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DE NOVO SYNTHESIS AND DEVELOPMENTAL CONTROL OF THE MULTIPLE GENE-CONTROLLED MALATE DEHYDROGENASE ISOZYMES IN MAIZE SCUTELLA

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Summary

In maize, both soluble and mitochondrial malate dehydrogenases (L-malate:NAD oxidoreductase, EC 1.1.1.37) exist in multiple molecular forms (isozymes). Biochemical studies and genetic analysis showed that the maize malate dehydrogenase isozymes are not interconvertable conformational forms of a single gene product. Instead, they are determined by multiple genes which may reside on different chromosomes. Therefore, developmental expression of the various malate dehydrogenase isozymes was studied.

During early development of the sporophyte (dry kernel to 10 days of germination), the total malate dehydrogenase activity in scutella increases through the first 5 days, peaks about the 6th day and decreases gradually thereafter. Both soluble and mitochondrial malate dehydrogenase isozymes exhibit similar activity profiles; however, the total mitochondrial malate dehydrogenase activity is only 60% of that in the cytosol. Density labeling experiments and atempts to detect the possible existence of "inactive malate dehydrogenase precursors" were performed. The results show that both soluble and mitochondrial malate dehydrogenase are synthesized in the scutella during germination and early growth of young maize seedlings. Accumulation of the maize malate dehydrogenase isozymes is probably controlled by synthesis as well as degradation instead of activation and inactivation of enzyme moieties. Correlation between the developmental pattern of the scutellar malate dehydrogenase isozymes and the physiological conditions of the young maize seedlings is discussed.

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Introduction

Malate dehydrogenases (L-malate:NAD oxidoreductase, EC 1.1.1.37) have several important physiological functions within the cell [1—4]. There exist at least two major classes of malate dehydrogenase. One class is restricted in occurrence to the mitochondria (m-MDHs) where it functions as a component of the Krebs cycle, while the other class (s-MDHs) occurs in the soluble fraction of the cell, where it may participate in malate shuttle [1] and other metabolic pathways [2,3]. Multiple molecular forms of both soluble and mitochondrial malate dehydrogenases have been shown to exist in various eukaryotic organisms [4—12]. In plants, malate dehydrogenases have also been found in glyoxysomes [5,13] and peroxisomes [14].

In maize, multiple electrophoretic forms of both s-MDHs and m-MDHs have been demonstrated [5]. We [15] showed previously that the maize malate dehydrogenase isozymes are strain specific and are not merely conformers of one basic form of the enzyme. Our recent genetic analysis (Yang, N.S. and Scandalios, J.G., unpublished) confirms the above results and shows that the maize malate dehydrogenase isozyme patterns are genetically determined and are controlled by multiple genes. Genetic control of maize malate dehydrogenases, which will be reported elsewhere, is briefly described under Discussion. These results and the occurrence of soluble mitochondrial and glyoxysomal malate dehydrogenase isozymes in maize [5] offers a good system for studying developmental processes within the cell. Not only does the expression of these genetically defined isozymes represent a mechanism by which cells may regulate a group of proteins (enzymes) with similar characteristics during development, but the subcellular distribution of malate dehydrogenase isozymes may be used as a probe to study, both genetically and biochemically the cooperation between organelles. In this communication, developmental expression of both soluble and mitochondrial malate dehydrogenases in maize scutella is studied. The possible intracellular site for the synthesis of both classes of isozymes will be discussed in a later report.

Materials and Methods

Growth of seedlings

Two inbred maize strains, W64A and 59 were used. Dry kernels were surface-sterilized with 5% sodium hypochlorite solution for 10 min, washed twice with sterilized deionized water and were then soaked for 5—8 h. After soaking, the seeds were germinated between moistened germination papers either in plastic trays (40 × 30 × 8.5 cm) or in petri dishes (10 cm in diameter, 7.5 cm in height) in the dark at 25°C. Age of the seedlings was counted from the time of soaking. At different developmental stages (1—10 days), various tissues were isolated and used for enzyme extraction or organelle preparation.

Identification of soluble, mitochondrial and glyoxysomal malate dehydrogenase isozymes in maize scutella

Isolation of soluble, mitochondrial and glyoxysomal fractions from maize scutella was performed by a modification of the method described by Longo and Longo [16].

Malate dehydrogenase isozymes in the various fractions were identified by starch gel electrophoresis and specific staining for the enzyme as described by Scandalios [17].

Preparation of crude malate dehydrogenase extracts

Scutella of the maize seedlings at different developmental stages were isolated. Five scutella were homogenized with 0.5 g sand in a chilled mortar with 1 ml 0.025 M glycylglycine buffer, pH 7.4. The homogenate was centrifuged at 25 000 × *g* for 30 min in the refrigerated centrifuge (Sorvall RC-2B; SS-34 rotor). The supernatant, containing more than 95% of total malate dehydrogenase activity found in crude homogenates, was taken as the crude extract and was used for measuring total malate dehydrogenase activity. This crude scutellar extract was also used as a source to prepare individual isozymes.

