BIOCHEMICAL AND DEVELOPMENTAL STUDIES OF THE GENETICALLY DETERMINED MALATE DEHYDROGENASE ISOZYMES IN MAIZE

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ABSTRACT

BIOCHEMICAL AND DEVELOPMENTAL STUDIES OF THE GENETICALLY DETERMINED MALATE DEHYDROGENASE ISOZYMES IN MAIZE

By

Ning-Sun Yang

Multiple molecular forms (isozymes) of malate dehydrogenase (MDH; L-malate: NAD oxidoreductase E.C.1.1.1.37) in maize have been identified by starch gel electrophoresis and the zymogram technique. Two major classes of MDH isozymes are observed. One class is restricted in occurrence to the mitochondria (m-MDHs), while the other class (s-MDHs) occurs in the soluble fraction of the cell. A third group has been associated with microbodies.

Genetic analysis indicates that the two major classes of MDH isozymes are coded by different structural loci. Within each of the two classes, multiple electrophoretic forms are observed. Two soluble MDHs and five mitochondrial MDHs are commonly observed in the various MDH phenotypes found among the 20 highly inbred lines examined. These MDH isozymes in maize are genetically determined and are not different conformational forms derived from the same primary structure.

Genetic control of the two soluble MDH isozymes has not been studied in detail, because variants of these two

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soluble forms are not available in the present investigation. The nuclear gene controlled mitochondrial MDH isozymes regulated by multiple structural loci. The five most anodal m-MDHs are controlled by two groups of loci. These two linkage groups, each with two closely linked loci are located on two different chromosomes. Genetic analysis of the expression of the m-MDH isozymes suggests that the m-MDH structural loci are likely controlled by "regulatory" genes.

The formation of a single type of hybrid MDH molecule from two types of subunits having different electrophoretic mobilities suggests that maize MDH isozymes (both s-MDHs and m-MDHs) are dimers in molecular structure. The two soluble and the five mitochondrial MDH isozymes in inbred strain W64A were separated and highly purified through six steps of purification procedures. Biochemical properties for each of the seven MDH isozymes were examined. The soluble and the mitochondrial MDHs differ in most of the physical and kinetic properties. In comparing these properties of the five mitochondrial MDH isozymes, it is found that m-MDHs can be classified into two groups, the two most anodal m-MDHs belong to one group, while the three less anodal m-MDHs belong to another. These results along with the genetic analysis suggest that gene duplications are involved in the evolution of maize mitochondrial MDH isozymes.

Expression of two soluble and five mitochondrial MDH isozymes in the development of young maize seedlings is

studied. All of the scutellar s-MDHs and m-MDHs exhibit similar activity profiles in the scutellum, however, the total m-MDH activity is only 60% of that in the cytosol. Density labeling experiments indicate that both s-MDHs and m-MDHs in the scutellum of developing maize seedlings are *de novo* synthesized. Effects of protein synthesis inhibitors, cycloheximide (CH) and chloramphenicol (CAP), on MDH activities and on protein synthesis in scutella are studied. The increases of both s-MDHs and m-MDHs are inhibited by CH, but not by CAP, suggesting that protein synthesis in the cytoplasm, but not in the mitochondria, is essential for the increase of both s-MDHs and m-MDHs activities during development. This result is consistent with the finding that mitochondrial MDHs in maize are controlled by nuclear genes and indicates that maize m-MDHs are synthesized in the cytoplasm and then become associated with the mitochondria.

BIOCHEMICAL AND DEVELOPMENTAL STUDIES OF THE GENETICALLY DETERMINED MALATE DEHYDROGENASE ISOZYMES IN MAIZE

By

Ning-Sun Yang

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Dedicated to my parents and Vina

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LIST OF ABBREVIATIONS

3-AP-NAD	3-acetyl pyridine analog of NAD
BSA	bovine serum albumin
CAP	chloramphonicol
CH	cycloheximide
DEAE-cellulose	diethylaminoethyl cellulose
deam-NAD	deaming analog of NAD
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gravitational force
HEPES	N-2-hydroxyethylpiperazine-N-2- ethane sulforic acid
Km	Michaelis-Meuten constant
MDH	malate dehydrogenase
s-MDH	soluble malate dehydrogenase
m-MDH	mitochondrial malate dehydrogenase
g-MDH	glyoxysomal malate dehydrogenase
mdh^m	genes coding for m-MDHs
mdh^s	genes coding for s-MDHs
MMC	megaspore mother cell
NAD, NADH	nicotinamide adenine
^{15}N-D_2O medium	10 mM $^{15}NH_4Cl$ in 70% D_2O
^{14}N-H_2O medium	10 mM $^{14}NH_4Cl$ in H_2O
nm	nanometer (10^{-9}cm)
OAA	oxaloacetic acid

PMC	pollen mother cell
PI	isoelectric point
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
TN-NAD	thionicotineamide analog of NAD
Tris	tris (hydroxymethyl) amino methane
Ve	elution volume in gel filtration
Vo	void volume in gel filtration

PART I

GENETIC CONTROL OF THE MULTIPLE ELECTROPHORETIC FORMS OF MAIZE MALATE DEHYDROGENASE (MDH)

Introduction

The occurrence of multiple molecular forms of enzymes (isozymes) is now known to be a common characteristic in most organisms (1, 2). Malate dehydrogenases (MDH), in various animal and plant tissues, have been shown to commonly exist in isozymic forms. Since malate dehydrogenase plays several physiological roles within the cell (3, 4) and was found both in soluble cytoplasm and in mitochondria (3), it would be important to know how the expression of MDH isozymes is controlled genetically.

Genetic variants of MDH isozymes have been observed in several animal tissues (5, 6, 7, 8, 9) and in plants (10). The mitochondrial MDH isozymes in maize (10) and in mouse (5) were found to be controlled by nuclear genes, however detailed genetic analysis of the mitochondrial MDH isozymes has not been reported in either study. The number of structural genes coding for soluble MDHs in vertebrates may vary. In reptiles, birds, and mammals, s-MDH typically exists as a single major anodal form (11, 12, 13) which suggests single gene control. In fishes and amphibians (8, 14), the s-MDHs appear to be controlled by two unlinked loci.

In the present study, electrophoretic variants and the formation of hybrid molecules of both soluble and mitochondrial MDH isozymes have been found in maize. Studies of the genetic control of maize MDH isozymes have been concentrated on mitochondrial forms. The reasons are: 1) Even though the variants of soluble MCH isozymes were found, inbred lines homozgous for such variants are still not available. 2) Several highly inbred lines carrying mitochondrial MDH variants were prepared. Back crosses and F_2 progenies made from these inbred lines were obtained in large quantities.

The following points were observed in this study: 1) The maize mitochondrial MDH isozymes are controlled by multiple loci which may reside on two different chromosomes. 2) Null alleles of m-MDH genes may exist widely in maize and they appear to be correlated to observed lethal effects. 3) "Regulatory" genes may be involved in the expression of m-MDH structural genes.

Materials and Methods

Maize Strains

Maize strains that were inbred for at least ten generations were used in all experiments. Back crosses and F_2 involving these inbred lines were made in 1972 and 1973. Maize ears were harvested between 16 and 20 days after pollination. MDH isozyme patterns in the fresh liquid ebos

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endosperm from individual kernels were checked. The maize ears were then stored, within 24 hours after harvest, in the freezer (-18°C). The thawed and fresh liquid endosperm were found to have the same MDH isozyme patterns, no distinguishable changes in zymogram were observed after freezing and thawing the immature maize kernels.

Open pollinated maize strains of unknown genetic background was also used for studying variants of soluble MDHs.

Identification of mitochondrial and soluble malate dehydrogenase isozymes in maize

Soluble and mitochondrial fractions of maize scutella were separated by modifying and method of Longo and Longo (18) and will be described in detail in Part III. The MDH isozymes in the two different subcellular locations are identified by starch gel electrophoresis and zymogram techniques. The organelle specific MDH isozyme patterns are consistent with those found by Longo and Scandalios (10).

Starch gel electrophoresis and MDH staining

The MDH isozymes were separated by electrophoresis on 12% starch gels according to the method fo Scandalios (15), with the Tris-citrate buffer system (pH 7.0) of Meizel and Markert (16). The 12% starch gel was used in all experiments.

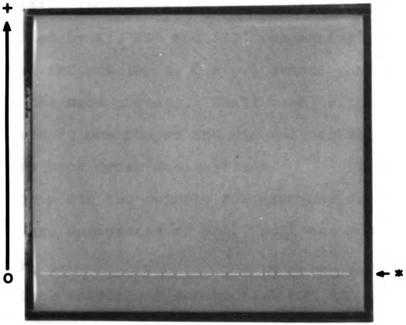
The liquid endosperm from individual kernels was squeezed onto a 4 mm x 6 mm piece of Whatman #3 mm filter paper and inserted into a vertical slot in the starch gel. About 25 samples were applied to each gel as shown in Figure 1. Horizontal starch gel electrophoresis was conducted at 5°C, under an applied voltage gradient of 8-10 V/cm for 16-18 hours. After electrophoresis, the gels were sliced horizontally and stained for malate dehydrogenase activity. The staining mixture described by Fine and Costello (17) and modified by Scandalios (15) is shown in the following:

100 ml Tris - HCl (0.2M, pH 8.3)
100 ml Na-Malate (0.2M, pH 7.0)
2.0 ml KCN (0.002M)
2.0 ml NAD (0.01M)
2.0 ml phenazine methosulfate (0.01M)
100 mg Nitro Blue Tetrazolium (from Sigma Company)

Staining was completed in approximately 50 minutes to 1 hour at 37°C. The gels were then washed several times with cold tap water and were photographed. In most cases, the gels were preserved for future reference in a solution of 40-50% glyceral.

To screen the soluble MDH isozyme variants, scutella in the open pollinated ears (dry seed) were used. Each scutella was ground, at 4°C, with 0.3 ml glycylglycine buffer (0.025M, pH 7.4). Aliquots of the crude scutellar extracts were subjected to starch gel electrophoresis and stained for MDH activity as described below.

Figure 1.--Starch gel prepared for screening MDH phenotypes. Wicks containing sample were prepared from liquid endosperm and were inserted into the gel for electrophoresis. Approximately 25 samples were run per gel. The gel was then subjected to electrophoresis and stained for malate dehydrogenase as described under "Materials and Methods."



*Slit to insert filter paper wicks

Scoring and symbols for the phenotypes and genotypes of soluble and mitochondrial MDH isozymes in maize

In the progenies of open pollinated plants, the phenotypes of mitochondrial MDH isozymes are designated as I, II, and III. Those carrying additional variants of s-MDHs are represented by I', II' and III' respectively.

Capital letters (A, B, C etc.) denote phenotypes observed in the back crosses. Small case letters (a, b, c etc.) indicate F_2 phenotypes and are not necessarily correlated to back cross designations.

The genes for the soluble MDH isozymes (s-MDH^1, s-MDH^2 and s-MDH^4) are designated as $\underline{mdh}_1^s$, $\underline{mdh}_2^s$ and $\underline{mdh}_4^s$) respectively.

The genes encoding mitochondrial MDH isozymes (m-MDH^1, m-MDH^2 and m-MDH^5, etc.) are designated as $\underline{mdh}_1^m$, $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$, etc. respectively.

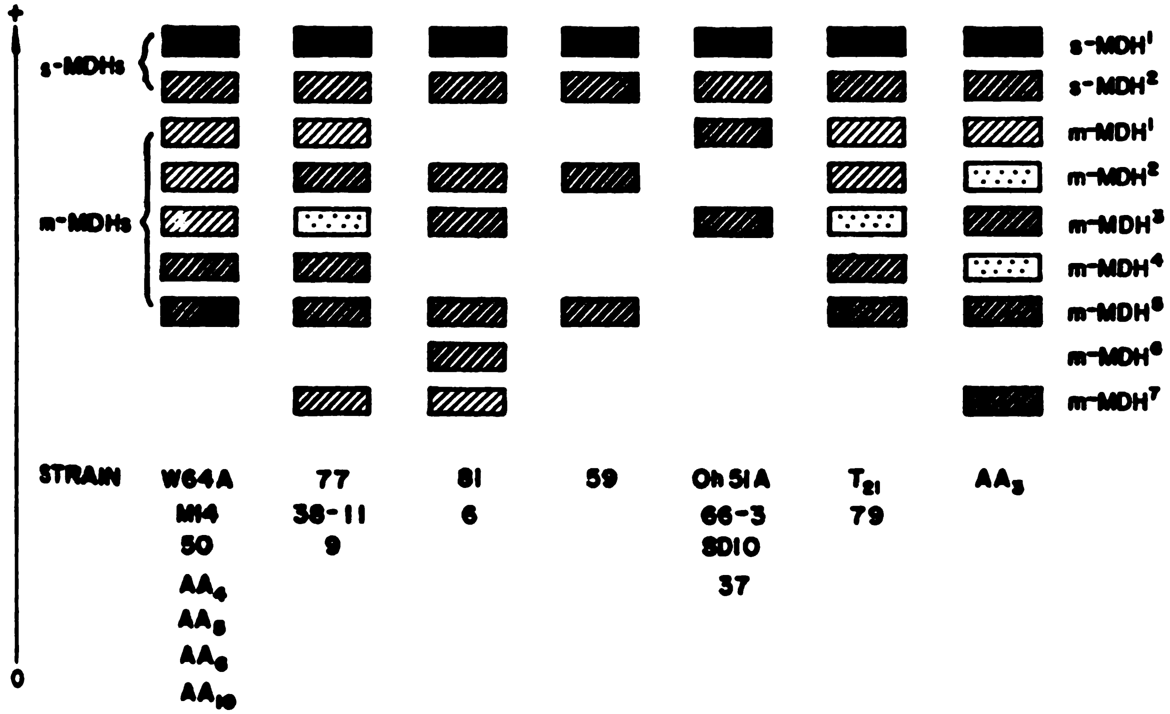
Linkage relationships between two linked loci as $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ is represented as "linkage group $\underline{mdh}_{1-3}^m$." Similarly, $\underline{mdh}_{2-5}^m$ denotes the two linked loci of $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$.

Results

Genetic variants of maize MDH isozymes

In maize, there are two major classes of malate dehydrogenase isozymes (Figure 2). The two soluble forms (s-MDHs) appear in all the inbred lines tested and are names as s-MDH^1 and s-MDH^2. Additional s-MDH isozyme

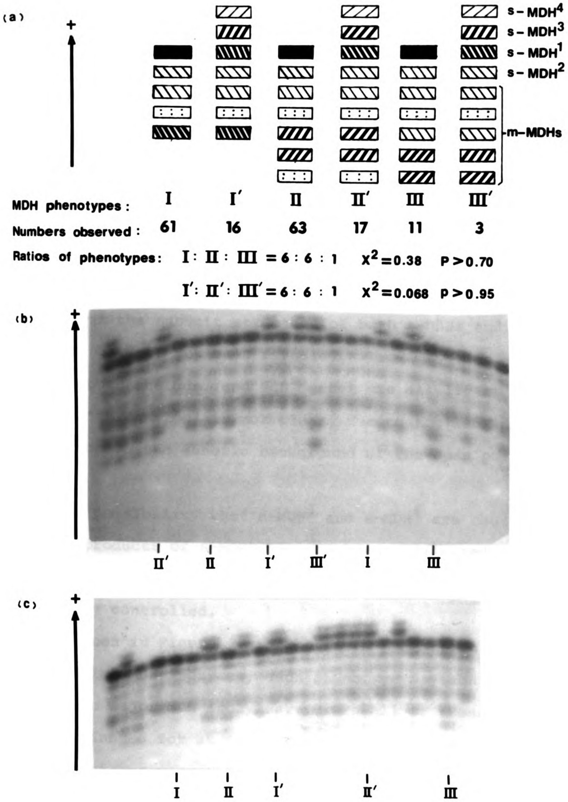
Figure 2.--Phenotypes of MDH isozymes observed in various inbred maize lines.


+
s-MDHs
m-MDHs
s-MDH¹
s-MDH²
m-MDH¹
m-MDH²
m-MDH³
m-MDH⁴
m-MDH⁵
m-MDH⁶
m-MDH⁷
STRAIN
W64A
MI4
50
AA₄
AA₅
AA₆
AA₁₀
77
38-11
9
81
6
59
Oh51A
66-3
SD10
37
T₂₁
79
AA₃
0

variants have been observed occasionally in inbred lines 59, 37 and T_{21}. These variants are more anodal compared to s-MDH^1. However, the lack of a consistent appearance of the s-MDH variants in these inbred lines made the genetic studies on s-MDHs impossible. Seven variants of the mitochondrial MDH isozymes (m-MDHs) were observed in the 20 highly inbred lines examined. The mitochondrial MDH isozymes are named from the anode toward to cathode as m-MDH^1, m-MDH^2, m-MDH^3 . . ., m-MDH^6 and m-MDH^7.

In the scutella of open pollinated corn, the s-MDH variants appear in a fairly high frequency (Figure 3). There is no correlation between the occurrence of soluble and mitochondrial variants in these plants, since the frequencies of the appearance of the s-MDH variants are the same in plants with three different m-MDH phenotypes (Figure 3). This result indicates that the s-MDHs and m-MDHs are probably under the control of different genes. The appearance of the two additional s-MDHs seem to be coupled with the decrease of activity found in s-MDH^1. The activity found in s-MDH^3 is always higher than that in s-MDH^4 (Figure 3). The simultaneous appearance of s-MDH^3 and s-MDH^4 coupled with the decrease in the activity of s-MDH^1 (Figure 3) suggests that s-MDH^3 is a hybrid molecule consisting of one subunit of s-MDH^1 and another one of s-MDH^4. Since the activity in s-MDH^1 is much higher than that in s-MDH^4, the association of s-MDH^4 with s-MDH^1 will "drive"

Figure 3.--The appearance of soluble MDH variants in the scutella of open pollinated indian corn. The expression of two more anodal soluble MDHs are not correlated to that of the specific mitochondrial MDH phenotype.



MDH phenotypes :	I	I′	II	II′	III	III′
Numbers observed :	61	16	63	17	11	3

Ratios of phenotypes: I : II : III = 6 : 6 : 1 $X^2 = 0.38$ $P > 0.70$

I′ : II′ : III′ = 6 : 6 : 1 $X^2 = 0.068$ $P > 0.95$

most of the available s-MDH^4 subunits to form s-MDH^3. Assuming s-MDH^1 is expressed twice the amount to that of s-MDH^4, the s-MDH^3 is derived from association of s-MDH^1 and s-MDH^4 in random, the expected dosage of the activities in s-MDH^1, s-MDH^3 and s-MDH^4 would be $(\frac{1}{2} + 1)^2 = \frac{1}{4} : 1 : 1 = 1 : 4 : 4$. The results in Figure 3 indicate that the activities in s-MDH^1 and s-MDH^3 are indeed similar and are much higher than that in s-MDH^4. The formation of a single hybrid molecule suggests that the soluble MDH isozymes in maize are dimers. Dimeric soluble MDH isozymes have been postulated in procine heart (19), and in salmon (8). Evidence of the subunit structure of both s-MDHs and m-MDHs in plants has not yet been revealed.

Whether the $\underline{mdh}_1^s$ and $\underline{mdh}_4^s$ genes are allelic or exist at two different loci is not clear, due to the lack of the information on the genetic background of the open pollinated ears.

The possibility that s-MDH^3 and s-MDH^4 are the modified products of s-MDH^1 has not been eliminated. However, if so, the mechanism(s) of modification may very likely be genetically controlled.

As seen in Figure 2, in the seven phenotypes of mitochondrial MDH isozymes, each phenotype may consist of two to five major MDH isozymes. Since all of these lines have been inbred for at least ten generations, and the MDH isozyme patterns were observed consistently, the isozymes in each of the specific inbred lines should not be the products of allelic genes. For example, in strain 59, m-MDH^2 and m-MDH^5 cannot be allelic isozymes, because they never segregate in the inbreds. This is also true for all the isozymes found in the other six MDH phenotypes. No single isozyme band is found to exist consistently in all the inbred lines examined.

The absolutely independent expression of each individual isozyme argues strongly against the possibility that any of the isozymes results from the modifivation of another. Since the isozymes also appear not to be allelic, I suggest that each m-MDH isozyme, except the possible hybrid molecules, is coded by a separate structural locus.

Genetic control of mitochondrial MDH isozymes

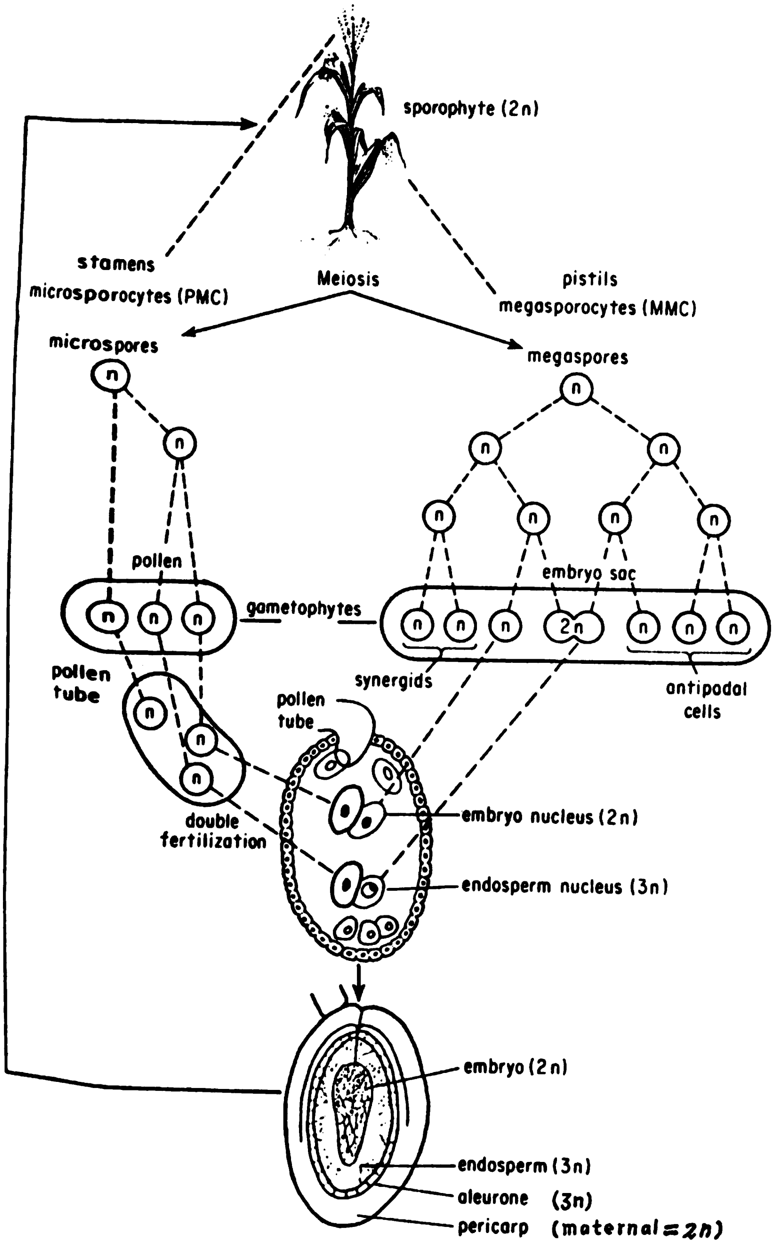
To study the genetic control of mitochondrial MDH isozymes, back crosses and F$_2$ progenies were made from several inbred maize lines seen in Figure 2. MDH phenotypes in the triploid (3n) liquid endosperm were studied. In order to give a better understanding of the results to be described later, the reproductive cycle in maize will be briefly described. In corn the two types of gametophytes are represented by small microspores in the stamens (tassels on top of the plant) for the male, and by large megaspores in the pistils (ears of the plant) for the female. In the stamens, single diploid microspore mother cells (pollen mother cell, or PMC) divide meiotically to yield four

haploid microspores, each becoming encapsulated as a pollen grain (the male gametophyte). The haploid pollen nucleus then divides mitotically to form a tube nucleus and a generative nucleus, the latter then divides once or more to form the two male gametic nuclei (Figure 4).

A similar succession of events occurs for each megaspore mother cell (MMC) in the pistils, except that only one of the four haploid megaspore nuclei becomes the functional occupant of the embryonic sac (the female gametophyte). The nucleus of this cell then divides mitotically into two daughter nuclei, which divide twice or more, forming a total of eight haploid nuclei, four at each end of the embryo sac. A single nucleus from each end group of four then unites at the center to form the diploid endosperm nucleus. Of the remaining six nuclei in the embryo sac, the group of three farthest away from the pollen-tube point of entry (micropyle) are called the antipodal cells, while the other group differentiates into a single female gametic nucleus and two synergids (Figure 4).

In the process of fertilization, one male gamete fertilizes the female gametic nucleus to form the diploid zygote and the other male gamete combines with the diploid endosperm nucleus to form the triploid (diploid + haploid) tissue that will furnish the embryo. Thus, this double fertilization forms two types of tissue, embryonic (2n) and endosperm (3n).

Figure 4.--Double fertilization of embryo nucleus and endosperm nucleus in maize.


sporophyte (2n)
stamens
microsporocytes (PMC)
Meiosis
pistils
megasporocytes (MMC)
microspores
megaspores
n
pollen
embryo sac
gametophytes
2n
synergids
antipodal cells
pollen tube
pollen tube
double fertilization
embryo nucleus (2n)
endosperm nucleus (3n)
embryo (2n)
endosperm (3n)
aleurone (3n)
pericarp (maternal = 2n)

It is of interest and importance to geneticists that these two tissues differ genetically only in the number of sets of chromosomes from the same female haploid nucleus. Therefore, in the endosperm, the genomes transmitted from the female parent consist of two sets of chromosomes from the same female haploid nucleus and have double doses compared to those from the male parent.

Segregation of $\underline{mdh}^{m}_{1-3}$ linkage group and $\underline{mdh}^{m}_{2-5}$ linkage group

Results of back crosses involving strain 59 and Oh51A are shown in Table I. Four distinguishable MDH phenotypes were observed and are shown in Figure 5. When Oh51A was used as ♀ parent and the heterogametic (59xOh51A) were observed with a 1 : 1 ratio. In these crosses, m-MDH^{1} and m-MDH^{3} were inherited as a unit. Segregation of these two m-MDHs was not observed. As mentioned earlier, m-MDH^{1} and m-MDH^{3} are not allelic isozymes. These results suggest that $\underline{mdh}^{m}_{1}$, and $\underline{mdh}^{m}_{3}$ are two closely linked loci. An additional m-MDH isozyme, m-MDH^{4}, which is not present in the inbred 59 (phenotype A) and Oh51A (phenotype B) appears in the heterogametic progenies (phenotype C) (Figure 5). With appearance of m-MDH^{4} there is a concomittant decrease in m-MDH^{5} (in type C) and in m-MDH^{3} (in type D, to be discussed later). This result suggests that m-MDH^{4} is a hybrid molecule formed of a m-MDH^{3} and m-MDH^{5} subunits. Additional evidence for this will be presented later on.

Table I. Summary of back crosses (between strains 59 and Oh51A) involving MDH phenotypes of maize liquid endosperm (3n)

Cross	Parents			Phenotypes of Progenies[a]							
	Female		Male	(A)	(B)	(C)	(D)	Total	Ratio of Phenotypes	X^2	P
1.	Oh51A	x	(59xOh51A)		118	103		221	B:C - 1:1	1.02	>0.30
2.	Oh51A	x	(Oh51Ax59)		73	74		147	B:C = 1:1	0.007	>0.95
3.	(59xOh51A)	x	Oh51A		85	41	38	164	B:C:D = 2:1:1	0.319	>0.20
4.	(Oh51Ax59)	x	Oh51A		118	60	57	235	B:C:D = 2:1:1	0.09	>0.95
5.	(59xOh51A)	x	59	56		126	59	241	A:C:D = 1:2:1	0.05	>0.50
6.	59	x	(59xOh51A)	25			81	106	A:D = 1:3	0.113	>0.20

[a] Phenotypes of the progenies and of the inbred lines, 59 and Oh51A, are shown in Figure 5.

Figure 5.--MDH phenotypes of the back crosses involving strains 59 and Oh51A.

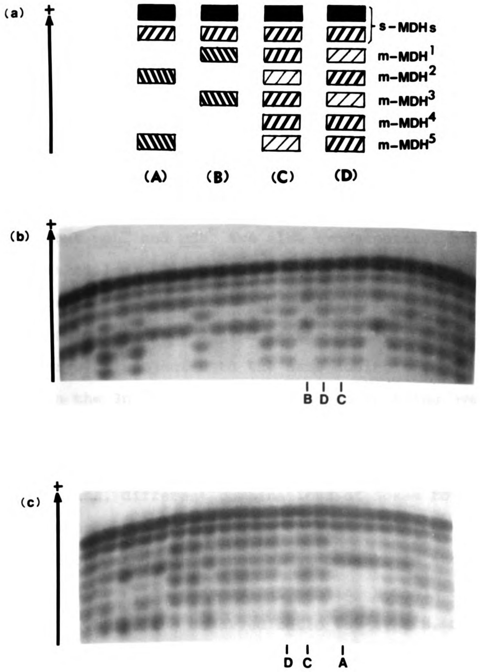
(See Table I)

(a) A schematic diagram of all four phenotypes observed in the back crosses described in Table I. Type A and type B are the same as those found in inbred lines 59 and Oh51A respectively.

(b) MDH zymogram of the progeny of the back cross, (Oh51Ax59) X Oh51A.

(c) MDH zymogram of the progeny of the back cross (59xOh51A) X 59. Type B, C and D were observed.

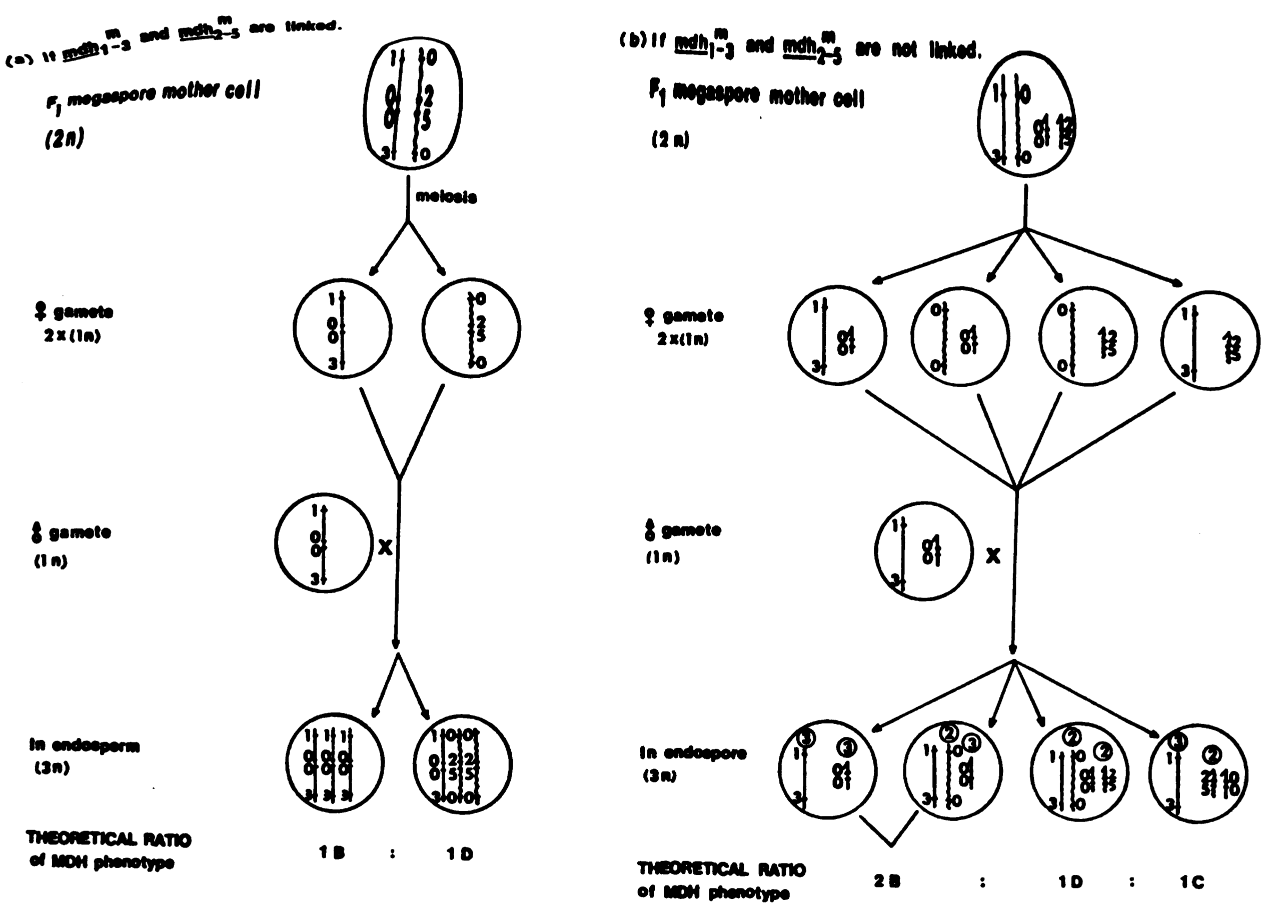
In (b) and (c) lettered channels indicate representative examples of the phenotypes in (a).


(a)
+
s-MDHs
m-MDH1
m-MDH2
m-MDH3
m-MDH4
m-MDH5
(A)
(B)
(C)
(D)
(b)
+
B
D
C
(c)
+
D
C
A

When heterogametic (59xOh51A) or (Oh51Ax59) was the female parent and Oh51A was the male parent, three MDH phenotypes, instead of two, were observed (Table I and Figure 4). Again, m-MDH^1 and m-MDH^3 do not segregate independently from each other, suggesting that $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ are two linked loci. MDH phenotypes C and D clearly indicate that they are derived from differential dosing of m-MDH^1 and m-MDH^3 or of m-MDH^2 and m-MDH^5. This suggests that $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$ are also two separate, but closely linked loci [Figure 5 (a), (b)].

According to the scheme of double fertilization in Figure 4, only the genes linked on the same chromosome should always be subjected to the same dosing effect observed in the 3n endosperm (assuming no crossing over). The genes localized on different chromosomes may segregate independently during meiosis, therefore, in the back cross progenies, different combinations of doses for these genes would be observed in the 3n endosperm. A schematic interpretation using $\underline{mdh}^m$ genes as an example is shown in Figure 6. In the back crosses, (59xOh51A) X Oh51A or (Oh51Ax59) X Oh51A, the observed ratio for type B, C and D is 2 : 1 : 1 (Table I). This ratio fits exactly the theoretical ratio assuming an $\underline{mdh}_{1-3}^m$ linkage group and an $\underline{mdh}_{2-5}^m$ linkage group on different chromosomes [Figure 6 (b)]. This result strongly suggests that two groups of m-MDH isozymes are

Figure 6.--Segregation of $\underline{mdh}^{m}_{1-3}$ linkage group and $\underline{mdh}^{m}_{2-5}$ linkage group in the back cross of (59xOh51A) X Oh51A. Chromosomes derived from strain 59 are represented by wavy lines, those from strain Oh51A are represented by straight lines. Numbers on the chromosome indicate the various genes coding for the mitochondrial MDH isozymes, the symbol "o" indicates the "Null" allele. For the purpose of better demonstration, the various genes were drawn in a random space and do not necessarily indicate the real distance of these genes on chromosomes.



OBSERVED RATIO of MDH phenotype = 85 B : 41 C : 38 D = 2B : 1C : 1D encoded by two groups of genes localized on two different chromosomes. The $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ belong to one linkage group while $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$ are linked on another chromosome.

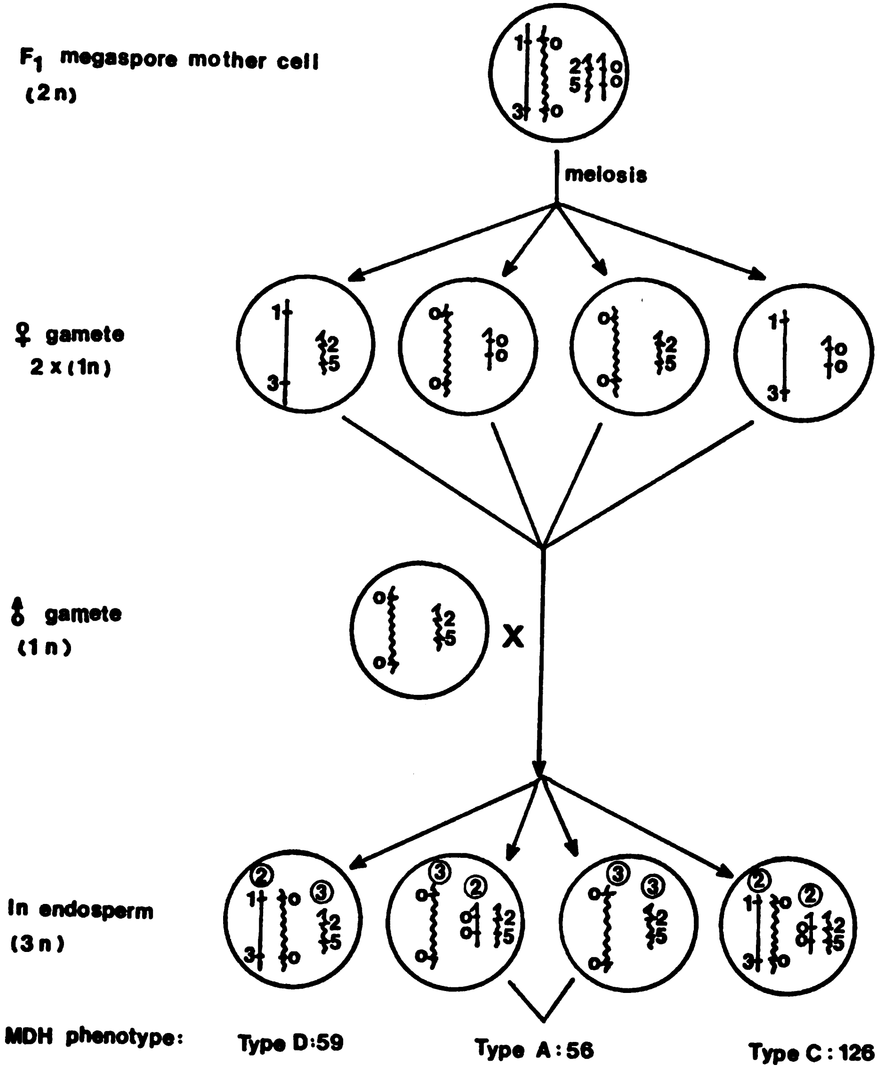
The back cross, (59xOh51A) X 59, turns out to be an interesting and important cross in the current investigation. As seen in Figure 5 (c) three phenotypes (A, C and D) were found. Hybrid molecule, m-MDH^4, was observed in heterozygotes in this cross. Segregation of m-MDH^2 from m-MDH^5 was not observed in this cross. As mentioned previously they also showed the same dosing effect observed in the 3n endosperm. As represented in Figure 5 (c), two sets of chromosome carrying $\underline{mdh}_1^m$, $\underline{mdh}_3^m$ and one set carrying $\underline{mdh}_2^m$, $\underline{mdh}_5^m$ will give a phenotype C in the endosperm. Similarly, two sets of chromosomes carrying $\underline{mdh}_1^m$, $\underline{mdh}_3^m$ and 3 sets of chromosomes carrying $\underline{mdh}_2^m$, $\underline{mdh}_5^m$ will give a phenotype D in the endosperm. These observations suggest again that $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$ genes are linked. Since m-MDH^2 and m-MDH^5 are not allelic gene products, the $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$ must be two different, but closely linked loci.

All the results observed in the above suggest that m-MDH^1 and m-MDH^3 are controlled by two different loci on the same chromosome and that m-MDH^2 and m-MDH^5 are controlled by two different loci linked to another chromosome. The m-MDH^4 isozyme is derived from random association of the subunits of m-MDH^3 and m-MDH^5. The fact that m-MDH^1 and m-MDH^3 are not expressed in strain 59, and that m-MDH^2 and m-MDH^5 are not expressed in strain Oh51A suggests that null alleles for $\underline{mdh}_1^m$, $\underline{mdh}_3^m$ exist in strain 59 and null alleles for $\underline{mdh}_2^m$, $\underline{mdh}_5^m$ exist in Oh51A (Figure 2). As shown in crosses 1 to 5 of Table I and in Figure 6 (b), the $\underline{mdh}^m$ genes and their corresponding null alleles segregate according to Mendelian rules. Data from F_2 progeny also support the hypothesized null alleles (Table II). In all these cases, the existence of null alleles appears to be the most reasonable explanation to account for the observed results.

Correlation between m-MDH phenotypes and viabilities of the kernel

As seen in cross 5 to Table I and in Figure 7, when (59xOh51A) was back crossed to 59, a ratio of 1A : 2C ; 1D for the three phenotypes was observed. But the expected theoretical ratio derived from the scheme in Figure 7 should be 2A : 1C : 1D. The fact that in this specific back cross, low recovery of the parental phenotype A has also been reported by Longo and Scandalios (10). However, when the same female parent (59xOh51A) was back crossed to male Oh51A (Cross 3 in Table I), the frequency of the parental phenotype of Oh51A was recovered as expected (Table I and Figure 6). These observations could be explained by a low viability of strain 59 in this specific back cross. A careful study of the four genotypes (shown in Figure 7) from this back cross indicates that increased numbers of null alleles for m-MDH^1 and m-MDH^3 in the genotype results in a decreased frequency of progeny. Similarly increased

Figure 7.--Segregation of $\underline{\underline{mdh}}^{m}_{1-3}$ linkage group and $\underline{\underline{mdh}}^{m}_{2-5}$ linkage group in the back cross of (59xOh51A) X 59. Symbols used are the same as described in Figure 6.



