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Low Temperature Effects on Soybean (*Glycine max* [L.] Merr. cv. Wells) Mitochondrial Respiration and Several Dehydrogenases during Imbibition and Germination[1]

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ABSTRACT

The influence of low temperature on soybean (*Glycine max* [L.] Merr. cv. Wells) energy transduction via mitochondrial respiration and dehydrogenases was investigated in this study during imbibition and germination. Mitochondria were isolated from embryonic axes of seeds treated at 10 and 23 C (control) by submergence in H_2O for 6 hours and maintenance for an additional 42 hours in a moist environment. Arrhenius plots of initial respiration rates revealed that those from cold-treated axes had respiratory control (RC) ratios of near 1.0 above an inflection in the plot at 8 C. Arrhenius plots of control axes mitochondrial respiration showed RC ratios of 2.8 above and 5.0 below an inflection temperature of 12.5 C. Energies of activation for mitochondrial respiration between 20 and 30 C for the cold and control treatments were 7.8 and 15.6 kcal/mole, respectively. These data indicate possible differences in mitochondrial membranes, degree of mitochondrial integrity, and mitochondrial enzyme complement between the two treatments.

Glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6P-DH), and NADP-isocitrate dehydrogenase (NADP-ICDH) were assayed from whole seeds and axes (after germination) during the 48 hours of temperature treatments. Activity of these dehydrogenases decreased during the first 6 hours with the exception of MDH. After germination at 23 C (48 hours) all five dehydrogenases increased in activity. Arrhenius plots of cotyledon dehydrogenase activities indicated that one inflection temperature between 6 and 18 C was present for each enzyme assayed. Differences were seen in Arrhenius plots of axes dehydrogenase activities with the two temperature treatments in the cases of GDH and MDH from mitochondrial pellets and with differences in enzyme extraction media. These data suggest that the temperature treatments yield differences in mitochondrial enzyme complement. There were no detectable inflection temperatures for the activities of G6P-DH and ADH extracted from axes. Arrhenius plots of NADP-ICDH activity indicated extreme cold sensitivity. The slopes of the plots for axes NADP-ICDH were very similar to those for mitochondrial respiration (23 C treatment) suggesting that this enzyme may limit mitochondrial respiration at low temperature in soybean tissues grown at moderate temperatures.

Plants have been categorized into those which may be irreversibly injured or fail to grow between the freezing point of living tissues and about 15 C (chilling-sensitive) and those which are not damaged and continue to grow at all temperatures within this range (chilling-resistant) (21, 26). The soybean is a chilling-sensitive plant in that a halt in growth and/or injury may occur at some point within this range of temperatures during all stages of development from germination to seed ripening (15). In addition to photoperiodic requirements, sensitivity to temperature has limited the growing season and geographic distribution of soybeans, although cultivar responses to both environmental parameters vary to some extent (15, 32).

Sensitivity of plants and animals to chilling temperature seems to be due to a combination of two factors: (*a*) changes in the molecular ordering of organelle membranes (26, 27); and (*b*) increases in the Arrhenius E_as[2] for enzymic reactions (26, 37), which may be associated with membrane phase changes (17, 29). Unlike chilling-sensitive organisms, chilling-resistant plants and animals lack inflections or discontinuities in Arrhenius plots for such processes as respiration (22, 26). Inflections in Arrhenius plots for a physiological process at low temperature are usually indicative of a higher E_a for an enzyme or enzymes below the inflection temperature and indicate an alteration of that process.

Because the removal of membrane-bound enzymes from their membranes may result in the disappearance of inflections or discontinuities in the Arrhenius plots for the activities of those enzymes (17, 29), it can be inferred that enzyme-membrane or enzyme-lipid interactions are accountable for variations in physiological function of chilling-sensitive and chilling-resistant plants. Differences in the lipid constituents of membranes rather than enzymes in these two groups of plants are usually considered to produce the observed variations in Arrhenius plots (26, 27). However, changes in isoenzymes occur with cold acclimation (development of resistance to freezing) and deacclimation (19) along with changes in the amounts of membrane-bound and water-soluble enzymes (12, 19). This suggests that, at least in part, the enzyme complement of tissues could influence responses to low temperature in some species.

Studies with legumes have shown that during imbibition and germination rapid changes occur in mitochondrial respiration (24, 33). Dehydrogenases are of particular importance in cold acclimation due to their production of reduced nucleotides necessary for energy transduction (1, 19). Such dehydrogenases as MDH have been implicated in controlling mitochondrial respiration (2). This study reports data on the effects of low

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[2] Abbreviations: GDH: glutamate dehydrogenase (EC 1.4.1.3); MDH: malate dehydrogenase (EC 1.1.1.37); ADH: alcohol dehydrogenase (EC 1.1.1.1); NADP-ICDH: NADP-isocitrate dehydrogenase (EC 1.1.1.42); G6P-DH: glucose-6-phosphate dehydrogenase (EC 1.1.1.49); α-KG: α-KG: α-ketoglutarate; E_a: energy of activation; RC: respiratory control.

temperatures as they relate to the aforementioned factors in soybeans.

