ratio of increase, but the values were considerably lower (table I). These data indicate that low temperature may cause an impairment of cellular permeability resulting in leakage from the cells.

The nature of the ions leaking from the tissues was investigated by flame photometry. The analyses showed only traces of manganese, iron, aluminum, sodium, and copper in samples from chilled and non-chilled tissues. Since none of these ions leaked from the tissues it seems that they have no relation to the chilling effect. However, there was considerable leakage of potassium ions from the chilled tissue slices (fig 1). Apparently the greater conductivity of the medium around chilled tissue was due entirely to leakage of potassium ions. It was surprising to find that more potassium leaked from the tissues in sucrose than in distilled water even though the conductivity of the sucrose solutions was lower than that of the distilled-water solutions (table I, fig 1). This is probably related to the effect of a non-polar molecule like sucrose on conductivity in an aqueous medium.

MITOCHONDRIAL ACTIVITY: Mitochondria isolated from chilled and non-chilled tissues were compared with respect to their oxidative and phosphorylative capacities. In complete assay systems, the oxidative rates of mitochondria from chilled and non-chilled tissue (table II) were approximately the same during the first four to five weeks. The mitochondria from the non-chilled tissue continued to maintain approximately the same level of oxidation throughout the 10-week period of the experiments. However, the mitochondria from the chilled tissue showed a marked decline after the 5th week and at the 10th week there was practically no activity at all.

Phosphorylation, as indicated by P/O ratios (table III), was reduced in the chilled tissue even before there was a reduction in oxidative activity. After the 5th week the decline in the P/O ratio became considerably more pronounced, and after the 8th week phosphorylation was practically eliminated.

These data indicate that if the oxidative pathway in the mitochondria was injured by chilling, the injury was reversible during the 1st four- to five-week period at low temperature. There was some decline in the phosphorylating system during this period, but only after five weeks were both oxidation and phosphorylation seriously degraded. It seems logical to suppose, therefore, that the initial injury caused by low temperature does not seriously affect the mitochondria per se but may affect other cellular components which ultimately have considerable influence on mitochondrial activity.

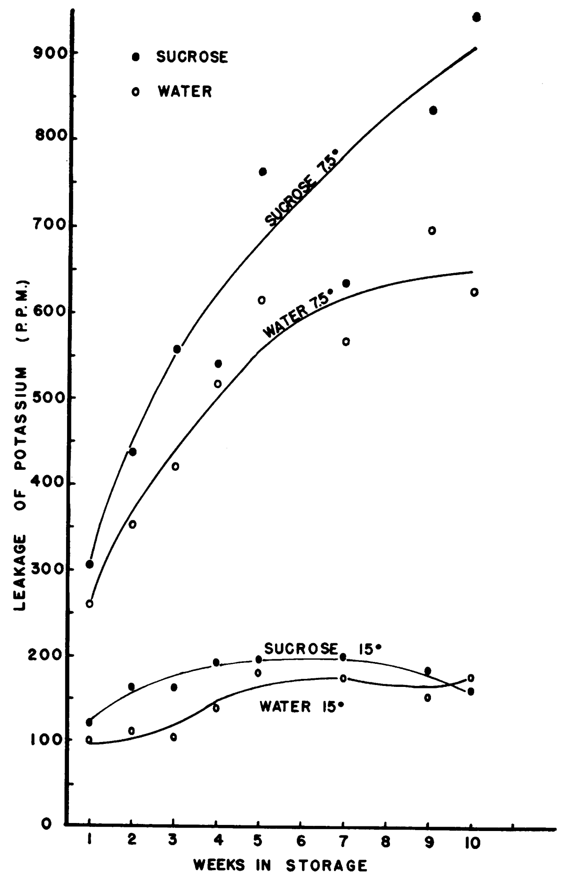

FIG. 1. Leakage of potassium ions from tissue slices of sweetpotato roots stored at a chilling temperature (7.5° C) and at a non-chilling temperature (15° C).

