SOME EFFECTS OF CHILLING ON RESPIRATION, PHOTOSYNTHESIS, AND NUCLEOTIDE COMPOSITION OF COTTON SEEDLINGS

By

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SEEDLINGS

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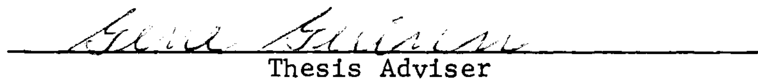

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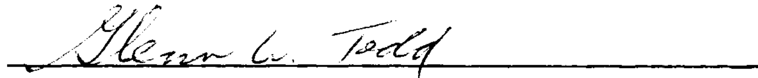

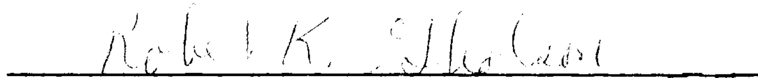

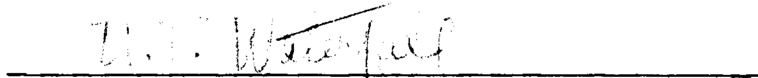

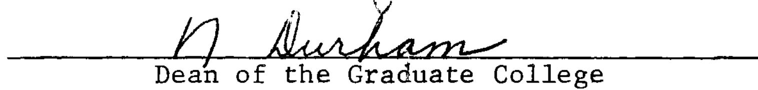

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CHAPTER I

INTRODUCTION

Many tropical and subtropical plants are sensitive to chilling temperatures between 0 and 10 C and are severely injured or killed if chilling is continued for any length of time. Cotton is one such plant which is of major economic importance in this and other countries. The detrimental effects of low, non-freezing temperatures on cotton seedling survival have been recognized for many years but the specific causes or site of the chilling injury has not been studied extensively. This is true for chilling injury in general since most biochemical research has been concerned with frost rather than chilling injury. As a consequence, there is an extensive amount of descriptive information concerning responses of several plant and cell parts to chilling, but little on the biochemical and/or physical nature of the chilling injury.

The present study was initiated to determine the effect of low, non-freezing temperatures on some of the processes involving high-energy phosphate compounds in cotton seedlings. Initial investigations were concerned with the response of respiration and photosynthesis to low temperature. Subsequently, the effects of chilling on adenosine triphosphate and other nucleotides were studied. Concluding investigations were concerned with the response of mitochondria to chilling injury.

CHAPTER II

REVIEW OF LITERATURE

A recent review (81) indicated that the detrimental effects of low, non-freezing temperatures on tropical and subtropical plants was recognized as far back as 1830 when it was reported that tropical plants, when exposed to 3 C for several days, developed black spots on the leaves followed by curling and abscission. The first physiological investigations into chilling injury were reported by Sachs in 1860. According to Vasil'yev (81), Sachs observed that wilting occurred in three cold-sensitive plants when the temperature was maintained at 2 to 4 C. If the atmosphere surrounding the plants was saturated with water to restrict transpiration, wilting did not occur. Sachs concluded that chilling-sensitive plants die because of desiccation when transpiration from the tops exceeds water absorption by the roots.

A brief historical sketch by Vasil'yev (81) outlines the development of ideas concerning chilling injury after the work of Sachs. Several reports suggested that death to chilling-sensitive plants was caused by accumulation of toxic materials. At higher temperatures these toxic substances would normally be converted into non-toxic substances but due to reduced metabolic activity become injurious at low temperature. Wilhelm discredited this idea and suggested that death was caused by a combination of dehydration and protein exhaustion. More immediate effects of chilling were observed by Biebl and others.

Tropical seaweed died within 10 minutes when exposed to temperatures around 2-3 C. With the plasmodium of Physarum polycephalum death occurred after 5 seconds when the temperature was dropped rapidly to 0 C.

The idea of toxins accumulating at low temperature continued to find supporters. Plank (67) suggested that low temperature decreased destruction of toxic material more than synthesis. Pentzer and Heinze (64) proposed that the toxic material was volatile and accumulated at low temperatures, but this was expelled by warmer temperatures, if accumulation had not proceeded too far. More definitive evidence was presented by Lieberman et al. (47), who showed that the polyphenols catechol and chlorogenic acid accumulated in chilled sweet potatoes. These substances inhibit oxidative phosphorylation (46).

Levitt (43) indicated that injury or death of chilling-sensitive plants may be caused by an imbalance between three major physiological processes: (a) a greater rate of transpiration than water absorption would result in desiccation; (b) higher respiration than photosynthesis would result in starvation; (c) increased protein hydrolysis over synthesis would disrupt normal metabolism and cause protein depletion.

The factors enumerated by Levitt would seem sufficient to explain ordinary chilling injury where the chilling-sensitive plant is slowly injured but does not show visible sympotms of injury or death until several hours or days at low temperature. However, in cases where injury is very rapid (c.f. above) his postulates are inadequate. Kavanau (35) postulated that inactivation of certain sensitive enzymes occurs because of an increase in intramolecular hydrogen bonding. Such over-bonding would cause active centers to lose their specific configuration. As a result the metabolism of the organism would be disrupted.

Respiration Response to Chilling

Most past work on the effects of chilling involved cold storage and post-harvest responses of chilling-sensitive plants and plant parts (3, 12, 13, 52, 64). In these studies the response of respiration to chilling has been used as an indication of the physiological effects. An indirect approach was used by Lewis (44) who observed protoplasmic streaming. In sensitive plants protoplasmic streaming ceased or was only slightly perceptile after 1 to 2 minutes at 10 C. Streaming stopped abruptly when the temperature was dropped to between 0 and 5 C. In cold-tolerant plants streaming continued down to 0 C. In the chilling-sensitive tomato resumption time of streaming in the trichomes at 20 C was inversely related to the time they remained at 0 C. After 2 days at 0 C some trichomes were plasmolyzed.

Direct measurements of respiration in cucumbers (12) indicated that the rate of CO_2 production increased, reached a plateau, then declined with time of storage at chilling temperature. The rise and cecline were associated with onset of injury and general death respectively. A similar response to chilling has been observed in sweet potato roots (45). Cucumbers (12), citrus fruit (11), and other chilling-sensitive vegetables (64) show a sharp increase in respiration at non-chilling temperatures after a low-temperature holding period. Non-sensitive plants generally do not show this sudden flush of CO_2 (11).

Woodstock and Pollock (83) found that temperatures of 15 C or lower during the first hour of inhibition by lima bean seeds immediately inhibited respiration. Inhibition of subsequent growth was correlated with the initial respiration inhibition. They suggested that the respiratory mechanism was too slow during low temperature inhibition to

supply sufficient energy for the growth of the membranes. Extensive membrane damage could lead to inhibition of growth and render the seedling more susceptible to attack by pathogens.

Similar results were obtained by Ibanez (30) with cacao seeds. Imbibitions of water at 4 C for 20 minutes made the seeds non-viable. Respiration determinations on isolated embryos indicated no difference between chilled and unchilled seeds. However, cotyledons isolated from chilled seeds showed an initial burst of O_2 uptake followed by a decline. Brownish pigment, thought to be oxidation products of polyphenols, occurred in the chilled cotyledons. Cytological examination of the chilled cotyledons indicated that the tannin cells were destroyed (31). The authors suggested that cold caused physical change in the cell membrane due to lytic or simple mechanical collapse of the cell.

Goodman and Wedding (18) studied the influence of temperature from 5 to 55 C on the respiration rate of discs cut from maturing cotton leaves. All tissue had the same optimum pretreatment before respiration rate determination at the given temperatures. Below 25 C there was some tendency for the respiration to increase with time over a 3-hour period. A preliminary report by Peynado and Amin (65) concerned the changes in respiration of cotton during chilling. At temperatures from 15 to 25 C discs from young cotton leaves had a higher respiration rate than discs from old leaves. At 5 C the reverse was true. Although the rate was low in both cases, the decrease in rate with decreasing temperature was greater in the young tissue than in the old. The CO_2/O_2 ratio was less than unity in all cases but the young tissue maintained their RQ over a broader range of temperatures.

Chilling Effects on Photosynthesis and Chloroplasts

Since tropical plants cease photosynthesis below about 5 C (14, 15) photosynthsis in these plants is apparently very sensitive to chilling. Yesipova (84) studied the effects of temperature on the photosynthesis of cotton. With respect to low temperature two zones were noted: (a) zone of depression of photosynthesis from -4 to 10 C and (b) zone of damage to the photosynthetic apparatus of the leaves from 2 to 5 C.

Structural changes have been observed in leaf cells in thermophilic plants at temperatures slightly above 0 C (37). Isolated leaves from _Cucumis sativa_ and _Zea mays_ kept at 1.7-2.0 C showed early decrease in photosynthetic ability, decrease in chlorophyll, and changes in chloroplast structure when the leaves were in the light. In the dark the photosynthetic disturbances were much slower to develop. Electron microscopic examination of cucumber chloroplasts indicated that the normal structure of the organelles gradually broke down under the action of low positive temperatures (39). When young cucumber plants were chilled in the dark at 3 to 5 C for 15 to 64 hours the subsequent rate of photosynthesis at room temperature was depressed. Longer treatments resulted in considerable or complete loss of photosynthetic ability (58).

A significant report concerning freezing injury to chlorplasts may have some relation to chilling injury. Heber (23) found that freezing of chloroplast membranes uncoupled photophosphorylation from electron transport. He suggested that the uncoupling was caused by interference with a structure involved in the formation of a non-phosphorylated high energy state of chloroplasts. The basic injury caused by freezing was

thought to be due to changes in the permeability of the membranes.

ATP and Other Nucleotides

A measure of adenosine triphosphate level has been used to measure the influence of a number of factors in a wide variety of organisms (10, 54, 75, 76, 78). However, little has been reported concerning the influence of chilling on nucleotides. Hannon and Rosenthal (22) reported that cold acclimated rats had 65% more reduced and total triphosphopyridine nucleotides than control rats. According to Nechaeva (56) nucleotides in the leaves of radish and sugar beets increased at low temperature. Low non-freezing temperatures harden these plants against freezing injury. The same result was obtained with *Chlorella pyrenodosa* grown at 4-6 C compared to 20-22 C.

A report by Ketellapper (36) indicated that nucleotides may be involved in certain low temperature-induced chemical lesions. Nicotinic acid stimulated the growth of tomato at 20 C day-14 C night. Cosmos was stimulated by a mixture of B vitamins when grown at 17 C day-10 C night. No stimulation was observed at optimum temperature. Jung and co-workers (34, 73) suggested that foliar application of purines and pyrimidines influence the hardening process in alfalfa. However, their work appears inconclusive in relating nucleotides to chilling resistance.

Mitochondrial Response to Chilling

Several papers suggest that mitochondria may be involved in chilling injury. Lieberman et al. (47) studied the mitochondrial activities of sweet potato roots which had been stored at 7.5 and 15 C.

During the first four weeks the mitochondria from chilled and non-chilled tissues showed approximately equal rates of oxidation and phosphorylation. After the fifth week of storage a rapid decline in both oxidative and phosphorylative activity in the chilled tissue occurred. By the tenth week mitochondria from chilled tissue were inactive. The activity of particles from non-chilled roots showed little change during the 10 weeks. Oxidative and phosphorylative activities of sweet potato mitochondria have also been studied by Minamikawa et al. (53). Mitochondria from tissue stored at 0 C gave a sharp decline in oxidative activity after 10 days. Particles from tissue stored at 20 C did not show this decrease in activity. The P/O ratio remained relatively constant in the chilled mitochondria in spite of the oxidative decrease.

Christiansen and Moore (8) determined the minimum temperature for hydrolysis of cotton lipid was around 15 C. The enzymes involved in lipid metabolism are associated with the mitochondrial structures (41). Changes in fatty acids of alfalfa roots during cold hardening were noted by Gerloff et al. (17). Unsaturated fatty acids, mainly linoleic and linolenic, increased during the hardening process. Reports of changes in membrane lipid unsaturation appear significant in view of recent mitochondrial swelling experiments. Richardson and Tappel (69) showed that mitochondria from a cold-blooded animal had the ability to swell rapidly at temperatures down to 0 C. Particles insolated from a warm-blooded animal could not swell rapidly at low temperatures. They also correlated swelling ability with degree of unsaturation of the mitochondrial fatty acids. Lyons et al. (49) extended this idea to chilling-sensitive plants. Again, mitochondria from chilling-resistant tissue were more flexible, as measured by their greater ability to

swell, than were mitochondria from chilling-sensitive tissue. They also found a higher concentration of unsaturated fatty acids in the mitochondria of the resistant plants than in those from sensitive plants. These authors suggested that the metabolic injury caused in chilling-sensitive tissues may be due to inability of the relatively inflexible mitochondria to function at low temperature.

As interesting report by Heber and Santarius (24) on freezing injury may also apply to chilling injury. Freezing inactivated the phosphorylating systems of chloroplasts and mitochondria isolated from spinach. Electron transport was apparently not affected by freezing so that the breakdown in ATP synthesis was considered to be an uncoupling effect. The oxidative phosphorylation of isolated mitochondria could be protected from freezing by sugars. This protection was reversed by salts. They suggested that alteration of lipoprotein was a main cause of frost injury.

Other reports indicate that individual enzymes in or on the mitochondria are inactivated by low temperatures. Pullman et al. (68) purified a soluble ATPase from beef heart mitochondria which was stable at room temperature but lost activity rapidly at 4 C. They subsequently reported (62) that the ATPase functioned as a factor which coupled phosphorylation to substrate oxidation by the submitochondrial particles. Both ATPase activity and coupling activity decreased markedly when the enzyme was incubated at 0 C for short periods of time. Dilution of the enzyme or addition of potassium salts, particularly KI and KNO_3, increased the rate of inactivation (63). Scrutton and Utter (72) indicated that highly purified pyruvate carboxylase from chicken liver mitochondria was inactivated very rapidly by exposure to low

temperature. Sucrose in high concentrations or certain reaction components, notably acetyl coenzyme A, protected against low-temperature inactivation.

CHAPTER III

MATERIALS AND METHODS

Source of Tissue

Two cultivars of cotton, _Gossypium hirsutum_ L., were used as the tissue source. 'Stoneville 62' was used for respiration and photosynthesis determination, while 'Parrott' was used for mucleotide and mitochondrial studies. Seeds were germinated in moist vermiculite in the greenhouse until the cotyledons emerged, usually 4 to 6 days. The seedlings were then transferred to 12-liter polyethylene buckets containing a modified Hoagland's solution (26). Each bucket was equipped with a wooden lid with 6 holes through which the seedlings were placed. Support for the plants was provided by stuffing cotton fiber into the holes around the stem of the seedling. A diffuser stone was placed in each bucket and connected to an air pump to supply aeration to the roots. The seedlings were grown under greenhouse or growth chamber conditions until use. Additional details and applicable modifications of the above procedure are described for individual experiments.

Determination of Respiration

Respiration at 5 Degree Intervals

Respiration was measured on roots, cotyledons and leaves. For cotyledon experiments plants were grown in the greenhouse for one week

after transplanting, while plants used for leaf and root experiments were gorwn for two weeks. Respiration was measured at 5 degree intervals from 5 to 25 C using standard Warburg manometric methods (80). Measurements were made on successive days using a different temperature each day. To ensure plants of similar size and age at the time of use, seeds were planted on each of five consecutive days for each experiment. For root respiration determinations, lateral roots of one plant were placed in a 20-ml Warburg flask. The volumes of the roots were determined by water displacement and subtracted from the volumes of the flasks. For leaf and cotyledon respiration determinations two-centimeter discs were cut from either side of the midrib of the blade with a cork borer. Five discs of a given tissue were leaned against the center well of each flask. In all respiration determinations 0.2 ml of 20% KOH and a strip of fluted filter paper were added to the center well of one-half the flasks. To determine RQ values, 0.2 ml of water was substituted for KOH in the remainder of the flasks. Oxygen consumption was usually measured for 3 hours, after which time the tissue was oven-dried and weighed. Final results were expressed as the average of six replications in ul O_2 consumed per milligram dry weight per hour.

Effect of Prechilling on Root Respiration

To determine the effects of prechilling on root respiration, plants were grown for two weeks from transplanting. The roots of one-half the plants were then chilled at 10 C for 36 hours in the greenhouse while roots of control plants were maintained at 30 C. Root temperature was maintained within ±0.8 C of the stated temperatures by placing the plant

containers in thermostatically controlled water tanks. The roots were harvested and the following respiration rate determinations made: (a) rate of control roots at 25 C, (b) rate of control roots at 10 C, (c) rate of chilled roots at 25 C and (d) rate of chilled roots at 10 C. The procedure described above were used for the manometric determinations.

Manometric Determination of Photosynthesis

Cotyledonary material used for photosynthetic determinations was grown as described for respiration. Cotyledons were harvested and two 1-cm discs punched from either side of the midrib. In each Warburg flask six discs were floated adaxial side down in 0.2 ml of modified Hoagland's solution (26).

In initial experiments Pardee's (60) CO_2 buffer was used to maintain a constant CO_2 atmosphere. This system was strongly temperature-dependent, however, and inadequate for the desired CO_2 concentration at the lower temperatures. Kreb's (38) system, supplemented with arsenite (71), was then used. Since the measurements were to be made at 5 degree intervals from 5 to 25 C, the buffer had to be equilibrated at the desired temperature of each determination. Five ml of 4 M diethanolamine solution containing 1 M sodium arsenite and 0.1% thiourea were saturated with 100% CO_2 at the temperature desired. The gas was cooled before bubbling through the solution by passing it through copper tubing submerged in a refrigerated water bath. An additional 10 ml of diethanolamine was mixed with the saturated solution and the total equilibrated with 1% CO_2 in air at the appropriate temperature. One ml of the equilibrated buffer, along with fluted filter papers, was added to the center well and side arm of each of the

Warburg flasks. The flasks were equilibrated with a stream of 1% CO_2 obtained by mixing 1 part cylinder CO_2 plus 99 parts air at the appropriate temperature for 20 minutes before the manometers were closed.

Photosynthesis proceeded in light supplied by six incandescent lamps giving approximately 1500 ft-candles at flask level. Alternating light and dark periods of from 30 to 60 minutes were used to measure O_2 evolution and consumption respectively. After each determination the tissue was washed, oven-dried and weighed. Final results were expressed as the average of six replications in microliters of O_2 evolved per milligram dry weight per hour. Two determinations were made at 5 degree intervals from 5 to 25 C.

Extraction and Assay of ATP

Preparation of Tissue

The initial experiment was designed to determine the effect of root temperature on the ATP level of roots and leaves. The plants were grown in the greenhouse for three weeks, then their roots subjected to 10, 15, 20, or 30 C for four days. Tops were maintained at ambient greenhouse conditions. Roots and leaves were harvested, frozen and lyophilized, then ground to pass a 60-mesh screen. The powdered tissue was stored at -26 C until it was assayed.