MATERIALS AND METHODS

Plant Material. Initial moisture content of soybean (*Glycine max* [L] Merr. cv. Wells) seeds (10 seeds/lot) was determined on a fresh wt basis by drying three lots at 90 to 95 C to constant weight. Initial seed moisture (fresh wt basis) was about 9 to 10%, which is near optimal for soybean imbibition at low temperture (14).

Imbibition was started by submerging seeds in distilled H_2O at 10 ± 0.1 C or 23 ± 0.1 C for 6 hr. Seeds were then placed on H_2O-saturated tissue paper in 150-ml beakers at their respective initial temperatures of imbibition. Each beaker contained about 5 g of seeds (initial fresh wt). Temperatures of the beakers were held constant by inserting them into a grid which held them partially immersed in a thermostatically controlled circulating H_2O bath. The entire grid was then covered with insulation. Seeds were checked every 12 hr after the onset of imbibition for germination, as indicated by emergence of the radicle through the ruptured testa. At 0, 3, 6, 12, 24, and 48 hr, seeds from three beakers (each treatment) were removed, blotted dry, and weighed. After 48 hr, embryonic axes were excised from cotyledons, and cotyledons and axes were weighed separately. Each beaker of seeds, cotyledons, and embryonic axes was held on ice for enzyme or mitochondria extraction.

Extraction of Mitochondria and Enzymes. One hundred embryonic axes (about 1.2 g at 10 C and about 2.7 g at 23 C) from seeds treated 48 hr were ground in a chilled mortar containing 6 ml of mitochondria extraction medium (0.4 M sorbitol, 0.02 M cysteine-HCl, 0.02 M K_2HPO_4, 0.1 M Trizma [Sigma Chemical Co.] at pH 7.5 and 0.5% BSA [w/v])/g of tissue with a small amount of acid-washed sand. The slurry was squeezed through three layers of cheesecloth and then centrifuged at 500*g* for 5 min to remove cellular debris and sand. The supernatant was then centrifuged at 20,000*g* for 30 min and the pellet containing mitochondria was resuspended in 5 ml of mitochondria extraction medium and immediately placed on ice and used in respiratory experiments.

Enzyme extraction of whole seeds or excised cotyledons was initiated by placing the contents of each beaker in 45 ml of enzyme extraction medium (0.4 M sucrose or 0.4 M sorbitol, 0.02 M cysteine-HCl, 0.1 M Trizma [pH 7.5], and 2% [w/v] purified egg albumin [Fisher Scientific Co.]) with 0.5 g polyvinylpolypyrrolidone (Sigma Chemical Co.)/g of tissue and homogenizing 3 for 5 min at near 0 C with a MSE "homogeniser" (Measuring and Scientific Equipment, London) set at highest speed. Embryonic axes from each beaker were ground in a chilled mortar with 10 ml of enzyme extraction medium. Slurries were washed from"homogeniser" Vortex beakers or mortars with 10 ml of enzyme extraction medium and centrifuged at 20,000*g* for 30 min. Pellets (20,000*g*) were resuspended in 10 ml of enzyme extraction medium and freeze-thawed three times to rupture intact mitochondria for GDH, MDH, and NADP-ICDH assays. Enzymes were precipitated by dissolving solid $(NH_4)_2SO_4$ to 60% saturation in 10-ml aliquots of each 20,000*g* supernatant at 2 to 3 C for 24 hr. Ammonium sulfate precipitants were collected by centrifugation at 20,000*g* for 20 min; pellets were redissolved in 10 ml enzyme extraction medium. Samples were stored at −10 C between assays.

Measurements of Respiration and Enzyme Activities. Mitochondrial respiration was measured polarographically with a Gilson K-ICO oxygraph utilizing a Clark electrode and a thermostated 1.5-ml reaction cell. O_2-saturated H_2O was used to calibrate the instrument. Reaction mixtures contained 0.2 ml of mitochondria preparation, 1.2 ml of mitochondria extraction medium, 0.1 ml of 0.3 M succinate (substrate for oxidation, state 4 respiration), 0.05 ml of 0.03 M ADP (state 3 respiration). All assay additions were saturated with O_2 at a temperature below assay temperature and then equilibrated to assay temperature before addition. Total time of assays (from highest to lowest temperature) was 5 to 6 hr with about 10% loss of activity at the high temperature over the course of the assay period. Respiratory activity is expressed as nmol of O_2 consumed min^{-1} g^{-1} fresh wt in that the addition of BSA to preparations made protein determination impossible.