Isolation and quantitative assay of the individual cytoplasmic or mitochondrial malate dehydrogenase isozymes

The malate dehydrogenase isozymes were separated by starch gel electrophoresis according to the method of Scandalios [17]. A 150-µl aliquot of the crude extract was subjected to electrophoresis for 14—16 h at an applied voltage gradient of 6—8 V/cm and 5°C. One horizontal slice was taken from the gel and stained for malate dehydrogenase activity. This stained slice was then used as template for excising single isozyme bands from the unstained portion of the gel. The stained slice typically comprised one-fourth of the total gel weight; this measurement allowed us to calculate the malate dehydrogenase activity lost in the stained gel template. Each excised band was then placed in a syringe and squeezed into a centrifuge tube. Glycylglycine (1 ml; 0.025 M) buffer pH 7.4 was added to dilute the mascerated gel. The suspension was then centrifuged at 45 000 × *g* for 1 h. The supernatant, containing a single isozyme, was used for quantitative assay of the enzyme activity. Approx. 70—75% of the malate dehydrogenase activity applied to the gel could be recovered in this manner.

Density labeling of the newly synthesized proteins in maize scutella

Inbred Line 59 was selected for density labeling experiments since it exhibits only two forms of mitochondrial malate dehydrogenase. Dry kernels were surface-sterilized and rinsed as before. They were then soaked in 10 mM $^{14}NH_4Cl$ in H_2O ($^{14}N/H_2O$) or in 10 mM (99 atom%) $^{15}NH_4Cl$ in 70% ^2H_2O ($^{15}N/^2H_2O$) for 8 h with aeration. The soaked seeds were germinated between moistened germination papers in petri dishes as described before. Since the growth rate of maize seed in $^{15}N/^2H_2O$ is about 70% of that in $^{14}N/H_2O$, the $^{15}N/^2H_2O$ scutella were taken for enzyme extraction after 7 days, and the $^{14}N/H_2O$ scutella were taken after 5 days; periods of comparable growth stages.

50 scutella from each treatment were homogenized with sand in a mortar and pestle in 2 ml of 25 mM glycylglycine buffer (pH 7.4). The homogenates were then diluted with 40 ml of the same buffer, stirred and centrifuged at 30 000 × *g* for 30 min. The crude supernatant was then partially purified by

ammonium sulfate fractionation. Proteins precipitated in the 45—70% fraction were collected by centrifugation at 25 000 × *g* for 10 min. The pellet was resuspended in 5 ml 0.025 M glycylglycine buffer, pH 7.4, and dialysized overnight against the same buffer. The partially purified malate dehydrogenase preparation was then used for electrophoresis. For preparative purposes, 2 ml enzyme extract was absorbed in thick filter paper wicks, and was applied to a single starch gel. Starch gel electrophoresis and isolation of each individual isozyme from the gel were performed as described before. Each invidual isozyme eluted from the starch gel was used directly for centrifugation in CsCl.

The procedure for density gradient centrifugation was essentially that of Filner and Varner [18]. Each tube contained 1 ml of satd CsCl with 10 mg/ml NAD, 2 ml of gel eluate and 20 μg of lactate dehydrogenase (EC 1.1.1.27; Sigma) as a marker, all uniformly mixed. NAD was found to prevent, very effectively, the loss of malate dehydrogenase activity in the conc. CsCl solution. The tubes were centrifuged at 40 000 rev./min for 72 h at 3°C in a Beckman L2-65B ultracentrifuge equipped with an SW-65 rotor. The tubes were punctured with a No. 22 needle and three-drop fractions were collected in the cold. About 90 fractions were collected from one tube. The refractive index of every tenth fraction was determined on a Bausch and Lomb Abbe-32 refractometer and converted to density units. The average mean density of the CsCl gradient at the end of the run was 1.28—1.29 g/cm^3. Recovery of enzyme activity from the gradient was about 80—90%, with no quantitative differences between isozymes.

Preparation and mixing of crude scutellar extracts (480 × g supernatant) at different developmental stages

Ten scutella were isolated from seedlings at each of the following developmental stages: 2-day-old, 4-day-old, 5-day-old and 8-day-old seedlings. 50 scutella were also isolated from seeds which had been imbided for only 5 h in water (0.2-day-old). Each of the five sets of scutella was ground with 0.5 g sand in 3 ml 0.025 M glycylglycine buffer containing 5 mM mercaptoethanol, pH 7.4. The crude homogenates were then adjusted to a final volume of 5 ml. The crude homogenates were thoroughly mixed and centrifuged at 480 × *g* for 10 min to pellet down the wall debris and unbroken cells. The supernatants were collected and used as crude extracts. For each of the crude extracts, 0.2-ml aliquots were mixed either with 0.2 ml 0.025 M glycylglycine buffer or with 0.2 ml of crude extract from another developmental stage. The 0.4-ml crude extracts, in small test tubes, were then incubated at 37°C in a water bath for 2 h. After incubation, the crude extracts were chilled to 4°C and the total malate dehydrogenase activities were measured spectrophotometrically.

Enzyme assays of malate dehydrogenase

All enzyme activities were assayed with a Gilford spectrophotometer (Model 2400) equipped with a recorder. Malate dehydrogenase was assayed in the direction of oxaloacetate reduction according to Ochoa [19]. With a total volume of 3 ml, the reaction mixture contained 0.025 M glycylglycine buffer, pH 7.4, 0.25 mM oxaloacetate (adjusted to pH 7.4 with NaOH) and 0.05 mM NADH. Assays were performed at 25°C by adding 10—20 μl enzyme prepara-

tion and following the decrease in absorbance at 340 nm during NADH oxidation. Initial rates were used in calculations of activities.