In subsequent experiments plants were grown for two weeks in the greenhouse or growth chamber after which time various chilling treatments were imposed upon the entire plant in a growth chamber. When the growth chamber was used, a 14-hour light period of approximately 3,000 ft-candles was provided by cool-white Sylvania and Ken-Rad fluorescent lights supplemented by incandescent lamps. Chamber temperature

conditions before chilling treatments were 30 C day, 20 C night. Each treatment was replicated four times and each replication placed in a deep-freeze within two minutes of harvest. All harvests were made at the same time of day. The frozen tissue was dried, ground and stored as described above.

Extraction of ATP

The luciferin-luciferase assay system (77) was selected for the specific estimation of ATP. For this method the ATP had to be quantitatively extracted from the plant tissue into a solution free of substances interfering with the enzyme. Preliminary efforts to extract ATP involved the use of cold 0.5 N perchloric acid (PCA) (50, 82). However, Bieleski and Young (4) reported that 11% of the total phosphate coprecipitated when the perchlorate was removed as $KClO_4$. Also, internal standards and slow response of the luciferase enzyme to the samples indicated that an interfering substance was present in the PCA extract. Consequently, a comparison of a number of extraction methods was made to find one suitable for the assay system. This included (a) 0.5 N PCA with the perchlorate removed as $KClO_4$, (b) 50% aqueous ethanol (32) with ethanol removed by rotary flash evaporation, (c) 0.2 N formic acid in 20% ethanol (4) with the organic solvents removed by lyophilization and (d) 5% (w/v) trichloracetic acid (TCA) with the acid removed by ether extraction. The PCA extract gave an ATP estimation 40% lower than the TCA extract. The ethanolic extractions were intermediate between the PCA and TCA.

Five percent TCA extraction was used for the estimation of root temperature effect on ATP level. Two hundred mg of the ground tissue

were extracted with 10 ml of ice cold 5% (w/v) TCA by grinding in a glass hand homogenizer. The homogenate was centrifuged and 8 ml of the supernatant fraction extracted with cold ether four successive times. A rotary flash evaporator was used to remove the excess ether. The pH of the extract was adjusted to 7.4 with KOH and made to a known volume. After centrifugation the supernatant fraction was used for ATP assay.

The luciferase response to the TCA extract was improved over the PCA extract but was still slower than response to standard ATP. In order to determine if the inhibition was due to a salt effect, the ATP in acid extracts (PCA or TCA) of 100 mg of tissue was sorbed onto 0.3 grams of activated charcoal. The charcoal containing the ATP was collected by filtration then washed with 25 ml of deionized ice water. ATP was eluted with 20 ml of 50% ETOH/1% NH_4 OH (28). The ethanolic extract was frozen and dried in vacuo. The dried residue was taken up in 10 ml of 0.05 M glycine buffer, pH 7.4, centrifuged and used for ATP assay. This procedure permitted a very good luciferase response and gave fairly reproducible results. However, for a large number of samples, sorption onto charcoal was very time consuming.

Belatedly, extraction of ATP with hot water (74) was compared with the acid-charcoal procedure. In this method 5 ml of boiling deionized water was quickly added to 25 mg of the dried, ground tissue and placed in a boiling water bath for three minutes. The extract was quickly cooled in an ice bath, then centrifuged to remove the insoluble residue. The supernatant fraction was used directly for assay. A comparison of the hot water and acid-charcoal procedure is given in Figure 1. Except for the experiment concerning the effect of root temperature on ATP, all ATP estimations are based on the hot water extraction.

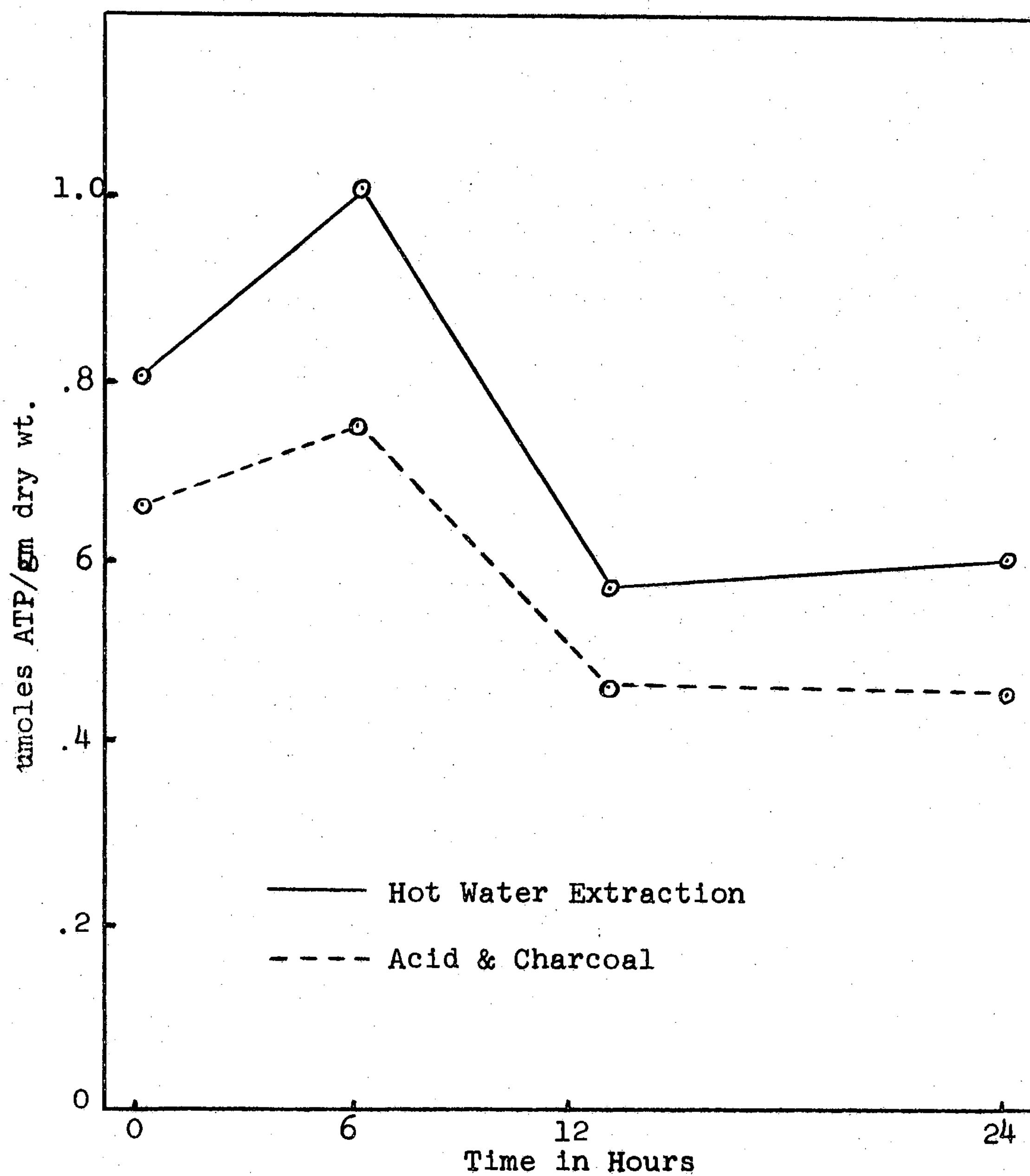

Figure 1. Comparison of ATP Extraction Methods on Four Treatments of Cotton Tissue. The Abscissa Represents Hours Cotton was at 5 C.

Preparation and Specificity of the Enzyme

A method similar to that of Strehler and Totter (77) was used for the ATP assay. Firefly lanterns were obtained commercially (Sigma, St. Louis) or taken from fireflies caught locally. In the latter case the fireflies (*Photuris pennsylvanica* De G.) were caught and placed alive in a lyophilizer and vacuum dried. The lanterns were cut off and stored at -26 C. Immediately before use, 20 lanterns were hand homogenized with a glass homogenizer in 5 ml of cold 0.1 M sodium arsenite buffer at pH 7.4. An additional 1 ml of buffer was used for wash and the combined homogenate and wash centrifuged 10 minutes at 27,000xg. The supernatant fraction was retained and mixed with 50 mg of $MgSO_4$. For assay of the hot water extracts, 130 mg (40-45 lanterns) were ground in 10 ml of buffer with 2 ml for wash. A 100-mg portion of $MgSO_4$ was added to the supernatant fraction and aliquots of this used directly as the enzyme in the assay.

Balfour and Samson (2) indicated that the crude enzyme preparation from firefly lanterns contained transphosphorylases which catalyze the reaction xTP + ADP —— ATP + xDP. Although interference by other triphosphates was not considered detrimental to the purpose of the experiments, it was considered desirable to know to what extent they contributed to the estimation. At equimolar concentrations with ATP the triphosphates of guanosine and cytidine gave increases in light emission of about 6% and 1% respectively over the ATP light emission. Response to ATP reached a maximum in less than 2 seconds. When GTP and ADP were mixed in equimolar concentrations the light response was about 10% of the ATP response within the two-second response time. CTP and ADP at equimolar concentrations gave a light emission of about 2% of the ATP.

The ADP alone gave no response. The maximum light emitted for the mixtures was greater than the percentages given above but was much slower to develop than the ATP light response. The maximum for GTP mixtures took about 12 seconds, while CTP mixtures took about 30 seconds. UTP was not available, but according to Balfour and Samson (2) equimolar concentrations do not reach maximum for about 20-25 seconds. Although other triphosphates give some response in the assay system, they would have to be present in high concentration to significantly change the ATP estimations.

Assay of ATP

Initial efforts to measure the light produced in the luciferase assay with a Turner fluorometer were unsatisfactory. Consequently, a Beckman DU-2 spectrophotometer with a recording attachment was adapted to the assay system. The reaction vessels were 4-ml cuvettes of 1 cm light path raised about 1 cm in the holder. This permitted maximum light detection of a small volume. The reaction mixture consisted of 0.6 ml of 0.05 M glycine buffer at pH 7.4, 0.2 ml of the enzyme preparation and 0.2 ml of the sample or standard containing from 0 to 1 microgram of ATP. The enzyme and buffer were mixed in the cuvettes then placed in the sample chamber of the spectrophotometer. The sensitivity was set between 6 and 8, shutter to the phototube opened, and the recorder adjusted to 0 to compensate for any endogenous fluorescence of the enzyme extract. The sample was then forcibly injected into the cuvette with an air-tight syringe through a small light-tight hole in the lid of the chamber. Maximum light intensity was recorded with a 10 millivolt recorder. The results were quantitated by inclusion of

reagent ATP standards with each assay. Peak height was correlated with concentration and linear responses were always obtained with standards. Figure 2 gives a typical standard curve obtained with reagent ATP.

Ion Exchange Chromatography of Nucleotides

A number of attempts were made to determine free nucleotides in cotton tissues by ion exchange chromatography. Initial efforts involving the method of Ingle (32) were plagued with technical difficulties in the system and resolution of the nucleotides was poor. Cherry and Hageman's (6) modification of the procedure reported by Hurlbert et al. (29) gave acceptable separation of most of the major nucleotides.

The Chromatographic System

Dowex 1x8, Cl^-, 200-400 mesh, anion exchange resin was prepared by washing it successively with water, acetone-water (1:1), acetone, ether, acetone, acetone-water (1:1), and water. The resin was then slurried into a large column and washed with 4 M sodium formate until the effluent gave a negative test for Cl^- (acidified silver nitrate). Excess sodium formate was washed out with deionized water and the resin stored in the cold until use. The chromatographic columns were packed by gravity in two-centimeter sections. For root nucleotide separations the columns were 40x0.8 cm, but for leaf nucleotide separation the columns were 50x1.2 cm. After the columns were formed they were washed with 3-4 bed volumes of 88% formic acid to remove an opaque material that contributed to the UV absorbing background. After being washed with deionized water the columns were ready for use.

The samples were loaded onto the columns by gravity then washed

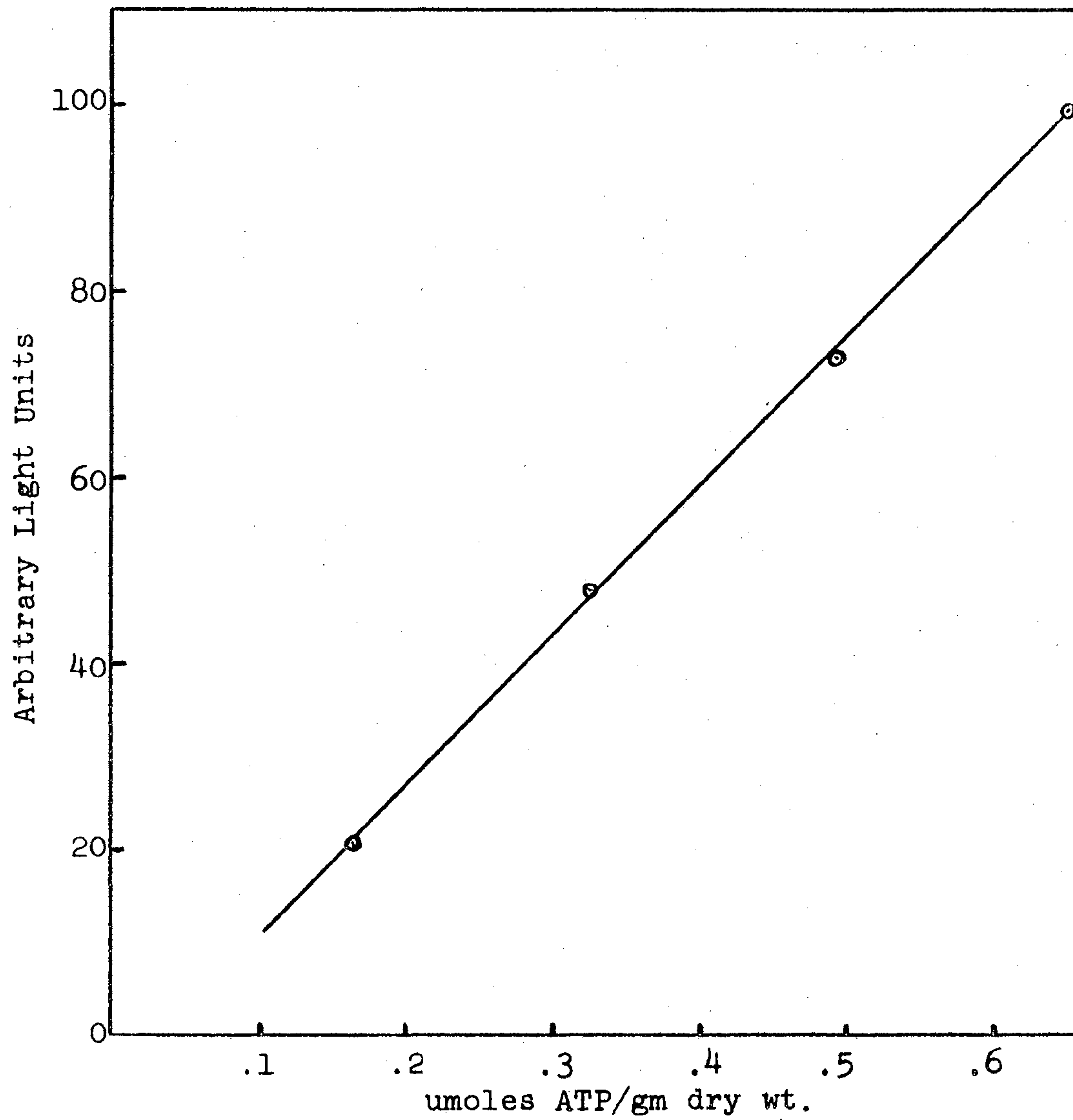

Figure 2. Typical Standard Curve Obtained with the Firefly Luciferin-Luciferase Assay System.

with water to remove cations and uncharged substances. Elution of the nucleotides was started by addition of 500 ml of 4 M formic acid to one of the reservoirs. This was followed by addition of approximately 800 ml of 4 M formic acid to the inverted reservoir and 1000 ml of 1.6 M ammonium formate in 4 M formic acid to the upright reservoir. Four to five ml fractions were collected at the rate of 0.8 to 1 ml per minute. This flow rate was maintained by a hydrostatic head of four feet. In some cases the fraction volume was increased to 6-7 ml after the start of the ammonium formate elution. The elution of UV absorbing material was followed by determining the absorption of each fraction at 260 nm.

Preparation of Extract for Chromatography

Major difficulty was encountered in identification of the nucleotides because of the high background of the chromatogram. The background was especially high for acid extracts from leaves and was probably due to the high content of phenolics, flavonoids and related compounds in cotton tissue. The presence of these materials prevented identification of individual peaks from the columns by absorption spectra. Little purification of the nucleotides could be obtained by activated charcoal because the interfering substances were sorbed and eluted as were the nucleotides. Paper chromatography was not especially useful because of the smearing and fluorescence of the material.

The background material was apparently held on the Dowex 1 resin by pi complex formation since its elution from the columns was essentially independent of eluent pH and ionic strength. This implied aromatic material, so a number of organic solvents, including toluene, chloroform, ether, heptane, amyl alcohol, and iso-amyl alcohol were

tested on acid extracts of leaves in an effort to remove some of the interfering material. Removal of the red pigment found in acid extracts of cotton leaves was used as the criterion for effectiveness of the solvents. Amyl and iso-amyl alcohol were most efficient in removing the red pigment. Amyl alcohol has been used as a general extractant of flavonoid compounds (70).

Leaf nucleotides were extracted from 6 grams of dried leaves with 120 ml of ice-cold TCA by homogenizing at full speed for 5 minutes in an Omni-Mixer. The homogenate was centrifuged at 27,000xg for 10 minutes and the supernatant fraction filtered. One hundred ml were extracted with 0.2 volumes of n-amyl alcohol four times. The extract was centrifuged briefly after each extraction to separate the phases. The TCA was extracted into the amyl alcohol. The residual alcohol was removed by extracting twice with 0.2 volumes of ethyl ether and residual ether removed *in vacuo* on a rotary evaporator. All operations were at 4 C or less. The pH of the extract was adjusted to 7.1 with 5 N KOH then centrifuged. The resulting supernatant liquid was applied to the column.

The acid extracts of root tissue contained less interfering material than those of leaf tissue. The method of extraction of nucleotides from root tissue was the same as for the leaves except 0.5 N PCA was used instead of TCA. After filtration the pH of a known volume was adjusted to 7.1 with 5 N KOH. After 15 minutes at 0 C the $KClO_4$ was removed by centrifugation and the resulting supernatant fraction was applied to the column.