All dehydrogenases were assayed spectrophotometrically utilizing a Gilford 2000 spectrophotometer equipped with a H_2O-jacketed cuvette chamber for temperature control. GDH and MDH were assayed as described before (9) with pellet and supernatant activites added to give total activity. ADH, G6P-DH, and NADP-ICDH assays were conducted with ammonium sulfate preparations from supernatants only in samples containing cotyledons. Ammonium sulfate precipitation of these enzymes was necessary due to endogenous reduction of nucleotides with the crude preparation. NADP-ICDH mixtures contained 2.5 ml of 0.1 M HEPES (pH 7.5), 0.1 ml of enzyme preparation, 0.2 ml of 0.03 M $NADP^+$, 0.1 ml of 0.15 M $MnSO_4$, and 0.1 ml of 0.15 M DL-isocitrate, with the last two listed additions incubated together for at least 5 min before their addition (16). ADH assay mixtures contained 2.6 ml of 0.1 M Trizma (pH 7.5), 0.1 ml of enzyme preparation, 0.2 ml of 10^{-3} M NAD^+, and 0.1 ml of 3 M ethanol. G6P-DH reaction mixtures contained 2.5 ml of 0.05 M HEPES, 0.1 ml of 0.15 M $MgCl_2$, 0.1 ml of enzyme preparation, 0.2 ml of 10^{-3} M $NADP^+$, and 0.1 ml of 0.04 M glucose-6-P. All assays except those of Arrhenius plots were conducted at 23 C. Activities of enzymes at 10 C were calculated for those extracted from cold-treated seeds (see Fig. 3) from the Arrhenius plots (see Fig. 4). Enzyme activities are expressed as nmol or μmol of nucleotide reduced or oxidized min^{-1} g^{-1} fresh wt or g^{-1} initial wt (weight before imbibition) and as ΔOD min^{-1} in Arrhenius plots. Again due to inert protein added to stabilize dehydrogenase activities (8), protein determinations of any reliability were impossible.

E_as were calculated as by Segel (34):

$$E_a = \frac{2.3\,RT_1T_2\,\log Q_{10}}{10}$$

with T_1 and T_2 for plots with two phases (*i.e.* all of Fig. 4) at 20 and 30 C for the upper phase and 0 and 10 C for the lower phase. This required extending plots in some cases (*i.e.* ADH, Fig. 4). All other E_as are calculated with 20 and 30 C for temperatures. All E_as are expressed in kcal/mol.

RESULTS AND DISCUSSION

Imbibition and Germination. Figure 1 indicates that from 0 to 3 hr, while submerged, imbibition at 23 C (24.5% increase in fresh wt/hr) was considerably faster than that at 10 C (16.7% increase in fresh wt/hr). Between 3 and 6 hr, both rates decreased. After removal from submergence (6 hr), rates of imbibition were almost equal (3.9 and 3.7% increase in fresh wt/hr, respectively, for 10 and 23 C treatments). Between 12 and 24 hr, the rate for 23 C imbibition was near zero while that at 10 C had slowed considerably. Because appreciable soybean germination occurred between 24 and 48 hr at 23 C, embryonic axes were separated from cotyledons at 48 hr and each part was weighed separately to determine its respective contribution to the marked increase in rate of weight gain during this period. At 48 hr, embryonic axes at 10 C weighed about 40% of those at 23 C. Weight gain at 10 C was very slow after 24 hr.

Germination studies consisted of 300 seeds each at 10 and 23 C. These two temperatures were chosen because screening for "cold tolerance" with soybean cultivars had been conducted by testing for germination at 10 C (20), and whole seedling

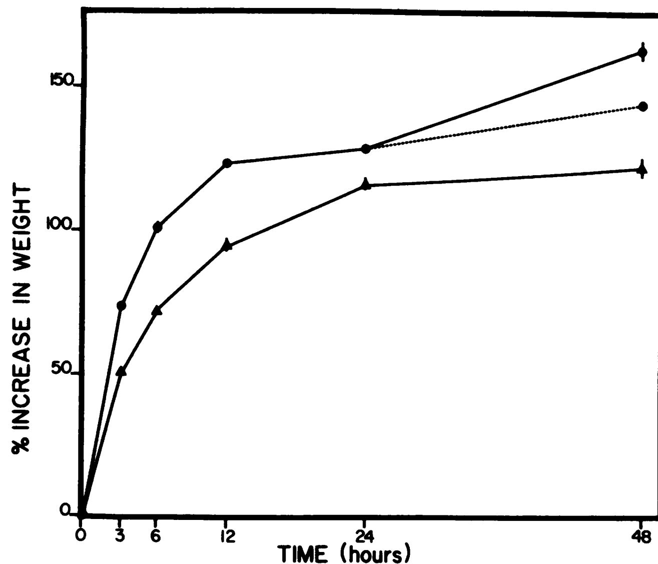

FIG. 1. Effect of temperature on soybean weight increase with imbibition and germination. Each point represents mean + SD of three (5 g each) groups of seeds. Seeds were imbibed at 23 C (●) and 10 C (▲) for 6 hr, submerged in distilled H_2O, and then maintained on H_2O-saturated tissue paper at these temperatures for the next 42 hr. (...): rate of weight increase less excised embryonic axes.

accumulation of dry matter appeared maximal between 20 and 25 C (32). At 23 C, 72% of the seed had germinated at 36 hr, whereas at 10 C this percentage was not achieved until after 144 hr (Table I). Wells appears to be among the slower soybean cultivars in germination at 10 C as compared to other cultivars (20). After germination at 10 C, hypocotyl extension was severely depressed and growth was slow and abnormal.

