

NUCLEAR GENE CONTROL OF MITOCHONDRIAL MALIC DEHYDROGENASE IN MAIZE*

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Abstract and Summary.—The isozymes of malic dehydrogenase (MDH) of maize have been separated by starch gel zone electrophoresis. The MDH isozyme pattern was the same in different organs and developmental stages. The subcellular distribution of the different isozymes was established by means of differential centrifugation. The MDH isozymes were found to be differentially distributed in the cytoplasm, the mitochondria, and the glyoxysomes. The latter proved the only exception to the pattern constancy among organs insofar as they are present only in the scutella. Among several inbred maize strains examined, only variants of the mitochondrial isozymes were found. Crosses between strains with different isozyme patterns gave Mendelian inheritance: thus, these mitochondrial isozymes are under nuclear-gene control.

The presence of DNA in the mitochondria of many different organisms is now well established. Evidence for a genetic function of this DNA has been brought forward by genetic and biochemical studies.¹ However, several investigators have questioned the complete genetic autonomy of mitochondria on the basis that the amount of DNA present in them may not be sufficient to code for all their proteins.¹⁻³ If this is true, one has to assume that some of the mitochondrial proteins are coded by nuclear DNA. This paper presents some genetic evidence for the coding of mitochondrial malic dehydrogenase (MDH) by chromosomal DNA. This enzyme is known to exist in several isozymic forms, some of which are specifically associated with mitochondria.⁴⁻¹⁰ We have identified these mitochondrial isozymes in maize scutella and have found variants among them by screening many different inbred strains. By means of appropriate genetic crosses, we have demonstrated that the isozymes of mitochondrial MDH are inherited according to Mendelian rules.

Materials and Methods.—Maize strains that were inbred for at least seven generations were used in all experiments. The plants were grown on moist vermiculite in darkness at 28°C.

The MDH isozymes were separated by starch gel electrophoresis according to the method of Scandalios,¹¹ but with the Tris-citrate buffer system of Meizel and Markert⁹ in place of Poulik's buffer system. The samples were subjected to electrophoresis for 16-18 hr at 200 v and 5°C. Horizontal gel slices were stained for MDH by following the method of Fine and Costello.¹²

Crude extracts from different organs of the young corn plant were run on starch gels in order to obtain the complete pattern of MDH isozymes. These extracts were prepared by homogenizing the different organs in a mortar with 0.025 M glycylglycine buffer pH 7.4 (2 ml of buffer were used for 1 gm of fresh weight of tissue). The homogenate was filtered through four layers of cheesecloth and centrifuged at 14,800 × *g* for 15 min. The supernatant was used for electrophoresis and for assaying quantitatively the MDH activity by the method of Ochoa.¹³ Protein was determined by the method of Lowry *et al.*¹⁴

Mitochondria were prepared from 15–20 gm of scutella of maize seeds soaked for 24 hr in water at 28°C. The scutella were chopped with razor blades and then gently ground in a mortar with 3–4 vol of the medium described by Breidenbach and Beavers.¹⁵ The crude mitochondrial pellet sedimenting between 480 and 10,500 *g* for 10 min was purified by running it on a 25–55% continuous sucrose gradient (4-hr spin at 25,000 rpm in the Spinco SW-25.1 rotor). Fractions were collected from the top of the gradient and assayed for cytochrome oxidase to determine the location of the mitochondrial band. The fractions containing the mitochondria were pooled, pelleted down, and resuspended in 0.5–0.6 ml of the grinding medium. Suspending the mitochondrial pellet in 0.025 *M* glycylglycine buffer or freezing and thawing the suspension before electrophoresis did not significantly improve the resolution of the MDH isozymes.

Genetic analysis was done partly on scutella (excised from seeds soaked for 24 hr) and partly on the liquid endosperm. In the latter case the seeds were harvested at the 16th day after pollination from plants grown in the field. The extract of a single scutellum in glycylglycine buffer (0.1 ml) was used for soaking each paper strip that was inserted in the gel. When liquid endosperm was used for genetic analysis, the liquid of a single kernel was squeezed directly onto the paper strip.

Results.—Preliminary observations of the isozymic patterns of MDH in different organs, at various stages of seedling development, showed that the number and position of the isozymes under study remained constant, the only apparent exception being the glyoxysomal MDH isozymes in scutellum extracts which will be described below.

