Comparison of Sensitive and Desensitized Forms of Maize Homoserine Dehydrogenase[1]

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ABSTRACT

The properties of homoserine dehydrogenase (EC 1.1.1.3) isolated from shoots of young etiolated seedlings of *Zea mays* L. var. earliking can be reversibly altered by dialysis against an appropriate buffer. Treatment with 500 millimolar potassium phosphate buffer (pH 7.5) in the absence of L-threonine results in diminished regulatory control such that the enzyme becomes less sensitive to feedback inhibition. The physical and regulatory properties of experimentally altered and unaltered enzymes are compared with those of enzyme isolated from shoots of older seedlings. Multiple forms of both sensitive and insensitive enzymes are identified, and a model which is consistent with the observed isozymes and the difference in regulatory properties of enzymes obtained from seedlings of different ages is proposed. The initially sensitive enzyme is postulated to undergo a conformational change followed by formation of insensitive multimeric aggregated forms. The experimental conditions which facilitate alteration of the enzyme are discussed in relation to conditions which could occur *in vivo*.

The biosynthesis of amino acids in plants is characterized by diverse regulatory mechanisms (2, 16). Recent results obtained in a number of laboratories suggest that temporal or developmental changes in specific regulatory enzymes occur during the growth of plants (4). Previous investigations of maize homoserine dehydrogenase (EC 1.1.1.3) indicated that this enzyme becomes progressively less sensitive to inhibition by the feedback modifier L-threonine during growth of etiolated or light-grown seedlings (14). These results were extended by demonstration of this phenomenon with enzyme obtained from intact chloroplasts isolated from leaves of different ages (6). In addition, the regulatory properties of the enzyme are tissue-specific (10). In all cases multiple forms of the enzyme were detected. Similar changes in the regulatory properties of soybean homoserine dehydrogenase have been reported (15) whereas the enzyme isolated from young or old leaves of *Vicia faba* showed no differences in regulatory properties (5).

The present investigation represents a continuation of the characterization of changes in homoserine dehydrogenase during the growth of maize. Enzyme obtained from young seedlings can be reversibly altered *in vitro*. The regulatory properties of the altered enzyme differ markedly from those of the native enzyme and are similar to those of enzyme isolated from older plants. Furthermore,

the physical and catalytic characteristics of the altered enzyme are indistinguishable from those of enzyme isolated from older maize seedlings. A model which accounts for multiple forms of homoserine dehydrogenase is proposed and the conditions required for experimental alteration are related to those which may facilitate changes of the enzyme *in vivo*.

MATERIALS AND METHODS

Materials. Substrates and coenzymes were obtained from Sigma. Reagents for polyacrylamide gel electrophoresis were PAGE[3] purity from Isolab. Gel filtration media were obtained from Bio-Rad or Pharmacia. Agarose-2',5'-ADP was purchased from P-L Laboratories. All other chemicals were the best reagent grade available from commercial sources.

Plant Material, Enzyme Preparation, and Assay. Etiolated seedlings of *Zea mays* L. var. earliking (Harris Seed Co., Rochester, N. Y.) were grown axenically for 72 h at 28 C. Shoots were excised and homoserine dehydrogenase was prepared as described previously (14). The enzyme was partially purified by conventional techniques (8) and by affinity chromatography with Agarose-2',5'-ADP. Homoserine and coenzymes were used at concentrations approximately $10 \times K_{m_{app}}$ during assays (1). The reaction was initiated by addition of either enzyme or coenzyme. One unit of enzyme is defined as the amount required for a change in A_{340} of 0.001/min at 25 C. Enzyme sensitivity was established by duplicate assays in the presence and absence of 10 mM L-threonine and is expressed as the per cent inhibition.

Protein Determinations. Protein was routinely measured spectrophotometrically by the methods of Kalckar (13) and Waddell (18). The protein content of crude extracts and highly purified preparations was also measured by the Bio-Rad protein assay (Technical Bulletin 1051).

Gel Filtration Chromatography. Upward flow gel filtration chromatography was performed at 4 C in columns (1.5 × 90 cm) containing Bio-Gel A 0.5m equilibrated in 50 mM K-phosphate containing 1.4 mM 2-mercaptoethanol, 1.0 mM EDTA, 5.0 mM L-threonine, and 20% (v/v) glycerol unless otherwise noted. Column fractions were assayed for NAD^+- and/or $NADP^+$-dependent enzyme activity.

Electrophoresis. PAGE was carried out at 5 to 7 C and the results analyzed as previously described (3). After electrophoresis, active forms of the enzyme were detected by incubation of the gels at 30 C (14). Coomassie brilliant blue R was utilized to stain gels for protein (3).

Sucrose Density Gradient Centrifugation. Five per cent and 20% sucrose solutions were prepared in either 50 or 500 mM K-phosphate (pH 7.5) containing 1.4 mM 2-mercaptoethanol, 1.0 mM EDTA, and 20% (v/v) glycerol. Threonine was included as indicated in the text. Linear gradients were prepared and centrifuga-

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[3] Abbreviation: PAGE: polyacrylamide gel electrophoresis.

tion was performed at 62,000 rpm in a Beckman SW Ti-65 rotor at 4 C. Catalase (Sigma) and *Leuconostoc mesenteroides* glucose 6-P dehydrogenase, supplied by H. R. Levy of this department, were added to the preparations of homoserine dehydrogenase as internal markers. Gradient fractions were collected from the bottom of the tube via a pressure system and all enzyme activities were measured by standard procedures.

