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GENETIC STUDIES ON MUTANT ENZYMES IN MAIZE: SYNTHESIS OF HYBRID ENZYMES BY HETEROZYGOTES

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The investigations on the genetic control of the abnormal hemoglobins in man have contributed greatly toward the elucidation of the problem of gene action.[1-3] Such a system, whereby mutant genes can be studied via differences in the proteins that they specify and in which both "immediate" gene products can be recognized in the heterozygote, is invaluable to the study of the relation between gene and protein. Study of the abnormal human hemoglobins, however, has the disadvantage of involving poor material for genetic investigations: long generation time, small progeny numbers, and the improbability of directed matings. The result is that the genetics has not kept pace with the biochemistry. As an example, it is established that hemoglobins *S* and *G* involve alterations of adjacent amino acids in the β polypeptide;[4] however, whether the genes Hb^S and Hb^G are allelic is still a much-debated question and in fact the pertinent available data consist of a single individual. Because of the low progeny number and the low frequency of these genes in the population, it is also difficult to distinguish between allelism and close linkage. This paper deals with a similar system involving the genetic control of an enzyme that has esterase activity. The material, maize, has the advantage of being excellent for cytogenetic analysis.

Materials and Methods.—Smithies' technique[5] of zone electrophoresis in starch gel was used in this study. Extracts from immature endosperm tissue were inserted in the gel on saturated strips of Whatman No. 1 filter paper. After the electrophoretic separations, the gels were sliced horizontally and stained for esterases by the histochemical method described by Markert and Hunter.[6] α-Naphthyl acetate was used as the substrate and fast blue RR salt as the dye coupler. The endosperm material was harvested 16 days after pollination and stored at $-20°$C. The esterases did not appear to lose any appreciable activity even after a year of storage under these conditions. In the early part of this study the endosperm tissue was macerated, centrifuged at $16{,}000 \times g$ to remove the cell debris, and the supernatant used for the electrophoretic separations. However, we found it possible to work with single endosperms simply by squeezing the juice out of individual kernels with a pair of pliers onto the piece of filter paper to be inserted in the starch gel. Only a fraction of the extract from a single kernel was

required to saturate the filter-paper strip. The tissue from germinating seedlings was handled in a similar manner except that here the tissue was squashed directly on the filter-paper strip, since a whole drop could not be squeezed out of the tissue.

Results.—The zymograms developed from the endosperm tissue showed a number of enzyme bands with esterase activity. In this paper we will limit ourselves to the investigations of a basic protein with esterase activity that migrates to the negative pole at pH 8.6, although differences in migration rates have also been observed for some of the other esterases. Three forms of this enzyme have been found in the genetic stocks investigated. These forms differ with respect to their rate of migration. The enzyme types are referred to as fast (*F*), normal (*N*), and slow (*S*) (Fig. 1*a*, *b*, *c*). The normal type has been so labeled because it is the type

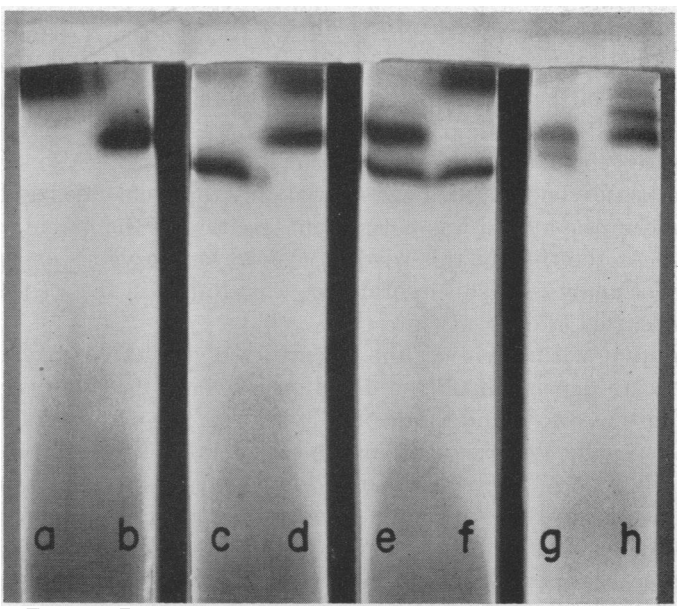

FIG. 1.—Zymograms of endosperm extracts showing the various esterase types: *a*, slow; *b*, normal, *c*, fast; *d*, mixture of normal and slow; *e*, mixture of normal and fast; *f*, mixture of fast and slow; *g*, N × F ♂ hybrid; *h*, N × S ♂ hybrid.

most commonly found in these studies, and occupies a position intermediate between the fast- and the slow-moving enzymes. Of the four highly-inbred lines tested, *W*-23, *Oh*-51, and *KYS* show only the N esterase, and *M*-14 has only *F*.

