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Genetic Control of Multiple Molecular Forms of Enzymes in Plants: A Review

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INTRODUCTION

Progress in the field of biochemical genetics of diploid organisms has been considerable in the last decade. Much of this has been made possible by the introduction of improved electrophoretic procedures utilizing various gel matrices for separation of mixtures of proteins. Investigators of genetic control of protein synthesis have effectively used this tool to estimate the number of genes involved in the production of a protein or enzyme. These methods have already proved useful in providing information as to the number of polypeptide subunits that make up a protein molecule in studies with microorganisms (Levinthal *et al.*, 1962), animals (Markert, 1963), and plants (Scandalios, 1965*a*). In conjunction with the high-resolution "zymogram" method for displaying enzyme activity on gels (Hunter and Markert, 1957), the gel electrophoretic procedures have afforded the geneticist a means to study mutations which presumably alter the structure of enzymes, resulting in differential electrophoretic mobilities of the molecules, while their catalytic activity is retained. This is a new and promising dimension for studying gene action, since before the advent of these techniques similar studies were essentially confined to enzyme variation due to alterations in total catalytic activity.

A large number of electrophoretic variants of enzymes have now been discovered (Shaw, 1965). With these findings came the knowledge that enzymes may exist in the same organism in more than one molecular form. Such multiple molecular forms of an enzyme in a single organism have been designated *isozymes* (Markert and Møller, 1959). Isozymes may differ in primary structure because they are encoded in different genes, either allelic or nonallelic. The primary structure may be further

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modified by conjugation of molecules with reactive groups, such as amino, carboxyl, or hydroxyl groups of the amino acid residues of the polypeptide chain.

The term *isozyme* is used here in the operational sense, as proposed by Markert and Møller (1959), to refer to multiple molecular forms of an enzyme, with similar or identical catalytic activities, occurring within the same organism. This definition embraces several distinct kinds of isozymes; their precise definition will depend on the progress of studies on their origin and nature. Thus, Markert (1968) has recently proposed that one might modify *isozyme* with such terms as allelic, nonallelic, homopolymeric, heteropolymeric, conformational, hybrid, conjugated; whereas Shaw (in press) has classified isozymes into two basic categories: (1) those which are distinctly different molecules and are presumably produced from different genetic sites and (2) those which result from secondary alterations in the structure of a single polypeptide species and may, in many cases, be *in vitro* artifacts. This discussion deals primarily with Shaw's first category. However, I also employ the term *isozymes* to denote a third type of multiple enzyme, i.e., the heterozygous state in genetically variant enzymes. Shaw (in press) prefers not to include these among the forms of isozymes, but concedes (personal communication) that the classification is arbitrary.

The number of studies of genetic control of isozymes in plants has been small in comparison with such studies in the animal kingdom (Wroblewski, 1961; Shaw, 1965). The earliest study dealing with genetic aspects of isozymes in higher plants was the work on the multiple esterases in maize endosperm (Schwartz, 1960). The genetics of other isozyme systems has also been mostly investigated in *Zea mays*. Some of these systems are "leucine aminopeptidase" (LAP) (Beckman *et al.*, 1964; Scandalios, 1964; 1965*c*), catalase (Scandalios, 1965*b*; 1968), amylase (Scandalios, 1966*a*), and alcohol dehydrogenase (Scandalios, 1966*b*; 1967*a*).

This review is not intended to cover all work on isozymes in plants. It is confined to studies which were undertaken in efforts to examine basic genetic principles applicable to all organisms. It is further hoped that the review will encourage the use of plant material by geneticists who are less concerned with the organism *per se* and more interested in resolving general genetic and molecular phenomena. Plants lend themselves well as experimental organisms for the solution of many of these problems. It is also the author's intention to stress, with examples, the usefulness of isozymes whose genetics is well understood as effective markers in developmental genetics, in studies on differentiation, and in studies on the physiological roles of specific isozymes. The possible physiological role of individual isozymes is one of the most important questions to be answered in our efforts to understand the significance of multiple molecular forms of enzymes in any organism.

It is toward these ends that the literature review is both selective and subjective.

The survey of literature for this review was concluded in April 1968. Any information where reference is not given indicates work from this laboratory which is either unpublished or in press.

AMYLASE

Amylases afford an excellent opportunity for the study of multiple molecular forms of enzymes. Both α-amylase and β-amylase are known to occur in higher plants, and

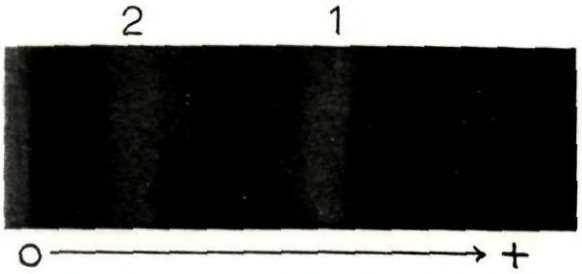

Fig. 1. Zymogram showing amylase isozymes in 3-day-old maize seedlings. Numerals indicate zones of amylase activity. Arrow indicates direction of migration.

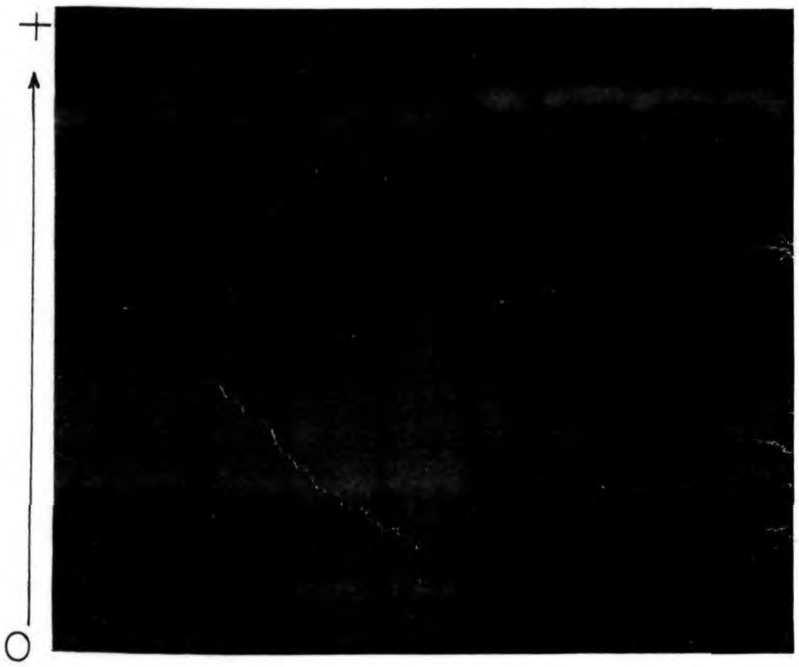

Fig. 3. Cyanogum-41 zymogram showing amylase variation in 16-day-old maize endosperm of several inbreds. Arrow indicates anodal migration.

their chemical and physiological activities are fairly well understood. Germinating cereal seeds are known to produce and secrete starch-hydrolyzing enzymes which have as their sole physiological function the digestion of the starch reserves of the storage tissues. This fact allocates the amylases a role in development and differentiation worthy of investigation. Recent results by Varner and his associates (see Varner and Johri, in press) on barley aleurone α-amylase render this enzyme system amenable to the investigation of hormonal effects on gene action, since they demonstrate *de novo* synthesis of α-amylase in response to treatment of the tissue with exogenous gibberellin.

In recent years, the existence of multiple molecular forms of plant amylases has been established in maize (Scandalios, 1965*a*), tobacco (Jaspars and Veldstra, 1965), barley (Frydenberg and Nielsen, 1965), and peas (Espiritu and Scandalios, unpublished).

Genetic Variants of Amylase in Maize

The first genetic studies of amylase isozymes in plants were reported by Scandalios (1966*a*) in inbred lines of *Zea mays*.

Using extracts from 3-day-old seedlings of several inbred lines of maize and starch gel electrophoresis, Scandalios found electrophoretic variants of multiple amylases (Fig. 1). Hybrid patterns obtained from F_1 crosses generally consist of the maximum number of bands present in either parent. However, in the F_1 hybrid (AA9♀ × AA2♂) and its reciprocal the slow zone appears to be intermediate to the parental enzymes, suggesting formation of a hybrid molecule (Fig. 2). Backcrosses yield segregation ratios which suggest that the amylases in zone 2 are controlled by codominant alleles. The formation of a hybrid molecule indicates that the active enzyme is a dimer composed of two subunits. More recently, a gel assay method has been developed in this laboratory (Chao and Scandalios, unpublished) utilizing Cyanogum-41 as the gel matrix instead of starch (see Appendix I). This method is more sensitive and allows resolution of amylase isozyme activity at the liquid endosperm stage of maize development. It further allows for easier manipulation (elution,

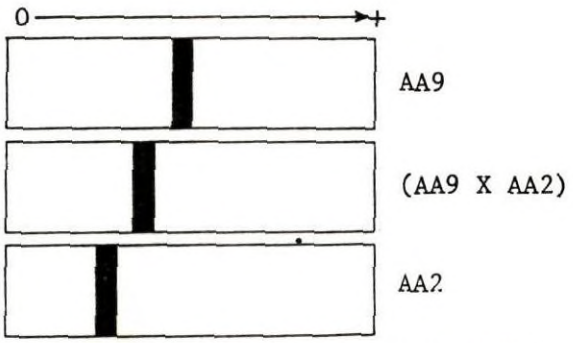

Fig. 2. Scheme showing the amylase variants detected in the inbreds AA9 and AA2 and their resulting heterozygote. Note the intermediate position of the single band in the heterozygote.

etc.) of individual amylase isozymes and thus offers distinct possibilities relating to the chemical characterization (i.e., distinction between α- and β-amylases) of these isozymes. A typical zymogram resulting from this procedure is shown in Fig. 3. It is a distinct advantage to be able to assay the amylases at the liquid endosperm stage of development, because it permits study of gene dosage effects and also shortens the generation time, since the kernels on the parent plant will be the F_1 progeny.

Genetic Variants of Amylase in Barley

Frydenberg and Nielsen (1965) investigated the occurrence of α- and β-amylase isozymes in germinating barley seeds in a search for genetic variants, analyzing single kernels of 98 barley varieties by means of agar gel zone electrophoresis. They found a *maximum of nine individual zones of amylase per kernel of each variety (Fig. 4).*

Of the nine zones, five proved to be α-amylases, two β-amylases, and two zones could not be identified by the properties examined (see Fig. 4). Although the authors found no electrophoretic variants of the nine bands, they were able to convert the various α-isozymes into either one band (the D band) or two bands (the D and C bands) on treating the seed extract with heat. Hence the C and D bands are seemingly the most "stable" α-amylases.