Identification of Nucleotides

Elution of nucleotides was monitored by determining UV absorption at 260 nm. Fractions common to a peak were pooled, shell-frozen and lyophilized. In some cases pooled fractions which contained ammonium formate were mixed with 0.3 grams of acid-washed activated charcoal. The charcoal was collected by filtration, washed with deionized water, and the nucleotides eluted with 50% ETOH/1% NH_4OH (28). The ethanol and ammonium were removed by flash evaporation, then the samples were lyophilized. The resulting residues were taken up in a small amount of water and an attempt made to identify the bases by their UV spectra. In some cases identification was possible, but in others interfering materials, some of which were fluorescent, prevented identification. The pooled fractions consequently were reduced to 1 ml and applied to a Sephadex G-10 column, 40x2 cm or 60x2.2 cm. Distilled water was used as eluent and 5 ml fractions were collected. Each fraction was monitored at 260 nm and spectra from 220 to 290 nm taken of peak tubes. Reasonably good spectra could usually be obtained from the peaks eluted from the Sephadex. Those peaks which showed nucleotide-type spectra were again freeze-dried, taken up in a minimum of water and co-chromatographed with standerds on thin layer plates of MN-cellulose 300 G using Pabst solvents I and III (59). Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were identified by the cyanide addition reaction (9). ATP was identified with firefly luciferase. Previously published results (5, 6, 29, 32) were relied upon heavily to determine elution sequence, and hence, subsequent identification of the nucleotides.

Isolation and Assay of Mitochondria

Problem of Isolation

Active mitochondria are not obtained from cotton (25) unless special precautions are taken. Throneberry (79) showed that bovine serum albumin (BSA) protected mitochondria of cotton hypocotyls from inactivation by endogenous inhibitors. Gossypol, a cotton polyphenol, was observed to inhibit some of the oxidative respiratory enzymes and to inhibit phosphorylation at concentrations which had little effect on oxidation (55). BSA reduced inhibition, provided it was present before the mitochondria were exposed to gossypol.

Initial attempts to obtain active mitochondria from cotton tissue involved the use of etiolated hypocotyls and were patterned after the method of Throneberry (79). Polyvinylpyrrolidone (27, 33) and Carbowax 6000 (40, 49) were tested but did not give mitochondria as active in malate oxidation as those isolated with BSA. The use of sodium m-bisulfite in the extracting medium was also found to be beneficial. This compound serves as a reducing agent to prevent oxidation of phenols and consequent inactivation of the mitochondria (1). The method developed for isolation of mitochondria from hypocotyls was used with only slight modification for root tissue.

Method of Isolation

For hypocotyl mitochondria, cotton seeds were germinated in the dark at 30 C for six days in vermiculite moistened with nutrient solution. Fifty grams of the etiolated hypocotyls were vacuum infiltrated with 200 ml of extracting medium which contained 0.4 M sucrose, 0.01 M

NaEDTA, 2% BSA, 0.02 M citrate, and 0.005 M Na m-bisulfite. The pH was adjusted to pH 7.5 with KOH. The hypocotyls were rolled between a pyrex dish and a glass roller at 3 C to express the mitochondria, and the liquid was strained away from the pulp with 4 layers of cheesecloth. Mitochondria were obtained by differential centrifugation as follows: 10 minutes at 1,000xg, pellet discarded; 10 minutes at 15,000xg, pellet retained. The pellet was resuspended in the extracting medium and the two centrifugations repeated. The final pellet was washed with a medium (24) which contained 4 M sucrose, 0.01 M KH_2PO_4, and 0.01 M KCl, pH 7.2 then centrifuged 10 minutes at 15,000xg. All operations were conducted as near to 0 C as possible. The resulting pellet was suspended in 0.25 M sucrose and used for oxygen uptake determination by manometric procedures.

Isolation of mitochondria from roots was the same as above with the following changes. Root tissue was obtained from plants grown in the greenhouse by hydroponics as described earlier. Only lateral roots were used. The washed tissue was cut with scissors into fine pieces in 4 volumes of the extracting medium then blended in an Omni-Mixer at full speed for a brief period. Initially this was for 20 seconds but later 5 seconds was found to be adequate. The homogenate was strained through 2 layers of fine mesh nylon hose to remove the brei. The centrifugations and washes were identical to those given above except 0.02 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was added to the wash medium as buffer.

Assay of Mitochondrial Activity

In initial experiments mitochondria activity was measured

manometrically at 30 C. The total mixture (2.5 ml) at pH 7.2 contained the following:

Sucrose	625 umoles
Glucose	125 umoles
Hexokinase	0.5 mg (Sigma, type V)
KH_2PO_4	125 umoles
$MgSO_4$	2.5 umoles
Cytochrome C	1 mg (Sigma, type III)
ADP	2.5 umoles
NAD	2.5 umoles
NADP	0.25 umoles
$MnCl_2$	0.25 umoles
TPP	0.25 umoles
Coenzyme A	0.25 umoles
Substrate	125 umoles
Mitochondria	0.2-0.4 mg protein N

All components were mixed in the main chamber of double side-arm manometric flasks except the substrate, which was added to one side arm. After a temperature equilibration period of about 10 minutes, the reaction was started by tipping in the substrate. Controls containing all the components except the substrate were included to correct for endogenous oxidations. Oxygen consumption was determined at 10 minute intervals for at least 1 hour. In experiments where an effort was made to determine phosphorylation, 0.5 ml of 20% TCA was added to one of the side arms. The reaction was stopped by tipping in the acid. Mitochondrial protein was determined by the method of Lowry et al. (48), and protein nitrogen estimated by dividing the protein concentration by 6.25 (66). When phosphorylation was determined, inorganic phosphate before and after reaction was estimated by the method of Fiske and SubbaRow (15).

With the acquisition of YSI Model 53 oxygen electrode (Yellow Spring Instr. Co.), O_2 uptake determinations were simplified. The method of mitochondrial isolation was changed only in that less tissue

was used (12 grams per sample) and the final pellet was suspended in a solution of 0.25 M sucrose, 125 mM KH_2PO_4 and 2.5 mM $MgSO_4$ at pH 7.2. The total reaction system was not changed.

In order to measure the activity of isolated mitochondria at different temperatures, two thermostatically controlled water baths were set up. One was maintained at 30 C and the other at the desired temperature from 5 to 25 C. A portable water pump was used to circulate the water around the reaction vessels of the O_2 electrode. The temperature in the reaction chambers could be changed and equilibrated from 5 to 30 C in less than 10 minutes. The vessels were first equilibrated at the lower temperature and O_2 uptake determined. The pump was then transferred to the 30 C bath and the reaction vessels allowed to equilibrate at that temperature (7-10 min.). The rate at 30 C was then determined on the same mitochondria. A different set of mitochondria were isolated for each 5 degree increment, but by expressing the results as percent of the 30 C rate, determinations were directly comparable.

CHAPTER IV

RESULTS AND DISCUSSION

Respiration

Respiration at 5 Degree Intervals

Sugars accumulated in young cotton plants when chilling conditions were imposed (19), and apparently root temperature was more important than air temperature in causing the accumulation (21). These results indicated that root respiration was more sensitive to chilling than leaf or cotyledon respiration. Consequently, the rate of respiration of cotton seedling roots, cotyledons, and leaves in relation to temperature was measured to determine if this were true. The rates of O_2 uptake from 5 to 25 C are given in Figure 3. Leaves and cotyledons did not differ significantly from each other in their response to temperature. However, root respiration was considerably higher than that of the green tissue at the warmer temperatures, but about the same at 5 C. In other words, there was a greater response to temperature by the roots than by the tops. The results of Figure 3 are also reflected in the Q_{10} values (Table I). All tissue had a greater Q_{10} from 5 C to 15 C than at higher temperatures but that of the roots was especially high. These data support the suggestion that root tissue is more sensitive to low temperature than leaf tissue.

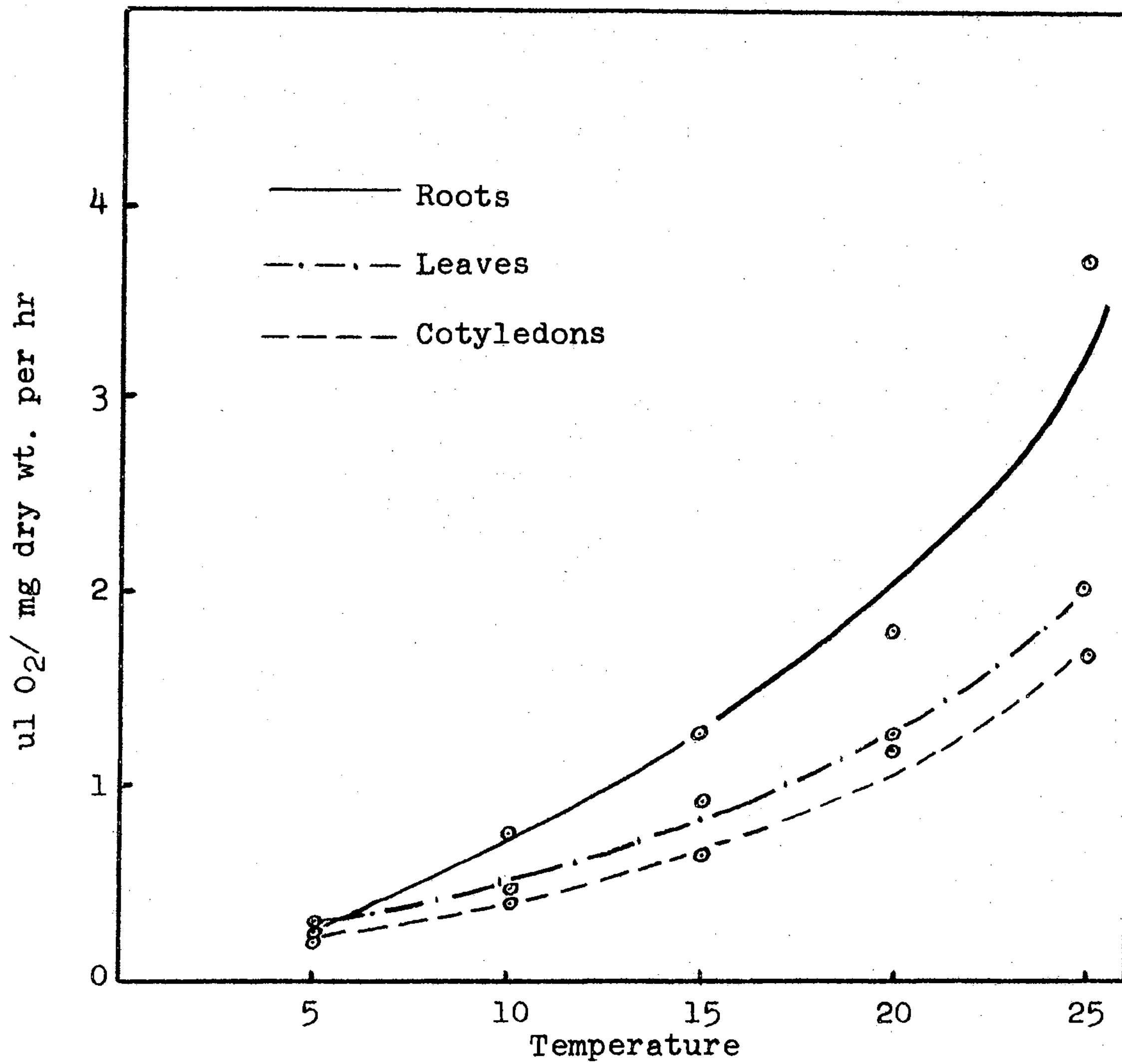

Figure 3. Effect of Temperature on Cotton Tissue Respiration.

TABLE I

Q_{10} VALUES FOR COTTON TISSUE RESPIRATION

Range	Roots	Leaves	Cotyledons
5-15	5.3	3.4	3.3
10-20	2.3	2.9	2.8
15-20	2.9	2.3	2.6

In a second experiment the respiration rates at 5 degree intervals were determined on the entire root systems of one-week old plants. The response of the whole root to temperature was not as great as the response of lateral roots. However, the rate of O_2 uptake apparently decreased with time at all temperatures except 25 C (Figure 4). The decline in respiration at 10 C was greater than at other temperatures. Visual observations indicate that chilling injury begins around this temperature in unhardened plants. The slower decline in rate at 5 C may be an indication of immediate inhibition of respiration. The differences in initial rates between 5-10 C and 10-15 C appear to support this suggestion. The decline in rate probably was not due to substrate starvation because the total consumed at 25 C greatly exceeded the O_2 consumed at any of the other temperatures. However, in view of Lewis' (44) work with protoplasmic streaming, local substrate depletion cannot be ruled out.

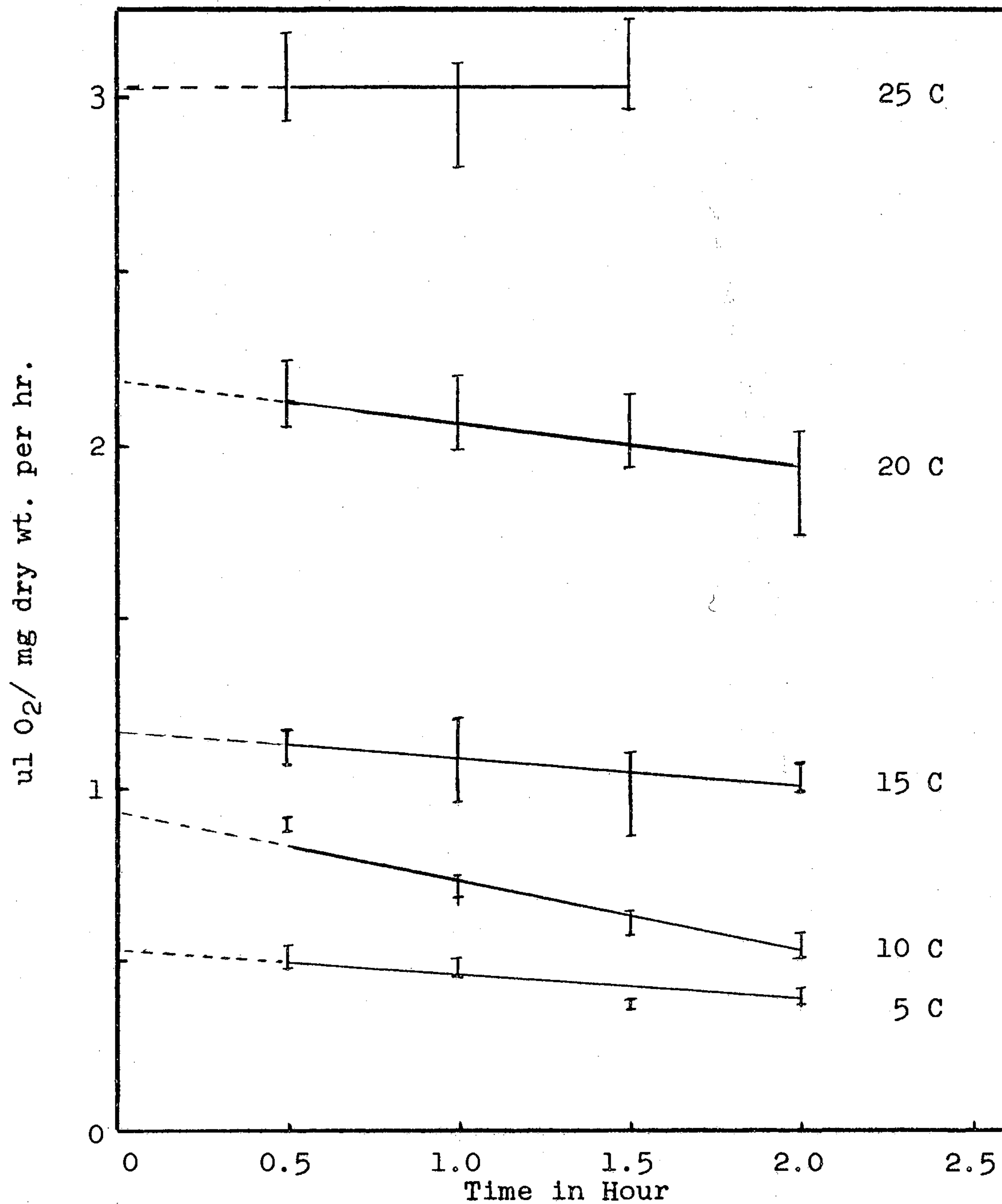

Figure 4. Change in Root Respiration with Time at Various Temperatures. The Lines are Least-Squares Fits of Six Determinations. Vertical Lines Represent the Standard Deviations of the Means.

Effect of Prechilling on Root Respiration

Although the respiration rate of roots decreased with time at chilling temperatures, the rate appeared to level off in a few hours. After 24 hours the rate was still about 50% of the original rate. Prechilling of root tissue before respiration determination, gave results similar to those reported for other chilling-sensitive tissue (11, 30, 45). Figure 5 gives the respiration rate at 10 and 25 C of roots subjected to temperature regimes of 10 and 30 C for 36 hours prior to measurement. At both 10 and 25 C the prechilled tissue had a higher O_2 uptake than non-chilled tissue. Eaks (11) suggested that the increased O_2 consumption observed at warm temperature after a holding period at chilling temperature was due to accumulation of excessive amounts of certain unspecified metabolic intermediates at low temperature. Guinn and Hunter (20) have shown that sugars accumulate rapidly at a root temperature of 10 C. Thus, the higher respiration rate at 25 C of prechilled tissue may be explained on the basis of increased substrate. The same phenomenon probably also applies to the higher rate at 10 C of prechilled roots. Since the chilled tissue was allowed to warm between time of harvest and time of O_2 uptake determination, any local depletion of substrate during the prechilling would be overcome.

Effect of Temperature on Photosynthesis

Since sugars accumulate in cotton plants at chilling temperatures (20), photosynthesis may continue at a relatively high rate compared to respiration. That is, photosynthesis may not be as temperature sensitive as respiration.

