CHILLING STRESS EFFECTS ON THE GROWTH, MITOCHONDRIAL ACTIVITY AND PROTEIN SYNTHESIS IN ETIOLATED MUNGBEAN SEEDLINGS

YIH-MING CHEN[1], HSIN-FEI LIU[1] and CHU-YUNG LIN[1]

Abstract: Mungbean (*Vigna radiata* L.), a chilling sensitive plant species, was used in this study. Seeds were germinated at 28°C in the dark for 32 h before cold treatment. The seedlings were chilled at 4°C for various durations. Growth, respiration rate, protein synthetic activity, cellular leakages and succinate dehydrogenase activity in isolated mitochondria were then determined.

Chilling at 4°C suppressed seedling growth, increased solute leakages, suppressed succinate dehydrogenase activity in isolated mitochondria, inhibited general protein synthesis and induced the synthesis of a set of proteins termed chilling-induced proteins. The molecular weights of these chilling-induced proteins were 110, 88, 78, 69, 58, 48, 38, 27, 22, 19, 14, 12.5 and 10.5 Kd, respectively and the proteins with molecular weights of 88, 69, 58, 48, 38, 14, 12.5 and 10.5 Kd were the major chilling-induced proteins of mungbean seedlings. These chilling-induced proteins were different from the heat shock proteins of mungbean seedlings.

INTRODUCTION

Temperature is one of the major environmental stress governing the growth, development and distribution of both wild and cultivated plants.

Tropical and subtropical plants exhibit a marked physiological dysfunction when they are exposed to low or nonfreezing tempertures below 10°C to 12°C. This dysfunction is referred to as chilling injury and has been studied of great concern for many years with harvested plant parts. Because lowered storage temperature is generally one of the effective means of extending the postharvest life of fruits and vegetables (Couey, 1982).

Various physiological and biochemical alterations occur in response to chilling stress in plants (Wang, 1982). The extent of these alterations and the ability of the plant to withstand these alterations determine whether the plant is sensitive or resistant to chilling at that temperature. Chilling-sensitive plants are those killed or seriously injured by temperatures above the freezing point of the tissue, up to about 15–20°C in some case. The severity of damage usually increases at lower temperatures and is time-temperature dependent (Lyons, *et al.*, 1979). All plants able to grow near 0°C are classified as chilling-resistant.

The physiological and biochemical responses of plants to chilling stress have been widely documented and reviewed recently (Graham & Patterson, 1982). These

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(1) 陳益明、劉心斐、林秋榮, Department of Botany, National Taiwan University, Taipei, Taiwan 10764, Republic of China.

changes involve in protein content and enzyme activities (Calderon & Pontis, 1985; Guy & Carter, 1984; Li, 1985; Robertis, 1982) metabolic modifications and changes in lipid composition (Roughan, 1985) and membrane structure (Sikorska & Kacperska-Palaez, 1979; Uemura & Yoshida, 1984; Yoshida, *et al.*, 1986), phosphorylation of thylakoid proteins (Moll & Steinbach, 1986), cyclosis and cytoplasmic structure (Wood, *et al.*, 1983), redistribution of intracellular calcium ion (Wood, *et al.*, 1983), cell leakage of electrolytes and amino acids (Yoshida & Niki, 1979), a diversion of electron flow to the alternate pathway (Yoshida & Niki, 1979), and gene expression (Benza-Basso, *et al.*, 1986; Eaks, 1960; Guy, *et al.*, 1985) etc.

Based on these results, a conclusive understanding of the mechanism of chilling stress is still lacking. In order to have a better comprehension of chilling responses to plants as a whole, the effects should be made to integrate various discrete aspects that were observed in chilling-sensitive and chilling-resistant plants, focus on the direct or indirect effects of chilling temperatures upon whole seedlings in chilling-sensitive or chilling-resistant plants including growth of seedlings, metabolic activity and gene expression. In this study, we are dealing mainly with the effects of chilling on the growth of seedlings, mitochondrial enzyme activity and protein synthesis in chilling-sensitive etiolated mungbean seedling.

MATERIALS AND METHODS

(1) Plant materials

Seeds of mungbean (*Vigna radiata* L.) were soaked in running tap water for 8 h at room temperature, then grown in rolls of moist tissue paper at 28°C in a dark chamber for 32 h. The seedling sample was divided into two batches. One of them was transferred to a cold growth chamber (4°C) for various duration (such as 24 h, 48 h, 72 h, etc, as chilling-treated sample). The other was continually incubated at 28°C as a control.

(2) Growth analysis

The seedlings were treated at 4°C for various duration and then were transferred to growth at 28°C for 72 h. The length of whole seedling (root plus hypocotyl) and hypocotyl were measured.

(3) Effect of chilling on the respiration rate of whole seedlings

The respiration rate of whole seedlings was measured by Warburg manometer at 25°C according to the method of Umbreit *et al.* (1972).

(4) Isolation of Mitochondria

Five grams of seedlings were chopped with blade razor in 20 ml of the medium containing 0.4 M mantiol, 25 mM MOPS(pH 7.8), 5 mM KCl, 8 mM cyseine, 1 mM EGTA and 0.1% (w/v) BSA. Isolation and purification of mitochondria was performed followed the methods as described by Forde *et al.* (1978) with minor modifications. Crude mitochondria were further purified by percoll gradient (28%, v/v, percoll, 0.3 M sucrose, 1 mM EGTA, 10 mM phosphate buffer, pH 7.2, 0.1% defatted BSA). The samples were centrifugated at 40,000 ×g for 30 min. The mitochondria fractions were collected by a fraction collector (Model 211 Super Rac monitered by LKB Model 2151 variable wavelength monitor). Purified mitochondria were subjected to enzymatic assay.

(5) Determination of succinate dehydrogenase activity in purified mitochondria

The activity of succinate dehydrogenase in purified mitochondria was assayed by the method of Cooperstein *et al.* (1950) with modifications. The enzyme activity was assayed in a reaction mixture containing 17 mM succinate, 34 mM phosphate buffer (pH 7.4), 1 mM KCN, 0.4 mM DCPIP at 25°C and the changes of the optical density at 600 nm in each assay was measured. The specific activity of succinate dehydrogenase was expressed as Δ OD 660/min/OD 280 (protein content).

(6) Quantitative estimation of amino acids and sugars

One gram of seedlings was incubated in a 100 ml flask containing 20 ml of deionized water and was shaking at room temperature in the speed of 120 rpm for 20 min. Blotting dry the seedings and they were continuely skaking in a 100 ml flask containing 20 ml deionized water at same speed for another 2 h. Amino acids that leaked from the seedlings into the incubation medium were estimated by the ninhydrin method as described as Moore and Stein (1954) using leucine as a standard. Soluble sugars in the incubation medium were estimated by the phenol-sulfuric acid reaction of Doubois *et al.* (1956) using glucose as a standard.

(7) In vivo labeling

To label proteins, ten 32–hour–old etiolated seedlings (control or cold-treated, appropriately 1 g in weight) were incubated in 5 ml of the incubation medium containing 15 μ Ci L-[^{35}S]-methionine (39.89 TBq/mmole or 1,078 Ci/mmole, NEN Research Product, Deupont) at 28°C for 2 h or at 4°C for 5 h as described by Key *et al.* (1981), Labeled seedlings were rinsed thoroughly with nonradioactive methionine (1 mM) before the extraction of proteins.