Expression of malate dehydrogenase activity

The malate dehydrogenase activity in crude extract is represented as μmol of NADH oxidized per min per mg protein. For quantitative measurements of individual isozymes, the volume of the gel extract and the relative weights of the stained and unstained gel were precisely measured. This allows us to calculate the activity of each isozyme per scutellum. Because 25—30% of the crude extract activity was always lost during electrophoresis and during extraction procedures, the activities of the different isozymes in scutella are relative rather than absolute values.

Data given under results are the mean values of two or three independent experiments. For each experiment, enzyme activity or protein concentration measurements were made in triplicate.

Results

In maize kernels, the embryo is tightly packed in the groove of the scutellum which is in turn surrounded by the starchy endosperm. After the maize kernel has been imbibed in water for 5 h, the scutellum becomes tender and the embryo starts to swell, while the endosperm is still very rigid and solid. 2 days after germination, the embryonic axis grows to about 1 cm and the scutellum becomes very tender and has reached its maximal fresh weight, however, the endosperm is only partially liquified. It is therefore believed that the scutellum, by acting as a nutrient furnishing organ, plays an important role in the early sporophytic development of maize seedlings. In fact, the specific activity of malate dehydrogenase is higher in scutellum than in any other organ examined.

In the scutellum of maize, inbred Strain W64A, there are seven major malate dehydrogenase isozymes, namely two soluble forms and five mitochondrial forms (Fig. 1). The two soluble forms appear in all the inbred maize strains we examined; the five mitochondrial forms are commonly observed in the seven mitochondrial malate dehydrogenase phenotypes we found [15]. A third class of isozymes, the glyoxysomal isozymes (g-MDHs) also exists in maize scutella. However, since the g-MDHs only move a short distance toward the anode and appear to be unstable during starch gel electrophoresis, it is difficult to separate them into clear bands. In addition, their concentrations are much lower than those of s-MDHs and m-MDHs, thus, they can only be observed when the enzymes are prepared in high concentrations. Therefore, scutella of Strain W64A were used to study the development of malate dehydrogenase isozymes in maize seedlings. The malate dehydrogenase isozymes in Strain W64A were named from the anode toward the cathode as s-MDH^1, s-MDH^2 (the two soluble forms), m-MDH^1, m-MDH^2, m-MDH^3, m-MDH^4, m-MDH^5, (the five mitochondrial isozymes) and g-MDHs (the glyoxysomal isozymes).

The specific activity of malate dehydrogenases in the scutellum is about 2—7-fold higher than those in other organs in the etiolated maize seedlings; the shoot, root, endosperm and pericarp follow with decreasing activities in that

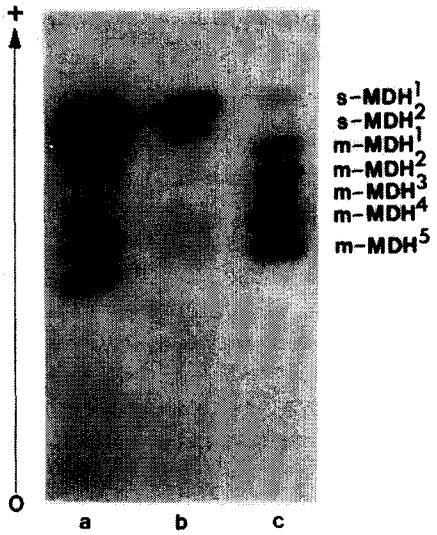

Fig. 1. Zymogram of maize malate dehydrogenase isozymes in subcellular fractions isolated from 4-day-old scutella of the inbred Strain W64A. Migration is anodal. 0 = point of sample insertion. (a) Crude scutellar extract (480 × *g* supernatant). (b) Soluble fraction (25 000 × *g* supernatant). (c) Mitochondrial fraction (isolated by sucrose gradient centrifugation).

order. In scutellum, s-MDH^2 is very dominant compared to the same isozyme in other organs. The glyoxysomal isozymes, were found only in scutella. Intermediate bands located between mitochondrial and glyoxysomal isozymes were occasionally observed but found to be non-specific dehydrogenases. Under the experimental conditions described here, activity of malate dehydrogenase prepared from pericarp of the young seedling was not high enough to show a clear pattern of isozymes. However, pericarp of the immature kernel (18 days after pollination) has a similar isozyme pattern as those of shoot and root.

Because the activities of glyoxysomal isozymes (g-MDHs) in scutella are low compared to the soluble and mitochondrial isozymes, our studies were centered on the latter forms of the enzyme.

Zymograms of scutellar malate dehydrogenase of W64A at various stages of seedling development, showed that the number and positions of the isozymes under study remained constant. In etiolated W64A seedlings, the total malate dehydrogenase activity increases through the first 5 days and peaks about the 6th day. This is followed by a gradual decrease in activity, and by the 10th day the level is the same as that of the 4th day (Fig. 2). Since there is an increase in "glycylglycine buffer extractable-protein" per scutellum (from 1.3 mg/scutellum at Day 0 to about 2.8 mg/scutellum at Day 6), the total malate dehydrogenase activity per scutellum increases much more dramatically than that per mg of protein.