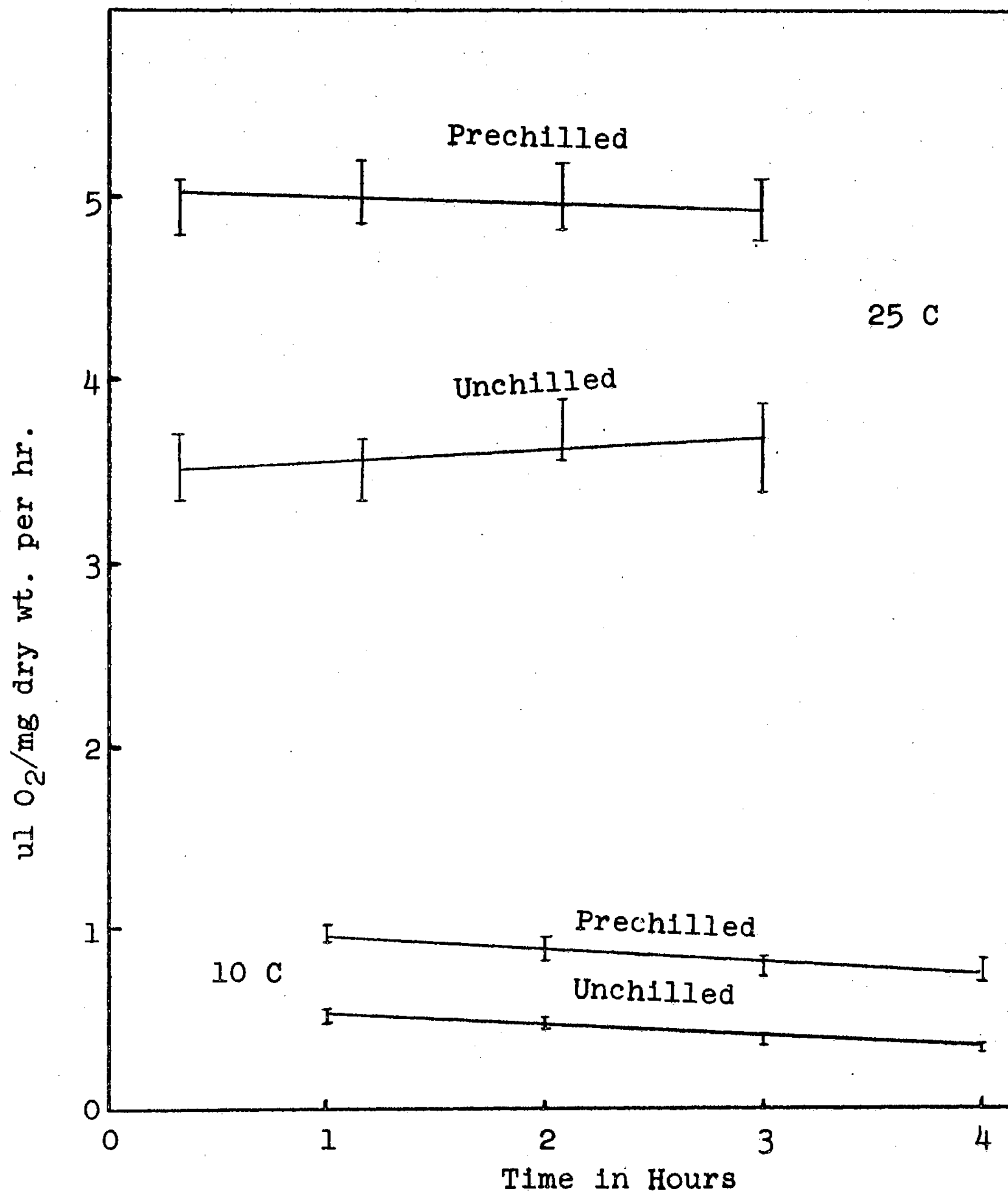

Figure 5. Effect of Prechilling on Root Respiration Rate at 10 and 25 C. The Lines are Least Squares Fits of Six Determinations. The Vertical Line Represents the Standard Deviations of the Means.

The effect of temperature on the rate of photosynthesis is given in Figure 6. The determinations were made in 1% CO_2 atmosphere so that the response to temperature would be independent of CO_2 concentration. Under natural conditions the CO_2 would probably be the limiting factor at temperatures above 15 C (16), and thus mask the temperature effect. The greatest decrease in photosynthetic rate due to temperature occurred between 15 and 25 C rather than between 5 and 15 C as was the case with respiration. The Q_{10} from 15 to 25 C was approximately 4 compared to 2 for the 5 to 15 C range.

Although the photosynthesis of cotyledons was very sensitive to chilling, the rate of O_2 production was still considerably higher than O_2 consumption at chilling temperatures. Sugars may increase because of relatively high photosynthesis, but these results are inconclusive when other factors are considered. Electron transport of the chloroplasts could be uncoupled from phosphorylation as suggested by Heber and Santarius (24), or the chloroplast structure may degenerate with continued chilling (37, 39). When whole cotton plants were chilled at 5 C in a growth chamber bleaching of the leaves was observed within two to three hours. Also unknown is the effect of temperature on the conversion of sugar to end products such as nucleic acid and protein. Therefore, an estimation of available energy should give a better indication of chilling injury than measurements of respiration or photosynthesis.

Effect of Chilling on the ATP Level

Adenosine triphosphate is the main phosphorylated compound in both oxidative and photophosphorylation and is involved in every major

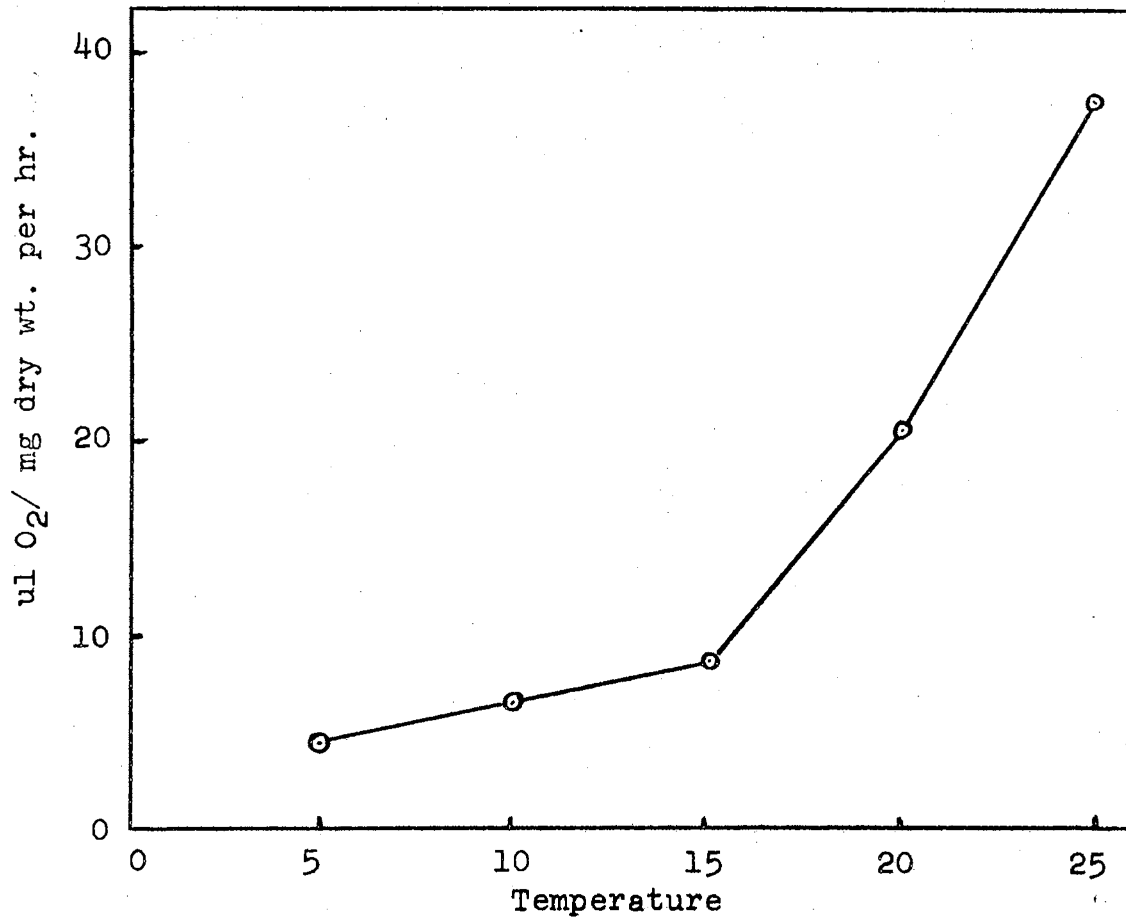

Figure 6. Effect of Temperature on the Rate of Photosynthesis in Cotyledon Discs in a 1% CO_2 Atmosphere. Points are Averages of 12 Determinations.

synthetic role we know. Any phenomenon which alters the supply of ATP will indirectly affect the whole organism. Therefore, a determination of the effects of chilling on ATP concentration is of interest in elucidating the nature of chilling injury.

Effect of Root Temperature

The roots of plants grown 1 week in nutrient solution were chilled for 4 days at 10, 15, 20 and 30 C and then the ATP content of the roots and leaves measured. The results are given in Figure 7. In roots the ATP level increased with decreasing temperature down to 10 C, while the ATP level in the leaves decreased with decreasing root temperature below 20 C. Apparently the low temperature decreased use of available ATP more than synthesis in the roots. In a separate experiment roots were chilled at 5 C for four days. In this case the ATP level in the roots at 5 C was lower than the level at 25 C and was only about 20% of the level found at 10 C in the experiment above. A root temperature of 5 C decreased the leaf ATP even more than 10 C. These results indicate that the injury to the ATP synthesizing system in the roots may occur below 10 C. However, since ATP does accumulate at chilling temperatures down to 10 C, one can assume that energy requiring processes such as absorption and transport of essential minerals to the leaves are considerably slowed by low temperature. A decrease in available phosphate and other minerals due to cold roots would result in a decrease in ATP synthesis in the tops even at warm leaf temperatures. Root temperature, therefore, is important to the welfare of the entire plant.

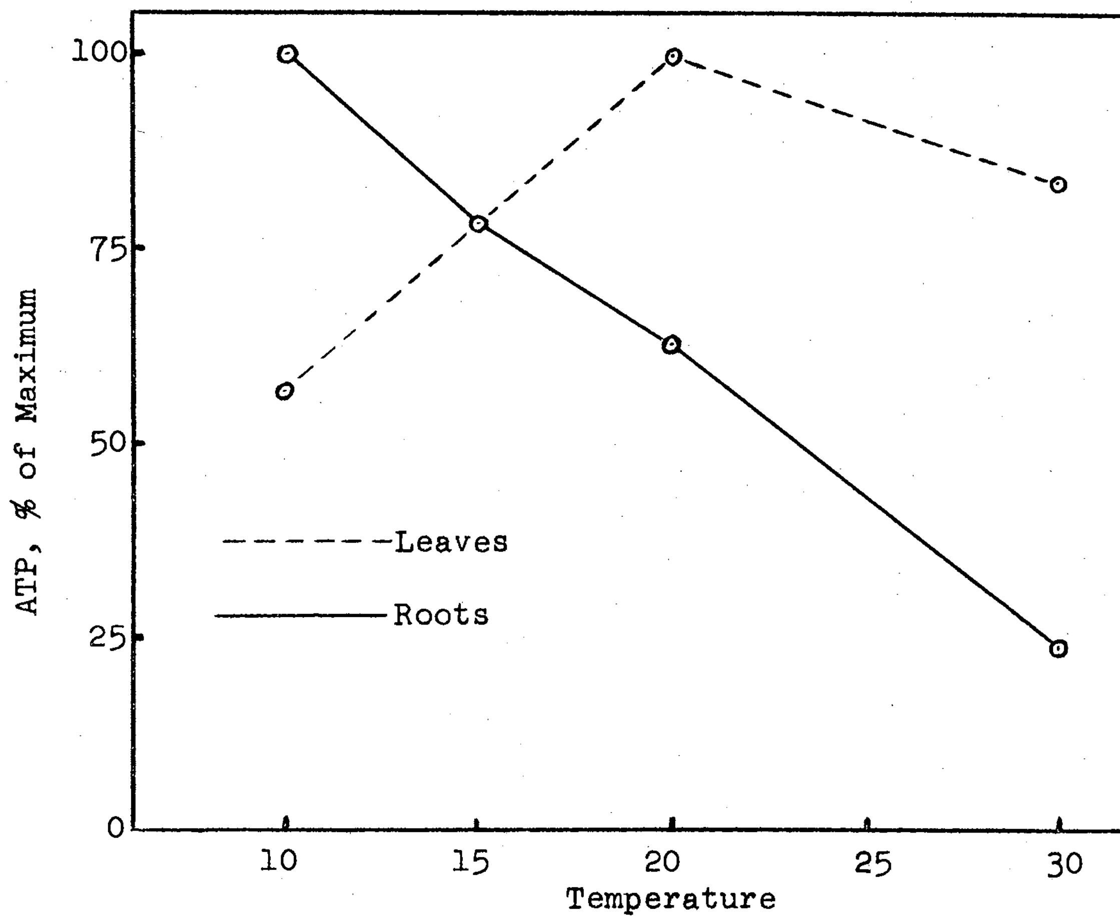

Figure 7. Effect of Root Temperature on ATP Level in Roots and Leaves. The Temperatures Were Maintained for Four Days and Four Replications Were Made. 100% Represents 254 and 276 ugm/gm Dry Weight for Leaves and Roots Respectively.

Response to Severe Chilling by Unhardened Plants

The decrease in ATP level in the roots at 5 C indicated that severe chilling influenced the relative rate of synthesis or use of ATP. When two-week old cotton seedlings were subjected to severe chilling treatment at 5 C in a growth chamber, the ATP content of the leaves began to decrease almost immediately. Figures 8 and 9 show the effects of chilling the entire plant for 6, 13, and 24 hours at 5 C compared to control plants maintained at 30 C day and 20 C night. For both control and chilled plants the light period began at 6:00 A.M. and ended at 8:00 P.M. The initial harvest was at 9:30 A.M. In the leaves and cotyledons (Figure 8) the consistent decrease in ATP concentration is apparent. This indicates that the chilling-mediated decrease in ATP occurred rather rapidly after chilling conditions were imposed. The increase in ATP in the control plants at 13 hours is probably due to the 20 C temperature of the dark period. Since the nutrient solution buffered the roots against sudden temperature changes, they went through an initial period of ATP increase as the solution cooled (Figure 9). After 6 hours the root temperature was about 10 C. By 13 hours the temperature was 5 C and the level of ATP declined. Some hardening may have occurred during the cooling period (cf. below).

Although the decrease in ATP was observed after relatively short periods of chilling, the plants were able to restore the initial concentration if chilling was not continued. Figures 10 and 11 indicate that the original level of ATP was regained within one day under greenhouse conditions after 24 hours of chilling. When the plants were chilled for two days, however, chilling injury was much more extensive, and the ATP concentration remained low when the plants were returned to

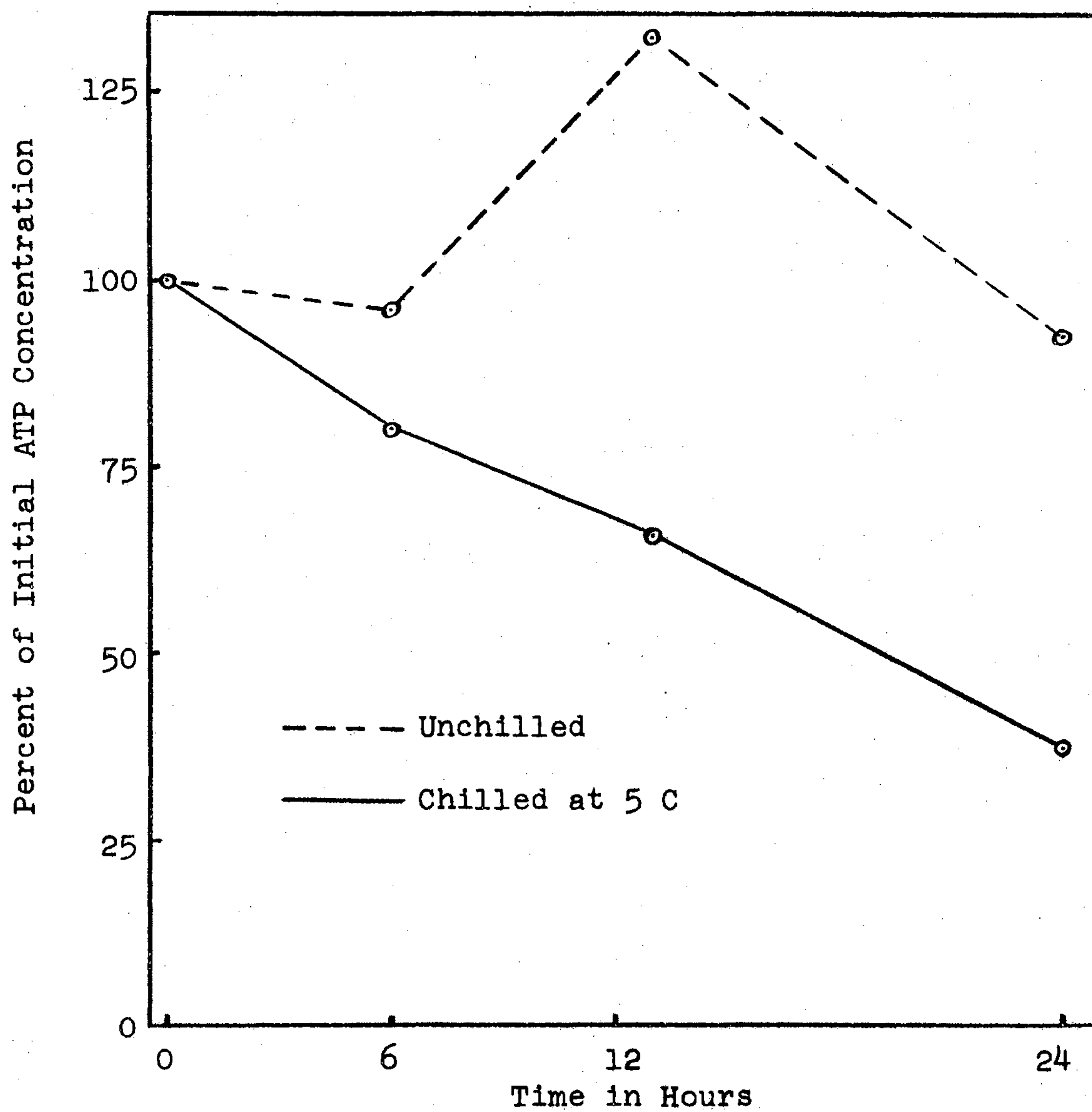

Figure 8. Immediate Effects of Chilling on Leaf ATP Level. Each Point Represents the Average of 4 Replications. 100% Represents .715 umoles ATP/gm Dry Weight.