INTER-RELATION OF POLYPHENOLS AND ASCORBIC ACID DURING CHILLING: One of the striking characteristics of chilled sweetpotato roots is the darkening of the tissue on exposure to oxygen. This rapid darkening occurs after a prolonged period at low temperatures and is therefore a manifestation of some of the final degradation reactions caused by low temperature.

During the mitochondrial studies of the badly chilled tissues we observed that the supernatants, obtained by high-speed centrifugation of the homogenates, turned black on standing. The supernatants from tissues that had been exposed to low temperature for shorter periods did not turn black. It therefore seemed that a substrate either accumulated or suddenly appeared as a result of chilling and that it was oxidized to form a dark pigment. Chlorogenic acid was a likely suspect since it is a polyphenol which can be oxidized to melanin-like pigments and has been identified in the sweetpotato root (15).

Comparisons of the chlorogenic acid content in the chilled and non-chilled tissues were made by extracting 100 g of sweetpotato roots (previously held for 12 weeks at 7.5° C) in a Waring blendor at top speed for five minutes with 150 ml of 95 % ethanol. The filtered extract was brought to 250 ml with 50 % ethanol and a 1-ml aliquot was diluted to 250 ml with distilled water. The absorption spectra of these diluted samples were read in the Beckman DU spectrophotometer from 260 to 380 mμ. The ultraviolet absorption spectra of these extracts, compared with the spectrum of authentic chlorogenic acid, are shown in figure 2. These data clearly demonstrate a considerable increase of chlorogenic acid, or related polyphenols which have similar absorption spectra, in the chilled tissues.

The original alcoholic extracts from the chilled and non-chilled tissues were spotted on Whatman no. 1 paper and co-chromatographed with authentic chlorogenic acid in butanol : acetic acid : water (4 : 1 : 5). When the paper was viewed under ultraviolet light, four fluorescent spots were visible from both extracts, indicating that the chilled and the non-chilled tissues contained essentially the same components capable of absorbing ultraviolet. However, the spots derived from the chilled tissues were considerably larger and

TABLE II

OXYGEN UPTAKE BY MITOCHONDRIA FROM CHILLED AND NON-CHILLED SWEETPOTATOES EXPRESSED AS Q_{O_2} (N) *

PERIOD IN STORAGE	1ST YEAR **		2ND YEAR **	
	NON-CHILLED TISSUE	CHILLED TISSUE	NON-CHILLED TISSUE	CHILLED TISSUE
None	494	494	550	550
3 weeks	523	518	1138	1180
4 weeks	345	353	717	851
5 weeks	500	500	745	342
7 weeks	458	347	740	532
8 weeks	530	207	778	218
9 weeks	552	142	920	219
10 weeks	398	11	937	64

* The assay system consisted of 0.01 M α-ketoglutarate, 0.001 M adenosinetriphosphate, 0.02 M glucose, 1 mg hexokinase, 3.3×10^{-4} M diphospho pyridine nucleotide, 6.6×10^{-5} M cocarboxylase, 6×10^{-3} M Mg, 0.02 M PO_4, and 0.5 ml mitochondria containing approximately 0.3 to 0.5 mg of nitrogen.

** The homogenizing medium for isolating the mitochondria differed in the two years. In the 1st year the medium contained 0.25 M sucrose, and 0.01 M EDTA and yielded mitochondria containing approximately 0.5 mg of N per aliquot. In the 2nd year the medium contained 0.25 M sucrose, 0.1 M phosphate buffer pH 7.0, and 0.01 M EDTA and yielded mitochondria containing approximately 0.3 mg of N per aliquot. This is believed to be responsible for the higher Q_{O_2} (N) values during the 2nd year.

TABLE III

AVERAGE P/O RATIOS FOR OXIDATIVE SYSTEMS USING MITOCHONDRIA ISOLATED FROM CHILLED AND NON-CHILLED SWEETPOTATO TISSUE *

PERIOD IN STORAGE	NON-CHILLED TISSUE	CHILLED TISSUE
None	1.0	1.0
3 weeks	1.2	0.9
4 weeks	1.8	1.1
5 weeks	1.9	1.0
7 weeks	1.3	0.6
8 weeks	1.2	0.6
9 weeks	1.9	0.1
10 weeks	1.4	0.1

* The data are averages for two years; same assay system as in table II.