(8) Extraction of in vivo-labeled proteins

[^{35}S]-methionine labeled tissue was homogenized at room temperature in 5 ml of grinding medium containing 50 mM Tris-HCl (pH 8.0), 2% SDS, 2% 2-mercaptoethanol and 1 mM PMSF. The homogenate was centrifuged at 12,100 $\times$g in a Sorvall SS34 rotor (Sorvall RC-2B) for 30 min and filtered through one layer of miracloth. A sample of each filtrate was blotted on a 3-mm filter paper as described by Mans and Novelli (1961).

(9) Gel electrophoresis and fluorography of in vivo synthesized proteins

Proteins prepared as described above were dissolved in a sample buffer containing 2.3% SDS and 5% 2-mercaptoethanol. One-dimensional SDS-PAGE was carried out according to the method of Laemmli (1970) and an equal radioactivity of protein samples were loaded in each well. Fluorography of the gels was accomplished using ENHANCE (New England Nuclear) and Kodak film (XAR-5) (Key, *et al.*, 1981).

RESULTS

1. **Effect of chilling on the growth of seedlings after various duration of chilling treatment at 4°C**

The 32 h-old etiolated seedlings were treated at 4°C for various duration and then grew in the dark at 28°C for 3 days, the results show in Figs. 1 and 2. The
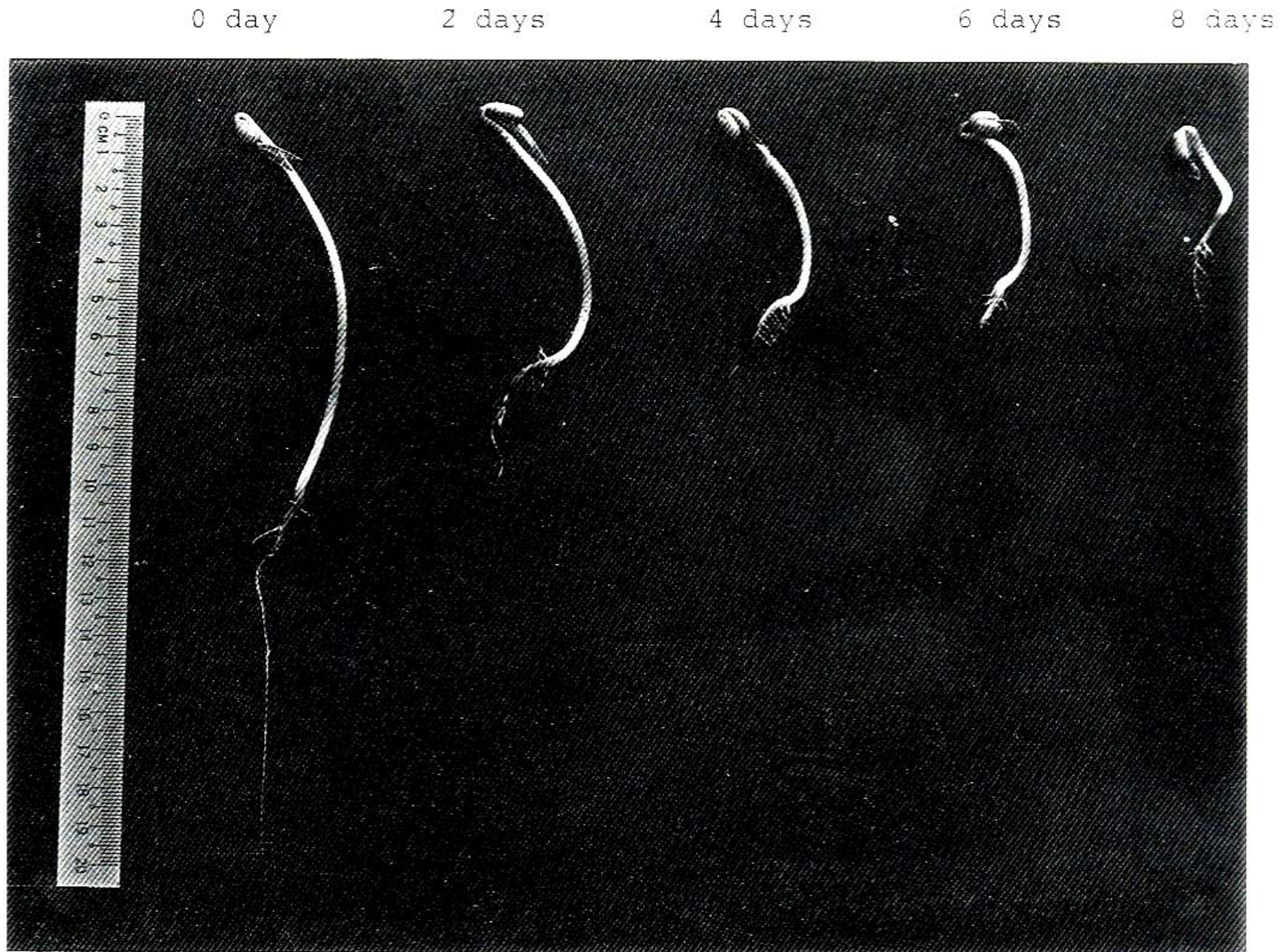
Fig. 1. The external features of growth of 32 h-old etiolated mungbean seedlings
pretreated at 4°C for various duration and then grew at 28°C in the dark
condition for 3 days.

results indicated that the longer chilling treatment at 4°C, the more growth
inhibition of seedings was observed. The chilling treatment causes the enlarge-
ments of hypocotyls and the injury of root in seedlings. After 8-day chilling
treatment and then grew at 28°C in dark for 3 days, the length of seedlings was
only 25% of the control (Fig. 2).

2. Effect of chilling on the respiratory rate of seedlings

When the seedlings pretreatment at 4°C for different duration and then measured
the respiratory rate by Warburg manometer at 25°C, the results showed in Fig. 3.
The short-term chilling treatment did not affect the respiration rate of the
seedlings, but the prolonged chilling treatment caused the increment of respiration
rate. After 6 days of chilling, the respiration rate of seeding increase about 80%.