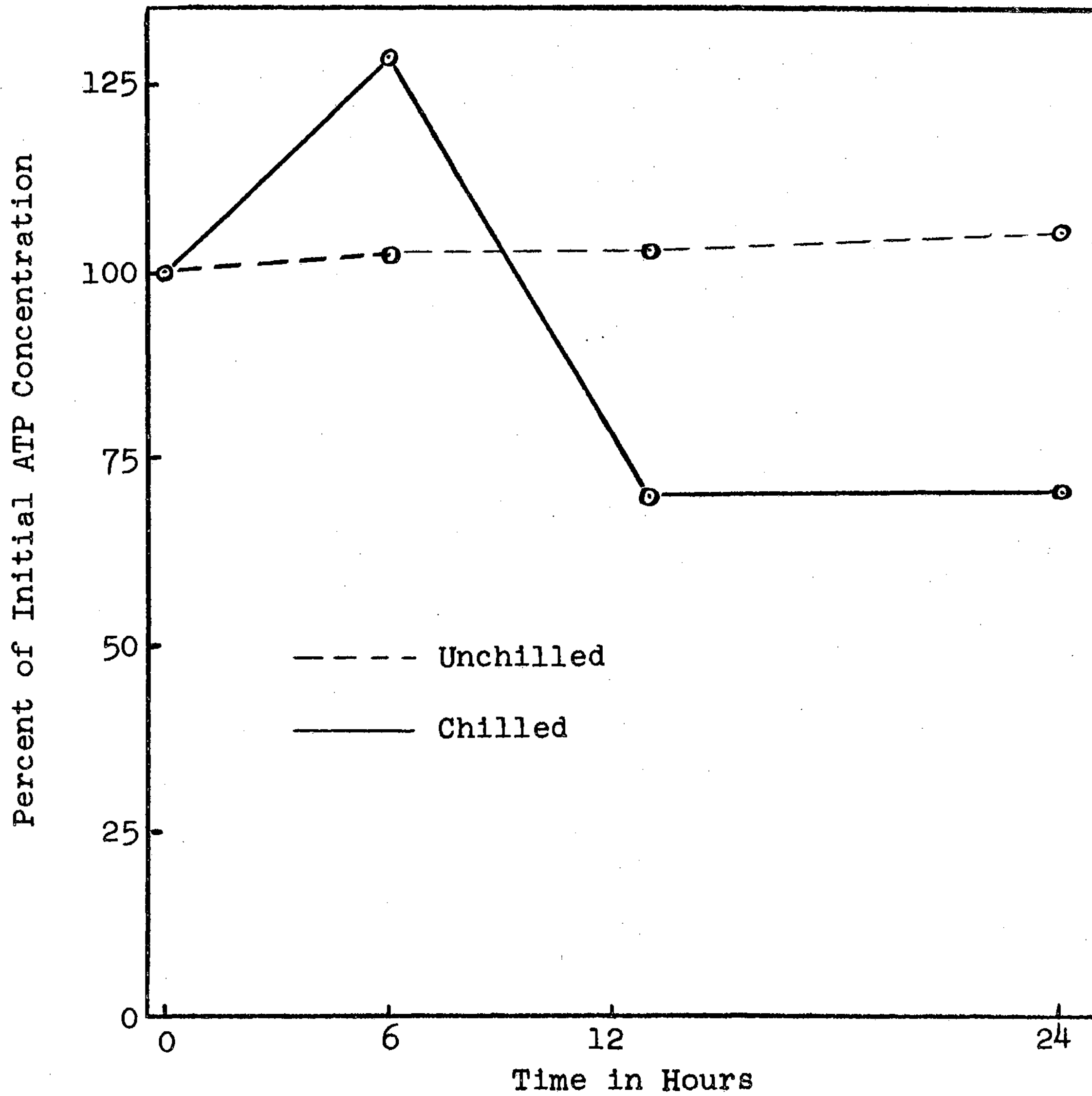

Figure 9. Immediate Effects of Chilling on Root ATP Level. Each Point Represents the Average of 4 Replications. 100% Represents .800 umoles ATP/gm Dry Weight.

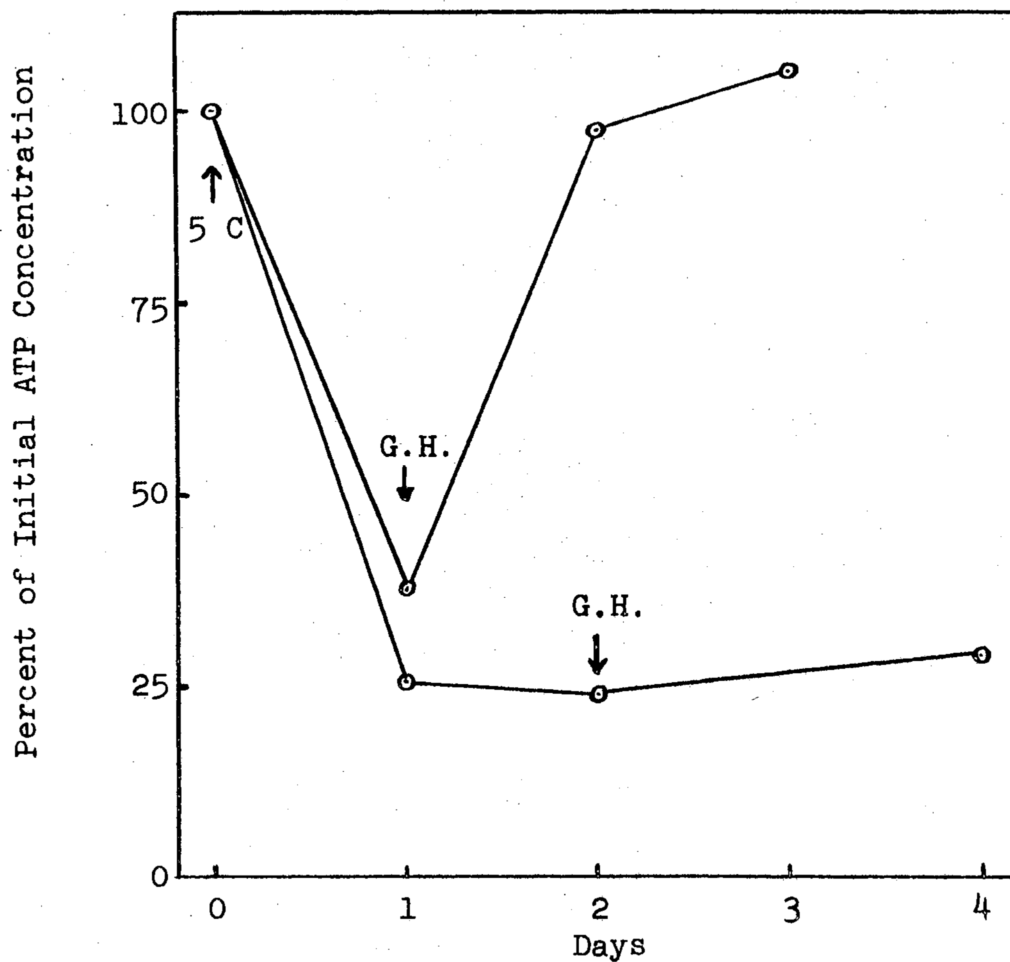

Figure 10. Effect of Chilling on Subsequent ATP Levels in Leaves. Plants Were Chilled One or Two Days at 5 C in the Growth Chamber Then Returned to the Greenhouse (G.H.) for Two Days. 100% Represents 1.4 and .788 umoles ATP/gm Dry Weight for the One and Two Day Treatment Respectively.

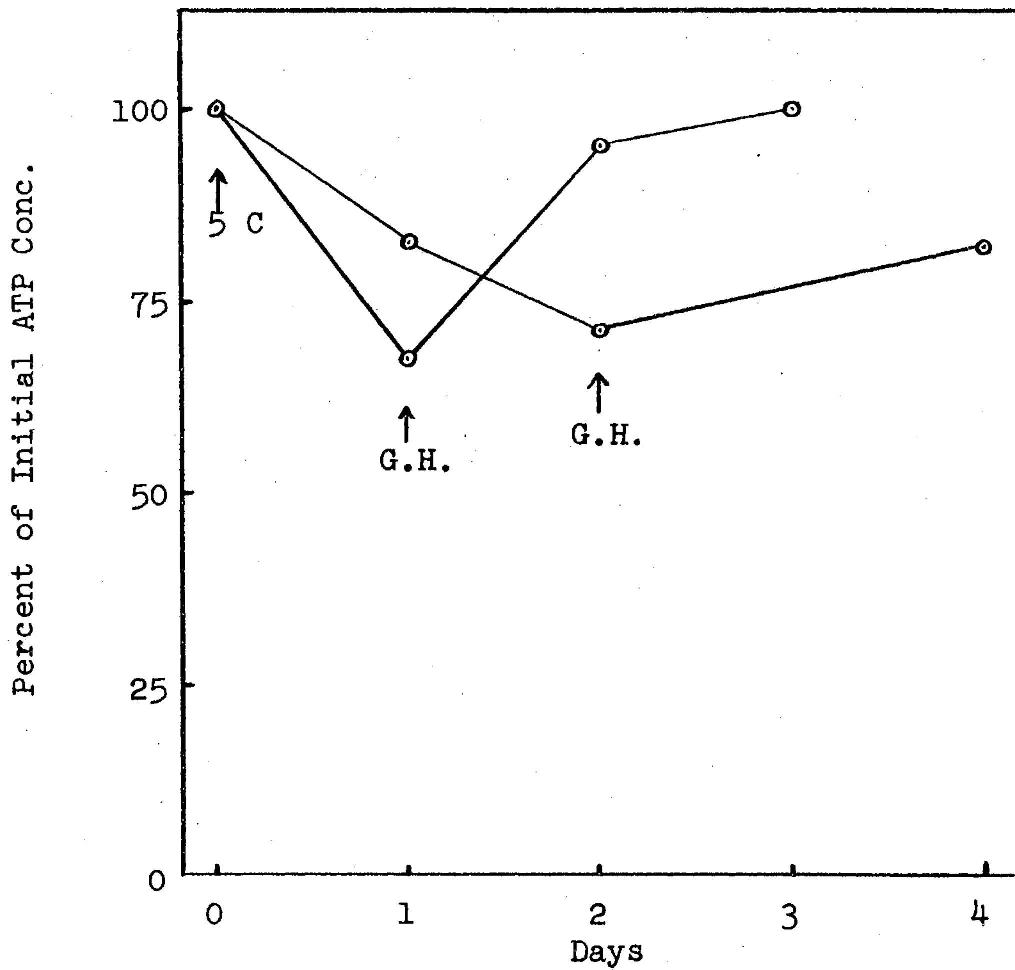

Figure 11. Effect of Chilling on Subsequent ATP Levels in Roots. Plants Were Chilled One or Two Days at 5 C in the Growth Chamber Then Returned to the Greenhouse (G.H.) for Two Days. 100% Represents .925 and .870 umoles ATP/gm Dry Weight for the Two and One Day Treatment Respectively.

the greenhouse for two days. The slight increase observed was probably due to the gorwing points which seemed best able to survive chilling conditions. Apparently a time factor is involved before permanent injury occurs.

Response to Severe Chilling by Hardened Plants

The results shown in Figures 8 through 11 were from plants that were grown under near optimum conditions before chilling conditions were imposed. Cotton seedlings can be hardened against chilling shock by subjecting them to a temperature of 15 C with 14 hour day length for two or more days immediately before the chilling treatment (G. Guinn and R. Muckel, personal communication). When the seedlings were hardened before the chilling treatment was imposed, the ATP concentration did not decrease in the leaves (Figure 12). In fact, an increase was observed. When the hardened plants were returned to the greenhouse after two days of chilling, the ATP level returned to normal. No visible damage could be detected.

The lower curve shown in Figure 12 is for comparative purposes to show the effect of prolonged chilling on unhardened plants. In this particular experiment the ATP concentration of the foliage after 4 days at 5 C was about 6% of the initial concentration. These plants were severely wilted and showed the necrotic areas typical of chilling injury.

As indicated in Figure 10, the ATP level continued to decrease with time of chilling in unhardened root tissue. After 4 days of chilling the concentration was less than 25% of the original value (Figure 13). Results with ion exchange chromatography of nucleotides

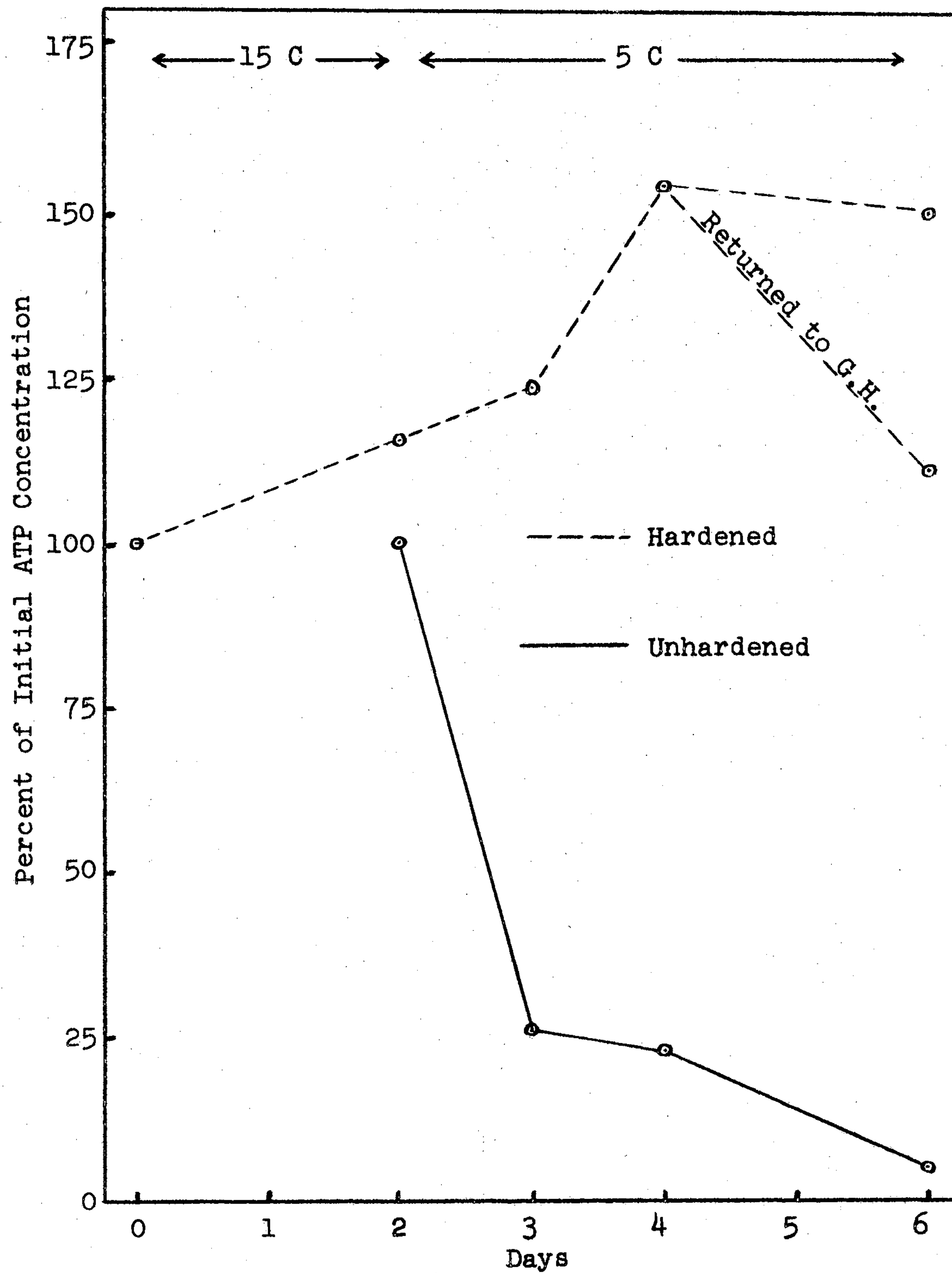

Figure 12. Effect of Severe Chilling on the ATP Content of Hardened and Unhardened Leaves. 100% Represents .95 and 1.4 umoles ATP/gm Dry Weight for Hardened and Unhardened Respectively.

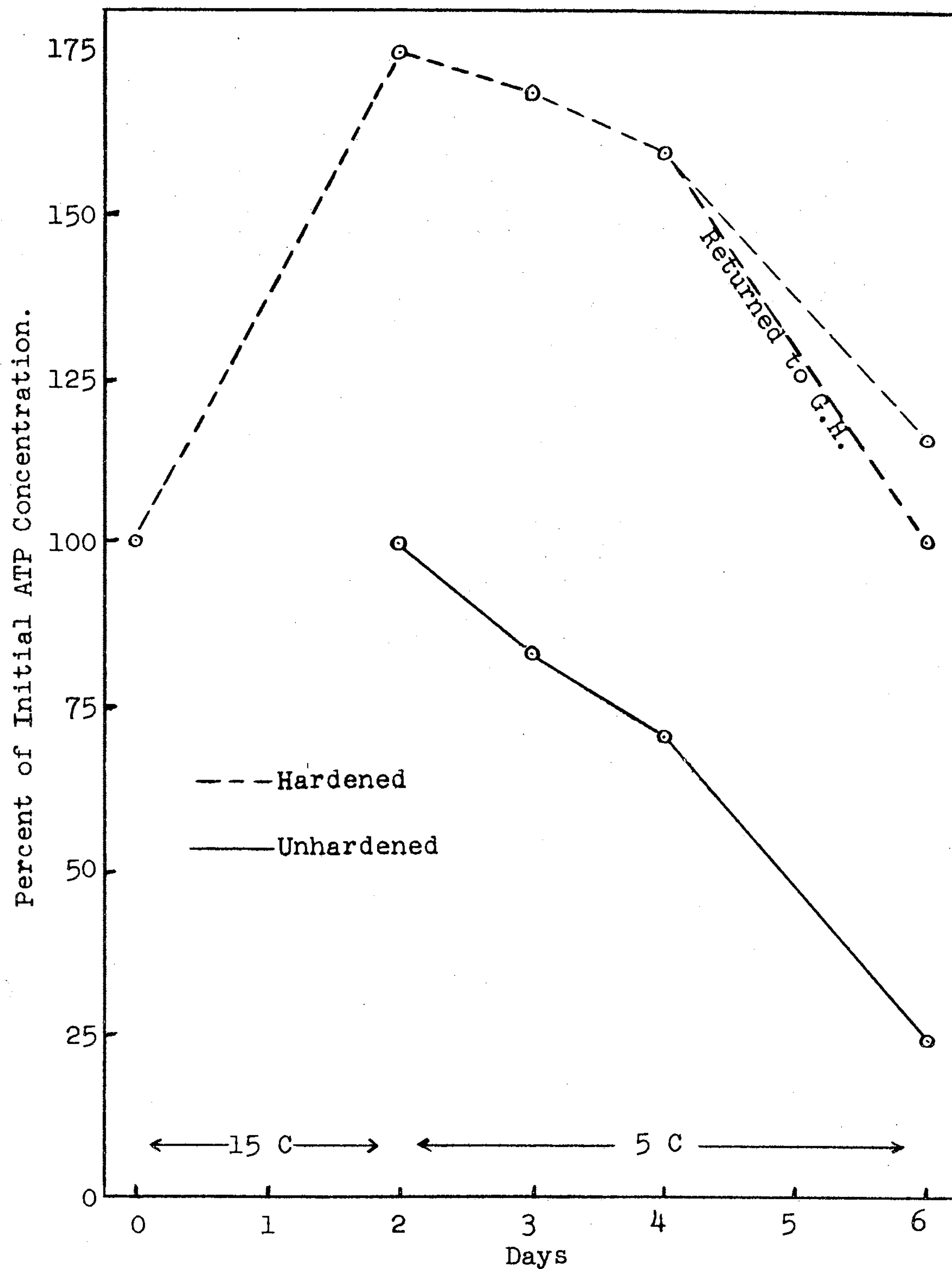

Figure 13. Effect of Severe Chilling on the ATP Content of Roots from Hardened and Unhardened Seedlings. 100% Represents .480 and .925 umoles per Gram Dry Weight for the Hardened and Unhardened Respectively.

indicates that this was a conservative estimate of the ATP decline. However, if the plants were hardened for two days, the concentration of ATP in the roots increased. Upon chilling the hardened plants at 5 C the level of ATP in the roots began to decrease. The actual decrease over the 4 day chilling period was approximately the same as for the unhardened plants. This may indicate that the protective action of hardening in roots is not as great as in the tops. Although longer periods of chilling were not studied, one might speculate that chilling damage would begin to occur after 4 days of chilling even though the plants were hardened. Two days of chilling apparently did not injure hardened roots since the ATP level returned to normal when the plants were returned to greenhouse conditions.