Mitochondrial Respiration. Embryonic axes were harvested chronologically at 48 hr for mitochondrial extraction because there were no comparable developmental stages at the two temperatures. Mitochondria from axes at 23 C had only one inflection point in the Arrhenius plot (12.5 C) for both state 3 and 4 respiration (Fig. 2). The RC ratios (ratio of state 3 to state 4 respiration, *cf.* Estabrook [10]) were increased to near 5.0 below this inflection, as compared to 2.8 above the inflection point. The E_a above the inflection temperature was 15.6 kcal/mol whereas the E_a below the inflection temperature was very large.

Our 10% loss of activity over the assay period compares favorably with the data of others (28) and does not explain the rapid drop in activity below the lowest inflection temperature for mitochondria extracted from axes of both treatments (Fig. 2). However, these substantial decreases in respiratory activity may be due to our measuring only initial state 3 and 4 respiration. Raison and Lyons have indicated that initial state 3 respiration of plant mitochondria is slow and becomes progressively slower as temperature is lowered; hence, they have either averaged states 3 and 4 over several cycles (22) or rejected initial state 3 values entirely (29). We could find no basis for rejecting initial state 3 or 4 respiration rates and feel that they may represent *in vivo* conditions more closely than rates obtained after several additions of substrate and ADP.

Endogenous nucleotide pools in mitochondria (13) may account for lower initial rates of mitochondrial respiration. Lower state 3 rates would be probable if these endogenous pools were inadequate for maximal ATP-ADP exchange (18). After several additions of ADP a supply of exchange nucleotides would accumulate within the mitochondria for potential exchange with the next ADP addition and rates would increase. This, however, might not represent the true physiological state of mitochondria when initially isolated.

The Arrhenius plot (Fig. 2) for respiration of mitochondria extracted from tissues at 10 C is discontinuous at 31 C and 8 C. Discontinuous three-phase plots seem to be normal for chilling-sensitive organisms (30); however, it is unusual that the phase at the higher temperature, as in our study, has a higher E_a than that of the next lowest phase (23.6 kcal/mol from 30-40 C and 7.8 kcal/mol from 20-30 C). The RC ratios for all phases were near 1.0. This could indicate a large endogenous supply of ADP within the mitochondria (13). Cold hydration of maize seeds has been shown to increase endogenous supplies of ADP (5). On the other hand this could reflect uncoupled mitochondria or ADP transport that is more cold-sensitive than respiration. In rat liver mitochondria, ADP transport is quite sensitive to low temperature, having an E_a of 28.5 to 34 kcal/mol below the Arrhenius inflection (18, 25). Following cold imbibition in maize, RC ratios were 1.0 and required several days for recovery when succinate was used as a respiratory substrate (5). Cold treatment greatly reduced RC ratios in *Triticum* (23) indicating that this may be a common response to cold treatment with plants. Respiration rates for the mitochondria of 10 C-treated axes were higher than those of 23 C-treated axes at all temperatures above 8 C.

Table I. *Germination of Soybeans at 10 and 23 C.*

Approximately 300 seeds were used at each temperature with germination determined by radicle emergence from the testa.

Time (hours)	Percent Germination	
	10 C	23 C
12	0	0
24	0	52
36	0	72
48	0	89
60	0	97
72	3	97
84	10	97
96	20	97
120	52	97
144	71	97
168	82	97
192	82	97

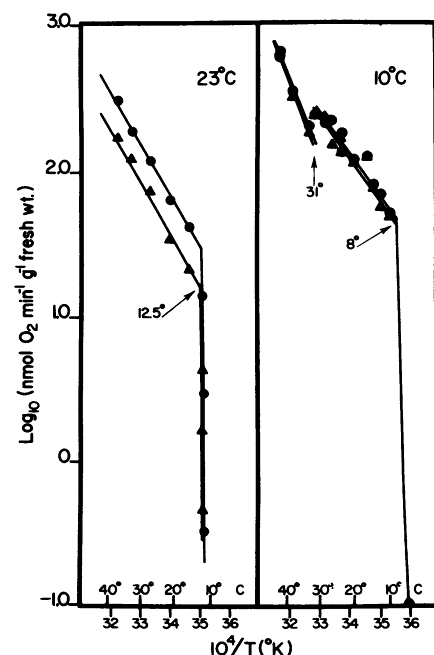

FIG. 2. Arrhenius plots of soybean axis mitochondrial respiration. Embryonic axes were excised from germinated seed (23 C for 48 hr) and ungerminated seed (10 C for 48 hr). State 3 respiration (●) and state 4 (▲) respiration were measured utilizing succinate as the respiratory substrate.