RESULTS

Experimental Desensitization. Dialysis of maize homoserine dehydrogenase against most media tested did not effect the enzyme's sensitivity to inhibition by the feedback effector, L-threonine (Table I). Only the conditions utilized for experiment 10 resulted in a stable enzyme which exhibited reduced sensitivity. This dialysis medium differs from the buffer in which the enzyme is normally stored in that it does not contain threonine and the concentration of K-phosphate is increased from 50 to 500 mM. Enzyme desensitized in this medium was comparatively stable during exposure to 50 C (*cf.* experiments 5, 6, 11 versus 10) and maintained the reduced sensitivity without loss of catalytic activity for at least 6 months at −20 C. Unless specified, experimental desensitization was therefore performed as indicated for experiment 10 in Table I.

During purification of homoserine dehydrogenase from young maize seedlings, native enzyme sensitivity was maintained, as was the ability to desensitize the enzyme experimentally (Table II). In all experiments, the loss of sensitivity, measured with either NAD+ or NADP+ as coenzyme, exceeded the loss of catalytic activity. In several cases, one of which is illustrated in Table II, no loss of activity occurred during desensitization. Thus, the experimental

Table I. Desensitization of Maize Homoserine Dehydrogenase

Enzyme preparations were isolated from shoots of etiolated seedlings as described previously (14), partially purified (8), and stored at −20 C in 50 mM K-phosphate (pH 7.5) containing 1.4 mM 2-mercaptoethanol (ME), 1.0 mM EDTA, 20% (v/v) glycerol, and 5.0 mM L-threonine. Enzyme was then dialyzed for 44 h at 5 C against an excess of the indicated buffer. One change of medium was made at 20 h. K-phosphate was utilized for experiments at pH 7.5 while Mes and Tris were used for the low and high pH experiments, respectively. One or all of the additives present in the storage buffer were omitted in individual experiments as indicated.

Experi-ment No.	Characteristics of Dialysis Media			Sensitivity[a]		Activity[b]	
	pH	mM	Omission	After dialysis	After heat-ing[c]	After dialysis	After heat-ing[c]
1	5.5	50	None	85.5	43.1	92.2	19.5
2	7.5	5	None	84.1	84.4	106	102
3	7.5	50	None	81.9	85.7	100	100
4	7.5	500	None	81.9	84.4	99.7	124
5	7.5	50	All	9.3		64.7	0
6	7.5	500	All	30.1		33.0	0
7	7.5	500	ME	85.6	89.1	85.4	65.6
8	7.5	500	EDTA	80.2	89.8	92.4	90.4
9	7.5	500	Glycerol	80.5	87.4	118	44.4
10	7.5	500	Threonine	57.6	47.9	97.9	75.0
11	7.5	50	Threonine	81.3	56.2	90.4	27.9
12	9.0	50	None	81.7	35.0	114	11.8

[a] Sensitivity is measured as described under "Materials and Methods" and is the per cent inhibition of NAD+-dependent activity by 10 mM L-threonine.

[b] Enzyme activity is expressed as a per cent of NAD+-linked activity measured before experimental treatment.

[c] Aliquots were heated for 10 min at 50 C, chilled, and analyzed.

Table II. Experimental Desensitization of Enzymes of Different Specific Activities

Enzyme isolated from young etiolated maize shoots (S.A. 50.6, NAD+/ 29.1, NADP+; N = 23) was partially purified by a series of conventional techniques which included differential fractionation with ammonium sulfate, treatment with calcium phosphate gel, ion exchange and gel filtration chromatography (8). Specific activity was calculated as described under "Materials and Methods," and the results of several independent experiments (N) were averaged.

Enzyme Preparations		Assay	Sensitivity[a]			Activity[b] Decrease
N	Specific activity		Initial	Final	Decrease	
					%	%
5	77.4	NAD+	81.3	46.5	42.8	29.7
	44.9	NADP+	51.0	33.8	33.7	26.5
5	129	NAD+	83.6	35.7	57.3	35.2
	81.6	NADP+	55.9	20.4	63.5	38.2
4	1,140	NAD+	92.1	38.4	58.3	29.7
	790	NADP+	69.8	33.2	52.4	25.8
1	5,090	NAD+	88.7	26.1	70.5	0
	2,710	NADP+	67.9	22.0	67.6	5.9

[a] Sensitivity of the enzyme to inhibition by 10 mM L-threonine was measured before (Initial) and after 45 h dialysis against desensitizing medium (Final) as described in Table I, experiment 10. All results are based on duplicate assays and corrected for nonspecific activity when necessary.

[b] Decrease in activity was calculated from measurements of total activity before and after desensitization.

alteration does not result from differential inactivation of a unique sensitive form of the enzyme. The process apparently does not depend upon an intrinsic factor which can be readily removed during purification. An enzyme preparation representing greater than 2,000-fold purification of the enzyme present in crude extracts has been experimentally desensitized.

Threonine as a Determinant of Enzyme Sensitivity. When a preparation of sensitive enzyme was passed through a small column of Sephadex G-25 equilibrated in desensitizing medium, less than 10% decrease in sensitivity was observed, initially or after storage at 5 or −20 C. However, dialysis of the gel-filtered enzyme preparation against desensitization medium resulted in the expected desensitization. These results suggested that some factor which contributes to the preservation of enzyme sensitivity was not removed during buffer exchange on G-25. To test whether this factor was threonine, aliquots of a sensitive enzyme were dialyzed for 45 h against two changes of desensitizing medium containing defined concentrations of threonine (Fig. 1). The extent of desensitization was clearly dependent upon the level of threonine in the dialysis medium. In addition, the reduction in sensitivity obtained at one threonine concentration could be enhanced by redialysis against desensitization medium containing a lower concentration of the effector. Based on a linear transformation of the results (Fig. 1 inset), it can be estimated that only 2 μM threonine is required to maintain half-maximal sensitivity of the enzyme under these conditions. Thus, the unexpected lack of desensitization during gel filtration was probably due to incomplete removal of threonine. This conclusion was reinforced by finding that the level of sensitivity obtained under the conditions used for the experiments illustrated in Table II could be further reduced by repeated changes of the medium and extended dialysis.