Discussion

Considering the decrease in ATP with chilling, oxidative and photophosphorylation must be more sensitive to low temperature inhibition than other synthetic systems which use ATP. In view of the results with chilled roots, there appears to be a critical temperature below 10 C at which oxidative phosphorylation activity of the mitochondria of unhardened roots ceases. This idea supports the suggestion of Lyon et al. (49) that the lipid membranes of mitochondria from chilling sensitive plants are inflexible at low temperature. However, the possibility that ATPase activity is increased at low temperature cannot be overlooked.

Any postulates, however, must recognize that cotton plants can be hardened to some extent against chilling injury. Unfortunately, the nature of this hardening process is not understood, but evidence with

other plants indicates that sugars are the protective agent (23, 57, 61). Heber and Santarius (24) reported that sugars protected the phosphorylating system of isolated mitochondria and chloroplasts. Sugars do accumulate at the hardening temperature (15 C) in cotton (20) but whether protection is afforded by these sugars remains to be seen.

There are at least three possibilities why ATP decreases with severe chilling: (1) inhibition of oxidative phosphorylation, (2) high ATPase activity, or (3) inhibition of adenosine monophosphate (AMP) synthesis. An investigation of chilling effects on all the nucleotides should determine if synthesis of AMP is inhibited or if dephosphorylation is higher than ATP synthesis. A measurement of activity of mitochondria isolated from chilled tissue should determine if oxidative phosphorylation is permanently damaged by chilling.

Effect of Chilling on Nucleotides

Root Tissue

Root tissue contained less material which interfered with identification of nucleotides than leaves, so the preliminary amyl alcohol extraction used with leaf tissue was omitted. Two-week old cotton seedlings were chilled at 5 C for 48 hours then harvested and dried. A set of non-chilled plants were harvested at the beginning of the chilling treatment. Nucleotides were extracted from 3 grams of dried tissue and an amount of extract equivalent to 2.4 grams of tissue applied to a Dowex 1 column. The elution chromatogram of root nucleotide from non-chilled tissue is given in Figure 14. Figure 15 gives the chromatogram for root nucleotides of chilled tissue. Peaks with the same letter represent the same nucleotide on the two chromatograms.

Identification of Root Nucleotides

Peak A: The materials in this peak were cations and non-charged substances at pH 7.1. Although the non-chilled tissue had more UV absorbing material in the water-wash than the chilled, the spectrum of the water-wash of chilled tissue indicated that it contained more nucleosides and free bases than the non-chilled. No additional work was done with the water-washes.

Peak B: The spectrum of this peak indicated that a mixture was present. When eluted through a Sephadex G-10 column, 3 peaks resulted. The first peak eluted from Sephadex had adenine spectral characteristics and co-chromatographed with standard NAD in Pabst solvent 1 (59). The second and third peaks from Sephadex had spectral characteristics of thymine. Additional identification of these was not attempted.

Peak C: This peak had an adenine-type spectrum. Based on previously published results (5), it was assumed to be adenosine monophosphate (AMP).

Peak D: The spectrum of this peak was unidentifiable until the material was passed through a Sephadex G-10 column. Two major peaks resulted, one of which had the adenine absorption spectrum. Based on location of elution from the Dowex 1 and the spectrum it was assumed to be NADP.

Peak E: This substance was present in too low a concentration for identification. After Sephadex purification a spectrum was obtained which resembled the acid spectrum of cytosine. The sample, however, was in distilled water at unknown pH. The maximum was at 281 nm and minimum at 254 nm. The minimum wave-length was probably not valid because of interfering material. Location of elution from the column and

spectrum indicate that the peak is cytidine diphosphate (CDP). Fluorescent material prevented confirmation by thin layer chromatography.

Peak F: The spectrum of this peak after Sephadex purification indicated a uridine compound. When chromatographed with solvent I two spots were obtained, one of which corresponded to standard uridine monophosphate (UMP). The other spot had an Rf value which corresponded to uridine and probably resulted from partial degradation of the UMP.

Peak G: After Sephadex fractionation an adenine type curve was obtained for one compound but this may have been due to contamination from the following peak.

Peak H: The spectral type for this peak was adenine. Co-chromatography with standards in solvent I indicated that it was adenosine diphosphate (ADP).

Peak I: After Sephadex purification a uracil type spectrum was obtained. Chromatography on thin layer plates indicated that it was not uridine diphosphate (UDP) or UDP hexose (UDPH). Elution from Sephadex indicated that it was a larger molecule than UDPH.

Peak J: This peak possessed the uracil spectrum and the Sephadex purified material corresponded to UDPH in solvents I and III.

Peak K: The material present in this peak was in low concentration. Sephadex purification was only partially successful, however, a spectrum was obtained which suggested cytosine. Published results (6) indicate that cytidine triphosphate (CTP) should be eluted at this point. Due to low concentration this could not be confirmed by chromatography with standards.

Peak L: The spectral type of this compound was adenine. The specific derivative was identified as adenosine triphosphate (ATP) with

firefly luciferin-luciferase.

Peaks M, N and O: These peaks were apparently non-nucleotide substances.

Peak P: After Sephadex purification a uracil spectrum was obtained. Site of elution from Dowex 1 and co-chromatography with standard uridine triphosphate (UTP) indicated that this was UTP.

A summary of the probable identification of the peaks is given in Table II.

TABLE II

IDENTIFICATION OF PEAKS FROM ANION EXCHANGE SEPARATION OF ROOT NUCLEOTIDES

Peak	Identity	Peak	Identity
A	Water-wash	I	Uridine derivative
B	NAD	J	UDPH
C	AMP	K	CTP?
D	NADP	L	ATP
E	CDP?	M	Non-nucleotide
F	UMP	N	Non-nucleotide
G	Unidentified	O	Non-nucleotide
H	ADP	P	UTP

Effect of Chilling on Root Nucleotides

A comparison of Figures 14 and 15 immediately shows that chilling reduced the nucleotide content at all levels of phosphorylation in the roots. Anion exchange separation of the nucleotides confirmed the results obtained with the luciferase assay for ATP. The triphosphates were especially decreased. The decrease in ATP is evident from the Dowex 1 chromatograms, but due to interfering materials the extent of decrease in UTP is not as evident. Figure 16 gives the elution from Sephadex G-10 of the UTP peaks from chilled and non-chilled treatments. Here the extent of UTP decrease due to chilling is more evident. Other uridine derivatives were decreased by chilling temperature, especially UDPH, but UMP was not changed appreciably. The ADP peak also decreased with chilling, but the AMP peak showed a slight increase. The increase in AMP could result from decline in phosphorylation or increase in ATPase activity but the amount of AMP present could hardly account for the almost total disappearance of ATP with chilling. The spectra of the water-washes indicated that the chilled root tissue possessed more material which had a maximum around 260 nm than the unchilled tissue. This may indicate that the nucleotides were hydrolyzed to the nucleosides. Unfortunately, the water-washes were not investigated further. Although the present data indicates that decreases in phosphorylation or increase in ATPase activity occurred, inhibition of AMP synthesis cannot be discounted without additional information.

Leaf Tissue

Plants were grown in the growth chamber for two weeks then harvested after the following treatments: (A) control, no chilling treatment,

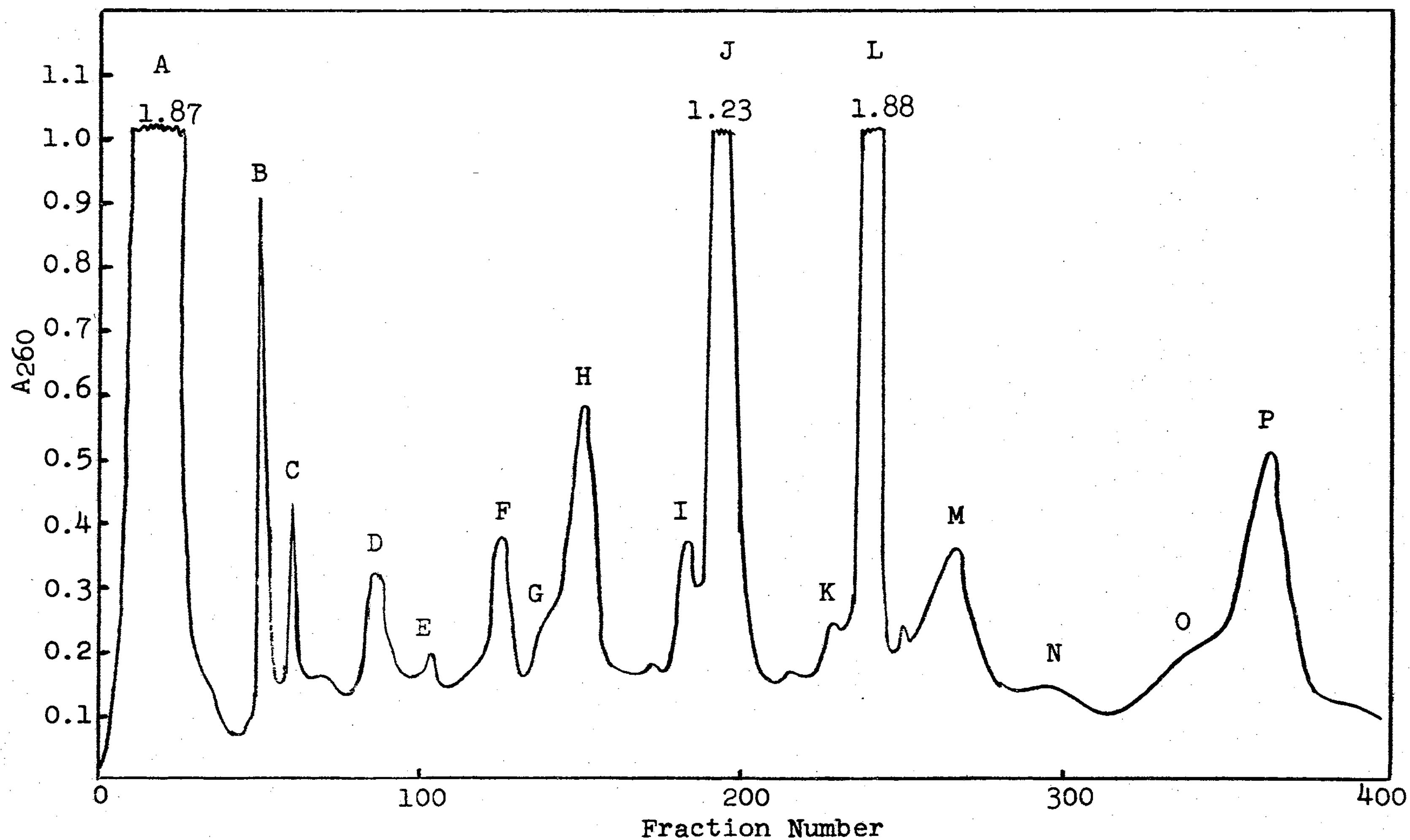

Figure 14. Ion-Exchange Elution-Chromatogram from Unchilled Cotton Roots. The Values (A) Represent the Nucleotide Content of 2.4 gm of Dried Material.

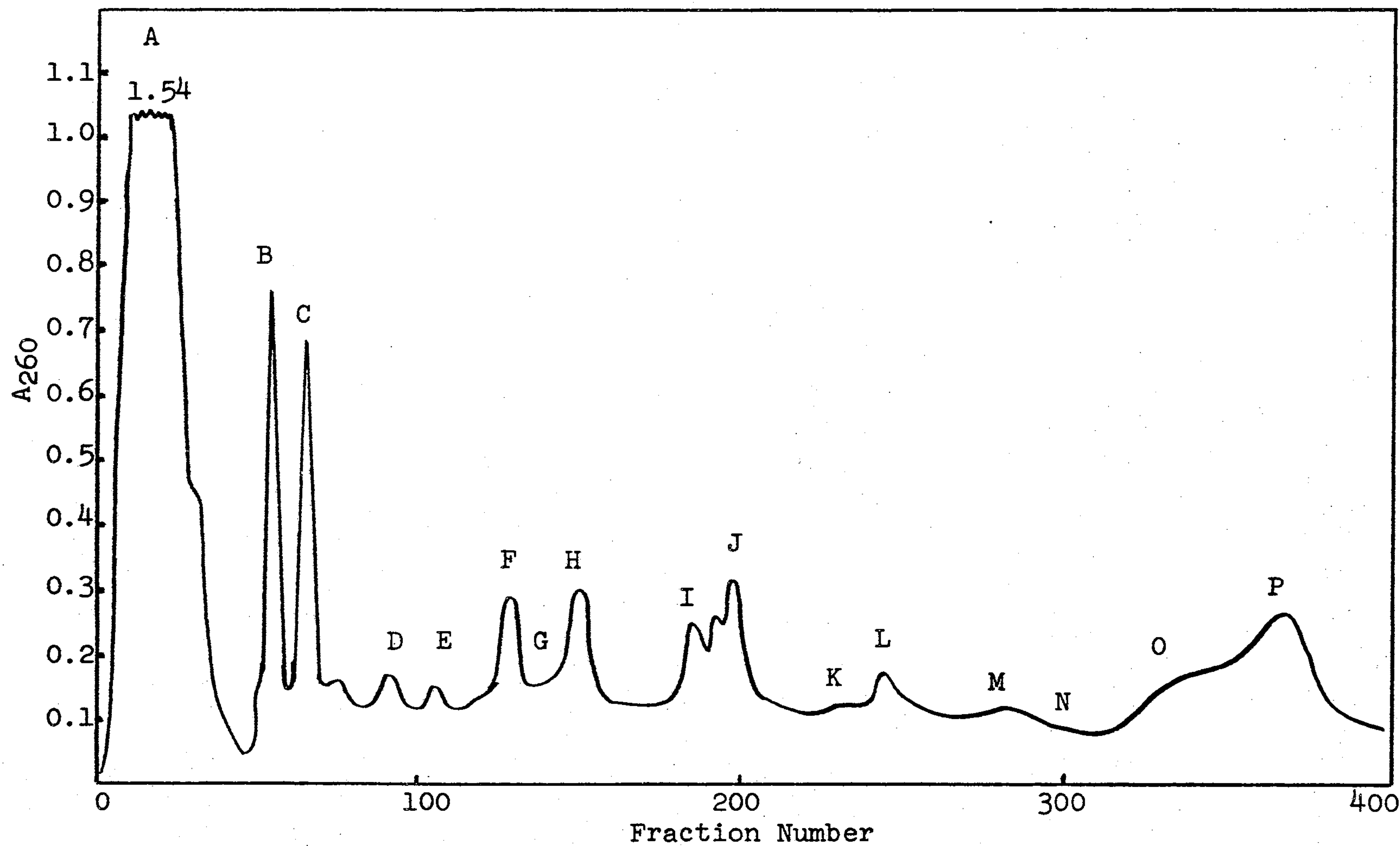

Figure 15. Ion-Exchange Elution-Chromatogram from Chilled Cotton Roots. The Values (A) Represent the Nucleotide Content of 2.4 gm of Dried Material.

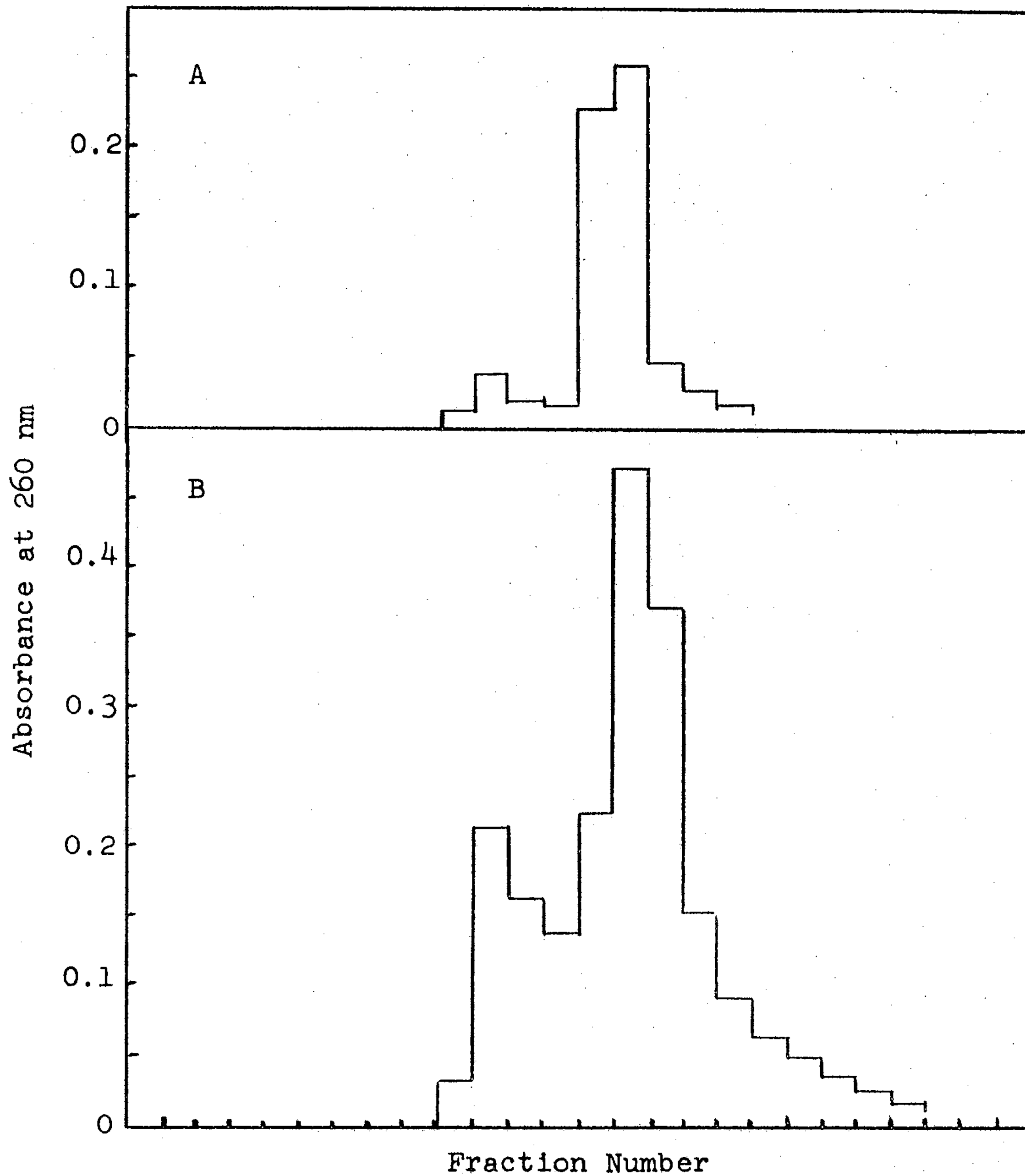

Figure 16. Elution Pattern of Peak P from Dowex-1 through Sephadex G-10. A is from Chilled Tissue; B is from Unchilled Tissue. The First Peak Eluted is UTP. The Second Peak is Non-nucleotide.