The time course of development of the two soluble isozymes is shown in Fig. 3. The s-MDH^1 has much higher activity than the s-MDH^2, especially during the late developmental stages. This is also observed in gel assays. In young scutella, the five mitochondrial isozymes exhibit similar developmental patterns (Fig. 4). The activity of all mitochondrial forms is less than that of the soluble forms. However, all the isozymes do not seem to follow exactly the

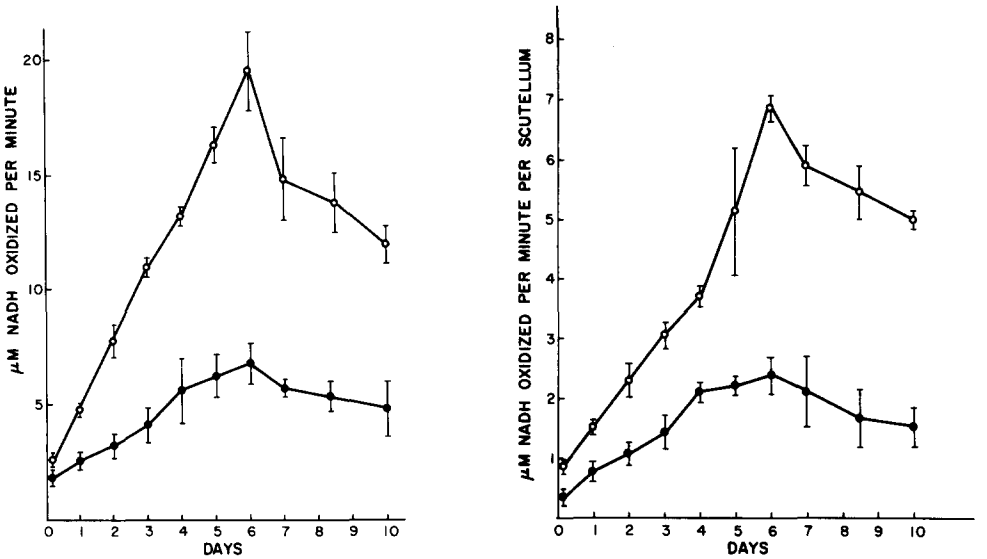

Fig. 2. Time-course of total malate dehydrogenase activity in scutella of germinating maize seeds (W64A): ○———○, activity per scutellum; ●———●, activity per mg of protein.

Fig. 3. Time-course of development of the two supernatant malate dehydrogenase isozymes in scutella of germinating maize seeds (W64A): ○———○, s-MDH^1; ●———●, s-MDH^2.

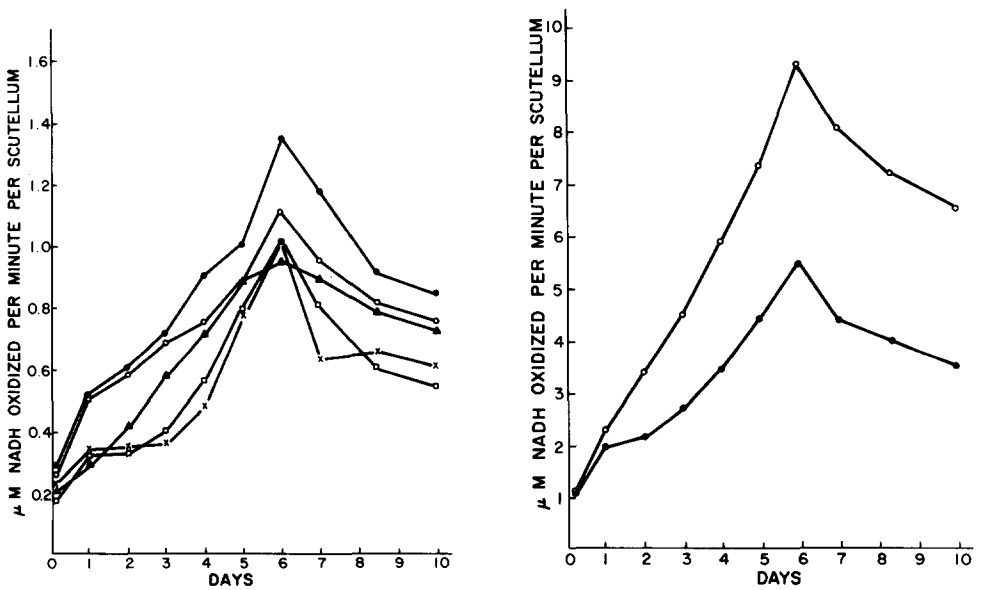

Fig. 4. Time-course of development of the five mitochondrial malate dehydrogenase isozymes in scutella of germinating maize seeds (W64A): ●———●, m-MDH^1; ▲———▲, m-MDH^2; X———X, m-MDH^3; ○———○, m-MDH^4; and □———□, m-MDH^5.

Fig. 5. Time-course of soluble and mitochondrial malate dehydrogenase activities in scutella of germinating maize seeds (W64A): ○———○, s-MDH; ●———●, m-MDH.

same kinetics of accumulation; some subtle regulation controlling the expression of each isozyme may be involved; this aspect will be discussed later on. Fig. 5 represents the total soluble and mitochondrial malate dehydrogenase activities at different developmental stages. The soluble activity is obtained by adding the activities of the two soluble isozymes together. The sum of the activities of the five mitochondrial isozymes gives the total mitochondrial activity of the enzyme.

