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Changes in Enzyme Regulation during Growth of Maize

I. PROGRESSIVE DESENSITIZATION OF HOMOSERINE DEHYDROGENASE DURING SEEDLING GROWTH[1]

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ABSTRACT

The sensitivity of homoserine dehydrogenase (EC 1.1.1.3) to inhibition by the feed-back modifier, L-threonine, was examined in preparations derived from etiolated shoots, roots, and light-grown tissues of *Zea mays* L. var. earliking. A progressive decrease in enzyme sensitivity was observed during seedling growth. Enzyme derived from internode tissue retained a greater sensitivity to the effector than enzyme derived from apical portions of etiolated shoots, whereas enzyme from root tips was characteristically more sensitive than that prepared from mature cells of the root. Enzyme desensitization occurred rapidly during culture of excised shoots and the activities of both homoserine dehydrogenase and aspartokinase (EC 2.7.2.4) declined during shoot culture under a variety of conditions. The initial enzyme levels and the characteristic sensitivity of homoserine dehydrogenase were preserved during culture at 5 to 7 C, but desensitization was not prevented by inclusion of cycloheximide in the culture medium.

Results of control experiments provide evidence that desensitization occurs *in vivo*. No alteration of the enzyme properties was detected during extraction or concentration of sensitive or insensitive enzyme or during coextraction of enzyme from mixed populations of different age shoots; nor was a differential distribution of inhibitors or activators indicated during assay of mixed preparations. The change in enzyme sensitivity was apparent under a variety of assay conditions and was not accompanied by changes in the apparent affinity of the enzyme for the substrate, homoserine. It is suggested that systematic changes in the regulatory characteristics of certain enzymes could be an important level of metabolic regulation during cellular differentiation.

Three forms of maize homoserine dehydrogenaase were detected after acrylamide gel electrophoresis of samples derived from 72-hr shoots. Similar analysis of samples from older shoots revealed a broad asymmetric band of enzyme activity, suggesting that changes in the relative distribution of specific forms of the enzyme could be related to the growth-dependent changes in the sensitivity of maize homoserine dehydrogenase.

Metabolite control of enzyme activities associated with amino acid biosynthesis has been documented in a variety of plants (4, 19). In contrast to microbial systems, however, evidence of end-product control of the concentration of a number of key biosynthetic enzymes has not been obtained in higher plants (2, 7, 13, 20, 22, 30). This may reflect significant differences in the range and extent of regulatory mechanisms which serve to balance metabolism in dissimilar organisms. A greater extent of metabolite compartmentation in structurally complex multicellular plants would be expected (24, 31), but unique mechanisms may also contribute to metabolic regulation in these organisms.

The present experiments are concerned with alterations in the activities of homoserine dehydrogenase (EC 1.1.1.3) and aspartokinase (EC 2.7.2.4) during development of *Zea mays* L. var. earliking. These enzymes contribute to the regulation of the biosynthetic pathway leading from aspartic acid to several amino acids in both microorganisms and higher plants (1, 3, 5, 9, 11). Regulatory properties of homoserine dehydrogenase were analyzed after extraction of the enzyme from tissues of seedlings at different stages of development. During germination and early seedling development of maize, degradation products of the endosperm serve as a major source of amino acids (15), and the effects of removal of this source of protein precursors on the enzyme activities were examined by culturing excised shoots in defined media.

MATERIALS AND METHODS

Materials. Seeds of *Zea mays* L. var. earliking were obtained from Joseph Harris Co., Rochester, N.Y. Substrates, coenzymes, nitro blue tetrazolium, phenazine methosulfate, and the majority of organic compounds used were purchased from Sigma. Vitamin-free casein hydrolysate and deproteinized coconut milk were supplied by Difco and Gibco, respectively. Sepharose 4B and Sephadex G-25 were purchased from Pharmacia. Cyanogen bromide and hexamethylenediamine were obtained from Aldrich. Electrophoretic materials were of P.A.G.E. purity from Isolab as was bromphenol blue. All other chemicals were reagent grade.

Growth Conditions and Measurements. Seeds were surface-sterilized with 0.52% (v/v) NaOCl for periods of 10 to 30 min, washed thoroughly with sterile H_2O, and germinated under axenic conditions or planted in vermiculite. Dark-grown seedlings were maintained at 28 C for periods of 72 to 168 hr. Seeds planted in vermiculite were irrigated regularly with a 1 g/l solution of Hyponex (Hydroponic Chemical Co.), and the seedlings were grown for 5, 7, 12, or 19 days at 26 C in a growth chamber on an 18-hr light/6-hr dark cycle. Illumination was provided by four 20-w fluorescent lights supplemented with a 15-w incandescent bulb (600 ft-c). Plants grown for 60 days were obtained from a garden plot. Length and fresh

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weight determinations were based on random samples of 50 shoots or roots.

Culture Conditions for Excised Shoots. After 72 hr of growth in the dark, the etiolated shoots were excised at the scutellar node. One hundred to 150 excised shoots were transferred to a 2.6-liter Fernback flask containing approximately 500 ml of liquid medium. In most of the experiments, the balanced salts medium of Nason (21) containing 2% (w/v) sucrose was utilized and was adjusted to pH 5.2 prior to autoclaving. Contamination was minimized by inclusion of 50 μg/ml each of penicillin G and streptomycin sulfate in the media and carrying out all operations in a sterile transfer room. Unless otherwise specified, the excised shoots were cultured at 28 C in the dark without shaking.