(B) hardened two days at 15 C and (C) unhardened, chilled at 5 C for two days. After harvest and lyophilization nucleotides were extracted from 6 grams of tissue as described under materials and methods. A volume of extract equivalent to 5 grams of tissue was applied to the Dowex 1 column. Elution chromatograms for the three treatments are given in Figures 17, 18, and 19 for the control, chilled and hardened leaves, respectively. Peaks common to all three chromatograms are assigned a letter, while peaks peculiar to one or two treatments is given a number.

Identification of Leaf Nucleotides

Water-wash: This peak contained material not absorbed onto the Dowex 1 column at pH 7.1, and includes free bases and some of the nucleosides. Absorption spectra and total optical density at 260 nm indicated that the water-wash from the chilled treatment contained more of these bases than the water-wash of the warm control. The hardened treatment contained slightly less than the chilled wash.

Peak 1: This peak did not occur in the warm control but did in the chilled and hardened series. The spectrum was characteristic of an adenine compound and chromatography in solvents I and II with standards indicated it was adenosine.

Peaks 2 and 3: These two occurred as one peak in the hardened series, and the spectral characteristics of 2 and 3 in the chilled series were very similar. In neutral solution the spectra strongly resembled that of nicotinic acid but in acid solution the inflections characteristic of niacin were not present. Upon resolution on Sephadex two major peaks resulted, one of which possessed the absorption spectrum

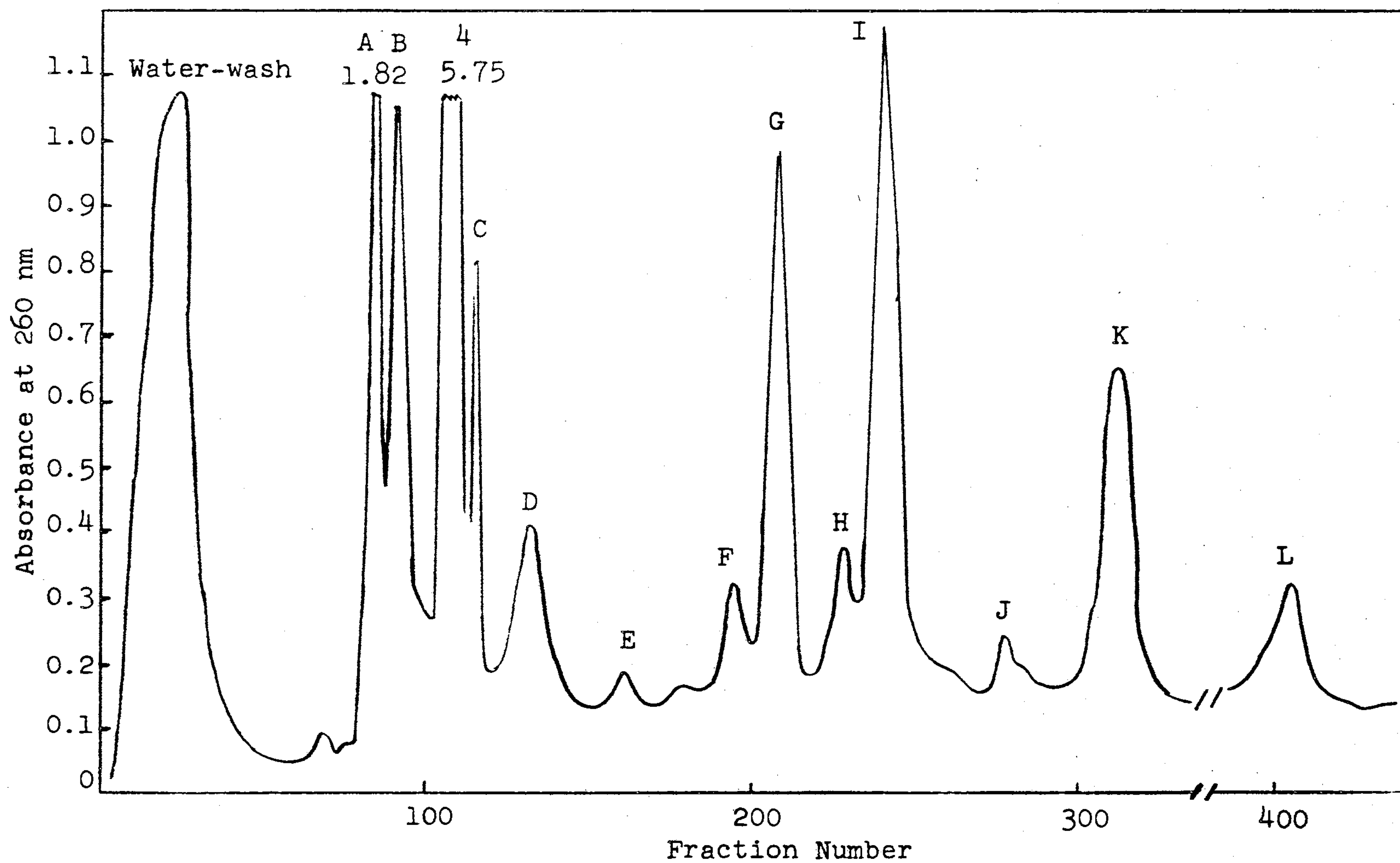

Figure 17. Ion-Exchange Elution-Chromatogram from Warm Control Cotton Leaves. The Values (A) Represent the Nucleotide Content of 5 gm of Dried Material.

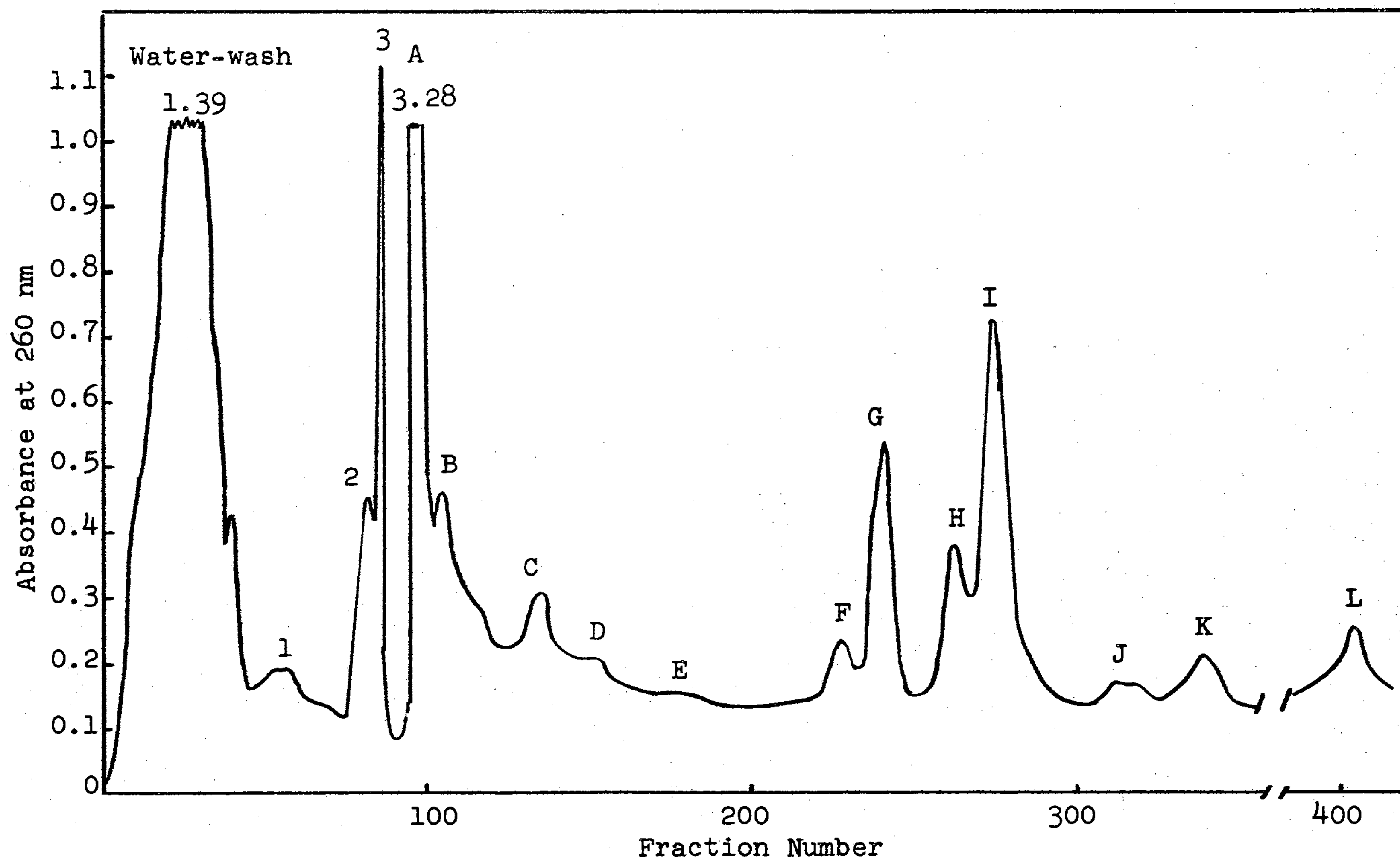

Figure 18. Ion-Exchange Elution-Chromatogram from Chilled Cotton Leaves. The Values (A) Represent the Nucleotide Content of 5 gm of Dried Tissue.

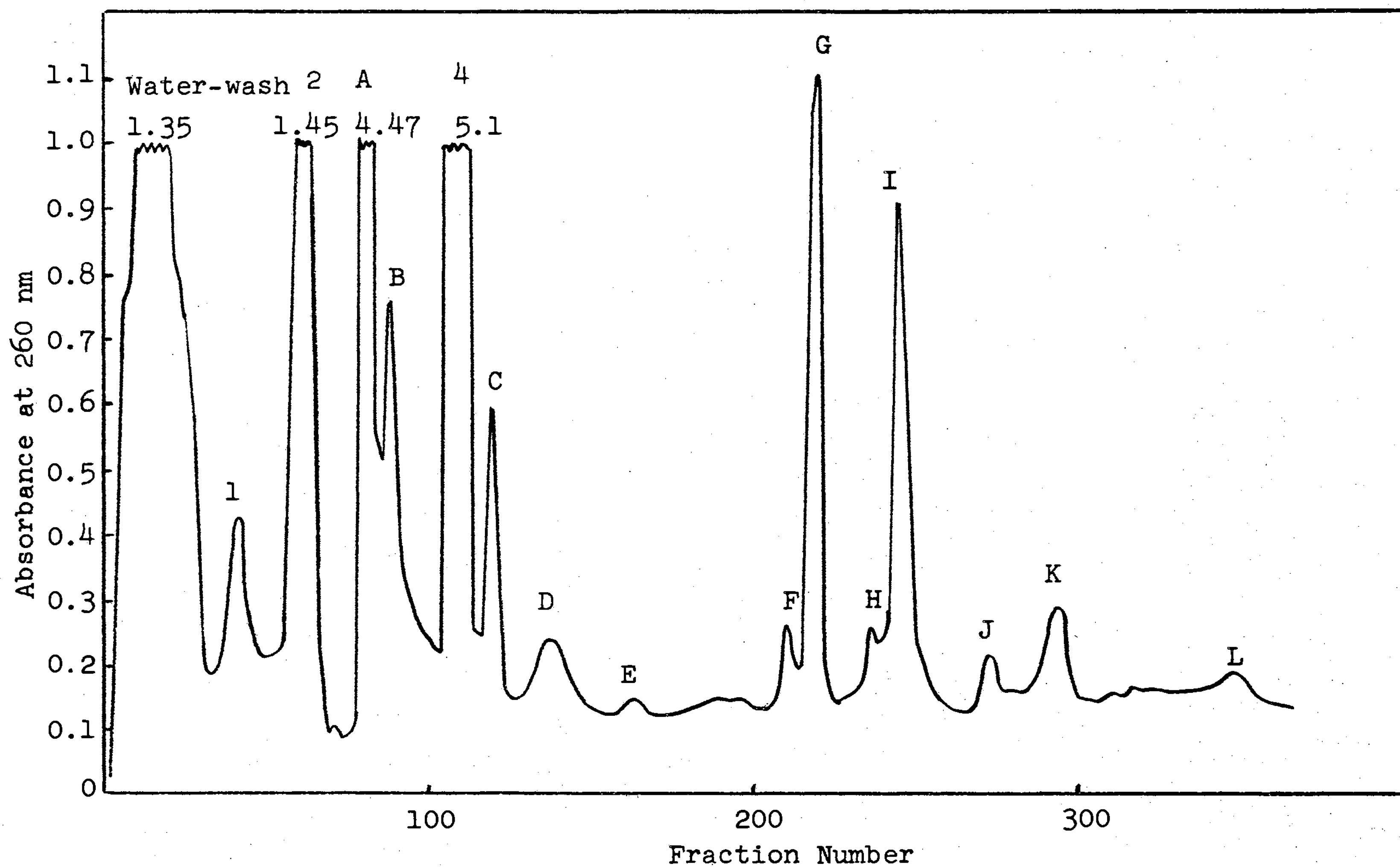

Figure 19. Ion-Exchange Elution-Chromatogram from Hardened Cotton Leaves. The Values (A) Represent the Nucleotide Content of 5 gm of Dried Tissue.

typical of tryptophan. The peaks also gave a ninhydrin positive reaction.

Peak A: Three peaks resulted from Sephadex fractionation of A. The major peak had an adenine spectrum and co-chromatographed with standard NAD in solvent I. A second spot was also present in the thin layer separation. This compound was not definitely identified, but was probably CMP. The cyanide addition product confirmed the presence of NAD in peak A.

Peak B: Adenine was the spectral type. Chromatography with standards in solvents I and II indicated the compound to be AMP.

Peak 4: The substance in this peak apparently was not a nucleotide. However, considering that it was present in high concentration in the control and hardened series and totally absent in the extract of chilled leaves, it could have some bearing on chilling injury. The absorption spectrum of peak 4 at neutral pH is given in Figure 20. Acid pH did not change the spectrum but alkaline conditions destroyed the 243 nm maximum and shifted the peak maximum to 300 nm.

Peak C: Non-nucleotide material comprised this peak. The texture of the dried material and the absorption spectrum suggested an inorganic salt.

Peak D: This peak was confirmed to be NADP by its adenine absorption spectrum and additional product with cyanide.

Peak E: Published reports (5, 29) indicate that CDP and inosine monophosphate (IMP) are eluted at this point. Due to low concentration of nucleotide and high concentration of fluorescent material, confirmation of identity could not be made.

Peak F: Complete identification of this peak was not made. The

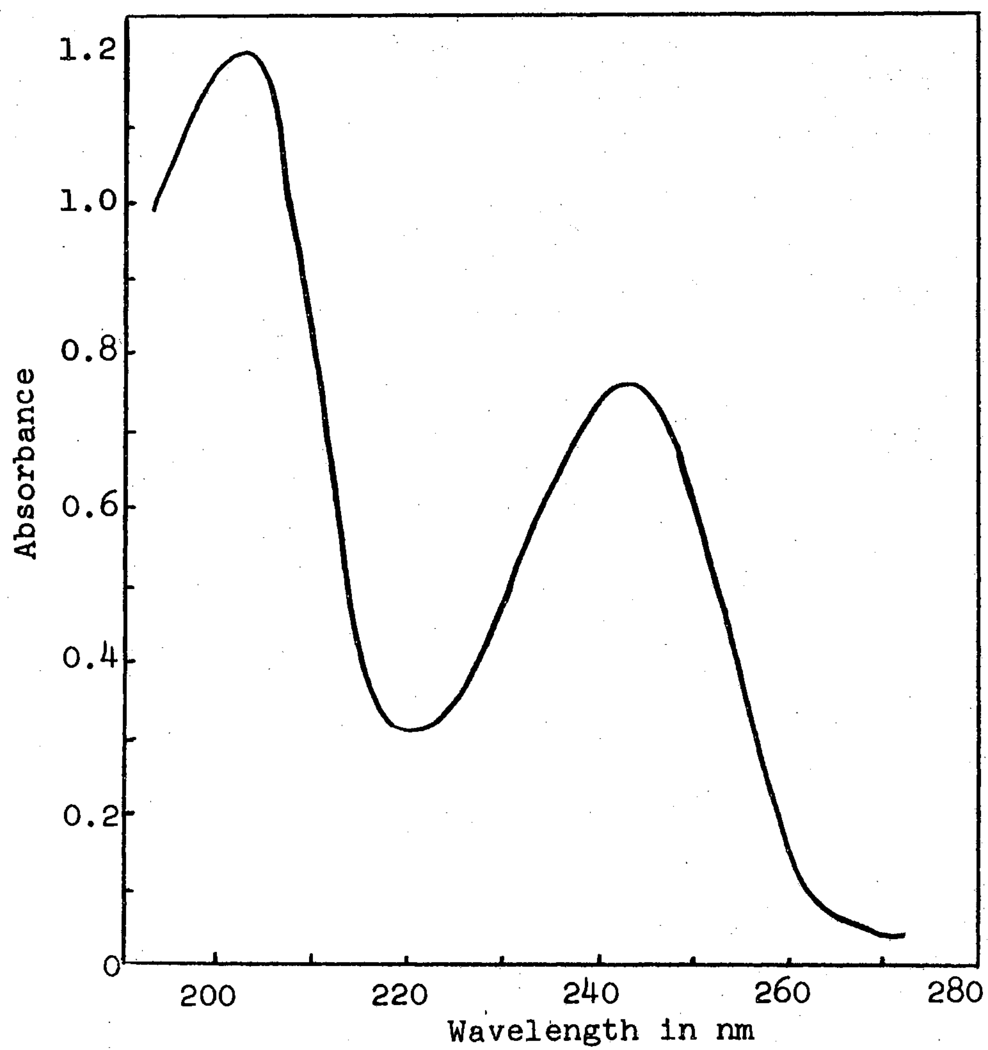

Figure 20. Absorption Spectrum of Material from Peak 4 at Neutral pH.

base component appeared to be either adenine or uracil but the unknown did not correspond to standards of ADP or UMP. Brown (5) indicated that the acid degradation product of NADH$^+$, adenosine diphosphate ribose (ADPR) elutes at this point.