The differential migration rate of these enzyme types persisted even after the extracts were partially cleaned up by dialysis, ammonium-sulfate precipitation, and precipitation of the nucleic acid with $MnCl_2$. The enzymes retained their differential migration rates in mixtures of extracts from the different endosperm classes (Figs. 1*d*, *e*, *f*). Mixtures of extracts from *F* and *S* endosperm types showed both the *F* and the *S* enzyme bands, of *N* and *S* showed both the *N* and *S* bands, and of *F* and *N* showed both the *F* and the *N* bands. It is important to stress that the zymograms of the mixtures showed only the bands found in each extract used in the mixture. No new bands appeared even when the extracts were mixed a few hours before the electrophoretic separation.

Crossing studies indicate that the migration rates of these enzymes are under genetic control. Crosses of $F \times F$, $N \times N$, and $S \times S$ gave plants that showed only the fast-, normal-, and slow-migrating esterases respectively. When two plants having esterases that migrate at different rates are crossed, however, the heterozygotes show both parental enzyme types, but in addition they show the presence of a hybrid enzyme that migrates at a rate intermediate between the two parental enzymes. Thus, in a cross of $N \times F$, the F_1 hybrid shows the N band, the F band, and a third band labeled fast-normal (FN) that occupies a position between F and N (Fig. 1*g*). An F_1 hybrid between N and S shows the N band, the S band, and also a third band intermediate between these two that is labeled normal-slow (NS) (Fig. 1*h*). There is no question but that the hybrid bands found in the heterozygotes represent new enzyme types not present in either parent. For example, in making an $N \times S$ hybrid, we used as the N parent the highly inbred line W-23. A large number of seeds from many different W-23 ears were tested, and all were of the N enzyme type. It is therefore safe to conclude that the W-23 plant used as the female parent in this cross was also N. The identical plant of the S enzyme type used as the male parent in the cross was also self-pollinated. All the tested kernels on the selfed ear showed only the S enzyme band. Thus the NS band found in the hybrid was not present in either of the parental plants used in this cross. At the time of this writing, $F \times S$ heterozygous endosperms were not available for analysis. This hybrid type was studied in the seedling, however, and will be discussed later.

In the majority of the hybrids, the intensities of the three enzyme bands are not equal and are dependent on the direction in which the cross is made. This is true for both the $N \times F$ and the $N \times S$ hybrids. The maternal and the hybrid bands are intense and the paternal band is considerably lighter. As an example, in the F_1 hybrid of an $N \times S$ ♂ cross, the S band is light but the N and NS bands are heavy, indicating greater enzymatic activity. In the reciprocal cross, $S \times N$ ♂, the N band is light and the S and NS bands intense. The same is true for the $N \times F$ reciprocal crosses. This situation does not always exist, however, and in some cases all the bands are of about equal intensity. Actually, even the heaviest bands in the hybrid are less intense than the single band found in the homozygote, suggesting that the total amount of this enzyme produced by a plant is fairly constant, and can occur as either a single type or be distributed among three different classes.

These results are consistent with, although not necessarily proof of, the hypothesis that these esterase enzymes exist as dimers. The dimers can be composed of two F, two N, two S monomers, or any combination of N, F, or S. On this basis, the NS and FN hybrid enzymes described would be composed of an N and an S monomer and an F and an N monomer, respectively. The migration rates of the hybrid dimers would be expected to be intermediate between the two homozygous parental types. Since the endosperm tissue is triploid, receiving two chromosome sets from the maternal parent and only one from the male, the hybrid would contain twice as many maternal genes as paternal and might conceivably produce twice as many maternal-type monomers as paternal. Random association of monomers would result in a ratio of 4 maternal (homozygous): 4 hybrid (heterozygous): 1 paternal (homozygous) dimers. If the hybrid enzymes are dimers, this maize system would

differ from the human hemoglobin system since $\beta^A\beta^S$ hybrid molecules are not found in A/S heterozygotes.[3]