Enzyme Extraction from Etiolated Seedlings. Freshly excised (hereafter referred to as intact) or cultured shoots were homogenized in a chilled Waring Blendor with buffer (0.2 M tris-HCl, pH 8.5, containing 0.1 M KCl, 1 mM EDTA, 1.4 mM 2-mercaptoethanol, 5 mM L-threonine, and 30% [v/v] glycerol) in a volume of 0.5 or 1 ml/g fresh weight. All subsequent operations were carried out at 5 to 7 C. Homogenates were centrifuged at 20,000*g* for 60 min, and the resultant supernatants were adjusted to pH 8.2 prior to concentration of the protein. Protein was concentrated by addition of a saturated solution of $(NH_4)_2SO_4$ to obtain 50 or 66% saturation and collected by centrifugation after 30 min of mixing. The precipitated protein was dissolved in a small amount of 50 mM potassium phosphate buffer, pH 7.5, containing 1.4 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM L-threonine, and 20% (v/v) glycerol. The soluble protein was dialyzed overnight against a large excess of the same buffer. Alternatively, dry Sephadex G-25 (1 g/5 ml) was added to the centrifuged extracts and allowed to absorb buffer. The concentrated enzyme preparation was removed from the swollen Sephadex by centrifugation through nylon mesh. This procedure was repeated until the desired enzyme concentration was achieved. Equivalent amounts of enzyme activity were obtained by each method but less protein was recovered when $(NH_4)_2SO_4$ at 50% saturation was used.

Preparation of Homoserine Dehydrogenase from Light-grown Seedlings. Plant material extending above the surface of the vermiculite (or soil) was harvested, and the enzyme was extracted as described above. Homogenates were pressed through nylon mesh to remove fibrous material prior to centrifugation at 20,000*g* for 60 min. The protein fraction of the resultant supernatant precipitating between 25 and 65% saturation with $(NH_4)_2SO_4$ was collected, dissolved in the potassium phosphate buffer, pH 7.5, and dialyzed overnight.

Enzyme Assays. Homoserine dehydrogenase activity was determined from the initial rates of coenzyme reduction in the presence of homoserine. Changes in absorbance at 340 nm and 25 ± 1 C were recorded using a Gilford Model 240 spectrophotometer attached to a Sargent Model SR recorder with full scale adjusted to 0.1 A_{340}. All rates were corrected for small amounts of nonspecific activity and were linear for at least 3 min. One unit of enzyme activity is defined as the amount of enzyme required to produce a change in A_{340} of 0.001/min. The reaction mixtures utilized for these measurements have been described previously (3). L-Homoserine and NAD^+ or $NADP^+$ were routinely employed at concentrations of 20 mM, 6.7 mM, and 0.48 mM, respectively, representing about 10 times K*m* in each case (3).

Aspartokinase activity was measured by the hydroxamate procedure described by Bryan *et al.* (5) with 50 mM L-aspartate and 25 mM ATP. One unit of aspartokinase activity is defined as the amount of enzyme producing 1 nmole of β-aspartyl phosphate/hr. Controls from which aspartate had been omitted from the reaction mixture were included in every experiment.

Protein Determinations. Protein was measured by the methods of Kalckar (17) and Waddell (28). Average values derived from these two spectrophotometric methods proved to be in good agreement with those based on the chemical method of Lowry *et al.* (18) (unpublished data).

Sepharose-HMD column chromatography. Sepharose 4B was activated by cyanogen bromide (250 mg/ml settled gel volume) and HMD was coupled to the activated Sepharose according to the method of Cuatrecasas (10). One-half to one ml samples representing about 40 mg protein were applied to columns containing 1.5 ml of Sepharose-HMD[3] and homoserine dehydrogenase was eluted with five column volumes of 50 mM potassium phosphate buffer, pH 7.5, containing the additions noted above, followed by consecutive washings with the same buffer containing 50 mM, 300 mM, or 1 M KCl. The bulk of the activity was recovered during elution with the first two buffers. Purification of up to 10-fold was achieved.

Polyacrylamide Disc Gel Electrophoresis. Enzyme samples derived from Sepharose-HMD column chromatography were analyzed by electrophoresis on 5% acrylamide gels according to the method of Davis (12). Gels were removed from the apparatus and placed in reaction mixtures consisting of 60 mM tris-HCl, pH 9, containing 0.15 mM EDTA, 0.21 mM 2-mercaptoethanol, 150 mM KCl, 32 mM L-homoserine, 0.266 mg/ml NBT, 0.025 mg/ml PMS, and 13.4 mM NAD^+ or 0.96 mM $NADP^+$. Gels were incubated in the dark at 30 C in a shaker bath until dye precipitation representing enzyme activity was apparent (10–90 min). Identical gels incubated in staining mixtures from which homoserine was omitted were utilized to detect nonspecific activity. Gels were scanned at 600 nm, utilizing a linear transport cell attached to a Gilford Model 240 spectrophotometer.

RESULTS

Growth and Time-dependent Changes in Enzyme Activities of Etiolated Shoots. The growth of intact and cultured shoots, defined by increases in length and fresh weight, is illustrated in Table I. The growth rate of cultured shoots was less than that of intact plants but both length and fresh weight doubled after 70 hr of culture (Fig. 1A). There was an initial decrease in extractable protein when shoots were transferred to liquid media (Fig. 1A). Although a reduced synthesis of protein was observed during culture of excised maize embryos (23), the relatively small amounts of protein obtained from shoots which were cultured in the presence of cycloheximide (Table II) suggest that substantial protein degradation may also occur during culture. Net increases in protein were not observed until after 48 hr of shoot culture.

The levels of both aspartokinase and homoserine dehydrogenase activity decreased progressively during culture (Fig. 1B). In contrast, the activity of homoserine dehydrogenase in intact shoots increased between 72 and 120 hr and remained relatively constant or decreased slightly between 120 and 168 hr of growth (Table I). The results of aspartokinase determinations in extracts of older intact shoots have proven to be quite variable. For example, values ranging from 67.1 to 155 units/shoot and 42.4 to 106 units/shoot have been obtained with preparations from shoots of seedlings grown for 120 and 168 hr, respectively. Although the data are not conclusive, it appears that both enzymes follow a similar pattern of change in intact shoots.

[3] Abbreviations: HMD: hexamethylenediamine; NBT: nitro blue tetrazolium; PMS: phenazine methosulfate.