At any given point in scutellar development, the total malate dehydrogenase activity in mitochondria is only about 60% of that in the cytoplasm. The test tube assays are consistent with the zymogram patterns which show that the s-MDHs stain more intensely than any of the mitochondrial forms.

The density labeling technique was used to determine whether the development of m-MDH and s-MDH isozymes in the scutella of developing maize seedlings is due to de novo synthesis of the enzyme moieties or to activation of the pre-existing enzymes. Fig. 6 shows that both s-MDHs (s-MDH^1 and s-MDH^2) and m-MDHs (the two m-MDHs of Strain 59, correspond to m-MDH^2 and m-MDH^5 of Strain W64A) become labeled after 5 days germination. This indicates that both classes of malate dehydrogenase isozymes are synthesized de novo. Therefore, the newly appearing isozymes in maize scutella are accumulated during the de novo synthesis of the enzyme moieties. However, do they turn over as they accumulate? The density labeling data indicate that the

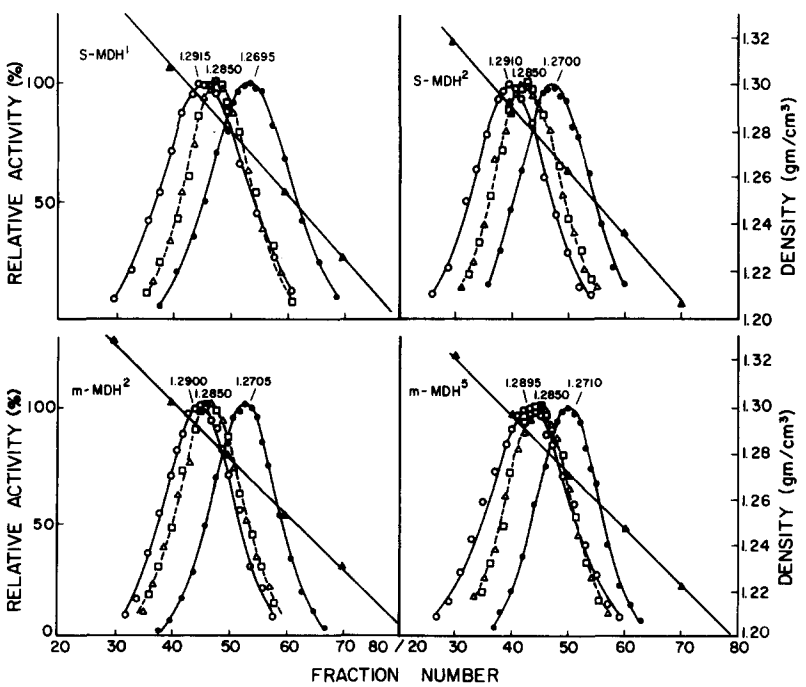

Fig. 6. Equilibrium distribution in CsCl gradients of scutellar s-MDHs and m-MDHs from seeds (Strain 59) grown on either $^{14}NH_4Cl$ in H_2O for 5 days (●———●) or $^{15}NH_4Cl$ in 70% ^2H_2O for 7 days (○———○). The activity of the lactate dehydrogenase marker in the labeled (△———△) and unlabeled (□———□) gradients have been superimposed and drawn as one. Relative activity means that all points on these curves are expressed as percentage of the highest point on each of the individual curves. Density of CsCl gradient (▲———▲).

malate dehydrogenase isozymes do turn over as they accumulate. Because the increase of malate dehydrogenase activity in the first 5 days is less than 6-fold over the ungerminated level (Figs 4 and 5); if there was no substantial turn over, then in Fig. 6 one would expect the presence of an unlabeled component, evident as a shoulder. However, the enzyme activities in the labeled gradients are symmetrical and no "shoulders" were observed. These results suggest that either the "fully unlabeled" malate dehydrogenase molecules are no longer present or they only exist in a very small amount which was not able to be picked up in our experiments.

Quail and Scandalios [20] observed that after 36 h of germination, maize catalase isozymes became fully density labeled. Furthermore, they showed that the time required for the fully labeled catalase isozymes to decrease by 50% of the density difference between the unlabeled and the fully labeled molecules is 22—44 h. Therefore, it is not surprizing that after 7 days in $^{15}N/^{2}H_2O$ medium, (the growth rate is equal to 5 days in $^{14}N/H_2O$ medium), no fully unlabeled molecules of malate dehydrogenase isozymes were detected.

As shown in Figure 6 and Table I, it is interesting that the band width at the half height for the labeled and unlabeled enzymes are the same for s-MDHs and m-MDH^2; however, the band width at the half height for the labeled m-MDH^5 is about 30% broader than that of the unlabeled m-MDH^5. The repeatable distributions of the lactate dehydrogenase marker in the labeled and unlabeled gradients clearly shows that the results are not artifacts.