3. Effect of chilling on the respiratory enzyme activity in mitochondria

To investigate the effect of chilling on the respiratory enzyme activity in
mitochondria, the 32-h old etiolated seedlings were pretreated at 4°C for 4 days
and then the mitochondria were purified. The succinate dehydrogenase activity
in the isolated purified mitochondia was measured. The results indicated that
chilling treatment only slightly affect the activity of succinate dehydrogenase in
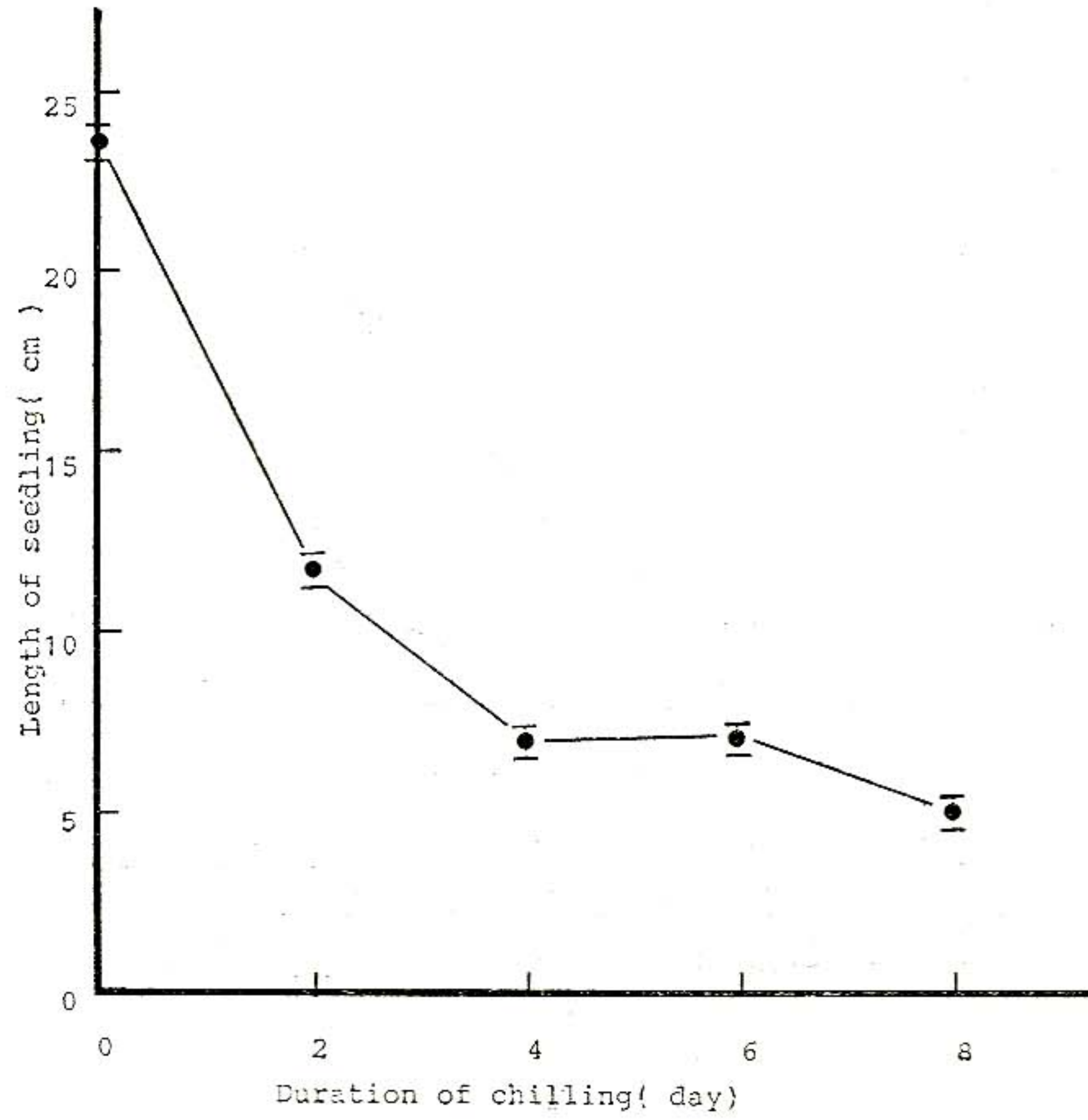isolated mitochondria. The maximum reaction rate of succinate dehydrogenase

Fig. 2. Effects of chilling treatment at 4°C on the growth of 32 h-old etiolated mungbean seedlings in the dark at 28°C for 3 days.
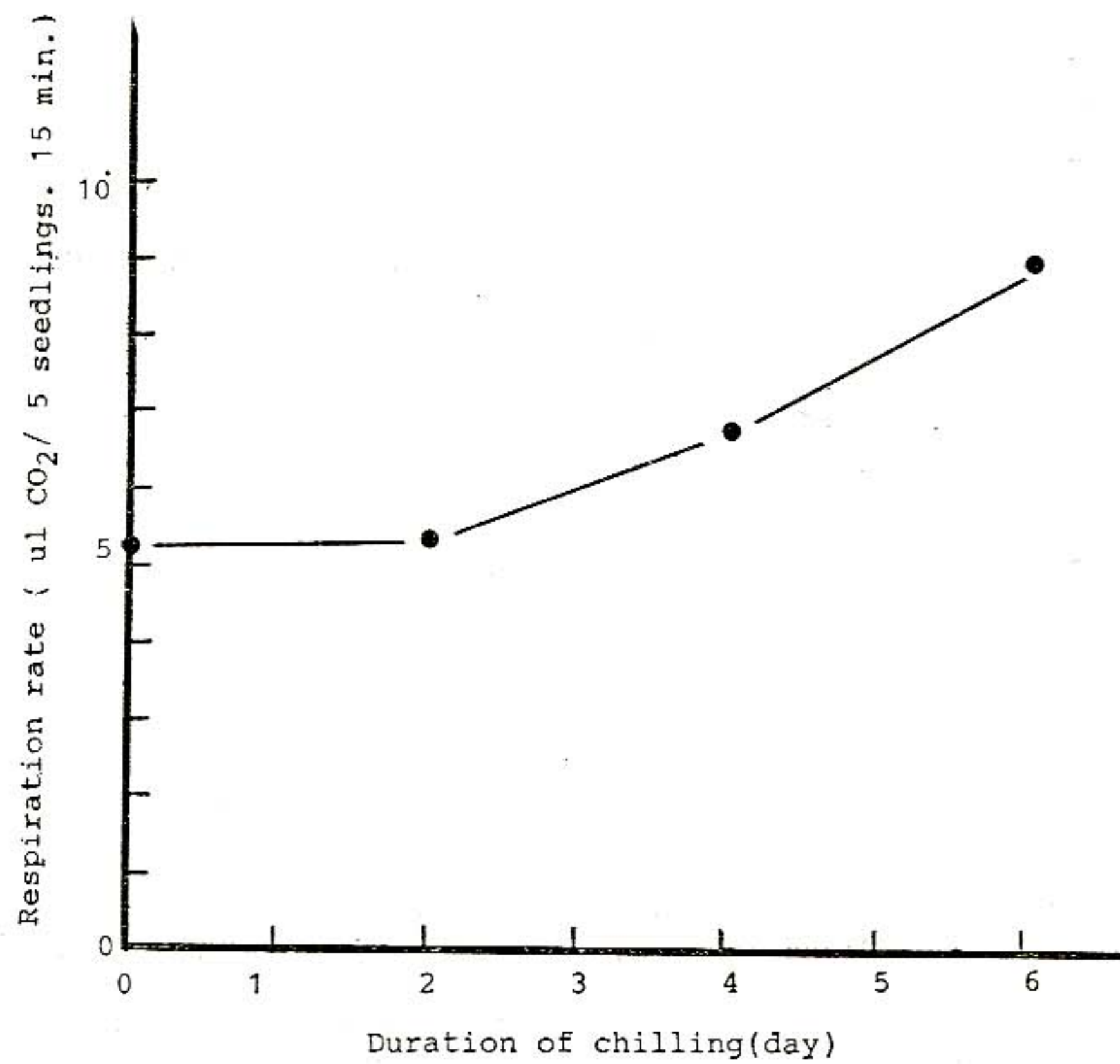