Peak G: Spectrum and chromatography with standards in solvents I and III indicated that this peak was ADP.

Peak H: Peak I of the roots given previously corresponds to this peak from leaves. Again, it is apparently a derivative of uridine.

Peak I: The uracil spectrum and co-chromatography with UDPglucose indicated that this was UDP hexose.

Peak J: Fluorescent material prevented identification of this peak by UV spectrum or chromatography on thin layer.

Peak K: Spectrum, co-chromatography with standards and firefly luciferase identified this peak as ATP.

Peak L: Although a poor spectrum was obtained with this peak due to fluorescence, chromatography in solvent III indicated two spots which corresponded to UTP and guanosine triphosphate (GTP).

A summary of the probable constituents of each peak of the elution chromatogram of leaf nucleotides is given in Table III.

Effect of Chilling on Leaf Nucleotides

The effect of chilling on leaf nucleotides was similar to the results obtained with root nucleotides. As indicated by the ATP assays given previously, the triphosphates were most noticeably decreased by chilling (Figures 17 and 18). With hardened tissue (Figure 19), however, the results were contrary to those obtained with the luciferase assay of ATP (cf. Figure 12). A comparison of the size of the ATP peaks

eluted from the Dowex 1 columns indicates that ATP decreased with hardening rather than increased as previous experiments with hardened tissue indicated. Luciferase assay of hot water extracts of the tissue used for nucleotide studies confirmed that ATP was less in the hardened tissue than in the control tissue. However, an additional observation with the luciferase assay indicated that the decrease in the ATP concentration with hardening was peculiar to that particular harvest. The concentration of ATP in the hardened tissue which was chilled at 5 C for two days was almost the same as in the unchilled control. Additional work needs to be done on this aspect of hardening before a conclusion can be reached.

TABLE III

IDENTIFICATION OF PEAKS FROM ANION EXCHANGE SEPARATION OF LEAF NUCLEOTIDES

Peak	Identity	Peak	Identity
1	Adenosine	#	Unidentified
2	Trypotphan	F	Adenosine derivative?
3	Tryptophan	G	ADP
A	NAD + CMP	H	UDPX
B	AMP	I	UDPH
4	Non-nucleotide	J	Unidentified
C	Non-nucleotide	K	ATP
D	NADP	L	UTP + GTP

Other nucleotides, including AMP, NADP, ADP, UDPH, UTP, and GTP, decreased when unhardened plants were chilled. The increase in peak A was probably not due to the NAD, but to the unidentified component in that peak. The general decrease in the nucleotides with chilling suggests that hydrolytic processes exceeded phosphorylation of the nucleotides. The occurrence of peak 1 in the chilled series supports this suggestion. Peak 1, which was identified as adenosine, and the water-wash indicated that nucleosides and free bases were present in higher concentration in the chilled series than in the control series. These probably resulted from a combination of hydrolysis and decrease in phosphorylation.

Two other major changes were evident with chilling, although these apparently involved non-nucleotide material. The substance in peak 4 occurred in high concentration in both control and hardened series, but was entirely absent in the chilled series. The identity of this compound should be established and its relation to chilling injury investigated. The other major change in chilled and hardened tissue was noted by the presence of peaks 2 and 3. These peaks were a mixture of substances, one of which appeared to be tryptophan. The increase of tryptophan with chilling may indicate that proteins are hydrolyzed or that synthesis of compounds for which trypotphan is a precusor (e.g., indole acetic acid) are inhibited. Uridine may have been present in peak 2 of the hardened series, but its identity was not definitely established.

Effect of Chilling on Mitochondrial Activity

Chilling decreases the ATP level in cotton seedlings, therefore

the mitochondria may be damaged by low temperature since mitochondria and chloroplasts are intimately involved in the synthesis of ATP. A number of experiments were conducted to determine the effect of chilling on the oxidative activity of cotton mitochondria.

Effect of Chilling on Hypocotyl Mitochondria

A comparison of the oxidative activities of mitochondria isolated from unchilled etiolated hypocotyls and from hypocotyls which were chilled at 3-4 C for two days indicated that activity was decreased by chilling. With malate as substrate the unchilled hypocotyl mitochondria had a Q_{O_2} (N) of 432 compared to 145 for mitochondria from chilled hypocotyls. No phosphorylation was detected in the chilled mitochondria while those from the unchilled plants had a P/O ratio of 0.51. When alpha-keto-glutarate was used as substrate the Q_{O_2} (N) was 330 for the unchilled hypocotyl mitochondria compared to 237 for chilled mitochondria. The P/O ratio in both chilled and non-chilled treatments was near unity. These results suggest that certain of the Kreb cycle enzymes may be more sensitive to chilling than others. However, the P/O ratios were low in all determinations and, therefore, may not be reliable.

Effect of Chilling on Root Mitochondria

The most striking results were obtained when the roots of three-week old greenhouse-grown plants were chilled at 5 C for 4 days. The Q_{O_2} (N) was 506 for mitochondria isolated from warm roots compared to 55 for chilled mitochondria with malate as substrate. The total number of mitochondria isolated, as measured by total protein, was

essentially the same for chilled and non-chilled tissue. There was no measurable phosphorylation in either case. The decrease in oxidative activity indicates that, directly or indirectly, chilling inactivates the malate oxidation by root mitochondria.

Previous history of cotton plants before chilling temperatures were imposed affected the degree of chilling injury. Plants grown in the greenhouse during winter months were very susceptible to low temperature and were frequently damaged at 15 C. Plants grown in the growth chamber were hardier and not as susceptible to chilling injury as those grown in the greenhouse. When cotton plants were grown in the growth chamber for two weeks then chilled at 5 C for 3 days, the decrease in malate oxidation by isolated mitochondria was again decreased, but not to the same extent as with greenhouse plants. The activity of mitochondria from chilled tissue was 64% of the activity of mitochondria from unchilled plants.

Hardening of the cotton seedlings at 15 C for 3 days apparently does not injure the mitochondria. There was no difference in the oxidative activity of mitochondria from hardened and unhardened roots when malate was used for substrate, i.e., 365 vs. 361. The results of a separate experiment in which hardened and unhardened plants were compared to control plants are given in Table IV. In this case succinate was used as substrate. Hardening did not decrease succinate oxidative activity but the activity of mitochondria from plants chilled 2 days at 5 C, whether hardened or not, was reduced about 20%. With 4 days of chilling, activity of mitochondria from unhardened plants decreased to about 50% of the control. The mitochondria from hardened plants had activity only slightly less than that after 2 days of chilling. The

decrease in activity of the mitochondria from hardened tissue after 2 days of chilling may indicate incomplete hardening. Another possibility is that chilling injury occurs in two phases: one which cannot be prevented by hardening and one which can be prevented by hardening.

TABLE IV

RATE OF O_2 CONSUMPTION BY MITOCHONDRIA FROM HARDENED AND UNHARDENED COTTON ROOTS AS AFFECTED BY CHILLING, USING SUCCINATE AS SUBSTRATE

Treatment	Q_{O_2} (N)	% of Control
Control	894	100
Control	920	
Hardened 2 Days	985	107
Control	801	
Chilled 5 C 2 Days		
Hardened	623	78
Unhardened	491	53

Considering the low concentration of ATP which results when cotton plants are chilled, it seemed advisable to determine the relative activity of mitochondria at different temperatures compared to activity at 30 C. The results of this experiment are given in Figure 21. Succinate was used as substrate in all cases. The decrease in activity was almost linear with decrease in temperature, with the rate at 5 C approximately 12% of the rate at 30 C. In agreement with respiration determinations

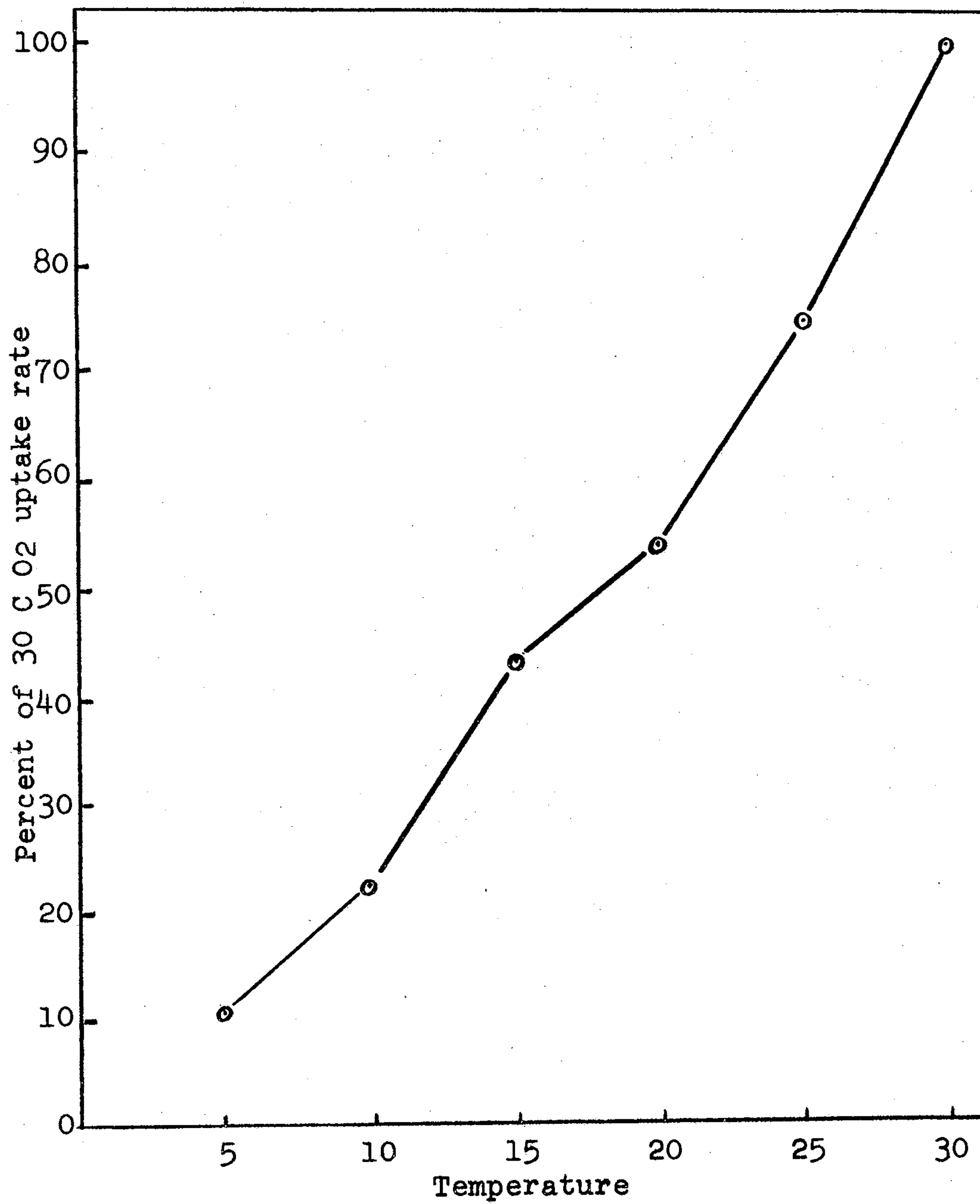

Figure 21. Relative Rate of O_2 Uptake at Different Temperatures by Mitochondria from 6-Week Old Cotton Roots. Succinate was Substate.

the Q_{10} was greatest from 5 to 15 C. In a similar experiment with malate as substrate the oxygen consumption rate at 6 C was approximately 7.7% of the rate at 30 C. Malate oxidation may be more sensitive to chilling than succinate oxidation.

Discussion - A Theory of Chilling Injury

The results obtained with respiration determinations confirmed the suggestion that roots were more sensitive to chilling than tops (21). The sensitivity of roots is also indicated by Christiansen's report (7) that the cortex tissue of day-old seedling radicles was killed by six days of chilling. Sloughing of the cortex was noted when the seedlings were returned to warm temperature. In three-week old tissue the rate of respiration in lateral roots decreased with time at 10 C. Apparently chilling injury was associated with time of exposure to low temperature. Results with the ATP determinations also indicated that a time factor was involved before permanent injury was sustained. When plants were chilled for one day only, they were able to regenerate the ATP lost during chilling, but after two days of severe chilling the plants were not able to regenerate the ATP to any appreciable extent. Isolation of mitochondria from prechilled root tissue indicated that the oxidative activity of these particles was not permanently damaged by short periods of chilling. The longer the plants were at low temperature, however, the less active were the mitochondria. These results are similar to those reported for other phenomena such as protoplasmic streaming (44), respiration in cucumbers (12), and mitochondrial activity in sweet potatoes (47).

The reports of Heber and Santarius (24), Richardson and Tappel

(69), and, in particular, Lyons et al. (49) may provide a clue to the nature of chilling injury and why a time period is usually involved before permanent injury results. Heber and Santarius (24) reported that freezing of mitochondria uncoupled phosphorylation from oxidation by mitochondria. If a similar phenomenon occurred at low non-freezing temperature in chilling-sensitive plants, ATP would be decreased with chilling as reported here. The uncoupling, however, would have to be reversible to account for the recovery of ATP when the plants are returned to warm temperature after short periods of chilling. The suggestion of Lyons et al. (49) may have more bearing than the results of Heber and Santarius on chilling injury. They observed that mitochondria from a number of chilling-sensitive plants were relatively inflexible at low temperature while mitochondria from chilling-resistant plants were still quite flexible at low temperature. They suggested that the metabolic injury caused in chilling-sensitive tissues may be due to inability of mitochondria to function at low temperature because of this inflexibility.

The phenomenon of membrane inflexibility could explain many of the results obtained with chilling in cotton. The decrease in ATP with chilling is most obvious, since synthesis of ATP is associated with swelling and contraction of the mitochondria (41). Inflexibility does not necessarily mean inactivation, so when plants are returned to warm conditions after short periods of chilling, the mitochondria may again be functional and resupply the ATP lost during chilling. Should chilling be continued, hydrolytic processes not associated with membranes would probably continue slowly (81) and consume the phosphorylated compounds present. Without a resynthesis of ATP a general

decrease in phosphorylated compounds would result. As stated earlier in this report, a general decrease in nucleotides was observed when cotton was chilled for two days. The gradual inactivation of the mitochondria which was observed could result from normal turnover or an inactivation of enzymes due to conformational changes induced by low temperature. Cases of conformational changes are well documented (63, 72) in animal tissue. Beyond a certain point of depletion of available energy, the tissue would no longer be able to maintain the metabolic integrity of the cytoplasm necessary for survival. Any structural damage in the lipid membranes or proteins associated with these membranes could not be repaired because of the lack of ATP which is necessary for protein and lipid synthesis. One would expect differential permeability to be lost as a result. Some evidence for this was provided by Lieberman et al. (47) who noted that chilling of sweet potatoes caused leakage of ions, particularly potassium.

In essence, this theory suggests that one of the main aspects of chilling injury is energy starvation; chilling temperature inhibits ATP synthesis while utilization proceeds, if but slowly. With depletion of the usuable cellular energy, general disorganization of cellular structure and metabolic processes occurs.

CHAPTER V

SUMMARY AND CONCLUSIONS

Determinations were made of the respiration rates in roots, leaves and cotyledons of cotton seedlings at temperatures from 5 to 25 C. The greatest inhibition of rate in all tissue was between 5 and 15 C. However, root tissue was more sensitive to low temperature inhibition of respiration than the tops. Respiration in lateral roots decreased with time of chilling but did not stop completely, even after 24 hours. Roots prechilled at 10 C for 36 hours had a higher rate of respiration at 10 and 25 C than unchilled control plants.

Photosynthetic rates of cotyledons in a 1% CO_2 atmosphere at temperatures from 5 to 25 C were determined. In contrast to respiration, inhibition of photosynthetic rate due to chilling was greater between 15 and 25 C than between 5 and 15 C.

Several experiments were conducted to determine the effect of chilling on the ATP level of cotton seedlings. When roots of cotton plants were chilled, ATP increased in the root down to a temperature of 10 C, but at 5 C the ATP content was decreased sharply. The ATP content of the leaves was progressively lower with lower root temperature. When the entire seedling was chilled at 5 C, decrease in leaf ATP was detected within 6 hours. Root ATP also decreased but not as rapidly as in the leaves because of the slow cooling of the nutrient solution. ATP concentration continued to decline with time at chilling temperature

in both leaves and roots. When the seedlings were returned to warm conditions after 1 day of chilling the original ATP level was regained. After two days of chilling the seedlings did not resynthesize the ATP lost during chilling. Hardening of cotton seedlings at 15 C for two days prevented the chilling-induced loss of ATP in the leaves for at least 4 days. Hardening caused an increase in ATP in both leaves and roots but the level of ATP in the roots began to decline when chilling conditions were imposed.

An investigation of cotton nucleotides by ion exchange column chromatography indicated that most of the common nucleotides were present. ATP and UDPH were present in highest concentration in unchilled tissue. Chilling of seedlings at 5 C for two days greatly reduced the amounts of triphosphates in both leaves and roots and reduced to varying degrees the amounts of other nucleotides. Spectra of water-washes and the occurrence of adenine indicated that nucleotides were hydrolyzed to nucleosides during chilling.

Isolation of mitochondria from chilled plants indicated that brief periods of chilling did not injure the mitochondria to any extent. With extended periods of time at chilling temperature, however, inactivation became progressively greater. Oxidation rates of different substrates by isolated mitochondria indicated that malate oxidation may be more chilling sensitive than succinate or alpha-keto-glutarate oxidation.

In view of the results obtained it is concluded that low nonfreezing temperatures decrease the level of phosphorylated material in cotton plants. This decrease is probably due to inability to snythesize ATP. It is suggested that continued low level of high-energy

phosphorylated compounds results in disorganization of metabolic processes.

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