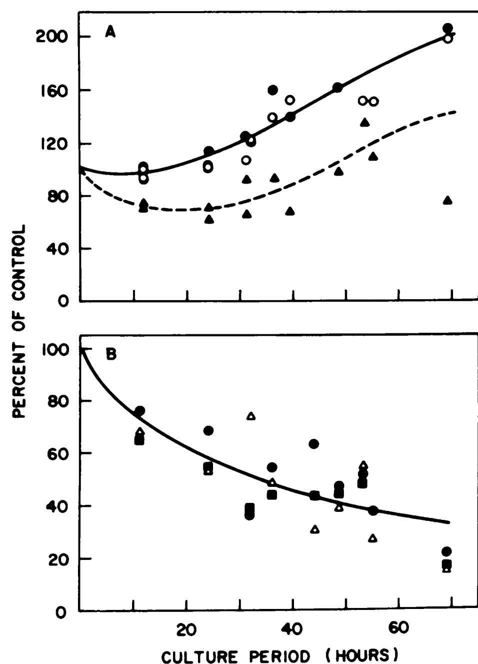

FIG. 1. Growth and enzyme activities of excised shoots during culture in liquid media. A: Length (○); fresh weight (●); protein (▲). B: NAD^+-dependent homoserine dehydrogenase activity (●); $NADP^+$-dependent homoserine dehydrogenase activity (■); aspartokinase activity (△). Shoots of seedlings grown for 72 hr were transferred to Nason's medium and cultured for the indicated times prior to growth and enzyme measurements. Freshly excised uncultured shoots served as the control (see Table I, 72-hr shoots for average values). For each experiment, results (per shoot) obtained from approximately 300 intact shoots were compared with those obtained from an equivalent number of cultured shoots.

The coordinate loss of both enzyme activities was prevented by culture of the excised shoots at low temperature. The activity of aspartokinase and homoserine dehydrogenase increased slightly during culture at 5 to 7 C for periods of 36 to 48 hr. Other attempts to improve shoot growth or preserve the initial levels of enzyme activity by supplementing the standard Nason's medium were unsuccessful. Supplements included 0.4 mg/ml casein hydrolysate, 5 and 20% (v/v) deproteinized coconut milk, 50 μg/ml *meso*-inositol, 2 μg/ml 2,4-dichlorophenoxyacetic acid, 10 μM GA_3, 10 μM kinetin, 0.2 M mannitol, a synthetic mixture of amino acids (23), and various combinations of the pathway products. Different plant tissue culture media were also utilized in other experiments. In no instance was growth improved nor were the enzyme levels significantly higher than those listed in Table I.

Cycloheximide inhibited growth but did not prevent loss of enzyme activities (Table II). Aspartokinase activity was essentially identical when shoots were grown in the presence or absence of cycloheximide, whereas less homoserine dehydrogenase activity was observed when cycloheximide was present. Such differential effects on these enzymes combined with the observation that aspartokinase and homoserine dehydrogenase activities can be partially separated during column chromatography (unpublished results of Bryan, Matthews, and DiCamelli) suggest that the enzyme activities are associated with different proteins in maize, unlike the analogous activities in *Escherichia coli* (8).

Changes in Regulatory Characteristics of Homoserine Dehydrogenase during Shoot Growth. The sensitivity of homoserine dehydrogenase to inhibition by its feedback modifier, L-threonine, was tested by inclusion of 10 mM threonine in the

Table I. *Changes in Growth and Enzyme Activity of Intact and Excised Shoots*

The data are averages of the results obtained in 3 to 10 separate experiments, each involving 100 to 1000 seedlings, and are presented as the mean ± SE: intact 72 hr, n = 8; 120 hr, n = 3; 168 hr, n = 6; excised, n = 10. Intact shoots were obtained from seedlings grown for the indicated periods. Excised shoots were derived from 72-hr seedlings and cultured in Nason's medium for 48 hr prior to preparation of the enzymes. Homoserine dehydrogenase inhibition was measured in the presence of 10 mM threonine with standard assay mixtures containing either 6.7 mM NAD^+ or 0.48 mM $NADP^+$ as coenzyme and 20 mM L-homoserine.

Measurements	Conditions and Duration of Growth (hr)			
	Intact 72	120	168	Excised 72 + 48
Growth				
Length (mm/shoot)	46.1 ± 3.5	93.5 ± 4.3	155 ± 8	72.0 ± 2.7
Fresh weight (mg/shoot)	182 ± 18	471 ± 43	788 ± 48	275 ± 10
Protein (μg/shoot)[1]	645 ± 54	827 ± 89	1040 ± 89	698 ± 38
Homoserine dehydrogenase:				
Units/shoot				
NAD^+	48.0 ± 4.0	75.0 ± 15.6	63.3 ± 7.6	17.7 ± 1.1
$NADP^+$	22.7 ± 2.8	43.5 ± 7.7	33.3 ± 3.8	10.7 ± 0.8
Units/mg fresh wt				
NAD^+	0.26	0.16	0.08	0.06
$NADP^+$	0.12	0.09	0.04	0.04
Units/mg protein				
NAD^+	74.4	90.7	70.7	26.2
$NADP^+$	35.2	52.6	32.0	15.5
Inhibition (%)				
NAD^+	81.7 ± 1.9	69.7 ± 3.8	34.6 ± 2.4	43.3 ± 5.9
$NADP^+$	53.9 ± 4.7	41.8 ± 5.2	24.7 ± 3.0	29.7 ± 4.1
Aspartokinase:				
Units/shoot	53.8 ± 4.6			20.7 ± 1.0
Units/mg fresh wt	0.32			0.08
Units/mg protein	83.4			30.2

[1] In all of the experiments involving intact shoots, protein was precipitated with 50% $(NH_4)_2SO_4$, whereas 66% saturation was employed with cultured material. These procedures yield equivalent amounts of enzyme but the latter results in recovery of slightly larger amounts of protein (approximately 10%). For comparison with intact material, the specific activities of enzyme derived from excised shoots would be slightly higher than those indicated.