These differences that we observed in mitochondrial respiration with the two sources employed (10 and 23 C, 48-hr axes) may indicate that some type of modification occurred in the lipids of the mitochondrial membranes of soybean embryonic axes with chilling. Phospholipids in mitochondrial membranes have been directly implicated as accounting for the slopes and inflection points of the Arrhenius plots for mitochondrial respiration with observed differences in respiration at various temperatures reportedly due to changes in membrane phospholipid constituency (26, 27). Also, soybean phospholipids have been reported to be modified by cold treatment (36). In that our study covered only a short period, it is not possible to determine whether the observed differences in soybean mitochondrial respiration from the two treatments were of a transitory or permanent nature and if the cold treatment caused permanent injury. The change in the temperature of inflection to 2 degrees below the treatment temperature of 10 C with the cold-stressed seeds would indicate that to some extent chilling resistance may have developed. At this inflection point mitochondrial lipids undergo a phase change from a liquid-solid state to a solid state (<8 C) (27). Apparently, damage may result to mitochondrial membranes when the lipid portions are in a prolonged solid state (21, 27).

Dehydrogenase Activities and Freezing. Freezing has various effects on dehydrogenases. With freezing, lactate dehydrogenase, triose-P dehydrogenase, NADP-ICDH, ADH, and G6P-DH reportedly lost activity (4, 19), MDH gained activity (9) and GDH lost, gained, or remained the same in activity (4, 9, 19). In this study our results were similar to the aforementioned in that we found G6P-DH, ADH, and NADP-ICDH to lose activity and GDH and MDH to gain activity with storage at -10 C. Differences among dehydrogenases in response to freezing may be due to rearrangement of subunits (4) or to the breakdown of oligomer units. All compared activities reported here reflect activities after the same number of freeze-thaws (usually two or three). Arrhenius plots from preparations frozen various numbers of times did not vary significantly.

Figure 3 indicates that there was a general decrease in GDH activity on a fresh and initial wt basis for 24 hr with both treatments. The plot of activity on a fresh wt basis appears as