The critical role of threonine in the alteration of the regulatory properties of the maize enzyme is further illustrated in Table III. For this series of experiments, a sensitive enzyme preparation was
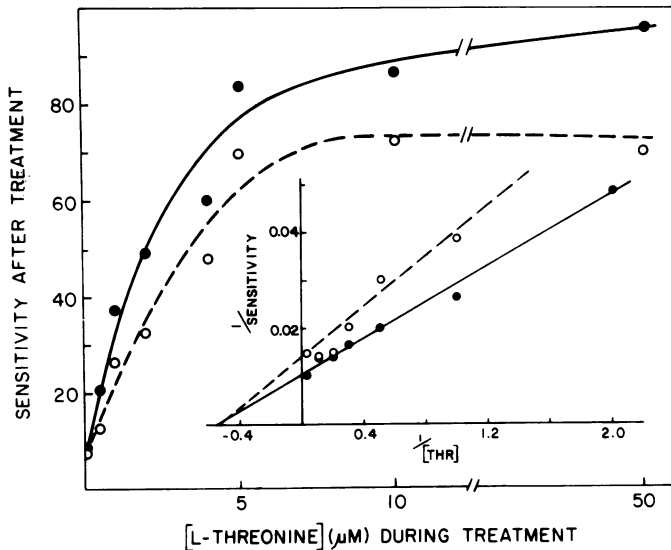
FIG. 1. Effect of threonine on regulatory properties of maize homoserine dehydrogenase. Enzyme was isolated from shoots of young seedlings and partially purified (S.A. 2140, $NAD^+/960$, $NADP^+$). Aliquots were dialyzed for 44 h against 0.5 M K-phosphate (pH 7.5) containing 1.4 mM 2-mercaptoethanol, 1.0 mM EDTA, 20% (v/v) glycerol, and the indicated concentrations of L-threonine. The ratio of buffer to sample was approximately 250:1 and two changes of medium were made during the period of dialysis. After treatment, enzyme sensitivity was measured. (O, ●): Measurements of $NADP^+$- and NAD^+-dependent activities, respectively.

divided into two portions, one of which was experimentally desensitized. Aliquots of both the sensitive enzyme and its desensitized counterpart were then treated as indicated. Treatment of the sensitive enzyme with desensitization medium to which threonine had been added resulted in the preservation of sensitivity (treatment B). However, identical treatment of the desensitized preparation resulted in an increase in sensitivity. The increase was slight immediately after dialysis but further increase was observed during storage at −20 C (or 5 C in separate experiments). Although DTT neither preserves sensitivity nor is essential for resensitization, it appears to enhance changes in sensitivity. In the absence of added threonine, inclusion of DTT in the desensitization medium resulted in a substantial loss of sensitivity of either enzyme preparation during treatment (Table III, treatment C). However, the sensitivity of both preparations subsequently increased during storage. Whether these preparations contained trace amounts of threonine which contributed to the slow resensitization during storage was not established. Inclusion of both threonine and DTT in the desensitization medium prevented desensitization of the sensitive enzyme and resulted in complete resensitization of the desensitized preparation (treatment D). The actual rate of resensitization under various conditions has not been established, but rapid resensitization has not been observed under any experimental conditions tested to date. Further experiments are in progress to obtain a better understanding of resensitization and the conditions which affect its rate and extent. It is evident that experimental desensitization does not irreversibly alter maize homoserine dehydrogenase.

Properties of Experimentally Desensitized Enzyme. The apparent K_m values for homoserine, NAD^+, and $NADP^+$ using desensitized enzyme (data not shown) were essentially equivalent to those obtained with enzyme from roots (1) or with enzyme from young or old shoots (14). In addition, the V_{max} of the altered enzyme does not differ significantly from that of the native enzyme since a major difference in V_{max} before and after treatment would be apparent at the near saturating levels of substrates utilized for routine measurements of enzyme activity (Table II). Thus, sensi-

tive and desensitized enzymes are indistinguishable on the basis of the catalytic parameters which have been measured.

The decreased sensitivity of experimentally desensitized enzyme is analogous to that observed with enzyme obtained from older seedlings. For example, the extent to which any given concentration of threonine inhibits enzyme from older plants or altered enzyme from young seedlings is proportionately decreased in comparison with inhibition of native enzyme isolated from young plants. Furthermore, the reduced sensitivity of experimentally altered enzyme is apparent under a wide range of assay conditions including measurements of the conversion of aspartate semialdehyde to homoserine (5).

Characterization of Multiple Forms of Sensitive and Desensitized Enzyme. PAGE has been routinely used to identify multiple forms of plant homoserine dehydrogenase (4). Initially three forms of the maize enzyme were identified using this technique (9, 14), whereas four forms have been detected in extracts of maize suspension cultures (19) and various tissues (10). Forms which differ only in net charge or in mol wt can be distinguished by the method of Hedrick and Smith (11). Three mol wt classes of enzyme detected during the present investigation are designated classes I, II, and III in order of increasing size. Class I is a naturally occurring form of maize homoserine dehydrogenase which is insensitive to inhibition by threonine and is characteristically a minor (15–25%) component of preparations isolated from young

Table III. *Reversible Alteration of Regulatory Properties of Homoserine Dehydrogenase*

Enzyme Preparation[a]	Experimental Treatment[b]	Sensitivity $(NAD^+/NADP^+)^c$		
		After treatment	After 3 weeks at −20 C	After 15 weeks at −20 C
Sensitive (94.0/80.7)	A (DM)	54.4/49.9	65.1/54.9	54.4/49.9
Sensitive (94.0/80.7)	B (DM+ THR)	95.0/80.5		92.3/75.1
Desensitized (54.4/49.9)	B (DM + THR)	64.8/51.9	80.2/72.9	77.1/67.1
Sensitive (94.0/80.7)	C (DM + DTT)	33.8/42.2	73.9/54.7	79.5/66.6
Desensitized (54.4/49.9)	C (DM + DTT)	11.1/19.9	56.4/48.6	80.6/68.7
Sensitive (94.0/80.7)	D (DM + THR + DTT)	95.1/78.4		94.6/83.2
Desensitized (54.4/49.0)	D (DM + THR + DTT)	66.5/53.5	87.6/77.4	91.1/73.3