Table II. *Effects of Cycloheximide on Growth and Enzyme Activity of Cultured Shoots*

Measurements were made with preparations derived from shoots of seedlings which had been grown for 72 hr, excised and cultured in Nason's medium containing the indicated concentrations of cycloheximide for 48 hr. The results are the average of duplicate determinations from experiments involving 150 to 300 excised shoots. Results obtained in the absence of cycloheximide are presented in Table I.

Measurement	Cycloheximide Concn. (μg/ml)			
	10	50	100	250
Growth				
Length (mm/shoot)	58.6	44.0	46.6	39.0
Fresh wt (mg/shoot)	210	180	185	160
Protein (μg/shoot)	550	390	340	240
Homoserine dehydrogenase				
Activity (units/shoot)				
NAD^+	16.4	11.6	11.3	10.7
$NADP^+$	9.2	7.2	6.9	6.7
Aspartokinase:				
Activity (units/shoot)	24.0	23.0	21.6	22.1

standard assay mixtures. The extent of inhibition under these conditions was significantly reduced in preparations derived from either cultured or older shoots (Table I). Inhibition of the maize enzyme is only partial and this concentration of threonine should be nearly saturating (3). A reduction of 82% to 46% in the extent of inhibition of the NAD^+-dependent activity suggests that more than one-half of the enzyme remaining after culture of excised shoots could be insensitive to threonine. This change in a regulatory characteristic is not due to excision and culture of the shoots as a clear progression of enzyme desensitization, measured with either NAD^+ or $NADP^+$ as coenzyme, also occurs during the growth of intact shoots.

The loss of sensitivity to inhibition by threonine occurs quite rapidly during culture of the shoots; enzyme isolated from shoots cultured only 12 hr was inhibited 48% with NAD^+ as coenzyme and 29% with $NADP^+$ compared with 82% and 54% prior to culture. Enzyme sensitivity, evaluated after 48 hr of culture, was not preserved during any of the experiments in which Nason's medium was supplemented with various compounds nor during treatment with cycloheximide. In the one treatment which prevented loss of enzyme activity, *i.e.* culture at low temperature, enzyme sensitivity was also preserved. Therefore, if loss of sensitivity can be experimentally dissociated from loss of activity during culture, the appropriate combination of conditions has not been identified.

All concentrations of threonine tested were effective in inhibiting both sensitive and insensitive enzyme preparations (Fig. 2). However, the difference between the sensitivity of individual preparations is greater at low effector concentrations.

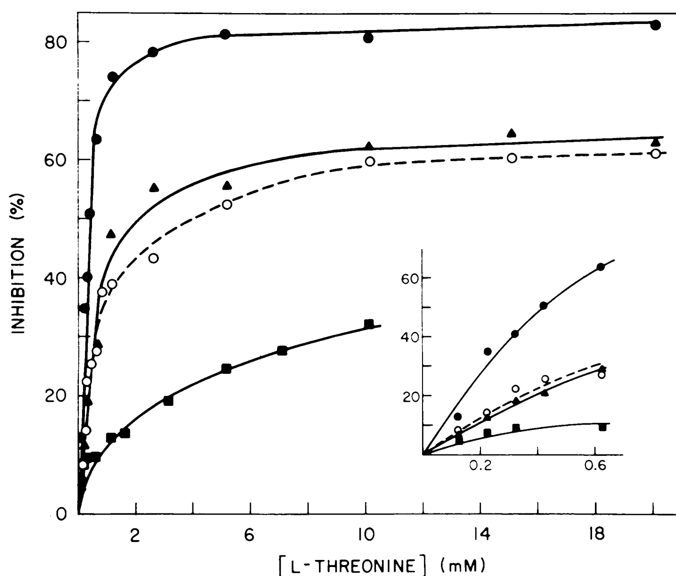

FIG. 2. Effect of threonine concentration on the activity of homoserine dehydrogenase isolated from maize shoots. Enzyme was derived from shoots of seedlings grown for 72 hr (●), 120 hr (▲), 168 hr (■), and from shoots which had been excised after 72 hr of growth and cultured in Nason's medium for 48 hr prior to isolation of the enzyme (○). Enzyme activity was measured with 6.7 mM NAD^+ as coenzyme and 20 mM L-homoserine. The velocities measured at low concentrations of L-threonine were extrapolated to those which would occur in the absence of threonine to correct for the small amount of inhibitor present (threonine was added to all buffers as a stabilizing agent during preparation of the enzyme). Inhibition was calculated using the extrapolated values as the controls. Results are averages of duplicate experiments utilizing 300 to 1000 seedlings. The inset illustrates the effects of low concentrations of threonine.

Table III. *Coextraction of Sensitive and Insensitive Homoserine Dehydrogenase from Mixtures of Plants*

Measurements	Mixed Preparations[1]							
	A		B		C		D	
	Predicted[2]	Observed[3]	Predicted	Observed	Predicted	Observed	Predicted	Observed
Activity (units)								
NAD^+	4900	4330	6200	5360	5870	6050	7080	7570
$NADP^+$	2790	2110	3510	3440	2820	2740	3370	3470
Specific activity (units/mg protein)								
NAD^+	31.6	33.9	38.7	34.0	110	107	73.3	78.1
$NADP^+$	18.0	16.7	21.9	22.0	52.9	48.4	34.9	37.1

[1] The following mixtures of plant materials were utilized: A: 20 g of intact shoots (96 hr) + 20 g of cultured shoots (84-hr + 48-hr culture); B: 30 g of intact shoots + 10 g of cultured shoots; C: 15 g of intact shoots (72 hr) + 15 g of intact shoots (168 hr); D: 17 g of intact shoots (72 hr) + 16 g of intact shoots (168 hr). Protein was concentrated by precipitation with 66% $(NH_4)_2SO_4$ with the exception of C where 50% $(NH_4)_2SO_4$ was utilized.

[2] Enzyme was independently isolated from samples of each component of a specific mixture, and the amount of activity obtained was used to calculate the predicted activity from the coextracted mixture.