Fig. 3. Effects of chilling treatment at 4°C on the respiration rate of etiolated mungbean seedlings after various duration of chilling treatment. The respiration rate was measured at 25°C.
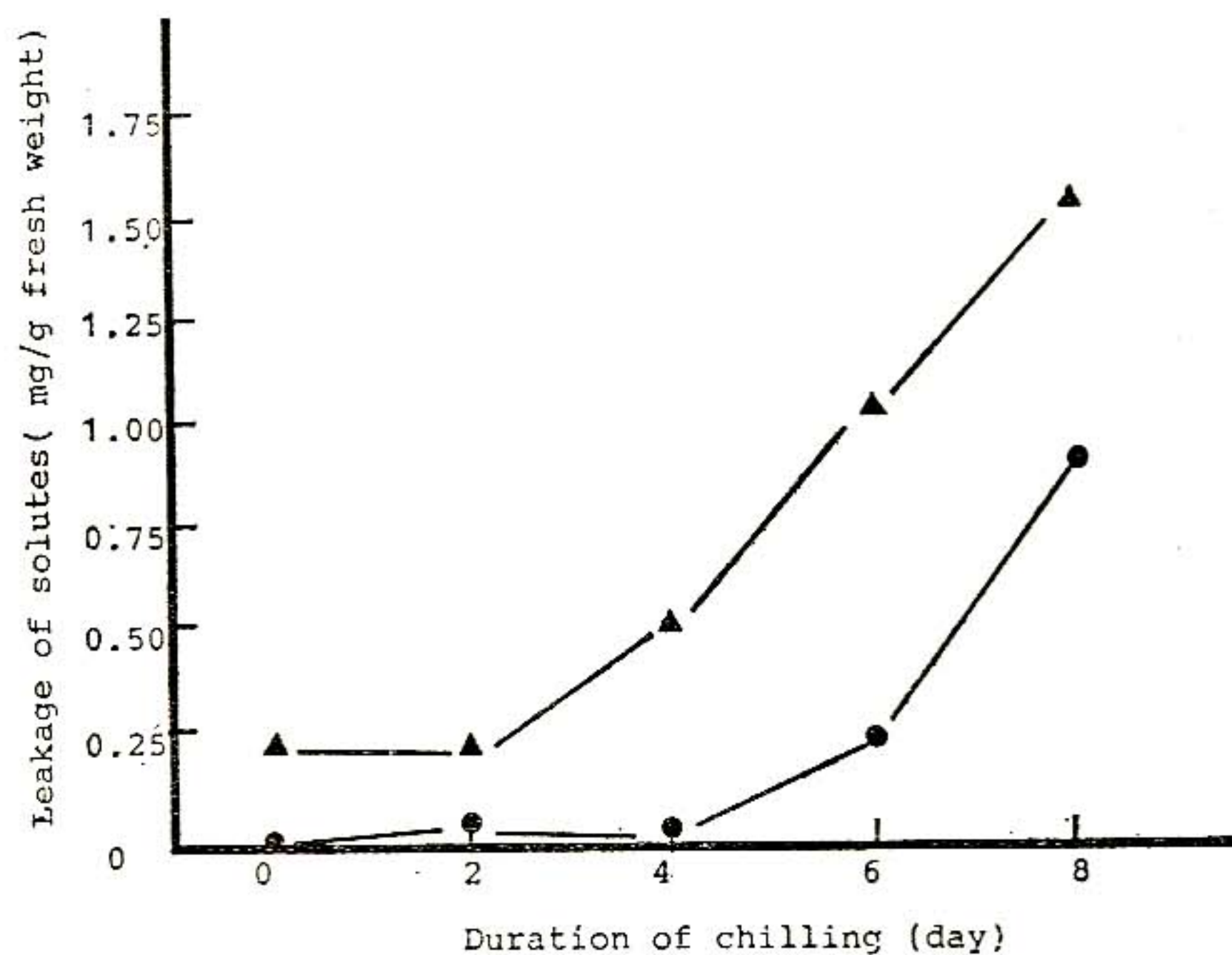
Fig. 4. Effects of chilling treatment at 4°C on the solute leakages from the etiolated mungbean seedlings to the shaking medium.
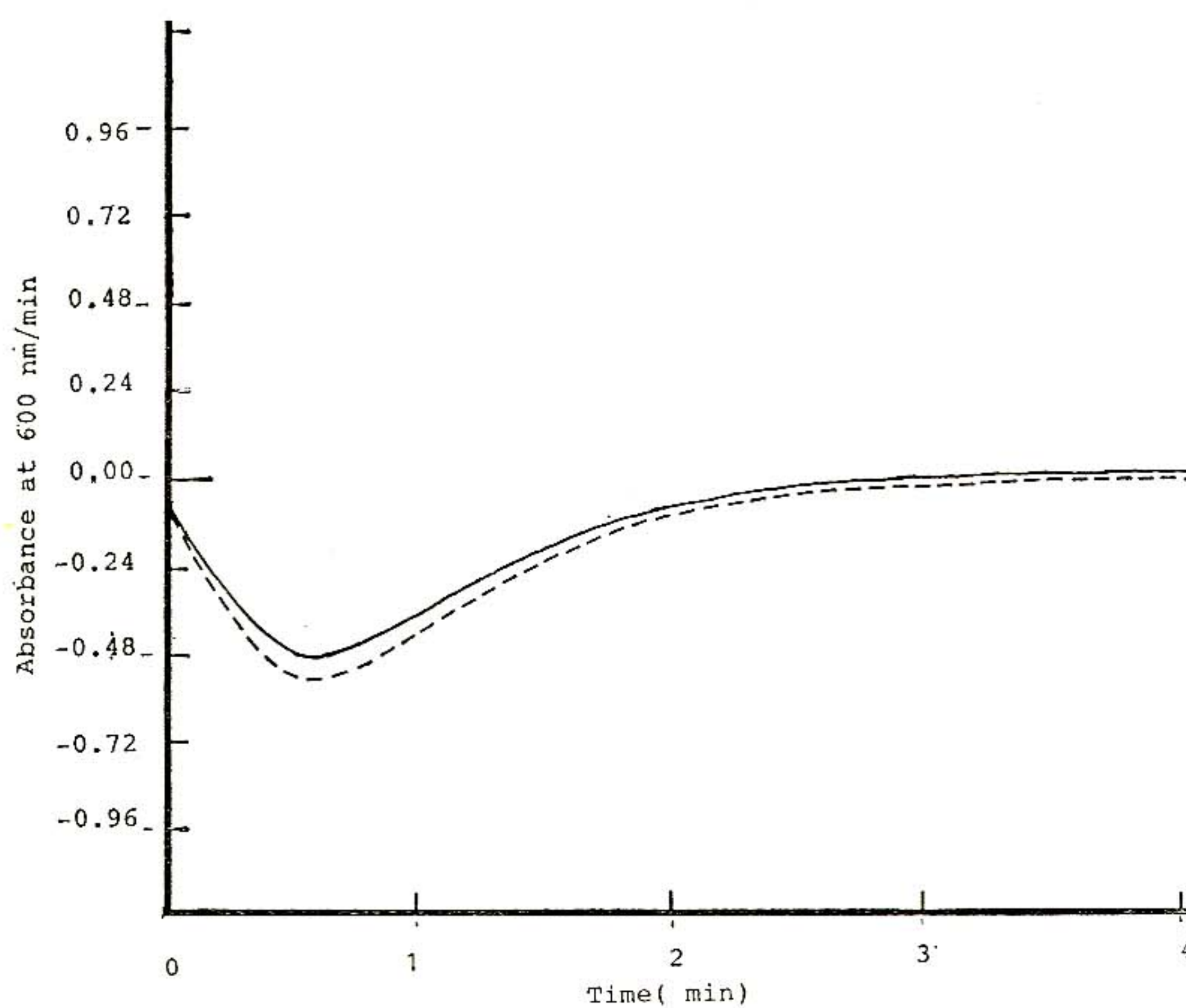
▲——▲ Sugars;　　　●——● Amino acids.