Using these heat-stable α-amylases as a basis, the authors re-examined all the 98 barley varieties and classified them as one- or two-banded types. No exceptions to these two types were found. From these data, the authors proposed three basic rules governing the inheritance of the alternative of having one or two stable α-amylases following heat treatment: (1) when a daughter variety has originated by mere selection or mutation, it has the same number of stable α-isozymes as the mother variety; (2) whenever two varieties with the same number of stable α-amylases have yielded a new variety by hybridization, the daughter variety has the parental type α-amylases; (3) when varieties with different numbers of stable α-amylases have been crossed to give new varieties, these are either of one or the other parental type. These types segregate in approximately a 1:1 ratio, suggesting a simple Mendelian inheritance for the stable α-amylases in barley.

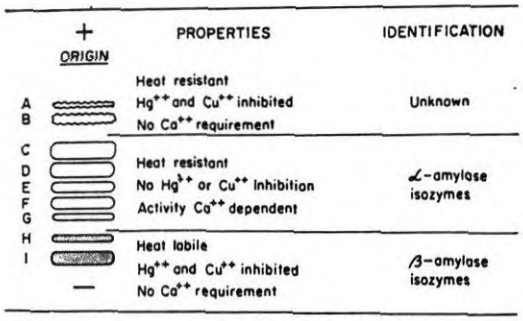

Fig. 4. Diagrammatic zymogram showing the nine zones of amylase activity of barley grains and their characteristic properties (from Frydenberg and Nielsen, 1965).

Although Frydenberg and Nielsen's data indeed suggest simple Mendelian inheritance, we must bear in mind that the variants were induced by an "unnatural" heat treatment. Thus they may not reflect the true genetic basis for the isozymes, but rather the genetic capacity of the α-amylases to be heat-labile or heat-stable when exposed to the proper environment. That is, heat stability may be controlled by another gene(s), rather than by the structural gene(s) for the specific amylases. It must also be pointed out that all of the barley amylase isozymes in this system were found to migrate toward the cathode during electrophoresis. It would normally be expected that the amylases would migrate toward the anode since their isoelectric points (5.7–5.8) are lower than that of the buffer used (*p*H 7.3; μ 0.02). The authors explain this cathodic movement as being the result of excessive endosmotic flow characteristic of agar gel electrophoresis. This flow apparently proceeds faster toward the cathode than do the amylases toward the anode, relative to the buffer. This high endosmosis may affect the individual isozymes in relation to each other, and this probability has not been excluded by Frydenberg and Nielsen.

Despite the reservations just stated, both the study of Scandalios (1966*a*) in maize and that of Frydenberg and Nielsen (1965) in barley strongly suggest simple Mendelian control of the amylases in the higher plants examined. Improved methods for the qualitative assay of genetic amylase types should give further information on the genetic control as well as the structure and possible genetic–structural relationships that may exist between the α- and β-amylases.

CATALASE

Catalase ($H_2O_2 : H_2O_2$ oxidoreductase; 1.11.1.6) is a heme protein which acts specifically on hydrogen peroxide. Catalases have four iron atoms per molecule attached to the protein and chelated to protoporphyrin IX. Catalase is widely distributed in animals, plants, and microorganisms. However, its biological role remains unknown, although its presence seems to be important as an accessory to the consumption of oxygen by organisms. Crystalline catalases have been obtained from a variety of animal sources (Dixon and Webb, 1964), but plant catalases have so far defied all attempts at purification and crystallization.

Electrophoretic Variants of Maize Endosperm Catalase

By methods previously described (Scandalios, 1964; 1968) and appended to this review, six distinct electrophoretic variants of catalase have been found in maize endosperm.[2] Five of these variants have been designated F, K, M, S, and V, in order of descending anodal electrophoretic mobility at *p*H 8.5 (Fig. 5). The sixth variant, V′, is distinct from the other five in that it has three bands of catalase activity—a major band corresponding to the V variant and two additional subbands. The frequency with which these variants were found to occur in 152 inbred lines of maize is shown in Table I.

[2] The endosperm of most angiosperms contains two maternal and one paternal set of chromosomes. Two alleles can therefore be combined in four different genotypes: $Ct^F/Ct^F/Ct^F$, $Ct^F/Ct^F/Ct^S$, $Ct^F/Ct^S/Ct^S$, $Ct^S/Ct^S/Ct^S$.

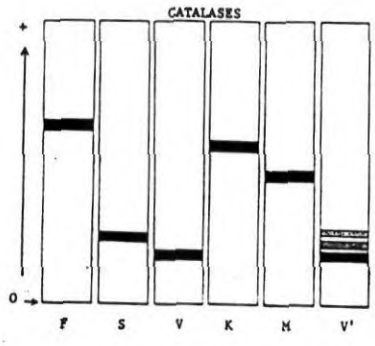

Fig. 5. Schematic zymogram showing the six electrophoretic variants of maize endosperm catalase. 0 = point of sample insertion. Letters indicate the different catalase variants.

Table I. Frequency Distribution of the Six Electrophoretic Variants of Maize Endosperm Catalase in a Population of 152 Inbred Strains of *Zea mays* Examined

Catalase phenotype	Observed	Frequency of occurrence
F	35	0.230
K	6	0.040
M	2	0.013
S	65	0.430
V	43	0.280
V'	1	0.007
Total	152	1.000

Genetic Control of the Catalases

In the heterozygote between any two of the electrophoretic variants, catalase is resolved into five bands—the parental types and three new "hybrid" molecules (Fig. 6). The heterozygote patterns differ with respect to the activity concentration (staining intensity) of individual bands, depending upon the direction in which the cross was made. This pattern of activity concentration in the heterozygote is undoubtedly the result of the double gene dose from the female parent due to the triploid nature of the endosperm.

Crosses between all lines with different catalase phenotypes were made, and Table II shows the results with the F and S variants. Similar data were obtained in crosses between all other variants; these data are to be found in a separate publication (Scandalios, 1968). From Table II it can be seen that backcrosses yielded parental and hybrid types (two types SH and FH, depending on the direction of the cross) in close agreement with the expected 1:1 ratio. In the F_2 progeny, four types were found—the two parental types and the two hybrid types (i.e., F, FH, SH, S).

Thus, all evidence to date suggests that maize endosperm catalase is regulated by six allelic genes as shown in Table III, the alleles acting without dominance.

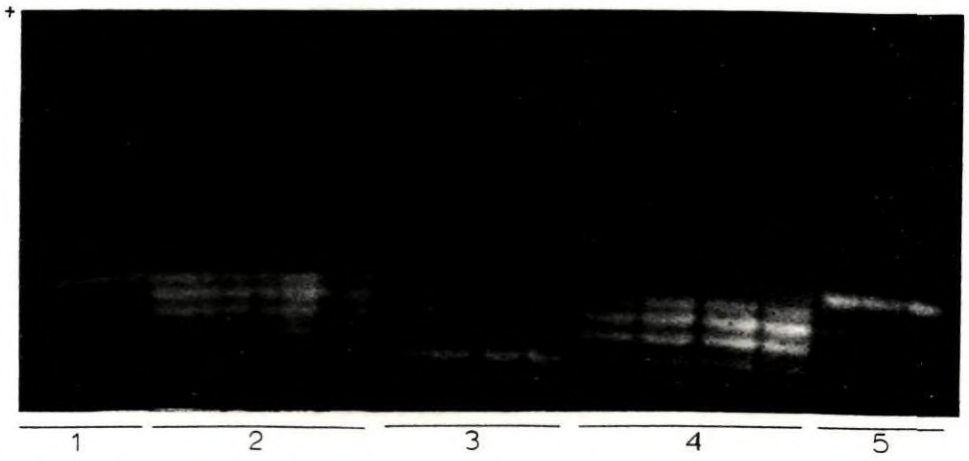

Fig. 6. Zymogram showing the two types of heterozygotes generated in reciprocal crosses between the F and V catalase variants in maize. 1 and 5 are F-type catalase variants and 3 is a V-type catalase variant, which served as the common parent in these reciprocal crosses. 2 is a heterozygote with activity concentration in the fast zone (FH); the F variant was the female parent. 4 is a heterozygote (VH) resulting when the V variant was used as the female parent.

Table II. Summary of Crosses Demonstrating the Inheritance of the Catalase Variants in Maize. The Parental Genotypes Are Deductions Fitting the Results (from Scandalios, 1968)

Parent		Catalase patterns of offspring				Total
♀	♂	F	FH	SH	S	
FF	FF	250	0	0	0	250
SS	SS	0	0	0	155	155
FF	SS	0	95	0	0	95
SS	FF	0	0	95	0	95
FS	FF	44	0	36	0	80
FF	FS	37	48	0	0	85
FS	SS	0	42	0	38	80
SS	FS	0	0	33	47	80
FS	FS	21	24	29	16	90

Table III. Summary of the Catalase Variants of Maize Endosperm and the Allelic Genes Which Specify Them

Catalase-I locus	
Enzyme phenotype	Allelic gene
Catalase-F specified by	Ct^F
Catalase-K „ „	Ct^K
Catalase-M „ „	Ct^M
Catalase-S „ „	Ct^S
Catalase-V „ „	Ct^V
Catalase-V′ „ „	$Ct^{V'}$

Genetic Evidence for the Structure of Catalase

The formation of the hybrid enzymes and the apparent gene dosage effects in the heterozygotes suggest that the maize catalases exist functionally as tetramers. Thus the hybrid enzymes would result from random association of two distinct kinds of subunits, coded by the respective alleles of the catalase gene. The expected isozyme patterns of both diploid and triploid (endosperm) tissues, based on expanding the appropriate binomial, are presented schematically in Fig. 7.

Chemical Evidence for the Subunit Structure of Catalase

In an effort to substantiate the tetrameric nature of the catalases of maize endosperm from a chemical viewpoint, *in vitro* hybridization experiments were done, following dissociation of the active enzymes (Scandalios, 1965*b*). The procedure involved mixing equal amounts of any two of the catalase variants in neutral phosphate buffer (*p*H 7.0)

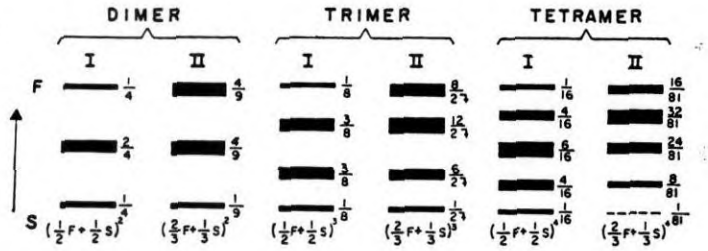

Fig. 7. Schematic presentation of the theoretically expected proportions of isozyme components for different multimers in heterozygotes. F and S represent the two variant types. In each case I = the expected pattern and proportions in diploic tissues; II = the expected pattern and proportions in triploid tissues. The patterns and proportions in each case can be obtained by expanding the binomial given at the bottom of each pattern (from Scandalios, 1968).