MDH patterns in different organs and organelles: The MDH isozyme pattern is the same in all the organs of the young maize plant (scutellum, root, shoot, coleoptile, and leaf), but the best resolution can be obtained with scutellum extracts (Fig. 1). In the scutellum at the seventh day of germination the specific activity of MDH is fivefold higher than in any other organ examined (Fig. 2). This offers an additional advantage in allowing the use of the extracts of one single scutellum for the electrophoretic analysis. Even scutella excised from dry seeds show a high MDH activity. This activity (expressed as reaction rate per scutellum) increases rapidly during the first five days of germination and begins to decrease thereafter. If the MDH activity is expressed per milligram

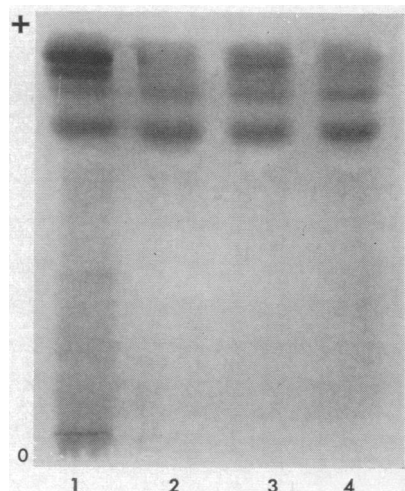


FIG. 1.—Zymogram showing the MDH isozymes from crude extracts of different organs from 7-day-old maize seedlings. 1, Scutellum; 2, root; 3, shoot; 4, coleoptile; O, origin or point of sample insertion.

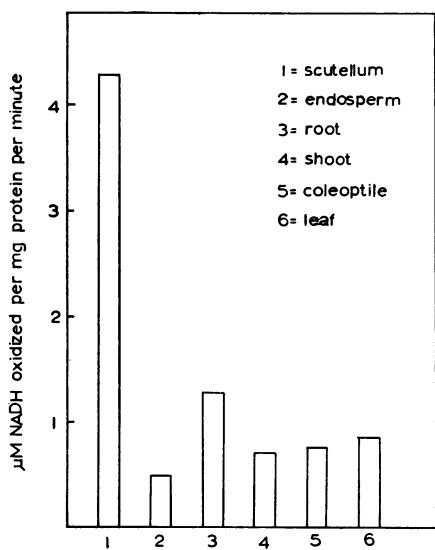


FIG. 2.—Specific activity of MDH in different organs from maize seedlings at the seventh day of germination.

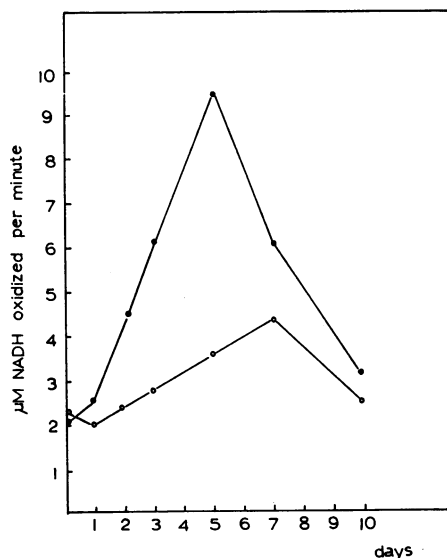


FIG. 3.—Time course of MDH activity in scutella of germinating maize seeds. —●—, Activity per scutellum; —○—, activity per milligram of protein.

of protein, the peak is reached at the seventh day. This is probably due to the decrease in soluble protein that occurs after the fifth day of germination (Fig. 3). In spite of these variations in enzymic activity during germination, the number and position of the bands remain constant over the first ten days (Fig. 4). For these reasons the scutellum seems to be the most suitable organ for the study of the MDH isozymes.

The glyoxylate cycle is known to be operating in maize scutella.¹⁶ The mitochondrial fraction isolated from this organ by differential centrifugation could

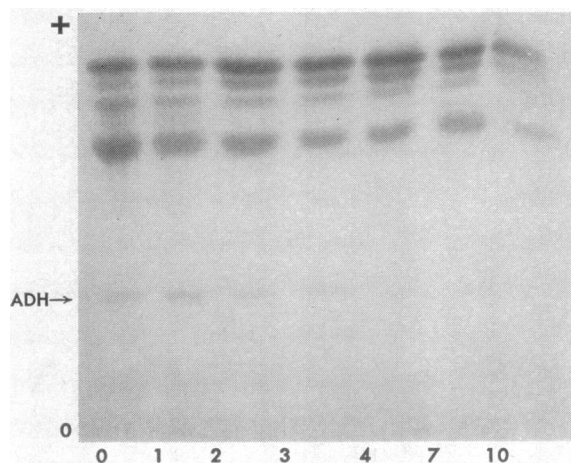


FIG. 4.—Zymogram showing the MDH isozymes from crude extracts of scutella at different days of germination. The numbers indicate the days. O, origin or point of sample insertion. (The zone of activity closest to the origin (ADH) is nonspecific alcohol dehydrogenase.)

