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GENETIC STUDIES ON MUTANT ENZYMES IN MAIZE, II. ON THE MODE OF SYNTHESIS OF THE HYBRID ENZYMES

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Maize tissue heterozygous for two different alleles of the *E* gene, which controls the specificity of a particular esterase enzyme, always forms a new enzyme in addition to the enzyme types specified by the two alleles when in homozygous condition.[1] These hybrid enzymes are found only in the heterozygotes. The esterase types specified by the different alleles are distinguishable by their electrophoretic mobilities. Three alleles, designated E^F, E^N, and E^S, were described in the earlier publication, and the three heterozygous combinations E^F/E^N, E^F/E^S, and E^N/E^S each form a different hybrid enzyme having an electrophoretic migration rate intermediate between that of the enzyme types specified by the parental alleles. Comparison on the relative intensities of the three enzyme bands of the heterozygotes in endosperm and seedling tissue, where the dosage relations of the two alleles can be varied, suggested that the enzymes are composed of two subunits. The subunits are considered to be identical in the enzyme types formed in homozygotes (*FF*, *NN*, and *SS*) but different in the hybrid enzymes (*FN*, *FS*, or *NS*). Since in our studies the heterozygotes are usually formed as F_1's by crossing two homozygotes, the *FF*, *NN*, and *SS* enzyme bands are referred to as the *parental types* for simplicity.

This paper deals with the timing of the event responsible for the doubled nature of the enzyme molecule; does it precede or follow synthesis? Specifically, the question asked is as follows: Is the enzyme synthesized as a double molecule or is it synthesized as a monomer with dimerization of two monomers occurring after synthesis is completed? The study was made possible by our finding that a part of

the esterase activity specified by the *E* gene is particle-bound. This will be shown to be newly formed enzyme affording us the opportunity to investigate the nature of the enzyme still attached to its template.

The isoelectric point of the esterase under investigation is around pH 7.5, as determined by zone electrophoresis on acrylamide gels where electroosmosis is negligible. Henceforth, this enzyme will be referred to as the pH *7.5 esterase* to distinguish it from the other esterases in maize tissue.

Methods and Materials.—Experiments were carried out on immature maize kernels harvested 16 days after pollination and on young maize seedlings. The washed particles were obtained following the procedure of Mans and Novelli.[2] All the steps were performed in the cold. The tissue was macerated with a mortar and pestle in a solution of 0.05 *M* Tris buffer at pH 7.5–0.45 *M* sucrose–0.001 *M* $MgCl_2$ about equal in volume to the water content of the tissue. The homogenate was centrifuged for 20 min at 20,000 $\times$ *g*, and the pellet was discarded. The supernatant was recentrifuged for 2 hr at 105,000 $\times$ *g* in 12-ml tubes. This supernatant was decanted and the pellets and centrifuge tubes rinsed with distilled water. The pellets were washed by resuspension with a Teflon homogenizer in the Tris-sucrose-$MgCl_2$ solution and a second centrifugation for 2 hr at 105,000 $\times$ *g*. After the supernatant was decanted, the washed pellets and centrifuge tubes were again rinsed with distilled water and the pellets were combined in a single tube and homogenized in 1 to 3 ml of 0.0025 *M* Tris buffer at pH 7.5 containing 0.25 per cent deoxycholate (DOC), depending upon the size of the combined pellets. The amount of deoxycholate solution added was kept at a minimum to decrease the dilution factor. DOC treatment liberated the particle-bound enzyme and the homogenate was either used as such in electrophoresis or after a 2-hr centrifugation at 105,000 $\times$ *g* to remove the nucleoprotein particles, or after dialysis against distilled water to remove the deoxycholate. All treatments gave the same results in electrophoresis.

In some experiments, the first 105,000 $\times$ *g* pellet from tissue carrying one enzyme type was homogenized in the first 105,000 $\times$ *g* supernatant of tissue of another enzyme type before going through the described procedure for liberating the particle-bound enzyme.

Electrophoretic analyses was carried out in starch gel (borate buffer pH 8.6, 0.1 μ), 1% agar gel (veronal buffer pH 8.6, 0.02 μ), and paper strips (veronal buffer pH 8.6, 0.05 μ).

Results.—When a 20,000 $\times$ *g* supernatant of a crude extract of maize tissue is run on paper electrophoresis and the paper strip stained for esterase activity, some of the activity is found to remain at the origin. This activity is associated with particles which do not migrate or migrate only very slowly during electrophoresis. The free enzyme can be separated from the particle-bound enzyme by centrifugation at 105,000 $\times$ *g* (Fig. 1). The relative intensities of the migrating and nonmigrating bands on paper electrophoresis are misleading in that they give the impression that most of the esterase activity in the extracts is associated with the particles. This is not so. The total activity of the 105,000 $\times$ *g* supernatant and pellet homogenate was analyzed using a modification of the Gomori technique for determining esterase activity in solution[3] using the same substrate and diazonium salt as were used to stain the paper strips. Results indicated that the activity in the pellet fraction was only about one twenty-fifth of the supernatant activity. This in-

tensity artifact is probably due to a rapid leakage of the free enzyme off the paper during the staining procedure.