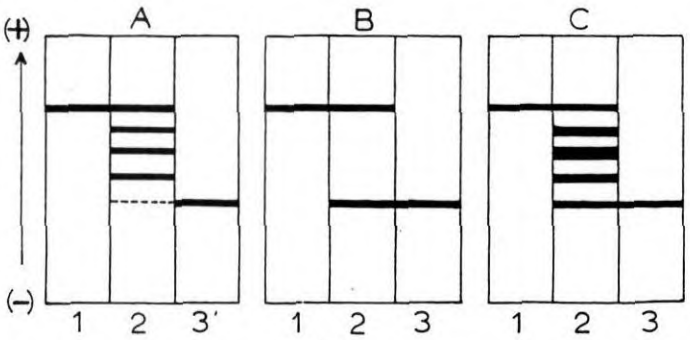

Fig. 8. Scheme of experiments done to establish the subunit structure of catalase. In all cases 1 = the F-catalase variant, and 3 = the V-catalase variant. A-2 = pattern from the F_1 hybrid, F × V; B-2 = pattern obtained from a mixtute of F and V in water; C-2 = pattern obtained by freezing and thawing a mixture of F and V in 1 M NaCl (from Scandalios, 1965*a*).

containing 1 M NaCl. The mixture was frozen, thawed, and subjected to electrophoresis. Mixture of the two variants used either in water or in buffer and extracts from the biological hybrids served as references. The results from such an experiment, using the F and V variants, are shown in Fig. 8. Simple mixtures of the two variants in water or buffer, or merely mixtures of the liquid endosperm, resulted in a pattern showing both parental types without any intermediate forms (Fig. 8B). The biological hybrid, with the apparent gene dosage effects, is shown in Fig. 8A; and the result of the freeze–thaw experiment in Fig. 8C. It is obvious that the five isozymes characteristic of the heterozygote can be effectively generated *in vitro* in the predicted binomial proportions. Note the lack of gene dosage effects in the *in vitro* hybrid, the pattern being characteristic of the distribution expected for diploid tissues (refer to Fig. 7). Hence, upon mild salt dissociation, the individual polypeptides retain their polymerizing

capacity and are capable of reassociating in a random fashion to generate the tetrameric molecules. These experiments further suggest that the primary gene product is the monomeric subunit, or single polypeptide, and that these aggregate in a random fashion to form the active tetramers.

That catalase in maize may also exist in other multimeric forms has been suggested by Scandalios (1968), on the basis of two genetic variants, at a locus other than that discussed above, which on crossing generate a total of four isozymes in the heterozygote—two parental forms and two hybrid forms. The proportions of these four isozymes show a close fit to a distribution of 8:12:16:1, expected for a trimeric situation. However, the genetic evidence alone is not sufficient to eliminate other possibilities and attempts at *in vitro* hybridization of these variants have thus far been unsuccessful.

Tissue Specificity of Catalase Isozymes

Isozyme patterns undergo marked changes in the course of the development of an organism. Such changes are found during the development of a particular organ, e.g., the endosperm, and are also reflected by differences in the isozyme patterns of different organs or tissues. For purposes of organization, these two aspects of the developmental variation of isozyme patterns will be treated separately in this review.

The distribution of catalase isozymes in various tissues of the maize sporophyte was investigated by Scandalios (1964; 1968). The major findings (see Fig. 9) were: (1) Electrophoretically identical isozymes may be present in different tissues, but varying in concentration, as seen in the case of the liquid endosperm *versus* the leaf.

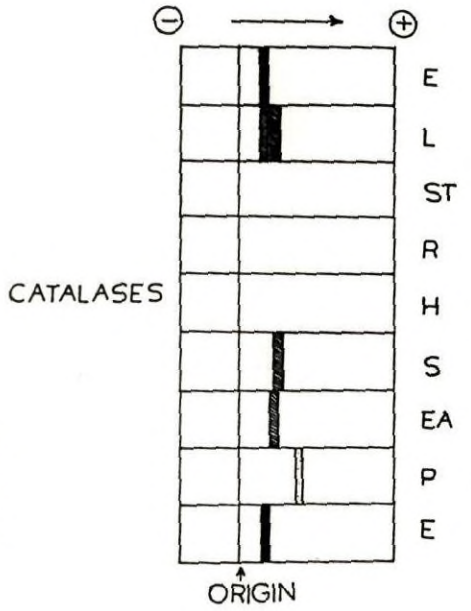

Fig. 9. Scheme showing catalase patterns of different tissues in an inbred of the F-type catalase. = 16-day-old endosperm; L = leaf; ST = stem; = root; H = husks; S = silks; EA = young ear; = pollen. Note absence of catalase in the root, em, and husk (from Scandalios, 1964).

(2) An isozyme may be absent at a specific developmental stage or occur in such low concentrations that it cannot be detected by the methods employed. (An example of this is the absence of catalase in stem, root, and husk extracts.) (3) Electrophoretically distinct isozymes may occur in the various tissues, as exemplified by pollen, silks, young ear, and leaf homogenates.

The distribution of catalase isozymes in different tissues, in conjunction with knowledge of the physiological roles of these tissues, will perhaps provide clues as to the possible biological function of the catalases.

Developmental Studies of Catalase Isozymes

During the differentiation and development of the maize sporophyte, shifts in number and intensity of catalase isozymes have been found in the early stages of development of the maize plant (dry kernel to 5-day-old seedling) and appear to be characteristic of the individual genetically inbred strain. An example is shown in Fig. 10. There is an apparent shift of one electrophoretic form to another during this stage of development. When the two extreme forms are isolated and subjected to *in vitro* hybridization experiments, a total of five isozymes is generated. This indicates dissimilarity of the subunit polypeptides of the two catalase forms and suggests separate genetic coding for each of the two catalase forms.

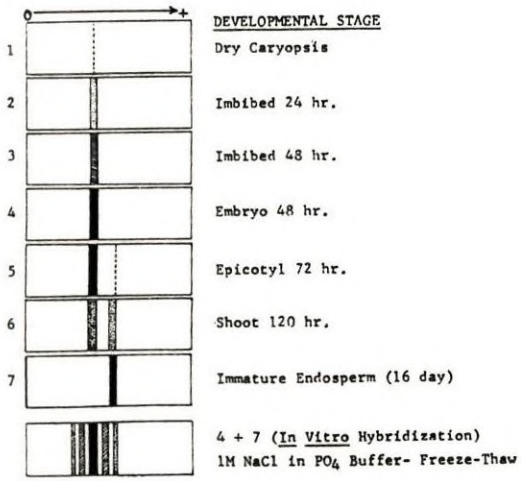

Fig. 10. Schematic zymogram showing developmental shifts of catalase isozymes in the course of development and differentiation of an F-type catalase variant in maize. When isozymes are isolated from stages 4 and 7 and subjected to *in vitro* hybridization, five isozymes are generated, indicating different subunit structure for the two developmental-stage specific catalases.

Such studies show that the catalase patterns observed in early sporophytic development do not correspond to those exhibited by tissues in the adult plant, at least in the majority of inbred strains examined. These findings thus suggest differential activation of either genes or gene products; that is, the apparent sequential shifts in catalase isozyme patterns may reflect either changes in relative activities of different genes, or differential assembly of gene products (i.e., polypeptide subunits) on the ribosomes. Whatever the case, the catalase system offers good possibilities for the study of developmental genetics in higher organisms.

ALCOHOL DEHYDROGENASE

Alcohol dehydrogenases (alcohol:NAD oxidoreductase, EC 1.1.1.1), enzymes widely distributed among animals, plants, and microorganisms, catalyze in the presence of nicotinamide-adenine dinucleotide (NAD) the following reaction:

$$RCH_2OH + NAD \rightleftarrows RCHO + NADH_2$$

The reaction is not highly specific with respect to substrate. ADH can react with a large number of normal and branched-chain aliphatic and aromatic alcohols (primary and secondary).

Isozymes of alcohol dehydrogenase (ADH) have been reported in a variety of organisms, with the first detailed genetic analysis having been done in *Drosophila* (Ursprung and Leone, 1965; Grell *et al.*, 1965). Somewhat later, isozyme variations in plants were reported by Scandalios (1966*a*; 1967*a*) and by Schwartz and Endo (1966).

In maize, alcohol dehydrogenase has been found in the endosperm of 16-day-old kernels, the scutellum of mature kernels, and the plumule and root of young seedlings.

Electrophoretic Variants of Alcohol Dehydrogenase

Two major zones of alcohol dehydrogenase activity are found in the scutellum of dry seeds and the 16-day-old endosperm of maize. Both zones were present in all maize strains examined and were anode-migrating at *p*H 8.0 (Fig. 11), but the relative rates of their respective electrophoretic mobilities in several inbred lines were found to differ. In addition to these variants, a third electrophoretic variant, called ADH-C, has been reported (Schwartz and Endo, 1966). However, we have to date examined over 200 inbred strains of maize and have not encountered the ADH-C variant; thus the gene determining this variant must be rare.

Genetics of Alcohol Dehydrogenase

Genetic data obtained by Scandalios (1967*a*) support the hypothesis that there are two closely linked loci controlling ADH production in maize. The most anode-migrating zone (ADH-1) and the slower zone (ADH-2) are controlled, respectively, by the genes Adh^1 and Adh^2. Each locus is diallelic, with Adh^{1F} specifying the fast variant and Adh^{1S} specifying the slow variant at the ADH-1 zone, and Adh^{2F} and Adh^{2S} specifying the fast and slow variants, respectively, at the ADH-2 zone; the alleles act without

dominance. The allelic products of the ADH-2 gene interact to generate a hybrid mole cule in the heterozygote, in addition to the parental-type molecules; however, there i no hybrid molecule generated in the F_1 relative to the ADH-1 gene, presumably due t lack of interallelic (intracistronic) interaction. There is apparently no intergeni (intercistronic) interaction.

The gene dosage effects, due to the triploid nature of the endosperm, and th generation of the intermediate hybrid molecule in the ADH-2 heterozygote (see Tabl IV and Fig. 12) support the hypothesis that ADH-2 exists functionally as a dimer whereas ADH-1 may exist as a monomer, or possibly a dimer with affinity for identica polypeptide chains.

The evidence for two loci, as stated above, is based primarily on the lack o chemical interaction between the products of the two systems (zones) and on th

Table IV. Results Arranged to Demonstrate the Two Types of Heterozygous Electrophoretic Patterns Obtained from Gene Dosage Effects (from Scandalios, 1967*a*)

Female parent	Male parent	ADH patterns in offspring				
		F	FH	SH	S	Total
F(F^1/F^1)	S(S^1/S^1)	0	90	0	0	90
S(S^1/S^1)	F(F^1/F^1)	0	0	90	0	90
F(F^2/F^2)	S(S^2/S^2)	0	90	0	0	90
S(S^2/S^2)	F(F^2/F^2)	0	0	90	0	90

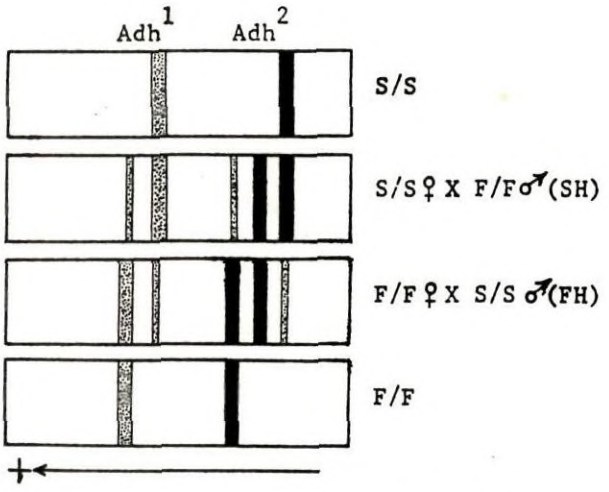

Fig. 12. Scheme showing the two types of ADH heterozygotes generated in reciprocal crosses as a result of gene dosage effects in the endosperm of maize.