[3] The average of duplicate assays of enzyme activity is presented in each case.

For example, at 20 mM threonine the extent of inhibition was 83% and 36% for enzyme derived from shoots of seedlings grown for 72 hr and 168 hr, respectively (NAD^+ as coenzyme); the comparable values in the presence of 1 mM threonine were 71% and 12%.

Preservation of Differential Enzyme Properties during Isolation. Efforts have been made to rule out a variety of possible experimental artifacts. The conditions of isolation were varied to maximize the yield of homoserine dehydrogenase and modifications of the standard procedures included inclusion of Polyclar AT (Bio-Rad) during enzyme extraction, use of different buffers and potential stabilizing agents, and concentration of the enzyme with Sephadex G-25 instead of $(NH_4)_2SO_4$. Although reduced yields were obtained under certain conditions, none of the variations affected the sensitivity of the enzyme to inhibition by threonine. Very sensitive enzyme was consistently obtained from young shoots and less sensitive enzyme was extracted from older intact or cultured shoots. Preparations could be stored at −20 C for several weeks without alteration of the enzyme properties. However, under the appropriate conditions maize homoserine dehydrogenase can be experimentally desensitized (3).

Potential effects of components of older or cultured shoots on the yield of enzyme from younger shoots were investigated by coextraction of homoserine dehydrogenase from mixed populations of plant material (Table III). The general agreement between the predicted and observed enzyme recoveries and specific activities tends to eliminate the possibility that a substantial portion of enzyme in older or cultured shoots is inactivated during isolation. To detect even subtle alterations in enzyme sensitivity, the inhibition of enzyme prepared from equal amounts of 72- and 168-hr shoots by threonine concentrations of 0.05 to 10 mM was calculated from data obtained from homogeneous material and compared with results obtained with preparations from mixtures of plants (Fig. 3). In these and similar experiments involving mixtures of freshly excised and cultured shoots, the observed measurements averaged 100.9% ± SE 3.1% (n = 58) of the expected values. Although properties of isolated enzymes should not be equated with *in vivo* characteristics without extreme caution, these results indicate that the differences between enzyme isolated

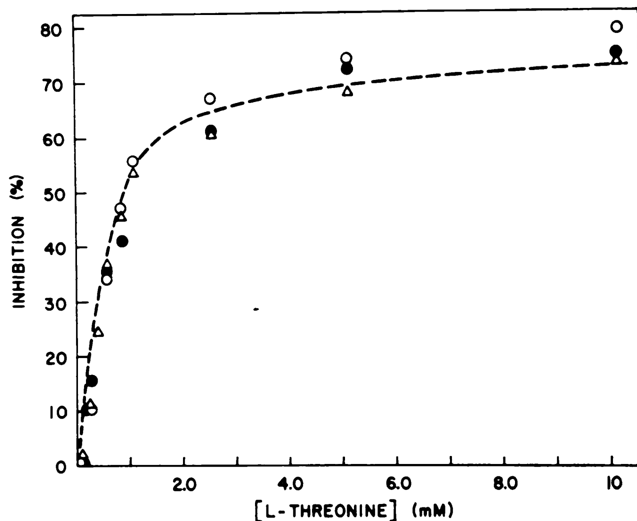

FIG. 3. Effect of threonine concentration of homoserine dehydrogenase prepared from mixtures of plant material. A theoretical curve (– – –) was calculated from results of NAD^+-dependent enzyme activity obtained independently with preparations of intact shoots which had been grown for 72 or 168 hr. Data were obtained with enzyme isolated from 1:1 (w/w) mixtures of 72-hr and 168-hr shoots. In one case (○), the pH of the soluble extract resulting from cohomogenization of the plants was adjusted to 8.2 prior to concentration of the enzyme with $(NH_4)_2SO_4$, and in the other (●) pH was not adjusted. (△) = 1:1 (v/v) mixture of enzyme extracts resulting from independent extraction of shoots of seedlings grown for 72 and 168 hr, respectively.

from various materials are not the result of differential modification of enzyme during extraction.

Variation of Enzyme Sensitivity in Different Tissues. The sensitivity of homoserine dehydrogenase derived from roots of 72- or 168-hr seedlings was identical, unlike the results obtained with shoots of different ages. The NAD^+-dependent activity of both root preparations was inhibited 60% by 10 mM threonine. Since the growth pattern of roots differs from that of shoots, enzyme derived from 1-cm apical root tips, which are characterized by actively dividing and elongating cells, was compared with enzyme prepared from the remainder of the root, composed of fully differentiated cells (Fig. 4). Homoserine dehydrogenase derived from root tips proved to be more sensitive to threonine than that prepared from the basal portions (Fig. 4). The lack of time-dependent changes in whole roots probably reflects a relatively constant proportion of enzyme contributed by apical and basal portions of roots at 72 or 168 hr due to development of secondary roots in older plants.

Enzyme derived from internode tissue and from the coleoptile and developing plumule of 72- and 168-hr shoots was also examined (Fig. 4). The apical and basal portions of 72-hr shoots contained enzyme of equal sensitivity. However, a major difference in the sensitivity of preparations of the apical and basal portions of 168-hr shoots was observed. A pronounced decrease in the sensitivity of homoserine dehydrogenase of the apical portion occurred during growth, while only a slight decrease in the sensitivity of the enzyme from internode tissue occurred.

Homoserine dehydrogenase derived from 120-hr etiolated shoots, whether prepared from segments or whole shoots, tended to exhibit more variability in the level of sensitivity than enzyme from younger or older plants. This may reflect a relatively rapid onset of desensitization occurring around 120-hr growth of shoots in the dark. Nevertheless, the characteristic properties of enzyme isolated from different tissues were consistently observed. Enzyme isolated from the internode tissue of 120-hr seedlings exhibited the same sensitivity as that prepared from internodes of 168-hr seedlings, whereas the enzyme derived from the apical regions of 120-hr seedlings was unusually insensitive (Fig. 5). No evidence of activators or inhibitors in the preparations of different tissues was obtained upon assay of mixtures of the enzyme preparations (Fig. 5).