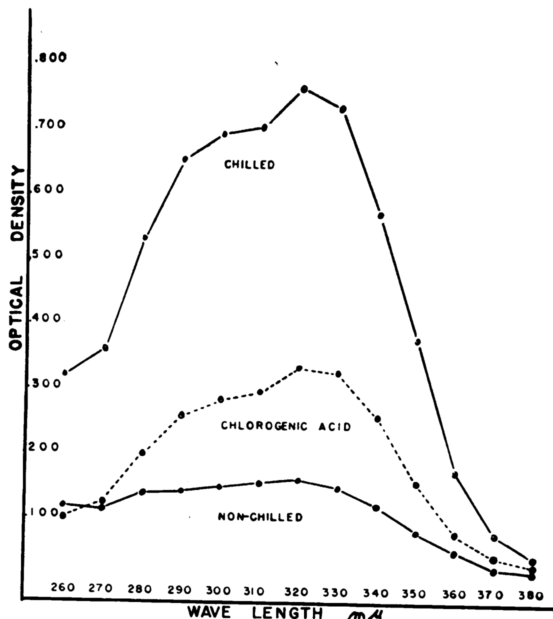

FIG. 2. Ultraviolet absorption spectra of extracts from chilled and non-chilled sweetpotato roots and of authentic chlorogenic acid (2×10^{-5} M).

TABLE IV

R_f VALUES OF SPOTS OBTAINED ON PAPER AFTER CHROMATOGRAPHING EXTRACTS FROM CHILLED AND NON-CHILLED SWEETPOTATO TISSUES

SPOT NUMBER	NON-CHILLED TISSUE		CHILLED TISSUE	
	R_f VALUE	BLUE-GREEN SPOT PRESENT AFTER FUMING WITH NH_3	R_f VALUE	BLUE-GREEN SPOT PRESENT AFTER FUMING WITH NH_3
1	.43	–	.43	–
2	.58	+	.58	+
3	.75	+	.75	+
4	.88	+	.88	+
Authentic cholorgenic acid	.75	+	.75	+

more dense in all cases, indicating higher concentrations. This is in agreement with the ultraviolet absorption spectra. One of the spots derived from both the chilled and the non-chilled tissue coincided with authentic chlorogenic acid. The R_f values for the four spots are shown in table IV.

The data in figure 2 and the density of the spots on the chromatograms indicate a considerable increase in chlorogenic acid in the chilled tissues. There also appears to be a like increase of the other substances (spots 1, 2, and 4) appearing on the paper. Spots 2 and 4 may be neochlorogenic acid and *iso*-chlorogenic acid, respectively, since the R_f values appear to be approximately the same as those reported for these substances (4) and the ultraviolet absorption spectra of these three chlorogenic acids are identical (19).

Ascorbic acid has been shown to reduce quinones to phenols (11) and also has been reported to have an inhibitory effect on the polyphenolase enzyme complex (1). Craft and Heinze (3) found no relation of ascorbic acid to chilling of tomatoes while Miller and Heilman (12) found that ascorbic acid declined when the pineapple (*Ananas comosus*) was subjected to low temperatures. It was, therefore, interesting to compare the ascorbic acid in chilled and non-chilled sweetpotatoes. Ascorbic acid concentrations were determined by the method of Loeffler and Ponting (10). The result of such a study (table V) showed that a marked decline in ascorbic acid occurred in chilled tissues whereas the ascorbic acid content of the non-chilled tissue remained approximately constant.