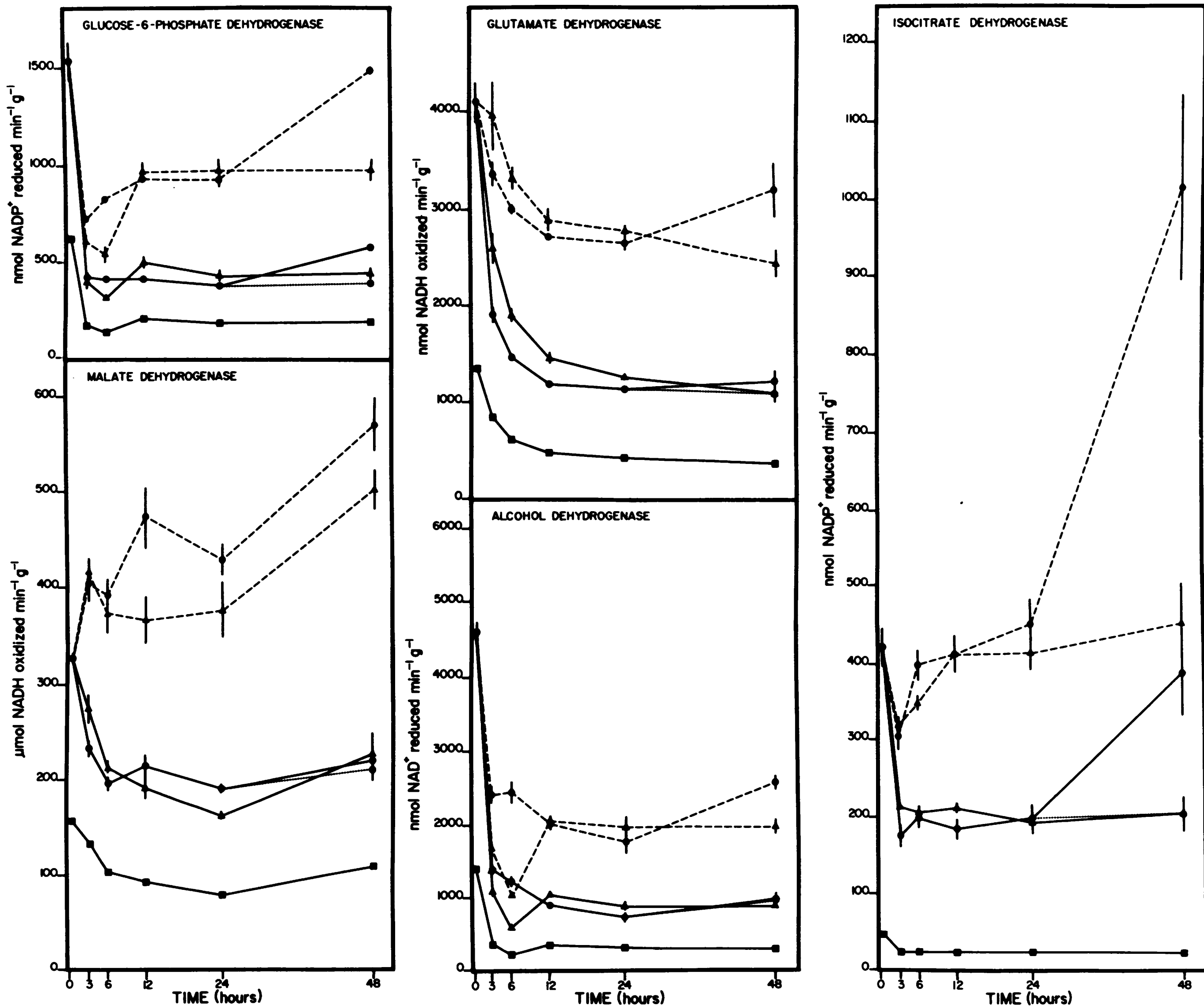

FIG. 3. Effects of temperature on dehydrogenase activities from soybean seed. Seeds (——) were maintained at 10 C (▲) and 23 C (●) with the first 6 hr fully submerged in H_2O. Each point represents mean + SD of three separate enzyme preparations. (——): activities on a fresh wt basis; (- - -): activities on an initial wt basis. Dehydrogenase activities for seeds of the 10 C treatment were calculated for 10 C (■) utilizing the Figure 4 Arrhenius plots (Fig. 4, 10 C activity/Fig. 4, 23 C activity × Fig. 3, 23 C activity for the 10 C treatments). Assays were conducted at 23 C. (——): activities of seeds less activities of excised embryonic axes.

an inverted mirror image of that for imbibition (Fig. 1). On an initial wt basis GDH began to increase in activity between 24 and 48 hr for the 23 C treatment. The GDH activity at 10 C for 10 C-treated seeds was calculated from Figure 4 to be about 35% of the 23 C activity (Fig. 3). The amount of GDH activity recovered from particulate (mitochondrial) fractions was 66.7 ± 8.8% and 36.1 ± 5.1% of the total activity for axes and cotyledons, respectively, after 48 hr at 23 C. In general, preparations increased in the percentage of mitochondrial dehydrogenases (GDH, MDH, and NADP-ICDH) recovered in the mitochondrial pellet (20,000g) over the course of the experiment (data not shown). This was true for both treatments and may have been due to increased mitochondrial integrity as has been shown to occur with imbibition in legumes (33). All GDH should have been mitochondrial in origin (7, 35) at this stage of development with any recovery of the enzyme in the supernatant due to mitochondrial disintegration.

The Arrhenius plot for cotyledon GDH activity (Fig. 4) has an inflection at 15 C. The E_a below the inflection point was twice that above it. Arrhenius plots of GDH activity from embryonic axes (Fig. 5) varied with the temperature at which axes were treated and with the extraction medium employed. E_as were 6.8 and 5.4 kcal/mol between 20 and 30 C for GDH

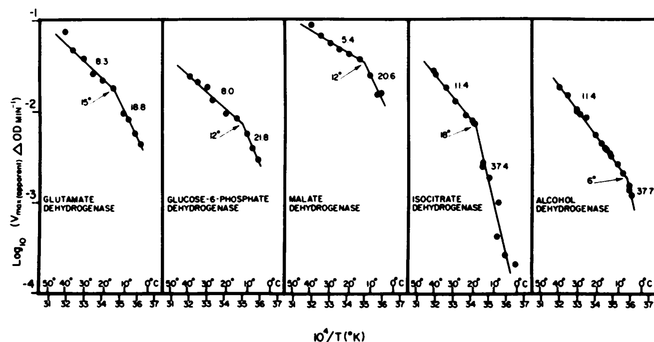

FIG. 4. Arrhenius plots of dehydrogenase activities from soybean cotyledons. Enzymes were extracted from cotyledons excised from seeds germinated at 23 C (48 hr). Temperatures of inflection points and E_as in kcal/mol are indicated. MDH and ADH activities are 0.01× and 0.1× actual activities, respectively. Assays were conducted with ammonium sulfate precipitants (ADH, G6P-DH, and NADP-ICDH) and mitochondrial pellets (GDH and MDH).

extracted from 23- and 10 C-exposed axes, respectively, when isolated in sucrose, and 5.2 and 2.8 kcal/mol, respectively, when isolated in sorbitol. The inflection point was 6 C for GDH from both treatments in sucrose and 7 C for the 23 C-treated axes in sorbitol. A discontinuity was observed at 17 C in the Arrhenius plot for GDH in sorbitol from axes of the 10 C treatment. Differences in GDH Arrhenius plots may have been due to changes in isoenzymes in germination at 23 C or to changes in polymerization and depolymerization (35) with the two treatments.

Glucose-6-Phosphate Dehydrogenase Activity. G6P-DH activity decreased rapidly during imbibition at both temperatures for the initial 3 hr (Fig. 3). During the next 3 hr, activity decreased at 10 C and increased at 23 C. Activities increased rapidly between 6 and 12 hr and then leveled for the duration of the experiment at 10 C. A rapid increase in activity due to axis activity was observed between 24 and 48 hr with the 23 C treatment.