THEORETICAL RATIO = 2A : 1C : 1D

OBSERVED RATIO = 1A : 2C : 1D

numbers of normal alleles for m-MDH^2 and m-MDH^5 in the genotype also results in a decreased frequency of progeny. Similarly increased numbers of normal alleles for m-MDH^2 and m-MDH^5 in the genotype also results in a decreased frequency of progeny. For example, in phenotype A, one genotype has three chromosomes of  and three , another genotype has three  and one  (Figure 7). The frequency of thie phenotype is much less than expected. However, phenotype C, with only one chromosome of  and one  occurs more frequently than expected. These observations suggest that there may be some incompatible combinations between the chrosomes carrying the mitochondrial MDH structural genes. Such incompatible combinations may decrease viability or may even be lethal. The effects appear to depend on gene dosage. If we assume the viability of the progenies in the back cross of (59xOh51A) X 59 to be as follows:

a) With more than two chromosomes of  (see Figure 7), the viability reduced to half.

b) With more than two chromosomes of  (Figure 7), the viability also reduced to half.

c) If a and b occur simultaneously, the progenies are lethal.

Then in Figure 7 the type C progenies will be viable as normal, one genotype of type A will be lethal and the other will have ½ viability, type C will have ½ viable progeny. The ratio of the three phenotypes would, therefore, be ½A : : 1C : ½D = 1A : 2C : 1D. This is indeed the phenotype ratio we observed (Table I). In cross 6 of Table I, inbred line 59 was used as the ♀ parent and (59xOh51A) was used as ♂ parent, a ratio of 1A : 3D was observed. In this case, due to a similar gene dosage effects, two heterogametic genotypes would show the same phenotype D and another two would show phenotype A. If the viabilities of the various genotypes are the same, the expected ratio would be 2A : 2D. However, if the same hypothesis on viability described above is adopted, the ratio would then be ½A : (½D+1D) = 1A : 3D. This ratio is again what we observed in Table I.

These observations agree with the fact that the phenotype A kernels are indeed smaller and the ears in the back corsses to strain 59 showed many aborted kernels.

To test the proposed existence of "Null alleles" for these mitochondrial MDH genes, F_2 kernels of (Oh51Ax59) X (Oh51Ax59) were screened for MDH phenotypes. The zymogram in Figure 8 shows the five distinguishable phenotypes observed. Gene dosage effects on the expression of mitochondrial MDH isozymes were clearly observed again in the triploid endosperm. As was found earlier, m-MDH^1 and m-MDH^3 were subjected to the same dosage effect as a unit, m-MDH^2 and m-MDH^5 as another unit. Expression of m-MDH^4 depends strictly on the doses of m-MDH^3 and m-MDH^5 and further suggests that m-MDH^4 is a hybrid molecule formed by random association of the subunits of m-MDH^3 and m-MDH^5.

Ratio of the phenotypes in the F_2 offsprings of (Oh51Ax 59) is shown in Table II. The corresponding proposed

Table II. F_2 progenies of (Oh51Ax59) involving MDH phenotypes of maize endosperm (3n)

F_1 parents: Female (♀) (Oh51Ax59) Male (♂) (Oh51Ax59)

*Phenotypes of the F_2 progenies

a.	b.	c.	d.	e.	Total
180	87	64	113	32	480

Ratio of the phenotypes:

Theoretical ratios: (if there is no lethal effect)

a:b:c:d:e:n = 5:2:2:3:3:1
(a+b+c):d:e:n = 9:3:3:1

Theoretical ratios: (if there are lethal effects as described in the text)

a:b:c:d:e:n = 4.5:2:1.5:3:1:0
(a+b+c):d:e:n = 8:3:1:0

Observed ratios:	X^2	P
a:b:c:d:e = 4.5:2:1.5:3:1	2.977	>0.50
(a+b+c):d:e = 8:3:1	2.71	>0.20

(*) Phenotypes of the F_2 progenies are shown in Figure 8. The corresponding genotypes of these phenotypes are shown in Figure 9.

genotypes of these five phenotypes are shown in Figure 9. The observed ratio for the five phenotypes is 4.5a : 2b : 1.5c : 3d : 1e or 8 (a+b+c) : 3d : 1e (Table II). Assume that there is no lethal effect as described above, the expected ratio would be 9 (a+b+c) : 3d : 3e : 1n (n, the null phenotype), the probability of the chi-square for the observed ratio to fit this ratio is much lower than 0.001. If there is a lethal effect as described above, then two genotypes designated as "x" in Figure 9 would be lethal. Relative viabilities of the other genotypes are given in Figure 9. The genotype denoted as "n,X" is the expected null mutant for all mitochondrial MDH isozymes. Since mitochondrial MDH is one of the enzymes of the Krebs cycle and plays a central role in intermediate metabolism, it is reasonable to suggest that complete absence of mitochondrial MDH should be a lethal condition. Therefore the null mutants for all m-MDH isozymes should not be recovered. Assuming these two lethal effects proposed above, the expected ratio of the F_2 phenotypes would be 4.5a : 2b : 1.5c : 3d : 1e or 8 (a+b+c) : 3d : 1e as shown in Figure 9 and Table II. These are indeed the ratios observed. The probabilities for the observed ratios to fit the two expected ratios are >0.50 and >0.20 (Table II). The suggestion that different null alleles for mitochondrial MDH loci exist in these inbred maize lines, is consistent with segregation patterns observed in F_2's and back crosses, and is more reasonable than any alternate explanation which could account for all of the observed data. Therefore, I suggest that either

Figure 8.--MDH phenotype of F_2 progenies of (Oh51Ax59) X (Oh51Ax59). Note that m-MDH^1 and m-MDH^3 are always subjected to the same dosing effect, m-MDH^2 and m-MDH^5 were dosed similarly. However these two types of MDHs were never dosed simultaneously in the triploid endosperm. Letter channels (a, b, c, etc.) represent those MDH phenotypes which have the genotypes shown in Figure 9.

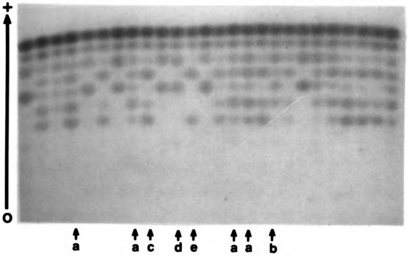
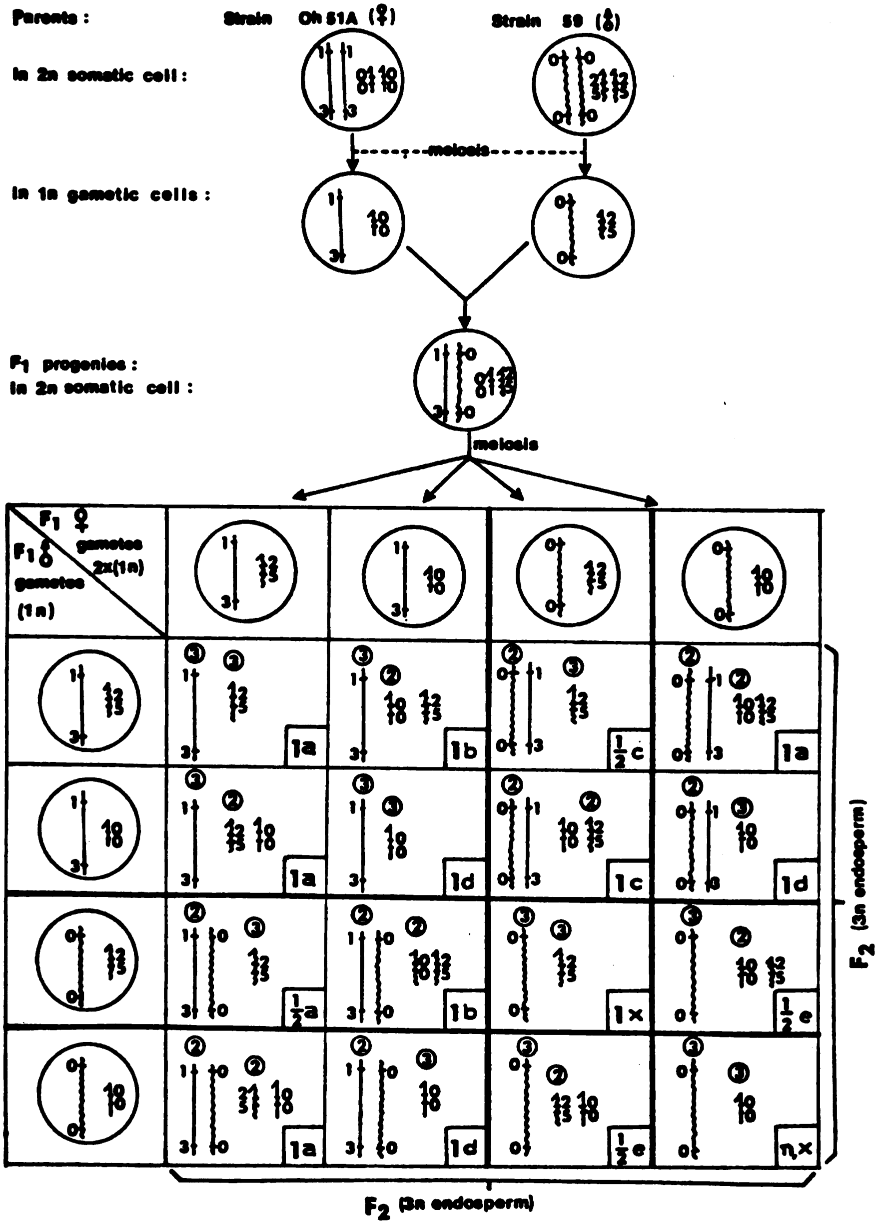

+
O
a
a
c
d
e
a
a
b

Figure 9.--Segregation and assortment of $\underline{mdh}^{m}_{1-3}$ linkage group and $\underline{mdh}^{m}_{2-5}$ linkage group in the F_2 progenies of (Oh51Ax59) X (Oh51Ax59). The genotypes could be classified into 6 types. Five distinguishable phenotypes (Figure 8) corresponding to the five groups of genotypes were marked as a, b, c, d, and e in the corner box of each possible genotype. Relative viability of each genotype (see text) is given by the number within the corner box. The "n" represent the theoretical "null" mutant. The symbol "x" indicates those genotypes which should be lethal (see text).


Parents :
Strain Oh 51A (♀)
Strain 59 (♂)
In 2n somatic cell :
meiosis
In 1n gametic cells :
F1 progenies :
In 2n somatic cell :
meiosis
F1 ♀ gametes 2x(1n)
F1 ♂ gametes (1n)
1a
1b
½c
1a
1a
1d
1c
1d
½a
1b
1x
½e
1a
1d
½e
n,x
F2 (3n endosperm)
F2 (3n endosperm)

null alleles of various mitochondrial MDH loci exist in a high frequency in maize system or the expressions of the various m-MDH loci are controlled genetically by some regulatory factors (see Discussion).

In Figure 10, phenotypes from the back crosses involving strain T_{21} and Oh51A are presented. The linkage relationships between $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$, and those between $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$ may also hold in these crosses (Figure 11, Table III). The gene dosage effects on m-MDH^1 and m-MDH^3 as a unit, and on m-MDH^2 and m-MDH^5 as another unit are again observed. Formation of the proposed hybrid m-MDH^4 isozyme was observed. The fact that four phenotypes, instead of three, are observed in these back crosses, and that gene dosage of m-MDH^1 and m-MDH^3 occurs independently of m-MDH^2 and m-MDH^5 further support the suggestion that $\underline{mdh}_{1-3}^m$ and $\underline{mdh}_{2-5}^m$ are on two separate linkage groups.

The third linkage group mitochondrial MDH genes

As observed in Figure 12, five MDH phenotypes were observed in the back crosses involving inbred strains 59 and 81. The phenotypes A and I were recovered in a 1 : 1 ratio in the back cross progenies of (59x81) X 81 and of (81x59) X 81. In these reciprocal back crosses, $\underline{mdh}_2^m$ and $\underline{mdh}_5^m$ segregate again as a unit indicating their linkage relationship is the same as observed earlier in other corsses. As seen in Figure 12 (c), m-MDH^6 and m-MDH^7 were clearly observed in phenotype H (the parental type of strain 81), while in phenotype I, m-MDH^6 and m-MDH^7 (especially m-MDH^7) are expressed to lesser

Figure 10.--MDH phenotypes of the back crosses involving strains T_{21} and Oh51A (Table III).

(a) A schematic diagram of all four phenotypes observed in the back crosses described in Table III. Type (B) and (E) are the same as those found in inbred lines, Oh51A and T_{21} respectively.

(b) MDH zymogram of the progenies of the back cross, (T_{21}xOh51A) X Oh51A. Lettered channels indicate representative examples of the phenotypes in (a).

(c) MDH zymogram of the progenies of the back cross, (Oh51AxT_{21}) X T_{21}. Lettered channels indicate representative examples of the phenotypes in (a).

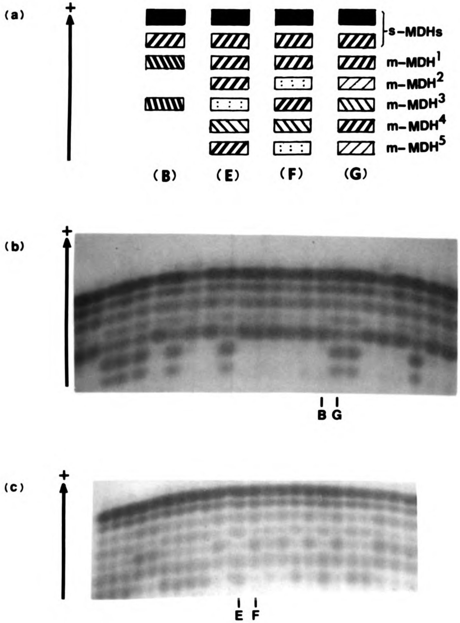
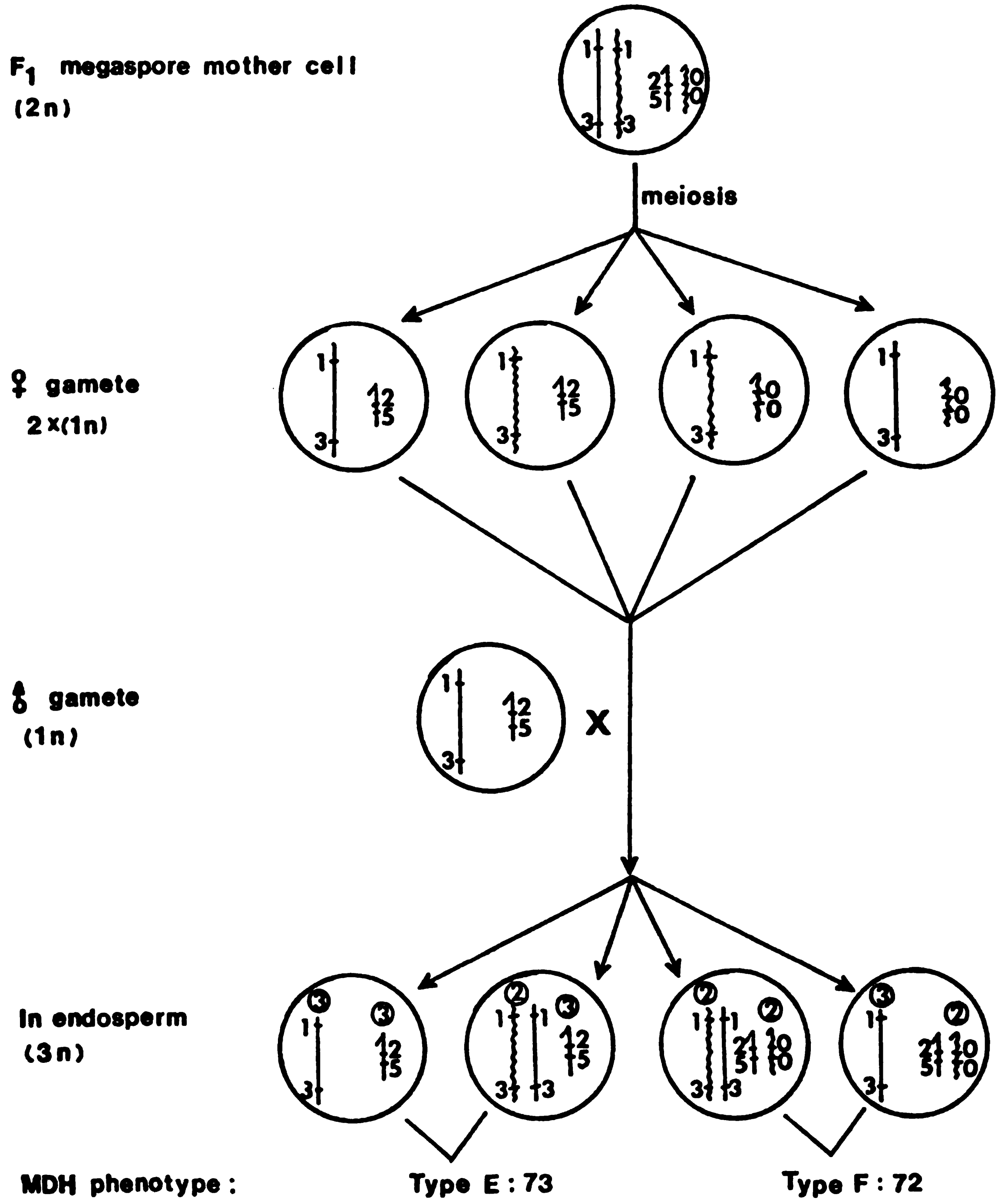

(a)
+
s-MDHs
m-MDH1
m-MDH2
m-MDH3
m-MDH4
m-MDH5
(B)
(E)
(F)
(G)
(b)
+
B
G
(c)
+
E
F

Table III. Summary of back crosses (between strains Oh51A and T_{21}) involving MDH phenotypes of maize liquid endosperm (3n)

Crosses	Parents		Phenotypes of Progenies[a]							
	Female	Male	(B)	(E)	(F)	(G)	Total	Ratio of Phenotypes	X^2	P
1.	(T_{21} X Oh51A)	X T_{21}		96	105		201	E:F = 1:1	0.41	>0.5
2.	(Oh51A X T_{21})	X T_{21}		73	72		145	E:F = 1:1	0.007	>0.90
3.	(Oh51A X T_{21})	X Oh51A	87			91	178	B:G = 1:1	0.09	>0.70
4.	(T_{21} X Oh51A)	X Oh51A	116			98	214	B:G = 1:1	1.514	>0.2
5.	Oh51A X (T_{21} X Oh51A)		92		86		178	B:F = 1:1	0.202	>0.5

[a] Phenotypes of the progenies and of the inbred lines, Oh51A and T_{21}, are shown in Figure 10.

Figure 11.--Segregation of $\underline{mdh}^{m}_{1-3}$ linkage group and $\underline{mdh}^{m}_{2-5}$ linkage group in the back cross of (Oh51AxT$_{21}$) X T$_{21}$. Chromosomes derived from strain T$_{21}$ are represented by wavy lines, other symbols are the same as described in Figure 6.



THEORETICAL RATIO = 1E : 1F
OBSERVED RATIO = 1E : 1F

extent. Whenever the m-MDH^6 and m-MDH^7 expression was reduced (in phenotype I), lower activity was also observed in m-MDH^3. However, when m-MDH^6 and m-MDH^7 expression was greater (in phenotype H), higher activity in m-MDH^3 was also found. These results suggest that m-MDH^3, m-MDH^6 and m-MDH^7 are subjected to the same gene dosage effects and thus are probably linked. It can be also noted that in the heterozygous phenotype I shown in Figure 12 (b), the m-MDH^7 can be easily observed, even though the activity is low. However, in the heterozygous phenotype I shown in Figure 12 (c), the activity observed in m-MDH^7 is very low, sometimes it appears to be absent. In this case, m-MDH^6 will also have lower activity. These differences again are due to the different gene doses for the isozymes. In Figure 12 (b), the heterozygotes have two doses of alleles from strain 81 and one dose from strain 59, while in Figure 12 (c), the heterozygotes show the opposite dosage patterns. There higher activity of m-MDH^7 in phenotype I would be observed in Figure 12 (b) and lower activity of m-MDH^7 in phenotype I would be observed in Figure 12 (c). Since both strains (59 and 81) have m-MDH^5, the result that m-MDH^6 always has higher activity than does the m-MDH^7 indicates that m-MDH^6 is a hybrid molecule of m-MDH^5 and m-MDH^7. When two doses of m-MDH^7 are expressed, as in type I of Figure 12 (b), subunits of m-MDH^7 will associate with m-MDH^5 and form m-MDH^6 in addition to the two parental molecules. When only one dose of m-MDH^7 is expressed, as in type I of Figure 12 (c), m-MDH^5 will drive most of the

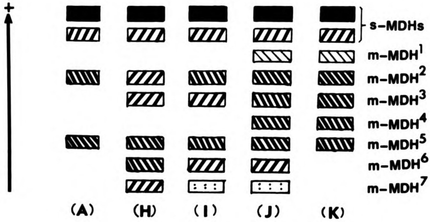
Figure 12.--MDH phenotypes of the back crosses involving strains 59 and 81 (Table IV).

(a) A schematic diagram of all five phenotypes observed in the back crosses as described in Table IV. Type (A) and type (H) are the same as those found in inbred lines, 59 and 81 respectively.

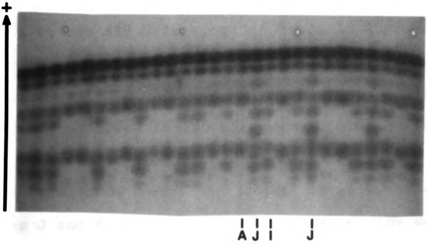
(b) MDH zymogram of the progenies of the back cross, (59x81) X 59. Lettered channels indicate representative examples of the phenotypes in (a).

(c) MDH zymogram of the progenies of the back cross, (81x59) X 81, lettered channels indicate representative examples of the phenotypes in (a).

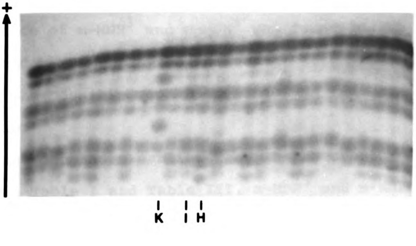
(a)


+
s-MDHs
m-MDH^1
m-MDH^2
m-MDH^3
m-MDH^4
m-MDH^5
m-MDH^6
m-MDH^7
(A)
(H)
(I)
(J)
(K)

(b)


+
A
J
I
J

(c)


+
K
I
H

available m-MDH^7 subunits to form hybrid m-MDH^6 and only small numbers of the m-MDH^7 subunits will form homodimers. These results and the above explanations suggest that m-MDH^6 is a hybrid molecule of m-MDH^5 and m-MDH^7.

Recombinant m-MDH phenotypes

As shown in Figure 2, m-MDH^1 and m-MDH^4 are not expressed in strains 59 and 81. However, in the back crosses involving these two strains, two phenotypes (J and K in Figure 12) carrying m-MDH^1 and m-MDH^4 were observed. Table IV shows that in the 335 offsprings of cross 1, 8 progenies with phenotype J and 2 progenies with phenotype K were observed. In cross 4, one progeny with phenotype K and no phenotype J were observed in 196 offsprings. Two interesting points were observed in these two crosses. First, the concomittant expression of m-MDH^2 and m-MDH^4 is always observed in phenotypes J and K. Under this circumstance, the activity in m-MDH^3 is always higher than that observed in phenotype I [Figure 12 (b)]. Secondly, in phenotype K [Figure 12 (c)], the appearance of m-MDH^1 and m-MDH^4 was found in the absence of m-MDH^6 and m-MDH^7. In the following, attempts are made to analyze the possible mechanisms involved in these two observations.

It has been shown earlier that in the crosses described in Table I and Table III, m-MDH^3 and m-MDH^5 may associate randomly to form hybrid m-MDH^5. However, when both m-MDH^3 and m-MDH^5 are expressed in phenotype H and

Table IV. Summary of back crosses (between strains 59 and 81) involving MDH phenotypes of maize endosperm (3n)

Crosses	Parents		Phenotypes of Progenies[a]								
	Female	Male	(A)	(H)	(I)	(J)	(K)	Total	Ratio of Phenotypes	X^2	P
1.	(59 X 81)	X 59	164		161	8	2	335	A:I = 1:1	0.03	>0.7
2.	(59 X 81)	X 81		93	102			195	H:I = 1:1	0.42	>0.5
3.	(81 X 59)	X 59	122		128			250	A:I = 1:1	0.144	>0.7
4.	(81 X 59)	X 81		103	92		1	196	H:I = 1:1	0.623	>0.3

[a] Phenotypes of the progenies and of the inbred lines, 59 and 81, are shown in Figure 12.

phenotype I (Figure 12), the hybrid m-MDH^4 is not observed in both phenotypes. Several reasons may account for the above result.

Hypothesis (1). The hybridization of m-MDH^3 and m-MDH^5 to form m-MDH^4 is controlled genetically, in strain 81 (with phenotype H), the control is changed by mutation such that hybrid formation of m-MDH^4 is no longer possible.

Hypothesis (2). Either m-MDH^3 or m-MDH^5 in strain 81 is not the same gene product as that in strain Oh51A or in strain 59 (Figure 2). For example, the m-MDH^3 in 81 and that in 59 are different isozymes (different gene products) but have the same electrophoretic mobility. The m-MDH^3 in strain 81 is not able to form hybrid m-MDH^4 by association with m-MDH^5 and therefore the m-MDH^4 is not expressed. Under this assumption, m-MDH isozyme at position 3 of strain 81 should be designated as m-MDH$^{3'}$. If the same event occurs for m-MDH^5, then the isozyme in strain 81 should be denoted as m-MDH$^{5'}$.

There may be other reasons which will also account for the observations described above. At present, the two explanations given previously are adopted in the attempts to analyze the possible mechanisms involved in the expression of m-MDH^1, m-MDH^3 and m-MDH^4 in phenotypes J and K. (Table IV and Figure 12).

If by some mechanisms (to be discussed later), the $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ are expressed in the heterozygous offspring shown in Figure 12 (b). The appearance of phenotype I may be explained if we accept "Hypothesis (2)" described above. The appearance of m-MDH^3 not only increase the MDH activity found in the same position of m-MDH$^{3'}$, but also the m-MDH^3 will associate with available m-MDH^5 to form hybrid m-MDH^4. Therefore, the MDH isozyme pattern would be observed as phenotype J in which m-MDH^1 and m-MDH^4 appeared, and the activity observed in the position of m-MDH$^{3'}$ increased.

If we accept "Hypothesis (1)" on explaining the phenotype of strain 81 (type H), then the expression of $\underline{mdh}_1^m$ must occur concomittantly with another mechanism which would permit m-MDH^3 and m-MDH^5 to form hybrid m-MDH^4. Under this situation, expression of m-MDH^1 and m-MDH^4 in phenotype J requires two mechanisms which would be more complicated. Even if we accept such a possibility, we would expect a decrease of the activity found in m-MDH^3, because some subunits of m-MDH^3 would be associated with those of m-MDH^5 to form hybrid m-MDH^4 isozymes. However, as seen in Figure 12 (b), an increase in activity, instead of decrease, was observed for m-MDH^3 in phenotype J [Figure 12 (b)].

The above analysis indicates that Hypothesis (2), instead of Hypothesis (1), is more likely the reason that m-MDH^4 is not expressed in strain 81 (phenotype H). In addition, it suggests that the m-MDH^3 in strain Oh51A is different from the isozyme having a same electrophoretic mobility observed in strain 81. The latter is therefore denoted as m-MDH$^{3'}$.

Based on the above suggestion then the appearance of m-MDH^1 and m-MDH^4 in phenotypes J and K is actually due to expression of m-MDH^1 and m-MDH^3 in the heterozygous progenies of the back crosses shown in Table IV. The m-MDH^3 associate with m-MDH^5 to form hybrid m-MDH^4. In addition, the appearance of homodimers of m-MDH^3, having the same electrophoretic mobility as that of m-MDH$^{3'}$ would make the m-MDH$^{3'}$ activity appear to be increased.

Since both m-MDH^2 and m-MDH^5 are expressed in strain 59 and 81, it is impossible to study their segregation from other m-MDHs in the back crosses described in Table IV. Figure 13 shows that $\underline{mdh}^m_{3'}$ and $\underline{mdh}^m_7$ are linked on the same chromosome. Because m-MDH^5 is always expressed in these back crosses, and m-MDH^5 may associate with m-MDH^7 to form hybrid m-MDH^6, the linkage between $\underline{mdh}^m_{3'}$ and $\underline{mdh}^m_7$ will therefore let m-MDH$^{3'}$, m-MDH^6 and m-MDH^7 be expressed dependently in the progenies.

The facts that m-MDH^2 and m-MDH^5 are expressed, and m-MDH^1 and m-MDH^3 are not expressed in both parental types (types A and H) makes it impossible to study the linkage relationship between linkage group $\underline{mdh}^m_{3',-7}$ and the other two linkage groups ($\underline{mdh}^m_{1-3}$ and $\underline{mdh}^m_{2-5}$).

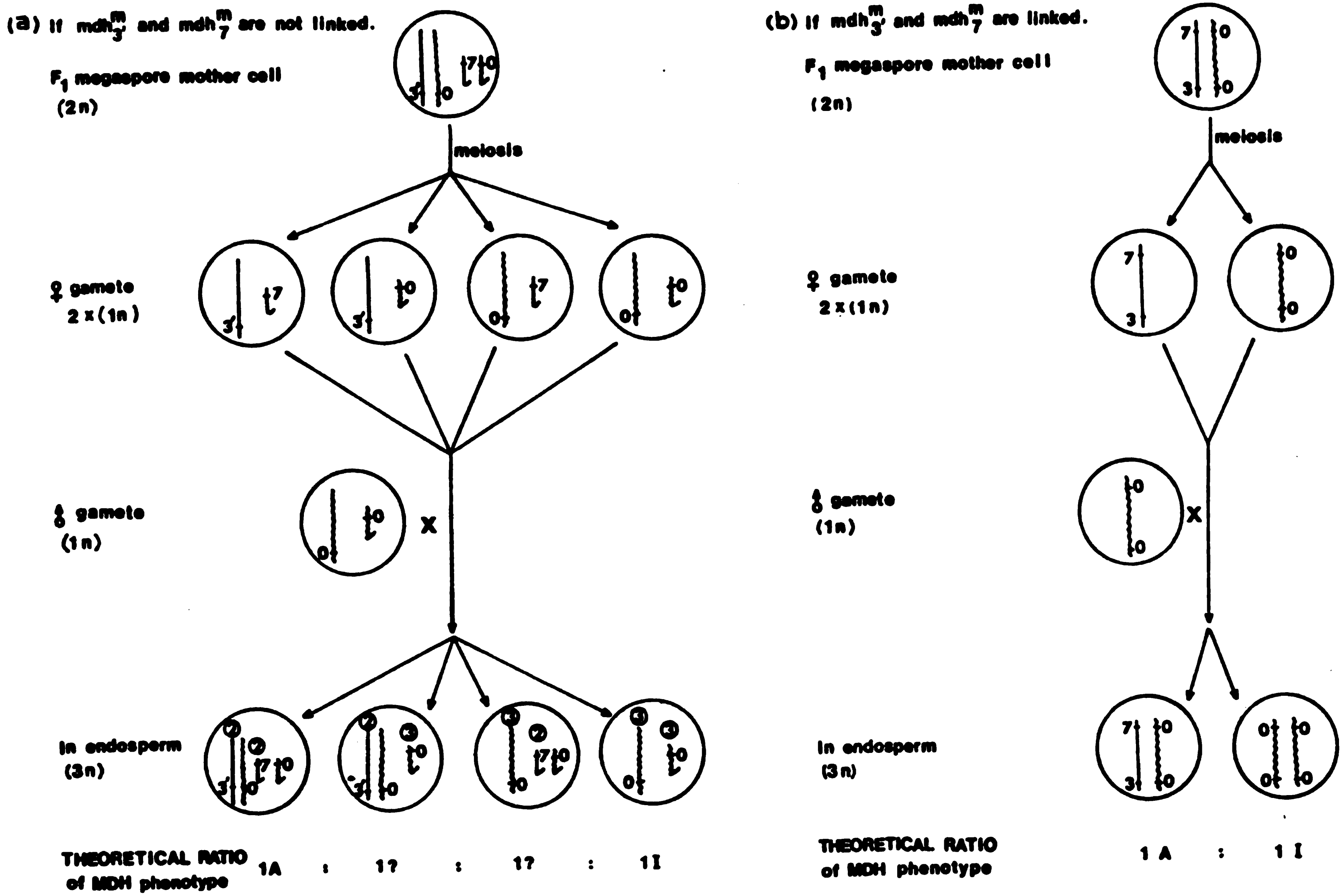
The possible "regulatory genes"

In the crosses shown in Table IV, the concomittant expressions of $\underline{mdh}^m_1$ and $\underline{mdh}^m_3$ are always observed in both phenotypes J and K (Figure 12), and that the two genes are not expressed in both parental phenotypes (type A and type H) suggest crossing over and regulatory mechanisms are

Figure 13.--Theoretical segregations of $\underline{\mathrm{mdh}}_3^m$, and $\underline{\mathrm{mdh}}_7^m$ in the back cross of (59x81) X 59. Chromosomes derived from strain 81 are represented by straight lines, the other symbols are the same as described in Figure 6.

(a) Assume that $\underline{\mathrm{mdh}}_3^m$, and $\underline{\mathrm{mdh}}_7^m$ are located on different chromosome.

(b) Assume that $\underline{\mathrm{mdh}}_3^m$, and $\underline{\mathrm{mdh}}_7^m$ are linked.



(a) If $mdh^m_{3'}$ and mdh^m_7 are not linked.

(b) If $mdh^m_{3'}$ and mdh^m_7 are linked.

OBSERVED RATIO of MDH phenotype = 164 A : 161 I = 1 A : 1 I

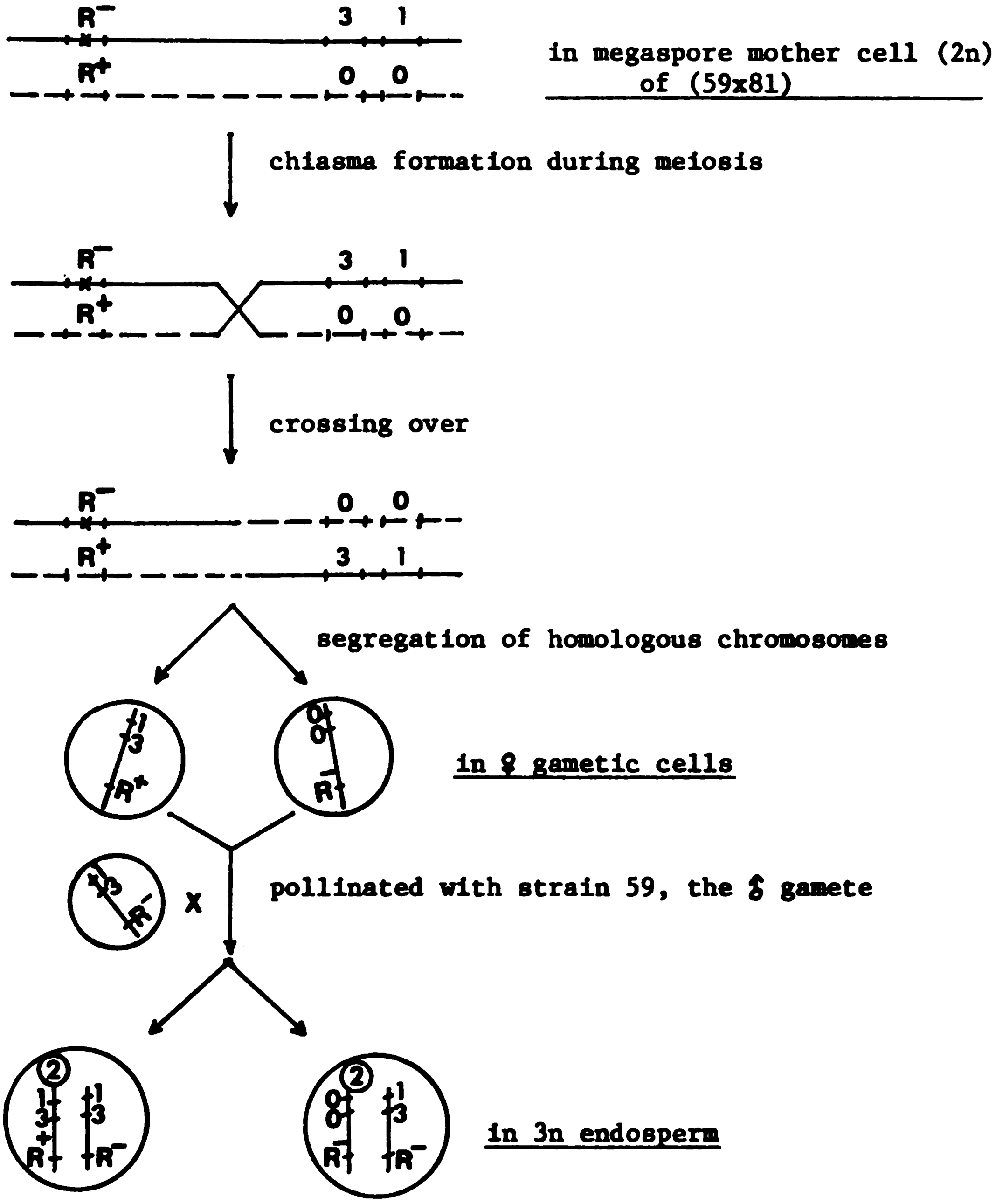
No segregation of $m\text{-}MDH^{3'}$ and $m\text{-}MDH^7$ as would expected in scheme (a) was observed in the back cross progenies (Fig. 12 and Table IV).

involved. As was indicated earlier, the two loci $\underline{\underline{mdh}}_1^m$ and $\underline{\underline{mdh}}_3^m$ are linked. The results in Table I to Table III indicate that the two genes are either expressed or not expressed concomittantly in all the progenies observed. This suggests that these two loci are closely linked. In order to explain the concomittant expression of m-MDH^1 and m-MDH^3 in heterozygous phenotypes J and K (Figure 12), two possible mechanisms are proposed and are shown in Figure 14 and Figure 15.

The observed frequency of the recombinant progenies (type J and type K) is $\frac{8+2}{335} = 2.98\%$ (Table IV). Because one of the two recombinant phenotypes shown in either Figure 14 or Figure 15 would have the same phenotype as phenotype I (in which m-MDH^1 and m-MDH^3 isozymes are not expressed), therefore the real number of recombinant progenies should be (8+2) X 2 and the frequency of cross over would be 5.96%. A recombination frequency of such a value favors the model of a single cross-over between the "regulatory" gene and structural genes of MDHs (Figure 14) and strongly against the double "intragenic" cross over model proposed in Figure 15. Intragenic cross over in higher eukaryotic organisms has not been clearly demonstrated yet, and double cross-overs of two closely linked loci would occur with a very low frequency. The frequency for the above two mechanisms to occur at the same time would be extremely low. Therefore, the results suggest that "regulatory" gene(s) may be involved in the expression of mitochondrial MDH

Figure 14.--Possible involvement of regulatory gene in the expression of m-MDH isozymes in the back cross (59x81) X 59 (see Table IV). The two homologous chromosomes carrying the proposed regulatory genes and structural genes for m-MDH^1 and m-MDH^2 are presented. R+ = active regulatory gene, which would let the structural m-MDH genes be transcribed. R- = inactive regulatory gene which would not let the structural m-MDH genes be transcribed. The numbers 1 and 3 indicate $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ respectively. "0" denotes the null alleles.

Hypothetical model for crossing over between "regulatory gene" and structural genes of mitochondrial MDH isozymes.
(Double stands of chromosomes are not shown)

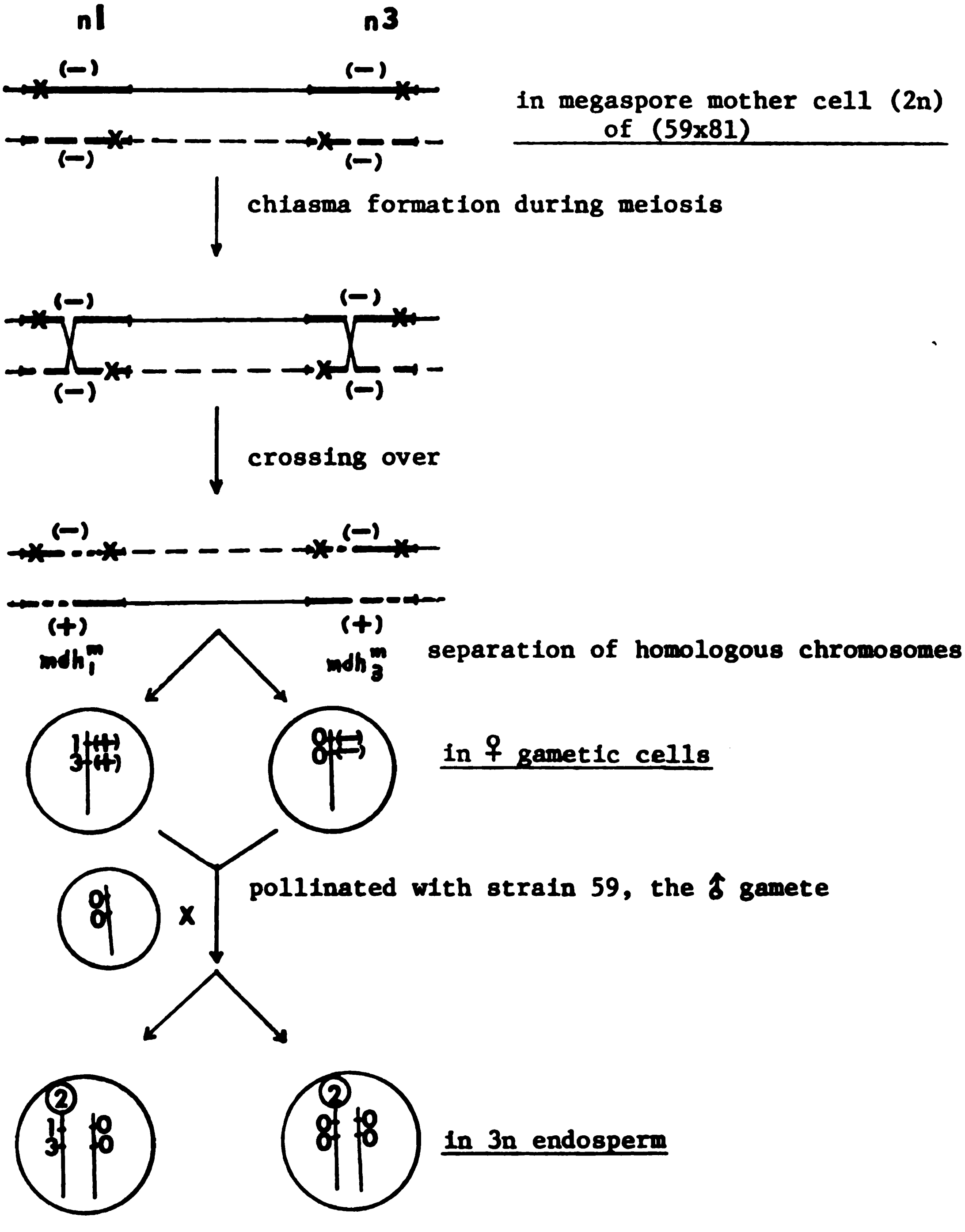


With phenotype J or K (m-MDH^1 and m-MDH^3 isozymes appear)

With phenotype A or I (no m-MDH^1 and m-MDH^3 isozymes are observed)

Figure 15.--Hypothetical intragenic double cross over within the null alleles of $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ in the back cross of (59x81) X 59 (see Table IV). The two homologous chromosomes carrying null alleles at $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ are presented. The two closely linked loci are exaggerated in the figure to give a better demonstration. The two loci are indicated by thick lines and the space between these two loci does not necessarily indicate the real distance on chromosomes. The "n1" denotes the null alleles at the $\underline{mdh}_1^m$ locus, "n3" designate the null alleles at the $\underline{mdh}_3^m$ locus. The symbol (-) indicates that the m-MDHs produced from these alleles are inactive, (+) indicates that normal m-MDHs are produced from these alleles, -X- indicates the mutation site, which make the allele to produce inactive m-MDHs.