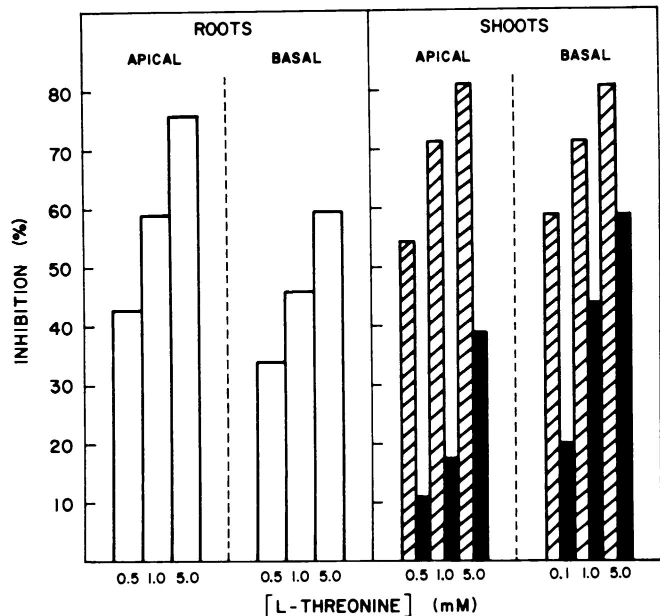

FIG. 4. Inhibition of homoserine dehydrogenase activity after isolation of the enzyme from different tissues of maize seedlings. Roots: 1-cm root tips (apical) and the remainder of the roots (basal) of 72-hr seedlings served as the source of the enzyme. Shoots: the tissue above the coleoptilar node (apical) and the material between the coleoptilar and scutellar nodes (basal) from shoots of 72-hr ▨ and 168-hr ■ seedlings were used. Inhibition of NAD^+-dependent enzyme activity measured as described in "Materials and Methods" at three concentrations of L-threonine is indicated.

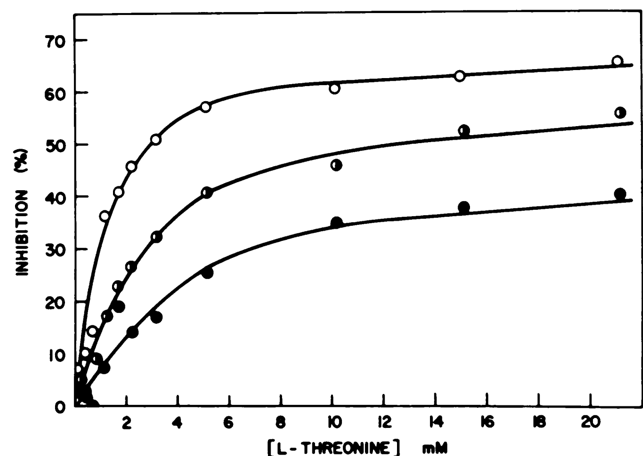

FIG. 5. Differential effect of threonine concentration on homoserine dehydrogenase isolated from different regions of maize shoots. Shoots of seedlings grown for 120 hr were harvested and divided into separate regions as described in the legend of Fig. 4. Enzyme was prepared from the coleoptilar portion (●) and internode tissue (○); results obtained with a 1:1 (v/v) mixture of the individual preparations (◑). Data are the average of duplicate determinations of NAD^+-linked enzyme activity.

Changes in Maize Shoots during Growth in Light. To establish whether the changes in the regulatory characteristics of homoserine dehydrogenase are restricted to dark-grown plants, enzyme was derived from seedlings which had been grown in the light for 5 to 60 days (Table IV). Enzyme activity per shoot was variable but consistently higher than that obtained from etiolated shoots (Table I). The enzyme sensitivity of 5-day light-grown shoots was equivalent to that of 72-hr dark-grown shoots. The greatest decrease in enzyme sensitivity, evaluated at high effector concentrations, occurred between 5 and 7 days in plants grown under either set of conditions. In light-grown plants, sensitivity remained at a relatively constant level after 7 days. This level is slightly higher than that observed for 168-hr etiolated shoots, perhaps reflecting a degree of senescence in the latter case. Nonetheless, growth-dependent losses in sensitivity of maize homoserine dehydrogenase to inhibition by the feed-back modifier, threonine, clearly occur under a variety of conditions.

Table IV. *Changes in Shoots during Growth of Maize Seedlings in Light*

Data are averages of the results of two to four independent experiments except for the data for plants grown for 60 days, which are derived from a single experiment. Threonine inhibition was measured in standard assay mixtures containing 10 mM threonine with either NAD^+ or $NADP^+$ as coenzyme. Plants were grown for the indicated periods under a light/dark regime as described.

Measurements	Duration of Growth (days)				
	5	7	12	19	60
Fresh wt (g/shoot)[1]	0.29	0.49	0.89	1.45	10.6
Protein (mg/shoot)	1.58	1.62	2.78	5.80	33.0
Homoserine dehydrogenase					
Units/shoot					
NAD^+	113	78.5	73.8	163	480
$NADP^+$	69.8	40.4	39.1	87.8	224
Units/mg fresh wt					
NAD^+	0.39	0.16	0.08	0.11	0.05
$NADP^+$	0.24	0.08	0.04	0.06	0.02
Units/mg protein					
NAD^+	79.0	49.3	27.1	28.6	14.5
$NADP^+$	48.8	24.5	14.1	15.2	6.7
Inhibition by 10 mM L-threonine (%)					
NAD^+	81.2	53.9	50.1	51.0	44.3
$NADP^+$	49.8	35.0	31.2	32.4	

[1] All plant material extending above the vermiculite substrate (soil for 60 day plants) was used for the experiments.