Arrhenius plots of cotyledon (Fig. 4) and axis (Fig. 5) G6P-DH activities indicated differences in the enzyme from the two sources. An inflection was observed at 12 C in the cotyledon Arrhenius plot whereas none was observed with the axis plots. However, G6P-DH plots revealed that deactivation of the enzyme occurred at higher temperatures in axis preparations. Decreases in activity began at 27 C and 30 C with preparations containing sucrose and sorbitol, respectively. E_as for the two axis preparations with both sucrose and sorbitol were similar in preparations of axes (13.9–14.4 kcal/mol).

Malate Dehydrogenase Activity. During imbibition at 23 C there was an increase in MDH activity on an initial wt basis for the first 3 hr and between 6 and 12 hr (Fig. 3). From 24 to 48 hr the activity of MDH from the 23 C-treated seed increased to nearly twice that of the initial activity on an initial wt basis. At 10 C activity increased for the first 3 hr and then decreased for the next 3 hr much like the 23 C treatment. Activities of MDH from the 10 C treatment leveled between 6 and 24 hr and then increased for the last 24 hr of treatment. The amount of activity recovered in particulate fractions at 48 hr was about 39% and about 12% of the total activity for the 23 C-treated axis and cotyledon preparations, respectively, indicating that at least one-third of the MDH in axes was mitochondrial. In general, an increase was observed in the percentage of particulate MDH with time in both treatments as with GDH.

Arrhenius plots of MDH activity for cotyledon preparations

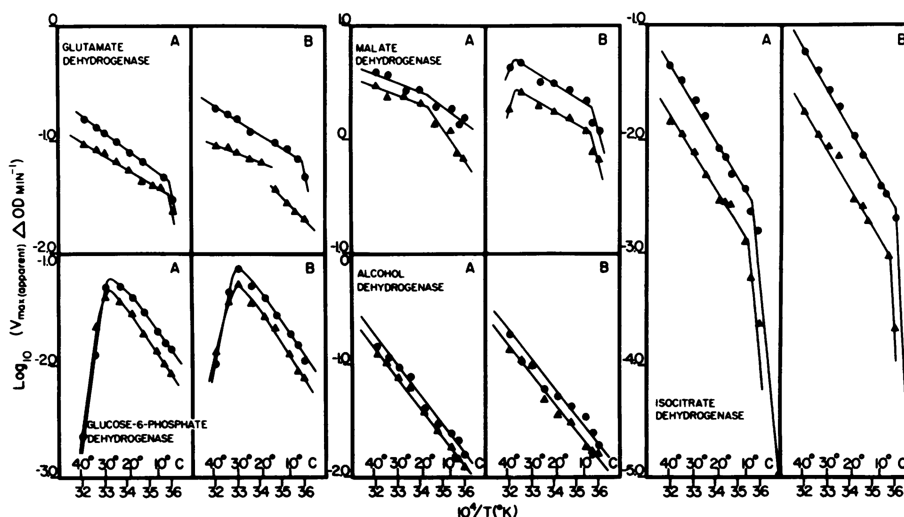

FIG. 5. Arrhenius plots of dehydrogenase activities from soybean axes. Enzymes were extracted from axes of seeds imbibed for 48 hr at 23 C (●) and at 10 C (▲). Enzymes were extracted in medium containing either 0.4 M sucrose (A) or 0.4 M sorbitol (B). GDH and MDH assays were conducted with mitochondrial pellets (20,000g) while all other enzymes were assayed with supernatant preparations (20,000g).

indicated an inflection temperature of 12 C (Fig. 4) whereas those for axis MDH indicated inflections at about 18 and 7 C for preparations with sucrose and sorbitol, respectively (Fig. 5). An apparent deactivation of MDH began at 38 C for preparations made with sorbitol. E_as between 20 and 30 C were higher in sorbitol (about 5.0 kcal/mol) than in sucrose (4.0 kcal/mol) for axis preparations. The same E_a was found between 9 and 10 C with MDH from axes prepared with sorbitol (20.1 kcal/mol) in contrast to one low (23 C preparation) and one high (10 C preparation) E_a for MDH extracted in sucrose. This would indicate that with the two temperature treatments there may be differences in MDH conformation or the proportion of mitochondrial MDH and cytosol MDH.

Alcohol Dehydrogenase Activity. ADH activity in many plants is apparently stimulated by anaerobic or submerged conditions (11); this appears not to be true during imbibition of soybeans in that we observed a considerable decrease in activity during the initial 6 hr of imbibition (Fig. 3). This decrease in ADH activity was somewhat greater for seeds imbibed at 10 C. After imbibition while submerged, activities of ADH at both temperatures leveled, to some extent, except for activity between 24 and 48 hr for the 23 C treatment where an increase was observed. This increase in ADH activity appeared to be due to increases in both cotyledon and axis activity because activities of the two tissues were much the same at 48 hr.

An inflection was observed at 6 C in the Arrhenius plot for cotyledon ADH activity (Fig. 4) while none was observed for plots of axis ADH activity (Fig. 5). The E_as for all plots between 20 and 30 C were nearly the same (11.2-11.5 kcal/mol for axis plots and 11.4 kcal/mol for cotyledon plots) indicating a possible homogeneity in enzyme complement of ADH with the two tissues and treatments.