Hypothetical model for double cross overs within the structural genes of mitochondrial MDH isozymes.
(Double strands of chromosomes are not shown)



With phenotype J or K (m-MDH^1 and m-MDH^3 isozymes appear)

With phenotype A or I (no m-MDH^1 and m-MDH^3 isozymes are observed) isozymes in maize. The earlier assumption of widely occurred null alleles in maize strain does not necessarily contradict the present hypothesis. Possible control mechanisms of the expression of m-MDH isozymes involving both null alleles and "regulatory" genes will be presented in "Discussion."

The reasons that m-MDH^6 and m-MDH^7 are absent in the presumed "recombinant" phenotype K (Figure 12 and Table IV) are not clear at this moment. Since m-MDH^1 and m-MDH^3 are expressed in this phenotype (probably due to cross over as described in Figure 14), whether m-MDH$^{3'}$ is still present or it has been lost concomittantly with m-MDH^7 could not be determined. If the m-MDH$^{3'}$ is still present, then there must be a cross over between $\underline{mdh}^{m}_{7}$ and $\underline{mdh}^{m}_{3'}$. However, if m-MDH$^{3'}$ is indeed absent in phenotype K (e.g., the MDH activity band between m-MDH^2 and m-MDH^4 is contributed only by m-MDH^3, instead of m-MDH^3 + m-MDH$^{3'}$), then the two closely linked $\underline{mdh}^{m}_{3'}$ and $\underline{mdh}^{m}_{7}$ would have to be under the control of some regulatory factors. By a similar mechanism as shown in Figure 14, the concomittant loss of both activities of m-MDH$^{3'}$ and m-MDH^7 would then be expected.

Discussion

Current evidence suggests that genes coded by mitochondrial and nuclear DNA share in supplying gene products for the structure, function and control of the mitochondria (20, 21). The only mitochondrial gene products that have positively been identified are ribosomal RNA and transfer RNA (20), although the coding capacity of mitochondrial DNA would be large enough to code for at least an additional 20 proteins. Maternal (cytoplasmic) inheritance would have prevailed if the genes were encoded in mitochondrial DNA. Some examples of cytoplasmic inheritance have been described in micro-organisms, Neurospora, insects and plants (22, 23, 24). However, there is no evidence that enzymes localized in mitochondria are coded by this genome. On the other hand, most workers agree that most proteins in mitochondria are coded by nuclear DNA. Mitochondrial leucyl-tRNA synthetase from Neurospora (25) and two mitochondrial peptide chain elongation factors from yeast (26) have been suggested to be coded by nuclear DNA. Mitochondrial malate dehydrogenase in maize (10), in man (27) and in mouse (5) have been shown to be coded by nuclear genes.

In the present study, genetic control of the multiple electrophoretic forms of both soluble and mitochondrial malate dehydrogenases in maize has been investigated. The two classes of malate dehydrogenase appear to be determined by separate loci since electrophoretic variants of soluble malate dehydrogenase do not alter the migration of the mitochondrial malate dehydrogenase, but it is not as yet known if the gene locus(loci) coding for s-MDHs is (are) linked to any of the linkage groups for m-MDH genes. At least two groups of unlinked loci are involved in the expression of maize mitochondrial MDHs. They segregate independently according to Mendelian rules. These findings further support that maize mitochondrial MDHs are controlled by nuclear genes.

Since genes encoding for m-MDH^2 and m-MDH^5 (in strain 59) are not linked to those for m-MDH^1 and m-MDH^3 (in strain Oh51A), this eliminates the possibility that these two groups of m-MDHs are conformers of a single gene product as observed for m-MDHs in chicken heart (27). Experiments to be described in Part II show that the various maize m-MDH isozymes are not interconvertable _in vitro_ and strongly suggest that maize MDH isozymes are not conformational forms derived from the same polypeptide coded by a single gene. These observations suggest that the polymorphism of maize MDH isozymes is genetically determined and is controlled by multiple loci.

Genetic control of the expression of mitochondrial MDH isozymes in maize has been suggested based on the occurrence of null alleles for the multiple m-MDH loci. No better alternatives other than the proposed null alleles would account for the observed results shown in Tables I, II and III. However, the occurrence of recombinant types shown in

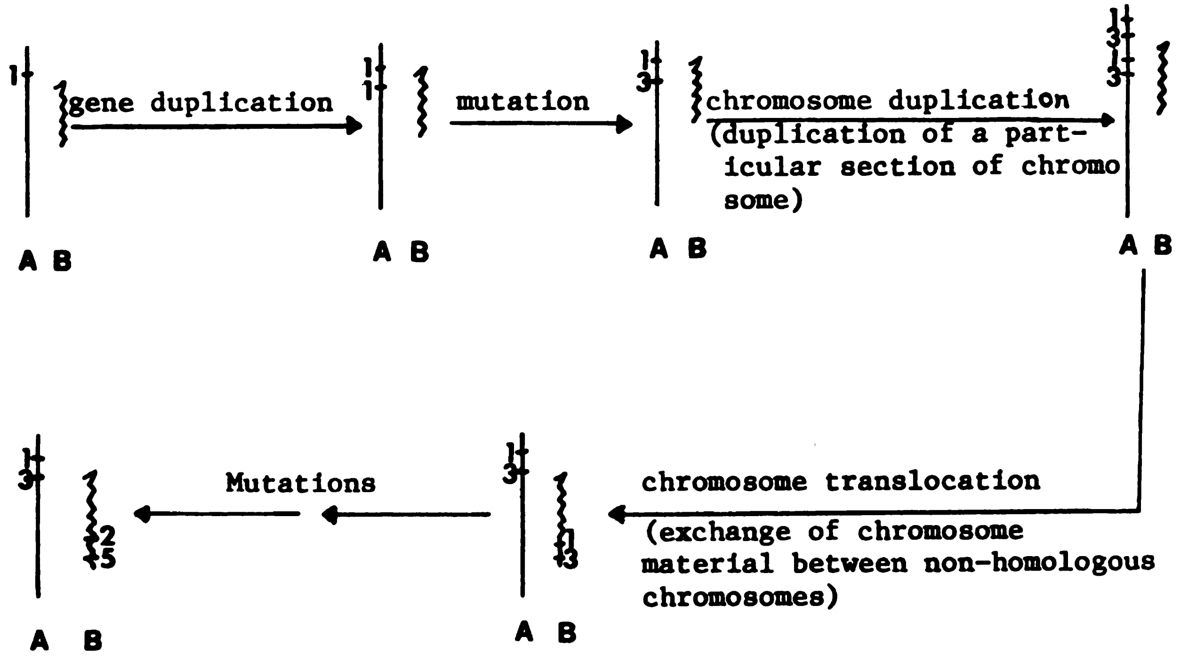
Table IV and the model shown in Figure 14 suggest that the existence of null alleles is not necessarily the only mechanism which may cause the absence of certain m-MDH isozymes in some specific strains.

Expression of mitochondrial isozymes in maize may also be controlled by some "regulatory" genes which may reside far apart from the m-MDH structural genes. In Figure 14, one may suggest that both allelic loci on the homologous chromosomes are not null mutants, (e.g., $\underline{mdh}_1^m$ and $\underline{mdh}_3^m$ on both homologous chromosomes are capable of coding the corresponding m-MDH isozymes) but are not expressed due to deficient "regulatory" genes on both chromosomes. If this is the case, then "intragenic" crossing over within the "regulatory" gene is required. The recombinant "regulatory" gene may then act as normal on structural loci and thus m-MDH isozymes are expressed in the recombinants. Again the observed frequency of recombination strongly against such hypothesis. Therefore, I suggest that both null alleles and m-MDH structural loci and "regulatory" genes for m-MDH isozymes may be involved in controlling the polymorphism of MDH isozymes in maize.

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 Davison (28) have proposed a gene regulation model and proposed that regulatory genes may also play important roles in gene expression of higher organisms. That there is a special class of genetic units that can regulate the action of genes was discovered in maize many years ago (29, 30, 31). More recently, McClintock (32) suggested that in maize, regulation of gene action was under control of two elements, an operator-like element at the gene locus, and a regulator element located elsewhere in the chromosome complement. Results of the present investigation on expression of mitochondrial MDH isozymes may add further support that gene actions in higher organisms are regulated by some genetic unit located elsewhere other than the structural gene loci.

As shown in the "Results," the nuclear gene controlled mitochondrial MDH isozymes are coded by multiple structural loci. Two linkage groups, each with two closely linked loci, are located on two different chromosomes. A third linkage group with another two loci may also exist in some inbred strains. Linkage relationships between the third linkage group and the other two have not yet been determined. The two loci within each linkage group appear to be closely linked. These results indicate that the multiple structural loci of maize m-MDH isozymes may be evolved by a series of gene duplications. Based on the physical and kinetic properties observed (described in Part II), five maize mitochondrial MDH isozymes can be classified into two groups:

the two most anodal m-MDHs (m-MDH^1 and m-MDH^2); and the three most cathodal m-MDHs (m-MDH^3, m-MDH^4 and m-MDH^5). However, the present genetic analysis shows that genes encoding for m-MDH^1 and m-MDH^3 are closely linked on one chromosome, those encoding for m-MDH^2 and m-MDH^5 are closely linked and located on another chromosome. The m-MDH^4 isozyme is a hybrid molecule of m-MDH^3 and m-MDH^5. These results lead me to suggest that the genes coding for the above five anodal m-MDHs in maize are possibly derived through evolution by the following scheme.



*A and B are two non-homologous chromosomes. The numbers denote the different MDH loci.

Ohno (33) has proposed that gene duplication may play an important role in evolution. Chromosome duplications (duplication of a particular section of chromosome) have also been found in studies of changes in chromosome structure (34). Stone (35) suggested many years ago that translocation and other chromosomal exchanges are involved in evolution and speciation. The present hypothesis suggests that these mechanisms are involved in the evolution of the maize m-MDH structural loci.

Genetic control of the polymorphic soluble MDH isozyme in animal cells have been studied in various organisms. Zee, et al (6) suggested that s-MDHs in Ascaris Suum are under the control of two separate genetic loci. Wheat, et al (14) have demonstrated that s-MDHs in fish are controlled by two unlinked loci. In salmon (8, 9) duplicate loci for each of the two unlinked loci have been suggested. It is interesting that there is a great similarity between the above results observed in animal s-MDHs and the present results observed in maize s-MDHs. Genetic control of mitochondrial MDH isozymes in animals has not been well demonstrated, probably due to the lack of appropriate genetic variants. In plants, multiple electrophoretic forms of both soluble and mitochondrial MDH isozymes have been observed in various organisms (36), but the present investigation is the first report in which genetic control of the polymorphic MDH isozymes are demonstrated.

Summary

Genetic control of the multiple forms of malate dehydrogenase in maize has been studied by using starch gel electrophoresis and zymogram technique. Open pollinated indian corn of unknown genetic background and several highly inbred lines were used in the present studies. The facts, that dosage effects on MDH phenotypes may be clearly observed in the triploid (3n) maize endosperm and that crude extracts of liquid endosperms can be easily obtained and subjected to gel electrophoresis, make the liquid endosperm an ideal material for the current investigation.

The following points were observed in the experiments described in this section.

1) The nuclear-gene controlled mitochondrial MDH isozymes in maize are regulated by multiple structural loci. Two linkage groups, each with two closely linked loci, were found to reside on two different chromosomes. A third linkage group, with another two loci, may also exist. The possible linkage relationship between the third linkage group and the other two could not be determined.

2) The formations of hybrid MDH molecules from two types of subunits having different electrophoretic mobilities suggest that the mitochondrial MDHs in maize are dimers in molecular composition. This may also be true for the soluble MDH isozymes, but further substantiation is necessary.

3) In the triploid (3n) maize endosperm, the m-MDH structural genes in each of the three sets of chromosomes appear to be expressed, since dosage effect on mitochondrial MDH isozyme expression were clearly observed in reciprocal crosses.

4) A high rate of lethality has been observed in crosses between strains 59 and Oh51A. Possible mechanisms of this lethality are discussed.

5) A model of m-MDH control has been proposed based on the occurrence of null alleles of the structural m-MDH loci. Evidence was also obtained for the existence of "regulatory" genes controlling linked m-MDH structural loci.

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PART II

PURIFICATION AND BIOCHEMICAL PROPERTIES OF THE GENETICALLY DEFINED MAIZE MDH ISOZYMES

Introduction

Multiple forms of malate dehydrogenase (L-malate : NAD oxidoreductase; E.C.1.1.1.37) have been shown to exist in a wide variety of eukaryotic organisms (1-20). 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 tricarboxylic acid cycle, while the other class (s-MDHs) occurs in the soluble fraction of the cell, where it may participate in the malate shuttle (1), in Crassulacean acid metabolism of plant tissues (21, 22), and other metabolic pathways (2). It is commonly observed that these two classes of MDH isozymes differ in their electrophoretic, physical and kinetic properties. In plant tissues, malate dehydrogenases have also been found in glyoxysomes (3, 17) and peroxisomes (18).

Within each of the two major classes, multiple electrophoretic forms are usually observed, even within a single tissue. Both posttranslational modification and genetic variants have been reported to account for such heterogeneity of malate dehydrogenase. Kitto, et al (4, 5) have shown that the multiple electrophoretic forms of chicken heart mitochondrial MDH have the same primary structure but differ in their conformation. Meizel and Markert (6) reported that the soluble MDH isozymes in the marine snail Ilyanassa might also be conformational isozymes. In *Neurospora crassa*, mitochondrial MDH forms a multiple molecular weight series *in vitro* (23). The multiplicity of these MDH isozymes results from different degrees of aggregation of the enzyme subunits. The state of aggregation is controlled by the ionic and PH conditions of the isozymes (23). On the other hand, genetic variation also contributes to the multiplicity of MDHs found in many animal cells (7-11).

In this part, I present data on comparative biochemical properties of malate dehydrogenase isozymes in maize for which many genetic variants are available. The following points were observed in my studies: 1) The multiplicity of the different electrophoretic forms is not caused by conformational conversions of the enzymes, rather, it is genetically determined. 2) Using highly purified enzyme preparations, I have studied the biochemical properties of each MDH isozyme in a highly inbred strain, W64A. These include PH optima, thermolability, molecular weight and isoelectric point, Michaelis constants of OAA, malate, NAD and NADH; substrate inhibition (OAA) and coenzyme inhibition (NAD); and the effects of various organic and amino acids, NAD analogs, chelating agent, reducing agents and metal ions on the enzymatic activity. I have found that not only are the s-MDHs and m-MDHs different in most of these kinetic and physical properties, but that the isozymes within each of the two major classes may also differ significantly in some of these properties. 3) According to their physical and kinetic properties and the genetic analysis discussed in Part I, I suggest that four groups of structural loci are involved in the determination of the polymorphism of maize MDH isozymes.

Materials and Methods

Identification of MDH isozyme patterns in maize liquid endosperm. Twenty maize strains were screened for malate dehydrogenase variants. They were all inbred for at least fifteen generations. Liquid endosperm from 16 to 20-day-old kernels was used since it could be applied to the gels directly, that is without further extraction. The liquid endosperm from individual kernels was squeezed onto a 6 mm x 10 mm piece of Whatman #3 MM filter paper and inserted into a vertical slot in the starch gel. Horizontal starch gel electrophoresis and specific staining for MDH were carried out according to the method of Scandalios (24) as described under "Materials and Methods" in Part I.

Culture of Seedlings. Inbred maize strain, W64A, was used. Seeds were surface sterilized with 5% sodium hypochlorite solution for 10 minutes, washed twice with deionized distilled water, and soaked in water for 5 to 8 hours. After soaking, the seeds were germinated between moistened germination papers in plastic trays (40 x 30 x 8.5 cm) in the dark at 25°C. After 4 to 5 days, various tissues were isolated and used for organelle preparation or enzyme extraction.

Identification of soluble, mitochondrial and glyoxysomal MDH isozymes in maize scutella. The soluble, mitochondrial and glyoxysomal fractions of maize scutella were separated on sucrose density gradients according to the method of Longo and Longo (25), as to be described in Part III. MDH isozymes in the various fractions were identified by starch gel electrophoresis and specific staining for MDH.

Enzyme assays and protein estimation:

Enzymatic activity of MDH was measured spectrophotometrically with a Gilford spectrophotometer (Model 2400) equipped with a digital absorbance meter and automatic recorder. For the forward direction (oxaloacetate + NADH→L-malate + NAD), the standard reaction mixture contained 25 mM glycylglycine buffer (pH 8.5), 125 uM oxaloacetate, 50 uM NADH and 10-50 ul enzyme in a total volume of 3.0 ml. This "standard forward reaction mixture" was used for measuring MDH activity in experiments on enzyme purification, determination of molecular weight, detection of the isoelectric point, thermostability; and on the effects of reducing agents, chelating agents and inorganic ions on the activity of MDHs. For the reverse direction (L-malate + NAD→oxaloacetate + NADH), the standard reaction mixture contained 25 mM glycylglycine buffer (pH 8.5), 5 mM malate, 0.75 mM NAD and 10-50 ul of enzyme in a total volume of 3.0 ml. This "standard reverse reaction mixture" was used for measuing the MDH activity in experiments concerning the efficiency of NAD analogs, and the effects of various naturally occurring metabolites on the activities of MDHs. Oxidation of NADH or reduction of NAD, or NAD-analog, was measured at 25°C by following the absorbance changes at 340 nm for NAD and deam-NAD, 365 nm for 3-AP-NAD and 400 nm for TN-NAD in cuvettes with a 1-cm light path. Initial rates were used in calculation of activities. All substrates were titrated with NaOH to the desired pH before use.

Thermostability of the MDH isozymes was measured at 53°C. 3 ml of each isozyme preparation was added to a small test tube and was heated at 53°C in a reservoir of a constant temperature bath. Samples (0.2ml), were withdrawn at various times, chilled immediately in an ice-water bath and subsequently assayed for MDH activity.

Substrate and coenzyme inhibitions of MDH isozymes were measured at three different pHs. Substrate (OAA) inhibition of MDH isozymes was measured in the forward direction (OAA + NADH→L-malate + NAD). Glycylglycine buffers (0.025 M) with three different pHs (pH 7.5, 8.5 and 9.5), 50 uM NADH and various concentrations of OAA, ranging from 0.0313 to 1 mM were used in the reaction mixture. Coenzyme (NAD) inhibition of MDH isozymes was measured in the reverse direction (L-malate + NAD→OAA + NADH). 0.025M glycylglycine buffers (pH 7.5, 8.5 and 9.5), 5mM malate (for pH 8.5 and 9.5) or 20mM malate (for pH 7.5; due to the much higher Kms for malate at this pH) and various concentrations of NAD, ranging from 0.19 to 2.0 mM were used in the reaction mixture.

Kinetic analysis for the estimation of Km values were performed by plotting s/v against s according to the equation, $s/v = Km/V + (1/V)S$ given by Hanes (26). Glycylglycine buffers at three different pHs were used. Substrate ranges were OAA: 0.025-0.25 mM, NADH: 0.0125-0.1 mM, malate: 0.625-10 mM, NAD: 0.03-0.5 mM. 50 uM. 50 uM of NADH, 125 uM OAA, 0.5 mM NAD and 5 mM malate (at pH 8.5 and 9.5) or 50 mM malate (at pH 7.5) were used when Kms for OAA, NADH, malate and NAD were tested respectively. Five to six points were used in the analysis of Km.

Protein determination: Protein concentration was determined by the phenol reagent method according to Lowry (27) with crystalline bovine serum albumin as the standard. Colorimetric readings were made at 660 nm. Specific activity of the enzyme is defined as units per mg of protein. In the eluted fractions of gel filtration or ion exchange column chromatography, the protein concentrations were estimated by the 280 to 260 nm method as described by Layne (28).

Purification and separation of malate dehydrogenase isozymes: All operations were performed at 0-4°C unless otherwise specified.

Step 1. Preparation of crude extract:

Scutella with the endosperms attached were collected by cutting off the shoots and roots from the 4- to 5-day-old maize seedlings. 400 g of scutella and endosperms are homogenized with 1200 ml of 0.02 M potassium phosphate buffer (pH 7.4) with 5mM 2-mercaptoethanol for 5 minutes in a Waring blender. The resulting homogenate was then squeezed through a four-layer cheesecloth, and centrifuged at 10,000 x g for 10 minutes. The supernatant fraction was taken as the crude extract of MDH.

Step 2. Treatment at pH 5.0:

The pH of the crude extract preparation was adjusted slowly to 5.0 with 0.05M HCl. The precipitate formed from the pH 5.0 treatment was removed by centrifugation at 15,000 x g for 30 minutes and discarded. The supernatant containing more than 90% of the original activity with about 2-fold increase in specific activity was adjusted to pH 7.0 with 0.05M KOH.

Step 3. Ammonium sulfate fractionation:

The pH 5 supernatant was brought to 50% saturation by slowly adding solid ammonium sulfate (29). The solution was stirred for 2 hours and the precipitate was removed and discarded by centrifugation at 15,000 x g for 30 minutes. The resulting supernatant was decanted and then brought to 65% saturation with ammonium sulfate. After stirring for 5 hours, the precipitate was collected by centrifugation at 15,000 x g for 30 minutes and dissolved in 0.02M potassium phosphate buffer, pH 7.0, containing 5mM mercaptoethanol. The resulting solution, about 40 ml, was then dialysed against 4 liters of the same buffer for 24 hours.

Step 4. Gel filtration through Sephadex G-150 column:

After ammonium sulfate fractionation and dialysis, the enzyme solution was concentrated by further dialysis in 50% aquacide (Calbiochem) solution. Seven to ten ml of the enzyme preparation containing 30 to 40 mg of protein was then applied to a Sephadex G-150 column (pharmacia, 2.5 x 95 cm) which had been equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, containing 5 mM 2-

mercaptoethanol and eluted with the same buffer at a flow rate of 30 ml per hour. For each fraction, 7.5 ml was collected. Enzyme activities and absorbance at 280 mμ were determined. Fractions with MDH activities were pooled.

Step 5. DEAE-cellulose column chromatography:

The enzyme preparation pooled in step 4 and brought to 80% saturation with ammonium sulfate. After stirring for 3 hours, the precipitate was collected by centrifugation at 15,000 x g for 30 minutes and dissolved in 0.02 M potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol. The resulting solution (25 ml) was then desalted by running through a Sephadex G-25 column (2.5 x 30 cm) with the same buffer described above. The 30 ml enzyme preparation eluted from a Sephadex G-25 column was applied to a DEAE-cellulose column (2 x 35 cm) which had been equilibrated with the same buffer used in gel filtration. After loading the enzyme solution on the column, the absorbent was washed with 10 ml of the same buffer with which it was equilibrated. A linear salt gradient (0.02 M KCl to 0.2 M KCl), prepared in the same buffer as previously mentioned, was applied at a flow rate of 25 ml per hour. Five ml fractions were collected. Three peaks of enzyme activity were obtained. The fractions of each peak were combined. The three peak fractions were separately concentrated by 80% saturated ammonium sulfate precipitation as described earlier in Step 5 and dialysed for 24 hours against 6 liters of 0.02M potassium phosphate buffer, pH 7.0, containing 5 mM mercaptoethanol. These three concentrated peak fractions were used in the studies of the physical characteristics of MDH isozymes such as: molecular weight, the possible convertibility of one electrophoretic form to another form by the treatments of mercaptoethanol, acid (ph 2) and 7.5 M guanidine hydrochloride.

Step 6. Starch gel electrophoresis and high speed centrifugation:

The three fractions from Step 5 were applied to three different starch gels and subjected to electrophoresis. For each gel, a sample of about 0.5 ml was applied by using three 6 mm x 5 cm filter papers. After electrophoresis, one horizontal slice was taken from the gel and stained for MDH activity. This stained slice served as a template for excising single isozyme bands from the unstained portion of the gel. Each excised band was then placed in a syringe and squeezed into a centrifuge tube. Glycylglycine buffer (1-3 ml; 0.025 M, pH 7.5) was added to dilute the macerated gels and to balance their weight. The suspension was then centrifuged at 45,000 x g for 1 hour. The supernatant, containing a single MDH isozyme, was diluted with glycylglycine buffer, pH 7.5, to obtain an activity of 0.01-0.02 absorbance per minute per 50 µl enzyme as measured with the "standard forward reaction mixture." The MDH isozyme preparations thus obtained (from Step 1 to Step 6) were quite stable in 0-4°C and were used for comparative kinetic studies.

Polyacrylamide disc gel electrophoresis:

Purities of the "highly purified" isozymes were checked by polyacrylamide disc gel electrophoresis. The method used in this gel electrophoresis was similar to that described by Ornstein (30) and Davis (31) with the following modifications. The glass tubes were 0.5 cm i.d. x 14 cm long. The height of the polyacrylamide gel columns were 10 cm and spacer gels were 2 cm. The concentration of all the running gels were 9% (w/v). The stock solutions were prepared as follows:

a) 48 ml of 1N HCl, 36.6 g of Tris, 0.23 ml of TEMED, and water to 100 ml.

b) 28.0 g of acrylamide, 0.735 g of BIS-acrylamide, and water to 100 ml.

c) 4 mg of riboflavin, and water to 100 ml.

d) 48 ml of 1N HCl, 5.98 g of Tris, 0.46 ml of TEMED, and water to 100 ml.

e) 10 g of acrylamide, 2.5 g of BIS-acrylamide, and water to 100 ml.

f) 40 g of sucrose and water to 100 ml.

The running gel contained 1 part (a), 2.4 parts (b), 1 part (c), and 3.6 parts water. The spacer gel contained 1 part (d), 1 part (c), 2 parts (e) and 4 parts (f). Buffer for electrodes contained 0.6 g of Tris, 2.9 g of glycine and water to 1 liter, pH 8.3.

The highly purified single isozyme preparations obtained through six steps of purification (the final elute from the starch gel in Step 6) were concentrated to about 5-fold by lyphogel. Samples, 0.2 ml of enzyme preparation containing approximately 2 µg protein, were layered onto the spacer gel by displacement of electrode buffer. Electrophoresis was performed at 4°C for 4 hours with a constant current of 0.8 mA per tube. On completion of the electrophoresis, the gels were carefully removed by use of a needle and air pressure. For protein stain, the gel was stained over night with 0.1% coomassie blue (prepared in 7.5% acetic acid, 5% methanal) and destained electrophoretically in 7.5% acetic acid. For specific staining of MDH, the gel was stained with the specific MDH staining reagent, containing malate, NAD and tetrazolium salt, as described by Scandalios (21).

Studies on interconvertibility of MDH isozymes. Six methods were used to study the possible interconversion of MDH isozymes.

Method 1. Crude extract, supernatant of the 10,000 x g for 10 minutes for the scutella of the 4-day-old maize seedlings, was used. After the first run electrophoresis, the gel was turned 90 degrees and a second sample serving as a control was inserted. The gel was then subjected to electrophoresis under the same conditions as the first gel. After the second electrophoresis, a thin slice of the gel was removed and stained for MDH.

Method 2. The same sample was used as that in Method 1. After electrophoresis in starch gel, a thin slice of the gel was removed and stained to serve as a guide for the location of individual isozymes. Pieces of starch containing these isozymes were then cut from the remaining starch gel and inserted in the sample slots of a second gel. Another crude MDH preparation was applied to a different channel to serve as a control. After the second electrophoresis was completed, the gel was stained for MDH and the mobilities of the individual isozymes were compared with their original mobilities after the first electrophoresis.

Method 3. (mercaptoethanol experiments) This experiment was carried out mainly according to the method described by Meizel and Markert (6). Three partially purified DEAE-cellulose MDH preparations as described under Step 5 in purification procedures were used. Mercaptoethanol was added to these preparations to a final concentration of 100 mM, and these solutions were sealed with parafilm within a tube and allowed to stand at 4°C. Controls without mercaptoethanol were also allowed to stand for the same period at 4°C. After 22 hours, samples of these solutions were electrophoresed and the gel was stained to determine the effect of 2-mercaptoethanol of the MDH isozyme pattern.

Method 4. Attempt to dissociate the MDH subunits by high ionic strength buffer was performed with some modification of the method of Scandalios (32). The three partially purified DEAE-cellulose MDH preparation (in 0.20M potassium phosphate buffer, pH 7.0) were mixed separately with equal volume of 2M NaCl in distilled water. The final concentration of the buffer is 1M NaCl in 0.01 M potassium phosphate buffer, pH 7.0. A mixture of the enzyme preparation with equal volume of distilled water only served as the reference. After 2 hours at room temperature, the mixtures were then frozen over night, thawed and subjected to starch gel electrophoresis. On completion, the gel was stained to study how the MDH isozyme patterns are affected by the treatment of freezing and thawing in high ionic strength.

Method 5. (pH 2 treatment) The MDH isozymes were treated at pH 2 by the method of Kitto et al (5). The three partially purified DEAE-cellulose MDH preparations mentioned in Method 3 were brought to 0.1M mercaptoethanol. An aliquot of 0.4 ml containing 0.5 to 1 mg protein / ml was carefully titrated to pH 2 with 0.1 N HCl. Experiments of denaturation and renaturation were performed at room temperature unless otherwise specified in a parafilm sealed tube for 20 hours. Renaturation was initiated by diluting the samples with 20 ml 0.5 M tris-citrate, pH 7.0, containing 100 mM mercaptoethanol and 1 mg/ml NADH. Approximately 5 hours after dilution, the renaturated enzyme solutions were dialysed extensively against 8 liters of 0.02 M tris-citrate buffer, pH 7.0, containing 5 mM mercaptoethanol for 20 hours. The dialysed enzyme solutions were then concentrated by pressure ultrafiltration using Amicon UM-10 membrane. Samples of these solutions along with the untreated samples were electophoresed and the gel was stained to determine the effect of acid treatment on the isozyme pattern. Untreated samples (controls) are those following the same procedure except that distilled water, instead of 0.1 N HCl, was added to the enzyme solutions. Both denaturation and renaturation were successful. After denaturation for 20 hours, no MDH activity was found by spectrophotometric assay but following 5 hours of renaturation, 30-38% of the original MDH activities were recovered.

Method 6. (7.5 M guanidine hydrochloride treatment). For reversible denaturation in guanidine hydrochloride, the three partially purified DEAE-cellulose MDH preparations were placed in 7.5 M guanidine hydrochloride made up in 0.1 M Tris-citrate pH 7.0 containing 0.1 M mercaptoethanol. The procedures for denaturation and renaturation were as described in Method 4.

Molecular weight determination:

1. Column chromatography on a calibrated G-150 Sephadex column:

The molecular weights of maize MDHs were estimated by chromatography on a Sephadex G-150 column calibrated for molecular weight determinations, as described by Andrews (33).

A Sephadex G-150 column was equilibrated as described in Step 4 of "Purification Procedures." The column was calibrated with eight combinations of non-enzymatic standard proteins; 8 mg protein / 2 ml buffer solution was applied for each of the marker proteins. After elution, fractions of 2 ml each were collected. Absorbance at 280 nm was measured to determine the elution volumes of these standard proteins. For each of the DEAE-cellulose MDH preparations (peak I, II and III), 6 mg protein / 2 ml was applied. The elution volume of MDHs was determined by the peak fraction of the MDH activity. The void volume (Vo) used in the calibration in the Ve of blue dextran. A standard plot of the correlation between log molecular weight and reduced elution volume (Ve/Vo) was used for the estimation of the molecular weights of MDHs.

2. Sucrose density gradient centrifugation.

The linear sucrose density gradient was prepared according to the method of Martin and Ames (34) by a device which consists of two chambers interconnected with each other when a needle valve is opened. The gradient was made in Beckman cellulose nitrate tubes and allowed to stand for 2 hours at 4°C, to smooth out before the sample was layered on the gradient. The three partially purified DEAE-cellulose MDH preparations (peak I, II and III) were used. An MDH preparation, 0.3 ml and containing 0.59-0.63 mg protein, was layered over a 5 to 20% sucrose density gradient. A swinging bucket rotor, SW 40 (Beckman), was used for the centrifugation which was performed at 35,000 rpm in a Beckman L-2-65B ultracentrifuge for 24 hours. Sixty fractions of 15 drops each were collected after needle puncture of the bottom of the tube. Beef liver catalase (mol. wt. = 250,000) was used as the marker for estimation of the relative molecular weight for the MDHs.

Electrofocusing column chromatography:

MDH preparations obtained from Step 3 of the purification (dialysed sample of the 50% to 65% ammonium sulfate cut) were electrofocused to determine the isoelectric points of MDH isozymes. An LKB model 8101 electrofocusing column with a total capacity of 110 ml was used. Electrofocusing was done according to the methods described in the LKB manual. Ampholyte, pH 3.0 to 6.0, was used in these experiments. The composition of gradient solution and electrode solution for the electrofocusing was as follows:

1. Dense gradient solution:
    - Ampholyte (40%)................1.9 ml
    - Sucrose........................28 g
    - Distilled water................to 55 ml
2. Less dense gradient solution:
    - Ampholyte (40%)................0.6 ml
    - Enzyme solution................varied
    - Distilled water................to 55 ml
3. Dense electrode solution:
    - NaoH...........................0.3 g

Sucrose......................18 g

Distilled water..............21 ml

4. Less dense electrode solution:

H_2SO_4 (conc.)................0.1 ml

Distilled water..............to 10 ml

Ten ml of MDH preparations containing 120 units of activity were added to the column. A constant potential of 300 volts was applied to the column with the aid of a Buchler model No. 3-1014A voltage and current regulated D.C. power supply. The temperature was maintained at 4°C by circulating water and methanol solution from a thermostat water cooler, Landa model WB-20/R (Brinkman Instruments) through the external and internal jackets. After 60 hours of electrofocusing, 180 fractions of 10 drops (0.6 ml) per tube were collected. pH values were measured immediately by an Orion digital pH meter and MDH activities were determined directly by using small amounts of enzyme solution (5 to 10 ml) of the eluted fractions. By using uniformly prepared filter paper Wicks (Whatman #3MM, 3x8 mm) each one holding 5 ml enzyme solution, the peak fractions were applied to a starch gel and subjected to electrophoresis. After electrophoresis, the gel was stained for MDH. The staining intensity was proportional to the activity applied to the gel and the pI of each isozyme was determined from the pH of the fraction which had the highest intensity in staining.

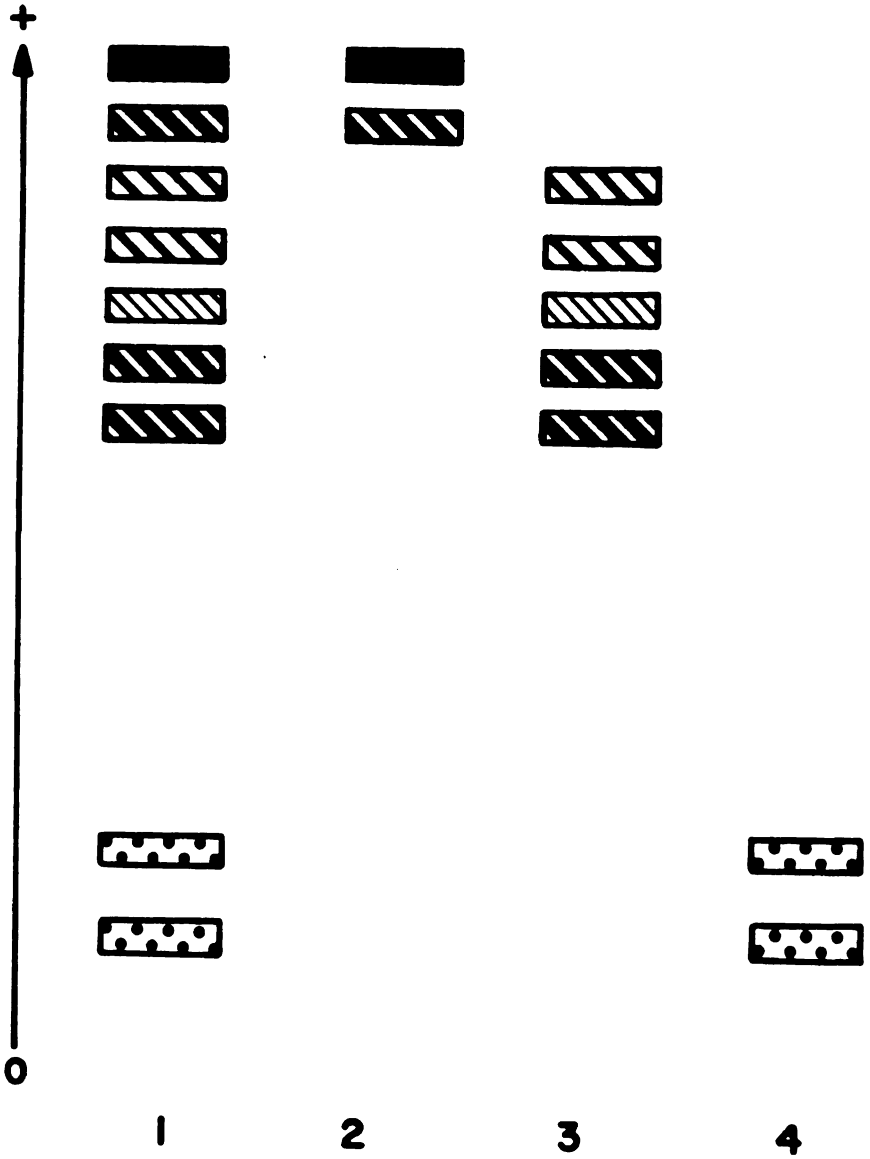
Results

Intracellular localization and isozyme pattern of malate dehydrogenase isozymes in maize.

In the inbred strain W64A, there are nine MDH isozymes including two soluble forms, five mitochondrial forms and two glyoxysomal forms (Figure 1). In the etiolated seedlings, all of the organs examined (endosperm, scutellum, root, shoot) had the same MDH isozyme patterns for both the soluble and the mitochondrial forms, while glyoxysomal MDHs were found only in the scutellum. The specific activity of MDH is higher in the scutellum than in any other organ examined. The soluble and mitochondrial MDH isozyme patterns in the scutellum of the etiolated seedlings were found to be the same as in the liquid endosperm of the immature kernel. Therefore, scutella were used as the material for MDH purification and for studies of the biochemical properties of MDH isozymes while liquid endosperms, which are very easy to apply to the starch gel, were used for screening of the MDH isozyme patterns in different inbred lines.

Twenty maize inbred lines have been tested for MDH isozymes in liquid endosperm. Two variants in the cytoplasmic bands and eight variants in the mitochondrial bands were detected (Figure 2). Strain W64A had the two s-MDHs and the five commonly observed m-MDHs, and was therefore chosen as a source of isozymes for the study of the biochemical properties of maize MDH isozymes. The MDH isozymes

Figure 1.--Schematic summary of the zymogram of MDH isozymes in subcellular fractions isolated from 4-day old scutella of the inbred strain W64A. The various fractions were separated by differential sucrose gradient centrifugation: (1) = crude extract (2) = soluble fraction (3) = mitochondria (4) = glyoxysomes.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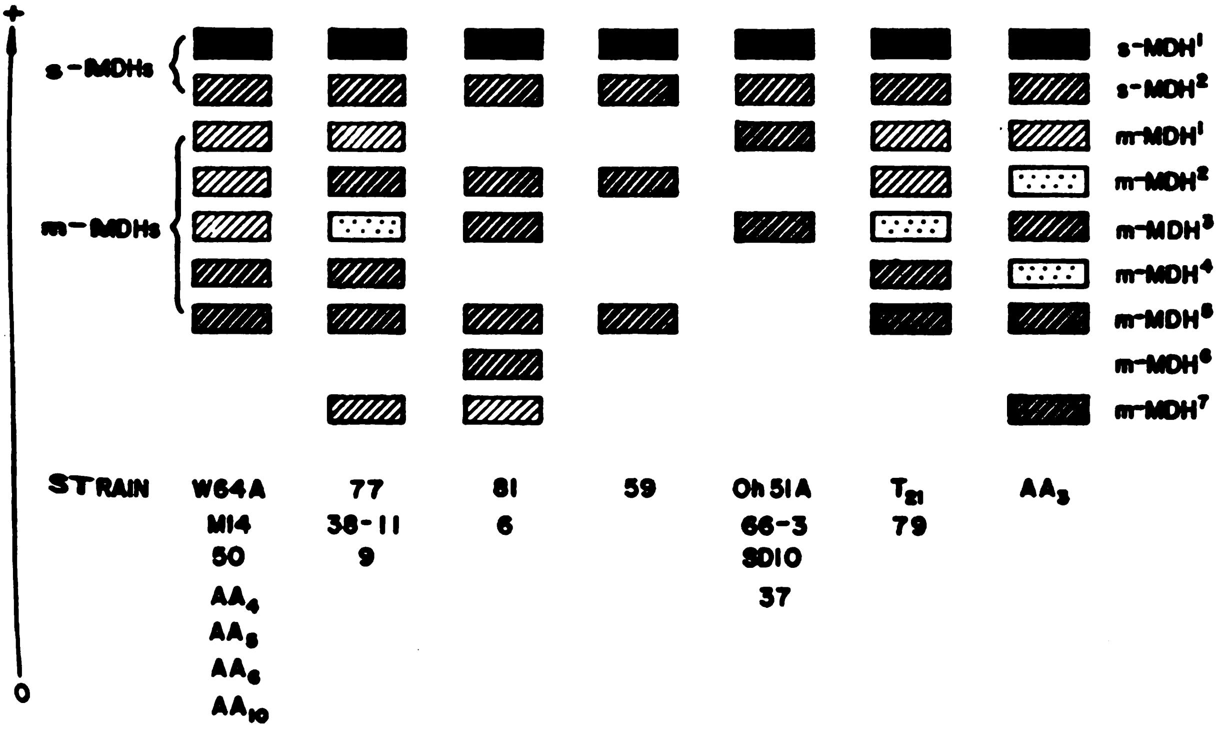
3
4

Figure 2.--Schematic diagram of soluble and mitochondrial MDH isozyme patterns in different maize strains. Liquid endosperms (18 to 22 days after pollination) of 20 maize strains were screened for MDH variants. Experimental details are described under "Materials and Methods."