Preliminary attempts to dissociate the enzymes have not been successful. No evidence for dissociation was obtained when the pH was reduced to 3 for as long as one hour. Since the pH of the extracts was brought back to neutrality before the electrophoretic separation, it was important to ensure in these studies that the negative results were not caused by reassociation. The treatments were carried out on a mixture of extracts of two enzyme types, *N* and *F*, and on the extract of a hybrid containing *F*, *FN*, and *N* enzymes. Dissociation without subsequent reassociation might not be detectable in the *F* and *N* mixture but would result in elimination of the hybrid enzyme band in the extract of the heterozygote. Alternatively, dissociation with subsequent reassociation might result in no noticeable change in the hybrid extract, but would result in the appearance of a hybrid band in the *F* and *N* mixture. The treatment caused no change in the zymogram pattern of the extracts.

The fact that zymograms can be developed with the extract of only single kernels makes it relatively simple to test the segregation of the three enzyme types, since all the kernels on an ear resulting from a particular cross can be tested and scored individually. The enzyme types appear to segregate in a straightforward Mendelian fashion. We have tested the F_2 progeny of a self-pollinated N/S heterozygote. The endosperms segregated in a 1:2:1 ratio (32 N:55 *N NS S*:36 *S*). Both types of heterozygotes were found, those with the *N* and *NS* bands being heavy, and others with heavy *NS* and *S* bands.

Some of the stocks tested show another slow-moving esterase band of varying intensity that migrates at about the same rate as the *S* band. This band is found in some of the *N* and *F* stocks and probably occurs in the *S* stocks as well but is masked by the *S* band. This band does not interfere with the analysis, however, since it can easily be distinguished from the *S* band by its failure to form the hybrid enzymes when in combination with an *N* or *F* enzyme. Further proof that this slow-moving esterase band involves a different enzyme and is not under the control of the gene or genes governing the *F*, *N*, and *S* bands comes from selfing experiments. If this slow-moving band were a mutant of the enzyme under consideration, so that plants that carried *F* or *N* and in addition the slow band were heterozygotes, selfing of such plants should produce progeny segregating for *F* or *N* alone, the slow-moving band alone, as well as progeny showing *F* or *N* in addition to the slow band. However, all the progeny of such self-pollinations showed both *F* or *N* and the slow-moving band.

The differential migration rates of the *F*, *N*, and *S* esterase types can also be demonstrated by agar gel electrophoresis, but the differences in migration rates of the three types are not so pronounced as in the starch gel.

This basic protein with esterase activity is found in the seedling as well as in the endosperm. The enzyme is either absent or present in only very low concentration in the radical, but its concentration in the plumule seems to be even higher than in the endosperm. The same enzyme types found in the endosperm also occur in the plumule. Stocks with *F*, *N*, or *S* type enzymes in the endosperm show the *F*, *N*, or *S* type enzymes in the seedlings, respectively. Each enzyme type from the seedlings forms a continuous band with the corresponding enzyme type

from the endosperm when the two are run side by side in the same gel in the overlapping test.[7] As is the case with the N/S and N/F endosperm heterozygotes, both the parental as well as the hybrid enzyme bands are seen in the seedling heterozygotes. The seedlings are diploid, and in the heterozygotes the hybrid band is usually more intense than the two parental bands. Selfing of the F_1 heterozygotes that show three bands results in progeny consisting of the two parental homozygous types and the heterozygotes. For example, the F_2 progeny of $F \times N$ crosses consisted of 141 F:292 *F FN N*:146 *N* seedlings; F_2 progeny of $N \times S$ was composed of 21 *N*:41 *N NS S*:20 *S* seedlings.

In the case of the seedlings, material was available for a study of hybrids resulting from crosses of $F \times S$. Such hybrids show the *F* and *S* bands and a third band in the normal position, which we interpret to be the *FS* hybrid dimer. This is the result expected if *F* and *S* are allelic. If the genes controlling *F* and *S* are not allelic and act independently, some *N* type enzymes should have been formed. Dimerization among the three types, *F*, *N*, and *S* would have resulted in five bands, *FF*, *FN*, *NN* (or *FS*), *NS*, and *SS*. The F_2 progeny of the $F \times S$ crosses tested segregated 139 *F*:314 *F N S*: 141 *S* seedlings. The gene is designated as *E* (for esterase) and the three alleles, E^F, E^N, and E^S.