therefore be expected to contain some glyoxysomes, the cell organelles in which the glyoxylate cycle enzymes are present.^{15, 17} As glyoxysomes contain MDH,^{15, 17} it is important to free the mitochondria from them. This was done by running the crude mitochondrial fraction on a sucrose density gradient. Two subfractions were obtained in this way: the lighter one has proved to be identical with mitochondria, the heavier one identical with the glyoxysomes. The latter were identified by their high content in isocitratase, malate synthetase, and catalase. Cytochrome oxidase was used as a marker for the mitochondria.

The MDH activity in the different subcellular fractions from maize scutellum is shown in Figure 5. The specific activity of the mitochondria is five times higher

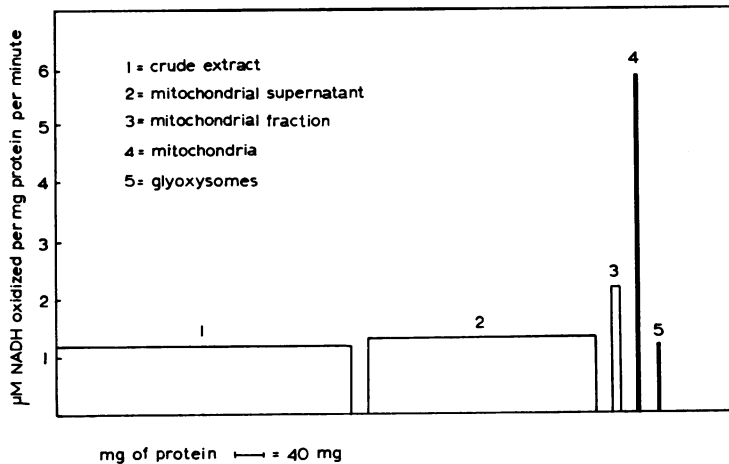
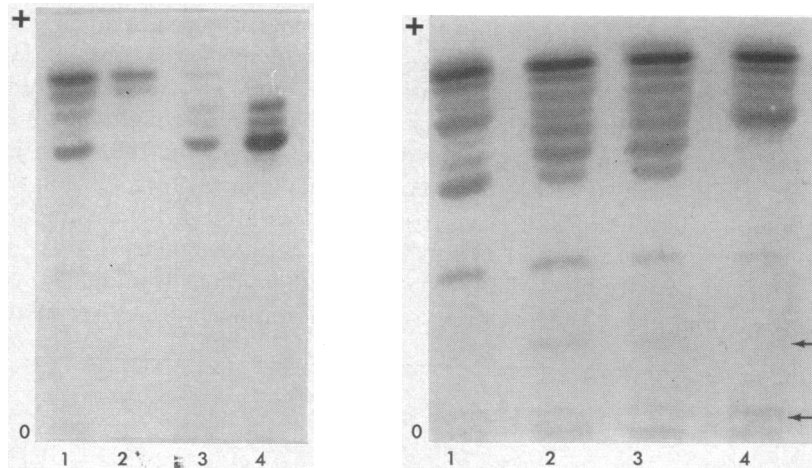


FIG. 5.—MDH activity in different subcellular fractions from 24-hr maize scutellum. The basis of the rectangles is proportional to the total protein content of the fraction.

than that of the crude extract, while the specific activity of the glyoxysomes is approximately the same as that of the crude extract. The contribution of the glyoxysomes to the total MDH activity of the tissue is thus rather small because these particles account only for a small fraction of the total protein. The isozymic pattern of the different subcellular fractions is shown in Figure 6. The two most anodic-migrating bands are present in the soluble fraction; the three middle bands are related to mitochondria. The glyoxysomal fraction shows two very slowly migrating bands which are fainter than any other band. These bands can be seen in Figure 7. They are detectable only in scutellum extracts, while the mitochondrial bands are present in all organs examined. A mitochondrial preparation from shoots shows the same bands as those present in mitochondria prepared from scutella. The mitochondrial bands seem thus to be constant in different organs and at different developmental stages.