Fig. 5. Comparison of DCPIP reductive rate of succinate dehydrogenase in mitochondria isolated from 28°C grown (control) and 4°C chilled etiolated mungbean seedlings for 4 days. The enzyme activity was assayed as described in the Materials and Methods. The DCPIP reductive rate of succinate dehydrogenase in isolated mitochondria was expressed as changes of the absorbance at 600 nm/min.

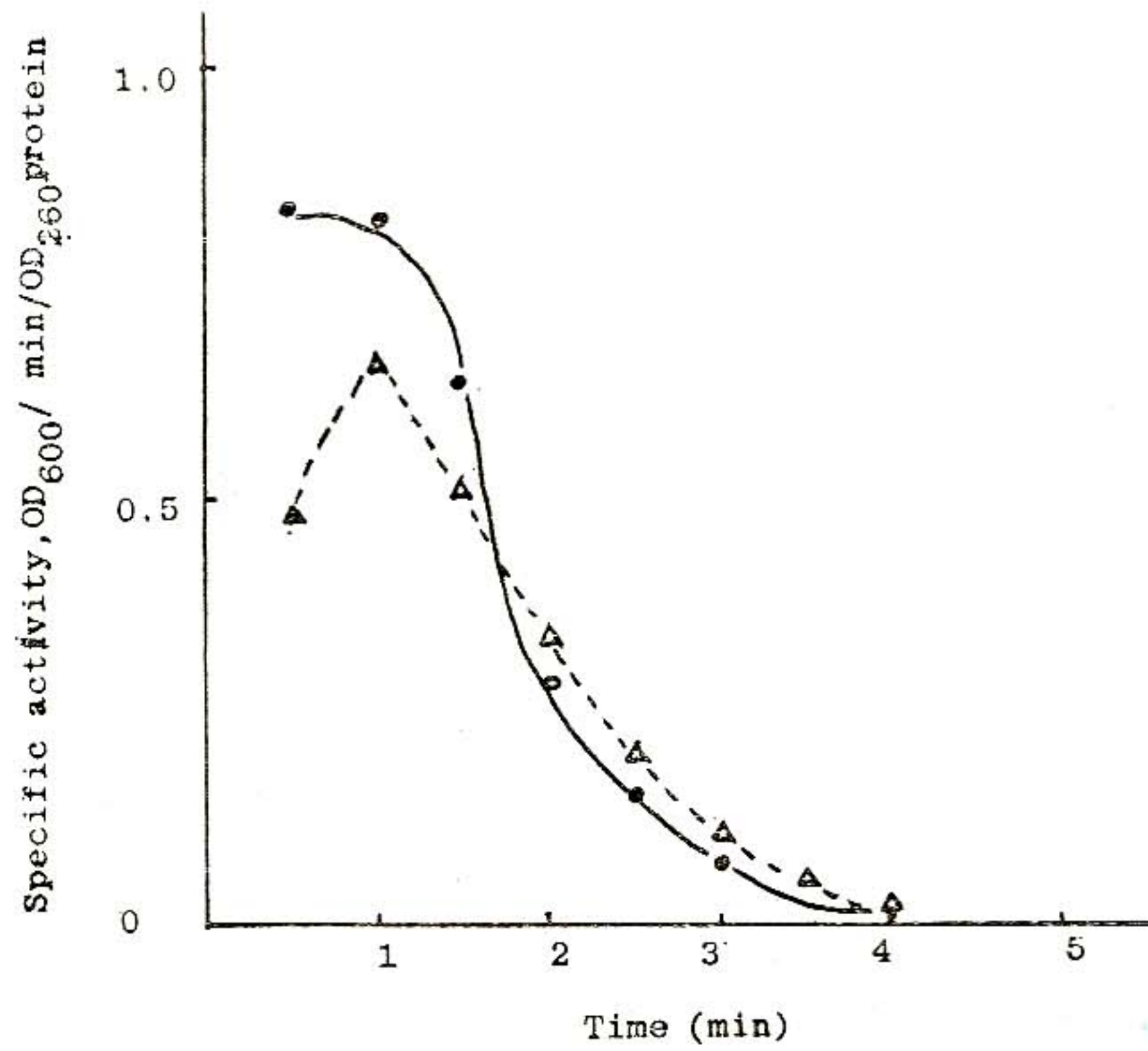————28°C control;　　　- - - - - - 4°C chilled.

Fig. 6. Comparison the specific activity of succinate dehydrogenase in mitochondria isolated from 28°C grown control and from 4°C chilled mungbean seedlings. ●——● Control; ▲——▲ 4°C chilled treatment.
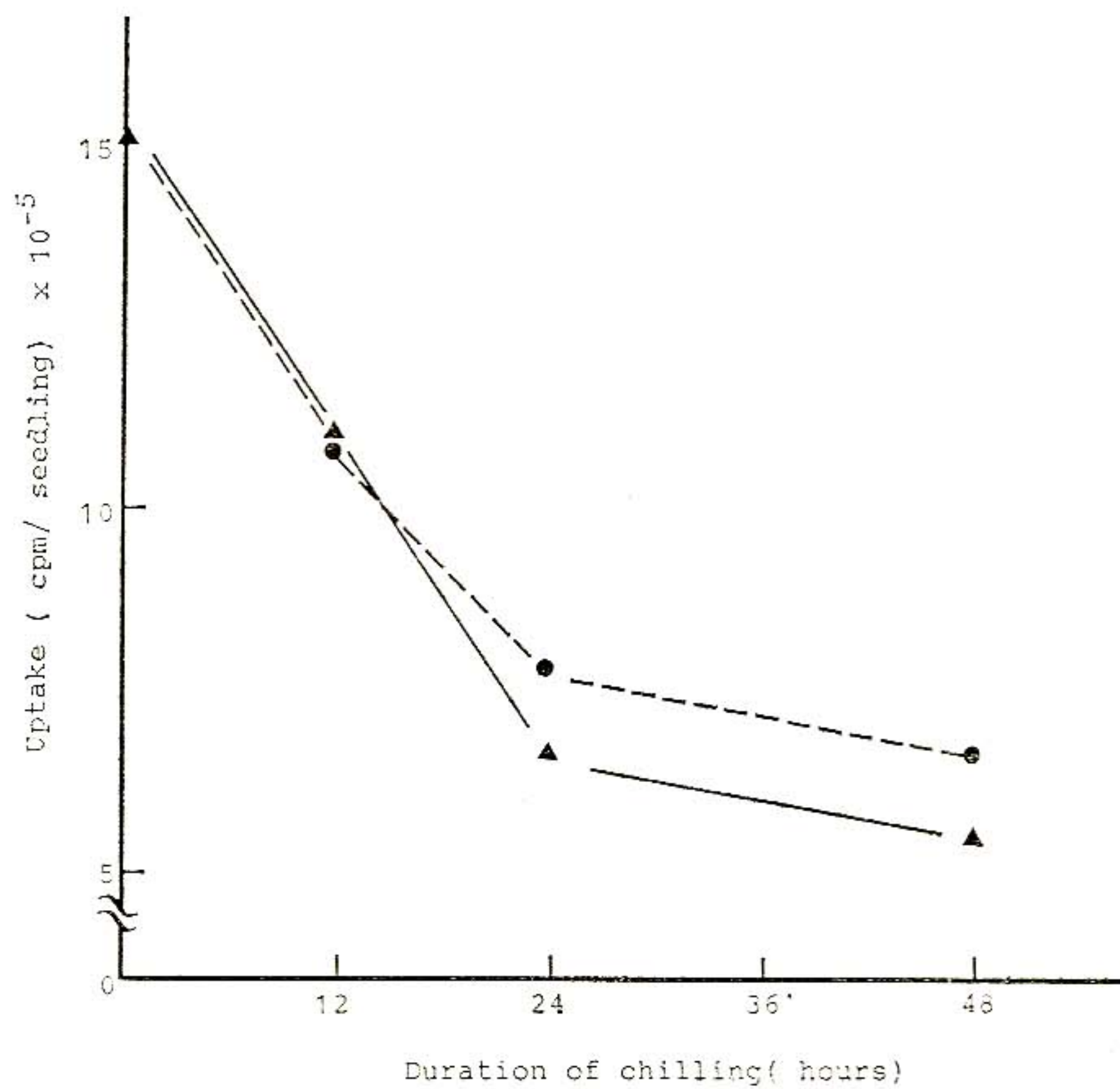