Two different mutant effects on enzyme control have now been described in maize. This paper deals with mutant genes, E^F and E^S, which result in the production of altered enzymes, whereas the sh_1 mutant[7] has been shown to be an amorph in that sh_1 homozygotes completely lack the protein produced in the presence of the dominant Sh_1 allele.

Synthesis of hybrid enzyme polymers has been postulated to explain interallelic complementation in Neurospora heterocaryons.[8, 9]

The formation of hybrid enzymes in gene heterozygotes such as described in this paper could be a factor in hybrid vigor. According to this hypothesis, the hybrid enzyme would be more active than either of the parental homozygous types. Such a comparison cannot be made in this study since we do not have an independent measure of enzyme concentration in the various bands. The parental and hybrid enzymes must be purified for determinations of specific activities.

The *E* gene has not been located on the genetic map. There is no easily detectable phenotypic alteration associated with these mutant genes; however, the mapping should be simplified by the finding that the E^S gene in the sh_4 stock is associated with a reduced functioning of the male gametophyte. An E^N/E^S heterozygote, synthesized by crossing *W*-23 $\times$ sh_4 ♂, was self-pollinated, and the F_2 endosperms were comprised of only two classes occurring in a 1:1 ratio, 23 $E^N/E^N/E^N$ (showing only the *N* enzyme band) and 18 $E^N/E^S/E^S$ (having the *N*, *NS*, and *S* bands with the *NS* and *S* being intense). These results indicate that none of the fertilizations were accomplished by pollen carrying the E^S allele so that all the heterozygotes received the E^S from the female side. The linkage relationship of the male gametophyte factor can be determined since it will result in aberrant F_2 ratios for linked marker genes and its location will serve to position the E^S gene.

Summary.—This paper describes genetic studies with three forms of a basic protein with esterase activity. These enzymes are under the control of three allelic genes, E^F, E^N, and E^S and show differential migration rates in starch gel electrophoresis. Heterozygotes are shown to contain a hybrid enzyme in addition to the

two parental types.

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*DIVERSE RATIOS OF MUTATIONS TO CHROMOSOME ABERRATIONS IN BARLEY TREATED WITH DIETHYL SULFATE AND GAMMA RAYS**

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The present report demonstrates that a solution prepared by adding diethyl sulfate to water (referred to as diethyl sulfate) induces a high frequency of mutations associated with relatively few chromosomal aberrations. These properties are quite unlike those previously shown for other alkylating agents and radiation.[1]

The mutagenic activity of diethyl sulfate on *Drosophila melanogaster* larvae was reported in 1947 by Rapoport,[2] and was shown recently[3, 4] to produce in barley a slightly higher frequency of mutations than Xrays. By providing different treatment conditions we have observed that the induced mutation frequency for diethyl sulfate may be more than double that previously reported.[3, 4]

Materials and Methods.—Resting barley seeds (*Hordeum vulgare* $2n = 14$, var. Himalaya C.I. 620) selected for uniformity of size and freedom from injury, were stored over a saturated solution of $NH_4Cl + KNO_3$ in a desiccator to stabilize their moisture content. The seeds contained approximately 15 per cent moisture at the time of treatment.

Seeds were treated by immersing them in saturated solutions of diethyl sulfate. The saturated solutions were prepared using 15 ml of diethyl sulfate per liter of oxygen-saturated distilled water at 30°C. The water was saturated with oxygen to assure repeatability of conditions. Recent work, however, has indicated that this factor is not important. After agitating the mixture frequently during a 90-min hydrolysis period, 100 ml aliquots were pipetted into 250-ml Erlenmeyer flasks containing approximately 260 seeds. Six replicates of seeds were immersed for 1-, $1^1/_2$-, and 2-hr treatment periods, then rinsed with distilled water and planted immediately on moist blotting paper. Each replication included: 50 seeds for an analysis of seedling injury; 200 seeds that were germinated 24 hr over moist filter paper, then sown in the field 2-in. apart in 40-ft rows for survival, fertility, and mutation studies; and 10 seeds for cytological examination.