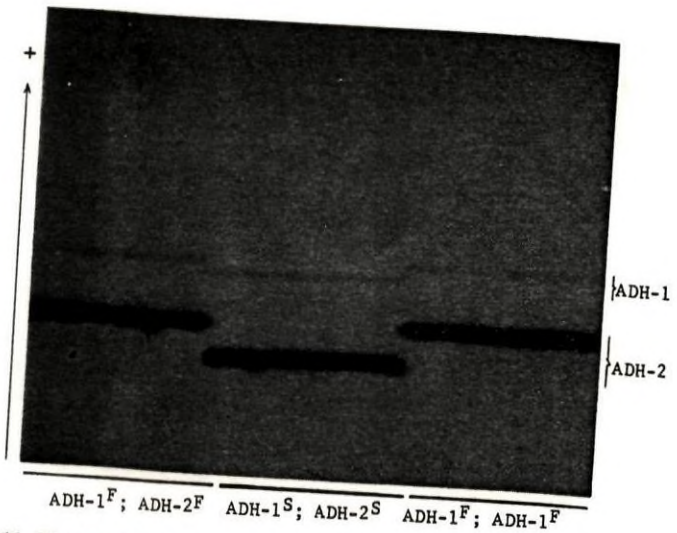

Fig. 11. Electrophoretic variants of maize alcohol dehydrogenase. The two distinct zones of activity, ADH-1 and ADH-2, are indicated on the right. The phenotypes for each zone are indicated at the bottom.

recovery of one (one kernel) recombinant (Adh^{2F}/Adh^{1S}) among the many progeny examined. That only one recombinant was recovered suggests very close linkage. Verification of the two-loci hypothesis must await further clarification at the genetic and chemical levels.

The suggestion that the products of the ADH-1 gene may be monomers has been rejected by Schwartz (1966) on two counts. Firstly he concluded that the ADH-1 and the ADH-2 enzymes did not differ significantly in molecular size. However, this conclusion was based only upon the altered starch concentration technique of Smithies (1962) for the estimation of molecular size, and this technique is not applicable in some situations. Moreover, Schwartz used significantly different electrophoretic conditions than those specified by Smithies (e.g., acid *versus* basic *p*H of gel). Gel filtration methods would probably have given more precise determination of molecular weight. Schwartz's second reason for rejecting the monomer–dimer hypothesis is based on the assumption that the relative migration rates of the faint and intense ADH zones among the variant stocks should be the same. He makes no mention of the possibility that the free monomers might undergo conformational changes which could lead to the observed differences. Schwartz (1966) has proposed a model fitting the dimer hypothesis. To do this, however, he hypothesized the existence of another locus, evidence for which he reports to have occasionally encountered on the zymograms, and which he calls ADH-X. This model is schematically presented in Fig. 13a and b. However, it must be pointed out that the model applies equally well to the monomer–dimer hypothesis (see Fig. 13).

In summary, there is no compelling evidence against the monomer–dimer model; nor is there absolute data supporting the two-loci hypothesis.

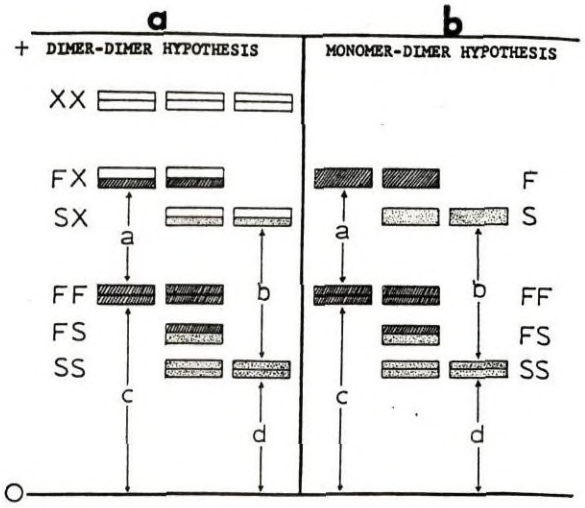

Fig. 13. The restriction that the relative distances (a/c and b/d) be the same if the monomer–dimer hypothesis is true applies equally well to the dimer–dimer hypothesis suggested by Schwartz.

ESTERASES

The esterases (E.C. 3.1) are a complex and heterogeneous group of enzymes. They show comparatively low substrate specificity in that they hydrolyze *in vitro* a very large number of different esters, although at different rates. However, all these enzymes hydrolyze specifically the ester link, according to the following general equation:

$$R-COOR'+HOH \rightleftarrows R-COOH+R'OH$$

It has been suggested (Myers, 1960) that the apparent overlap in substrate specificity between the different esterases may be due to an indiscriminate bond-breaking mechanism which involves any ester which can approach the active enzyme center. In addition, enzymes such as carbonic anhydrase, trypsin, and chymotrypsin have often been classified as esterases since these also attack α-naphthylacetate, one of the artificial esterase substrates. The natural substrates and *in vivo* actions of most of the esterases are not known.

There has been a considerable amount of work done upon the genetics, tissue specificity, and chemical properties of the esterases, but most of the work has been with animals (Augustinsson, 1958; Ogita and Kasai, 1965; Tashian, 1965). The only instance in which plant esterases have been investigated with a definitiveness approaching that in the work upon the animal esterases is the work of Schwartz and co-workers.

Electrophoretic Variants of Esterases

Schwartz (1960) reported the occurrence of qualitatively different esterases in maize, employing α-naphthyl acetate as the substrate and Fast Blue RR salt as the dye-coupler for the zymogram display of the enzymes. Schwartz has described three distinct loci, E_1, E_2, and E_3, controlling maize esterases, but his further efforts have been concentrated on the genetics of the E_1 esterase locus. The enzyme product of this locus has been designated by Schwartz as the *p*H 7.5 esterase, since the enzyme remains at the origin, upon electrophoresis, at this *p*H. Since the *p*H of the gel may vary during the electrophoretic run, it is questionable whether the true isoelectric point of the enzyme is at *p*H 7.5.

Genetic Control of the Esterases in Maize

Genetic analyses showed that there are seven alleles at the E_1 locus, each of the alleles specifying an esterase with a characteristic electrophoretic mobility. Schwartz *et al.* (1965) have labeled these alleles, in descending order from the cathode, as E^F, E^L, E^N, E^R, E^S, E^T, and E^W. Most of the formal genetics has been done using the E^F, E^N, and E^S alleles. When crosses were made between plants carrying any two of these alleles, the resultant heterozygotes produced not only the esterases characteristic of the alleles of the parents, but in addition an esterase of intermediate electrophoretic mobility (Fig. 14).

On the basis of these data Schwartz has proposed the hypothesis that the functional maize esterases are dimers. No chemical support (e.g., *in vitro* hybridization) for this hypothesis is so far available.

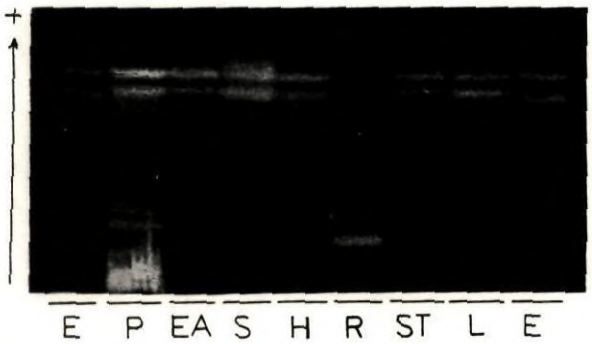

Fig. 15. *Zymogram showing esterase patterns in various tissues of maize.* E = 16-day-old endosperm; L = leaf; ST = stem; R = root; H = husk; S = silks; EA = young ear; P = pollen. All tissues except pollen and young ear were taken at the same time of development (from Scandalios, 1964). Photograph taken of negative by transillumination for clear visualization.

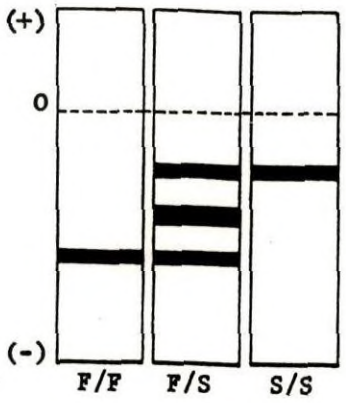

Fig. 14. Schematic zymogram depicting the F and S variants of the E_1 esterase in maize and the resulting heterozygote. Note the hybrid enzyme in the heterozygote (after Schwartz, 1960).

Tissue Distribution of the Maize Esterases

Endo and Schwartz (1966) reported that some of the E_1 esterases (N-type) were present in the developing endosperm, in the plumule, and in lower concentrations in the radicle of maize seedlings. Although the same allelic products are found in different tissues, their sensitivity to denaturation by urea seems to vary, and Schwartz has proposed the possibility that this difference in response to urea treatment may be due to differences in conformation of the esterases in different tissue environments. However, since the enzymes employed in this investigation were not purified preparations, the variation with respect to urea treatment cannot be interpreted as unequivocal proof of differences in the quaternary structure of the enzymes.

Tissue specificity studies have also been reported (Scandalios, 1964) for the *anodic* esterases of an esterase-F inbred. The zymograms show a fast migrating zone with little variability among the different tissues (Fig. 15). The endosperm has three distinct bands in this zone, one of which is also present, in varying concentrations, in all other tissues. The roots and leaves have a common slow band, but with distinct concentration differences. Pollen shows a unique pattern of three slow-moving bands, none of which is found in any of the other tissues. Other minor bands with even slower mobility were also found, but were obscured in this zymogram by gel deterioration at the origin, due to the presence of amylase activity. Other bands are faint, but apparent, in the zymogram.

In the course of these investigations, utilizing different inbred strains, it has become apparent that the anode-migrating esterases follow simple Mendelian inheritance. However, there are no detailed data at present as to the number of genes involved in the inheritance of these enzymes and the possible relationships which may exist with the E_1 system.

"LEUCINE" AMINOPEPTIDASE

Leucine aminopeptidase (LAP) is operationally defined as an enzyme that hydrolyzes the chromogenic substrate L-leucyl β-naphthylamide HCl. Its activity is accelerated by

the presence of either Mg or Mn ions. Chemically, LAP can be defined as being an exopeptidase which hydrolyzes peptide linkages adjacent to the free α-amino groups of a peptide. The reaction results in a free amino acid and a smaller peptide, and the process may be repeated since the smaller peptide would also contain a free amino group (Dixon and Webb, 1964).

