GENETIC STUDIES ON MUTANT ENZYMES IN MAIZE.
VI. ELIMINATION OF ALLELIC ISOZYME VARIATION BY
GLYCERALDEHYDE TREATMENT¹

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THE E_i gene in maize specifies the pH 7.5 esterase. The enzyme occurs as a dimer composed of homologous subunits. Dimerization occurs at random between the various monomeric forms specified by the allelic genes, and in heterozygotes both autodimers and allodimers or hybrid enzymes are formed (SCHWARTZ 1960). Hybrid enzyme synthesis has been described for a number of other enzymes in various organisms (LEVINTHAL, SIGNER, and FETHEROLF 1962; ALLEN, MESCH and MORRISON 1963; SHAW and BARTO 1965; SCANDALIOS 1965; SCHWARTZ 1964a), and differs from the situation in hemoglobin synthesis where only autodimers are formed for both the alpha and beta polypeptides (SINGER and ITANO 1959). Seven alleles of the E_i gene have been found. These alleles are distinguishable in that they specify enzyme forms with different electrophoretic migration rates. Earlier studies with sodium borohydride treatment (SCHWARTZ 1964b) suggested that the charge differences between the allelic isozymes were not due to differences in the net charge of the amino acids comprising the protein but to variation in the number of charged conjugated groups. In vitro treatment with the borohydride converts a single isozyme into a number of new enzyme forms (the number depending on the isozyme treated), with migration rates corresponding to allodimers and autodimers specified by other alleles of this gene. The present paper is concerned with the in vitro modification of this enzyme by glyceraldehyde. As a result of this treatment, the charge differences between the seven allelic isozymes are eliminated, and all of the isozymes are converted to more acidic forms with identical migration rates in starch gel electrophoresis.

A number of cases have been reported where the structure of an enzyme was modified by chemical treatment with no or only partial loss of activity (see FRAENKEL-CONRAT 1959). The case reported here is of particular interest, since the modification involves those portions of the enzyme which are differentially specified by the various alleles. A number of other esterases specified by non-allelic genes occur in maize, but the glyceraldehyde conversion is limited to the pH 7.5 esterase.

MATERIALS AND METHODS

The materials used in these studies were extracts from plumules of 3-to 5-day old seedlings (seeds soaked for 4 hours and germinated on moist filter paper at 30°C in the dark), and immature

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kernels harvested 16 days after pollination. The kernels were cut off the cobs and stored in plastic bags at -20°C until thawed for use. The pH 7.5 esterase is synthesized in both plant parts. Identical results were obtained with enzyme preparations from the two sources. The tissues were ground in a mortar and centrifuged at $20,000 \times g$ for 20 minutes to remove most of the particulate material. The crude supernatant was used for most of the experiments, but partially purified enzyme preparations were also employed. The purification procedure involved lowering the pH of the crude supernatant to 5.0 by addition of 0.5 M phosphoric acid, centrifuging at $20,000 \times g$ for 20 minutes, and raising the pH of the supernatant to 7.0 with 2 M NaOH. The 55 to 85% ammonium sulfate fraction was dialyzed against 0.005 M sodium phosphate buffer at pH 6.4 and adsorbed on a carboxymethyl cellulose column. A linear gradient was applied to the column with 0.005 M sodium phosphate buffer pH 6.4 in the mixing flask, and 0.03 M sodium phosphate buffer pH 7.5 in the inlet flask. The fractions were assayed for the enzyme by starch gel electrophoresis. The pH 7.5 esterase was eluted as a sharp peak in the early fractions. The fractions which contained this enzyme were free of the other esterases, but contained some contaminating protein which we have not as yet been able to separate from the esterase.

Glyceraldehyde was added to buffered (0.03 M phosphate pH 7.7) enzyme preparations to a concentration of 0.2 M and incubated at 37°C for 24 hours. In some runs the glyceraldehyde concentration, the incubation time, and temperature of incubation were reduced. The buffered enzyme solutions without the glyceraldehyde were used as controls.

The techniques of starch gel electrophoresis and staining of the gels for the esterase bands are described in an earlier publication (SCHWARTZ 1960). In the two-dimensional electrophoresis experiments, enzyme preparations were subjected to electrophoresis, and the gels cut into longitudinal strips about 3 mm wide. One strip was assayed to locate the position of the esterase bands, and a second was cut into one-inch lengths (the width of the gel molds), and inserted at the origin in a second set of gels for electrophoresis in the second dimension at right angles to the original run.