A number of different esterases are present in the maize tissue, and it was therefore necessary to establish that the pH 7.5 esterase, which is specified by the E gene, was bound to the particles. This was done using the overlapping test in starch gel electrophoresis.[4] Homogenization of the washed pellets in the deoxycholate solution breaks down the particles and liberates the esterases. The DOC pellet homogenate was run alongside the supernatant, which contained the pH 7.5 esterase, in the overlapping tests. Those esterases migrating to the negative pole in each half of the gel joined to form a continuous band, indicating homology. In all cases, the enzyme liberated from the particles was identical to the free enzyme type found in the supernatant. *The DOC particle homogenate from kernels heterozygous for two different alleles contained three enzymes, the two parental enzyme types and the hybrid, in the proportion in which they are found in the supernatant.* For example, DOC homogenate of particles from $E^F/E^F/E^S$ endosperm showed heavy FF and FS (hybrid) bands and a light SS band. DOC homogenates from $E^N/E^S/E^S$ endosperm showed a light NN band and heavy NS (hybrid) and SS bands.

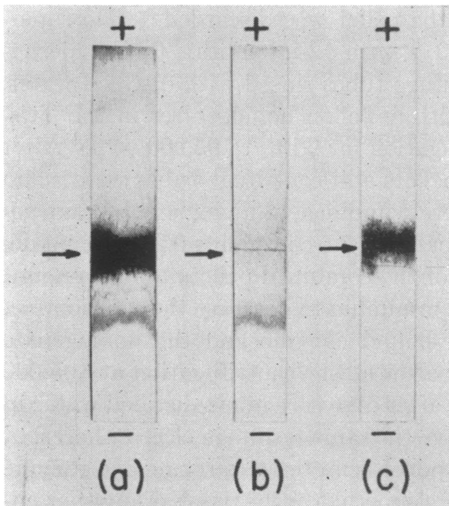

FIG. 1.—Paper strip electrophoresis of crude extract from immature maize kernels (veronal buffer pH 8.6, 0.05 μ). (*a*) 20,000 × *g* supernatant, (*b*) 105,000 × *g* supernatant, (*c*) 105,000 × *g* pellet homogenized in 0.1 *M* Tris buffer (pH 8.6) and taken up to original volume. Arrows indicate starting point.

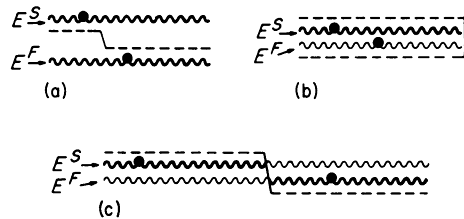

FIG. 2.—Possible models for hybrid enzyme synthesis. Bracketed letters refer to descriptions in the text. Dotted lines represent synthesized proteins and the solid wavy lines represent messenger-RNA specified by the E^S and E^F alleles; heavy and light portions indicate "sense" and "nonsense" complementary strands, respectively.

These results suggested that the hybrid enzymes are synthesized as hybrids on the particles and do not result from dimerization of two monomers. Two alternative explanations for the observed results were considered and ruled out experimentally to our satisfaction: (1) the particle-bound enzymes do not represent newly synthesized protein still attached to the templates but result from nonspecific adsorption of free enzymes onto the particles, and (2) the enzymes are synthesized as monomers on the templates but dimerize *in vitro* immediately upon being stripped off the

particles by the deoxycholate. Mixtures of free enzymes in the supernatants do not give rise to hybrids and, according to this scheme, would already exist in the form of dimers.

The first alternative was ruled out in two ways:

(*a*) The first 105,000 $\times$ g pellet from $E^S/E^S/E^S$ kernels was homogenized in 105,000 $\times$ g supernatant from $E^N/E^N/E^N$ kernels. The particles were again spun down at 105,000 $\times$ g, and the washing and regular procedure were followed for liberating the particle-bound enzyme. Only *SS* type enzyme was liberated from the particles. If the particle-bound enzymes were free enzymes absorbed onto the particles, *NN* type enzyme from the added supernatant should have been adsorbed on the particles and the liberated enzyme should have been of type *NN* alone or a mixture of *NN* and *SS*. This experiment was also repeated using particles from endosperm of a mutant which synthesizes practically none of this esterase in the endosperm.[5] As before, particles were homogenized in $E^N/E^N/E^N$ 105,000 $\times$ g supernatant. No detectable enzyme was liberated from the particles.