[a] A portion of partially purified enzyme (S.A. 1340, $NAD^+/715$, $NADP^+$) which was inhibited 94.0% (NAD^+) and 80.7% ($NADP^+$) by 10 mM threonine was desensitized under the experimental conditions designated A and described in Table I. The enzyme was inhibited 54.4% (NAD^+) and 49.9% ($NADP^+$) after desensitization. Portions of the sensitive and desensitized enzyme preparations were subsequently treated under conditions B, C, and D.

[b] Experimental treatment consisted of dialysis for 44 h as described in Table I. A = desensitization medium (DM) and was 500 mM K-phosphate containing 1.4 mM 2-mercaptoethanol, 1.0 mM EDTA, and 20% (v/v) glycerol (pH 7.5). B = DM plus 5.0 mM L-threonine (THR). C = DM plus 10 mM DTT. D = DM plus 5.0 mM threonine and 10 mM DTT. More than 80% of the enzyme was recovered after each experimental treatment and all preparations were stable during storage.

[c] Sensitivity was determined independently with each coenzyme. The results are expressed as per cent inhibition by threonine and are based on duplicate assays in the presence and absence of 10 mM threonine. Sensitivity was measured immediately after treatment, and after storage of each preparation at −20 C for 3 and 15 weeks.

seedlings (9) or suspension cultures (19). The results in Figure 2 demonstrate a relationship between class II and class III. When 1.0 mM threonine was included in the gel and upper reservoir buffers (———), the major form of the enzyme exhibits reduced electrophoretic mobility in comparison to that of enzyme subjected to electrophoresis in the absence of threonine (- - -). The difference in mobility is a function of acrylamide concentration (Fig. 2, A and C) indicating a change in mol wt (11). Comparison of the average results of two equivalent experiments with those obtained with a series of standard proteins (3) indicates that the predominant form in the presence of threonine and the minor form in the absence of threonine have mol wt of 332,000 (class III). In contrast, the mol wt of the predominant form in the absence of threonine and the minor form in the presence of this effector is 159,000 (class II). This marked difference is not due to differential enzyme stabilization since altered electrophoretic mobility was observed when the gels were stained for protein (Fig. 2D) as well as for active enzyme (Fig. 2B). The presence or absence of threonine does not affect the mobility of the two minor protein contaminants of the enzyme preparation (Fig. 2D) nor of protein standards (data not shown).
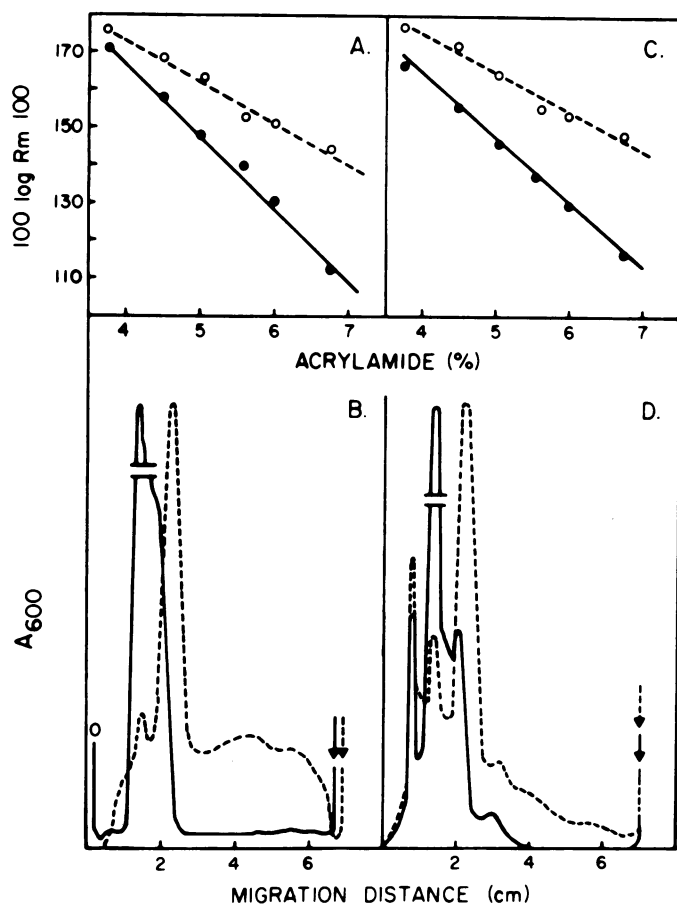