RESULTS AND DISCUSSION

Incubation of the pH 7.5 esterase with glyceraldehyde for 24 hours results in the conversion of the enzyme to a more acidic form. The untreated isozymes specified by six of the seven alleles at this locus migrate to the cathode in starch gel electrophoresis at pH 8.5. After glyceraldehyde treatment, the enzymes shift in the direction of migration from the cathodal to the anodal side (Figure 1). With 24-hour incubation, the acidic form of the enzyme migrates as a fairly sharp band. At shorter periods of incubation the enzyme migrates as a smear or a very broad band. The broad band shows a gradual shift from the cathodal to anodal migration as the incubation time is increased. At the intermediate incubation periods, the enzyme band straddles the origin with the band partly on the cathodal side and partly on the anodal side of the origin. Thus as the incubation time is increased from zero to 24 hours, the band changes from a narrow band to a smear, and then narrows again to a fairly sharp band concomitantly with a change in direction of migration as well as rate. These results indicate that a large number of steps are involved in the conversion of the enzyme by glyceraldehyde, since the enzyme can exist in a number of intermediate active forms (represented by intermediate migration rates) between the basic native form and the acidic converted form. The number of intermediate steps in the conversion must be large, since the enzyme band formed by intermediate incubation periods appears as a uniform smear rather than as a number of discrete bands. The conversion from the native to the acidic form can be stopped at any of the inter-

FIGURE 1.—Zymogram of crude seedling extract showing the alteration in migration of the NN pH 7.5 esterase with time of incubation in 0.2 M glyceraldehyde. O denotes the origin. a = untreated control, b-j = glyceraldehyde treated: b = 24 hours, c = 6 hours, d = 5 hours, e = 4 hours, f = 3 hours, g = 2 hours, h = 1 hour, i = 0.5 hour, j = no incubation prior to electrophoresis. The other esterase bands seen in the zymogram are specified by other nonallelic genes. The slower migration rates observed for these bands in the two gels on the right result from nonuniformity of contact between the gel and the sample saturated filter paper strips inserted at the origin.

mediate stages by dialysis to remove the glyceraldehyde. The rate of conversion can be decreased by lowering the incubation temperature or the concentration of glyceraldehyde. The glyceraldehyde conversion does not cause any appreciable inactivation of the pH 7.5 esterase. Partially purified esterase preparations were incubated at 37°C for 24 hours in the presence and absence of 0.2 M glyceraldehyde. Enzyme activity was determined by the Gomori method (Gomori 1953) colorimetrically measuring the amount of α -naphthol released by hydrolysis of α -naphthyl acetate. The enzyme preparation incubated with glyceraldehyde showed about 96% of the activity of the glyceraldehyde-free control (394 *vs.* 412 μ moles α -naphthol per ml.).

The two-dimensional electrophoresis runs of enzyme preparations incubated for 3 hours with 0.2 M glyceraldehyde verify that the broad enzyme band represents a heterogeneous population of enzyme forms with varying migration rates, rather than only a few enzyme forms which for some reason do not migrate uniformly and overlap each other, appearing as a smear in the zymograms. If the latter were the case, i.e., few enzyme forms of different charge migrating as smears, the activity should appear in a series of stepwise overlapping bands after electrophoresis in the second dimension. If however, the broad enzyme band formed in the first electrophoretic run represented a continuous spectrum of

FIGURE 2.—Two-dimensional electrophoresis zymogram of enzyme preparation incubated for 3 hours with 0.2 M glyceraldehyde (see text). O_1 and O_2 indicate position of origin in first and second electrophoretic runs.

FIGURE 3.—Zymogram showing the glyceraldehyde conversion of purified FF isozyme of the pH 7.5 esterase. Left: untreated control; right: 24-hr incubation with 0.2 M glyceraldehyde. The arrow indicates the origin.

enzyme forms with different charges, the second dimension run should form a sharp straight band extending through the origin from the cathodal to the anodal side. This is clearly the case, as is seen in Figure 2. The sharpness of the band in the second dimension electrophoretogram points to the stability of the partially converted enzyme forms in the absence of glyceraldehyde.

The alteration of the enzyme appears to be a consequence of the direct action of glyceraldehyde on the esterase, and not an indirect effect resulting from the activation of some other enzyme by the glyceraldehyde, since the conversion was found to occur even when the purified enzyme preparation was treated (Figure 3). Furthermore, the conversion also resulted when the electrophoretically separated esterase in the gel is incubated with glyceraldehyde. The native enzyme migrates to the cathode in starch gel at pH 8.5. If a longitudinal slice of the cathodal half of the gel is rotated 90° and inserted at the origin of another gel for an electrophoretic run in the second dimension, the pH 7.5 esterase migrates to its characteristic position on the cathodal side. However, when the gel is incubated in a 0.2 M glyceraldehyde solution prior to electrophoresis in the second dimension, the esterase migrates to the anode, indicative of conversion of the electrophoretically separated pH 7.5 esterase band in the starch gel.

In order to test the possibility that the glyceraldehyde does not act directly to alter the enzyme but merely serves as substrate for growth of bacteria which cause the electrophoretic shift, glyceraldehyde incubation was carried out in the presence of 200 $\mu\text{g}/\text{ml}$ of chloramphenicol. Subsequent plating on enriched medium revealed no sign of bacterial growth in the incubated mixture but the electrophoretic shift still occurred.