(*b*) The pH 7.5 esterase is synthesized in the young seedling as well as in the immature kernel.[1] The enzyme is not actively synthesized in the older seedling. It is not now possible to determine precisely the stage at which synthesis stops. However, at about the time the first leaf emerges from the coleoptile, the enzyme level in the seedling begins to decrease appreciably. Experiments using particle-bound enzymes similar to those described were conducted with seedling tissue in place of immature kernels. The seedlings used were at the stage where the enzyme level was still high, but we believed synthesis of new enzymes had already stopped. If the particle-bound enzyme is free enzyme nonspecifically adsorbed to the particles, the DOC treatment should liberate enzyme from the particles, since the first supernatant shows a high content of free enzyme. However, if the particle-bound enzyme is indeed newly synthesized enzyme still attached to the template, no enzyme should be attached to the seedling particles, since synthesis had been halted and none should be liberated by the DOC treatment. None was found in the DOC homogenate. On the other hand, the pH 7.5 esterase was found in the DOC homogenate when the experiment was repeated using very young seedlings in which the enzyme was still being synthesized.

The second alternative of *in vitro* dimerization upon liberation from the particles was ruled out by the following mixing experiment. A mixture of kernels homozygous for the E^N and E^S alleles was macerated and the regular procedure followed for liberating the particle-bound enzymes. If the hybrid enzymes are formed by dimerization of monomers stripped from the particles by deoxycholate, such hybrid enzymes should be found in the mixture of the two homozygotes just as it occurs in the heterozygote. The result of the mixture experiment was that only *NN* and *SS* enzyme types—no hybrids—were found after the DOC treatment.

In view of these results, we conclude that in the heterozygotes the hybrid enzymes are synthesized as such and do not result from dimerization of free preformed monomers.

Discussion.—With the finding of two new alleles of the *E* gene in teosinte,[5] we now have five alleles which specify an equal number of enzyme types with different electrophoretic migration rates. Five heterozygous combinations forming five different hybrid enzymes have been made, and in each case the hybrid enzyme

shows a migration rate intermediate between those of the parental enzyme types. Thus, the hybrid enzymes appear to be under the influence or control of both alleles in the heterozygotes. According to the commonly accepted hypothesis for the sequence of events leading to protein synthesis, the DNA of the gene specifies messenger-RNA, which becomes associated with the ribosomes where it functions as the information template for synthesis of proteins. Synthesis of hybrid enzymes would require either interaction between genes to form hybrid messenger-RNA or interaction between messenger-RNA molecules. Although gene interaction is not ruled out, the discussion will be limited to messenger-RNA interaction, and various models for such interaction will be considered.

If double-stranded RNA is the template for protein synthesis with the amino acids specified by H-bonded base pair sequences, then a hybrid template composed of two RNA strands specified by different alleles could conceivably form a new enzyme different from those produced when both strands in the RNA template are made by the same allele. However, there is no reason to expect that the new enzymes produced in all of the heterozygotes should show a migration rate intermediate between the parental enzyme types.

On the basis of a single-stranded RNA template, the hybrid could result from association of two messenger-RNA molecules with the enzyme synthesized partly on one strand and then completed on the second (Fig. 2*a*). The electrophoretic observations from heterozygotes are not compatible with this copy choice model. The model requires that E^S, E^N, and E^F involve mutations at different sites and thus four enzyme bands would be expected in the heterozygotes: the two parental enzymes and two new enzymes resulting from the two possible types of "crossover." One new type would arise from synthesis begun on A template and then swtiched to B. The second new type would result from synthesis begun on B then switched to A. One of these two new types should be present in all heterozygotes regardless of which alleles were involved, a situation similar to wild-type recombinants arising from mutant heterozygotes. In fact, only one new (hybrid) enzyme occurs in heterozygotes and it occupies a different position in all five heterozygous combinations. Also the relative intensities of the three esterase bands in the heterozygote do not fit the expectations based on the model. Heterozygous endosperms, which carry two doses of the maternal allele and one of the paternal, contain essentially equal amounts of maternal and hybrid enzyme types with considerably less of the paternal-type enzyme. Heterozygous seedlings carry one dose of each allele and show a higher concentration of hybrid enzyme than either parental type. With random association of messenger-RNA molecules, the concentration of the hybrid would be expected to be lower than either parental type, according to the copy choice model for hybrid enzyme synthesis. Association of two messenger-RNA molecules synthesized by the same allele could give only parental-type enzyme, while association of messenger-RNA molecules synthesized by two different alleles could give rise to either parental type or hybrid enzymes, depending upon whether or not switching occurs during synthesis. Finally, hybrid enzymes formed in this manner could not all show migration rates intermediate between the two parental types. Some of the hybrids would have migration rates slower or faster than the parental form enzymes.