Inheritance of mitochondrial MDH isozymes: Several maize inbred lines have been tested for MDH isozymes in scutella and liquid endosperm. No variants in the cytoplasmic bands were found among these lines, whereas several variants were detected in the mitochondrial bands. The total number of inbred lines



(Left) FIG. 6.—Zymogram showing the MDH isozymes in different subcellular fractions from 24-hr maize scutellum.

1, Crude extract. 2, Mitochondrial supernatant. 3, Mitochondrial fraction. 4, Mitochondria purified on a sucrose density gradient.

(Right) FIG. 7.—Zymograms showing the MDH isozymes from extracts of scutella of the two variants chosen for genetic analysis and of their hybrids derived from reciprocal crosses.

1, Variant "type A". 2, Hybrid between A and B (B is the female parent). 3, Hybrid between A and B (A is the female parent). 4, Variant "type B."

The arrows indicate the glyoxysomal MDH bands.

tested was 25, and five variant types for mitochondrial MDH were found. Among these variants we chose two types for the crosses. One, which we call the "B type," has the same isozymic pattern as the strain used in the previous experiments; the other, called the "A type," shows two deeply staining mitochondrial bands, one of which is faster and one slower than the slowest strong band of the other parent. The variant "A type" is the inbred line 59, the "B type" the inbred line Oh 51A. To demonstrate the inheritance of these variations the two reciprocal crosses between A and B have been examined. The isozymic patterns in the imbibed scutella of the two hybrids are identical and their pattern is intermediate between those of the parents (Fig. 7). In the hybrid all the parental bands are inherited so that the mitochondria have a total of five bands. The isozymic pattern of the different fractions obtained from the parents and the hybrid are shown in Figure 8.

TABLE 1. Results showing the mode of inheritance of mitochondrial MDH variants A and B.

Parent		Phenotypes in Offspring			Total
Female	Male	A	AB	B	
A	A	500	0	0	500
B	B	0	0	500	500
A	B	0	100	0	100
B	A	0	100	0	100
AB	AB	18	62	20	100
AB	A	48	112	0	160
A	AB	19	61	0	80
AB	B	0	66	54	120
B	AB	0	41	39	80

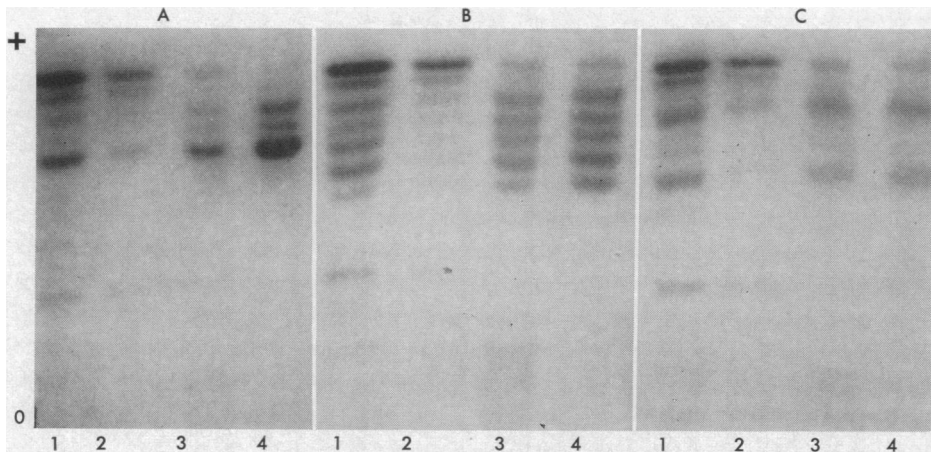
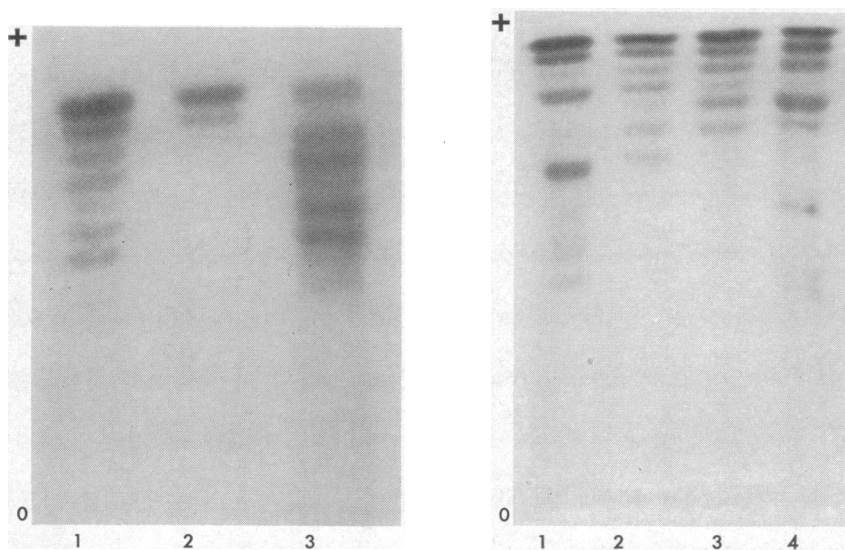