Fig. 7. Effects of chilling treatment at 4°C on the uptake of ^{3}H-leucine by the etiolated mungbean seedlings. The 32 h-old etiolated mungbean seedlings were pretreated at 4°C for various duration and they were labeled with ^{3}H-leucine at 28°C for 2 h. The radioactive in the homogenate was determined. ▲——▲ Chilling at 2°C; ●——● Chilling at 4°C.

in mitochondria from control and chilled-seedlings were-0.54 OD_{660}/min and -0.50 OD_{660}/min at 0.6 min, respectively (Fig. 5). The specific activity of succinate dehydrogenase in mitochondria from control and chilling-treated seedling was shown in Fig. 6. The specific activity of succinate dehydrogenase in chilling treated seedlings is lower than in control, when it was assay at beginning 1.5 min interval.

4. Effect of chilling on the leakage of solutes in seedlings

For the investigation of the injury of chilling in the seedlings, the leakages from the seedlings, such as sugars and amino acids, were measured. These results were shown in Fig. 4. After 4 days of chilling treatment, the leakage of sugars and amino acids increased significantly, especially after 8 days of chilling treat-ment. These results also showed the chilling treatment caused the injury of seedlings when the chilling treatment was longer than 4 days.

5. [35S]-methionine labeling for measurement of the protein synthetic activity in the chilled seedlings

For determintion of protein synthetic activity in the seedlings after different duration of chilling treatment, the [35S]-methionine or [3H]-leucine was used to label the proteins. The seedlings were pretreated at 4°C for different duration and then were labeled with 100 μ Ci L[35S]-methionoine or [3H]-leuciene. The total uptake of [3H]-leucine into the seedlings were shown in Fig. 7. Chilling treatment caused a significantly decrease in [3H]-leucine uptake into the seedlings. After
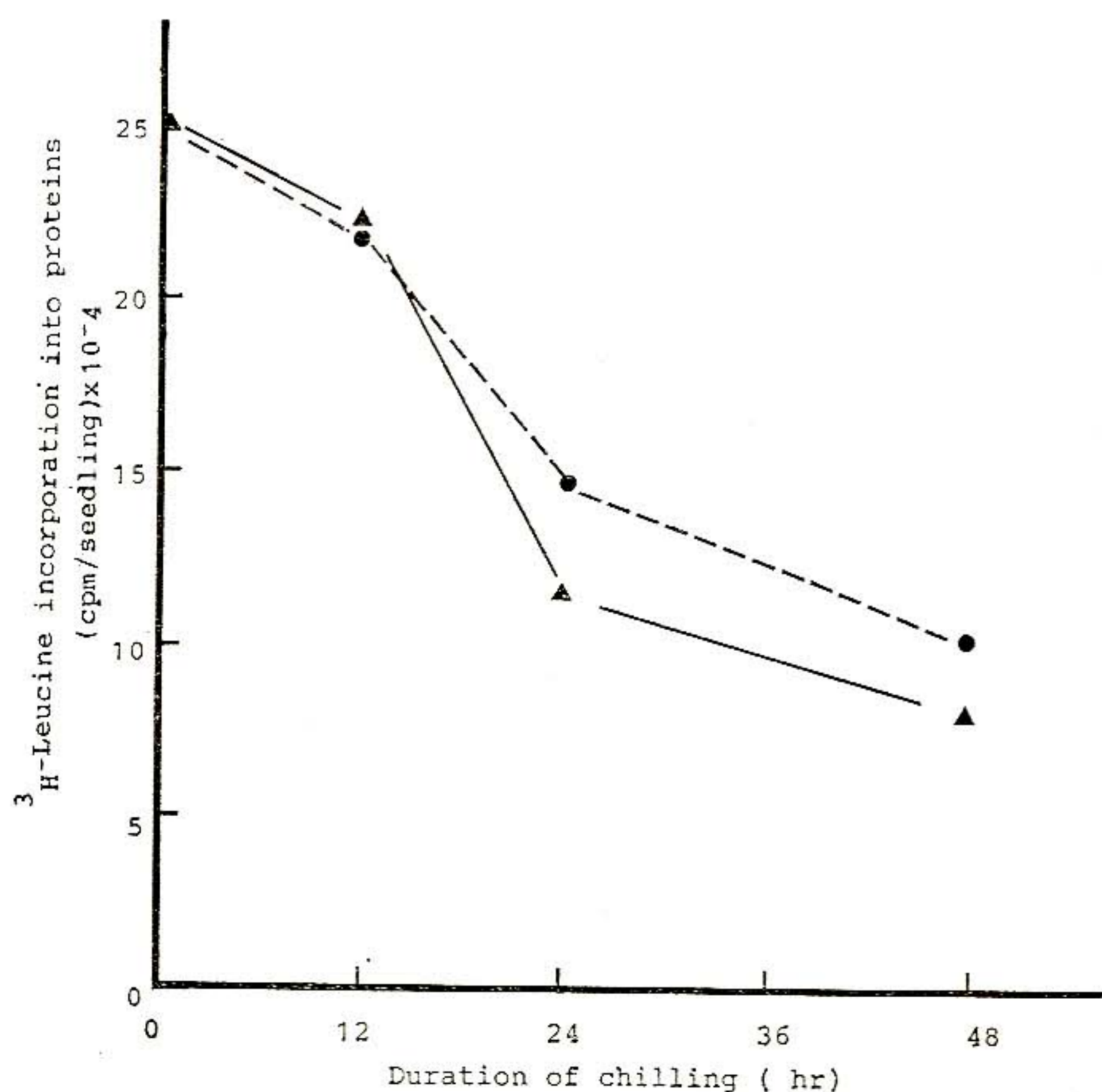

Fig. 8. Effects of chilling treatment on the 3H-leucine incorporation into proteins in the etiolated mungbean seedlings.

▲——▲ Chilling at 2°C;   ●——● Chilling at 4°C.