FIG. 2. Effect of threonine on homoserine dehydrogenase during PAGE. Enzyme was extensively purified from shoots of young seedlings (S.A. 130,000, NAD^+/64,000, $NADP^+$) and subjected to electrophoresis in the presence (———) or absence (- - -) of 1.0 mM L-threonine. Two-μg samples were used for measurements of active enzyme (A, B) and 6-μg samples were utilized for detection of protein (C, D). Tracings of the electrophoretic patterns obtained using 6% acrylamide gels are presented in the lower panels. In B, the origin of the gel containing threonine is indicated by (0) and arrows indicate the migration of bromphenol blue for each of the four gel patterns illustrated. The relative mobility of the predominant peak of enzyme activity (A) or protein (C) was measured after electrophoresis at six gel concentrations and analyzed by the method of Hedrick and Smith (11).
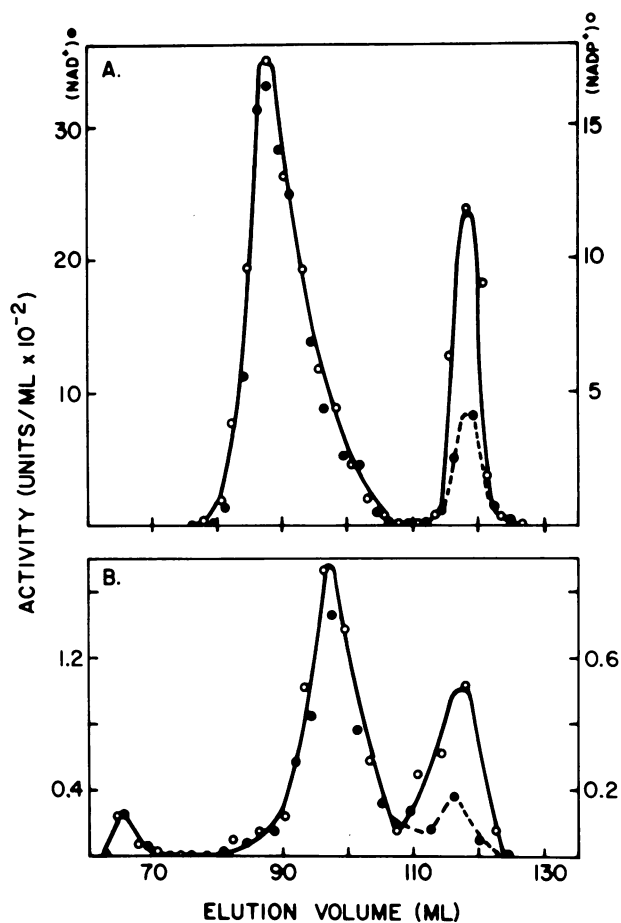

FIG. 3. Gel filtration chromatography of sensitive and experimentally desensitized homoserine dehydrogenase. Upward flow chromatography on Bio-Gel A 0.5m was performed. The elution profile of partially purified untreated enzyme (S.A. 502, NAD^+/347, $NADP^+$) is illustrated in A. The results obtained when a small portion of the enzyme was experimentally desensitized as indicated in Table I (experiment 10) and chromatographed on the same column under identical conditions are illustrated in B. (O, ●): Measurements of $NADP^+$- and NAD^+-linked activities, respectively.

Systematic differences between the electrophoretic patterns of native and experimentally altered enzyme have not been observed. However, two active forms of both class II and class III enzyme, differing only in net charge, were occasionally detected in both sensitive and desensitized preparations. Charge isomers of class II enzyme were reported previously (9). Although such isomers could represent sensitive and insensitive species of the enzyme, the lack of consistent results and the inability to obtain quantitative measurements of enzyme activity on gels preclude identification of either the nature or the origin of these electrophoretic variants.

To obtain additional quantitative information on sensitive and desensitized enzyme preparations, attempts to separate classes of enzyme by gel filtration chromatography were carried out. Class I can readily be separated from the other classes (Fig. 3A). The asymmetry of the elution profile of the remainder of the enzyme suggests a lack of resolution of class II and III enzymes. The elution profile of a portion of the same enzyme preparation which had been experimentally desensitized is shown in Figure 3B. The elution of class I was similar during both experiments. In contrast, the retarded, symmetrical elution of the desensitized enzyme would correspond to class II. However, this enzyme proved to be moderately sensitive to inhibition by threonine (67.7% inhibition of the NAD^+-dependent activity by 10 mM threonine compared to 94.0% inhibition of the class II/III mixture illustrated in Fig. 3A). Upon further analysis it was determined that a significant fraction

of the insensitive enzyme was inactivated during chromatography in the 50 mM K-phosphate. This result is consistent with the finding that the higher K-phosphate concentration of desensitization medium contributes to the stability of the experimentally altered enzyme (Table I). Attempts to carry out equivalent chromatographic experiments in desensitizing medium were hampered by slow flow rates and enzyme diffusion. Nevertheless, the results discussed above indicate that class II enzyme can be inhibited by threonine and, therefore, cannot exclusively represent desensitized enzyme.

The analytical problems encountered with gel filtration were overcome by use of sucrose density gradient centrifugation. First it was established that sensitive enzyme preparations exhibit similar sedimentation profiles in either 50 mM buffer used for column chromatography or in desensitizing medium containing threonine (Fig. 4, A and B). Class I enzyme had been removed from these preparations by gel filtration prior to centrifugation. Therefore, each preparation would be composed of a mixture of class II and class III enzyme. However, the highly symmetrical sedimentation pattern of the enzyme as reflected by measurements of both NAD^+- and $NADP^+$-dependent activities provides no evidence of multiple forms. This pattern has been obtained for all sensitive enzyme preparations from which class I has been removed, including resensitized enzyme. When class I enzyme was present in either native or treated preparations, its rate of sedimentation was slower than that of glucose 6-P dehydrogenase.

The sedimentation profile of desensitized enzyme preparations proved to be quite variable. In contrast to the results with sensitive enzyme, one or more peaks of activity sedimented ahead of catalase and variable amounts of activity were detected in fractions between the marker enzymes. A typical result in which two forms

of the enzyme are apparent is illustrated in Fig. 4C. In no case was class I enzyme observed after desensitization of preparations from which it had been removed. Thus, as in the case of in vivo alteration, experimental desensitization does not result in dissociation of the enzyme to a low mol wt form. In contrast, the appearance of rapidly sedimenting enzyme is clearly correlated with diminished sensitivity (Fig. 5; ref. 9). Insensitive forms of the enzyme were identified by measurement of enzyme activity in the presence of 10 mM threonine (Fig. 5, O- - -O). Three regions of insensitive enzyme activity were detected after sedimentation of this preparation which had been extensively desensitized (less than 20% inhibition by threonine). The most rapidly sedimenting form is almost totally resistant to inhibition, whereas the activity sedimenting ahead of catalase is either partially sensitive or composed of a mixture of sensitive and insensitive forms. The peak of activity sedimenting between the markers could be composed of a greater proportion of insensitive enzyme, while the remaining activity appears to be sensitive and sediments at a rate closer to that of catalase.