To explain this result, we suggest that the turn over of m-MDH^5 is controlled separately from that of m-MDH^2. Both soluble and mitochondrial malate dehydrogenases in porcine heart [21] have been shown to be dimers in molecular structure. Our genetic results (Yang, N.S. and Scandalios, J.G., unpublished) suggest that this is also true for the maize isozymes. In Fig. 6 and Table I, the same band width for the unlabeled and labeled m-MDH^2 indicate that the malate dehydrogenase molecules in each case are "homogeneous". That is, both subunits of malate dehydrogenase in the unlabeled gradient are not labeled, while the two subunits in the labeled gradient are both labeled to the same extent. Therefore the labeled m-MDH^2 gives the same band width as does the unlabeled m-MDH^2. For m-MDH^5, the band width of the molecules is 30% broader than that of the unlabeled molecules. This result suggests strongly that the molecules in the labeled m-MDH^5 peak are "heterogeneous". The m-MDH^5 peak in the labeled gradient may be composed of two types of

TABLE I

WIDTH AT HALF-HEIGHT FOR MALATE DEHYDROGENASE AND LACTATE DEHYDROGENASE AFTER ISOPYCNIC EQUILIBRIUM CENTRIFUGATION IN CsCl

Sample	Width at half-height (drops)				
	S-MDH^1	s-MDH^2	m-MDH^2	m-MDH^5	Lactate dehydrogenase
Labeled	50	45.5	41	51	45 (in labeled gradient)
Unlabeled	49.5	45	40.5	39	45 (in unlabeled gradient)
Labeled/unlabeled (%)	101	101	101	131	100

dimers. In one kind of dimer, both subunits are $^{15}N/^{2}H$ labeled, while in the other type of dimer, one subunit is labeled (heavy), while the other subunit is not labeled (light). A "half-labeled" m-MDH^5 molecule would therefore have a density higher than 1.2710 (Fig. 6) and a lower density than that of the malate dehydrogenase molecules with both subunits being labeled. Therefore, when both types of dimers are present, a broad activity peak with a density between them would then be expected. As seen in Fig. 6, the density shift (1.2710 to 1.2895) for m-MDH^5 is indeed less than those observed for the other three isozymes tested.

Therefore we suggest that the $^{15}N/^{2}H$ labeled s-MDHs and m-MDH^2 are "homogeneous" in the CsCl gradient shown in Fig. 6, while m-MDH^5 is "heterogeneous" and is composed of two types of malate dehydrogenase subunits as suggested above. If this is the case, then the developmental expression of maize malate dehydrogenase isozymes may be regulated as described in the following. First, turn over (synthesis and degradation) of m-MDH^5 and m-MDH^2 are likely controlled independently. Second, the degradation of maize malate dehydrogenase molecules may be regulated at the subunit level. According to Fig. 6, the turn over of m-MDH^2 increased to about 4-fold after germination for 5 days (Fig. 5). The absence of a "shoulder" of the fully unlabeled pre-existing malate dehydrogenase molecules and the same band width for both labeled and unlabeled enzymes (Fig. 6, Table I) indicate the turn over for m-MDH^2 must be fast, because the fully labeled malate dehydrogenase molecules with both subunits being labeled are the only type of malate dehydrogenase molecules detected in the CsCl gradient. On the other hand, the activities found in m-MDH^5 also increased to about 4-fold after germination for 5 days (Fig. 5); however, it is found that most activities are accumulated between the 3rd and 5th day. Therefore, if the rate of degradation for m-MDH^5 is very low, and the unlabeled molecules of m-MDH^5 can be dissociated into subunits in vivo and reassociated again with the newly synthesized subunits, then the "half-labeled" malate dehydrogenase molecules, having one heavy subunit labeled with $^{15}N/^{2}H$ and the other one unlabeled, would be formed. Since the m-MDH^5 increased to about 4-fold after 5 days germination, under these conditions, the distributions of non-labeled, "half-labeled" and "fully labeled" m-MDH^5 molecules would be $(1/4 + 3/4)^2 = 1/16{:}9/16$. The close densities of the "half-labeled" and the "fully labeled" m-MDH^5 molecules and the 6 : 9 distribution of these two types of molecules would therefore contribute the broad band width as shown in Fig. 6 and Table I. The lack of a "shoulder" for the 6% (1/16) fully unlabeled m-MDH^5 suggests that there is still turn over, even though the rate may be very slow.

The density labeling data on malate dehydrogenase isozymes presented here and results observed on catalase isozymes [20] suggest that the developmentally interesting changes in the enzyme content in germinating maize scutella result from regulation of synthesis as well as degradation.

It has been reported recently that the yeast chitin synthetase can be isolated in an inactive or zymogen state [22]. In addition, a protease extracted from yeast was found to act as an activating factor for the activation of zymogen [23]. In order to test whether the increase of the de novo synthesized malate dehydrogenase activity may be due to activation of the "inactive en-

TABLE II

ABSENCE OF IN VITRO DETECTABLE MALATE DEHYDROGENASE ACTIVATOR OR INHIBITOR IN MAIZE SCUTELLA

Crude extracts* of scutella isolated from different developmental stages	Calculated for additive summation	Total malate dehydrogenase activity observed ($\Delta_{340\,nm}$/min · 10 µl)
0.2 day + buffer**		0.66
2 day + buffer		0.27
4 day + buffer		0.43
5 day + buffer		0.52
8 day + buffer		0.37
0.2 day + 2 day	0.93	0.94
0.2 day + 4 day	1.07	1.09
0.2 day + 5 day	1.18	1.15
2 day + 4 day	0.70	0.67
2 day + 5 day	0.79	0.81
4 day + 8 day	0.80	0.78
5 day + 8 day	0.89	0.92

* Preparation of crude extracts described under "Materials and Methods".