LAP had earlier been shown, in animals, to have some degree of overlapping substrate specificity. Recently we have found a large degree of overlapping substrate specificity for pea and maize "LAP" (Table V); more data will be presented elsewhere. Because of this finding the general term "aminopeptidase" is perhaps more appropriate than the more restrictive term "leucine aminopeptidase," but the latter is being used in this review in keeping with previous work, although strictly on the basis of the operational definition.

LAP is widely distributed in animal tissues and intestinal secretions (Beckman, 1966; Scandalios, 1967*b*), as well as in plants (Scandalios, 1965*a*; Scandalios and Espiritu, unpublished).

Electrophoretic Variants of Maize LAP

Four electrophoretic LAP zones were found in extracts of liquid endosperm of several inbred strains of maize. These zones were arbitrarily called A, B, C, and D, in order of decreasing anodic electrophoretic mobility at *p*H 8.5. Electrophoretic variants of the A and D zones were found in several of the strains (Beckman *et al.*, 1964). To study

Table V. The Substrate Specificity of the LAP Bands of Maize Endosperm Resolved by Starch Gel Electrophoresis[a]

Substrate	Hydrolyzed by LAP band			
	A	B	C	D
DL-Alanyl β-naphthylamide	+	+	+	+
L-Leucyl β-naphthylamide HCl	+	+	+	+
L-Methionyl β-naphthylamide	+	+	+	+
L-Leucyl 4-methoxy β-naphthylamide HCl	+	+	+	+
L-Isoleucyl β-naphthylamide HBr	+	−	+	−
L-Valyl β-naphthylamide	−	+	−	+
L-Seryl β-naphthylamide	−	−	+	+
L-Threonyl β-naphthylamide	−	−	+	−
Glycyl β-naphthylamide	−	−	−	+
L-Arginyl β-naphthylamide HCl	+	+	+	+
L-Lysyl β-naphthylamide carbonate	+	+	+	+
L-Phenylalanyl β-naphthylamide	+	+	+	+
L-Tyrosyl β-naphthylamide	−	+	−	−
L-Tryptophyl β-naphthylamide	+	−	+	−
L-Prolyl β-naphthylamide HBr	−	−	+	−
L-Ornithyl β-naphthylamide	+	−	+	+

[a] Quantitative differences in activity are found, but will be reported in a subsequent publication. This table is merely intended to show that "LAP" is not specific for "leu" residues. Only substrates which were hydrolyzed by at least one of the LAP isozymes are reported.

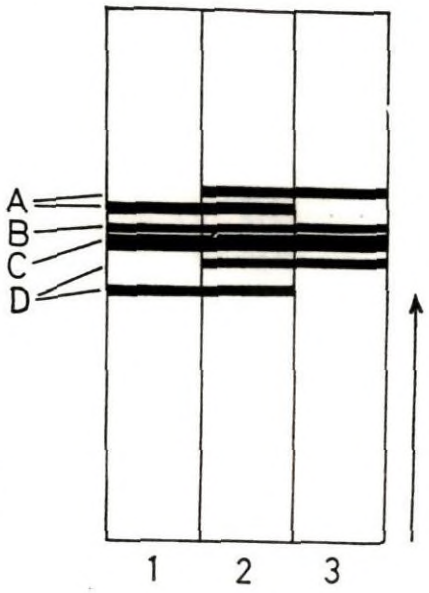

Fig. 16. Schematic drawing showing the electrophoretic LAP patterns in maize endosperm. 1 and 3 are variants for the A and D zones, and 2 is the heterozygote. Arrow indicates direction of migration toward the anode.

the inheritance of these variants, two variant inbred lines were crossed and the results are shown in Fig. 16. Detailed crosses made to determine the mode of inheritance of the LAP variants A and D are summarized in Tables VI and VII.

Genetic Control of LAP-A and LAP-D

In the heterozygote resulting from a cross between inbreds with a fast and a slow LAP variant, with respect to both the A and D zones, both parental types (F+S) are

Table VI. Results of Crosses Made to Demonstrate the Genetic Control of the LAP-A Enzyme Variants. The Genotypes Noted Within Parentheses Are Deductions Fitting the Results

Female parent	Male parent	LAP-A zones in offspring			
		F (*F*/*F*)	F+S (*F*/*S*)	S (*S*/*S*)	Total
AA4 (*F*/*F*)	AA4 (*F*/*F*)	60	0	0	60
P39 (*S*/*S*)	P39 (*S*/*S*)	0	0	60	60
AA4 (*F*/*F*)	P39 (*S*/*S*)	0	60	0	60
P39 (*S*/*S*)	AA4 (*F*/*F*)	0	60	0	60
(AA4 × P39) (*F*/*S*)	AA4 (*F*/*F*)	28	32	0	60
AA4 (*F*/*F*)	(AA4 × P39) (*F*/*S*)	33	27	0	60
(AA4 × P39) (*F*/*S*)	P39 (*S*/*S*)	0	31	29	60
P39 (*S*/*S*)	(AA4 × P39) (*F*/*S*)	0	26	34	60
(AA4 × P39) (*F*/*S*)	(AA4 × P39) (*F*/*S*)	20	22	18	60

Table VII. Results of Crosses Made to Demonstrate the Genetic Control of the LAP-D Enzyme Variants. The Genotypes Noted Within Parentheses Are Deductions Fitting the Results

Female parent	Male parent	LAP-D zones in offspring			
		F (*F*/*F*)	F+S (*F*/*S*)	S (*S*/*S*)	Total
AA4 (*F*/*F*)	AA4 (*F*/*F*)	60	0	0	60
P39 (*S*/*S*)	P39 (*S*/*S*)	0	0	60	60
AA4 (*F*/*F*)	P39 (*S*/*S*)	0	60	0	60
P39 (*S*/*S*)	AA4 (*F*/*F*)	0	60	0	60
(AA4 × P39) (*F*/*S*)	AA4 (*F*/*F*)	32	28	0	60
AA4 (*F*/*F*)	(AA4 × P39) (*F*/*S*)	29	31	0	60
(AA4 × P39) (*F*/*S*)	P39 (*S*/*S*)	0	33	27	60
P39 (*S*/*S*)	(AA4 × P39) (*F*/*S*)	0	28	32	60
(AA4 × P39) (*F*/*S*)	(AA4 × P39) (*F*/*S*)	16	29	15	60

present, as seen in Fig. 16. Backcrosses to the fast (F) parent yielded offspring of the F-type and the (F + S)-type, and backcrosses to the slow (S) parent resulted in individuals of the constitution (S) and (F+S), in either case in approximately 1:1 proportions. In the F_2 generation, all three types were found in proportions showing a close fit to a 1:2:1 distribution (refer to Tables VI and VII).

Data presented in Table VIII show recombination between the genes controlling LAP-A and those controlling LAP-D. Among 320 progeny in the backcross, 42 (13.1%) were recombinants.

These results support the interpretation that LAP-A is controlled by one genetic locus and LAP-D by another. The electrophoretic variants of each zone are controlled by allelic genes acting without dominance. Hence, homozygotes for the LpA^F allele show only the fast LAP-A band, and homozygotes for the LpA^S allele only the slow Lap-A variant, while heterozygotes have both bands. Likewise, the LAP-D locus is diallelic, and the alleles act without dominance; the fast LAP-D variant is specified by the LpD^F allele and the slow LAP-D variant by the LpD^S allele. The two loci are on the same chromosome.

Variation in the B and C LAP Zones

Recent data obtained in this laboratory show the existence of electrophoretic variants of the B and C zones of LAP in maize endosperm. These variants have been detected in other than the inbred lines previously described and are summarized in Fig. 17. Genetic crosses involving these variants suggest that the B and C enzymes are controlled by distinct genetic loci, and, as in the case of the A and D enzymes, that the variant bands in each zone are controlled by alleles acting without dominance. Hence, each of the four LAP isozymes (A, B, C, and D) found in 16-day-old endosperm of maize is controlled by a separate genetic locus. The possible homozygous genotypes would be 16, and the possible number of heterozygotes involving all four loci is 65.

Table VIII. Data Showing Recombination Between the *LpA* and *LpD* Genes

Backcross		Offspring			
Female parent	Male parent	*LpA*	*LpD*	Number	Recombinants (%)
$\frac{LpA^F}{LpA^S}\ \frac{LpD^F}{LpD^S}$	$\times\ \frac{LpA^F}{LpA^F}\ \frac{LpD^F}{LpD^F}$	$\frac{F}{F}$	$\frac{F}{F}$	41	15.0
		$\frac{S}{F}$	$\frac{S}{F}$	27	
		$\frac{F}{F}$	$\frac{S}{F}$	6	
		$\frac{S}{F}$	$\frac{F}{F}$	6	
$\frac{LpA^F}{LpA^F}\ \frac{LpD^F}{LpD^F}$	$\times\ \frac{LpA^F}{LpA^S}\ \frac{LpD^F}{LpD^S}$	$\frac{F}{F}$	$\frac{F}{F}$	34	12.5
		$\frac{S}{F}$	$\frac{S}{F}$	36	
		$\frac{S}{F}$	$\frac{F}{F}$	8	
		$\frac{F}{F}$	$\frac{S}{F}$	2	
$\frac{LpA^F}{LpA^S}\ \frac{LpD^F}{LpD^S}$	$\times\ \frac{LpA^S}{LpA^S}\ \frac{LpD^S}{LpD^S}$	$\frac{F}{S}$	$\frac{F}{S}$	33	11.3
		$\frac{S}{S}$	$\frac{S}{S}$	38	
		$\frac{F}{S}$	$\frac{S}{S}$	2	
		$\frac{S}{S}$	$\frac{F}{S}$	7	
$\frac{LpA^S}{LpA^S}\ \frac{LpD^S}{LpD^S}$	$\times\ \frac{LpA^F}{LpA^S}\ \frac{LpD^F}{LpD^S}$	$\frac{F}{S}$	$\frac{F}{S}$	27	11.8
		$\frac{S}{S}$	$\frac{S}{S}$	42	
		$\frac{F}{S}$	$\frac{S}{S}$	3	
		$\frac{S}{S}$	$\frac{F}{S}$	8	

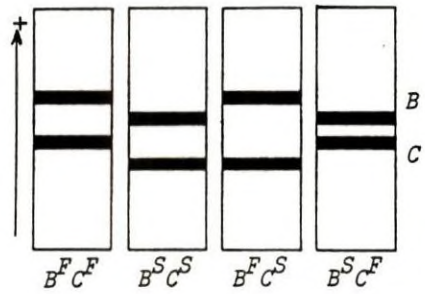

Fig. 17. Schematic zymogram showing variation in the B and C aminopeptidase zones of maize endosperm, 16 days after pollination. Phenotypes are indicated at the bottom.