Each of the analytical procedures described was utilized to obtain estimates of the mol wt of the individual forms of maize homoserine dehydrogenase by construction of appropriate standard curves with proteins of known mol wt, or, in the case of sucrose density gradient centrifugation, by independent comparisons with the rate of sedimentation of the two marker enzymes (Table IV). In the latter procedure it was determined that the sedimentation rate of neither marker was affected by any of the experimental conditions utilized. Class I enzyme was found to have a mol wt between 70,000 and 80,000 with a mean value of 74,000. The mean values for class II and III enzyme are 170,200 and 320,200, respectively. Estimates of the mol wt of the larger
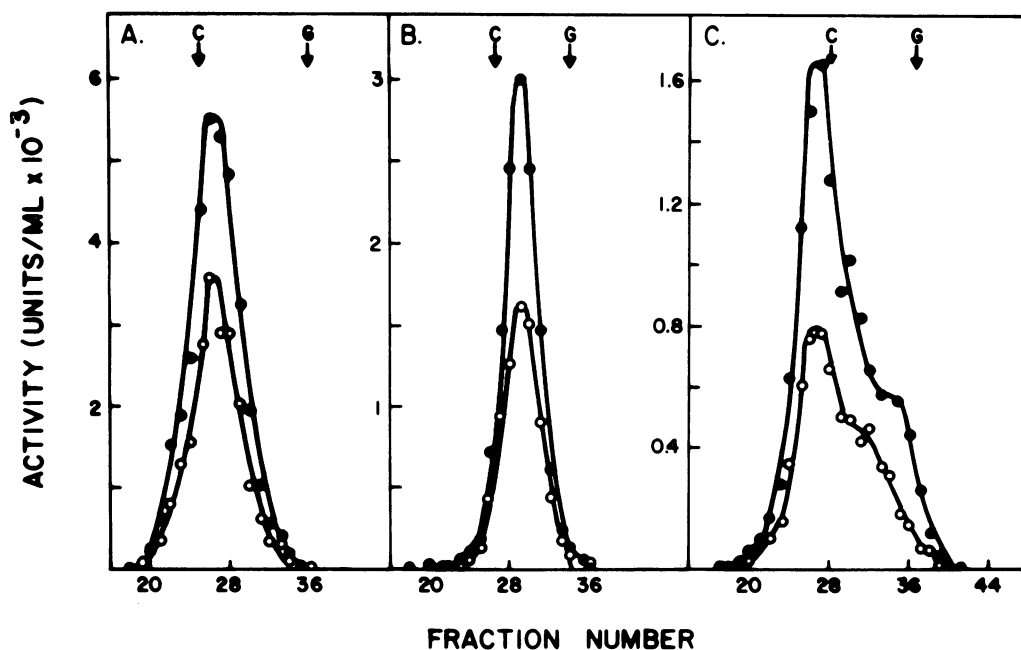

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FIG. 4. Sedimentation of homoserine dehydrogenase preparations on sucrose density gradients. Centrifugation through 5 to 20% linear gradients was performed. Sedimentation was from right to left and the position of the marker enzymes, catalase (mol wt 240,000) and glucose 6-P dehydrogenase (mol wt 103,000), is indicated by the arrows designated C and G, respectively. Class I homoserine dehydrogenase was removed from the enzyme preparations prior to centrifugation. The sedimentation profiles of sensitive enzyme preparations are illustrated in A and B, and that of a partially desensitized preparation in C. For the experiment illustrated in A, the gradient was prepared in 50 mM K-phosphate (pH 7.5) containing 1.4 mM 2-mercaptoethanol, 1.0 mM EDTA, 20% (v/v) glycerol, and 5.0 mM L-threonine. The enzyme (S.A. 1960, NAD^+/980, $NADP^+$) was inhibited 95.3% (NAD^+) and 82.1% ($NADP^+$) by 10 mM threonine. The gradient buffer used for experiment B was 500 mM K-phosphate (pH 7.5) containing the same additions as the buffer used in experiment A. The enzyme (S.A. 1090, NAD^+/580, $NADP^+$) was inhibited 95.0% (NAD^+) and 80.3% ($NADP^+$) by 10 mM threonine. Threonine was omitted from the 500 mM buffer used to prepare the gradient illustrated in C. The enzyme (S.A. 980, NAD^+/570, $NADP^+$) was partially desensitized by dialysis against this buffer as described in Table I. After treatment, the enzyme was inhibited 54.4% (NAD^+) and 49.9% ($NADP^+$) by 10 mM threonine. (O, ●): Measurements of the $NADP^+$- and NAD^+-dependent activities, respectively. Enzyme activity recovered after centrifugation was 77.0, 100, and 92.7% from A, B, and C, respectively.