48 h chilling treatment at 4°C, the uptake of [³H]-leucine decreased to 56% of the control. The protein synthetic activity in the seedlings after different chilling treatment showed in Fig. 8. The protein synthetic activity in the 4°C chilling-treated seedlings decreased about 56%.

6. Changes in protein synthetic patterns in the chilling seedlings

The 32 h-old etiolated mungbean seedlings were pretreated at 4°C for different duration and then were labeled with [³⁵S]-methionine at 28°C for 2 h, the protein synthetic patterns were analyzed by SDS-PAGE. There are 9 major newly protein bands were synthesized in the chilling-treated seedlings. The molecular weight of these protein bands are 110, 88, 78, 58, 48, 42, 27, 22 and 10.5 KD, respectively (unpublished data). The pretreatment of seedlings at 4°C for 4 days and then labeled with [³⁵S]-methionine at 4°C for 5 h, the protein synthetic pattern was shown in Fig. 9. There are 13 major newly protein bands were synthesized at
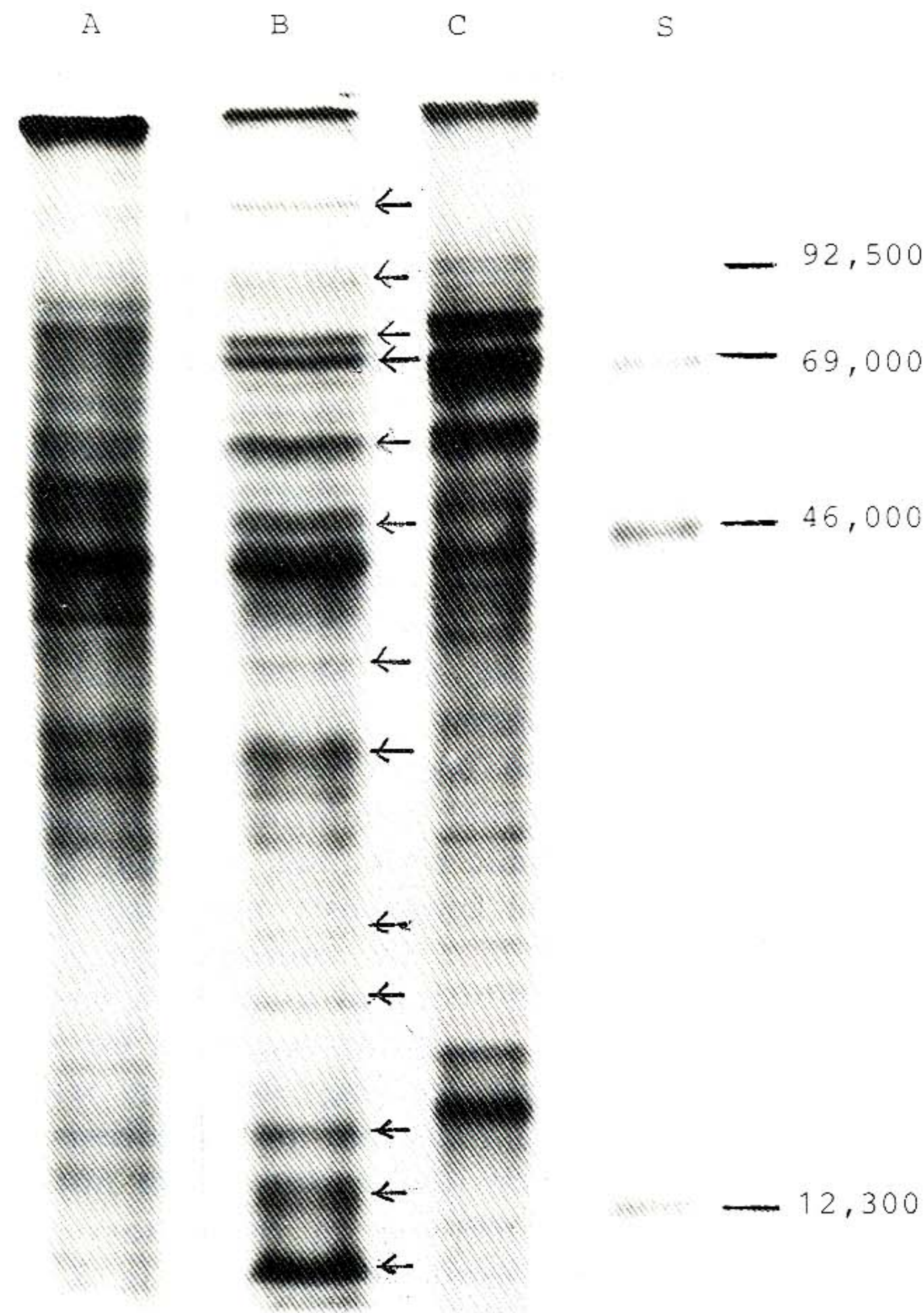

Fig. 9. The comparison of chilling-induced proteins and heat shock proteins isolated from etiolated mungbean seedlings and analyzed by in 12.5% SDS-PAGE and then fluorography.
A. 28°C control;   B. Chilling-induced proteins;
C. Heat-shock induced proteins;   S. Protein molecular weight standard.

4°C in the pretreated seedlings. The molecular weight of these chilling proteins were 110, 88, 78, 69, 58, 38, 27, 22, 19, 14, 12.5 and 10.5 KD, respectively. The major chilling-induced proteins bands were in the molecular weight of 88, 69, 58, 48, 38, 14, 12.5 and 10.5 KD. These chilling-induced proteins were quite different from heat shock proteins of mungbean seedlings (Fig. 9).

DISCUSSION

Mungbean a chilling-sensitive plant, when 32 h old etiolated seedling was treated at 4°C for 2 days, the growth of seedlings was inhibited (Fig. 1 and 2), and the leakage of solutes, such as sugars and amino acids also significantly increased (Fig. 4). This is due to the chilling injury of seedlings. The degree of chilling injury of seedlings is dependent on the duration of chilling. The longer period of chilling treatment, the more severe injury in seedling was observed. When the seedlings have been chilled, but not killed, they become stunted in shape. This phenomenon is similar to that of tomato (Kemp, 1968) and cucumber in chilling treatment (Robertis, 1982) or after heat shock (40°C) treatment for 2 h and then growth at 28°C (Chen *et al.*, 1986). The increment of solutes leakage from the chilling injury seedlings may be due to dehydration of membrane and then changes the structure of membrane, physical states and membrane composition etc. Lyons and Raison (1970) first suggested that *transition* of membrane from a flexible liquidcrystalline to a solid-gel structure could be the primary response to low temperature. Prologed exposure of chilling-sensitive species to low temperature, the primary event could lead to loss of membrane integrity, increased the leakage of solutes, decreased the energy supply and ultilization (Pomeroy & Andrews, 1986), decreased metabolic and photosynthetic rate, increased ethylene release (Wang & Adams, 1980), increased accumulation of plant growth hormones and toxic substances in the tissue (Levitt, 1972). Such physiological changes can be harmful to the tissues. Finally, it can result in the development of symptoms of chilling injury. Chilling-resistant plants did not show physical phase transition of membranes or sudden change of enzyme activity at temperatures in the chilling range. The alteration of membrane properties induced by chilling temperature are not confined to plasmalemma, other membrane in various organelles, such as mitochondria, chloroplast, endoplasmic reticulum, vacuole, cytoskeletons and the metabolic processes of membrane biogenesis are also influenced by chilling temperatures (Yoshida, *et al.*, 1986). The chilling injury of 32 h-old etiolated mungbean seedlings includes the inhibition of growth in hypocotyl and breakdown of root tissues. If 7-day-old green mungbean seedlings were exposed to light at 4°C, the primary symptom of injured seedlings was the wilting of leaves after 24 h of chilling treatment. But the 7-day-old pea seedlings (a chilling-resistant species) could tolerant to 4°C-chilling temperature (data not shown).