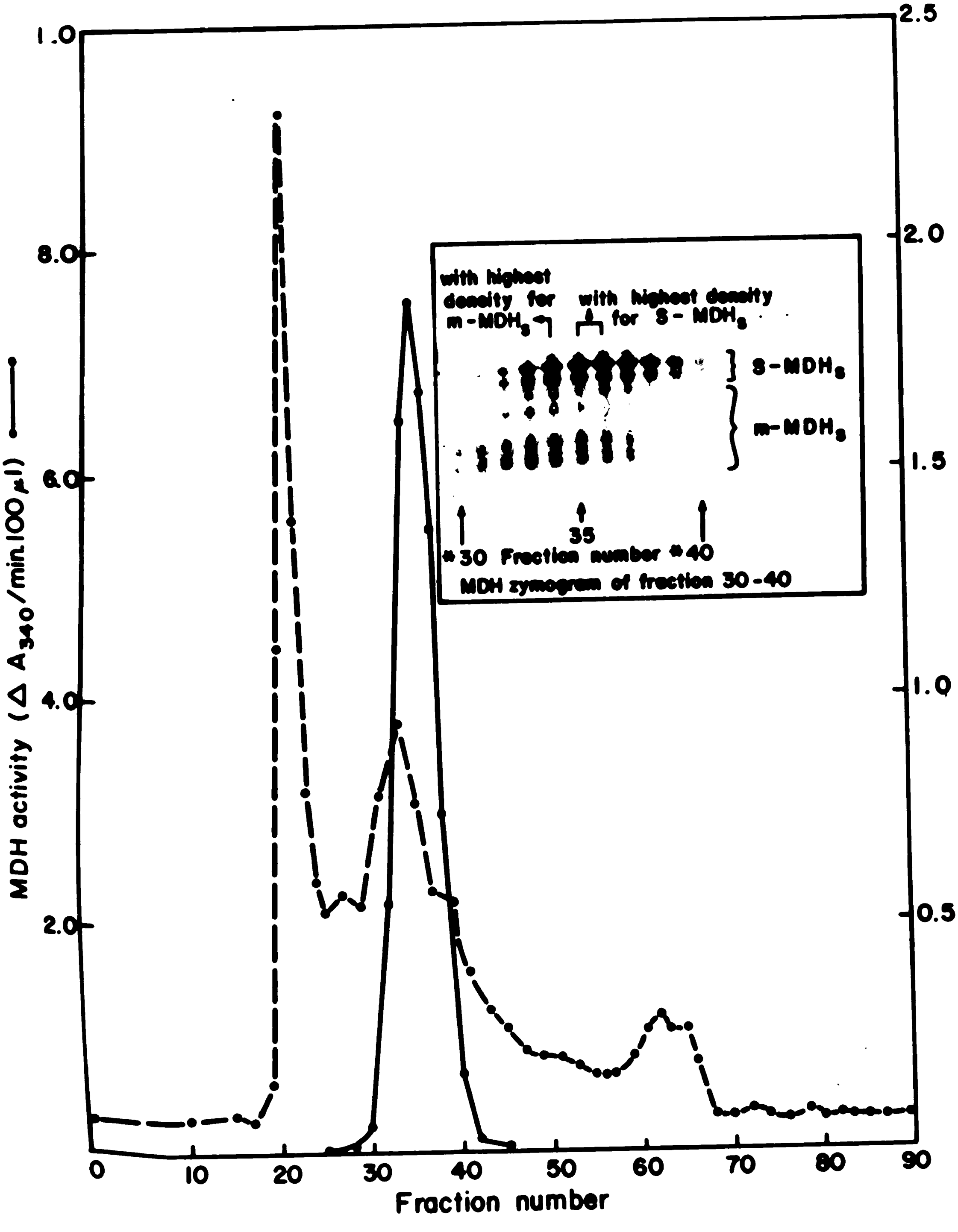

+
s-MDHs
m-MDHs
s-MDH^1
s-MDH^2
m-MDH^1
m-MDH^2
m-MDH^3
m-MDH^4
m-MDH^5
m-MDH^6
m-MDH^7
STRAIN
W64A
M14
50
AA$_4$
AA$_5$
AA$_6$
AA$_{10}$
77
38-11
9
81
6
59
Oh 51A
66-3
SD10
37
T$_{21}$
79
AA$_3$
0

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-MDH^1, g-MDH^2 (the two glyoxysomal isozymes). Because the activities of glyoxysomal MDH isozymes in scutella are very low compared to the soluble and mitochondrial MDH isozymes, we concentrated on the latter two forms of the enzyme.

Purification of maize MDH isozymes. Maize MDHs are stable in acid (pH 5.0), while many other maize proteins are denatured and precipitated at this pH. Therefore, pH 5 treatment was found to be a useful step for purification of maize MDHs. The "pH 5 soluble" MDH fraction was selectively precipitated by 50-60% saturated ammonium sulfate. There is no observable difference between s-MDHs and m-MDHs in their solubility in ammonium sulfate.

The s-MDHs and the m-MDHs are eluted as a single peak during gel filtration on Sephadex G-150 column (Figure 3). However, detailed analysis of the peak fractions by starch gel electrophoresis and MDH zymogram indicates that the m-MDHs were eluted from the column a little earlier than were the s-MDHs (inset of Figure 3). Therefore, a significant difference of the molecular weights of s-MDHs and these of m-MDHs may exist. Linear elution of the G-150 MDH peak fractions from a DEAE-cellulose column with buffers containing a salt gradient ranging from 20 to 200 mM KCl

Figure 3.--Gel filtration of maize scutellum malate dehydrogenase on G-150 column. An amount of 7.5 ml resuspension of 50% to 65% saturated ammonium sulfate precipitation fraction containing 30 mg of protein was applied to a column (2.5x95 cm) which had been equilibrated with 0.02M potassium phosphate buffer (pH 7.0) and eluted with the same buffer in 7.5 ml fractions. The flow rate was maintained at 30.8 ml/hr. Enzyme assays were performed as described under "Materials and Methods." Protein concentration was measured as the absorbance at 280 nm. As shown in the inset, starch gel electrophoresis indicated that m-MDHs (fraction 34) were eluted from the column a little earlier than were the s-MDHs (between fraction 35 and fraction 36).


1.0
8.0
6.0
4.0
2.0
MDH activity (Δ A340/min.100 µl)
2.5
2.0
1.5
1.0
0.5
A280
0
10
20
30
40
50
60
70
80
90
Fraction number
with highest density for m-MDHs
with highest density for S-MDHs
S-MDHs
m-MDHs
35
#30 Fraction number #40
MDH zymogram of fraction 30-40

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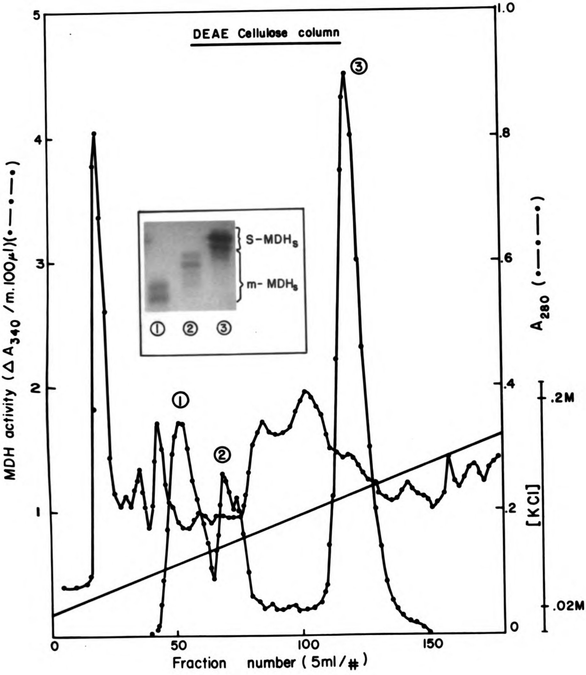
resulted in three distinct malate dehydrogenase peaks (Figure 4). Starch gel electrophoresis showed that Peak I consisted of the two most cathodal m-MDHs (m-MDH^4, m-MDH^5); Peak II, the three less cathodal m-MDHs (m-MDH^1, m-MDH^2, m-MDH^3); Peak III, the two s-MDHs (s-MDH^1, s-MDH^2) (inset of Figure 4). These three peaks separated by DEAE-cellulose column chromatography are symbolized as the following: DEAE-I MDHs (m-MDH^4, m-MDH^5), DEAE-II MDHs (m-MDH^1, m-MDH^2, m-MDH^3), DEAE-III MDHs (s-MDH^1, s-MDH^2). The MDH isozyme patterns observed in various fractions of the purification steps are shown in Figure 5.

By starch gel electrophoresis and high speed centrifugation, isozymes in each peak fraction were further separated and highly purified. A summary of the purification of the MDH isozymes of maize is shown in Table I. With a 9% polyacrylamide gel at pH 8.3, purity of the MDH isozymes purified through 6 steps of purification were checked by gel electrophoresis. In this experiment, three MDH isozymes, namely s-MDH^1, m-MDH^2 and m-MDH^5 were chosen to represent the three groups of DEAE-cellulose MDH isozymes. As shown in Figure 6, the s-MDH^1 isozyme preparation was observed as a single protein band which is associated with MDH activity. The m-MDH^5 isozyme preparation exist as a single protein band corresponding to the major MDH activity band. However, the slightly stained minor band shown by MDH activity stain does not seem to have a detectable corresponding protein

Figure 4.--Elution profile of malate dehydrogenase activities from DEAE-cellulose column chromatography. The MDH preparation partially purified from step 1 to step 4 (see Materials and Methods) was used. An amount of 30 ml enzyme preparation (about 25 mg protein/ml) was applied to a DEAE-cellulose column (2.5x35 cm) previously equilibrated with 2.5 liters of 0.02 M potassium phosphate buffer (pH 7.0) at a flow rate of 25 ml/hr. After layering the enzyme preparation on the column and washing with 10 ml of the same buffer, a linear salt gradient (0.02 M KCl) prepared in the same buffer with 5 mM mercaptoethanol was applied at the same flow rate as during equilibration. 5 ml fractions were collected. Three peaks of MDH activities were observed and the corresponding isozyme patterns are shown in the inset.



DEAE Cellulose column
MDH activity (ΔA340 /m.100µl)(•—•—•)
A280 (•—•—•)
[KCl]
Fraction number (5ml/#)
0
50
100
150
1
2
3
4
5
0
.2
.4
.6
.8
1.0
.02M
.2M
①
②
③
S-MDHs
m-MDHs

Figure 5.--Photographs of MDH zymogram showing the MDH isozyme patterns observed in various purification steps. (1) Crude MDH preparation (10,000 xg supernatant). (2) MDH selectively precipitated between 50% to 65% saturated ammonium sulfate. (3) MDH pooled from the single peak (fraction 30 to 40 in Figure 3) eluted from gel filtration on Sephadex G-150. (4) MDH pooled from the first peak (Peak I) eluted from DEAE-cellulose ion exchange column. (5) MDH pooled from the second peak (Peak II) eluted from DEAE-cellulose ion exchange column. (6) MDH pooled from the third peak (Peak III) eluted from DEAE-cellulose ion exchange column.

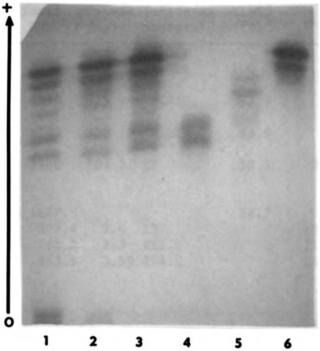

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Table I. Purification of Maize Malate Dehydrogenase Isozymes

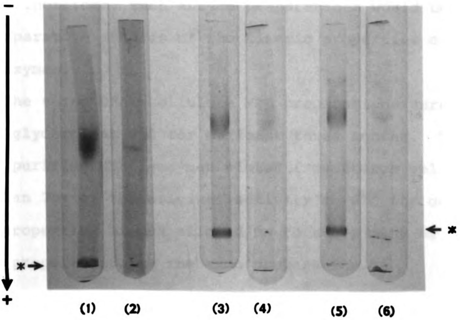
Steps	Total MDH	Total protein (mg)	Specific activity* (units/mg protein)	Yield (%)	Purification factor (fold)
1. Crude extract	5677	5600	1.016	100	1.0
2. pH5 treatment	5548	2475	2.258	97.7	2.22
3. $(NH_4)_2SO_4$,50-65%	4193	520	8.129	73.9	8.0
4. Sephadex G-150	2968	106.2	27	52.3	26.5
5. DEAE-Cellulose fractionation	1627.5			28.7	
Peak I	377.4	2.8	133		130.9
Peak II	266.2	2.3	115.6		113.8
Peak III	983.9	3.59	274.2		269.9
6. Starch gel electrophoresis					about 230-450

*Assayed for OAA reduction by using "forward standard reaction mixture" as described under "methods."

Figure 6.--Homogeneity of the highly purified MDH isozymes checked by 9% polyacrylamide disc gel electrophoresis. Protein and enzymatic staining of the highly purified MDH isozymes on a polyacrylamide gel after electrophoresis.

The MDH enzymatic staining pattern of (1) s-MDH^1 (3) m-MDH^2 (5) m-MDH^5.

The protein staining pattern of (2) s-MDH^1 (4) m-MDH^2 (6) m-MDH^5. For each sample, a 0.2 ml of lyphogel concentrated MDH isozyme preparation containing approximately 5 ug protein was applied to a 9% polyacrylamide gel. Details were described in "Materials and Methods."



*** Position of bromophenol blue, the marker dye.**

band. One major and two minor protein bands with the major one corresponding to the MDH activity stain were found for m-MDH^2. It is evident that these MDH isozymes purified and separated through 6 steps of purification are highly purified and some of them were obtained in a homogeneous state. Therefore, such enzyme preparations would be ideal for comparative studies of the kinetic properties of the MDH isozymes.

The three DEAE-cellulose MDH preparations were stable in 20% glycerol at 0°C for at least three months. The seven highly purified MDH isozymes eluted from starch gel maintained more than 70% of the original activity at 0°C for one month. These properties of MDH allowed us to study many of the enzyme properties with the same preparation.

Possible interconversion of the maize MDH isozymes.

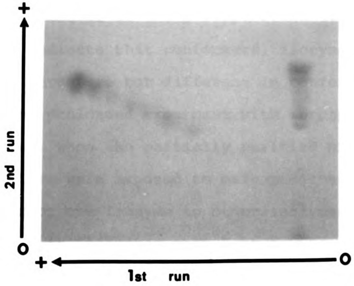
Results of Method 1 and 2. As can be seen in Figure 7, after second run electrophoresis, the isozymes retain their original mobilities relative to each other. No interconversion of the isozymes was observed.

Results of Method 3. Kirkman and Hanna (35) have shown that a single glucose 6-phosphate dehydrogenase isozyme from human erythrocytes was converted to another isozyme upon treatment with mercaptoethanol. Meizel and Markert (6) observed that all of the supernatant MDH isozymes of Ilyanassa were apparently of the same molecular weight and were all convertible to a single form by prolonged

Figure 7.--MDH zymograms showing a second run electrophoresis. All isozymes retained their original mobilities relative to each other.

(a) After the first run electrophoresis of a crude MDH preparation, the gel was turned to 90 and a second sample serving as a control was inserted. The gel was then subjected to electrophoresis under the same condition as the first gel. (b) The pieces of starch containing particular MDH isozymes were cut from the gel after first run electrophoresis of a crude MDH preparation and placed in the slots of a second gel and subjected to a second run electrophoresis is under the same condition as in the first.

(a)



(b)

exposure to mercaptoethanol. In both cases, conversion was reversible by removal of the mercaptoethanol. These observations indicate that conformers, isozymes with the same primary structure but different in conformation, may be detected by prolonged treatment with mercaptoethanol.

In maize, when the partially purified DEAE-cellulose MDH preparations were exposed to mercaptoethanol for 22 hours, no conversion of one isozyme to other isozymes occurred (Figure 8). This indicates that both s-MDHs and m-MDHs of maize are not mercaptoethanol convertible, conformational isozymes.

Results of Method 4. Markert (36) and Scandalios (32) reported that high ionic strength buffer accompanied by freezing and thawing, enzymes were dissociated again randomly so that hybrid enzyme molecules were able to form *in vitro*. Similar techniques were used in an attempt to dissociate and reassociate MDH isozyme molecules. As observed in Figure 9 the MDH isozyme patterns remain the same after freezing and thawing in 1M NaCl in phosphate buffer. Relative doses of the isozymes are also maintained after the treatment. These results indicate that after dissociation and reassociation of the MDH subunits, the isozymes retain their original mobility. In addition, it also suggests that, unlike the mitochondrial MDH isozymes in Neurospora (23), the isozyme patterns of both soluble and mitochondrial MDHs in maize are not affected by ionic concentration *in vitro*.

Figure 8.--Starch gel electrophoresis of maize MDH preparations after a 22-hour exposure to 100 mM 2-mercaptoethanol. Experimental procedures are described in detail under "Methods."

(1) Untreated DEAE-I MDHs (m-MDH^4, m-MDH^5).

(2) DEAE-I MDHs treated with 100 mM 2-mercaptoethanol.

(3) Untreated DEAE-II MDHs (m-MDH^1, m-MDH^2, m-MDH^3).

(4) DEAE-II MDHs treated with 100 mM 2-mercaptoethanol.

(5) Untreated DEAE-III MDHs (s-MDH^1, s-MDH^2).

(6) DEAE-III MDHs treated with 100 mM 2-mercaptoethanol.

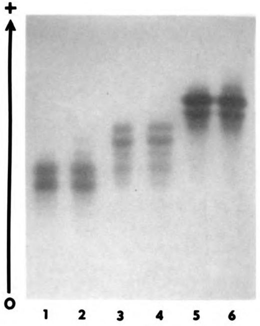

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Figure 9.--Starch gel electrophoresis of maize malate dehydrogenase subjected to freezing and thawing in 0.01 M potassium phosphate buffer (pH 7.0) with or without 1M NaCl. Experimental details are described under "Materials and Methods."

(1) Untreated Sephadex G-150 MDH isozymes.

(2) Sephadex G-150 MDH isozymes treated with 1M NaCl.

(3) Untreated DEAE-I MDHs (m-MDH^4, m-MDH^5).

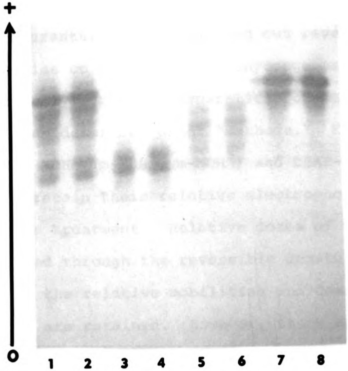
(4) DEAE-I MDHs (m-MDH^4, m-MDH^5) treated with 1M NaCl.

(5) Untreated DEAE-II MDHs (m-MDH^1, m-MDH^2, m-MDH^3).

(6) DEAE-II MDHs (m-MDH^1, m-MDH^2, m-MDH^3) treated with 1M NaCl.

(7) Untreated DEAE-III MDHs (s-MDH^1, s-MDH^2).

(8) DEAE-III (s-MDH^1, s-MDH^2) treated with 1M NaCl.


+
O
1
2
3
4
5
6
7
8

Results of Methods 5 and 6. Kitto et al (5) showed that studies on reversible denaturation provide a useful test of the conformer hypothesis of multiple electrophoretic forms of isozymes. Using both acid and guanidine hydrochloride as denaturants, we have carried out reversible denaturation studies on maize malate dehydrogenase isozymes.

Three partially purified preparations of DEAE-cellulose MDH were treated as described under "Methods." Figure 10 shows that DEAE-I MDHs (m-MDH^4, m-MDH^5) and DEAE-II MDHs (s-MDH^1, s-MDH^2) retain their relative electrophoretic mobility after the treatment. Relative doses of the isozymes are also maintained through the reversible denaturations. For DEAE-II MDHs, the relative mobilities and doses of m-MDH^1, m-MDH^2 and m-MDH^3 are retained. However, there appear to be an increase for the activities observed in the "contaminated" s-MDH^1 and m-MDH^5. Since both DEAE-I MDHs and DEAE-III MDHs are not able to convert into any of the isozymic forms of DEAE-II MDHs, the slightly increased activities observed for s-MDH^1 and m-MDH^5 in the denaturant treated DEAE-II MDH preparation should not be due to a simple "conversion" of one isozyme form to another. Possible explanation will be offered in the Discussion.

From all these tests of the conformer hypothesis for maize MDHs, it is suggested that the maize MDH isozymes (both s-MDHs and m-MDHs) are not conformational isozymes.

Physiochemical properties of maize MDHs. The pH optima of the soluble and mitochondrial MDHs were measured

Figure 10.--Starch gel electrophoresis of maize malate dehydrogenases subjected to reversible denaturation by acid (pH 2) and stained for enzyme activity. Samples were treated with denaturant for 20 hours. Same result was observed for maize MDHs reversibly denaturated with 7.5M guanidine hydrochloride. For details of the experimental procedures, see "Materials and Methods."

(1) Untreated DEAE-I MDHs (m-MDH^4, m-MDH^5)

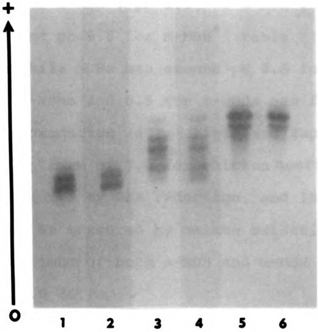
(2) DEAE-I MDHs reversibly denatured in acid.

(3) Untreated DEAE-II MDHs (m-MDH^1, m-MDH^2, m-MDH^3).

(4) DEAE-II MDHs reversibly denaturated in acid.

(5) Untreated DEAE-III MDHs (s-MDH^1, s-MDH^2).

(6) DEAE-III MDHs reversibly denaturated in acid.


+
O
1
2
3
4
5
6

by the assay system described under "Methods." Glycylglycine buffer (25mM) was used between pH 7 and 9, glycine-NaOH buffer (25mM) between pH 8.5 and 10.5. The pH optimum of both s-MDHs and m-MDHs with OAA as the substrate was 8.5. When malate was used as the substrate, the optimum was pH 9.0 for s-MDH^1, pH 9.3 for s-MDH^2, m-MDH^1, m-MDH^2, and m-MDH^3 and pH 9.5 for m-MDH^4 (Table II). The pH optimum of Drosophila MDHs was around pH 8.5 for OAA reduction, 9.0 for s-MDHs and 9.5 for m-MDHs was found as measured for malate oxidation (37). Kitto and Kaplan (30) have reported a pH optimum of 7.8 for chicken heart s-MDH, 7.6 for s-MDH as measured by OAA reduction, and 10.0 for both s-MDH and m-MDH as measured by malate oxidation. In Ilyanassa, the pH optimum of both s-MDH and m-MDH with OAA as a substrate was 7.9 (6).

The isoelectric point of the maize MDHs was determined by electrofocusing followed by starch gel electrophoresis. As seen in Figure 11, the MDH isozymes formed a broad peak by spectrophotometric assays. They were not able to be separated into distinguishable peaks under our experimental procedures. However, the result shows that maize MDH isozymes have isoelectric points ranging from 4.8 to 5.2. The peak fractions (fraction 110 to 150) were then subjected to electrophoresis and the approximate isoelectric point of each MDH isozyme was determined as described in "Materials and Methods." The results are shown in Table II.

Figure 11.--Elution profile of maize malate dehydrogenases from an electrofocusing. Partially purified MDH preparation containing both s-MDHs and m-MDHs was applied to the column. As tested by spectrophotometric assays, the MDH isozymes formed a broad peak and were not separated into different fraction. Ten drops were collected for each fraction. Detailed experimental procedures are described under "Materials and Methods."

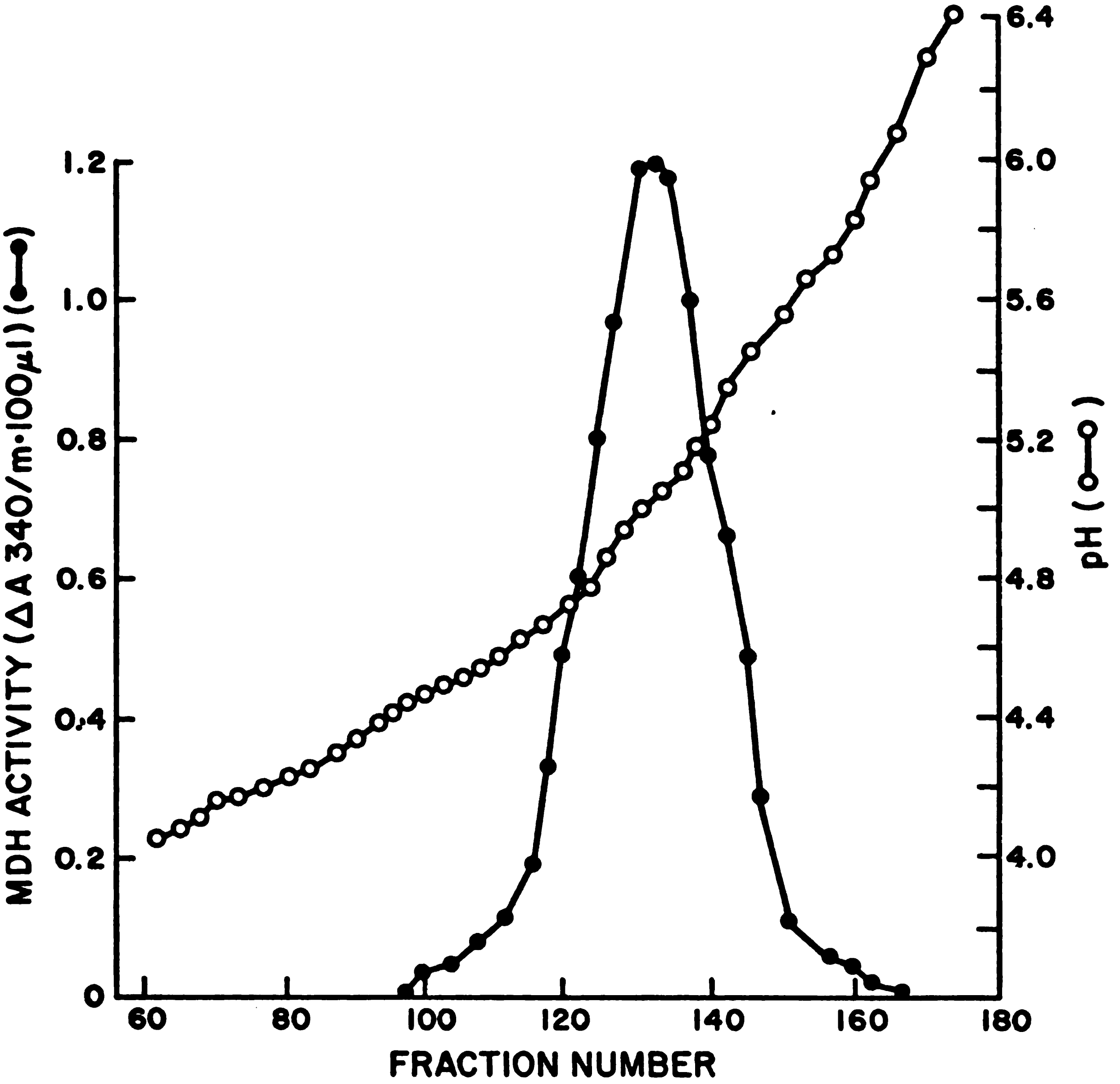

MDH ACTIVITY (ΔA 340/m·100μl) (●—●)
pH (○—○)
FRACTION NUMBER
0
0.2
0,4
0.6
0.8
1.0
1.2
4.0
4.4
4.8
5.2
5.6
6.0
6.4
60
80
100
120
140
160
180

Table II. Physiochemical Characteristics of Maize Soluble and Mitochondrial Malate Dehydrogenase Isozymes

	Isozymes						
Characters	s-MDH^1	s-MDH^2	m-MDH^1	m-MDH^3	m-MDH^3	m-MDH^4	m-MDH^5
Molecular Weight from sucrose density gradient centrifugation	70010		71000			73500	
from gel filtration (Sephadex G-150)	70800		74000			75000	
Isoelectric point (pH)	4.927	4.093	5.045	5.074	5.092	5.126	5.170
pH optima for OAA reduction	8.5	8.5	8.5	8.5	8.5	8.5	8.5
for malate oxidation	9.0	9.3	9.3	9.3	9.3	9.5	9.5

Molecular weight determination.

A Sephadex G-150 column with 40-120 bead size was used as described in "Materials and Methods." Eight non-enzymatic proteins with known molecular weight were used as the standard proteins to calibrate the column. A typical plot of the correlation between log molecular weight and reduced elution volume (Ve/Vo) is shown in Figure 12. The molecular weight of maize MDH isozymes in relation to their reduced elution volume were then estimated by the calibration curve of Figure 12 and are shown in Table II. The molecular weight differences between s-MDHs (m.w. = 70,000) and m-MDHs (m.w. = 74,000-75,000) seem to be significant. Because, when both classes of MDH were eluted simultaneously from the Sephadex column, the m-MDHs had a lower elution volume than did the s-MDHs (inset of Figure 3). Molecular weight determinations of maize MDH isozymes have also been conducted by 5-20% sucrose density gradient centrifugation according to the method of Martin and Ames (34). The elution profile of the soluble MDH isozymes from sucrose density gradient centrifugation is shown in Figure 13. The molecular weights of both soluble and mitochondrial MDH isozymes are shown in Table II. Both methods indicate that both soluble and mitochondrial maize MDH isozymes have fairly close molecular weights between 70,000 to 75,000.

Drosophila MDHs have been reported to have a molecular weight of about 68,000 (29). A molecular weight of 67,000 was determined for chicken heart MDH (30).

Figure 12.--Calibration curve for molecular weight determination on Sephadex G-150 column (2.5x95cm) was equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, 10 mM mercaptoethanol, at a flow rate of 25 ml/hr at 4°C. The column was calibrated with various combinations of non-enzymatic standard proteins. 8 mg protein/2 ml buffer solution was applied for each of the marker protein. 6 mg protein/2 ml was applied for each of the three MDH preparations (Peak I, II and III) separated by DEAE cellulose column. 2 ml was collected for each elution fraction. The void volumn (Vo) used in the calibration is the Ve of blue dextran. A typical plot of the correlation between log molecular weight and reduced elution volume (Ve/Vo) is shown in this figure. Apparently, the s-MDHs have a molecular weight about 70,800, while m-MDHs have 74,000-75,000. For further details, refer to the description in the text and Table II.

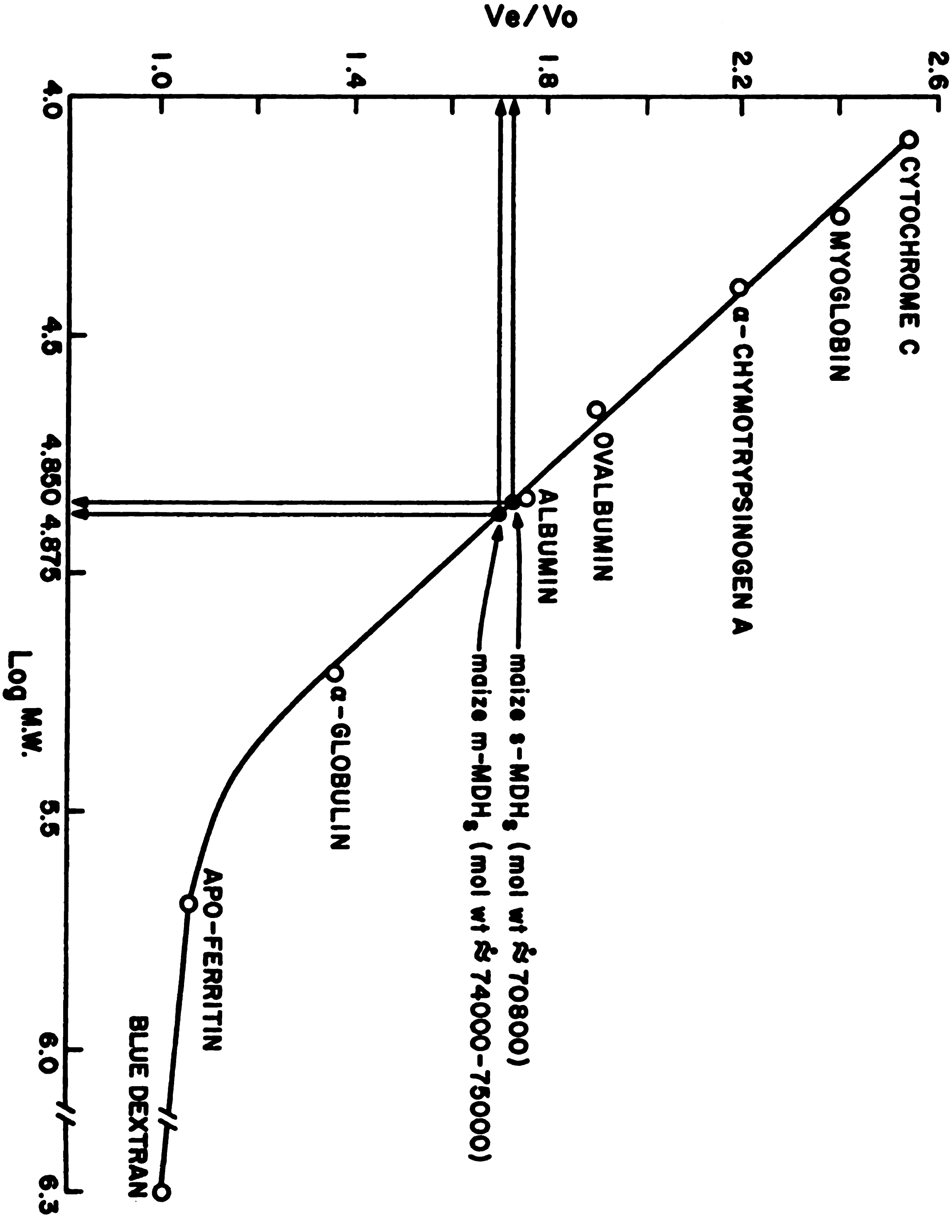
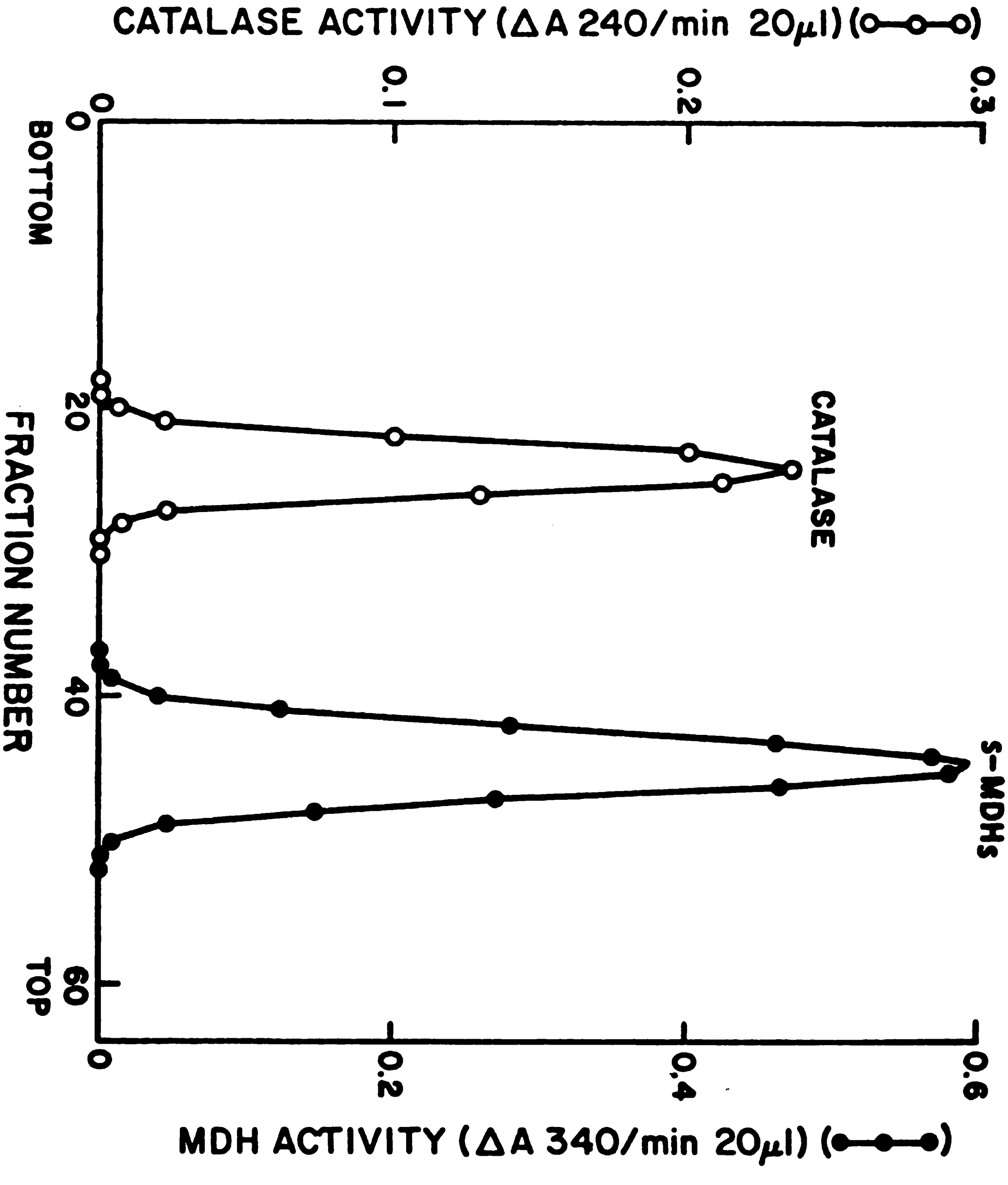

Ve/Vo
2.6
2.2
1.8
1.4
1.0
4.0
4.5
4.850
4.875
5.5
6.0
6.3
Log M.W.
CYTOCHROME C
MYOGLOBIN
α-CHYMOTRYPSINOGEN A
OVALBUMIN
ALBUMIN
α-GLOBULIN
APO-FERRITIN
BLUE DEXTRAN
maize s-MDH$_s$ (mol wt ≈ 70800)
maize m-MDH$_s$ (mol wt ≈ 74000-75000)

Figure 13.--The elution profiles of s-MDHs activities from sucrose density gradient centrifugation. Partially purified enzyme preparation (s-MDHs, peak III, eluted from DEAE cellulose column chromatography), 0.3 ml, containing 0.59 mg protein was layered over a 5 to 20% sucrose density gradient. Centrifugation was performed as described in "Materials and Methods." 15-drop fractions, total of 60 fractions were collected. Beef liver catalase (Mol. Wt. = 250,000) was used as the marker for estimation of the relative molecular weight for MDHs. In this specific run, a molecular weight of 70,600 was observed for s-MDHs. Determination of molecular weights of m-MDHs by sucrose density gradient centrifugation was performed the same way as the one shown here and the results are shown in Table II. For further details, refer to the description in the text.


CATALASE ACTIVITY (ΔA 240/min 20μl) (o—o—o)
0
0.1
0.2
0.3
CATALASE
S-MDHS
0
BOTTOM
20
FRACTION NUMBER
40
60
TOP
0
0.2
0.4
0.6
MDH ACTIVITY (ΔA 340/min 20μl) (●—●—●)

Catalytic properties.

Heat inactivation. The results of thermal inactivation are shown in Figure 14. Generally speaking, the s-MDHs are more thermolabile than the m-MDHs. This was found to be true also in Drosophila (27) and Opuntia (20), but the opposite was observed for chicken heart MDHs (38).

Coenzyme analogs. The maize s-MDHs and m-MDHs can be distinguished easily on the basis of their ability to use analogs of NAD (Table III). On the other hand, no significant differences were observed between the different isozymes within the same subcellular location. All the isozymes appear to be less active in the presence of the deamino analog of NAD (deam-NAD) than in the presence of NAD, but a two-fold difference with deam-NAD as a cofactor was observed between the s-MDHs and m-MDHs. The 3-acetyl pryidine analog of NAD (3-AP-NAD) serves as well as NAD for the s-MDHs but appears to be a better substrate for all m-MDHs than the natural coenzyme NAD. The thionicotineamide analog of NAD (TN-NAD) may serve as a better substrate for the s-MDHs than NAD, however, it is less effective than NAD as a substrate for m-MDHs. Our results are different from those found in Drosophila (37) and Opuntia (20).