forms characteristic of desensitized enzyme preparations were obtained only from sucrose density gradient centrifugation. In five experiments such as the one in Figure 5, a prominent rapidly
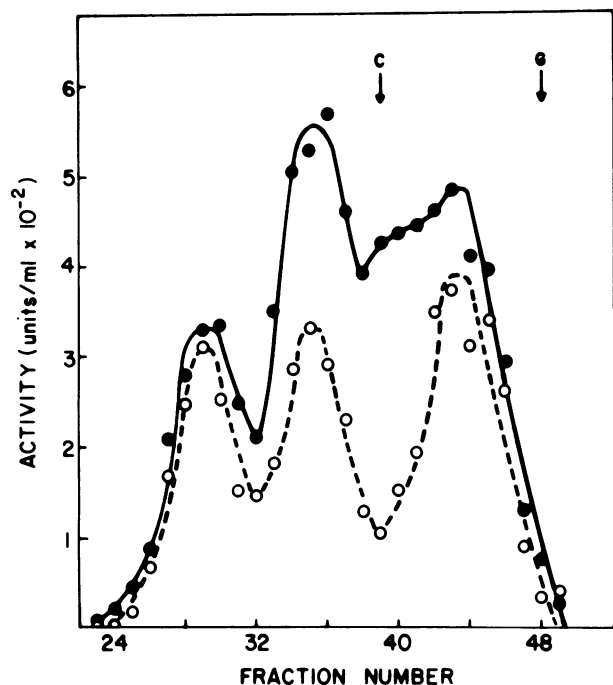

FIG. 5. Measurement of sensitive and insensitive homoserine dehydrogenase activity after sucrose density gradient centrifugation. Centrifugation was carried out under the conditions described for experiment C, Figure 4. The enzyme (S.A. 2140, $NAD^+/960$, $NADP^+$) was extensively desensitized as described in the legend of Figure 1, and was inhibited 20.7% (NAD^+) and 12.4% ($NADP^+$) by 10 mM threonine. (O): Measurements of NAD^+-linked activity in the presence of 10 mM threonine; (●): measurements of this activity in the absence of threonine. Enzyme activity recovered after centrifugation was 72.6%.

sedimenting form had an apparent mol wt of 402,000. However, small amounts of enzyme with an apparent mol wt of 661,200 were also observed in three experiments.

Sensitive enzyme preparations behaved somewhat anomalously during sucrose density gradient centrifugation, giving an apparent mol wt of 207,300. Since a species of this apparent size was not detected during any of the other procedures, we interpret these results as being indicative of the dissociation of class III enzyme to class II under the high pressures generated during centrifugation. Dissociation, accompanied by a tendency to reassociate, permits two protein species to sediment as an apparently single form with a mol wt which would be some weighted average of the individual proteins (12). This interpretation would be consistent with the predominance of class III enzyme during column chromatography (Fig. 3A). Although classes II and III do not appear to be in rapid equilibrium as judged by the results of large zone chromatography (data not shown) analyzed by the method of Winzor and Scheraga (20), this would not preclude the possibility of pressure induced dissociation.

Although there is variation among the mol wt estimates obtained by different procedures as well as within the results obtained from a single technique, the results collectively suggested a multimeric relationship among the classes of maize homoserine dehydrogenase. To examine this possibility, each class was assigned a multimeric value and these were plotted against the estimates of mol wt (Fig. 6). Regression analysis yields a line with an intercept near zero and a slope of 78,000. When class I was excluded from the calculations (see below) the intercept was increased and the slope was 75,900.

DISCUSSION

Among the multiple forms of maize homoserine dehydrogenase, class I is inherently resistant to inhibition by threonine (9, 19) and appears to be a true genetic isozyme differing in subunit composition from the other forms (19). Its presence in extracts of different maize tissues could account, in part, for the wide range of enzyme sensitivity which has been observed (6, 10, 14). However, during

Table IV. Estimates of Molecular Weights of Homoserine Dehydrogenase

Method[a]	Class of Enzyme[b]				
	I	II	III	Others	
A. Gel filtration					
Mol wt	69,300	169,800	352,300	—[c]	—
SE	5,400	—	26,200		
N	4	1	4		
B. PAGE					
Mol wt	68,600	173,100	325,600	—	—
SE	2,400	2,500	13,700		
N	19	64	27		
C. Sucrose gradients					
Sensitive enzyme					
Mol wt	79,200	—	207,300[d]	—	—
SE	2,700		3,700		
N	2		14		
Desensitized enzyme					
Mol wt	78,900	167,600	282,600	402,800	661,200
SE	3,300	3,500	8,900	12,900	7,400
N	3	9	7	5	3

[a] Upward flow chromatography in columns (1.5 × 90 cm) of Bio-Gel A 0.5m was utilized for gel filtration. PAGE was employed using the methods described by Bryan (3). Sucrose density gradient centrifugation is described under "Materials and Methods." SE is the standard error of the mean of N determinations.

[b] Three classes of enzyme were distinguished by several methods and are designated I, II, III on the basis of increasing mol wt. Forms detected only during sucrose density gradient centrifugation of desensitized enzyme and designated as others.

[c] In cases where mol wt are not included, the class of enzyme was either not detected or the method did not permit an estimate to be obtained.