Characteristics of Sensitive and Insensitive Homoserine Dehydrogenase. Inhibition of maize homoserine dehydrogenase does not result in sigmoid kinetics, and threonine is apparently competitive with respect to homoserine (Figs. 2, and 6). This proved to be true for enzyme derived from either cultured (Fig. 6B) or young shoots (Fig. 6A). However, this form of inhibition can only be demonstrated at less than saturating concentrations of threonine. When excess threonine was present linear noncompetitive kinetics were observed. The apparent K*i* for threonine is increased with enzyme from cultured shoots, but no changes in the catalytic characteristics of insensitive enzyme have been detected. The apparent K*m* for homoserine estimated from the double reciprocal plots of velocity *versus* substrate concentration illustrated in Figure 6B is similar to that determined for enzyme derived from uncultured shoots (Fig. 6A) or roots (3). Homogeneous preparations have not been obtained which precludes evaluation of V_{max} for either sensitive or insensitive enzyme. The ability of either NAD^+ or $NADP^+$ to serve as the coenzyme during catalysis of homoserine oxidation was also characteristic of all preparations of the maize enzyme, as was the differential inhibition of these two activities by equivalent concentrations of threonine.

Homoserine dehydrogenase was partially purified from plants of different ages by chromatography on Sepharose-HMD columns. Column fractions were assayed and subjected to disc gel electrophoresis. Scans of gels exhibiting typical patterns are illustrated in Figure 7. Enzyme preparations of 72-hr shoots (Fig. 7A) consistently contained three forms of homoserine dehydrogenase, designated I, II, and III. Electrophoresis of enzyme fractions from 168-hr shoots (Fig. 7B) revealed one major peak corresponding to form II and a more diffuse region of activity corresponding to form III was observed in most gels. Form I was not detected in preparations of older plants,

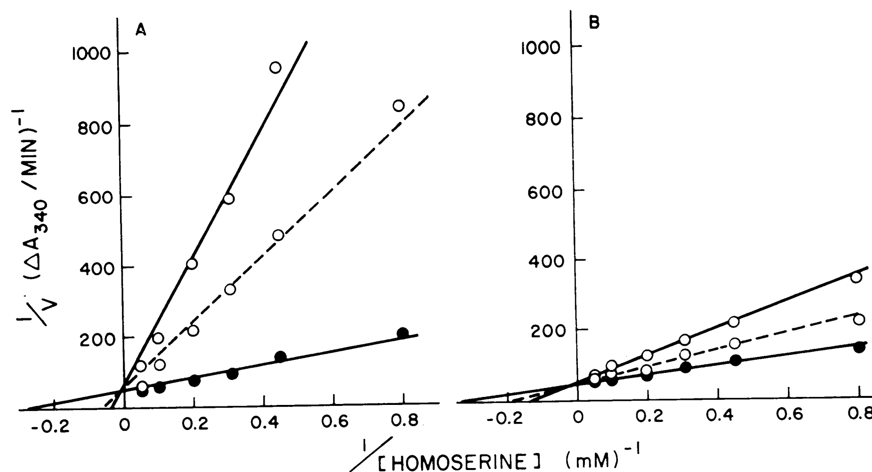

FIG. 6. Effects of L-homoserine concentration on the activity of homoserine dehydrogenase in the presence of different concentrations of threonine. A: Enzyme from uncultured shoots grown for 72 hr. L-Threonine = 0.05 mM (—●—); 0.55 mM (···○···), and 1.05 mM (—○—). B: Enzyme from shoots cultured 48 hr in Nason's medium after 72 hr normal growth in the dark. L-Threonine = 0.125 mM (—●—); 1.125 mM (···○···), and 5.125 mM (—○—). Assays were performed as described with 6.7 mM NAD^+ as coenzyme.

representing a clear age-dependent difference in the enzyme patterns.

DISCUSSION

There are many examples of experimental desensitization of regulatory enzymes by physical or chemical treatments *in vitro* (27), but the present results suggest that desensitization may also occur *in vivo*. The results of detailed control experiments designed to detect desensitization during isolation of the enzyme were uniformly negative. Homoserine dehydrogenase derived from intact etiolated shoots, excised shoots, or light-grown seedlings exhibited growth-dependent loss of sensitivity to the pathway product, threonine. Although the loss of enzyme sensitivity was correlated with a loss of enzyme activity during shoot culture, enzyme activity increases in intact seedlings while sensitivity decreases. Thus, the phenomenon of desensitization was not dependent upon a net loss of enzyme activity nor was it prevented by cycloheximide during shoot culture.

Culture of developing maize shoots resulted in the progressive loss of the activity of two enzymes required for the synthesis of several amino acids. Since neither homoserine dehydrogenase, nor aspartokinase activity was elevated by removal of the supply of amino acids derived from the endosperm, nor did inclusion of exogenous amino acids in the culture medium result in further reduction of enzyme levels, evidence of enzyme repression was not obtained. The present data would be consistent with enzyme repression only if excision stimulated endogenous proteolysis such that amino acid levels were sufficiently elevated to cause maximum repression. Nevertheless, similar negative results have been obtained in other investigations (2, 7, 13, 20, 22, 30) suggesting, at least, that end-product repression of enzymes associated with amino acid biosynthesis does not appear to be a common regulatory mechanism in multicellular plants. Depending upon the specific molecular mechanisms involved it is possible that enzyme desensitization could serve a function similar to that of derepression.

Growth appears to be a pre- or corequisite of enzyme desensitization in maize and two observations suggest that this phenomenon may occur during specific stages of cellular development. First, the rate of desensitization varies with growth conditions. For example, the same degree of sensitivity was exhibited by enzymes isolated from 120-hr light-grown and 72-hr dark-grown shoots. Second, the extent of desensitization is tissue specific. Thus, the more mature portion of roots, internode tissue or etiolated seedlings, and expanding leaves of light-grown seedlings all contain enzymes which exhibit characteristic degrees of insensitivity. In contrast, younger tissues, *i.e.* root tips and 72-hr etiolated shoots, contain enzyme which is extremely sensitive to inhibition by threonine. The changes in enzyme properties therefore appear to occur during cellular maturation or differentiation in a variety of different cell types. Verification of this possibility will require examination of more uniform and synchronously growing material.