Kinetic constants. Michaelis constants (Km) were determined for the seven isozymes for all four substrates (NADH, OAA, NAD, malate) at three pH values (Table IV). In general, the data indicate that the Km values for the coenzymes (NAD and NADH) of the m-MDHs increase with an

Figure 14.--Rate of heat-inactivation for s-MDHs (a) and m-MDHs (b). Small test tubes containing 0.5 ml of gel extract were incubated at 53°C for varying times, cooled to 0°C for two minutes and assayed immediately with "forward standard MDH reaction mixture" as described under "Methods."

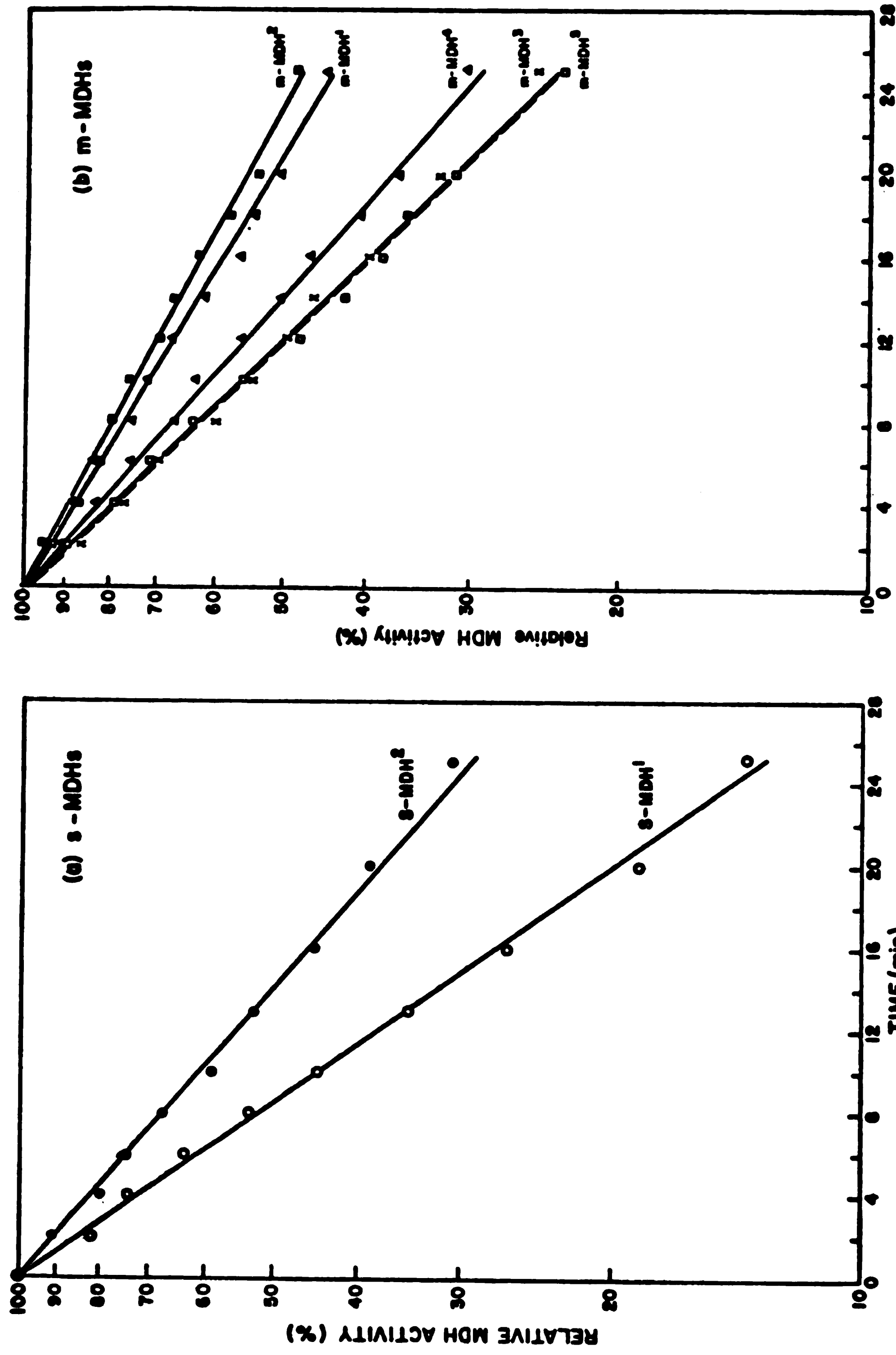

(a) s-MDHs
RELATIVE MDH ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
TIME (min)
0
4
8
12
16
20
24
28
s-MDH²
s-MDH¹
(b) m-MDHs
Relative MDH Activity (%)
Time (min.)
m-MDH²
m-MDH¹
m-MDH⁴
m-MDH³
m-MDH⁵

Table III. Catalytic Activity of Maize Malate Dehydrogenase Isozymes in the Presence of NAD or NAD Analogs

	Isozyme* (Relative activity)						
Coenzymes	s-MDH^1	s-MDH^3	m-MDH^1	m-MDH^2	m-MDH^3	m-MDH^4	m-MDH^5
NAD	100	100	100	100	100	100	100
Deam-NAD	54.6	44.8	20.7	28.5	24.4	26.5	21.1
3-AP-NAD	96.9	100.6	188	200	216.8	192.3	182.8
TN-NAD	241	253	18.3	21.4	21.8	21.1	21.5

*The data are the percentage of the reaction rates relative to NAD. Spectrophotometric assays were conducted with 0.75 mM NAD or analog at 5 mM malate, 0.025 M Glycylglycine buffer pH 8.5.

Table IV. Michaelis Constants (Km) for MAize Malate Dehydrogenase Isozymes at Various pH Values

Coenzyme or Substrate	pH	Km value (mM) for MDH isozymes						
		s-MDH^{1}	s-MDH^{2}	m-MDH^{1}	m-MDH^{2}	m-MDH^{3}	m-MDH^{4}	m-MDH^{5}
NADH	7.5	0.030	0.016	0.067	0.074	0.078	0.088	0.098
	8.5	0.023	0.015	0.080	0.088	0.096	0.086	0.094
	9.5	0.011	0.006	0.098	0.119	0.105	0.165	0.205
OAA	7.5	0.016	0.012	0.024	0.017	0.034	0.025	0.027
	8.5	0.028	0.019	0.037	0.023	0.043	0.032	0.029
	9.5	0.146	0.099	0.075	0.045	0.053	0.052	0.060
NAD	7.5	0.102	0.094	0.056	0.068	0.154	0.168	0.144
	8.5	0.097	0.086	0.100	0.164	0.272	0.316	0.300
	9.5	0.080	0.090	0.270	0.460	0.340	0.420	0.450
Malate	7.5	4.5	5.8	11.7	10.6	6.5	27	27
	8.5	1.5	1.25	1.3	2.0	2.4	2.0	2.2
	9.5	0.95	0.85	1.05	1.3	1.6	1.5	1.65

Assays for MDH under various conditions and estimation of Km values are described under "Methods."

increase in pH from 7.5 to 9.5 Km's for OAA of both s-MDHs and m-MDHs increase with an increase in pH. Km's for NADH of both s-MDHs decrease with an increase in pH, Km's for NAD of the s-MDHs do not change with an increase in pH (Figure 15). These results are quite different from those found in Opuntia (20).

Substrate and coenzyme inhibition. Using L-malate as substrate no inhibition of maize MDHs was observed even at a concentration of 0.1 M. When NADH was used as the coenzyme, no inhibition of maize MDHs could be observed at high concentrations of NADH (5 mM). However, as has been found for other MDHs, maize MDHs are susceptible to inhibition by high concentrations of OAA (Figure 16). This inhibition of maize MDHs by OAA is pH dependent, both s-MDHs and m-MDHs being more susceptible to inhibitions at lower than at higher pHs. The kinetics of OAA inhibition for s-MDHs and s-MDHs are clearly different, while no significant difference could be observed for isozymes of the same subcellular location.

Inhibitions of MDHs by OAA have been widely reported, but inhibitions of MDHs by NAD have not, at least to my knowledge. I found that both s-MDHs and m-MDHs in maize were inhibited by their coenzyme, NAD (Figure 17). Inhibitions of maize MDHs by NAD also pH dependent with greater inhibitions at higher pH values.

Effects of reducing agents, chelating agent, metal ions and some naturally occurring metabolites on the MDH activity.

Figure 15.--Michaelis constants of maise MDH isozymes as a function of pH. Some of the data shown in Table IV are plotted in this figure.

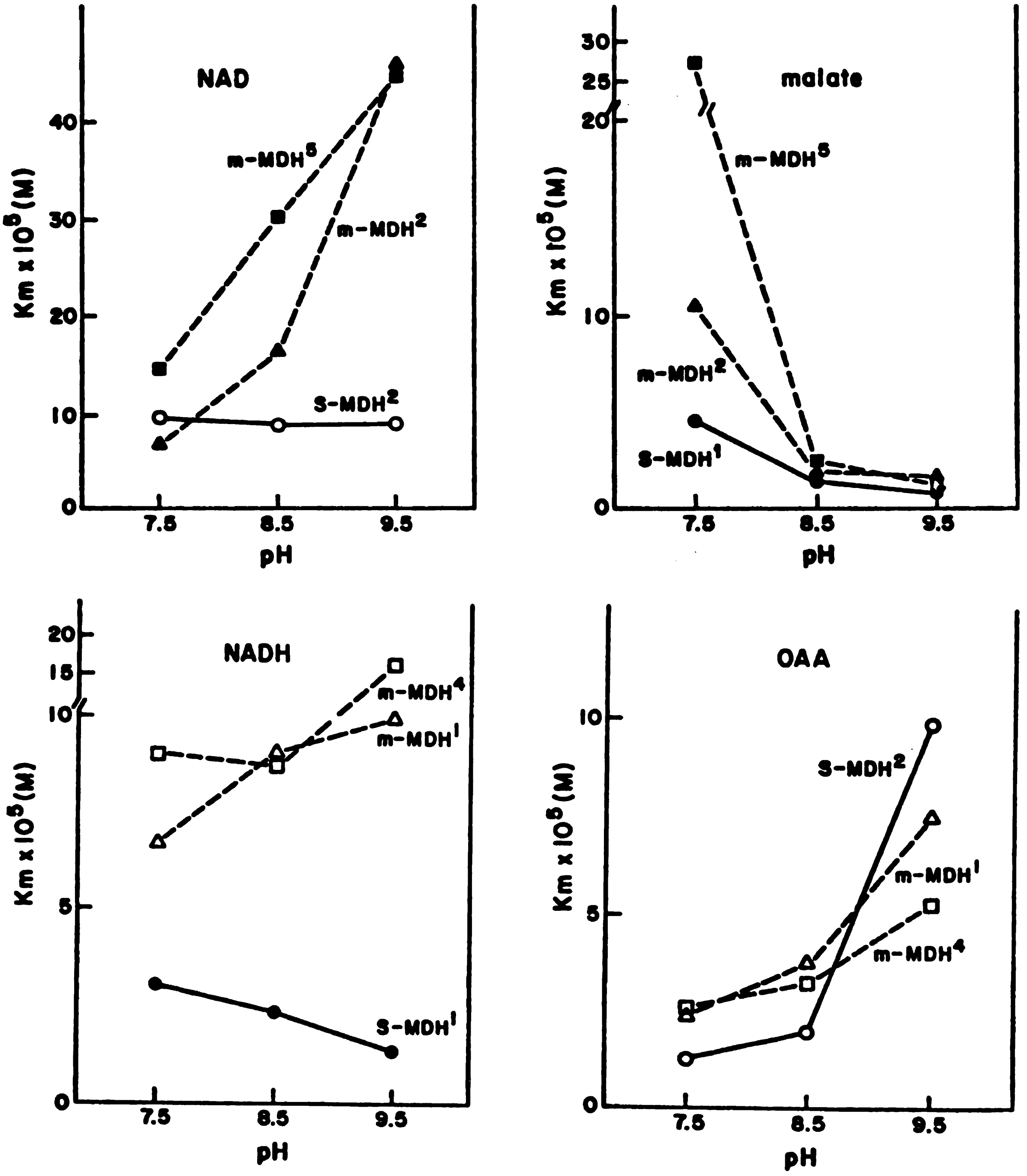

NAD
m-MDH5
m-MDH2
S-MDH2
Km x 10⁵ (M)
0
10
20
30
40
7.5
8.5
9.5
pH
malate
m-MDH5
m-MDH2
S-MDH1
Km x 10⁵ (M)
0
10
20
25
30
7.5
8.5
9.5
pH
NADH
m-MDH4
m-MDH1
S-MDH1
Km x 10⁵ (M)
0
5
10
15
20
7.5
8.5
9.5
pH
OAA
S-MDH2
m-MDH1
m-MDH4
Km x 10⁵ (M)
0
5
10
7.5
8.5
9.5
pH

Figure 16.--Substrate inhibition of the soluble malate dehydrogenases (s-MDHs) and mitochondrial malate dehydrogenases (m-MDHs) as a function of pH (25 mM sodium glycylglycine buffer, pH 7.5 to pH 9.5).

(a) pH 7.5 (b) pH 8.5 (c) pH 9.5

——●—— s-MDH^1 ——○—— s-MDH^2 ---△--- m-MDH^1
---▲--- m-MDH^2 ---□--- m-MDH^3 ---x--- m-MDH^4
---■--- m-MDH^5

Assays were conducted spectrophotometrically at 340 mμ with OAA adjusted with NaOH to the proper pH, 50 μM NADH, and buffer to give a total volume of 3 ml.

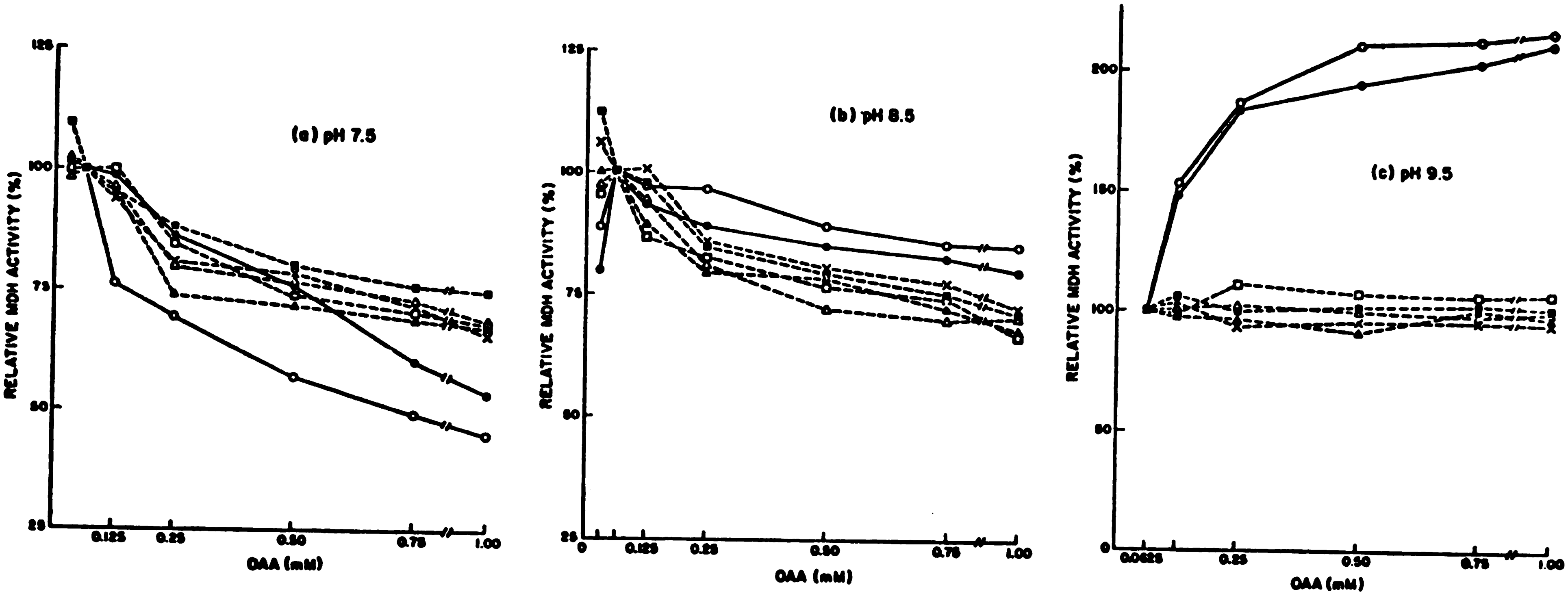
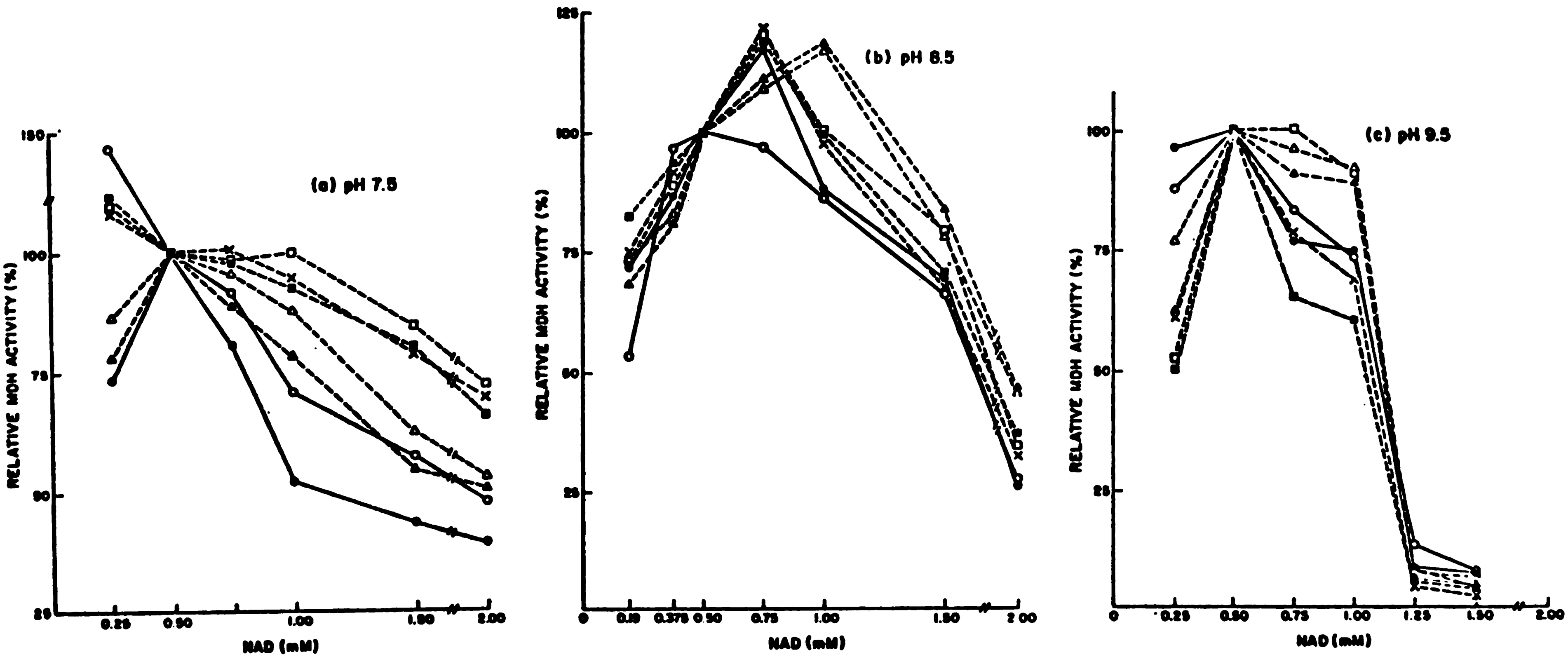

(a) pH 7.5
(b) pH 8.5
(c) pH 9.5
RELATIVE MDH ACTIVITY (%)
OAA (mM)
125
100
75
50
25
0
200
150
0.0625
0.125
0.25
0.50
0.75
1.00

Figure 17.--Coenzyme inhibition (NAD) of soluble malate dehydrogenases (s-MDHs) and mitochondrial dehydrogenases (m-MDHs) as a function of pH (25 mM sodium glycylglycine buffer, pH 7.5 to pH 9.5).

(a) pH 7.5	(b) pH 8.5	(c) pH 9.5
___●___ s-MDH^1	___○___ s-MDH^2	---△--- m-MDH^1
---▲--- m-MDH^2	---□--- m-MDH^3	---x--- m-MDH^4
---■--- m-MDH^5		

Assays were conducted spectrophotometrically at 340 mµ with malate adjusted with NADH to the proper pH and buffer to a total volume of 3 ml. 5 mM malate was used at pH 8.5 and 9.5, however, due to the increased Km for malate at pH 7.5 (see Table IV), 20 mM malate was found to give comparable activities and was used in pH 7.5 assays.


(a) pH 7.5
RELATIVE MDH ACTIVITY (%)
150
100
75
50
25
0.25
0.50
1.00
1.50
2.00
NAD (mM)
(b) pH 8.5
RELATIVE MDH ACTIVITY (%)
125
100
75
50
25
0
0.19
0.375
0.50
0.75
1.00
1.50
2.00
NAD (mM)
(c) pH 9.5
RELATIVE MDH ACTIVITY (%)
100
75
50
25
0
0.25
0.50
0.75
1.00
1.25
1.50
2.00
NAD (mM)

As seen in Table V, catalytic activities of both s-MDHs and m-MDHs are not, or only very slightly affected by reducing agents, a chelating agent, and monovalent metal ions, all at a concentration of 10 mM. On studies of procine malate dehydrogenases, Humphries et al (39) suggested that two essential sulfylhydryl resideus are located at or near the enzymatic center of m-MDHs. But not such "active center" cysteine was found for s-MDHs (39). In my studies, the lack of an effect of the reducing agents on both s-MDHs and m-MDHs in maize may be resulted for two reasons: 1) a lack of "active center" cysteine in maize MDH isozymes; 2) the "active center" cysteine in maize MDH isozymes are protected during the purification procedures. The divalent Zn^{++} ions (10 mM) inhibited both s-MDHs and m-MDHs. The inhibition of s-MDHs was around 80%, that of m-MDH^4 and m-MDH^5 35%-40% and that of m-MDH^1, m-MDH^2 and m-MDH^3 only 25%. At a concentration of 10 mM, Ca^{++} and Mg^{++} ions did not affect the activities of s-MDHs but enhancements of about 20% were observed for m-MDHs.

The effect of some TCA cycle acids and amino acids on the activity of MDHs was also tested (Table VI). L-glutamic acid, L-aspartic acid and succinic acid had no significant effect on the activities of both s-MDHs and m-MDHs. The m-MDHs were inhibited by citric acid and DL-isocitric acid, while the s-MDHs were not. Cis-aconitic acid and -ketoglutaric acid inhibit both s-MDHs and m-MDHs. The m-MDHs are more susceptible to inhibition by these two acids than

Table V. Effect of Reducing Agents, Chelating Agent and some Inorganic Ions on the Activity of Maize Malate Dehydrogenase Isozymes

Addition* (10 mM)	Relative activity (%) of MDH isozymes						
	s-MDH^1	s-MDH^2	m-MDH^1	m-MDH^2	m-MDH^3	m-MDH^4	m-MDH^5
None	100	100	100	100	100	100	100
Mercaptoethanol	97.9	106.6	94.2	102.2	98.8	102	104
Dithiothreitol	104.1	105	101.9	95.6	103.1	100	97.3
EDTA	97.4	103.3	103.8	98.9	98.	96.9	100
NaCl	95.8	94.7	109.5	101.1	103.4	102.1	105
KCl	97.9	104	107.7	104.4	96.2	104.5	101.3
$ZnCl_2$	20.4	19.1	80.0	75.9	74.5	61.8	66.6
$CaCl_2$	104.0	104.8	124.4	119.6	127.7	119.2	122.2
$MgCl_2$	108.8	101.9	123.2	119.8	123.4	121.1	127.7

*All reagents were adjusted to pH 8.5 and were added, at zero time, along with MDH assay reaction mixture to give a final concentration of 10 mM. Assays were conducted with 25 mM Glycylglycine buffer (pH 8.5), 50 μM NADH and 125 μM OAA.

Table VI. Effects of Various Organic and Amino Acids on the Activity of Maize Malate Dehydrogenase Isozymes

	Relative activity (%) of MDH isozymes						
*Addition	s-MDH^1	s-MDH^2	m-MDH^1	m-MDH^2	m-MDH^3	m-MDH^4	m-MDH^5
None	100	100	100	100	100	100	100
Succinic acid	100	101.5	106	97.6	97.2	102	100
L-glutamic acid	100	100	107	112.1	105.5	105.9	104.7
L-aspartic acid	102.5	103.8	100	104.8	91.6	108.8	96.5
Citric acid	97.5	88	68.9	69.5	69.4	74.5	64
DL-Isocitric acid	96	90.8	72.4	71.9	63.9	68.6	65.1
Cis-Aconitic acid	70	69.2	60.1	70.7	54.2	63.7	55.8
α-Ketoglutaric acid	65	69.3	44.8	62.2	47.2	53.9	48.8
**Cis-Aconitic acid + α-Ketoglutaric acid	48.4	50	34.7	40.9	30.5	31.9	26
***(a) Cis-Aconitic acid + α-Ketoglutaric acid	45.5	47.8	27	43.9	25.6	34.3	27.2
***(b)Cis-Aconitic acid + α-Ketoglutaric acid	35	36.4	4.9	32.9	1.4	17.6	4.6

*All acids were adjusted to pH 8.5 with NaOH, and were added at zero time, with a final concentration of 5 mM, along with the MDH reaction mixture. The reaction mixture contains 5 mM malate, 0.75 mM NAD and 25 mM glycylglycine buffer, pH 8.5, in a total volume of 3 ml.

**Observed experimental values for the combined inhibitions.

***Predicted values for the combined inhibitions:
(a) cumulative inhibition
(b) additive inhibition

Definitions of additive and cululative inhibitions are given in the text.

the s-MDHs; no preferential inhibitions were observed between the MDH isozymes from the same subcellular location. Because cis-aconitic acid and -ketoglutaric acid inhibited the MDH isozymes more than other metabolites tested, the combined inhibition of these two metabolites was studied. Two types of inhibition, namely, cumulative inhibition and additive inhibition have been found for combined inhibitions. For cumulative inhibition, the total residual enzyme activity in the presence of several inhibitors is equal to the product of the fractional activities observed when each of the inhibitors is tested along (40). In additive inhibition, the combined inhibition is the sum of individual inhibitions produced by the two inhibitors. The data shown in Table VI indicate that the combined inhibition of cis-aconitic acid and α-ketoglutaric acid appeared to be cumulative rather than additive. This result suggests that these two metabolites bind to the maize MDHs at sites distinct from each other.

Discussion

The genetic basis of soluble and mitochondrial MDH isozymes in maize.

Longo and Scandalios have reported that the mitochondrial MDH isozymes of maize are inherited according to Mendelian rules and thus are under control by nuclear genes (3). The MDH isozyme patterns in these highly inbred maize lines (Figure 2) were found to be constant through several generations of selfing (3). Detailed analysis of the genetic control of maize MDH isozymes are described in Part I. All of these results suggested that multiple genes are involved in the expression of maize MDHs.

An alternative way to account for the polymorphism of the MDH isozymes in maize would be that they are "conformers." For maize MDH isozymes, this explanation is however quite unlikely, for the following reasons:

1) The genetic results indicate that the MDH isozyme patterns are strain specific and constant in any given inbred line. The MDH isozyme patterns are inherited according to Mendelian rules (3). As described in Part I the multiplicity of maize MDH isozymes are genetically controlled. The soluble and mitochondrial MDH isozymes appear to be controlled by separate loci. The mitochondrial MDHs are coded by multiple loci located on two different chromosomes.

2) After 2nd run electrophoresis and after treatments of isozyme preparations with mercaptoethanol (100 mM), freezing and thawing in 1 N NaCl, acid buffer (pH 2 HCl solution) and guanidine hydrocholoride (7.5 M), the various maize MDH isozymes retain their relative electrophoretic mobilities. Conversion of one isozymic form to another was not observed.

Based on the above two reasons, I suggest that the maize MDH isozymes are not "conformers," instead, they are genetically determined.

As seen in the control sample of DEAE-II MDH preparation (Figure 10), the three more anodal m-MDHs are contaminated by m-MDH^4, m-MDH^5 and trace of s-MDH^1. After acid denaturant treatment the relative mobilities of m-MDH^1, m-MDH^2 and m-MDH^3 were retained. However, the "contaminated" s-MDH^1 and m-MDH^5 appear to have higher activities in the denaturant treated samples than in the control (Figure 10). But when DEAE-I MDHs and DEAE-III MDHs were mixed and then subjected to acid denaturation and renaturation, same results were observed in the samples treated with or without denaturant (zymogram not shown). Both DEAE-I and DEAE-III MDHs retain their relative mobilities, conversion of these isozymes to the isozymic forms of DEAE-II MDHs (m-MDH^1, m-MDH^2 and m-MDH^3) have not been observed. This result suggests that the slightly increased activities of s-MDH^1 and m-MDH^5 found in the acid denaturant treated DEAE-II MCH preparation are not due to a simple "conversion" of one esozymic form to another. Some other reasons may be involved in the observed result.

Genetic analysis in Part I suggests that m-MDH^4 is very likely to be a hybrid molecule of m-MDH^3 and m-MDH^5. It is therefore possible that during denaturation, the m-MDH^4 molecules have been dissociated into subunits of m-MDH^3 and m-MDH^5. Upon renaturation some of the m-MDH^5 subunits associate to form homodimers and therefore more activity in m-MDH^5 was observed. The lack of a concomittant increase in the activity observed in m-MDH^3 may be due to a lower stability of m-MDH^3 homodimers in the denaturation and renaturation processes. On the other hand, the slightly increased s-MDH^1 in the denaturant treated DEAE-II MDH preparation (Figure 10) does not seem to be derived from conversion of any of the m-MDHs. A higher stability of s-MDH^1 in the denaturation and renaturation procedures would account for the slightly increased activity of s-MDH^1 observed in the denaturant treated DEAE-II MDH preparation. The above hypothesis has not been studied in the present investigation, but it seems to be a likely explanation to account for the observed zymogram patterns shown in Figure 10.

Horecker and his coworkers (42) were able to convert liver fructose 1,6-diphosphate from "neutral" to "alkaline" forms and thus change the catalytic and allosteric properties of the enzyme. This was carried out by removal of the NH_2 - terminal region of the enzyme by digestion with subtilisin, or changing the conformation in this region of the protein by exposure to low concentrations of urea (42). Similarly, post-transcriptional and post-secretory modifications of salivary amylase (Amy) isozymes in human has been suggested by Karn et al (43). Their results indicate that glycosidation, deamination and deglycosidation of a single gene produce may be the mechanisms which cause the multiplicity of amylase. The two possible mechanisms mentioned above have not been ruled out yet in the maize MDH system. However, if these mechanisms are indeed involved in the expression of the polymorphism of maize MDH, these mechanisms themselves must be genetically controlled, such that the maize MDH isozyme patterns may still be strain specific and follow the inheritance patterns as described in Part I.

As shown in the results, the s-MDHs and m-MDHs are apparently different in most of the biochemical properties tested. These results further support the genetic result that the s-MDHs and m-MDHs are controlled by two different groups of structural genes. The two s-MDHs were also found to be significantly different in their thermolability and their kinetics of substrate (OAA) and coenzyme (NAD) inhibition. In comparing the biochemical properties of the m-MDH isozymes, we found that the maize m-MDHs could be classified into two groups. The m-MDH^1 and m-MDH^2 belong to one group, while m-MDH^4 and m-MDH^5 belong to another. The m-MDH^3 has some properties similar to m-MDH^4 and m-MDH^5, and others similar to m-MDH^1 and m-MDH^2. The two groups of m-MDHs are significantly different in their thermolability, kinetics of NAD inhibition, Kms for the substrates and Km dependency on pH. Recently, Curry et al (44) also purified one soluble and two mitochondrial MDH isozymes from maize seed. Antibodies prepared against one of the mitochondrial forms cross-reacts with the other form from the mitochondria. The antibodies against the mitochondria forms show no cross-reactivity with the soluble form (44). Their results indicate that the two mitochondrial forms are similar in immunological properties but different from those of the soluble form. Their immunological studies are coincident with our biochemical studies in that the maize m-MDHs have similar properties which are quite distinct from those of s-MDHs.

According to the different biochemical properties of the maize MDH isozymes and the genetic analysis of these isozymes (described in Part I), we suggest that four groups of loci, two for s-MDHs and two for m-MDHs, are involved in the genetic determination of maize malate dehydrogenase isozymes.

Possible metabolic roles of soluble and mitochondrial MDHs in maize.

Delbruck et al (45) and Kaplan (46) suggested a hydrogen shuttle between mitochondria and soluble cytoplasm. It is now accepted that a malate shuttle transfers reducing equivalents (NADH) across the mitochondrial membranes (1, 47). In addition, gluconeogenesis from TCA Cycle intermediate is mediated by the cooperation of mitochondrial and soluble malate dehydrogenases. The TCA Cycle intermediates may undergo oxidation to malate via m-MDHs, malate may then leave the mitochondria and undergo oxidation to oxaloacetate by s-MDHs in the extra mitochondrial cytoplasm, where phosphoenol pyruvate is formed and gluconeogenesis may then take place (48). Thus, s-MDHs and m-MDHs may be envisaged to carry out their physiological functions in a cooperative manner.

In maize, the soluble MDH isozymes seem to function to a large extent in the nonautotrophic CO_2 fixation pathway and Crassulacean acid metabolism (21, 22).

Among TCA Cycle enzymes, isocitrate dehydrogenase is known to be inhibited by NADH and stimulated by NAD (49). We show in this paper that, in addition to substrate (OAA) inhibition, both the soluble and mitochondrial MDH isozymes of maize are inhibited by high concentrations (1-2 mM) of NAD. A recent report by Dounce and Bonner (50) indicates that in plant mitochondria, Krebs Cycle oxidations might be controlled by oxaloacetate. Their results showed that inhibitions of the Krebs Cycle oxidations were caused by oxidation of a common pool of NADH, reduced by dehydrogenases, during the conversion of added oxaloacetate to malate.

From the above observations, we suggest that substrate and coenzyme inhibitions of maize malate dehydrogenase may play a role in the regulation of Krebs Cycle oxidations in maize tissues.

In addition to substrate (OAA) and coenzyme (NAD) inhibition, activation of MDH by substrate (malate) and inhibition of MDH by adenine nucleotides were reported recently. Telegdi et al (51) showed that, at concentrations between $3x10^{-2}$ and $2x10^{-1}$ M, porcine mitochondrial MDH was activated by its substrate, malate. The mechanism of activation apparently involves malate binding at other than the catalytic site, with the induction of a 10-fold decrease in Km for OPN (51). Oza and Shore (52) observed that ATP, ADP and AMP were able to inhibit pig heart mitochondrial MDH by competing for the coenzyme (NADH) binding sites on the enzyme. No such studies have been reported in plant materials yet. However, these findings and the results of my present studies suggest that both soluble and mitochondrial MDH isozymes may be regulated in a complicated manner by their substrates, coenzymes and some naturally occurring coenzyme analogs.

It is also found that some TCA Cycle acids and amino acids were able to affect the activities of maize MDH isozymes. This result suggests that in addition to pH, metal ions, substrate and coenzyme concentrations, some metabolites may also be able to influence the activities of maize MDH in vivo.

Summary

Malate dehydrogenase (MDH) of maize exists in multiple molecular forms (isozymes). In strain W64A, two soluble forms (s-MDH), five mitochondrial forms (m-MDH), and two glyoxysomal form (g-MDH) were found in etiolated seedlings. The s-MDHs and m-MDHs were prepared in highly purified form. Using these purified isozymes, experiments with reducing agents (100 mM mercaptoethanol), low pH (2.0) and high salt conc. (1M NaCl or 7.5 M guanidine-HCl), along with genetic data, have eliminated the possibility of conformational

alterations as an explanation for MDH multiplicity in maize; the MDH isozymes are genetically determined. Biochemical properties for each of the seven MDH isozymes were examined. Molecular weight, pI, pH optimum, thermolability, and Km for OAA, malate, NAD and NADH at different pHs were determined for each isozyme. Different kinetics of substrate inhibition (OAA) and coenzyme inhibition (NAD) were observed for the different isozymes. Effects of NAD analogs, chelating agents, reducing agents, metal ions, and TCA cycle acids on the enzymatic activity of these isozymes were tested. Based on the physical and kinetic properties observed, the maize MDH isozymes can be classified into four groups: s-MDH^1; s-MDH^2; the two most anodal m-MDHs; and the three most cathodal m-MDHs. Since strain W64A is highly inbred, our data along with the genetic analysis (shown in Part I) suggest that multiple genes are involved in the expression of maize MDH isozymes.

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PART III

DEVELOPMENTAL STUDIES OF MAIZE MDH ISOZYMES IN THE SCUTELLA OF YOUNG MAIZE SEEDLINGS

Introduction

Refined electrophoretic procedures, coupled with the zymogram technique, have afforded the developmental geneticist a powerful tool for studying developmental processes at the molecular level. The differentiation of protein (enzyme) patterns underlines onto-genetic changes and differences of tissues, hence, the enzyme complement of a tissue may be indicative of its stage of development and physiological function.

Malate dehydrogenases (MDH:E.C. 1.1.1.37) have several important physiological functions within the cell (1, 2, 3, 4). Multiple molecular forms of malate dehydrogenase have been shown to exist in various animal and plant tissues. Developmental studies of multiple forms of malate dehydrogenase have been reported in sea urchin (5), in rabbit liver (6), in bacteria (7), in amphibia (8), in pig heart (9), in frog embryos (10), and in mouse kidney (11). Developmental stage specific and tissue specific MDH isozymes have been observed in some of these studies.

In Part I and Part II, the genetic and biochemical studies have shown that the maize MDH isozymes are genetically determined and are not merely conformers of one basic form of the enzyme. The occurrence of soluble, mitochondrial and glyoxysomal malate dehydrogenase isozymes in maize (12) offers a good system for studying developmental processes within the cell. Not only does the expression of these isozymes represent a mechanism by which cells may regulate a group of proteins (enzymes) with similar characteristics during development, but the subcellular distribution of MDH isozymes may serve as a model to study, both genetically and biochemically, the cooperation between organelles.

The developmental control of the s-MDHs and the m-MDHs in maize has been studies using the highly inbred strains W64A and 59. Very similar developmental patterns of both soluble and mitochondrial isozymes have been observed. Density labeling experiments indicate that both s-MDHs and m-MDHs in the scutella of developing maize seedlings are syntheszed _de novo_. Differential effects of chloramphenicol and cycloheximide on the synthesis of proteins extracted from various subcellular fractions of scutella were observed. Chloramphenicol has no inhibitory effect on the expression of either s-MDH or m-MDH activities in scutella. However, both soluble and mitochondrial forms are strongly inhibited by cycloheximide. These results are consistent with the findings that maize mitochondrial MDHs are controlled by nuclear genes (12) and suggest that the m-MDHs are synthesized in the cytoplasm and then become associated with the mitochondria.

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 minutes, washed twice with sterilized deionized water and were then soaked for 5 to 8 hours. After soaking, the seeds were germinated between moistened germination papers either in plastic trays (40 x 30 x 8.5 cm) or in petri dishes (10 cm in diameter, 7.5 cm in height) in the dark at 25°C. Ages of the seedlings were 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 MDH isozymes in maize scutella:

Isolation of soluble, mitochondrial and glyoxysomal fractions from maize scutella was performed by modifying the method described by Longo and Longo (13). Thirty maize scutella (2.5 gm) from etiolated 4-day old seedlings, inbred line W64A, were minced with a razor blade and gently homogenized with a mortar and pestle with 5 ml of grinding medium containing 0.4 M sucrose (Sigma, Grade I), 0.05 M Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer pH 7.5, 0.1% BSA, 0.01 M KCl, 1×10^{-3} M EDTA, 1×10^{-4} M $MgCl_2$, 1×10^{-2} M dithiothreotol (14). The homogenate was then diluted with another 35 ml grinding medium and filtered through four layers of cheesecloth, and centrifuged in a Sorvall SS-34 rotor, for 10 minutes at 480 xg. The supernatant was recentrifuged at 12,000 xg for 10 minutes. On completion, the 12,000 xg supernatant was centrifuged again at 25,000 xg for 30 minutes and the supernatant obtained after centrifugation was taken as soluble fraction (the 25,000 xg supernatant). The 12,000 xg pellet containing mostly mitochondria and glyoxysomes, was carefully resuspended with 5 ml and 35% sucrose prepared in 10mM Hepes buffer, pH 7.5, 1mM EDTA and 1 mM dithiothreotol. Five milliliters of this suspension containing about 6 mg/ml of protein was layered on a continuous, 35 to 65% (W/V), sucrose gradient with a 5 ml cushion of 65% sucrose and centrifuged in the Beckman SW-25.1 Spinco rotor at 24,000 rpm and 2°C for 4 hours. The sucrose used for the gradient was dissolved in a medium containing 10mM Hepes, pH 7.5, 1mM EDTA and 1mM dithiothreotol.

Under these conditions the mitochondrial band comes to equilibrium half way down the centrifuge tube while the glyoxysomes form a yellow-greenish pellet (13). The mitochondrial band was collected from the top with a syringe. The dense particular pellet was resuspended in 1 ml of 10mM Hepes buffer, pH 7.5, unless otherwise indicated. All steps of the above procedures were performed at 0-4°C.

MDH isozymes in the various fractions were identified by starch gel electrophoresis and specific staining for MDH (15) as described in Part I.

Preparation of crude extract and quantitative assay of total MDH activity:

Scutella of the maize seedlings at different developmental stages were isolated by using a small spatula. Five scutella were homogenized with 0.5 g sand in a chilled mortar with 1 ml 0.0025M glycylglycine buffer, pH 7.4. The homogenate was centrifuged at 25,000 xg for 30 minutes in the refrigerated centrifuge (Sorvall RC-2B; SS-34 rotor). The supernatant, containing more than 95% of total MDH activity found in crude homogenates, was taken as the crude extract. MDH activity and protein in the crude extract were described later.