That is, having a case such as this, where there are four loci and each locus has the same number of alleles, the number of homozygotes and heterozygotes to be expected can be deduced from simple expansions, such as the following: (1) The total number of genotypes (homozygotes and heterozygotes) to be expected by simultaneously taking all loci having the same number of alleles is

$$T_{(\mathrm{Ho+He})} = (X)^n$$

where X is the number of genotypes per locus and n is the number of loci. For LAP, $T_{(\mathrm{Ho+He})} = (3)^4 = 81$. (2) The numbers of homozygous combinations to be expected from the above is

$$T_{\mathrm{Ho}} = (X')^n$$

where X' is the number of alleles per locus. For LAP, $T_{\mathrm{Ho}} = (2)^4 = 16$. (3) The total number of heterozygous combinations then becomes

$$T_{\mathrm{He}} = (X)^n - (X')^n$$

For LAP, $T_{\mathrm{He}} = (3)^4 - (2)^4 = 65$.

To date, we have not detected *all* the possible homozygote types, but the probability of doing so seems fairly high. More detailed data concerning the B and C variants of maize LAP will be forthcoming in a separate publication (Scandalios *et al.*, unpublished).

Tissue Specificity of Maize Peptidases

Leucine aminopeptidase was found to have a relatively homogeneous distribution in the major tissues of the maize sporophyte (Fig. 18). Using the four isozymes found in the 16-day liquid endosperm as a standard for comparison, it was found (Scandalios, 1964) that most tissues had the LAP-A and LAP-B isozymes. The C isozyme was found to occur in the 16-day-old endosperm and in the embryo at all developmental stages studied. The D isozyme, in contrast, has so far been found only in the endosperm. Silk was found to be the only tissue apparently lacking the B enzyme. These

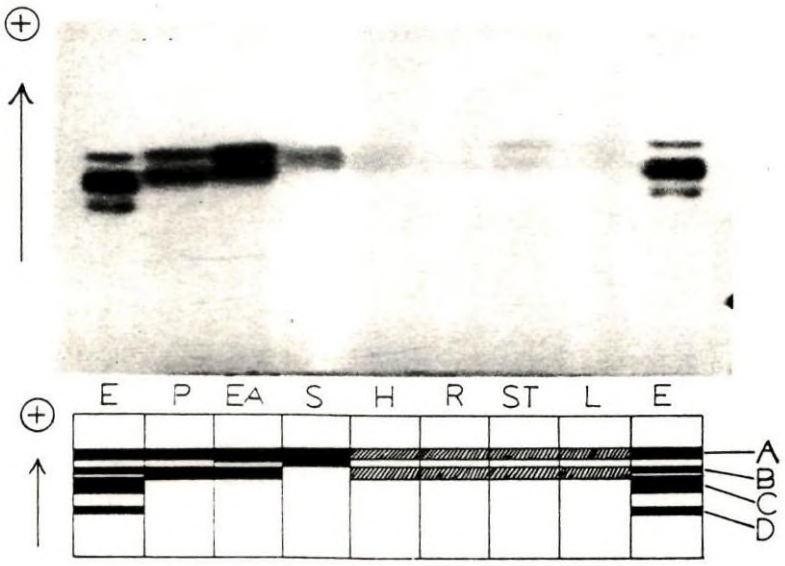

Fig. 18. Zymogram and schematic drawing showing the LAP patterns in various tissues of maize. E = endosperm; P = pollen; EA = young ear; S = silks; H = husk; R = root; ST = stem; L = leaf. Letters to the right of the drawing (A, B, C, D) indicate the four LAP bands. The arrow shows the direction of migration toward the anode (from Scandalios, 1964).

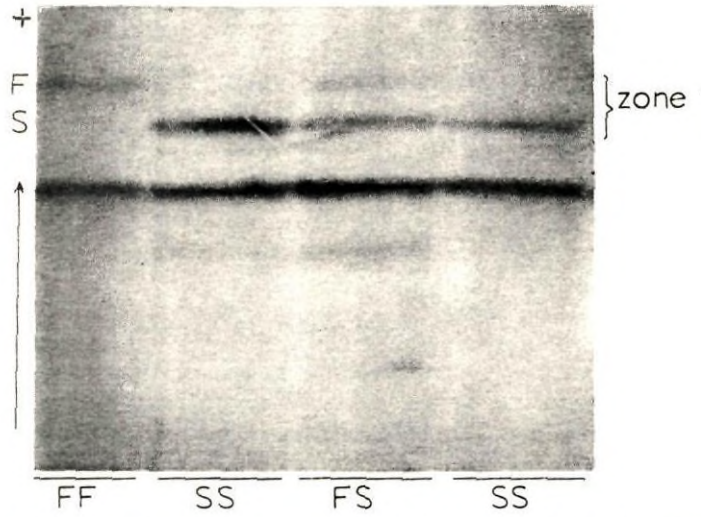

Fig. 20. Peroxidase isozyme variation in maize (zone 1). Note the paren types (F and S) and the resultant heterozygote pattern in the F_1 (FS). Ger types are given at the bottom.

data were based on detailed tissue examination from several Hawaiian maize inbred lines. In these variants pollen was found to have only the A and B isozymes; however, in several other variants examined, pollen also has the C isozyme.

LAP Isozymes in Development

As mentioned above, LAP-D was found to be characteristic of the endosperm. On considering this point, a series of experiments were performed in order to examine the fate of LAP-D at different stages of endosperm development (Scandalios, 1965*a*). Individual kernels were taken from the same ear, beginning with the fifth day after pollination, and the endosperm extract was immediately subjected to electrophoresis. The results obtained from such experiments are summarized in Fig. 19.

It is apparent from these data that LAP-D has a transient existence correlated with endosperm development. Whether this reflects intermittent activity of the *LpD* gene is still an open question. However, the system lends itself well to studies of differential gene activity in higher organisms, and it is presently pursued to this end.

PEROXIDASES

Peroxidases (donor: H_2O_2 oxidoreductase; 1.11.1.7) oxidize a wide range of substances, including phenolic substances, cytochrome *c*, nitrite, leucomalachite green, but not H_2O_2 itself or alcohols. However, like catalase, the peroxidases are hemoprotein enzymes which contain ferriprotoporphyrin (hematin) prosthetic groups. Thus, peroxidases are unspecific with respect to the hydrogen donor, but are highly specific with respect to the peroxide grouping.

Isozymes of peroxidase have been reported in a variety of plants. Peroxidases have further been shown to be tissue- and organ-specific in several plant systems (Scandalios, 1964; Evans and Alldridge, 1965; Macnicol, 1966). The physiological significance of peroxidase in plants is not yet well understood. Peroxidase has been implicated in the oxidation of the plant hormone auxin (indole 3-acetic acid = IAA) (Ockerse *et al.*, 1966), but there is no unequivocal evidence that this activity occurs *in vivo* and involves all or most of the isozymes of peroxidase. It is also possible that "IAA-oxidase" is quite another enzyme contaminated by peroxidase or having some peroxidatic

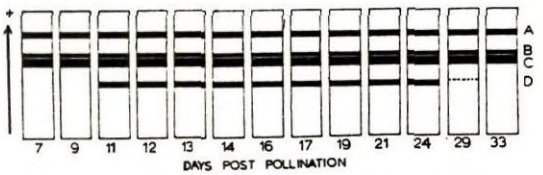

Fig. 19. Scheme showing the variations in LAP-D activity during the development of maize endosperm. Note the presence of zones A, B, and C throughout the developmental stages examined.

activity. Further, because of the apparent molecular heterogeneity of this enzyme system it is essential that purification and thorough kinetic comparisons be made of each isozyme before functional roles are assigned to any of the isozymes. It has also been speculated (Siegel and Galston, 1967) that the existence of isozymes increases the biochemical versatility of the organism and helps to protect the organism against loss of function as a result of mutational or environmental stresses. This argument can hardly be applied to the peroxidases, since so little is known of both their genetic control and biological function.

Genetic studies with peroxidases have been very limited, due to the complexity of the system. However, tissue specificity and ontogenetic studies aimed at simplifying the system to a point where formal genetic analyses could be attempted have been pursued.

Genetics of Peroxidase Isozymes

Genetic variants of peroxidase isozymes have been found in pollen and liquid endosperm of several inbred maize strains. The number of isozymes in the liquid endosperm ranges from a minimum of 8 to a maximum of 12, and the range in pollen is from 3 to 6 isozymes, depending upon the inbred strain. The large number of isozymes in the endosperm poses a problem in elucidating the genetic basis of all variants encountered; however, in the pollen the situation is more simple. To date, only one zone of peroxidase activity in pollen has yielded to successful genetic analysis. This is the most anodically migrating peroxidase, at *p*H 8.5. The electrophoretic analysis of parent and heterozygote pollen extracts shows that the variants in this zone are inherited according to simple Mendelian rules and are apparently determined by codominant alleles at one locus (Fig. 20). The genetic evidence on hand further supports a monomeric structure for the peroxidase products of this locus, although the possibility of dimers with affinity for identical polypeptide chains cannot yet be discounted. In addition to these variants, five other zones of peroxidase activity are present in the pollen zymograms; these isozymes appear to segregate in a manner suggesting other diallelic loci. Altogether, these preliminary studies suggest that the multiple peroxidases of maize are not all coded by the same cistrons, but are the products of more than one structural gene. The resulting polypeptides probably have distinct functions *in vivo* but exhibit some degree of overlapping substrate specificity *in vitro*. The situation is rather reminiscent of the esterases, where such enzymes as carbonic anhydrase, trypsin, and choline esterase, to name a few, attack the same artificial substrate, α-naphthyl acetate, and only because of this have often been classified as esterases. It is for similar reasons that the peroxidases should perhaps be classified as a "family of enzymes" rather than as isozymes, except in cases of multiple forms which are the products of allelic genes, or in cases where there is intergenic (intercistronic) interaction resulting in isozymes which show a high degree of chemical–physiological relationships.

Recently, Pandey (1967) has claimed that the peroxidases in *Nicotiana sp.* are specified by the S-incompatibility genes; this conclusion is based on the fact that the isozyme patterns were found to vary with different combinations of the S alleles. Pandey proposes a complex model in which identical dimers, one each from the pollen and

style, combine to form a tetramer which is physiologically active in producing incompatibility, dimers being inactive in this respect. This model is however without factual foundation. The possibility that any other of hundreds of enzymes might show similar variations was ignored. It might have been more profitable to consider the S genes as markers to establish the possible genetic bases for the peroxidases in *Nicotiana*.

Tissue Specificity of Peroxidases

In several plant species, peroxidases have been shown to be tissue- or organ-specific. In maize, Scandalios (1964) demonstrated that cathodic peroxidases show a large variability between tissues. Using an inbred strain, it was found that the endosperm, stem, husk, and young ear have one common band with equal intensity; the silks and pollen have the same band, but with lower intensity. The leaves have a unique, fast-moving component and a slower band which is also present in roots and husk. A slow zone is found in the homogenates of the stem, silks, and young ear, with the fast band of the zone being common, in varying intensities, to all three tissues. The slowest band is characteristic of the stem.