The electrophoretic assays on the treated pH 7.5 esterase clearly point to a change in the net charge of the enzyme molecule and cannot be explained by a change in size resulting from polymerization or dissociation into smaller subunits. The rate of migration of proteins in starch gel depends on both size and charge. However, a change in net charge must be involved, since the enzyme shifts in direction of migration from the cathodal to the anodal side. Also, in the course of conversion of the pH 7.5 esterase from the native to the acidic form, the enzyme molecules undergo a change in migration rate, first decreasing so that they show less migration away from the origin on the cathodal side, and then increasing in migration rate as they change direction and migrate to the anode. If a change in size were involved, one would have to postulate that during the course of conversion the enzyme first becomes larger, then smaller. This is highly unlikely, especially since treated WW isozyme, which in the native form migrates to the anodal pole, does not show this shift in migration rate. The WW isozyme shows a gradual increase in migration rate during the course of conversion to the acidic form (Figure 4).

Additional evidence that the glyceraldehyde conversion does not involve a striking change in molecular size is obtained from sucrose gradient centrifugation experiments with purified native and the converted esterase. The native and converted forms of the esterase migrate differently in starch gel electrophoresis, so that it is possible to mix the two forms and find their relative position in the same gradient. A mixture of partially purified enzyme preparation and 24-hour glycer-

FIGURE 4.—Conversion of the WW isozyme with time of incubation with 0.2 M glyceraldehyde. Two samples were run on each gel. Untreated control is on the extreme left. Proceeding to the right, the samples were incubated for 0, 0.5, 1, 2, and 3 hours prior to electrophoresis.

aldehyde treated crude extract was centrifuged in a 5 to 25% linear sucrose gradient for 23 hours at $63,500 \times g$ in a Spinco Model L with an SW 25.1 rotor; 124 four-drop fractions were collected, subjected to electrophoresis, and the gels stained for esterases to determine the fractions with the highest activity for each form, the native and the converted. The enzyme bands overlapped but the peak of activity for the native enzyme was in fractions 87 to 89, while the glyceraldehyde-converted enzyme peak was at 91 to 93. Thus the two enzyme forms are found in almost the same position in the sucrose gradient, with the converted form being either slightly lower in molecular weight, or changed in configuration so that it sediments at a slightly lower rate.

Six of the *E*_i isozymes migrate at different rates to the cathodal pole in starch gel at pH 8.5 and one migrates to the anode. Upon incubation with glyceraldehyde, all seven isozymes are converted into acidic forms with identical migration rates in both horizontal and vertical starch gel electrophoresis. Whatever the effect of the glyceraldehyde, it eliminates the genetically determined charge difference that exists between the allelic isozymes. Figure 5 compares the banding

FIGURE 5.—Zymograms showing the position of the FF, LL, TT, and WW pH 7.5 esterase isozymes, (a) before, and (b) after incubation with glyceraldehyde. All of the isozymes are converted to acidic forms with identical migration rates.

pattern of the native and converted enzymes for four of the most extreme isozymes. The converted isozymes may still differ in primary structure, but all have the same net charge.

Formaldehyde has been postulated to react with many different protein side groups. Acetaldehyde can react with indole and primary amino groups (PUTNAM 1953). The chemical nature of the glyceraldehyde conversion is not known, but its elucidation should be of considerable help in establishing how the allelic isozymes differ. The conversion to a more acidic form could presumably result from an unmasking of negative charges on the protein, a masking of positive charges, or the stripping off of positively charged side groups bound to the protein. Glyceraldehyde does not convert all enzymes to more acidic active forms. The other esterases in the maize extracts which are specified by nonallelic genes are not affected in a like manner (Figures 1 and 4). The intensities of these esterase bands in the zymogram decrease with increased incubation time with glyceraldehyde, but this is not associated with an alteration in migration rate (see Figure 1). After 3 hours of glyceraldehyde treatment, the pH 7.5 esterases have already undergone considerable shift toward the acid side, but the other esterases are unaffected either in activity or migration.

The effect of both the borohydride and glyceraldehyde treatments is to cause a unidirectional change in the net charge of the enzyme. The change induced by the borohydride is more limited, giving rise to relatively few slightly altered forms of the enzyme which migrate as discrete bands in electrophoresis. Glyceraldehyde causes a much more drastic change in the net charge of the enzyme, and gives rise to a highly heterogeneous population of enzyme forms. Both treatments must affect the charged groups which are responsible for the difference in migration rate between the allelic isozymes, as the borohydride treatment converts one enzyme form into another, and glyceraldehyde eliminates the charge differences between the allelic isozymes. However, glyceraldehyde probably affects additional groups over and above those involved in the isozyme variation. Evidence will be presented in a forthcoming paper (ENDO and SCHWARTZ, in preparation) showing that the glyceraldehyde converted isozymes are not identical even though they show the same migration rates in starch gel electrophoresis. The difference in urea sensitivity between the NN and FF isozymes persists even in the converted forms.

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SUMMARY

Incubation of the pH 7.5 maize esterase with glyceraldehyde results in the conversion of the enzyme to an acidic form. The alteration to the fully converted acidic form is a gradual process, and the enzyme can be stabilized in any of the intermediate forms by removal of the glyceraldehyde. The treatment eliminates the genetically determined charge differences which distinguish the seven allelic isozymes of the pH 7.5 esterase. All the isozymes are converted to acidic forms with identical migration rates.

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