Isolation and quantitative assay of the individual cytoplasmic or mitochondrial MDH isozymes:

The MDH isozymes were separated by starch gel electrophoresis according to the method of Scandalios (15) as described in Part I. A 150 µl aliquot of the crude extract was subjected to electrophoresis for 14-16 hours at an applied voltage gradient of 6-8 V/cm and 5°C. One horizontal slice was taken from the gel and stained for MDH 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 ¼ of the total gel weight; this measurement allowed us to calculate the MDH 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 x g for 1 hour. The supernatant, containing a single MDH isozyme, was used for quantitative assay of the MDH activity. Approximately 70-75% of the MDH activity applied to the gel could be recovered in this manner.

<u>Density labeling of the newly synthesized proteins in maize scutella</u>:

Inbred line 59 was selected for density labeling experiments since it exhibits only 2 forms of m-MDH. 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 10mM (99 atom %) 15NH_4Cl in 70% D_2O (^{15}N-D_2O) for 8 hours 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-D_2O is about 70% of that in ^{14}N-H_2O, the ^{15}N-D_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.

Fifty 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 minutes. 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 minutes. The pellet was resuspended in 5 ml 0.025 M glycylglycine buffer pH 7.4 and dialysized over night against the same buffer. The partially purified MDH 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 MDH isozyme from the gel were performed as described before. Each individual isozyme eluted from the starch gel was used directly for centrifugation of CsCl.

The procedure for density gradient centrifugation was essentially that of Filner and Varner (16). Each tube contained 1 ml of saturated CsCl with 10 mg/ml NAD, 2 ml of gel eluate and 20 µg of lactate dehydrogenase (E.C. 1.1.1.27: Sigma) as a marker, all uniformly mixed. NAD was found to prevent, very effectively, the loss of MDH activity in the concentrated CsCl solution. The tubes were centrifuged at 40,000 rpm for 72 hours 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 MDH activity from the gradient was about 80%-90%, with no quantitative differences among isozymes.

<u>Preparation and mixing of crude scutellar extracts (480 xg supernatant) at different development stages</u>:

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. Fifty scutella were also isolated from seeds which had been imbibed for only 5 hours 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 xg 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 glyclyglycine buffer or with 0.02 ml 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 two hours. After incubation, the crude extracts were chilled to 4°C and the total MDH activities were measured spectrophotometrically.

Enzyme assays:

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 (17). 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 preparation and following the decrease in absorbance at 340 mu during NADH oxidation. Initial rates were used in calculations of activities. Cytochrome c oxidase was assayed by following the decrease in absorbance at 550 mμ during cytochrome c oxidation (18). Catalase activity was assayed according to Chance and Maehly (19) by following the decrease in absorbance at 240 mμ during H_2O_2 decomposition.

Expression of MDH activity:

The MDH activity in crude extract is represented as umoles of NADH oxidized per minute 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% to 30% of the crude extract activity was always lost during electrophoresis and ensuing extraction procedures, the MDH activities of 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.

Cultivation of excised scutella in nutrient medium and dosage of the antibiotics applied to the medium:

In order to penetrate antibiotics into maize scutella which are enclosed in the endosperm, direct contact of scutella with medium solutions containing antibiotics are desired. Scutella in intact one-day-old seedlings were isolated by removing the attached axis and endosperm, the excised scutella were cultivated in a nutrient medium with or without antibiotics against protein synthesis (CH or CAP). Composition of the liquid nutrient medium was described in the following:

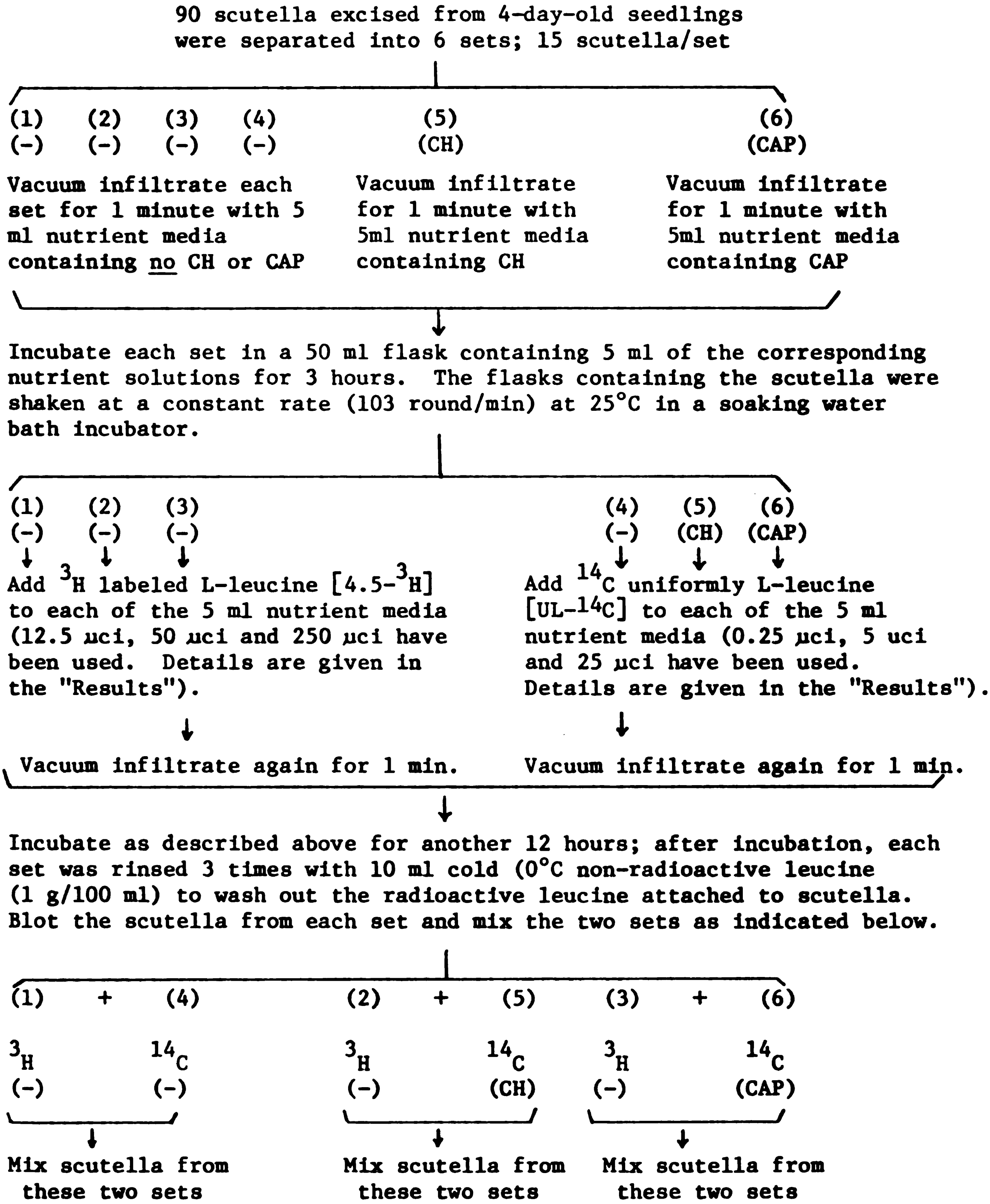
Stock Solutions			Volume or weight to be added to 100 ml	Final concentration in medium
A. Nitrogen source:				
1M KNO_3			1 ml	10 mM
B. Salts:				
Stock I:				
$MnSO_4.H_2O$.	.1 g	in 100 ml dist. water		1.0 µg/l
$CuSO_4.5H_2O$	20 mg each			0.02 µg/l
$ZnSO_4.7H_2O$				0.02 µg/l
KI				0.02 µg/l
H_3BO_3				0.02 µg/l
$(NH_4)_6MO_7O_{24}.4H_2O$				0.02 µg/l
Stock II:				
Stock I	1 ml	in 1000 ml	10 ml	-
KCl	149 mg			14.9 µg/l
KH_2PO_4	408 mg			40.8 µg/l
$CaCl_2$	440 mg			44.0 µg/l
$CaSO_4.2H_2O$	136 mg			13.6 µg/l
$MnSO_4.7H_2O$	492 mg			49.2 µg/l
C. Sucrose: 6.85 g			6.85 g	6.85%

The nutrient medium thus prepared was then autoclayed and stored at room temperature. Right before using penecillin and stryptomycin were added aseptically at concentrations of 1000 units/ml and 0.5 µg/ml nutrient solution respectively. The nutrient solution prepared as described above served as a "control." Cycloheximide (CH) at the final concentrations of 2 or 10 µg/ml and chloramphenicol (CAP) at 0.5 mg or 2 mg/ml were prepared separately in the "control" nutrient solution.

In a sterile box, scutella excised from one-day-old seedlings were surface sterlized with 2% sodium hypochloride (NaOCl) for 1 minute and rinsed thoroughly with sterilized distilled water. Thirty scutella were then vacuum infiltrated in a 50 ml flask containing 20 ml of nutrient solutions with or without protein synthesis inhibitors (CH or CAP). Vacuum infiltration was conducted by suction force of running water. After 2-minutes of vacuum infiltration, scutella from the various treatments were transferred to sterile pertri plates for culture on germination paper saturated with 10 ml of the corresponding nutrient media (control, + CH + CAP). The cultures were maintained in the dark at 25°C. The total MDH activity and the specific activity of each isozyme in the excised scutella were determined at different developmental stages.

Inhibition of protein synthesis in scutella by cycloheximide and chloramphenicol:

The effects of cycloheximide and chloramphenicol on protein synthesis in scutella was studied by measuring their effects on the incorporation of radioactive leucine into the scutellar protein. Preparation of nutrient media with or without antibiotics (CH or CAP), preparation of excised scutella and vacuum infiltration of scutella have been described above. Application of antibiotics, incorporation of ^{3}H-leucine and ^{14}C-leucine into scutella and measurement of TCA insoluble counts in various subcellular fractions of scutellar cells were performed as shown in the next page.



90 scutella excised from 4-day-old seedlings were separated into 6 sets; 15 scutella/set

(1) (-) (2) (-) (3) (-) (4) (-)
Vacuum infiltrate each set for 1 minute with 5 ml nutrient media containing no CH or CAP

(5) (CH)
Vacuum infiltrate for 1 minute with 5ml nutrient media containing CH

(6) (CAP)
Vacuum infiltrate for 1 minute with 5ml nutrient media containing CAP

Incubate each set in a 50 ml flask containing 5 ml of the corresponding nutrient solutions for 3 hours. The flasks containing the scutella were shaken at a constant rate (103 round/min) at 25°C in a soaking water bath incubator.

(1) (-) (2) (-) (3) (-)
Add ^{3}H labeled L-leucine [4.5-^{3}H] to each of the 5 ml nutrient media (12.5 µci, 50 µci and 250 µci have been used. Details are given in the "Results").
Vacuum infiltrate again for 1 min.

(4) (-) (5) (CH) (6) (CAP)
Add ^{14}C uniformly L-leucine [UL-^{14}C] to each of the 5 ml nutrient media (0.25 µci, 5 uci and 25 µci have been used. Details are given in the "Results").
Vacuum infiltrate again for 1 min.

Incubate as described above for another 12 hours; after incubation, each set was rinsed 3 times with 10 ml cold (0°C non-radioactive leucine (1 g/100 ml) to wash out the radioactive leucine attached to scutella. Blot the scutella from each set and mix the two sets as indicated below.

(1) + (4)
^{3}H (-) ^{14}C (-)
Mix scutella from these two sets

(2) + (5)
^{3}H (-) ^{14}C (CH)
Mix scutella from these two sets

(3) + (6)
^{3}H (-) ^{14}C (CAP)
Mix scutella from these two sets

The mixed scutella from the two sets were ground; soluble fractions (25,000 xg supernatant), mitochondrial fractions (in sucrose gradient) and glyoxysomal fractions (pellets in sucrose gradient) were isolated as described above. Equal volumes of the soluble fractions (40 ml) and mitochondrial fractions (10 ml) were collected from the three different preparations. The glyoxysomal pellets were resuspended with 10 ml Hepes buffer, pH 7.5. For determination of incorporation of radioactive leucine into proteins of these fractions, 50 µl of 0.1% bovine serium albumin (BSA) was added to 1 ml aliquots of each fraction and served as a carrier. Ten ml of cold (0°C) trichloroacetic acid (TCA) was added to the 1.05 ml aliquots of each fraction to a final concentration of 10%. The TCA precipitates were stirred thoroughly with a Vortex mixer and stored in the refrigerator overnight. The TCA isoluble materials were collected then on one layer of glass fiber (Whatman GF/C, 2.4 cm, presoaked in 10% TCA) and washed twice with 5 ml of cold 10% TCA. The glass fiber discs were then dried in a 70°C oven for 2 hours and counted in 10 ml of toluene based scintillation fluid. The scintillation fluid was prepared by adding 30.3 g Butyl PBD and 1.89 g PBBO into 3.78 liter toluene and stirring overnight. A refrigerator equipped Packard Tri-carb liquid scintillation spectrometer, model 3390, was used throughout all the experiments. Triplicate samples were counted for each fraction in each independent experiment.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the proteins in various subcellular fractions of maize scutella:

SDS polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (20) as modified by Flint (21). Detailed experimental procedures and modifications made in my experiment are described in the following:

Preparation of stock solution:

A. 26.7 g acrylamide, 0.72 bis per 100 ml Dist. H_2O.

B. 0.75 M Tris-HCl, pH 8.8, 0.2% SDS, 0.1% TEMED.

C. 0.25 M Tris-HCl, pH 6.8, 0.2% SDS, 0.1% TEMED.

D. 0.25 M Tris, 1.92 M glycine, pH 8.3.

E. 0.15 g $(NH_4)_2S_2O_8$ per 10 ml.

F. 0.125 M Tris-HCl, pH 6.8, 4% SDS, 8M urea, 10% mercaptoethanol.

Stock solutions A, B, C, D, were stored at 0-4°C. Stock E was always prepared in fresh. Stock F was stored at room temperature. Final concentration of gels and buffers:

Resolving gel; 0.375 M Tris-HCl pH8.8, 0.1% SDS, TEMED 12.5% acrylamide, 0.32% bis.

Stocking gel; 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% TEMED 3.5% acrylamide, 0.093% bis.

Electrode buffer; 0.025 M Tris, 0.192 M glycine pH 8.3, 0.1% SDS.

Sample buffer; 0.0625 M Tris-HCl pH 6.8, 2% SDS, 4 M urea, 5% mercaptoethanol.

To silate tube:

1) Immerse glass tubes (15 cm x 6 mm i.d.) in chromic acid for 12 hours or overnight.
2) Rinse with distilled H_2O and dry for several hours.
3) Place dry tubes in a closed vessel containing enough of dichlorodimethylsilane to cover the bottom one inch of the tubes.
4) Leave for about 24 hours.
5) Remove and dry in 40°C oven. Wash with hot 2% SDS solution; rinse with distilled water and dry at room temperature.

To make gels:

1) Mix 1 part B stock solution with 0.9 part A, warm solution to room temperatures and degas.
2) Add 0.01 part of E and add solution to glass tubes to a depth of 11 cm.
3) Immediately layer dist. H_2O to a depth of 4 mm. Let stand over night to insure complete polymerization.
4) Remove water and ungeled acrylamide solution.
5) Mix 1 part A with 4 parts C, 2.5 parts dist. H_2O and 0.25 parts E. Add this solution to the top of the polymerized gels to a depth of 2.5 cm and layer with distilled H_2O again.
6) Let the stocking gel to polymerize for 2 hours before using.

To prepare samples:

Subcellular fractions of maize scutella treated with or without protein synthesis inhibitors (CH or CAP) were obtained as described above. Three fractions, the soluble, the mitochondrial and the dense particulate fraction (the glyoxysomes contaminated with mitochondrial inner membranes) were prepared by adding equal volumes of sample solutions and stock solution F. As described above, stock solution F contains 0.125 M Tris-HCl pH 6.8, 4% SDS, 8 M urea and 10% mercaptoethanol.

1. Soluble fraction:
   One ml aliquot of the 25,000 xg supernatant (40 ml) was mixed with 1 ml of stock solution F.
2. Mitochondrial fraction:
   The mitochondrial fraction [10 ml in about 46% (W/V) sucrose-Hepes solution] isolated from sucrose gradient was diluted to 18% isotonic sucrose solution by adding slowly 35 ml of 10% sucrose solution prepared in 10 mM Hepes, 1 mM DTT and 1 mM EDTA. The mitochondrial preparation were then centrifuged at 25,000 xg for 30 minutes. The pellets containing more than 95% protein and radioactivity were resuspended in distilled H_2O to make the final volumes of the resuspension to 0.5 ml. Then 0.5 ml of stock solution F was added to each of the mitochondrial samples.

3. Dense particulate fraction:

   The pellets were resuspended with distilled H_2O to make the final volumes of the resuspension to 0.5 ml. Equal volumes (0.5 ml) of stock solution F was added to each of the sample fractions.

The final buffer concentrations of these sample solutions are 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 4 M urea and 5% mercaptoethanol. Glass tubes containing these sample solutions were sealed with parafilm stored at 30°C incubator for two days before electrophoresis.

To run gels:

1. Eighty ml of stock solution D, 0.8 g recrystalized SDS powder, and 20 drops of 0.1% Bromophenol blue (marker dye) were added to 720 ml dist. H_2O. After stirring for 15 minutes it was added to the cathode chamber. For the anode chamber, 1700 ml of 10 fold diluted stock D was added.
2. Samples with the concentration of 0.4-0.8 mg/100-200 µl were layered onto the stocking gel after the buffers have been added to the chambers.
3. The D. C. power supply (Buchler Instrument Company) was used for electrophoresis. Run samples into stocking gels at low current (about 2 ma/tube) for about 1.5 hours, then increase the current to about 8 ma/tube with a voltage of 90-100 volts. Let the gels run for 6-7 hours at the constant

voltage until run is complete. Completion of the run was indicated by reaching of the cromophenol blue dye to 0.5 cm from the bottom of the gel.

To stain and destain the gels:

1. After the gels have been removed from the tube, they are immersed into the fixing solution containing 7.5% acetic acid, 5% methanol and 10% isopropanol. Leave over night.
2. The gels were then stained overnight in 0.2% coomassic brilliant blue prepared in 7.5% acetic acid and 5% methanol and destained in 7.5% acetic acid and 5% methanol only.

Procedures for counting ^{3}H and ^{14}C in SDS polyacrylamide gels:

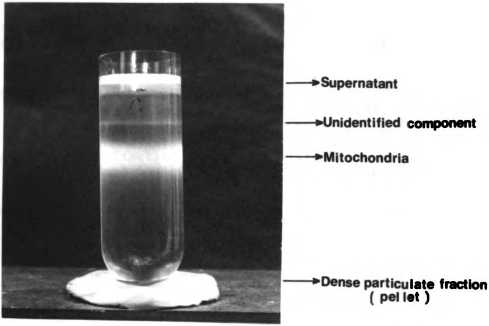
From the cathode end toward the anode end, the destained gels were cut into one hundred 1.0 mm slices. The gels were frozen with dry ice for 5 minutes and were then sliced with gel slicer. The gel slices weighing approximately 40 mg were put into counting vials. A 0.75 ml of 9:1 NCS tissue solubilizer-water solution (22) was added to each counting vial. Cap the vial and heat to 50°C for 5 hours. Cool and add 10 ml scintillator solution. Mix well. The scintillator solution has a final concentration of 6 g PPO and 75 mg POPOP per liter of toluene (22). The samples thus prepared were stored at 4°C in dark for 24 hours before counting.

Results

Subcellular fractionation of the crude particulate fraction of maize scutellum

A typical separation of the components in the crude particulate fraction (12,000 xg pellet) of maize scutellum is shown in Figure 1. The mitochondria banded in the middle of the 35% - 65% sucrose gradient. A dense particulate pallet was found at the bottom of the gradient. An unknown component with less density (1.19 g/c.c) than that of mitochondria (1.22 g/c.c) formed a very thin band around 41% (W/V) sucrose. Table I shows the distribution of three enzymes in the various maize fractions separated by sucrose gradient centrifugation. Catalase activity was recovered both on top of the gradient (the supernatant) and at the bottom of the gradient (the pellet). The glyoxysomes in the dense particulate fraction (13) are the only organelle in maize known to contain catalase (13, 23). By spectrophotometric assays (Table I) and zymogram staining (Figure 2), the mitochondrial fraction was judged to be free of contamination by glyoxysomes due to the absence of catalases. However, the dense particulate fraction appears to contain some other components other than glyoxysomes. Cytochrome oxidase (the marker of mitochondrial inner membranes) was recovered mainly (84%) in the mitochondrial fraction, but about 16% was found in the dense particulate fraction, no activity was recovered on the top of the gradient. Malate dehydrogenase was found

Figure 1.--A typical separation of the crude particulate fraction (12,000 xg pellet) from 4-day-old maize scutellum on a sucrose gradient.



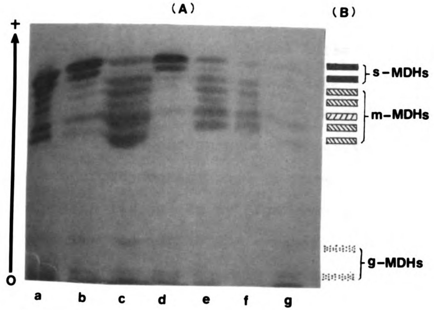
Supernatant
Unidentified component
Mitochondria
Dense particulate fraction
(pel let)

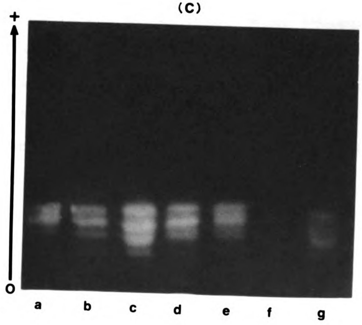
Table I. Distribution of three enzymes in the various maize fractions separated by sucrose gradient centrifugation.

Fractions in sucrose gradient	Percentage (%) activities recovered in the corresponding fractions		
	Catalase	Cytochrome Oxidase	Malate Dehydrogenase
Supernatant fraction (on top of gradient)	70%	0%	36%
Mitochondrial fraction (band in gradient)	0.5	84	62
Dense particulate fraction (pellet in gradient)	29.5	16	2

in all three fractions of the sucrose gradient, zymogram staining in Figure 2 shows that the mitochondrial MDH isozymes are slightly contaminated by the soluble MDH isozymes. However, as judged by staining intensity in the MDH zymogram (not shown), the MDH isozymes in the top of the sucrose gradient (supernatant fraction) appear to be composed of 2/3 of soluble MDHs and 1/3 of mitochondrial MDHs. The MDH activities recovered in the dense particulate fraction contains only about 2% of the total activity found in the gradient. Since the mitochondrial malate dehydrogenase is the well known marker of the matrix proteins of mitochondria, and cytochrome oxidase, the marker of mitochondrial inner membrane, the finding of mitochondriam MDH isozymes in the top of the gradient and the recovery of cytochrome oxidase in the dense particulate fraction indicate that around 15% (Table I) of the mitochondria are broken during the preparation. Most of the soluble matrix proteins (as m-MDHs) in the mitochondria may have been released and are recovered on the top of the gradient. The broken mitochondrial membranes, especially inner membranes having a higher density (24, 25) would therefore pellet down to the bottom of the gradient. Lardy and his coworkers (25) showed that the outer mitochondrial membrane would band in a density of 0.76 M (÷ 42%) sucrose, while the inner membrane of mitochondria would pass 1.32 M (÷ 74%) sucrose and pellet down to the bottom of the centrifuge tube. These results further support the idea that the cytochrome oxidase activity recovered in the dense

Figure 2.--Zymogram of MDH and catalase isozymes in subcellular fractions isolated from 4-day-old scutella of the inbred strain W64A. The fractions were separated differential and sucrose gradient centrifugations. Aliquots of the various fractions were subjected to starch gel electrophoresis. On completion, two horizontal slices were made from the gel, one was stained for MDH, the other for catalase. The subcellular fractions are indicated by (a) = crude extract; (b) = 12,000g supernatant; (c) = 12,000 g pellet; (d) = 25,000 g supernatant; (e) = 12,000-25,000 g pellet; (e) = mitochondria; (g) = glyoxysomes. Migration is anodal. O = point of sample insertion. (A) Zymogram of MDH isozymes. (B) Schematic summary of the MDH zymogram s = soluble fraction; m = mitochondria; g = glyoxysomes. (C) Zymogram of catalase isozymes.


(A)
(B)
+
O
s-MDHs
m-MDHs
g-MDHs
a
b
c
d
e
f
g


(C)
+
O
a
b
c
d
e
f
g

particulate fraction (Table I) came from the inner membranes of broken mitochondria. In addition, the unidentified component banded around 41% sucrose shown in Figure 1 may very likely be the broken mitochondrial outer membranes which was reported to band around 42% sucrose (25).

From the above observations, I conclude that the dense particulate fractions containing glyoxysomes (as judged by catalase) are cross-contaminated by inner membranes of broken mitochondria (as judged by high cytochrome oxidase activity and low mitochondrial MDH activity). Therefore, in this paper, the pellet fraction which contains glyoxysomes and mitochondrial inner membranes is named as "dense particulate fraction." However, the two MDH isozymes which appear only in this pellet fraction, not in mitochondrial fraction, are called glyoxysomal MDHs.

MDH activities in the germinating maize seedlings

In the scutellum of maize, inbred strain W64A, there are nine malate dehydrogenase (MDH) isozymes, namely two soluble forms, five mitochondrial forms and two glyoxysomal forms (Figure 2). The specific activity of MDH is higher in the scutellum than in any other organ examined. Therefore, scutella of strain W64A were used to study the development of MDH isozymes in maize seedlings. As seen in Figure 2, the MDH isozymes in glyoxysomes are the two most cathodal bands. The traces of soluble and mitochondrial MDH isozymes present in the glyoxysomal fraction are likely due to contamination of these isozymes non specifically associated with the broken mitochondrial inner membrane. The MDH 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-MDH^1, g-MDH^2 (the two glyoxysomal isozymes).

The specific activity of malate dehydrogenases in the scutellum is about 2 to 7 fold higher than those in other organs in the etiolated maize seedlings (Figure 3). In scutellum, s-MDH^2 is abundant compared to the same isozyme in other organs (Figure 4). The glyoxysomal MDH isozymes, were found only in scutella. Intermediate bands located between mitochondrial and glyoxysomal isozymes were occasionally observed but found to be nonspecific dehydrogenases. Under the experimental conditions described here, activity of MDH prepared from pericarp of the young seedling was not high enough to show a clear pattern of MDH isozymes (Figure 4). However, pericarp of the immature kernel (18 days after pollination) has a similar MDH pattern as those of shoot and root (Figure 4).

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

Figure 5 shows the stained template from one of the preparative gels used for isolation of single isozymes (see Methods). The two glyoxysomal isozymes can be distinguished.

Figure 3.--Specific activity of MDH in different organs from etiolated maize seedlings (W64A) at the 4th day of germination. (a) scutellum; (b) endosperm; (c) shoot; (d) pericarp; (e) root.

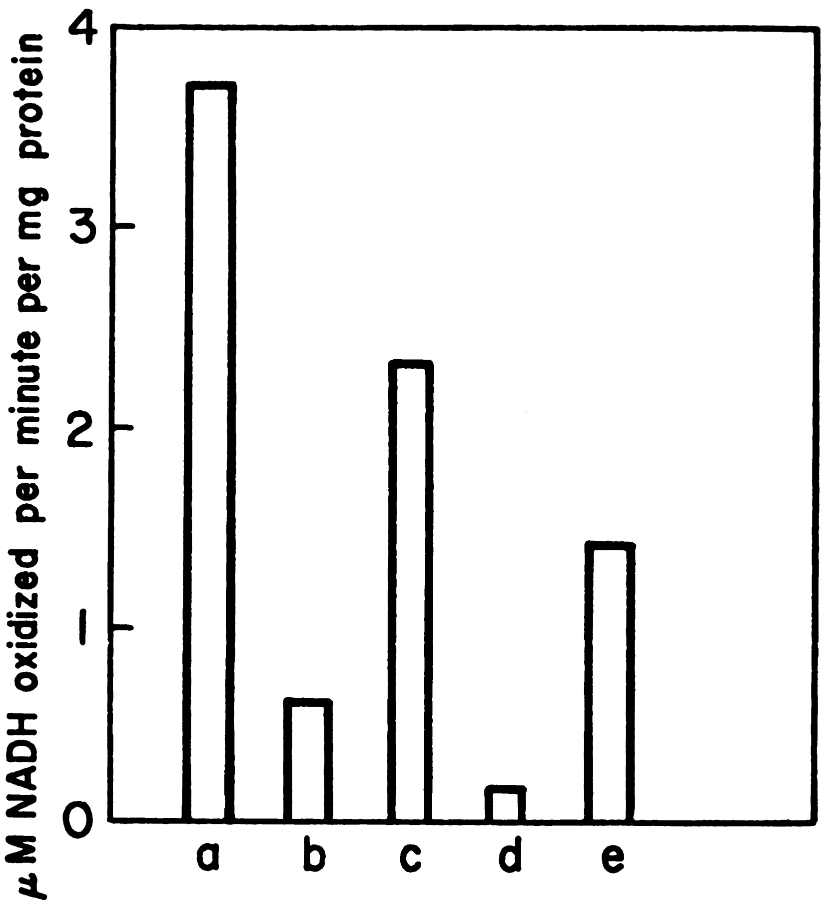

μM NADH oxidized per minute per mg protein
4
3
2
1
0
a
b
c
d
e

Figure 4.--Zymogram showing the MDH isozymes from crude extracts of different organs from etiolated maize seedlings (W64A) at the 4th day of germination. (a) endosperm; (b) shoot; (c) scutellum; (d) root; (e) pericarp; (f) pericarp of the immature kernel (18 days after pollination). The same MDH pattern as that of the shoot was observed. The extremely low MDH activity in pericarp of the seedlings has not allowed us to get a clear pattern in this zymogram. Note presence of glyoxysomal MDH only in the scutellum. Migration is anodal. O = point of sample insertion.

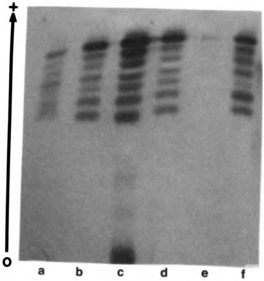
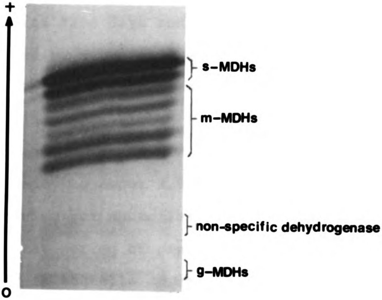

+
o
a
b
c
d
e
f

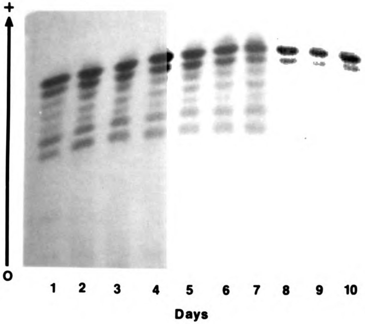
Figure 5.--Zymogram of MDH isozymes from maize scutellum (W64A). For quantitative separation of each individual isozyme, 150 µl of crude extract was subjected to electrophoresis in preparative starch gels as shown here. The ADH is a nonspecific alcohol dehydrogenase. O = point of sample insertion. Migration is anodal.


+
s–MDHs
m–MDHs
non-specific dehydrogenase
g–MDHs
O

Zymograms of scutellar MDH of W64A at various stages of seedling development, showed that the number and positions of the isozymes under study remained constant (Figure 6). In etiolated W64A seedlings, the total MDH activity increases through the first five 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 (Figure 7). Since there is an increase in "glycylglycine buffer extractable" protein per scutellum (from 1.3 mg/scutellum at 0-day to about 2.8 mg/scutellum at 6-day), the total MDH activity per scutellum increases much more dramatically than that per mg of protein. The developmental changes of MDH in scutella appear to be 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 to 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 longate. The high levels of MDH activities observed in the scutella of the 5th-7th 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.

The time course of development of the two soluble MDH isozymes is shown in Figure 8. The s-MDH^1 has much higher

Figure 6.--Zymogram showing the MDH isozymes from crude extracts of scutella (W64A) at different days of germination. O = point of sample insertion. Migration is anodal.



+
O
1
2
3
4
5
6
7
8
9
10
Days

Figure 7.--Time course of total MDH activity in scutella of germinating maize seeds (W64A). ———○———, activity per scutellum; ———●———, activity per mg or protein.

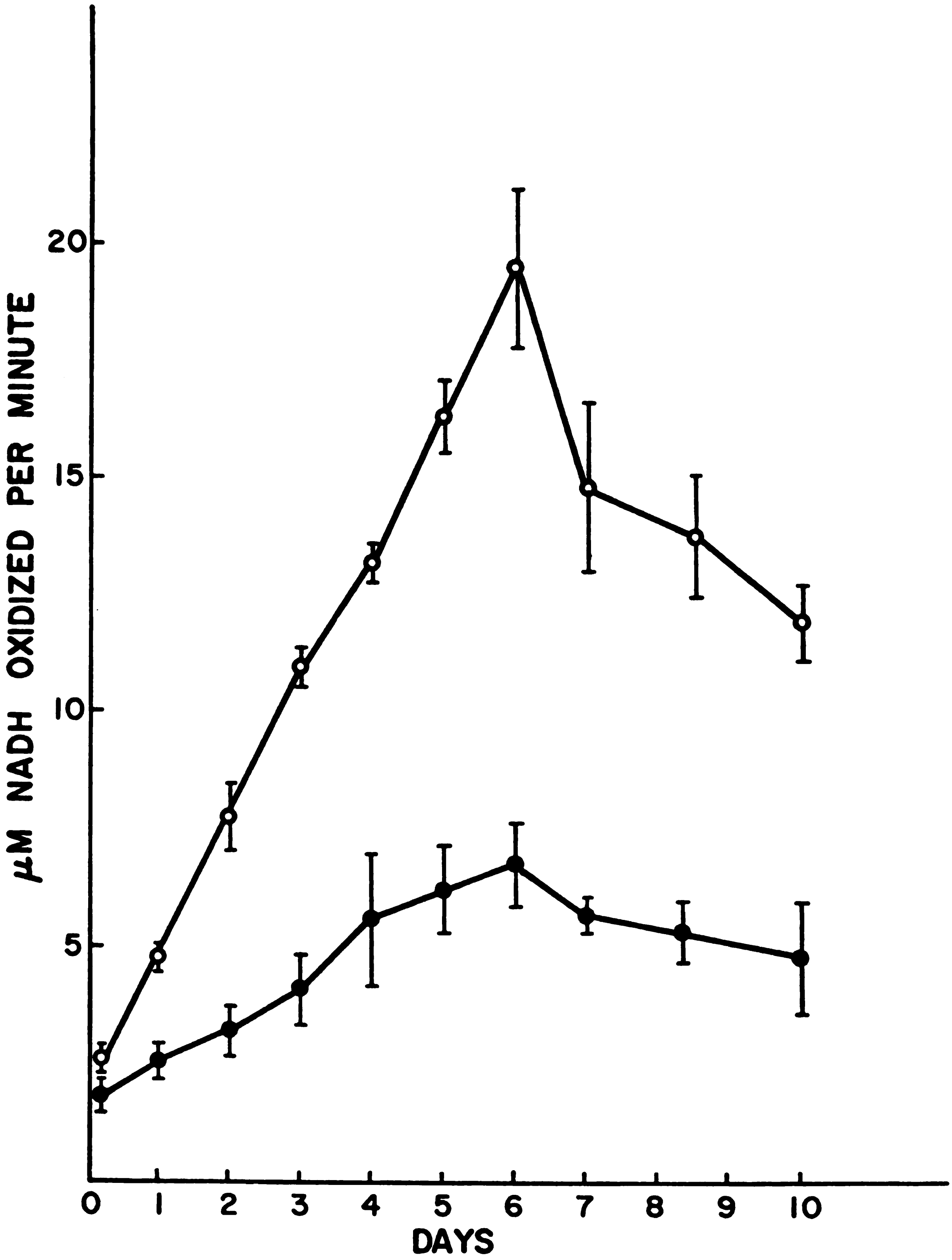
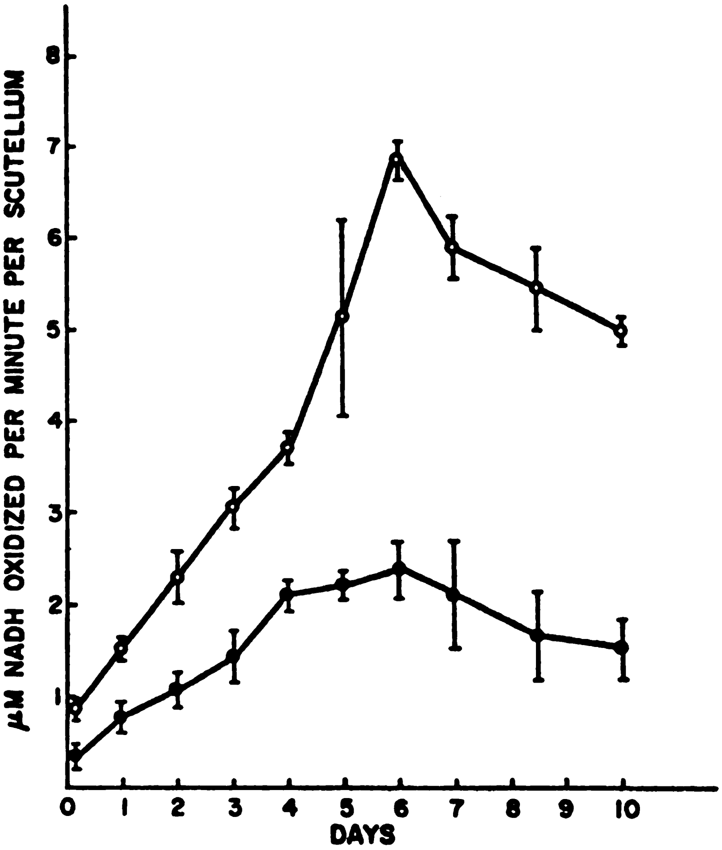

µM NADH OXIDIZED PER MINUTE
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
DAYS

Figure 8.--Time course of development of the two soluble MDH isozymes in scutella of germinating maize seeds (W64A) ———○———, s-MDH^1; ———●———, s-MDH^2.


μM NADH OXIDIZED PER MINUTE PER SCUTELLUM
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
DAYS

activity than the s-MDH^2, especially during the late developmental stages. This is also indicated by gel assays (Figure 6).

In young scutella, the five mitochondrial MDH isozymes exhibit similar developmental patterns (Figure 9). The activity of all mitochondrial forms is less than that of the soluble forms. However, all the isozymes do not seem to follow the same kinetics of accumulation. All the observed difference is likely not due to variability of the method, and some subtle regulation controlling the expression of each isozyme may be involved; this aspect has not been meaningfully examined at this point. Figure 10 represents the total soluble MDH activity and the total mitochondrial MDH activity at different developmental stages. The soluble MDH 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 MDH activity.

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

De novo synthesis of MDH isozymes

The density labeling technique was used to determine whether the development of m-MDH and s-MDH isozymes in the

Figure 9.--Time course of development of the five mitochondrial MDH isozymes in scutella of germinating maize seeds (W64A). ————●————, m-MDH^1; ————△————, m-MDH^2; ————x————, m-MDH^3; ————○————, m-MDH^4; ————□————, m-MDH^5.