Different peroxidases have been reported in tissues and organs of dwarf ($d^{\times}$) and normal (d^{+}) tomato plants (Evans and Alldridge, 1965). Peroxidase activities of the pith, cortex, and leaf of the dwarf were found to be about three times greater than those of the corresponding tissues of the normal; in contrast, roots exhibited similar activity in both the normal and the dwarf. Zymogram analysis showed further that the tissues possessed different complements of peroxidase isozymes, but no electrophoretic differences were apparent on comparing the peroxidase zymograms of the $d^{\times}$ and d^{+}.

Multiple forms of peroxidase have also been found to vary with different organs in *Petunia hybrida* (Hess, 1967), as well as in peas (Siegel and Galston, 1967).

Peroxidase Isozymes in Development

Zymograms from different stages in development of the maize sporophyte show distinct variation in the peroxidase content of the different tissues. In some instances totally new isozymes appear, while existing forms disappear (Fig. 21). However, the most common phenomena are shifts in enzymatic activity (i.e., progressive increase or decrease in activity) of specific isozymes. Similar developmental shifts in peroxidase

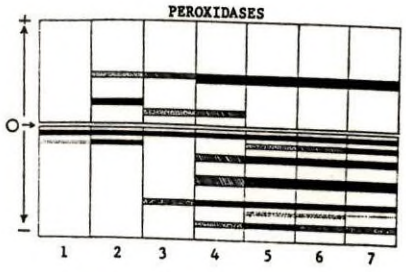

Fig. 21. Zymogram showing changes in the patterns of peroxidase isozymes during the course of development and differentiation of the maize sporophyte (mutant I-1). 1 = dry kernel; 2 = imbibed 24 hr; 3 = imbibed 24 hr plus 24 hr in sand; 4 = 72-hr seedling; 5 = 96-hr seedling; 6 = 120-hr seedling; 7 = 144-hr seedling.

isozymes have been reported during the development of pea cotyledons (Siegel and Galston, 1967), and in bean leaves, using preparative electrophoresis (Racusen and Foote, 1966).

Such changes tempt one to speculate on possible differential gene activities in development. However, the possibility that cellular environments may vary from tissue to tissue and that such differences may in turn alter specific enzymes should be borne in mind, and any interpretations must be proposed with due caution. Extreme caution is especially needed in interpreting tissue differences of isozymes whose genetic background and function are doubtful.

DISCUSSION AND PROSPECTS

The results reported in this review should make it clear that isozymes provide a natural "built-in" marker system for a wide range of investigations in the biochemistry, genetics, and developmental biology of plants. Since isozymes whose genetic control has been resolved reflect, in most cases, mutations which have altered the structure of an enzyme without affecting the catalytic activities of these molecules, they afford a unique opportunity to examine gene activity in a changing cellular or tissue environment.

Using genetically variant isozymes as built-in markers it is now possible for the geneticist to study the interaction of products of naturally occurring alleles (interallelic complementation) and the possible molecular bases for heterosis and differential gene action in the development of higher organisms. Gene dosage effects can be studied by using the triploidy of the endosperm and also by employing many of the known polyploid systems in higher plants. The usefulness of isozymes as markers in population studies is exemplified by the elegant work of several *Drosophila* investigators; so far little use has been made of isozymes in genetic studies with plant populations, but several groups have recently begun such investigations.

It is common knowledge today that the total enzyme repertory of an organism changes during development and differentiation. Gradual shifts of isozyme patterns in samples of a particular organ or tissue, taken in the course of development, demonstrate the appearance or disappearance, or both, of individual isozymes. Such changes suggest that genes involved in the synthesis of these enzymes are differentially activated in development. However, unless gene activity can be tested more precisely, one cannot discount other possible interpretations.

Although the presence of isozyme activity may be sufficient evidence for gene activity, it does not necessarily follow that the particular structural gene is active at the specific developmental stage at which the enzyme is detectable. It may also reflect activation of presynthesized polypeptide subunits, or the multimerization of subunits which are inactive in the form of monomers. In either case, the gene which ultimately controls the synthesis of these units may not be active at the developmental stage when the isozyme appears. Even if it can be conclusively demonstrated that a particular isozyme is indeed synthesized at a specific developmental stage, the particular gene controlling this enzyme does not necessarily have to be acting at this specific time. It has been suggested that the messenger RNA required for protein synthesis during

early sea urchin development is present, in a "masked" form, in the unfertilized egg and is activated at fertilization (Tyler, 1965). Special cytoplasmic particles, called "informosomes," may serve to stabilize and transport such stored templates. Such particles have been described in fish and sea urchin embryos (for review see Nemer, 1968). Hence, it is possible that mRNA is coded (gene action) at one stage in development, and translation (synthesis) occurs at another stage. It is especially in trying to resolve such problems that the developmental biologist, biochemist, or physiologist would be most prudent in using highly inbred lines and in determining the mode of inheritance of the isozyme system being investigated. Only by use of such genetically well-known stable material can he minimize the risk of introducing into his data unrecognized variation of unknown genetic origin. It thus becomes more meaningful to follow the ontogeny of isozymes which are known to be regulated by specific genes. For example, the most unequivocal proof for differential gene action is selection of recombinants. Since one may be dealing with two or more genes, each operative at different developmental stages, this may be a difficult task, but it is not an impossible one, provided the organism is well-known genetically and lends itself well to genetic analysis. Among animals, *Drosophila* is certainly the choice organism, due to our thorough knowledge of its genetic background as well as the unique delineation of its developmental stages. However, in the plant kingdom, there is also abundant opportunity for developmental genetic studies due to the fact that the life cycle of higher plants is clearly divided into distinct stages and there are sufficient time gaps between the stages to allow for thorough investigation. Among the higher plants the organism *par excellence* is *Zea mays*, for reasons similar to those stated for *Drosophila*.

The cautions stated above are not intended to minimize the effectiveness of isozymes as markers in studying developmental processes. The gap between gene (DNA) and gene product (protein) is relatively small when enzymes are used as the phenotypic markers, rather than secondary morphological characters which are further removed from the gene and subject to more genetic–environment interplay in their final expression. Whether stage-specific isozyme variations represent gene action directly (transcription) or indirectly (translation or activation), they nevertheless indicate the utilization of specific pieces of genetic information at specific periods of development. The challenge to the physiological geneticist is in defining the cellular environment in which genes or their products can be differentially activated during the course of development and differentiation.

The electrophoretic pattern obtained from heterozygotes will depend largely on the quaternary structure of the enzyme being investigated. The patterns to be expected from genetic variations involving monomers are as shown in Fig. 22A, although this pattern can also be obtained from crosses involving dimers where there is strict affinity for identical polypeptide chains. As discussed, the variation of LAP in maize may be interpreted in this light. The electrophoretic patterns to be expected from random combination of mononer subunits into enzymatically active dimers would be as shown in Fig. 22B; this situation is exemplified by the ADH-2 locus and the esterases in maize. Another model involving dimer formation in heterozygotes could result in cases where there is preferential affinity for nonidentical polypeptides (Fig. 22C); the only case, to our knowledge, where this model may apply are the maize

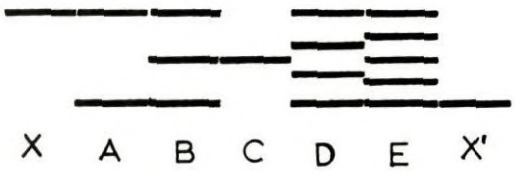

Fig. 22. Diagram of electrophoretic patterns obtainable from heterozygotes which reflect the subunit structure of the enzyme. A = monomeric (or dimeric); B = dimeric; C = dimeric; D = trimeric, and E = tetrameric molecules. X and X′ represent the normal and variant parental molecules.

amylases in the cross AA9 × AA2. In the case of trimeric molecules as the active enzyme form, the pattern in heterozygotes would be that shown in Fig. 22D. No firmly established examples of trimeric variants are known, with the possible exception of the two isolated catalase variants discussed. The most common multimeric structure so far known is that of tetramers, as shown in Fig. 22E. This model encompasses the LDH isozymes of mammals (Appella and Markert, 1961), the acid phosphatases of *Tetrahymena* (Allen, 1965), and the catalases of maize (Scandalios, 1965*b*). Other more complex multimeric structures are certainly possible. For example, rabbit muscle aldolase is believed to be enzymatically active as a hexamer composed of six identical subunits (Hass, 1964).

For cases where random association of subunits occurs in the heterozygote, Shaw (1964) proposed a formula which relates the number of isozymes (i) to the multimer size (p) and the number of kinds of subunits (s):

$$i = \frac{(s+p-1)!}{p!\,(s-1)!}$$

This discussion should demonstrate that effective use of genetic isozyme variants and their resultant heterozygotes can be of great help to protein chemists in analyzing enzyme structure.

An important question regarding the formation and existence of hybrid enzymes concerns their functional significance. Although suggestions have been made that hybrid proteins are of advantage to the organism, there is no real evidence that such hybrid molecules function in a way superior to the homomultimer, parental-type molecules. As early as 1954 Haldane suggested that, in general, homozygotes may be able to produce only single kinds of enzymes for each gene; this may restrict the occurrence of specialized reactions to a narrow range of environmental conditions. Haldane thus implied that the superior fitness of some heterozygotes is based, at least in part, upon a biochemical complexity resulting from the joint action of related, but somewhat different, alleles and their products. This would mean that heterosis is not the total vigor of the heterozygote, but consists of "single-gene heterosis" based on interaction of dissimilar alleles of a given gene locus. If hybrid enzymes are employed as markers in studies of single-gene heterosis or overdominance, they should be enzymes whose

biological function and genetic control are well understood, so that when functional aspects of homomultimers and heteromultimers are compared the parameters for comparison are well defined. Often such comparisons are based on enzymatic activity but are made with artificial substrates since the biological substrates and therefore the function(s) of the enzyme are not known. Only if the *in vivo* role of a protein is well understood, and its genetic control deciphered, may it be justifiable to speak of heterosis of the hybrid *versus* the parental molecules. It is perhaps for such reasons that only one case, in our opinion, can be considered as a fairly sound example for heterosis of a hybrid protein. That is the fish hemoglobin polymorphism reported by Manwell *et al.* (1963), who demonstrated that the hybrid molecules are more efficient in their oxygen transport capacity than either of the pure homomultimers from which the heteromultimers are derived. The hybrid hemoglobin shows greater heme–heme interaction and lower oxygen affinity. Hence, heterozygotes with respect to the hemoglobin gene are at an apparent advantage by being endowed, as a consequence of their heterozygosity, with more efficient molecules essential for maintenance of their physiological homeostasis.

Another aspect of gene function which can be effectively examined by using isozymes as both qualitative and quantitative markers is gene dosage effects. Relationships between genes affecting the same enzyme can be studied by examining the effect of the dosage of these genes on the specific isozymes. Examples of such dosage effects are apparent in the catalase and alcohol dehydrogenase zymograms presented in this review. Plants lend themselves particularly well to studies of gene dosage due to the range of ploidy available. Most angiosperms have a triploid endosperm. In addition, many can be maintained in the haploid state (Kimber and Riley, 1963), while other individuals may represent ploidy situations up to octoploidy. It would be of great interest from both a genetic and a biochemical viewpoint to demonstrate whether increasing gene dosage has an optimum with respect to qualitative and quantitative shifts of a particular isozymic system. Of all plants, maize offers the best opportunity in such studies mainly because of its large persistent endosperm and the excellent genetic stocks available.