** In order to detect the possible existence of "latent malate dehydrogenase" or "inactive malate dehydrogenase precursors" in dry seed, crude extract of scutella from seeds imbibed for 5 h was prepared five times the concentration (50 scutella/5 ml homogenate) used for the other crude homogenate (10 scutella/5 ml homogenate).

zymes", the crude scutellar homogenates were centrifuged at 480 × *g* for 10 min; the supernatants should contain membrane fractions, soluble macromolecules and micromolecules. By using such crude extracts, instead of fractionated subcellular fractions, we can then insure that we won't loose the "activators" (if they are) in our preparations. Results shown in Table II indicate that the malate dehydrogenase activities in the mixtures of crude extracts isolated from various developmental stages are the summation of activities as they are measured separately. Under the current experimental conditions, not only is there no "activation" of malate dehydrogenase activity when the crude extracts of 0.2-day- or 2-day-old scutella were added with those of 4-day- or 5-day-old scutella, but there is also no "inactivation" of malate dehydrogenase activities when the crude extracts of 4-day- or 5-day-old scutella were added with those of 8-day-old scutella. Therefore, we suggest that the increase and decrease of malate dehydrogenase activities observed in the scutella of germinating seedlings result from regulation of synthesis and degradation of the malate dehydrogenase isozyme moieties instead of activation or inactivation of pre-existing malate dehydrogenase isozyme.

Discussion

Longo and Scandalios [5] showed that the mitochondrial malate dehydrogenase isozymes in maize are controlled by nuclear genes. Our biochemical studies [15] of treating highly purified malate dehydrogenase isozymes with reducing agents (100 mM mercaptoethanol), low pH (pH 2 treatment), or high salt concentration (7.5 M guanidine · HCl), and the genetic analysis (Yang, N.S.

and Scandalios, J.G., unpublished) of the malate dehydrogenases have eliminated the possibility that conformational alterations could account for malate dehydrogenase multiplicity in maize. Detail genetic analysis (Yang, N.S. and Scandalios, J.G., unpublished) and the studies of the physical and biochemical properties of these isozymes [15] suggest that s-MDHs and m-MDHs are coded by separate loci, the five commonly observed m-MDHs are controlled by two groups of structural loci residing on two separate chromosomes. The genes coding for m-MDH^1 and m-MDH^3 are linked on one chromosome, while m-MDH^2 and m-MDH^5 are linked and located on another chromosome. The m-MDH^4 is a hybrid molecule of m-MDH^3 and m-MDH^5. Therefore, it would be interesting to study how the various maize malate dehydrogenase isozymes coded by different loci are expressed during development.

In the course of germination of young maize seedlings, the soluble and the mitochondrial malate dehydrogenase isozymes exhibit similar developmental patterns in scutella. The activities of s-MDH isozymes and m-MDH isozymes increase simultaneously and rapidly during the first 5 days, peak about the 6th day and decrease slowly thereafter.

The developmental changes of malate dehydrogenases in scutella are correlated to the growth of the young maize seedlings. During the first 5 days, the etiolated seedlings grew at a fairly constant rate and reached a state in which the shoots are about 5—6 cm long. Between the 5th and the 7th day, the shoots protruded the coleoptile, the scutella and endosperms became highly liquified. Then the leaves and the stems started to elongate. The high levels of malate dehydrogenase activity observed in the scutella of the 5—7-day-old seedling may indicate that the scutella have reached a state for maximal supply of nutrient and energy to the etiolated seedlings and are ready to be degraded thereafter.

Mitochondrial malate dehydrogenase is one of the Krebs cycle enzymes which play a central role in intermediate metabolism. Therefore, developmental changes of mitochondrial malate dehydrogenase activities may be a "developmental indicator" for the metabolic activities of the mitochondria. On the other hand, the soluble malate dehydrogenases transfer reducing equivalents (NADH) across the mitochondria and indicate the metabolic activities in the soluble cytosol. Based on these physiological functions of both soluble and mitochondrial isozymes and on the observed correlation between the growth of the young maize seedlings and the developmental changes of malate dehydrogenases in scutella, we suggest that the combined activity of both soluble and mitochondrial malate dehydrogenases may serve as an index for the physiological conditions of the maize tissues.

The increased activities of soluble and mitochondrial malate dehydrogenases during germination and early growth are due to de novo synthesis of these enzyme moieties themselves rather than a process which activates preexisting malate dehydrogenase molecules. The use of ^2H_2O as one of the density labels raises the possibility that, were the malate dehydrogenase a glycoprotein, the density shift could be entirely the result of deuteration of a carbohydrate moiety without synthesis of the protein moiety [20,24]. However, the low inherent density of the malate dehydrogenase molecules (1.2695—1.2710 g/cm^3) and the density shift of up to 0.02 g/ml upon labeling

are evidence that, at most, only a small part of the density shift could be due to carbohydrate. To obtain a density shift of 0.020 g/ml by deuteration of a postulated carbohydrate moiety without synthesis of the protein moiety, the malate dehydrogenase would need to be at least 50% carbohydrate. A density of 1.270 g/ml for the unlabeled enzyme renders this possibility unlikely. For example, horse radish peroxidase A [25], known to be only 20% carbohydrate [26], has a density of 1.349 g/ml [20]. A similar argument indicates that deuteration of a lipid moiety of malate dehydrogenase does not explain the observed density shift. Therefore, we conclude that both the soluble and mitochondrial malate dehydrogenase isozymes in the scutella of developing maize seedlings are synthesized de novo.