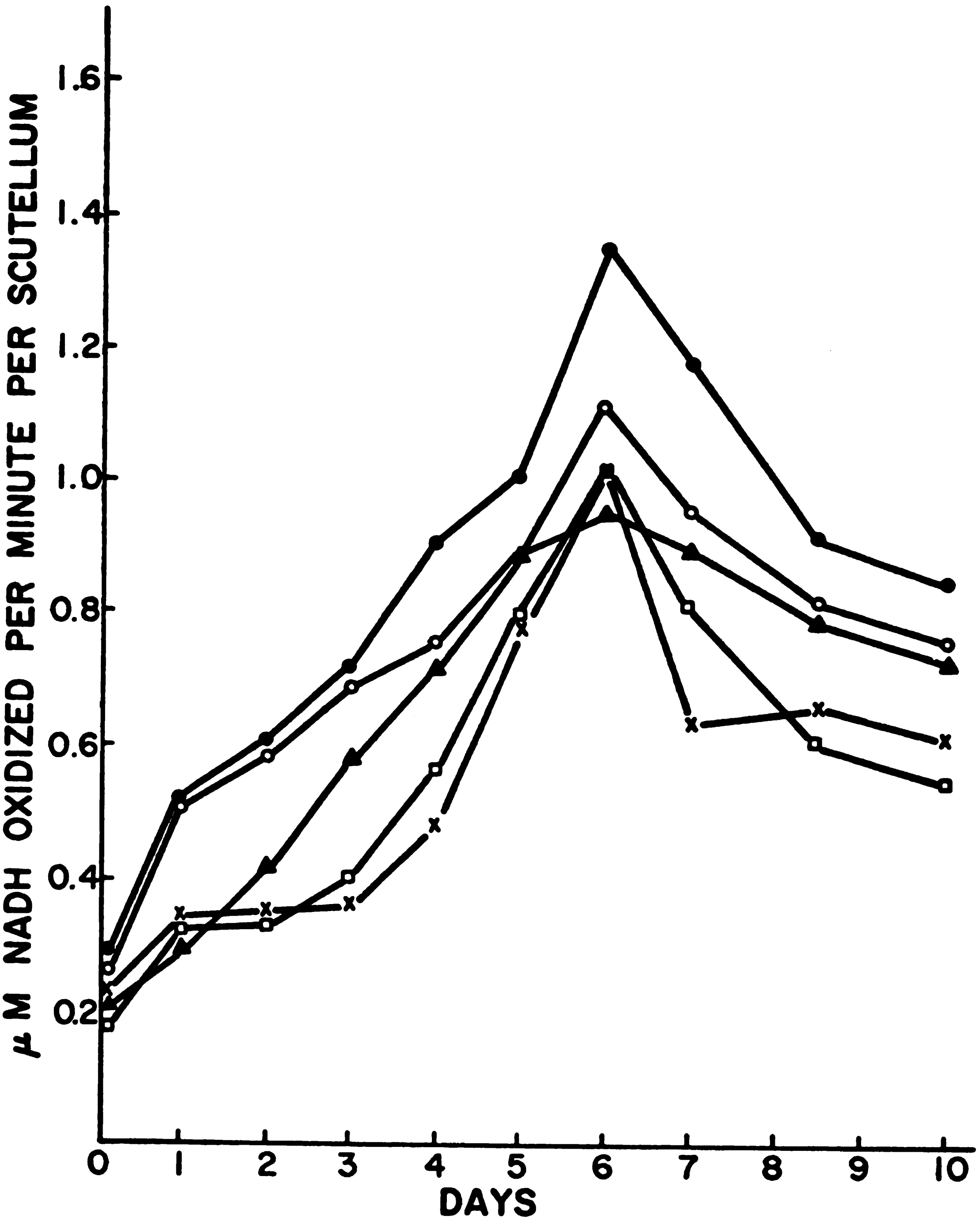
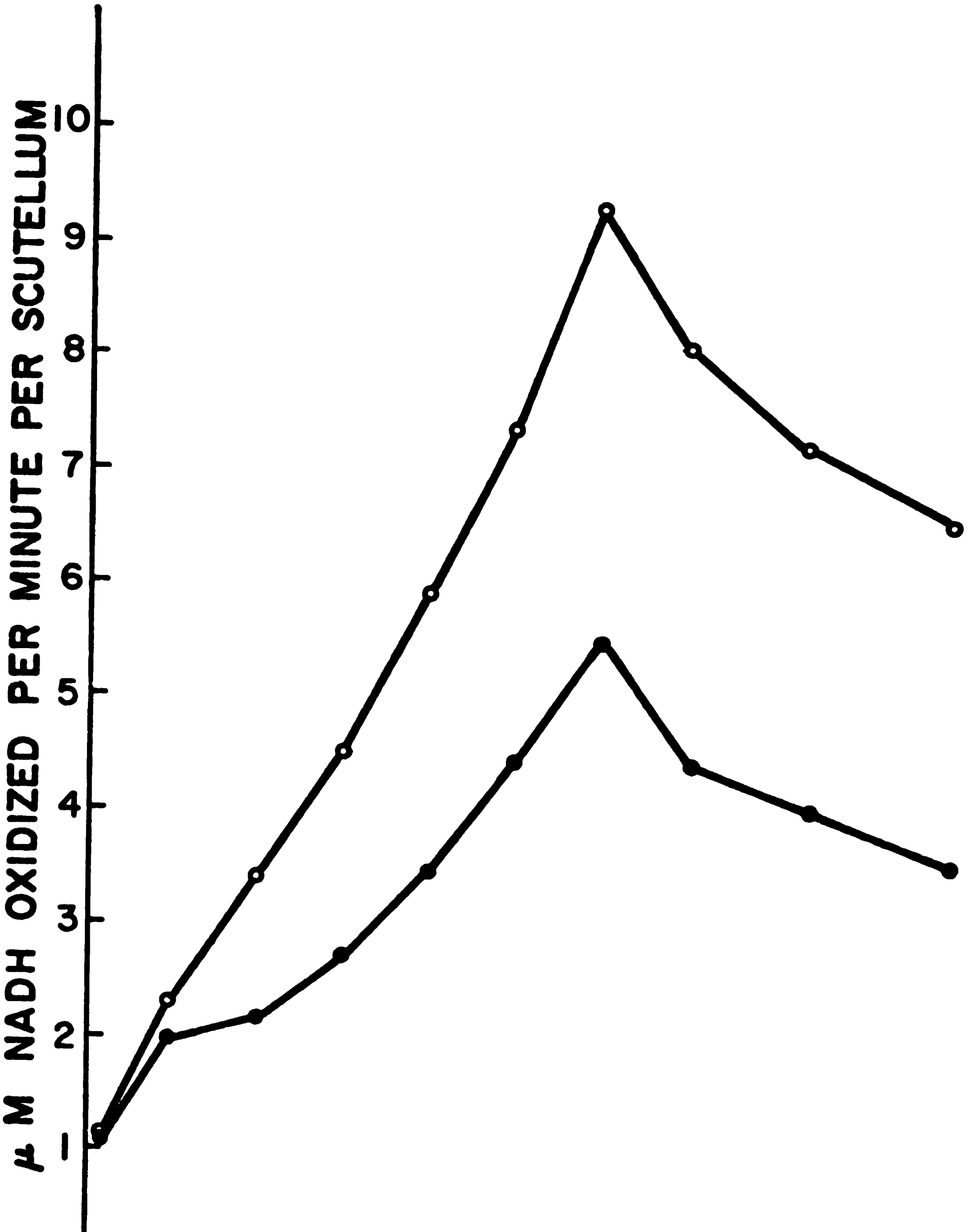

μM NADH OXIDIZED PER MINUTE PER SCUTELLUM
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
DAYS

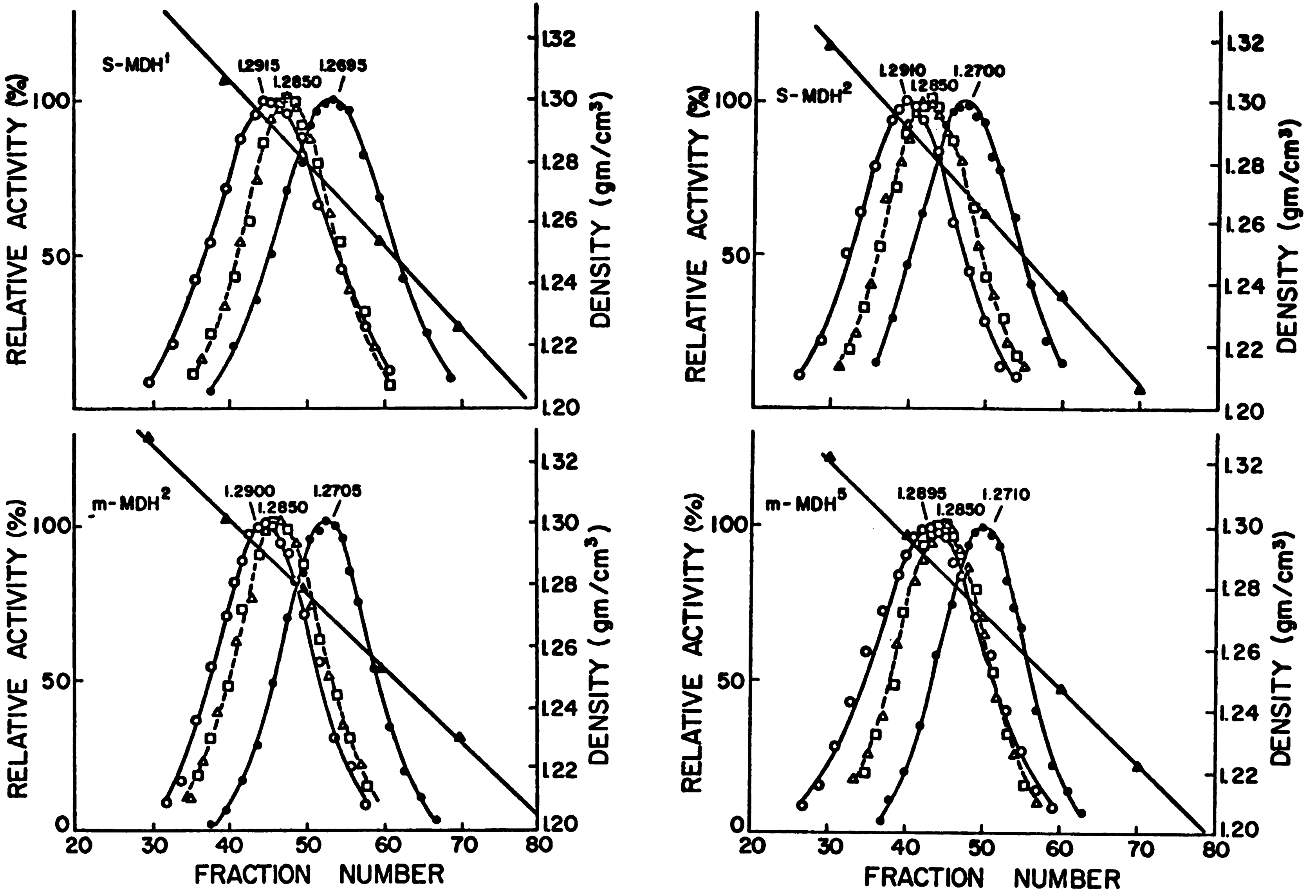
Figure 10.--Time course of soluble and mitochondrial MDH activities in scutella of germinating maize seeds (W64A). ———○———, s-MDH; ———●———, m-MDH.


μ M NADH OXIDIZED PER MINUTE PER SCUTELLUM
1
2
3
4
5
6
7
8
9
10
0
1
2
3
4
5
6
7
8
9
10
DAYS

scutella of developing maize seedlings is due to de novo synthesis of the enzyme moieties or to activation of the pre-existing enzymes. Figure 11 shows that both s-MDHs (s-MDH^1 and s-MDH^2) and s-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 of germination. This indicates that both classes of MDH isozymes are synthesized de novo. Therefore, the newly appearing MDH 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 turnover of these MDH isozymes must be rapid, since pre-existing molecules are not detected in CsCl gradients after 7-days. Because the increase of MDH is less than 10-fold over the ungerminated level, one might have expected the presence of an unlabeled component, evident as a shoulder at least, in the profiles of Figure 13, in the absence of substantial turnover.

Quail and Scandalios (26) observed that after 36 hours 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 hours. Therefore, it is not surprising that after 7 days in $^{15}N-D_2O$ medium, (the growth rate is equal to 5 days in $^{14}N-H_2O$ medium), no pre-existing molecules of MDH isozymes were detected.

Figure 11.--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% D_2O for 7 days (○______○). The activity of the LDH 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 (▲_____▲).


S-MDH^1
1.2915
1.2850
1.2695
S-MDH^2
1.2910
1.2850
1.2700
m-MDH^2
1.2900
1.2850
1.2705
m-MDH^5
1.2895
1.2850
1.2710
RELATIVE ACTIVITY (%)
0
50
100
DENSITY (gm/cm^3)
1.20
1.22
1.24
1.26
1.28
1.30
1.32
FRACTION NUMBER
20
30
40
50
60
70
80

The density labeling data on MDH isozymes presented in this chapter and results observed on catalase isozymes (26) 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 yeast chitlin synthatase can be isolated in an inactive or zymogen state (27). In addition, a protease extracted from yeast was found to act as an activating factor for the activation of such zymogen (28). In order to test whether the increase of the *de novo* synthesized MDH activity may be due to activation of the "inactive MDH precursors" which are continuously synthesized during development attempts were tried to test the possible existence of "activator(s)". The crude scutellar homogenates were centrifuged at 480 x g for 10 minutes; 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 MDH 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 condition, not only is there no "activation" of MDH 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"

Table II. Absence of in vitro Detectable MDH Activator or Inhibitor in Maize Scutella

*Crude extracts of scutella isolated from different developmental stages		Total MDH activity (ΔA_{340}/min. 10 µl) Observed
**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
	Calculated for additive summation	Observed
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 is described under "Materials and Methods."

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

of MDH activities when the crude extracts of 4-day or 5-day-old scutella were added with those of 8-day-old scutella. Therefore, it appears that the increase and decrease of MDH activities observed in the scutella of germinating seedlings result from regulation of synthesis and degration of the MDH isozyme moieties instead of activation or inactivation of pre-existing MDH isozyme.

Effects of pretein synthesis inhibitors on the development of MDH isozymes

Chloramphenicol and cycloheximide, two known inhibitors of protein synthesis, were used to study the intracellular site of MDH isozyme synthesis. Cycloheximide, in concentration of 2 μg/ml or 10 μg/ml, strongly inhibits the increase of MDH activity in scutella (Figure 12). Eight hours after the addition of cycloheximide, around 75% and 100% of the increments of total MDH activity are inhibited by CH at concentrations of 2 μg/ml and 10 μg/ml respectively. After 30 hours of treatment in cycloheximide at the concentration of 2 μg/ml, complete inhibition was also observed (Figure 12). The increase of both s-MDH and m-MDHs are inhibited by cycloheximide (Figure 13). Each of the m-MDH and s-MDH isozymes is inhibited by cycloheximide, with no preferential inhibition for any of the seven MDH isozymes (Table III).

Chloramphenicol, in concentrations of 0.5 mg/ml or 2 mg/ml, does not inhibit the increase in MDH activity during the first 40 hours of treatment (Figure 12). Within

Figure 12.--Effects of cycloheximide (CH) and chloramphenicol (CAP) on MDH activity during development of excised scutella. Scutella from 16 hours old seedlings were isolated and transferred to nutrient medium; they were then allowed to grow in the medium with or without antibiotics. ______○______, excised scutella in nutriend medium only; ______△______, excised scutella grown in nutrient medium with 0.5 mg/ml CAP; ______▲______, excised scutella grown in medium with 2.0 mg/ml CAP; ______□______, excised scutella grown in medium with 2 μg/ml CH; ______■______, excised scutella grown in medium with 10 μg/ml CH.

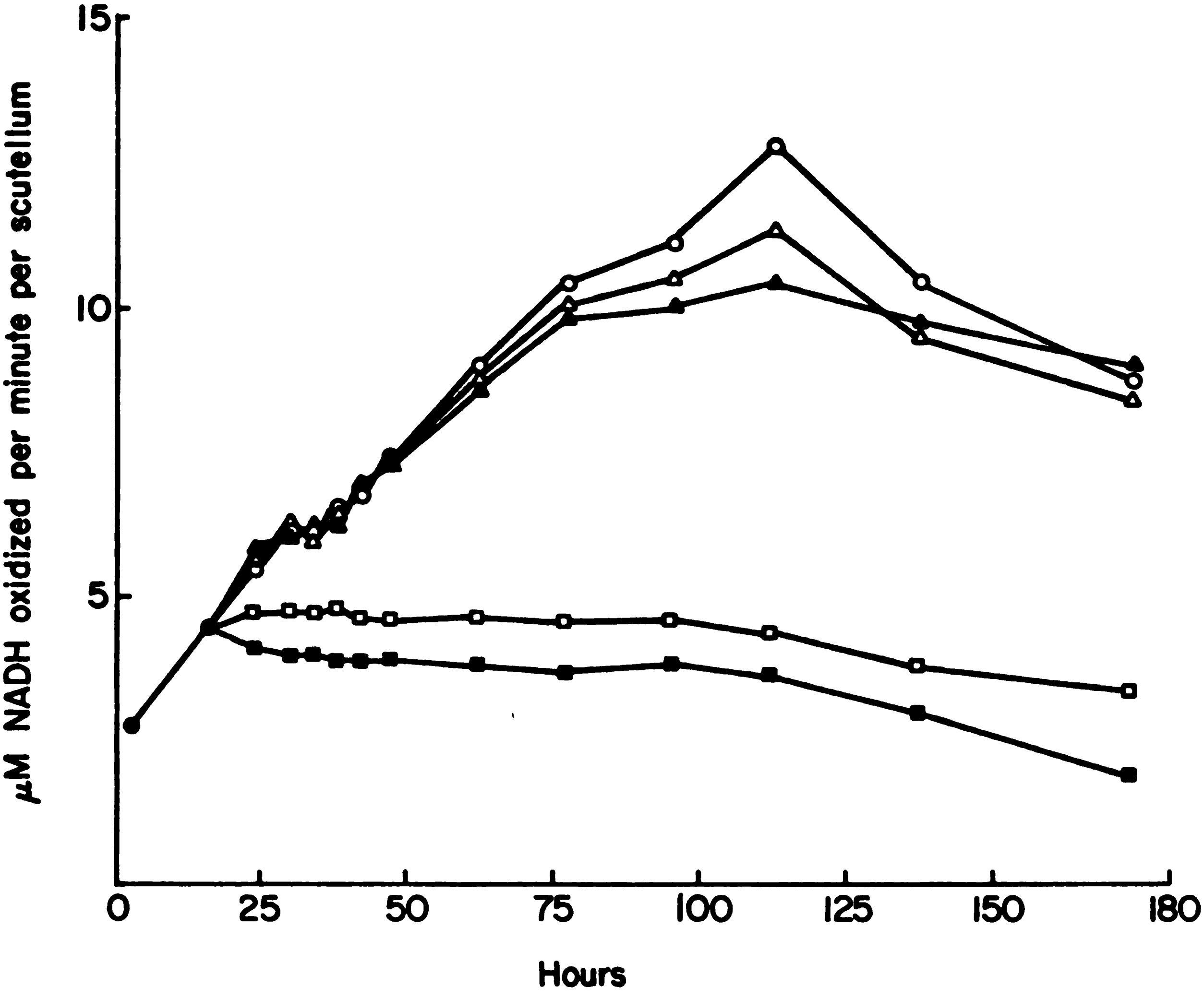
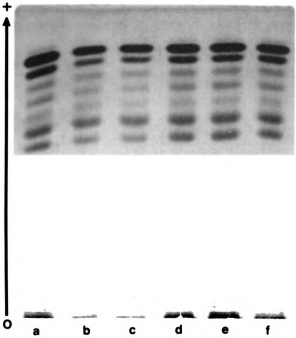
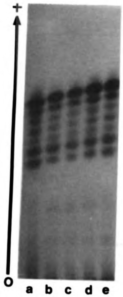

μM NADH oxidized per minute per scutellum
15
10
5
0
25
50
75
100
125
150
180
Hours

Figure 13.--Zymogram showing the effects of cycloheximide (CH) and chloramphenicol (CAP) on each of the soluble and mitochondrial MDH isozymes. (a) control (untreated); (b) + 2 µg/ml CH; (c) + 10 µg/ml CH; (d) + 0.5 mg/ml CAP; (e) + 2 mg/ml CAP; (f) control. A = 30 hr in medium; B = 96 hr in medium; Migration is anodal. O = point of sample insertion.



A. 30 hr in medium



B. 90 hr in medium

Table III. Inhibitory effect of cycloheximide [a](CH) on the increase of both soluble and mitochondrial MDH isozymes in maize scutella[b].

	% of inhibition of the increment of each individual MDH isozyme in CH treated scutella as compared to controls[c].	
MDH isozymes[d]	8 hrs in CH[d]	30 hrs in CH[d]
s-MDH^1	70 (%)	100 (%)
s-MDH^2	79	97
m-MDH^1	76	95
m-MDH^2	64	98
m-MDH^3	80	100
m-MDH^4	72	92
m-MDH^5	85	100

[a] CH: in a concentration of 2 µg/ml

[b] Scutella excised from 16 hours old maize seedlings were used as shown in Figure 12.

[c] Percent of inhibition is calculated on a per scutella basis, Inhibition of the increment of MDH activity of cycloheximide is determined as described in the following. At 0 hr in medium (just before the excised scutella were transferred to medium). Activities were measured and were taken as "Cont$_1$". After a certain period of time (as 8 hrs or 30 hrs) in medium, the activities observed in the excised scutella treated with and without CH were taken as "CH" and "Cont$_2$" respectively. The % of inhibition was calculated by the formula of $\left(1 - \frac{\text{CH}-\text{Cont}_1}{\text{Cont}_2-\text{Cont}_1}\right) \times 100\%$. See Figure 12 for reference.

[d] Isolation of indivdual isozymes, treatment of scutella with or without CH are described under "Materials and Methods."

Table IV. Effect of chloramphenicol [a](CAP) on the increase of soluble and mitochondrial MDH isozymes in scutella[b].

MDH isozymes[d]	Residual activity (%) of each individual MDH isozyme in CAP treated scutella compared to the activity in the control[c]. 30 hrs in CAP[d]	 96 hrs in CAP[d]
s-MDH^1	102 (%)	83 (%)
s-MDH^2	96	78
m-MDH^1	98	75
m-MDH^2	102	84
m-MDH^3	97	77
m-MDH^4	103	85
m-MDH^5	95	80

[a] CAP: in a concentration of 2 mg/ml

[b] Scutella excised from 16 hours old maize seedlings was used as shown in Figure 12

[c] Residual activity calculated on a par scutellum basis. A 100% residual activity means the activity of specific MDH isozyme found in the scutellum treated with or without CAP is the same; a 80% residue activity indicates that only 80% activity is observed in the CAP treated scutellum as compared to the scutellum treated without CAP.

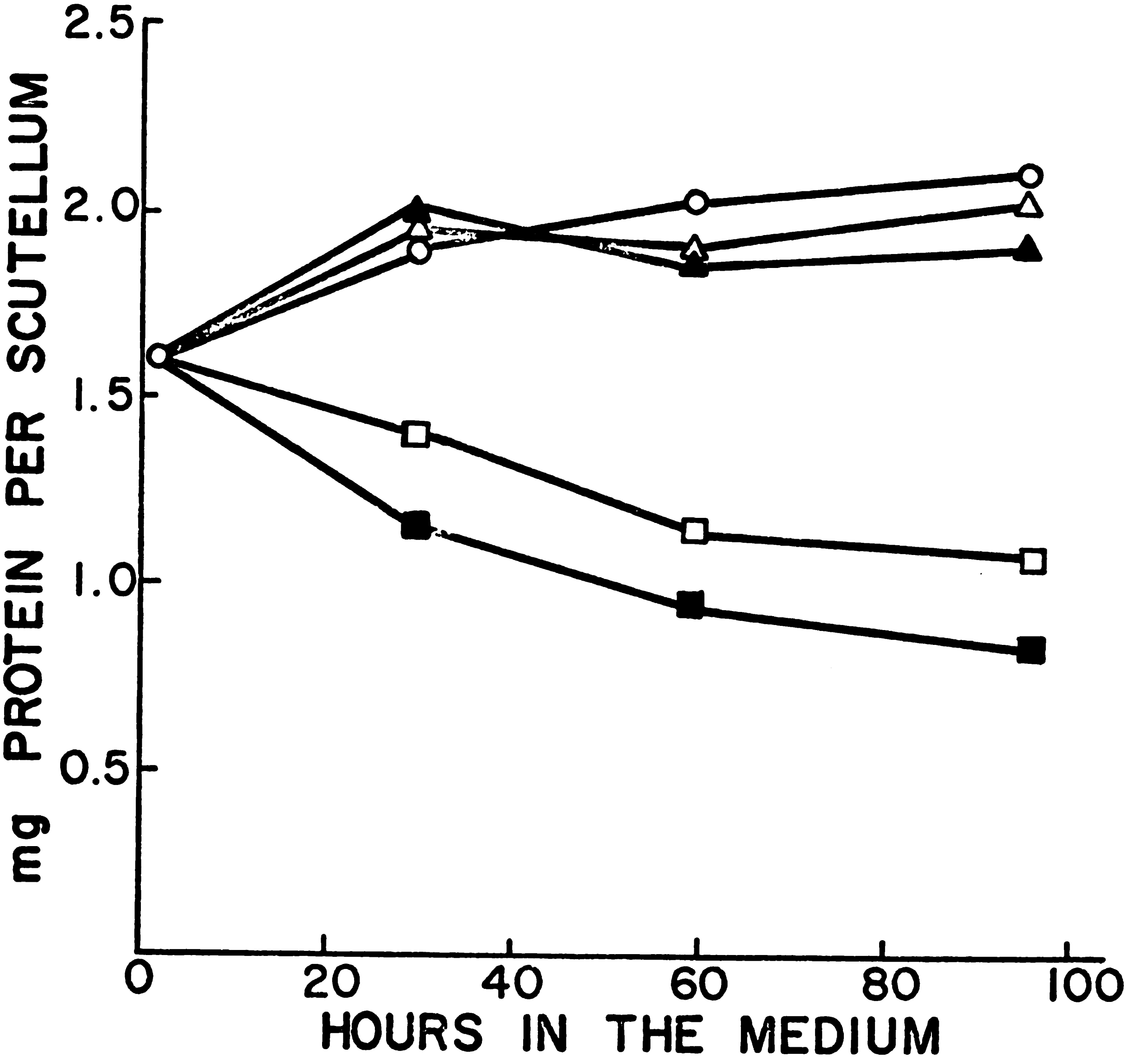
[d] Isolation of individual MDH isozymes, treatment of scutella with or without CAP are described under "Materials and Methods."

this period, neither s-MDHs nor m-MDHs are inhibited (Figure 13 and Table IV). The lack of an effect of CAP is not because that the inhibitor does not penetrate into the cells. This point will be proved later on. About 60 hours after the addition of CAP, inhibitory effects of the increase of MDH activities started to be observed. After 96 hours in chloramphenicol, about 10% and 20% inhibitions of the increments of the total MDH activity are observed at the concentrations of 0.5 mg/ml and 2.0 mg/ml respectively (Figure 12). Under such long period of treatment, the increases of both s-MDHs and m-MDHs are inhibited by chloramphenicol (Figure 13). Activities found in each of the s-MDH and m-MDH isozymes appear to decrease to a similar extent (Table IV). These inhibitions may be caused by long term non-specific side effects of chloramphenicol. As shown in Figure 14, 30 hours after the addition of cycloheximide, protein concentration in the extract of excised scutella was greatly reduced. While those in the chloramphenicol treated scutella did not decrease and appeared to increase slightly. After sixty hours in the chloramphenicol, 5-10% reduction of the protein concentration in scutella was observed.

Effects of cycloheximide and chloramphenicol on protein synthesis in maize scutella

Cycloheximide and chloramphenicol were employed to determine their effects on protein synthesis in maize scutella and to see if they exert a positive control on MDH

Figure 14.--Effects of cycloheximide (CH) and chloramphenicol (CAP) on the protein content of crude scutellar extracts (W64A). ———○——— control (medium only); ———△———, + 0.5 mg/ml CAP; ———▲———,+2.0 mg/ml CAP; ———□———, + 2.0 µg/ml CH ———■———, + 10 ug/ml CH.


mg PROTEIN PER SCUTELLUM
2.5
2.0
1.5
1.0
0.5
0
20
40
60
80
100
HOURS IN THE MEDIUM

activity during development. In these experiments, 5 µg/ml and 1 mg/ml were used for cycloheximide and chloramphenicol respectively. As described under "Materials and Methods," the excised scutella were double labeled for 12 hours. One set of scutella were labeled with ^{3}H-leucine, the other set with ^{14}C-leucine. These two sets were mixed and ground. Various subcellular fractions were isolated by differential and sucrose gradient centrifugation. The ^{3}H - labeled counts were all recovered from scutella not treated with antibiotics and served as an internal reference to compare the ^{14}C - labeled counts which were treated for 15 hours with antibiotics (CH or CAP) or without antibiotics (control). The ^{3}H/^{14}C ratio is then an indication of how the incorporation of ^{14}C-leucine was affected by the two antibiotics.

As can be seen in Tables V, VI and VII, cycloheximide (5 µg/ml) inhibits almost completely (∻ 97%) the incorporation of ^{14}C-leucine into TCA insoluble counts found in the soluble fractions. Chloramphenicol (1 mg/ml), on the other hand, inhibits less than 5% of the incorporation of ^{14}C-leucine into TCA precipitable materials recovered from the soluble fraction.

For the synthesis of proteins found in the crude particulate fraction (12,000 xg pellet), only about 80-85% was inhibited by cycloheximide; while about 30-35% was also inhibited by chloramphenicol (Table VII).

In order to make more detailed studies on the inhibitory effects of cycloheximide and chloramphenicol on the synthesis

Table V. Effects of cycloheximide and chloramphenicol on protein synthesis in maize scutella[a].

cpm[b]	Control			Cycloheximide			Chloramphenicol		
Sub-cellular fractions	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
Soluble fractions									
Exp. 1	2220	4521	0.491	1890	112	16.81	2281	4364	0.524
Exp. 2	3072	6606	0.465	2183	129	16.90	2872	5920	0.485
Exp. 3[c]	2632	6178	0.426	--	-	-	2504	6229	0.402
	(2632	6178	0.426)	--	-	-	3029	6839	0.443
Mitochondrial fractions									
Exp. 1	1483	738	2.01	1178	50.7	23.23	1371	557	2.46
Exp. 2	1126	593	1.90	1226	22.7	25.05	1045	462	2.26
Exp. 3[c]	922	663	1.39	--	-	-	860	508	1.69
	(922	663	1.39)	--	-	-	912	477	1.91

[a] Scutella excised from 4-day old seedlings were used. Cycloheximide and chloramphenicol were used at concentrations of 5 μg/ml and 1 mg/ml respectively. ^{3}H-leucine and ^{14}C-leucine were added at the final concentrations of 12.5 μci/5 ml and 1.25 μci/5 ml medium solutions respectively. Experimental details are described in "Materials and Methods."

[b] TCA insoluble counts in 1 ml aliquot of 40 ml soluble fractions or in 1 ml of 10 ml mitochondrial fractions.

[c] In experiment 3, two independent sets of scutella were treated with CAP; experiments on the treatment of CH were not performed (indicated by -----). Therefore, data on control were repreated to compare both preparations of CAP treated scutella.

Table VI. Effects of cycloheximide and chloramphenicol on protein syntehsis in maize scutella[a]

cpm[b]	Control			Cycloheximide			Chloramphenicol		
Sub-cellular fractions	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
Soluble fractions									
Exp. 4	10016	20789	0.482	10271	694	14.80	10652	20969	0.508
Exp. 5	11250	18639	0.60	9622	467	20.6	11015	17703	0.65
Mitochondrial fractions									
Exp. 4	5907	3215	1.89	5053	137	36.87	5943	2406	2.47
Exp. 5	5599	2731	2.05	4983	124	40.3	5885	2172	2.71
Dense particulate fraction									
Exp. 4	3403	661	5.14	3472	208	17.04	3248	319	10.17
Exp. 5	3045	565	5.39	3246	205	15.8	3078	332	9.26

---

[a] Experimental conditions are the same as those in Table IV, except 50 µci/5ml nutrient solutions were the final concentrations for ^{3}H-leucine and ^{14}C-leucine respectively.

[b] TCA insoluble counts in 1 ml aliquot of 40 ml solution fractions, or in 1 ml of 10 ml of mitochondrial fractions, or in 0.1 ml of the 1 ml resuspension of dense particulate fractions.

Table VII. Effects of cycloheximide and chloramphenicol on protein synthesis in maize scutella[a]. (Experiment 6)

cpm[b]

Sub-cellular fractions	Control			Cycloheximide			Chloramphenicol		
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
Soluble fractions (25,000xg supernatant)	45892	72101	0.637	41702	1897	21.98	47679	71349	0.668
Crude particulate fractions (12,000xg pellet)	48558	9042	5.37	47497	1665	28.52	50300	5945	8.56
Mitochondrial fractions (band in sucrose gradient)	25810	11894	2.17	26374	711	37.13	26684	9170	2.91
Dense particulate fraction (pellet in sucrose gradient)	22286	4464	4.99	19971	1452	13.75	20523	2098	9.78

[a] Experimental conditions are the same as those in Table IV, except 250 µci/5ml and 5 µci/5ml nutrient solutions were the final concentrations for ^{3}H-leucine and ^{14}C-leucine respectively.

[b] TCA insoluble counts in 1 ml aliquots of 40 ml soluble fractions, in 0.2 ml of 5.0 ml crude particulate fractions, in 1 ml of 10 ml mitochondrial fractions and in 0.1 ml of 0.7 ml dense particulate fractions.

of protein found in particulate fractions, mitochondria and dense particulate fractions were further isolated by sucrose gradient centrifugation. As shown in Table V, VI and VII, cycloheximide strongly inhibits the synthesis of proteins found in mitochondrial fraction, however, these inhibitions ($\doteqdot$ 94%) were always to a less extent as compared to the inhibitions found in soluble fraction. This can be easily observed by comparing the $^3H/^{14}C$ ratios of the soluble fractions treated with or without cycloheximide to those of the mitochondrial fractions treated with or without cycloheximide. Inhibitory effects of cycloheximide on the synthesis of proteins recovered from dense particulate fractions are greatly reduced. Table VI and VII show that for the dense particulate fractions, only 65-70% of the incorporation of ^{14}C-leucine was inhibited by cycloheximide.

Chloramphenicol inhibits 15% to 25% of the incorporation of ^{14}C-leucine into TCA insoluble materials recovered from mitochondrial fractions (Table V, VI, VII). As shown in Table VI and VII, about 40% to 50% of the synthesis of proteins found in dense particulate fractions were inhibited by chloramphenicol. The results shown in Table V, VI and VII suggest that in maize, cycloheximide and chloramphenicol exhibit differential inhibitory effects on the synthesis of proteins located at different subcellular fractions.

The reduced inhibitory effect of cycloheximide and the increased inhibitory effect on the synthesis of proteins recovered in the dense particulate fraction further support the conclusion that the dense particulate fraction containing glyoxysomes are contaminated by mitochondrial inner membranes. The reasons are: 1) less than 10% of the mitochondrial proteins are synthesized on the mitochondrial ribosomes (29, 30), this fraction consists mostly of hydrophobic proteins located in the inner membrane (29, 30, 31, 32). Synthesis of these proteins are chloramphenicol sensitive and cycloheximide resistant. 2) Development of microbody enzymes into plants are much more sensitive to cycloheximide than to chloramphenicol (33). 3) There is no independent protein synthesis machinery in the microbodies. 4) No chloroplasts or proplastids have been observed in maize scutella (13), and therefore mitochondria are the only organelles in maize scutella to have a ribosome machinery. Based on these observations, and the recovery of cytochrome oxidase activity (the marker enzyme of the inner mitochondrial membrane) from the dense particulate fraction as discussed at the beginning of "Results," the effect of CH and CAP on the dense particulate fraction must be caused by their effects on the synthesis of mitochondrial inner membranes. The results that the synthesis of inner membranal proteins of mitochondria recovered in the dense particulate fraction are more sensitive to chloramphenicol are consistent with the findings (29, 30, 34) that in yeast, Neurospora and many animal cells, some proteins of inner mitochondrial membrane are synthesized by mitochondrial ribosomes. Even though such evidence has not been provided in high plant tissues, the result of the present study may be an indication that these observations found in yeast and animal cells may be also true in higher plant cells.

Effects of cycloheximide and chloramphenicol on the synthesis of polypeptides found in various subcellular fractions

Based on these observations, it would be important and interesting to know whether some protein species are specifically inhibited by cycloheximide or chloramphenicol, while others are not. As shown in Figure 15, the proteins in various subcellular fractions were separated by SDS polyacrylamide gel electrophoresis. Since higher concentration (12.5%) of acrylamide and stacking gel were used, the proteins were able to be separated into rather sharp bands. The samples prepared from scutella treated with or without antibiotics (CH or CAP) had the same protein staining profiles and were not distinguishable from one another (data not shown). As described under "Materials and Methods," proteins isolated from three subcellular fraction of excised scutella treated with or without antibiotics (CH or CAP) were subjected to SDS gel electrophoresis. On completion, the gels were sliced and radioactivities (^{3}H and ^{14}C) in the 1 mm gel slices were counted. The samples used for these experiments were prepared from the same experiments (Exp. 6) as shown in Table VI.

Figure 16 (B) shows that the synthesis of four groups of polypeptides found in soluble fraction are affected by

Figure 15.--SDS polyacrylamide gel electrophoresis of proteins isolated from various subcellular fractions of maize scutella. Samples containing 0.1-0.2 mg of protein in 50-100 µg of sample solutions were applied to a 12.5% polyacrylamide gel and subjected to electrophoresis at room temperature for 8 hrs. Then the gels were stained for protein with 0.2% coomassie brilliant blue. Experimental details are described under "Materials and Methods." The three gel profiles shown in this figure are the polypeptides isolated from (a) soluble fraction, (b) mitochondria, (c) dense particulate fraction.

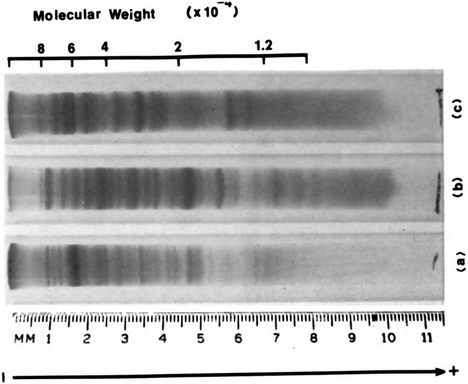
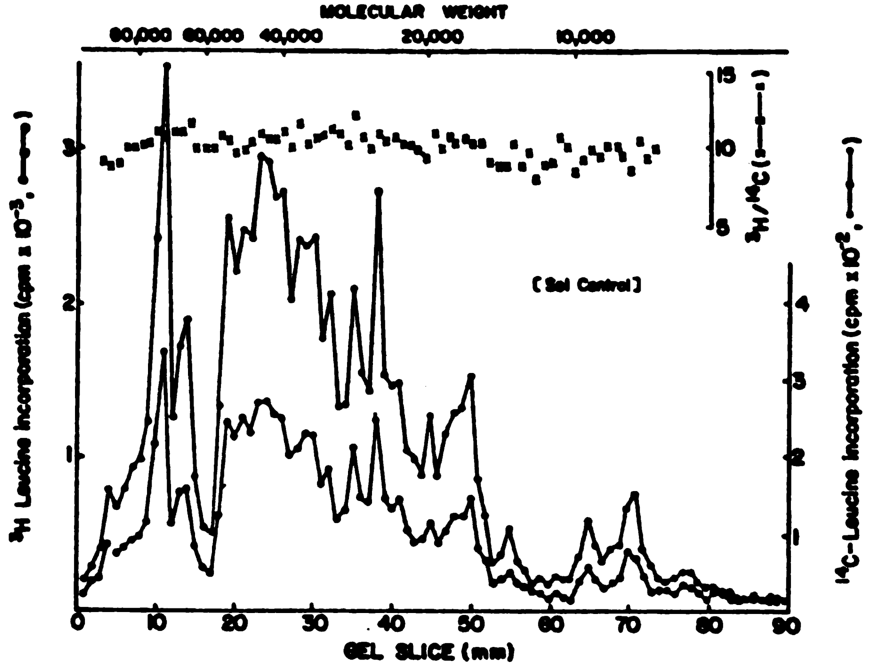
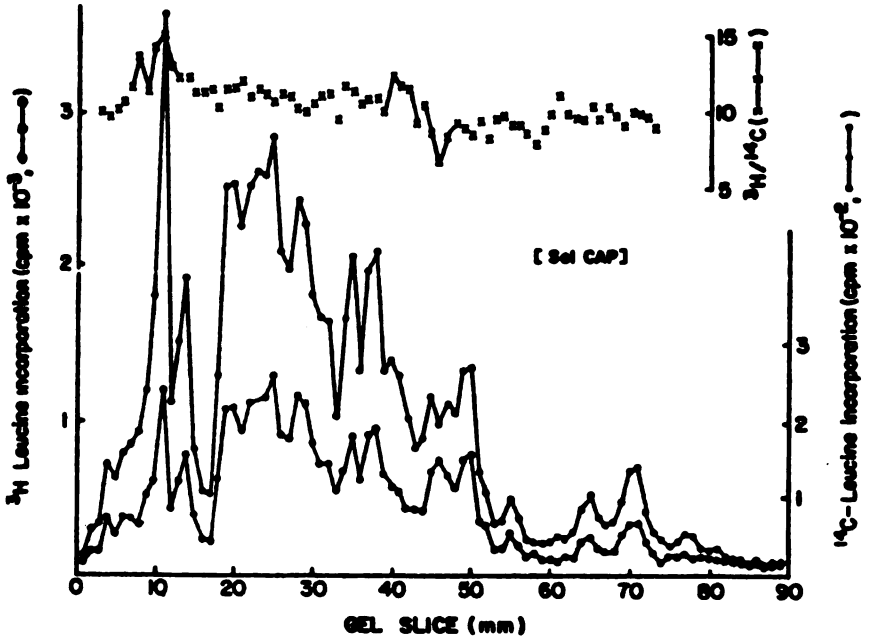

Molecular Weight (x10^{-4})
8
6
4
2
1.2
(c)
(b)
(a)
MM 1 2 3 4 5 6 7 8 9 10 11
-
+

Figure 16.--The double label SDS gel profiles of polypeptides in soluble fractions extracted from maize scutella treated (A) in the absence of antibiotics against protein synthesis, (B) in the presence of 1 mg/ml of chloramphenical (CAP), (C) in the presence of 5 µg/ml of cycloheximide (CH). All ^3H counts were extracted from scutella incubated in nutrient media only (without CH or CAP) and served as an internal standard for each gel. The ^{14}C counts were extracted from scutella incubated in the absence of CH and CAP (control), or in the presence of CAP or CH. Therefore, the increase and decrease in $^3H/^{14}C$ ratios in CAP or CH treated samples indicate their effects of inhibition and enhancement respectively. The $^3H/^{14}C$ ratios in the CAP treated samples, (B), significantly differed from those in the control, (A), are connected by solid lines. The $^3H/^{14}C$ ratios in the CH treated samples, (C), are not plotted, since inhibitions are almost complete. Application of antibiotics (CH or CAP), double labeling of radioactive leucine (^3H and ^{14}C) into excised scutella, isolation of fractions, SDS polyacrylamide gel electrophoresis, and counting the radioactivity in the gels have been described in detail under "Materials and Methods." The protein staining profile of the gel is the same as shown in Figure 15.