TABLE V

CONCENTRATION OF ASCORBIC ACID DURING STORAGE OF SWEETPOTATOES AT NON-CHILLING AND CHILLING TEMPERATURES

PERIOD IN STORAGE	NON-CHILLED TISSUE	CHILLED TISSUE
10 weeks	22.7	9.2
11 weeks	21.1	5.3
12 weeks	20.9	7.6
13 weeks	20.1	8.3

These data were taken after a prolonged period at the chilling temperature (7.5° C), and they indicate the general course of the final degradative processes. It appears that in chilled sweetpotato tissue there may be a decrease in ascorbic acid accompanying the increase in polyphenols.

DISCUSSION

After observing increased leakage in tissue slices from roots held three weeks at 7.5° C, it was surprising to find normal oxidative activity in the mitochondria isolated from the roots stored for more than four weeks at 7.5° C. These data may be explained by assuming that the mitochondria per se are not affected by low temperature, but that the nature and the concentration of the cofactors, which control the mitochondrial activity in vivo, are affected. Since in our assay system all the known cofactors were liberally supplied, these experiments offer no test of limitation of cofactors for mitochondrial activity in vivo. For example, potassium which we know leaked from chilled tissues, was adequately supplied with the phosphate buffer. In an alternate explanation, one may assume that the degree of injury to the mitochondria caused by 7.5° C for three or four weeks is considerably less than that due to the normal isolating procedure. Therefore, no effect on the mitochondria is observed after chilling the tissue for a short period at 7.5° C.

We may consider five to six weeks at the chilling temperature (7.5° C) as the "breaking point" in chilled sweetpotatoes. It is after this period at low temperatures that the mitochondria are severely injured, and their activity cannot be restored by the addition of cofactors. Also, at about this time chlorogenic acid begins to accumulate and ascorbic acid starts to decline. The data for these last observations are not reported in this paper, but we have observed this in a later study to be published elsewhere.

The loss of potassium from the chilled cells may be one of the factors concerned in the deterioration of oxidative phosphorylation after five or six weeks of chilling the roots. Lardy (5) has suggested that potassium ions may be involved in uncoupling oxidative phosphorylation. However, several observers (2, 17) reported that potassium ions are released from cells as a general effect of exposure to radiation. The release of potassium from chilled cells may thus be a non-specific effect of cellular injury.

Shichi and Uritani (18) suggested that a gradual coagulation of the lipid components of the sweetpotato occurs at low temperatures and damage to cellular structures eventually results. Support for this hypothesis may be derived from the long term chilling requirement in sweetpotatoes as evidenced by the five to six week "breaking point" (at 7.5° C) previously discussed. On the other hand the rapid leakage of potassium from chilled cells and the observations by Lewis (7) of the rapid cessation of cyclosis at low temperatures militates against this hypothesis.

More work is needed to explore additional physiological and biochemical aspects of low temperature injury.

Summary

Chilled and non-chilled sweetpotato roots were compared with respect to leakage from tissues, and mitochondrial activity during 10 weeks of storage at 15 and 7.5° C (the chilling temperature).

There was approximately five times as much leakage from the chilled tissue slices as from the non-chilled tissue slices. Almost all the leakage consisted of potassium ions.

During the 1st four weeks, the mitochondria from chilled and non-chilled tissues, when assayed in complete systems at 25° C, showed approximately equivalent rates of oxidation and phosphorylation. However, after the 5th week there was a rapid decline in oxidative and phosphorylative activity in the chilled tissues and at the 10th week the mitochondria from the chilled tissues were practically inactive.

In the late stages of degeneration there was a build up of chlorogenic acid (and similar polyphenols) and a decline of ascorbic acid in the chilled tissue.

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AUXIN ACTIVITY OF SOME INDOLE DERIVATIVES [1,2]

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During the past 20 years, in numerous laboratories, very large numbers of compounds have been synthesized and tested for their activity as auxins or as modifiers of auxin action. The majority of these have been acids of the aryloxy series. Curiously enough, although indoleacetic acid (IAA) is the most widely occurring natural auxin, very few of the compounds tested have been indole derivatives. Kostermans in 1934 tested a number of indole derivatives, but only in the Avena curvature test with agar blocks, where the ability of the substance to be transported largely controls its activity. More recently,

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