The fact, that both soluble and mitochondrial isozymes follow a similar developmental pattern is interesting in two aspects. (a) First, this may indicate that the structural genes coding for the various soluble and mitochondrial malate dehydrogenase are "expressed" simultaneously in maize scutella during early sporophytic development. Mechanisms for "expression" of these genes may be independent or they may be integrated. Gene regulation in higher eukaryotic organisms has not yet been well demonstrated. However, there are indeed indications that the nuclear mechanisms controlling the action of gene expression are complex and perhaps highly integrated. Britten and Davidson [27] have proposed a gene regulation model and suggested that regulatory genes may also play important roles in gene expression of higher organisms. In fact, our genetic studies (Yang, N.S., and Scandalios, J.G., unpublished) suggest that the expression of maize mitochondrial malate dehydrogenase isozymes appear to be regulated by another genetic unit which is located elsewhere on the chromosome. Therefore, it is possible that the developmental "expression" of the various genes coding for maize malate dehydrogenase isozymes is also regulated or integrated. Of course, it is also possible that the various malate dehydrogenase structural genes are not "expressed" in the scutella of young maize seedlings, instead, their mRNAs for the various malate dehydrogenase isozymes are synthesized independently prior to the maturation of the dry kernel. During seed germination, these mRNAs are stable and long-lived and therefore the same developmental patterns of maize malate dehydrogenase isozymes may still be observed as shown in the results. At this moment, we have not ruled out this possibility. However, stable mRNA species in maize have not been demonstrated and even in other eukaryotic organisms, they are still not commonly observed. Therefore, we suggest that this possibility is more unlikely. (b) If we assume that the "expression" of malate dehydrogenase structural genes is regulated simultaneously, it is still possible that the cellular activities of malate dehydrogenase isozymes are controlled at translational and post-translational levels. Therefore, even though the mitochondrial malate dehydrogenases appear to follow a similar developmental pattern, the synthesis and degradation of each isozyme may still be controlled independently.

As mentioned in the Results, Fig. 6 and Table I indicate that turn over of m-MDH^5 is under the control different from that of m-MDH^2. These results suggest that in the cell, regulations of isozyme activities may be just as complicated as regulations of different enzyme activities. The regulatory mechanisms may occur at transcriptional, post-transcriptional, translational and post-translational levels.

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References

1 Lehninger, A.L. (1970) in Biochemistry, Edn. 1, Chapter 18, p. 408, Worth Publishers, Inc.
2 Douce, R. and Bonner, Jr, W.D. (1972) Biochem. Biophys. Res. Common. 47, 619—624
3 Ting, I.P., Sherman, I.W. and Dugger, Jr, W.M. (1966) Plant Physiol. 41, 1083—1084
4 Ting, I.P. (1971) in Photosynthesis and Photorespiration. (Hatch, M.D., Osmond, C.B. and Slayter, R.O., eds), pp. 169—185, Inter Science, New York
5 Longo, G.P. and Scandalios, J.G. (1969) Proc. Natl. Acad. Sci. U.S. 62, 104—111
6 Kitto, G.B., Wasserman, P.M. and Kaplan, N.O. (1966) Proc. Natl. Acad. Sci. U.S. 56, 578—585
7 Meizel, S. and Markert, C.L. (1967) Arch. Biochem. Biophys. 122, 753—765
8 Shows, T.B., Chapman, V.M. and Ruddle, F.H. (1970) Biochem. Genet. 4, 707—718
9 Bailey, G.S., Wilson, A.C., Halver, J.E. and Johnson, C.E. (1970) J. Biol. Chem. 245, 5927—5940
10 Shannon, L.M. (1968) Ann. Rev. Plant Physiol. 19, 187—210
11 Wright, D.A. and Subtelny, S. (1971) Dev. Biol. 24, 119—140
12 Scandalios, J.G. (1974) Ann. Rev. Plant Physiol. 19, 187—210
13 Breidenbach, R.W. and Beevers, H. (1967) Biochem. Biophys. Res. Commun. 27, 462—469
14 Yamazaki, R.K. and Tolbert, N.E. (1969) Biochim. Biophys. Acta 178, 11—20
15 Yang, N.S. and Scandalios, J.G. (1974) Arch. Biochem. Biophys. 161, 335—353
16 Longo, C.P. and Longo, G.P. (1970) Plant Physiol. 45, 249—252
17 Scandalios, J.G. (1969) Biochem. Genet. 3, 37—79
18 Filner, P. and Varner, J.E. (1967) Proc. Natl. Acad. Sci. U.S. 58, 1520—1526
19 Ochoa, S. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds), Vol. II, pp. 735—739, Academic Press, New York
20 Quail, P.H. and Scandalios, J.G. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1402—1406
21 Devenyi, T., Rogers, S.J. and Wolfe, R.G. (1966) Nature 210, 489—491
22 Gabib, E. and Farkas, V. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2052
23 Gabib, E. and Ulane, R. (1973) Biochem. Biophys. Res. Commun. 50, 186—191
24 Anstine, W., Jacobson, J.V., Scandalios, J.G. and Varner, J.E. (1970) Plant Physiol. 45, 148—152
25 Shannon, L.M., Kay, E. and Lew, J.Y. (1966) J. Biol. Chem. 24, 2166—2172
26 Shannon, L.M. (1970) Isozyme Bull. 3, 19
27 Britten, R.J. and Davidson, E.H. (1969) Science 165, 349—357