What can be said about the possible physiological role of isozymes? By definition, all isozymes of a particular enzyme system perform essentially the same catalytic function. Yet it might seem wasteful for an organism to produce multiple molecular forms of any enzyme when one molecule is sufficient to perform that function. More broadly stated, the question is: What is the basis for the existence of isozymes? Attempts at answering these questions will be most promising of success if one deals with enzymes whose roles in the physiology of the organism are well understood and then compares the multiple forms on defined parameters. Perhaps the best example to date is the lactate dehydrogenase (LDH) system of isozymes in vertebrates. This system has been fully described in the literature (Markert, 1964; Kaplan, 1964; Vesell, 1965), and we need only to mention that the five isozymes of LDH were found to have somewhat different kinetic properties. Some parameters in which they differ are substrate concentration optima, *p*H optima, K_m, and relative activity with analogues of NAD. It was further shown that LDH-1 isozymes are predominantly found in heart muscle and other highly oxygenated tissues with low lactate production; in

contrast, the isozymes at the LDH-5 end of the spectrum are most abundant in tissues where lactate production is high and oxygen levels are low, such as skeletal muscles (Cahn *et al.*, 1962). Such findings strengthen the view that isozymes do indeed have a reason for being, and may reflect distinct metabolic activities of the tissues of which they are characteristic.

Unfortunately, in many isozymic systems which have been established the physiologic function of the enzyme is not well enough appreciated to allow meaningful functional conclusions. In such cases, it would perhaps be prudent first of all to attempt investigations on the possible physiologic functions of the enzyme. As a matter of fact, the tissue, cellular, and subcellular localization of isozymes may provide clues about functional aspects of specific isozymes, since these may reflect specific metabolic requirements of tissues, cells, or subcellular organelles. Organelle-specific isozymes can further be used to answer questions relating to the coding of specific enzymes. For example, one might pose the question of whether the DNA in mitochondria or chloroplasts is sufficient to code all or most of the enzymes associated with these organelles. The question can be effectively answered by determining whether such organelle-specific isozymes follow Mendelian segregation or not. In our laboratory we have initiated such an approach, and the results so far are promising (see Fig. 23). It scarcely needs to be said that in such investigations organisms which are well known and can be well manipulated genetically, such as maize, should be employed.

Lastly, mention must be made of the need for and usefulness of chromosomal localization of genetic enzyme variants. Chromosomal localization and linkage relationships of the genes are important in answering questions pertaining to the evolutionary origin of multiple forms of a protein (Smithies, 1964) and in establishing bases for the existence and maintenance of isozymes in populations.

The author has attempted to point out some of the questions relating to the nature and use of isozymes in plants for studies in genetics, biochemistry, and physiology. He has also tried to stress the suitability of higher plants for studies relating to differential gene activity and the genetics of development as a whole. It is indeed rare that abundance of material is coupled with such unique advantages as clear, distinct developmental stages, large numbers of mutants, existence of a triploid tissue and of haploid and polyploid individuals of similar genetic background, highly inbred strains, and amenability to thorough genetic and biochemical analyses.

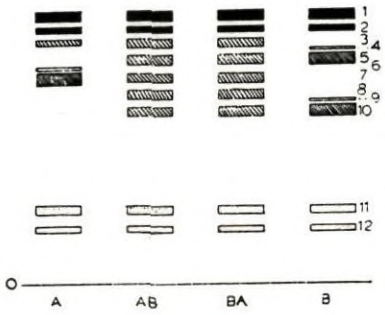

Fig. 23. Malate dehydrogenase isozymes of maize. Isozymes 1 and 2 are found in the soluble fraction; isozymes 3 through 10 are mitochondrial, and isozymes 11 and 12 are glyoxysomal. Only mitochondrial MDH variants have been found, and these follow strict Mendelian inheritance. A and B are parental types; AB and BA are the reciprocal heterozygotes. Migration is anodal (from Longo and Scandalios, in press).

APPENDIX I: PREPARATION OF GELS

(A) Starch Gels

The starch gel is prepared by heating 36.0 g of hydrolyzed potato starch (Connaught Laboratories) in 300 ml of the appropriate buffer for a 12% gel commonly employed in our studies. The starch–buffer suspension is vigorously swirled over a reducing flame until it is just at the point of boiling. The hot liquid is then aspirated to remove the bubbles, and, after a minute of cooling at room temperature, the mixture is poured into a lucite tray (18 by 20 by 0.7 cm). The gel is then allowed to cool and solidify for 20 min at room temperature or for 8–10 min at 5 C.

The samples to be subjected to electrophoresis are then absorbed onto pieces of filter paper (Whatman #3, usually) and inserted into a slot cut into the gel (or samples may be placed into wells formed in the gel by a template). After completion of the electrophoretic run, the gel is cut horizontally by a gel slicer and then stained with the appropriate stain.

(B) Acrylamide Gels

The acrylamide gel is prepared by weighing the appropriate amount (for the percent gel required) of the gelling agent Cyanogum-41 (American Cyanamid Co.) and allowing it to dissolve in the appropriate buffer to be used. After it is dissolved, it is filtered through Whatman #1 filter paper. Just prior to pouring the gel ingredients in its mold, 0.3 ml of TEMED (*N, N,N′, N′*-tetramethylethylenediamine) and 1.5 ml of 10% w/v ammonium persulfate are added; these quantities are for a 10% gel, and the order of their addition to the mixture is very important. The liquid mixture is then immediately poured into a gel tray (20 by 20 by 0.3 cm). The liquid is covered with a precoated (1% "siliclad" solution) glass plate and allowed to gel for approximately 15 min. The gel is then kept at 4 C for approximately 2 hr before use. Application of samples is similar to the procedure described above for starch gels.

More general procedures have been recently described by Shaw and Koen (1968).

APPENDIX II: BUFFERS

(A) Lithium–Borate Buffer (*p*H 8.3, 0.2 M)	
1. Lithium hydroxide	1.2 g
2. Boric acid (anhydrous)	11.89 g
3. Water	1000 ml
(B) Tris–Citric Buffer (*p*H 8.3, 0.2 M)	
1. Tris	6.2 g
2. Citric acid	1.6 g
3. Water	1000 ml
(C) Phosphate Buffer (*p*H 4.3, 0.2 M)	
1. NaH_2PO_4	27.8 g
2. Water	1000 ml
(D) Phosphate Buffer (*p*H 9.2, 0.2 M)	
1. Na_2HPO_4	53.63 g
2. Water	1000 ml
(E) Tris–Maleate Buffer (*p*H 3.3, 0.2 M)	
1. Tris	24.2 g
2. Maleic anhydride (or maleic acid)	19.6 g (23.2 g)
3. Water	1000 ml
(F) Sodium Hydroxide Buffer (*p*H > 14, 0.2 M)	
1. NaOH	7.99 g
2. Water	1000 ml
(G) Tris–Glycine Buffer (*p*H 8.7, 0.2 M)	
1. Tris	3.0 g
2. Glycine	14.4 g
3. Water	1000 ml

(H) Acetate Buffer (pH 4.0, 0.2 M)

1. 0.2 M acetic acid	410 ml
2. 0.2 M sodium acetate	90 ml
3. Water	500 ml

(I) Tris–HCl Buffer (pH 8.0, 0.2 M)

1. Tris	12.1 g
2. Water	1000 ml

3. Adjust pH with HCl

APPENDIX III: ENZYME STAINING ON THE GELS

Note: An effective buffer system for assaying most plant isozymes discussed in this review is the following: (a) Gel buffer: Use 9 parts of buffer B and 1 part of buffer A. (b) Electrode trays: Use only buffer A. For a continuous buffer system, use only buffer G in both the gel and the trays.

I. Leucine Aminopeptidase (LAP)

1. Distilled water	30 ml
2. L-Leucyl β-naphthylamide HCl	10 mg
3. Black-K salt	20 mg
4. Buffer E	50 ml
5. Buffer F	20 ml

Incubate 45 min at 37 C; wash and fix in 5:5:1 (water: methanol:acetic acid).

II. Esterases

1. Distilled water	40 ml
2. α-Naphthyl acetate (1% in 1:1 acetone–water)	2 ml
3. Fast Blue RR salt	40 mg
4. Buffer C	50 ml
5. Buffer D	10 ml

Incubate for 45 min at 37 C; wash and fix.

III. Catalase

1. Pour a solution of 0.5% H_2O_2 (Merck) over cut surface of gel and allow to stand for about 30 sec.
2. Wash gel by rinsing with running distilled water.
3. Pour 1.5% KI solution, acidified with glacial acetic acid (12 drops/30 cc), over surface of gel.
4. Photograph immediately.

IV. Alkaline Phosphatase

1. Tris–citric acid buffer (pH 8.5, 0.1 M)	100 ml
2. α-Naphthyl acid phosphate-Na salt	100 mg
3. Fast Blue RR salt	100 mg
4. 10% Aqueous $MgCl_2$	10 drops
5. 10% Aqueous $MnCl_2$	10 drops

Incubate at 25 C for approximately 8 hr.

V. Acid Phosphatase

1. Buffer H	100 ml
2. α-Naphthyl acid phosphate	100 mg
3. Fast Garnet GBC	100 mg
4. 10% Aqueous $MgCl_2$	10 drops

Incubate at room temperature (25 C) for approximately 5 hr.

VI. Peroxidase

1. Prepare benzidine solution (1.0 g into 9 ml acetic acid, heated to 50 C to dissolve; add 36 ml distilled water and keep in brown bottle).

2. Mix equal amounts (1:1) of benzidine solution and 3% H_2O_2.
3. Paint the mixture, with a brush, onto the cut gel surface.
4. Photograph immediately.

VII. Alcohol Dehydrogenase

1. Buffer I	50 ml
2. Distilled water	130 ml
3. KCN (0.002 M)	2 ml
4. NAD (0.01 M)	2 ml
5. PMS (0.01 M)	2 ml
6. Ethanol (100%)	1 ml
7. NBT (nitro blue tetrazolium)	50 mg

Incubate at 37 C for approximately 30 min.

VIII. Malate Dehydrogenase

1. Buffer I	50 ml
2. Distilled water	50 ml
3. DL-Malic acid (0.2 M)	1.38 g
4. KCN (0.1 M)	0.4 ml
5. NAD (0.01 M)	1 ml
6. PMS (phenazine methosulfate)	1.6 mg
7. NBT	50 mg

IX. Amylases

1. Following electrophoresis on acrylamide (cyanogum) gels, incubate the gel in 1% soluble starch (Baker or Merck) made up in 0.02 M K-phosphate buffer (*p*H 7.3) for approximately 12 hr at 25 C.
2. Then wash the gel in distilled water and flood with a solution of I_2-KI.
3. Photograph immediately.

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