Differentially regulated forms of homoserine dehydrogenase-aspartokinase are characteristic of several species of bacteria (9) and two forms of homoserine dehydrogenase, only one of which is associated with aspartokinase activity, have been recently identified in pea seedlings (1). Preliminary results obtained during the present studies suggest that neither form I nor form III of the maize enzyme possesses aspartokinase activity but whether the multiple forms of maize homoserine dehydrogenase are analogous to those of either peas or bacteria remains to be established. However, the existence of multiple forms may provide an explanation of growth-dependent desensitization in maize. If one (or more) of the forms is insensitive to inhibition, then differential distribution of this form(s) relative to the total enzyme population in specific tissues could account for preparations which differ with respect to sensitivity.

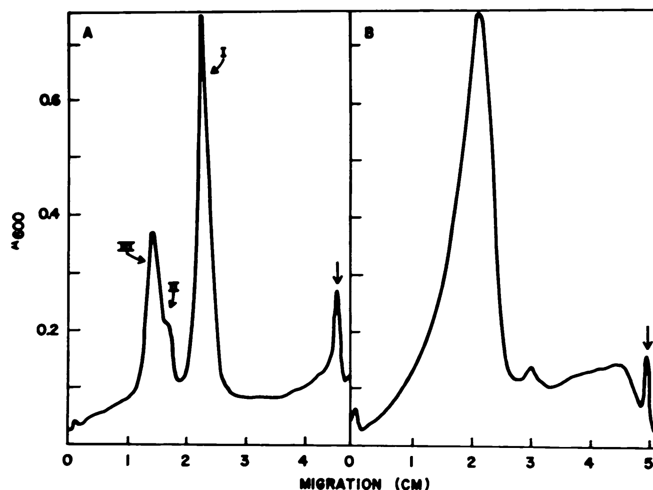

FIG. 7. Recorder traces of acrylamide gels which had been stained for homoserine dehydrogenase activity. Electrophoresis and visualization of enzyme activity were carried out as described. Enzymes were eluted from Sepharose-HMD columns with potassium phosphate buffer, pH 7.5, containing 50 mM KCl. A: Enzyme from shoots of 72 hr seedlings; the sample was 88 μg of protein. B: Enzyme from 168 hr seedlings; the sample was 168 μg of protein. Arrows indicate the migration of bromphenol blue. I, II, and III represent three different forms of maize homoserine dehydrogenase. These bands were not detected when duplicate gels were incubated in the absence of homoserine after electrophoresis. The broad asymmetric band illustrated in B is located in the region of the gel corresponding to forms II and III. The small band in B represents nonspecific activity as judged by the results of control experiments.

Evaluation of the sensitivity of most enzyme preparations was based on assays of enzyme activity in the presence of 10 mM L-threonine. This procedure probably underestimated the actual extent of desensitization. For example, the difference between the apparent inhibitor constants (K_i) derived from the results presented in Figure 6 suggested that the enzyme from cultured shoots was 25-fold less sensitive than that from uncultured shoots. Threonine appears to interact at a regulatory site distinct from the catalytic site (3) and, therefore, may not represent a true expression of the affinity of either sensitive or insensitive enzyme for threonine. Other quantitative expressions of sensitivity such as the effector concentration required to achieve 50% inhibition are also difficult to apply to maize homoserine dehydrogenase, since enzyme derived from 168-hr shoots was not inhibited 50% under any of the conditions tested. Nevertheless, significant differences between the activities of sensitive and insensitive enzymes clearly would be manifested under an extremely wide range of substrate and inhibitor concentrations. Although homoserine dehydrogenase activity was measured in the direction of homoserine oxidation, earlier results including *in vitro* enzyme desensitization suggest that only quantitative variations would be observed upon measurement of aspartic semialdehyde reduction (3).

The observed differences between the properties of sensitive and insensitive homoserine dehydrogenase can be considered analogous to the differences between enzymes in wild-type and certain regulatory mutants in bacteria (26). The general, but not necessarily exclusive, effect of such mutations affecting enzyme sensitivity is an overproduction of threonine. Depending upon the nature of other regulatory parameters, isoleucine

production may also be stimulated since threonine serves as a precursor of this amino acid. Furthermore, the unregulated activity of homoserine dehydrogenase could result in lysine limitation by virtue of a depletion of the branch point intermediate aspartic semialdehyde. The *in vivo* activity of any enzyme clearly depends upon a large number of factors, but the concentrations of both threonine and isoleucine do increase while levels of a number of other amino acids decrease during the growth of maize seedlings (29). Increased amino acid biosynthesis resulting from changes in metabolic regulation could be of importance during normal development even though the exact physiological consequences are not predictable at this time. In certain multicellular plants, profound developmental alterations have been attributed to amino acid imbalance (14) and a variety of metabolic effects resulting from amino acid excess or limitation have been reported in bacterial and mammalian cells (see ref. 16 for example).

The low sensitivity of assay procedures suitable for use with crude enzyme preparations and the difficulties encountered in assay of aspartokinase activity from older shoots have hampered detailed examination of the characteristics of this maize enzyme. Preliminary results suggest that the enzyme from older or cultured shoots, like that of young shoots (5, 6), is inhibited by L-lysine but the effects of low and presumably more physiological concentrations of lysine have not been studied. Oaks *et al.* (25) suggested that the biosynthesis of proline becomes progressively more resistant to end-product inhibition during the maturation of maize root cells. Their investigations involved analysis of tracer data, since direct examination of the relevant enzymes was not possible. Similar conclusions regarding the phenomenon of *in vivo* enzyme desensitization have been reached, based on entirely different methodology and relating to two different pathways of amino acid biosynthesis. Transition from stringent regulatory to less sensitive modulation of the activity of key biosynthetic enzymes may represent an important level of metabolic regulation during growth of multicellular plants.

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