[d] The apparently anomalous behavior of sensitive enzyme under these conditions is discussed in the text.
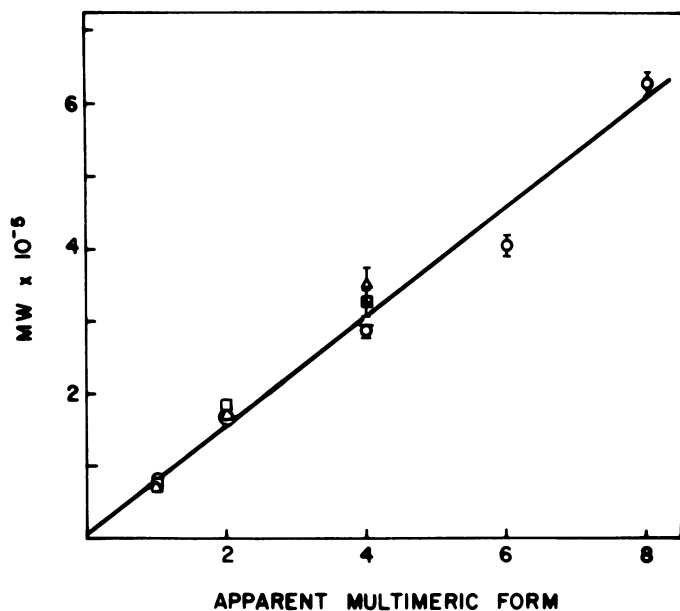
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FIG. 6. Relationships among various forms of maize homoserine dehydrogenase. Estimated mol wt of enzymes listed in Table IV were plotted against an assigned multimeric value. Results obtained by sucrose density gradient centrifugation (O), PAGE (□), and gel filtration (△) are indicated.
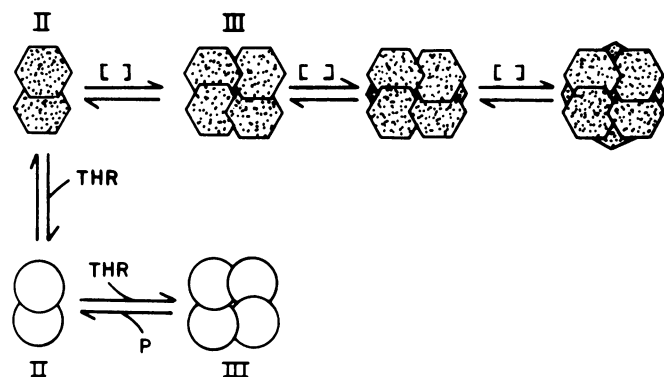

FIG. 7. Proposed multimeric states of sensitive and insensitive forms of maize homoserine dehydrogenase. Stippled forms are envisioned to be insensitive to inhibition by the feedback modifier, L-threonine. This metabolite (THR) is considered to favor a highly sensitive tetrameric state of the enzyme which is subject to pressure (P)-induced dissociation to a sensitive dimer. In the absence of threonine, a conformational change resulting in the loss of regulatory control can occur. High ionic strength appears to aid in stabilizing the insensitive forms and enzyme concentration (in brackets) may favor the formation of higher mol wt multimers of the insensitive dimer.

shoot growth class I enzyme appears to be replaced by higher mol wt forms while the bulk of the enzyme becomes increasingly resistant to inhibition by threonine (9). Based on the criteria which can presently be evaluated, experimentally desensitized enzyme is indistinguishable from that isolated from older seedlings. These criteria include: the lack of involvement of class I enzyme, equivalence of catalytic and regulatory properties of enzymes which exhibit comparable reduced sensitivity to inhibition by threonine, and formation of high mol wt aggregates.

It is evident that experimental desensitization does not result from inactivation of a sensitive form of the enzyme, since the enzyme can be altered without loss of catalytic activity. It is equally unlikely that the alteration is due to covalent modification based on the mild conditions required for desensitization, its

reversibility, and the fact that enzymes of different purity can be altered with equal facility. Another possibility, that of the dissociation of a small regulatory subunit during desensitization, is not supported by preliminary analysis of nearly pure preparations after electrophoresis under denaturing conditions. In addition, homoserine dehydrogenase isolated from maize suspension cultures has been reported to be composed of subunits with apparent mol wt of 89,000 and 93,000 (19). These observations severely restrict the range of known molecular mechanisms which could account for desensitization.

Our present concept of the multiple states of the regulated maize homoserine dehydrogenase is illustrated in Figure 7. The sensitive species of class II and class III are considered to exist in an equilibrium in which class III is strongly favored by threonine. This is supported by the results obtained with PAGE and gel filtration, and is consistent with the presumptive pressure-induced dissociation of the enzyme during density gradient centrifugation. A dimer is the major insensitive species detectable after minimal desensitization, but evidence for both sensitive and insensitive dimeric and tetrameric forms has been obtained. The insensitive tetramer would represent one of a series of even-numbered multimeric aggregates formed upon extensive desensitization. Desensitization would be accomplished in the absence of threonine by an unspecified type of conformational change of the sensitive dimer. Neither the possibility of an altered tertiary structure nor that of exchange of heterologous subunits (19) is excluded. Although a number of aspects of this model remain to be established, it is compatible with all of the available data. For example, if DTT acts to dissociate the insensitive multimers, then its enhancement of resensitization would be explicable in terms of an effective increase in the concentration of the insensitive dimer, which could revert to a sensitive conformation in the presence of threonine.

Confirmation of the equivalence of the in vitro and in vivo processes of desensitization will require detailed comparisons of highly purified enzymes obtained from both young and old plant material. During a preliminary experiment, slight but significant increases in the sensitivity of homoserine dehydrogenase were observed after succeeding stages of purification of enzyme from older plant material. This result would be consistent with slow resensitization but could also reflect reduced stability of insensitive forms of the enzyme. Preservation of the native properties of enzyme from different tissues of the same plant or from plants of different ages may require alternative experimental conditions. Nevertheless, neither the results of earlier control experiments (6, 14) nor our current knowledge of the properties of maize homoserine dehydrogenase provides any evidence that sensitive enzyme is being desensitized during extraction or purification.

Extremely low levels of L-threonine are effective in influencing the regulatory characteristics of the enzyme in vitro (Fig. 1). Although these levels are significantly lower than those reported to occur in maize leaves (7), reduced localized concentrations may occur, or other factors may function to reduce the effectiveness of threonine in vivo. In this context, it is of particular interest that the threonine concentration within maize chloroplasts decreases 12.5-fold during development and maturation (7). This decrease parallels the developmental changes observed for homoserine dehydrogenase within these subcellular organelles (6). In addition, estimates of the ionic concentration of plant cytoplasm and chloroplasts range between 100 and 300 mM (17), which could be adequate for stabilization of the enzyme during desensitization. To the extent that experimental alteration of homoserine dehydrogenase reflects processes which occur in vivo, the present results suggest that changes in the concentration of critical effectors may have enduring effects on metabolic regulation during plant development.

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