NADP-Isocitrate Dehydrogenase Activity. Figure 3 indicates that NADP-ICDH activity decreased with the initial 3 hr or imbibition at both 10 and 23 C with a subsequent recovery in activity over the next 18 hr. Between 24 and 48 hr, initial wt activity of seeds at 23 C increased rapidly to about 2.5 times the initial activity. Much of this activity increase may be due to germination (Table I) and subsequent increases in axis activity because cotyledon activity alone is near that for the 10 C-treated seed. The NADP-ICDH activity calculated for 10 C (Fig. 3) from Figure 4 for 10 C-treated seeds was only 10% of the activity at 23 C.

The inflection temperature of 18 C in the Arrhenius plot of cotyledon NADP-ICDH activity (Fig. 4) was very high when compared to axis plots (Fig. 5). Axis plots had inflections at 7.7 and 9.4 C in sucrose and 4.6 and 7.0 C in sorbitol with the lower inflection temperatures in each case determined with NADP-ICDH from the 10 C treatment. E_as for axis activity between 20 and 30 C were slightly lower for the enzyme extracted from seeds at 23 C (14.7-15.1 kcal/mol) as compared to those of the enzyme extracted from seeds at 10 C (15.7-15.9 kcal/mol). E_as between 0 and 10 C were about 35 kcal/mol for all preparations. It should be noted that E_as for NADP-ICDH were similar to those for mitochondrial respiration at 23 C (Fig. 2) indicating a possible relationship between it and mitochondrial respiration in soybeans. We found all extractable ICDH activity from mitochondria to be NADP-linked as have others working with legume mitochondria (2). However, there is some controversy over the nature of NADP-ICDH in that some researchers have found a large amount of this enzyme in plant mitochondria (2, 6) and others have claimed or implicated that only a small proportion is associated with mitochondria (16). We found that as much as 30% of NADP-ICDH could be found in particulate preparations of axes indicating that in soybeans the enzyme may be to some extent mitochondrial in nature. These data indicate that if NADP-ICDH is the citric acid cycle ICDH in legumes it could limit respiration at low temperature.

Dehydrogenases and Cold Treatment. The importance of energy transduction and the production of reduced nucleotides in cold acclimation has been recognized (1, 19); however, little is known as to how these factors are influenced by cold sensitivity. Brown (3) has shown that during imbibition and germination of *Pisum* there is a gradual increase in the proportion of reduced nicotinamide nucleotides in the form of NADPH (26% for dry seeds, 29% for imbibed seeds, and 39% for germinated seeds) indicating that NADP-linked dehydrogenases may gain importance in energy transduction during these processes. Our data indicate that NADP-ICDH and to a lesser extent G6P-DH could account for increases in the percentage of NADPH during imbibition and germination. Both of these enzymes were drastically affected by cold. The effect on NADP-ICDH was kinetic in that its activity was greatly lowered at 10 C whereas the effect of G6P-DH may have been both kinetic and a reduction in the total amount of enzyme in that a reduction in activity at both 10 and 23 C was observed (Fig. 3). ADH was similar to G6P-DH in its response to cold imbibition. During germination, both NADP-linked dehydrogenases assayed increased in activity significantly, and to a greater extent than NAD-linked dehydrogenases.

The differences in Arrhenius plots that we observed with cotyledon and axis dehydrogenase activities may have been due to the degree of association with lipids. Removal of phospholipids from such enzymes as ATPase or succinate dehydrogenase results in a one-phase (constant slope) Arrhenius plot such as we observed with axis ADH and G6P-DH, and the addition of lipids yields a two-phase (inflection in the slope) Arrhenius plot (17, 29). The nature of the lipid added will determine the slopes of the Arrhenius plots. Because soybeans contain large amounts of phospholipids, especially in cotyledons, it seems reasonable to assume that such enzymes as MDH, which is known to be associated with mitochondrial membranes, could respond like succinate dehydrogenase (2, 29) or ATPase in the presence of various lipids. All dehydrogenases are similar in physical characteristics (31) and might respond to various lipid environments in a similar manner. Our data indicate that all of the dehydrogenases tested had inflections in Arrhenius plots for activity when extracted from cotyledons whereas one-phase plots were observed for such cytosol enzymes as ADH and G6P-DH when extracted from seed axes. This could be indicative of lipid interactions with all cotyledon enzymes assayed and the absence of lipid interactions with cytosol axis enzymes assayed. Preliminary experiments with nonionic detergent extraction of these dehydrogenases indicate that this is the case (data not shown). As with mitochondrial respiration, differences in Arrhenius plots of enzymes from the 10 and 23 C treatments such as GDH and MDH may have been due to cold modification of soybean mitochondrial phospholipids (36). Arrhenius plot differences of dehydrogenase activities with the two extraction media employed may have been due to changes in solute-H_2O interactions, direct solute effects on the enzymes, or solute-lipid interactions.

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