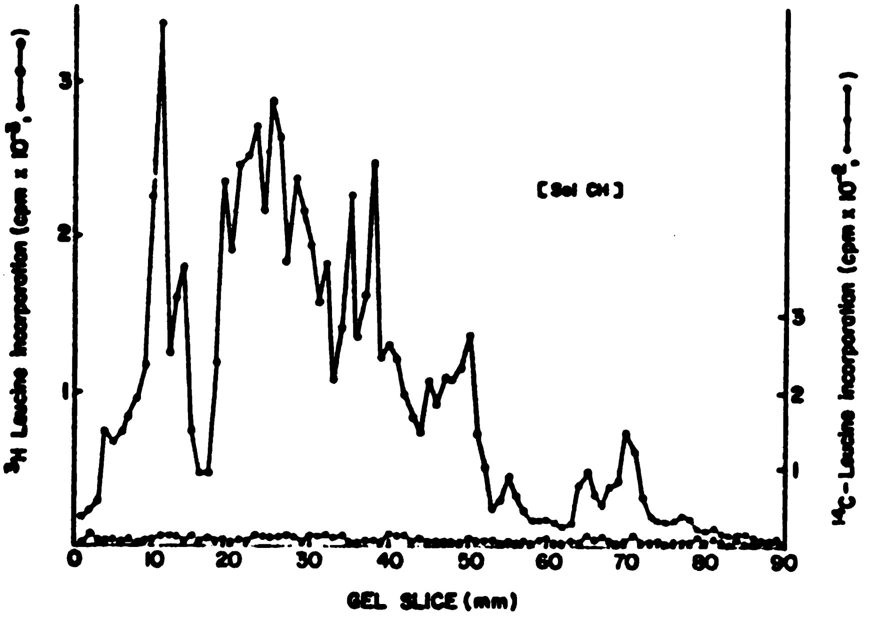
(A)


MOLECULAR WEIGHT
80,000
60,000
40,000
20,000
10,000
3H Leucine Incorporation (cpm x 10-3, ○—○)
3H/14C (■—■)
14C-Leucine Incorporation (cpm x 10-2, ●—●)
[Sol Control]
1
2
3
5
10
15
4
0
10
20
30
40
50
60
70
80
90
GEL SLICE (mm)

(B)


3H Leucine Incorporation (cpm x 10-3, ○—○)
3H/14C (■—■)
14C-Leucine Incorporation (cpm x 10-2, ●—●)
[Sol CAP]
1
2
3
5
10
15
0
10
20
30
40
50
60
70
80
90
GEL SLICE (mm)

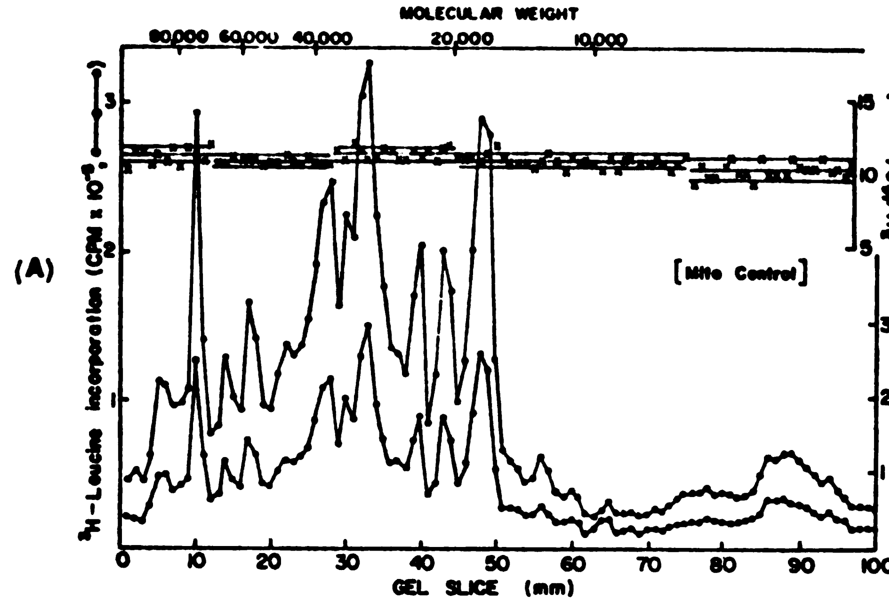
(C)

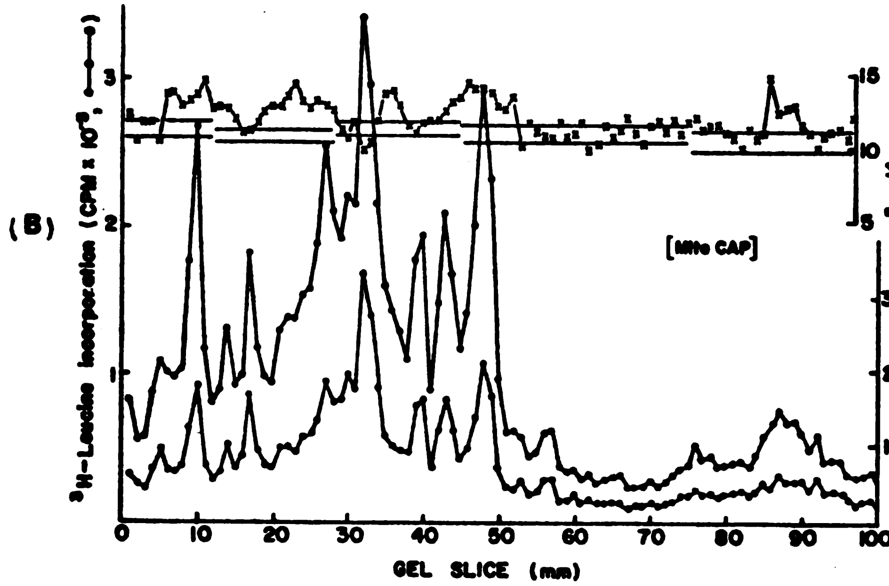

3H Leucine Incorporation (cpm x 10-3, ○—○)
14C-Leucine Incorporation (cpm x 10-2, ●—●)
[Sol CH]
1
2
3
0
10
20
30
40
50
60
70
80
90
GEL SLICE (mm)

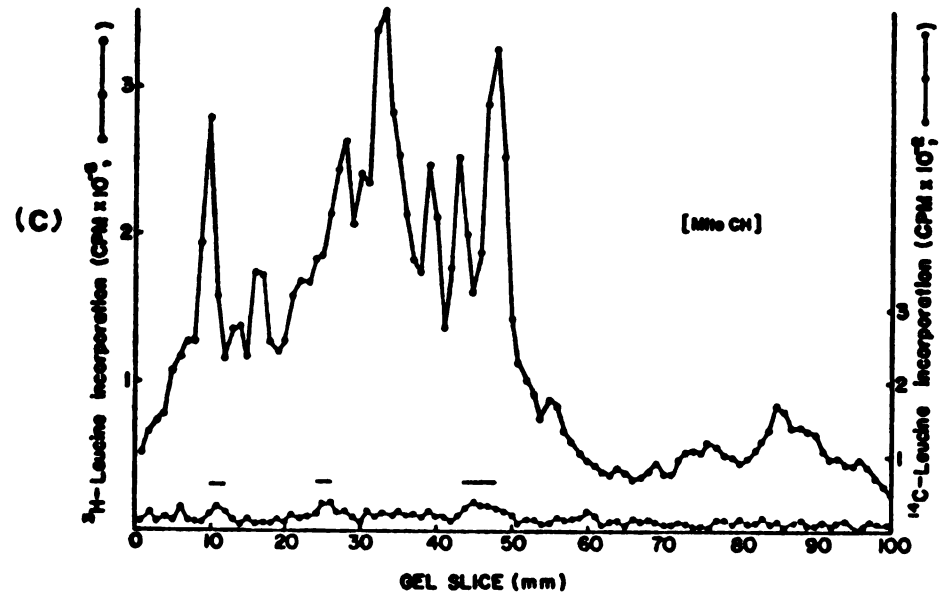
chloramphenicol, three were inhibited, one was enhanced. This result indicates that chloramphenicol may inhibit and enhance specifically some proteins extracted from soluble fractions. Cycloheximide, on the other hand, completely blocked the synthesis of proteins found in soluble fractions. No significant incorporation peaks were observed.

It can be seen in Figure 17, the synthesis of many (but not all) polypeptides localized in mitochondria are affected by chloramphenicol. About 20% to 35% inhibitions were observed. However, the synthesis of the polypeptide(s) having a molecular weight around 30,000 (the peak with the highest incorporation of ^3H) appears to be enhanced by chloramphenicol. Enhancement of the synthesis of mitochondrial and soluble proteins by chloramphenicol is not too surprising, similar results have been reported in Neurospora (35). The possible mechanisms involved have been proposed by Kuntzel and his coworker (35). Cycloheximide strongly inhibits the synthesis of polypeptides found in mitochondrial fraction (Figure 17). However, some polypeptides are less affected and are indicated by the three "bars" shown in Figure 17 (c). In these "bar" fractions, the $^3H/^{14}C$ ratios are about 40-70, and correspond to some of the peaks inhibited by chloramphenicol [Figure 17 (B)], while the ratios of the other fractions are about 150-250 [Figure 17 (C)]. The specific inhibitions and enhancement by CAP shown in Figure 17 (B) and the less inhibitory effect on some specific proteins by CH shown in Figure 17 (C) should not be taken as artifacts. The experiments

Figure 17.--The double label SDS gel profiles of polypeptides in mitochondria extracted from maize scutella treated (A) in the absence of antibiotics against protein synthesis, (B) in the presence of 1 mg/ml of chloramphenicol (CAP), (C) in the presence of 5 μg/ml of cycloheximide (CH). In order to make a better comparison, the mean values of $^{3}H/^{14}C$ and their standard deviations shown in the control gel, (A), are given in the CAP treated set, (B). The $^{3}H/^{14}C$ ratios in CH treated set are not plotted. Fractions with ^{14}C counts higher than 30 cpm are indicated by the bars (——) over the ^{14}C profile and the $^{3}H/^{14}C$ ratios for these fractions are about 40 to 70. The $^{3}H/^{14}C$ ratios for the fractions having less than 30 cpm for ^{14}C counts were between 150-300. Other details of the figures are the same as given in Figure 16.


(A)
MOLECULAR WEIGHT
80,000
60,000
40,000
20,000
10,000
[Mito Control]
³H-Leucine incorporation (CPM x 10⁻⁵, ●—●)
³H/¹⁴C (x—x)
¹⁴C-Leucine incorporation (CPM x 10⁻⁵, ●—●)
GEL SLICE (mm)
0 10 20 30 40 50 60 70 80 90 100

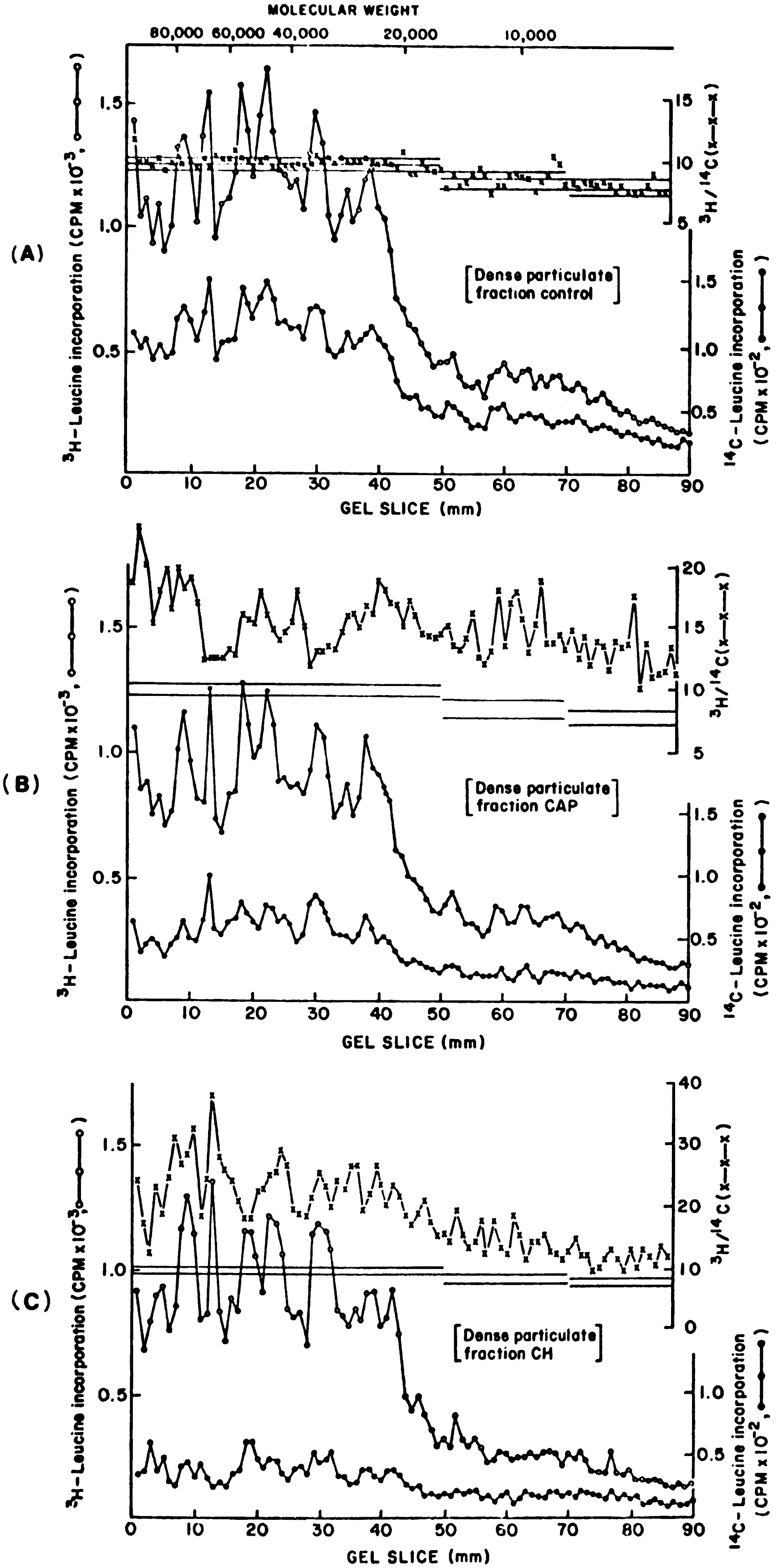

(B)
[Mito CAP]
³H-Leucine incorporation (CPM x 10⁻⁵, ●—●)
³H/¹⁴C (x—x)
¹⁴C-Leucine incorporation (CPM x 10⁻⁵, ●—●)
GEL SLICE (mm)
0 10 20 30 40 50 60 70 80 90 100


(C)
[Mito CH]
³H-Leucine incorporation (CPM x 10⁻⁵, ●—●)
¹⁴C-Leucine incorporation (CPM x 10⁻⁵, ●—●)
GEL SLICE (mm)
0 10 20 30 40 50 60 70 80 90 100

in Figure 17 have been repeated again, and similar results were obtained (data not shown). The lack of clear residual ^{14}C incorporation peaks in the cycloheximide treated mitochondrial fractions may be due to the following reasons: 1) The majority of the mitochondrial proteins (more than 90%) are synthesized in the soluble cytoplasm (29) and thus are much more sensitive to cycloheximide than to chloramphenicol. 2) Without further submitochondrial fractionation, the high protein concentrations in the whole mitochondrial preparation [Figure 15 (B)] prevented us from loading more radioactivity onto the gel. Therefore, the cycloheximide resistent incorporation of ^{14}C-leucine were masked. 3) Since the synthesis of the majority of the mitochondrial proteins including those needed to assemble mitochondrial membranes, are blocked by cycloheximide, the mitochondria in the CH treated scutella may be more easily to get broken during the experimental procedures. Therefore, more inner membranial components of mitochondria may be recovered in the dense particulate fraction. If so, more clear residual ^{14}C incorporation peaks should be observed in dense particulate fraction and this is true as shown in Figure 18.

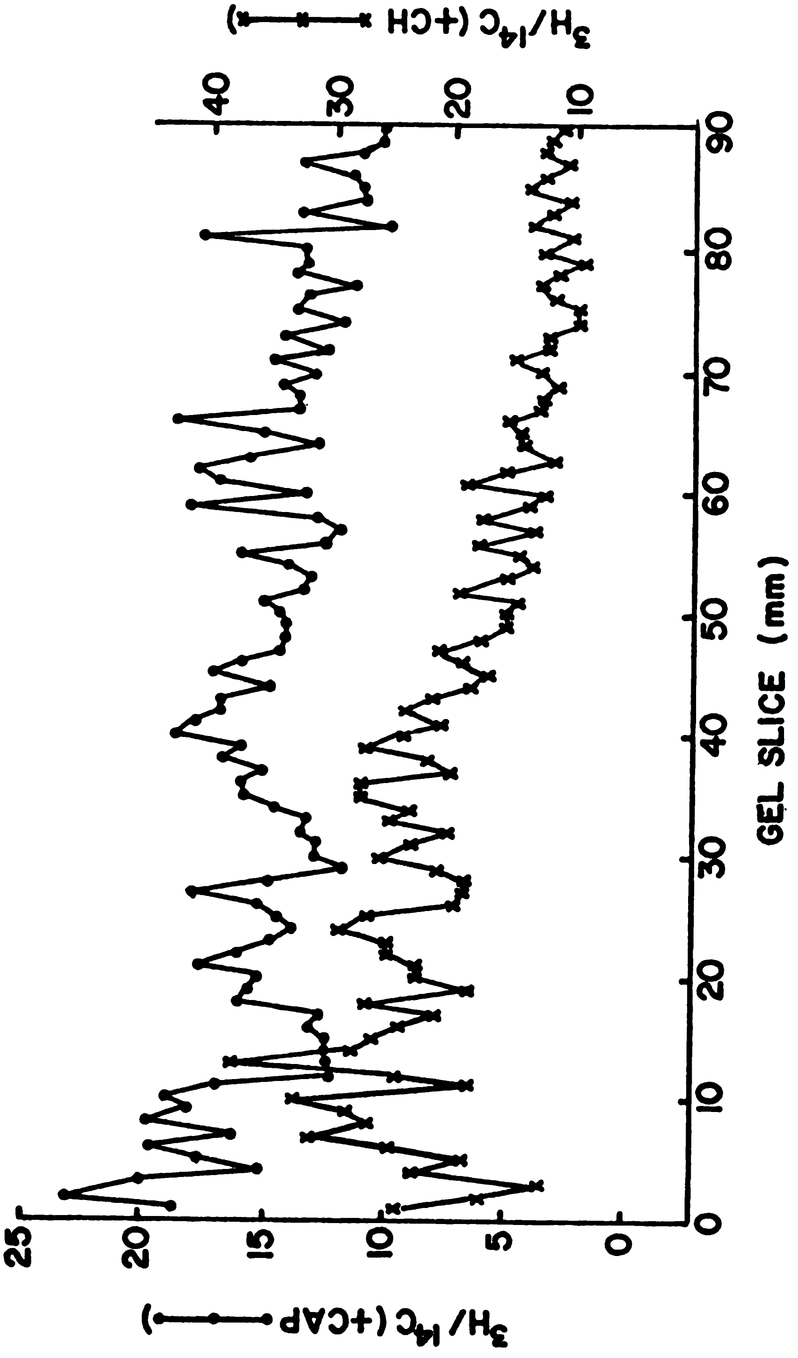
Effects of cycloheximide and chloramphenicol on the synthesis of polypeptide found in the dense particulate fractions are shown in Figure 18. Synthesis of many polypeptides recovered in the dense particulate fractions are severely inhibited by chloramphenicol. Such inhibitory

Figure 18.--The double label SDS gel profiles of polypeptides in dense particulate fractions extracted from maize scutella treated (A) in the absence of antibiotics against protein synthesis, (B) in the presence of 1 mg/ml of chloramphenicol (CAP), (C) in the presence of 5 µg/ml of cycloheximide (CH). In order to make a better comparison, the mean values of $^{3}H/^{14}C$ ratios and their standard deviations in the control gel (A) are given in the CAP treated (B) or CH treated (C) sets. Notice that different scales for $^{3}H/^{14}C$ ratios are used for set (B) and set (C). Other details of the figures are the same as given in Figure 16.


MOLECULAR WEIGHT
80,000 60,000 40,000 20,000 10,000
(A)
^{3}H-Leucine incorporation (CPM x 10^{-3}, o—o)
1.5
1.0
0.5
^{3}H/^{14}C (x—x—x)
15
10
5
[Dense particulate fraction control]
^{14}C-Leucine incorporation (CPM x 10^{-2}, ●—●)
1.5
1.0
0.5
0 10 20 30 40 50 60 70 80 90
GEL SLICE (mm)
(B)
^{3}H-Leucine incorporation (CPM x 10^{-3}, o—o)
1.5
1.0
0.5
^{3}H/^{14}C (x—x—x)
20
15
10
5
[Dense particulate fraction CAP]
^{14}C-Leucine incorporation (CPM x 10^{-2}, ●—●)
1.5
1.0
0.5
0 10 20 30 40 50 60 70 80 90
GEL SLICE (mm)
(C)
^{3}H-Leucine incorporation (CPM x 10^{-3}, o—o)
1.5
1.0
0.5
^{3}H/^{14}C (x—x—x)
40
30
20
10
0
[Dense particulate fraction CH]
^{14}C-Leucine incorporation (CPM x 10^{-2}, ●—●)
1.0
0.5
0 10 20 30 40 50 60 70 80 90
GEL SLICE (mm)

effects range from 30% to 50% [Figure 18 (B)]. However, there are some major ^{14}C incorporation peaks which are much less effected by chloramphenicol. For the dense particulate fraction, about 30% to 35% incorporation of ^{14}C-leucine was not inhibited by cycloheximide. These residual incorporations are not equally distributed throughout the SDS gels. As shown in Figure 18 (C), synthesis of some polypeptides are less inhibited by cycloheximide while others are more inhibited. A close comparison of the $^{3}H/^{14}C$ ratios of the cycloheximide treated sample to those of the chloramphenicol treated sample shows that the inhibitory effects of cycloheximide and chloramphenicol in the dense particulate fraction appear to be compensatory (Figure 19). That is, the fraction which are strongly inhibited by cycloheximide, are comparatively less inhibited by chloramphenicol and vice versa. The higher background of $^{3}H/^{14}C$ ratios in the CH treated samples over those in the control [Figure 18 (C) and Figure 19] may be caused by the non-specific inhibition of the synthesis of glyoxysomal proteins. As seen in Figure 18, the species of polypeptides found in the dense particulate fractions are much less than those observed in the mitochondrial fraction. Therefore, the broken mitochondrial inner membranes which formed the pellet with glyoxysomes are actually "partially purified." This may very well be the reason that the higher inhibitory effect of CAP and lower inhibitory effect of CH are magnified.

Figure 19.-- A close comparison of the $^{3}H/_{^{14}C}$ ratios of the double label SDS gel profiles of polypeptides in dense particulate fraction extracted from maize scutella treated in the presence of 1 mg/ml of chloramphenicol or in the presence of 5 µg/ml cycloheximide. $^{3}H/_{^{14}C}$ ratios in (B) and (C) of Figure 18 were superimposed to show the comparisatory inhibitory effects of CH and CAP on the dense particulate fractions. Notice that different scales are used for CH treated and CAP treated set.


GEL SLICE (mm)
0
10
20
30
40
50
60
70
80
90
³H/¹⁴C (+CAP ●—●—●)
0
5
10
15
20
25
³H/¹⁴C (+CH ×—×—×)
10
20
30
40

Results shown in Figure 18 and Figure 19 clearly indicate that synthesis of some specific proteins in the dense particulate fraction are more sensitive to chloramphenicol and more resistent to cycloheximide than are the protein synthesis carried out on 80S cytoplasmic ribosomes. Studies on the biosynthesis of mitochondrial membranes (25, 29, 30, 31, 32, 34) and on the development of microbodies (33), accompanied with the present observations, strongly suggest that the inner membrane of mitochondria, instead of glyoxysome, is the component responsible for the differential inhibitory effects of CH and CAP observed in the dense particulate fraction. Therefore, in maize, some protein components of the mitochondrial inner membrane appear to be synthesized on mitochondrial ribosomes.

The results shown in Figure 16, 17 and 18 indicates that, within 15 hours, chloramphenicol may inhibit the synthesis of some specific proteins, probably some components of the inner mitochondrial membrane, found in mitochondria and dense particulate fractions. It has very little effect on the synthesis of proteins found in the soluble cytoplasm. The inhibitory effect of chloramphenicol on the protein synthesis of maize scutella also support that, within 15 hours, chloramphenicol does penetrate into scutella cells under the present experimental conditions. Cycloheximide on the other hand, completely blocked the synthesis of proteins recovered in the soluble cytoplasm, it also strongly inhibited the synthesis of most proteins found in mitochondrial and dense particulate fractions. But cycloheximide is not able to block the synthesis of some specific proteins, probably some components of the inner mitochondrial membranes, found in mitochondrial and dense particulate fractions.

Effects of cycloheximide and chloramphenicol on MDH activities in scutella excised from 4-day-old seedlings

The effects of antibiotics (CH or CAP) on protein synthesis in maize scutella were studied by using scutella excised from 4-day-old seedlings. To make a better comparison, effects of chloramphenicol and cycloheximide on the development of MDH isozymes have also been studied again by using scutella excised from 4-day-old seedlings. The excised scutella were incubated with or without antibiotics (CH or CAP) for 15 hours under the same experimental conditions as were used for studying the effects of antibiotics on protein synthesis. Results in Table VIII show that the total MDH activities observe in the control and the CAP treated samples are the same. No inhibitory effect of CAP was observed. However, 15 hours after the addition of cycloheximide, about 30% of the MDH activity found in the control was lost. Table IX shows that neither s-MDH isozymes nor m-MDH isozymes are affected by the treatment of chloramphenicol for 15 hours. However, when scutella are incubated with cycloheximide for the same period of time, both soluble and mitochondrial MDH isozymes decrease to a similar extent (Table IX). These results are consistent with those shown in Table III and IV.

Table VIII. Effects of cycloheximide (CH) and chloramphenicol (CAP) on MDH activities in scutella excised from 4-day old seedlings.

Treatments[a]	Total MDH Activity (µM NADH oxidized/min. scutella)
Intact scutella (4 day):	10.90 ± 0.11
Excised scutella (4.0 day-4.62 day): control	10.96 ± 0.13
+ CH (5 µg/ml)	7.68 ± 0.06
+ CAP (1 mg/ml)	11.01 ± 0.07
Intact scutella (4.62 day)	13.05 ± 0.06

[a] Each fifteen scutella isolated from 4 day-old seedlings were transferred to nutrient media containing cycloheximide (5 µg/ml), or chloramphenicol (1 mg/ml), or no antibiotics against protein synthesis. After vacuum infiltration for two minutes, the samples were incubated in a water bath shaker at 25°C for 15 hrs. After incubation, MDH activities in these three sets of scutella and in scutella just isolated from 4.0 day and 4.62 day-old (4 day and 15 hrs) seedlings were determined.

Table IX. Effects of cycloheximide (CH)[a] and chloramphenicol (CAP)[b] on the development of the individual in maize scutella excised from 4-day old seedlings.[c]

MDH isozymes	Comparative MDH Activity[d] (%) 15 hrs in nutrient medium only (control)	15 hrs in CH	15 hrs in CAP
s-MDH^1	100	65	101
s-MDH^2	100	69	97
m-MDH^1	100	72	96
m-MDH^2	100	62	102
m-MDH^3	100	74	95
m-MDH^4	100	67	106
m-MDH^5	100	70	98

[a] CH: in a concentration of 5 µg/ml

[b] CAP: in a concentration of 1 mg/ml

[c] Treatments of the scutella are the same as that described in Table VII.

[d] Comparative MDH activity is calculated on a per scutellum basis. Isolation of each individual MDH isozyme is described under "Materials and Methods."

The results of all the experiments dealing with the effects of chloramphenicol and cycloheximide on protein synthesis and on MDH development in maize scutella indicate that, protein synthesis in the cytoplasm is necessary for the increase of both soluble and mitochondrial MDH activities which are observed in the course of sporophytic development. Protein synthesis in the mitochondria is not responsible for the increase of mitochondrial MDH activities.

Discussion

The maize s-MDH and m-MDH isozymes are controlled by two different groups of nuclear genes (Part I). Results of treating highly purified MDH 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 of the MDHs have eliminated the possibility that conformational alterations could account for MDH multiplicity in maize (Part II). Therefore, it is quite clear that maize MDH isozymes, both s-MDHs and m-MDHs, are genetically determined and are controlled by multiple genes. Detailed genetic analysis and sutdies of the physical and biochemical properties of these MDH isozymes suggest that s-MDHs and m-MDHs are coded by separate loci, the five commonly observed m-MDHs are controlled by two gruops of unlinked loci. Therefore, it would be interesting to study how the various maize MDH isozymes coded by different loci are expressed during the development.

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

The increased activities of s-MDHs and m-MDHs are due to *de novo* synthesis of these enzyme moieties themselves rather than a process which activates pre-existing MDH molecules. The use of D_2O as one of the density labels raises the possibility that, were the MDH a glycoprotein, the density shift could be entirely the result of deuteration of a carbohydrate moiety without synthesis of the protein moiety (26, 36). However, the low inherent density of the MDH 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 MDH 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, horseradish peroxidase A (37), known to be only 20% carbohydrate (38), has a density of 1.349 g/ml (26). A similar argument indicates that deuteration of a lipid moiety of MDH does not explain the observed density shift. Therefore, I conclude that both the soluble and mitochondrial MDH isozymes in the scutella of developing maize seedlings are synthesized de novo.

There is increasing evidence that mitochondria are able to synthesize by themselves some of their proteins (29, 30), however it has also been observed that many mitochondrial proteins are not synthesized in mitochondria (25, 29, 30, 39, 40). When yeast are incubated in medium containing cycloheximide and radioactive amino acids cytoplasmic protein synthesis is effectively blocked and virtually all of the labeled protein products are found in the inner membranes of mitochondria (34, 41). Similar results have been reported for liver tissue (42, 43) suggesting that the bulk of soluble matrix proteins of the mitochondria are synthesized in the cytoplasm. Recent studies (29, 30, 31, 32) indicate that in yeast, some subunits of the two inner membrane enzymes of mitochondria, namely ATPase and cytochrome oxidase are synthesized on the mitochondrial ribosomes. The studies of mitochondrial biogenesis in higher plant tissues are still very young and no such studies have been reported to my knowledge. Present studies on maize scutella, suggest that chloramphenicol may inhibit the synthesis of some specific mitochondrial proteins; on the other hand, cycloheximide does not inhibit non-specifically the synthesis of all proteins in scutellar cells, some specific proteins (likely some components of the inner membrane of mitochondria) are inhibited to a much lesser extent. The inhibitory effects of cycloheximide and chloramphenicol on the synthesis of such proteins appear to be compensatory. These results are consistent with those reported in yeast or animal cells (29, 30). In addition, they provided positive control for studying the effects of CH and CAP on the development of soluble and mitochondrial MDH isozymes. The increases of both s-MDHs and m-MDHs in scutella observed in the early sporophytic development were inhibited by cycloheximide but were not inhibited by chloramphenicol. A similar extent of inhibition by cycloheximide was observed for both soluble and mitochondrial MDH isozymes. It is thus apparent that protein synthesis on the cytoplasmic ribosomes is essential for the increase seen in both s-MDH and m-MDH activities during development. Mitochondrial protein synthesis, however, is not responsible for the increase of mitochondrial MDH activities. Since, as discussed earlier, the s-MDH and m-MDH isozymes are synthesized de novo, the above results suggest that not only s-MDHs, but also m-MDHs are synthesized on cytoplasmic ribosomes.

Longo and Scandalios (12) showed that the mitochondrial isozymes of MDH in maize are inherited in accordance with Mendelian rules and thus are controlled by nuclear genes. The results presented in this paper are entirely consistent with this finding and suggest that the nuclear gene controlled maize m-MDHs are synthesized in the cytoplasm and then become associated with the mitochondria. Whether the s-MDHs and m-MDHs are synthesized from a common amino acid pool or from separate amino acid pools is a very meaningful and important question. If they are synthesized from a common amino acid pool, it will further support our suggestion that both s-MDHs and m-MDHs are synthesized in the cytoplasm. Synthesis from separate amino acid pools might be a mechanism by which the s-MDHs and m-MDHs occur in different subcellular locations. For example, it may be that the s-MDHs are synthesized on free polysomes and are released into the cytoplasm once they are synthesized, while the m-MDHs are synthesized on rough microsomes and then transferred to mitochondria and incorporated into them. Several studies have shown that protein synthesized on microsomes can be transferred to mitochondria and incorporated into them. Several studies have shown that protein synthesized on microsomes can be transferred to mitochondria and incorporated into them (29, 39, 44, 45). At present studies, no indication was observed as which of the above mechanisms is actually operating in the maize scutella.

Another important question concerning the processing of the two classes of MDH isozymes after they are synthesized is: What is (are) the mechanism(s) by which only m-MDHs are incorporated into mitochondria, while the s-MDHs stay in the soluble cytoplasm. In the 4-day maize scutella, where the activities of m-MDHs and s-MDHs are rapidly increasing, we found very little m-MDH activity in the soluble fraction. That m-MDH activity which was found was very likely contamination from mitochondria broken during extraction procedures (Figure 2). Does this mean that even if the m-MDHs are synthesized in the soluble cytoplasm, they are not functional unless they are incorporated into mitochondria, or that after the m-MDHs are synthesized and before they are incorporated into mitochondria, certain modification(s) of the m-MDH molecules is (are) required? What kind of modifications could these be? Occurrence of glycosylations, methylations or phosphorylations have been found for numerous proteins after they are synthesized. Is one of these modifications required for m-MDHs, or are m-MDHs synthesized in the soluble cytoplasm only as zymogen molecules, and activated by some proteolytic enzymes once they are incorporated into the mitochondria.

It is also possible that the two s-MDHs are precursors of m-MDHs, once s-MDHs are modified, they will be able to be incorporated into mitochondria. This model would require that this modification be genetically controlled, since the genetic data (Part I) show that the m-MDHs are under independent genetic control, and that this modification should occur in mitochondrial membranes. Right after modification, the m-MDHs will be incorporated into mitochondria and will not appear in the soluble cytoplasm again.

Another possibility which might account for the organelle distribution of MDH isozymes may be the distinct characteristics of the s-MDH and m-MDH isozymes. The conformations of the s-MDHs and m-MDHs may be such that only m-MDH, and not s-MDHs, are capable of incorporation into mitochondria. Once m-MDHs are incorporated into mitochondria, the conformation of m-MDHs changes and they become functional.

None of the above possibilities has been studied in the present investigation, however, these would certainly be the important problems need to be solved in the near future.

Summary

Malate dehydrogenases (MDH) in maize have been classified according to their subcellular location; those found in the soluble fraction (s-MDH), those associated with the mitochondrial fraction (m-MDH), and those associated with glyoxysomes (g-MDH). The results shown in Part I and Part II indicate that the maize MDH isozymes are genetically determined, and are not conformers of a single gene product. Therefore, it would be interesting to study how the MDH isozymes are expressed during the development of young maize seedlings.

The developmental control of the two s-MDHs and the five m-MDHs has been studied using the inbred strain W64A. During early development of the sporophyte (dry kernel to 10 days of germination), the total MDH activity in scutella increases through the first five days, peaks about the 6th day and decrease gradually therefater. All of the scutellar s-MDHs and m-MDHs exhibit similar activity profiles in the scutellum, however, the total m-MDH activity is only 60% of that in the cytosol. In order to test whether the increased MDH activities in the developing scutella result from activation of pre-xisting MDH molecules or from *de novo* synthesis of the MDH molecules, density labeling experiments were performed. Both s-MDHs and m-MDHs extracted from scutella of seeds grown in H_2O with 10 mM $^{14}NH_4Cl$ had buoyant densities of 1.29 ± 0.0015 gm/cm^3. Attempts to detect the possible existence of "latent MDH isozymes" or "inactive MDH precursors" in the scutella were not successful. This finding indicates that both s-MDHs and m-MDHs in the scutella of developing maize seedlings are de novo synthesized.

Effects of protein synthesis inhibitors, cycloheximide and chloramphenicol, on MDH activities and on protein synthesis in scutella were studied. Within 40 hours of treatment, chloramphenicol (CAP; 0.5-2.0 mg/ml) does not inhibit the increase of MDHs. The increase of both s-MDHs and m-MDHs are not inhibited. Eight hours after the addition, cycloheximide (CH; 2-10 µg/ml) inhibits more than 70% of the increment of MDH activity, the increase of MDH activity is completely blocked thereafter. The increments of both soluble and mitochondrial MDH isozymes were inhibited to a similar extent.

Short term (15 hours) effects of chloramphenicol and cycloheximide on protein synthesis were studied by measuring their effects on incorporation of radioactive leucine into TCA insoluble materials. Cycloheximide (5 µg/ml) inhibits almost completely (about 97%) the incorporation of leucine into proteins found in soluble fraction, whereas, chloramphenicol (1 mg/ml) inhibits less than 5% of the synthesis of proteins found in soluble fraction. For the synthesis of proteins found in crude particulate fraction (12,000 x g pellet), only about 80-85% was inhibited by cycloheximide; while about 30-35% was also inhibited by chloramphenicol. Detailed analysis by SDS gel electrophoresis indicates that some proteins found in dense particulate fraction (likely the proteins of mitochondrial inner membrane) are specifically inhibited by chloramphenicol and cycloheximide. The results indicate that under the present experimental conditions, chloramphenicol has penetrated into scutella and inhibited the synthesis of some specific mitochondrial proteins; on the other hand, cycloheximide does not inhibit nonspecifically the synthesis of all proteins in scutella, some specific proteins (likely mitochondrial membrane proteins) are inhibited to a much lesser extent.

These results and the finding that the increases of both s-MDHs and m-MDHs are inhibited by CH, but not by CAP, suggest that protein synthesis in the cytoplasm, but not in the mitochondria, appears to be essential for the increase of both s-MDHs and m-MDHs activities during development. This result is consistant with the earlier findings that mitochondrial MDHs in maize are controlled by nuclear genes (12). Since both s-MDHs and m-MDHs in the scutella of developing maize seedlings are *de novo* synthesized, the above observations may indicate that maize m-MDHs, which are controlled by nuclear genes, are synthesized in the cytoplasm and then become associated with the mitochondria.

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PART IV

GENERAL DISCUSSION

An isozyme is defined as an enzyme existing in multiple molecular forms having identical or similar catalytic activities, and occurring within the same organism. In general, these isozymes differ from each other on the basis of their molecular sizes, charges and conformations as differentiated by means of electrophoresis, chromatography, immunochemistry and solubility. This molecular diversity of an enzyme has been found in nearly all organisms. Some one hundred different enzymes in various organisms have been observed to exist in isozymic forms. These multiplicities can be either genetically controlled or due to epigenetic effects.

The significance of isozyme does not lie in the multiplicity of an enzyme per se, but their role in cellular physiology and their adaptation in the organisms which carry the isozymes along the course of development. The isozymes, once genetically defined, may serve as useful intracellular markers for both genetic and developmental studies.

The expression of these isozymes may reflect directly the activities of their encoding genes. In addition, it may be a result of regulation of the activities among different genes (e.g., the interactions between "regulatory" genes and "structural" genes). By tracing these markers during the development, one may relate their cellular content, catalytic activity, and cellular location to their fuctional role(s) at specific developmental stages of the organism.

The physiological functions of the multiple forms of an enzyme have not been well demonstrated. There are two possibilities: 1) the various isozymes may have different physiological roles; 2) the multiple forms of an enzyme may work as a team and carry out certain physiological roles which could not be accomplished by a single form of enzyme. To study the physiological significance of the multiple molecular forms of an enzyme, one must first investigate how the various isozymic forms differ in their biochemical properties. The differences and similarities in their biochemical properties may then be used as a tool to study the possible physiological functions of isozymes described above.

There are several advantages for chosen maize as the experimental material in these investigations. It has a relatively short life cycle, well established genetic information, and is amenable to conditions for controlled pollination. Of particular interest is the fact that maize offers a system with monoploid tissue (pollen), diploid tissues, and triploid tissue (endosperm); with which one may study gene dosage effects on their enzymes and the possible interactions between isozymes coded by different genes.

In eukaryotic organisms, malate dehydrogenase (MDH) have been found both in mitochondria and in soluble cytoplasm. Those in mitochondria functions as a component of the Krebs cycle which plays a central role in the intermediate metabolism. The soluble malate dehydrogenase is a component of the malate shuttle which transfers reducing equivalent (NADH) across the mitochondrial membranes. In maize, isozymic forms of both soluble and mitochondrial malate dehydrogenases were observed. As discussed above, isozymes may serve as useful markers for studying many problems in biology, it would therefore be important and interesting to study the following aspects of maize malate dehydrogenase: 1) how the various maize MDH isozymes are controlled genetically; 2) how they differ in their biochemical properties; and, 3) how the various MDH isozymes are expressed during the development.

In Part I, genetic control of the expression of maize MDH isozymes have been demonstrated. The soluble MDHs and mitochondrial MDHs are likely to be controlled by two groups of different structural loci. The nuclear gene controlled mitochondrial MDH isozymes are coded by multiple structural loci residing on two different chromosomes. The present investigation is the first report in which genetic control of the first report in which genetic control of the polymorphic mitochondrial MDH isozymes are demonstrated. It is also found that certain genes which may reduce the viability of the kernel appear to be linked with the chromosomes carrying mitochondrial MDH isozymes. In addition, the genetic results suggest that "regulatory" genes are involved in the "expression" of MDH structural genes. These two findings have offered a good opportunity for future studies on "gene action in eukaryotic cells."

The isozymes within each of the two major classes may also differ significantly in their physiochemical and catalytic properties. This result suggests that, under variable conditions, the multiple isozymes having different biochemical properties may serve as a better enzyme system than a single enzyme form would do.

To my knowledge, the present study is the first case in which the mitochondrial MDH isozymes are genetically defined and their comparative biochemical properties are studied. Results of these studies suggest that gene duplication and chromosome translocation are probably involved in the evolution of maize mitochondrial MDH isozymes.

Developments of the maize MDH isozymes coded by different genes have been studied in the scutella of young seedlings. Both s-MDHs and m-MDHs exhibit a similar developmental pattern. Differential expression of the various MDH isozymes (which may reflect differential gene activation) has not been observed. Since the various MDH structural loci reside on, at least, two different chromosomes, a concomittant synthesis of these MDH isozymes may indicate that "expression" of their corresponding genes are integrated and are controlled by the same regulatory mechanism(s).

A clear separation of the density labeled MDH and the unlabeled MDH was observed in the density labeling experiments (Part III). In the future, it would be interesting to study the turnover (the synthesis and degradation) of each maize MDH isozyme by performing pulse and chase experiments.

The simultaneous increases and decreases of both soluble and mitochondrial MDH isozymes appear to be correlated to the growth conditions of the young maize seedlings. It is therefore suggested that in maize the malate dehydrogenase may be used as a marker enzyme for measuring the intracellular metabolic activities during the maize development.

Studies of the intracellular cite of the synthesis of mitochondrial MDH isozymes suggest that m-MDHs are synthesized in the cytoplasm and then become associated with the mitochondria. The result is consistent with the findings that maize mitochondrial MDHs are controlled by nuclear genes. Possible mechanisms involved in the subcellular compartmentation of soluble and mitochondrial MDH isozymes are the important and interesting problems for future studies and were briefly discussed in Part III.