FIG. 8.—Zymograms showing the MDH isozymes in different subcellular fractions from scutella of the two variants chosen for genetic analysis and their hybrid. *A*, Variant “type B”; *B*, hybrid between *A* and *B*; *C*, variant “type A.” 1, Crude extract; 2, mitochondrial supernatant; 3, mitochondrial fraction; 4, mitochondria purified on a sucrose density gradient; *O*, origin or point of sample insertion.

The presence of a hybrid isozyme pattern in the mitochondria of the heterozygote and the identical results obtained with the reciprocal crosses in the F_1 give a strong indication that the mitochondrial isozymes of MDH are under chromosomal control and are not coded by the mitochondrial DNA. However, in order to determine in an unequivocal way the nuclear control over this mitochondrial enzyme, we have examined also the F_2 and the backcrosses to both parents.

In the F_2 generation the two parental and the hybrid phenotypes are present as expected for a Mendelian type of inheritance. In backcrosses to the B parent, half the offspring were of the B type and half were the hybrid type (AB). In backcrosses to the A parent the two phenotypes expected (A and AB) were present, but the A type showed a lower frequency than expected. Also in the F_2 the frequencies of the three phenotypes were significantly different from the 1:2:1 distribution and the deviation was due to a low number of the “A type.” These observations could be explained with a lower viability of the “A type.” “A-type” kernels are indeed smaller and the ears in the F_2 and the backcrosses to A showed several aborted kernels. The results of the crosses are shown in Table 1.

The genetic experiments employing the liquid endosperm from kernels harvested on the 16th day after pollination gave the following results. Differential centrifugation of this material yields a mitochondrial fraction having a high cytochrome oxidase activity and an isozyme pattern identical to that of mitochondria from scutella (Fig. 9). As the liquid endosperm is triploid, a gene dosage effect should be expected to occur in the hybrid. This was indeed the case, as shown in Figure 10. When A was the female parent, the A bands stained much stronger than the B bands in the F_1 zymograms. The opposite happened when B was chosen as the female parent.



(Left) FIG. 9.—Zymogram showing the MDH isozymes in different subcellular fractions from the liquid endosperm of the hybrid between A and B types.

1, Crude extract. 2, Mitochondrial supernatant. 3, Mitochondrial fraction. O, Origin or point of sample insertion.

(Right) FIG. 10.—Zymogram showing the MDH isozymes from the liquid endosperm of the variants chosen for genetic analysis and the hybrids derived from reciprocal crosses.

1, Variant "type A." 2, Hybrid between A and B (A is the female parent). 3, Hybrid between A and B (B is the female parent). 4, Variant "type B." Note the gene dosage effect in the reciprocal crosses. O, Origin or point of sample insertion.

Discussion.—Our data strongly suggest that the mitochondrial isozymes of MDH in maize which we have studied are inherited in accordance with Mendelian rules. The dosage effect in the triploid tissue is additional evidence of the nuclear control over this mitochondrial enzyme. If mitochondrial MDH were coded by mitochondrial DNA, a cytoplasmic type of inheritance should occur and the two reciprocal crosses in the F_1 should be different, while not all three of the phenotypes should be found in the F_2 .

Similar genetic evidence for nuclear control over a mitochondrial enzyme has been found for β -hydroxybutyric dehydrogenase in *Paramecium*¹⁸ and for malic dehydrogenase in man.¹⁹ In the latter case, however, a reliable genetic analysis was impossible because of the small number of variant subjects found. Further support for the incapability of mitochondria to synthesize all their proteins comes from the biochemical findings of several research workers who have shown that protein synthesized on microsomes can be transferred to mitochondria and incorporated into them.²⁰⁻²² There is indeed increasing evidence that mitochondria are able to synthesize by themselves only their structural protein. It is not surprising, therefore, that malic dehydrogenase which is rather loosely bound to the mitochondrial structure²³ is not synthesized in mitochondria.

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