Chilling treatment caused the increase in respiratory rate in the etiolated mungbean seedlings (Fig. 3). The results shows in. Fig. 3 are similar to other many chilling-sensitive crops, such as citrus fruits (Eaks, 1960), cucumber (Eaks & Morris, 1956), snap beans (Watada & Morris, 1966) and sweet potatoes (Lewis & Morris, 1956) etc. The respiratory response to chilling stress included tissue-level studies, isolated mitochondria and detached organs (Leopold & Musgrane, 1979). The mechanism of stimulation is still unknown. But is was presumed to be due to uncoupling of oxidative phosphorylrtion (Eaks, 1980; Lewis & Workman, 1964;

Shichi & Uritani, 1956). The sustained increase in the respiratory rate after prolonged chilling treatment might be an indicative of the irreversible metabolic disturbance and accumulation of the oxidizable intermediates (Eaks, 1980).

Most of the enzyme systems are dramatically affected by chilling temperatures and these enzymes have been found to be associated with membranes. Because the Arrhenius plots of the membrane-bound enzyme systems in sensitive plants show a "break" at the same temperature where the membrane undergoes a phase transition from the liquid-crystalline to the solid-gel state (Lyons, et al., 1979; Raison, 1974). The activity and specific activity of succinate dehydrogenase in isolated mitochondria from chilled or non-chilled seedlings showed the maximum reaction rate was decreased slightly (Fig. 7 and 8). This may be due to the increase of enzyne activation energy below 9°C in chilling-sensitive plant tissues (Lyons, et al., 1979; Raison, et al., 1971). But chilling-resistant plant tissues showed a constant activation energy over the usual range of biological temperatures. However, there is an increased synthesis of phenolic compounds at low temperatures. Two enzymes of phenolic metabolism, PAL (phenylalanine lyase) and CQT (hydroxy cinnamoyl CoA quinate hydroxycinnamoyl transferase) have been studied extensively. The Km of sweet potato PAL for phenylalanine changes between 10°C and 40°C, with a minimum value around 20°C (Tandka & Uritani, 1977). PAL catalyzed the first step in the synthesis of compounds such as chlorogenic acid from phenylalanine. The extractable amount PAL increase when many plant species or their detached parts are chilled, such as apple fruit (Tan, 1980), potatoes (Rhodes, et al., 1979) and sweet potato tuber (Raison, 1974). In chilled apple fruit, as PAL increases, a factor which destroys PAL in vitro decreases. PAL is turned over rapidly at normal temperatures (Forde, et al., 1978) and it seems likely that the level of PAL increases during chilling because the rate of synthesis is decreased less by cold than in the rate of degradation. So that when pea seedlings grow at 4°C for more than two weeks, the color of leaves and stem appeared in red color (unpublished data).

Chilling treatment not only decreased the total uptake of [^{35}H]-leucine into the seedlings, but also decreased the protein synthetic activity in the seedlings (Fig. 7 and 8). This phenomenon is similar to the heat shock stress response in animals and plants (Key, et al., 1981; Schlesinger, et al., 1982). Protein synthesis responds rapidly and dramatical to heat stress in a wide range of organisms from bacteria to man (Schlesinger, et al., 1982). After a shift of soybean seedlings from the normal growing temperature (about 28°C) up to 40°C, there is a significantly decrease in protein synthetic activities, especially the temperature up to 42.5°C, under heat shock condition, the synthesis of normal proteins is greatly decreased and a new set of proteins called "heat shock proteins" is induced. Up to present, the effects of low temperatures on gene expression still have not been analyzed in detail. In most situations it is not clear whether the changes in protein content and enzyme activity resulted from inactivation or activation of preexisting enzymes, or from changes in mRNA abundance, translation efficiency, or other mechanisms. From Fig. 9 showed that is 13 protein bands were newly synthesized at 40°C in the prechilled mungbean seedlings. The major proteins synthesized at 4°C were 88, 69, 58, 48, 38, 14, 12.5 and 10.5 Kd. Benza-Basso et al. (1986) reported that when the seedlings of Brassica napus was chilled at 0°C and then labeled with [^{35}S]-methionine, they found 14 protein spots increases and 5 protein spots decreases in 2-D gel fluorograms. But they did not

calculate the molecular weight of these chilling-induced proteins, either from 1-D gel or from 2-D gel. Guy & Carter (1984) reported that exposure of spinash (*Spinacia oleraceia* L.) to a constant 5°C for several days could induce a greater tolerance to extracellular freezing. This treatment induced 5 newly synthesized protein bands in the leaves. The molecular weight of these proteins are 110, 82, 66, 55 and 13 Kd, respectively. The major chilling-induced proteins were different from mungbean heat shock proteins (Fig. 9). The molecular weight of heat shock proteins in mungbean seedlings are 92, 84, 72, 62, 27, 22, 18 and 15 Kd, respectively. The physiological roles of heat shock proteins may play the protection of organisms from injury under high temperature. These heat shock proteins can associate with plasmalemma and cellular organelles, such as ribosomes, mitochondria, chloroplasts and nuclei etc. under high temperature. The physiological roles of chilling-induced proteins are still unknown (Scott, *et al.*, 1985). Whether chilling-induced proteins are universal to exist in all organisms and also can associate with cellular organells to play some physiological functions are needed to further investigation.

ACKNOWLEDGEMENT

ABBREVIATION

MOPS: 3-(N-morpholino) propanesulfonic acid (Sigma)
BSA: bovine serum albumin (Sigma)
DCPIP: 2,6-dichlhrophenol indophenol (Sigma)
PMSF: phenylmethylsulfonyl fluoride
PAGE: polyacrylamide gel electrophoresis
NaOAc: sodium acetate
TCA: trichloracetic acid

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低溫逆境對綠豆白化幼苗之生長、粒線體活性及其蛋白質合成之影響

陳 益 明　劉 心 斐　林 秋 榮

摘　　要

本研究以對低溫敏感的植物——綠豆（*Vigna radiata* L.）為材料。在黑暗中萌芽 32 小時的綠豆幼苗，經 4°C 處理不同時間後，分析其生長、粒線體活性及蛋白質合成能力等；其結果顯示下列各種現象皆受到抑制：㈠幼苗的生長，㈡粒線體的 succinate dehydrogenease 活性；㈢幼苗的蛋白質合成能力。但低溫處理會導致幼苗在室溫下呼吸速率增快，且幼苗的組織也受到傷害，因此組織內溶質的漏出物增加。在 4°C 低溫處理下所合成的 ^{35}S- 蛋白質，經 SDS-膠體電泳分析及 fluorography 後，其中有 13 種蛋白質帶（protein band）是低溫誘導出的蛋白質（chilling induced proteins）；其分子量是 110，88，78，69，58，48，38，27，22，19，14，12.5 及 10.5 KD，其中 88，69，58，48，38，14，12.5 及 10.5 KD 是主要的低溫誘導蛋白質。這些蛋白質完全與綠豆的熱休克蛋白質不同。