The nature of the esterase which best fits the data is a double-sized molecule com-

posed of two either identical or homologous subunits synthesized on two messenger-RNA templates. The subunits can be linked by covalent bonds or some other stable linkage. Random association between the messenger-RNA molecules specified by the alleles of the *E* gene in heterozygotes would account for the observed relative concentrations of the three enzyme types. However, since the pH 7.5 esterase represents only a small fraction of the total protein of the maize tissue, only a small fraction of the messenger-RNA in the cells would be that specified by the *E* alleles. Therefore, there must exist some mechanism for specific association of two messenger-RNA molecules that can be synthesized by allelic genes on different chromosomes. It should be pointed out that there is no evidence for somatic pairing in maize.

One possible mechanism is that the messenger-RNA molecules pair specifically with a third element, such as a ribosome. This is unlikely unless the specificity of both the ribosome and its associated messenger-RNA were under the control of the same locus. A second possibility is 2 × 2 pairing between homologous messenger-RNA molecules. RNA specificity is believed to reside only in the sequence of bases, and the paired messenger-RNA molecules would be expected to be of complementary types. Since RNA association is postulated to account for the synthesis of two identical or homologous subunits on two templates, each of the complementary RNA molecules would have to be template for the synthesis of essentially identical polypeptides (Fig. 2*b*). It would seem quite unlikely that complementary messenger-RNA molecules would specify different enzymes with different amino acid sequences but having the same enzymatic activity and the same net charge. This model requires a degeneracy in the genetic code such that complementary base sequences code the same amino acid. Recent work of Nirenberg and Matthaei,[6] and Lengel, Speyer, and Ochoa[7] argue against such degeneracy in the code. Uracil sequences code phenylalanine while the complementary adenine sequences do not code any amino acids.

The requirement for this degeneracy in the code can be overcome if it is postulated that the messenger-RNA is a duplicate, composed of two complementary sequences joined end to end, tandem or mirror image, and only one of the sequences carries information, the complement being nonsense. If in such a duplicate strand we set the first half as sense and the second half nonsense, then on the complement of this strand the second half is sense and the first nonsense. Pairing between complementary duplicate messenger-RNA molecules specified by the same allele would give rise only to parental type enzymes, *FF*, *NN*, or *SS*, while pairing between RNA molecules specified by different alleles would produce the hybrid enzymes. Template switching is obligatory to make an enzyme molecule composed of two homologous subunits, and each RNA pair could synthesize only one type of enzyme (Fig. 2*c*). Duplicate messenger-RNA does not necessarily require a similar duplication of the gene DNA. The mirror-image duplicate type of messenger-RNA could be formed by the modification of the Watson-Crick DNA model[8] in which the DNA double helix consists of a single strand coiled back up on itself.[9, 10]

Summary.—An analysis of the newly synthesized particle-bound esterase in immature kernel and young seedling tissue indicates that the new enzyme found in maize plants heterozygous for two alleles of the *E* gene is synthesized as a hybrid and does not result from dimerization of preformed monomers. A number of mod-

els are considered for the interaction between messenger-RNA molecules required for synthesis of the hybrid enzyme.

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ASYNCHRONOUS DUPLICATION OF HUMAN CHROMOSOMES AND THE ORIGIN OF SEX CHROMATIN

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The sexual dimorphism in diploid intermitotic nuclei first demonstrated in many species of mammals by Barr and his associates[1] has been extensively studied in man and widely utilized in the investigation of anomalies of sex.[2, 3] A heteropycnotic body, the sex chromatin is present in a proportion of human somatic cells of the female and is lacking in those of the male. The sex chromatin is usually planoconvex with its flattened side located against the inner surface of the nuclear membrane. It measures about 1 micron in diameter, stains positively for DNA (deoxyribonucleic acid), and, in some nuclei, has a bipartite structure.

The origin of sex chromatin in man has been a subject of considerable speculation. Barr and his associates[1, 4] originally proposed the useful and widely held theory that sex chromatin represents the somatically associated heterochromatic segments of two X-chromosomes. However, recent evidence obtained by the direct examination of chromosomes is inconsistent with this view and strongly suggests that the sex-chromatin mass in somatic interphase nuclei is derived from a heteropycnotic segment of a single X-chromosome.[5-8]

Based on this evidence that one X-chromosome of the female forms the sex chromatin and from the patterns of mosaicism observed in the expression of sex-linked loci in the mouse, Lyon[9] proposed that the cytological manifestations coincide with a genetic inactivation. To explain the patterns observed, she assumes random inactivation of either the maternally or the paternally derived X-chromosome in an early stage of embryological development. Once the change is induced, the de-