Inheritance of an Erythrocyte Acetylesterase Variant in Man

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ENZYMES WHICH CATALYZE CARBOXYL ESTER CLEAVAGE (esterases*) have been demonstrated in human erythrocytes by histochemical technics (Wachstein and Wolf, 1958; Davis, 1959), and several molecular forms of these esterases have been resolved from human red blood cells by combined electrophoretic and dye-coupling procedures (Tashian, 1961). Examination of these patterns of enzyme activity in a series of human subjects has shown them to be both reproducible and constant. Acquired alterations from the normal patterns, however, have been observed in certain disease states. The finding of a distinct esterase variant in the red cell lysates from both members of a pair of identical twins has suggested the existence of a genetically determined variation in some of these enzyme forms (Tashian, 1961). A family study of these twins has now been completed and the results comprise the present report.

METHODS

Stromata-free erythrocyte lysates were prepared from heparinized venous blood samples as previously described (Tashian, 1961) and subjected to vertical starch gel electrophoresis by the method of Smithies (1959). Because the presence of even small amounts of stromata in hemolysates may result in considerable streaking of the electrophoretic pattern, it is important that hemolysates be thoroughly extracted with toluene and centrifuged prior to insertion in the gels. An increased resolution of diffuse or closely running bands of enzyme activity was achieved by the use of 1/32″ sample-insertion templates; 1/16″ templates were only used to locate bands of weak activity. As some bands showed differential responses of resolution and activity at different pH values, hemolysates were run in gels buffered at both pH 8.4 and pH 8.6 to insure a satisfactory composite of the esterase bands. The addition of NaCl to the bridge buffer has the effect of increasing the anodal migration of the *B* esterase, thereby separating it from the A_2 esterases (see next section). A final concentration of 0.03 *M* NaCl was usually used.

After the gels were sliced, the zones of enzyme activity were visualized by incubating with α-naphthyl acetate (Eastman Organic Chemicals) as the substrate and diazotized 4′-amino-2′, 5′-dimethoxy-benzanilide (Blue RR salt, Allied Chemical Corporation) as the dye coupler. The incubation mixture followed the proportions suggested by Markert and Hunter (1959) of substrate: 1.0 per cent in acetone, 2 ml.; buffer: 0.4 *M* tris (hydroxymethyl) aminomethane, pH 7.0, 10 ml.; and dye: 100 mg. made up to 100 ml. with distilled water.

Although aging or frequent freezing and thawing of hemolysates resulted in the formation of some altered bands of enzyme activity, it is possible to store freshly frozen samples

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*The hydrolytic enzyme forms described in this paper are referred to as esterases only on the basis of their reaction with the synthetic substrates used.

up to at least two months at —10° C. without marked alteration of the enzyme patterns. The enzyme forms were seemingly unaffected qualitatively by the sex or age of the subject.

RESULTS

Characterization of the normal pattern

In a preliminary report (Tashian, 1961) all of the red cell esterases which hydrolyzed the acetate ester of α-naphthol at a higher rate than the corresponding butyrate or propionate esters were tentatively referred to as *A* esterases. It has since been shown that these acetylesterases are composed of at least four types which may be characterized chemically. These are diagrammed in Fig. 1 and are designated as: *C* esterases, A_1 esterases (A_1a – A_1d), A_2 esterases (A_2a – A_2d), and carbonic anhydrases (CA I, CA II); these were formerly referred to (Tashian, 1961) as: A_1A_2, A_3–A_6, A_7, and A_8 respectively. Comparative chemical studies relating to the esterase and dehydrase activity of the carbonic anhydrase forms, as well as more detailed chemical characterization of these red cell esterases, will be the subject of later reports.

Because the molecular forms of the A_1 and A_2 complexes share certain chemical properties which have been described as characteristic for acetylesterases variously referred to as A-esterases by Aldridge (1953) or arylesterases (Augustinsson, 1961), they have been tentatively placed in this category. These properties include: (1) hydrolysis of aromatic acetate esters at a higher rate than aromatic propionate or butyrate esters, (2) inhibition by low concentrations of iodoacetamide and *p*-chloromercuribenzoate (possibly indicating sulfhydryl groups at the active site), and (3) resistance to 10^{-1} *M* eserine and 10^{-2} *M* diisopropylfluorophosphate.

Characterization of the atypical pattern

The pattern given by the esterase variant can be distinguished from the normal pattern at both pH 8.4 and pH 8.6. Comparisons of the patterns at the two pH's are shown in Fig. 2. In addition to the new, fast migrating component, the variant differs from the normal in the reduced activity of bands A_1a, A_1b, and A_1d and the increased activity of band A_1c. No alteration in the other enzyme forms is apparent. The variant pattern was constant in all affected individuals, whose ages ranged from 4 to 70 years.

Effect of pH

The differences in esterase migration patterns in gels buffered at pH 8.4 and pH 8.6 are shown in Figs. 1 and 2. At pH 8.4 CA I and hemoglobin have remained essentially at the origin and there is a greater separation within the forms of the A_1 and A_2 bands. The best resolution of bands A_1c and A_1d occurs at pH 8.6.

Alterations acquired in vitro

An anodal migration of what seems to be some molecules of the A_1a and A_1b esterases begins to take place in fresh hemolysates which have been frozen

and thawed about four or five times. Subsequent treatment of this type results in the gradual appearance of two distinct advanced bands which are apparently composed of the altered A_1a and A_1b molecules (Fig. 2: normal altered). Al-

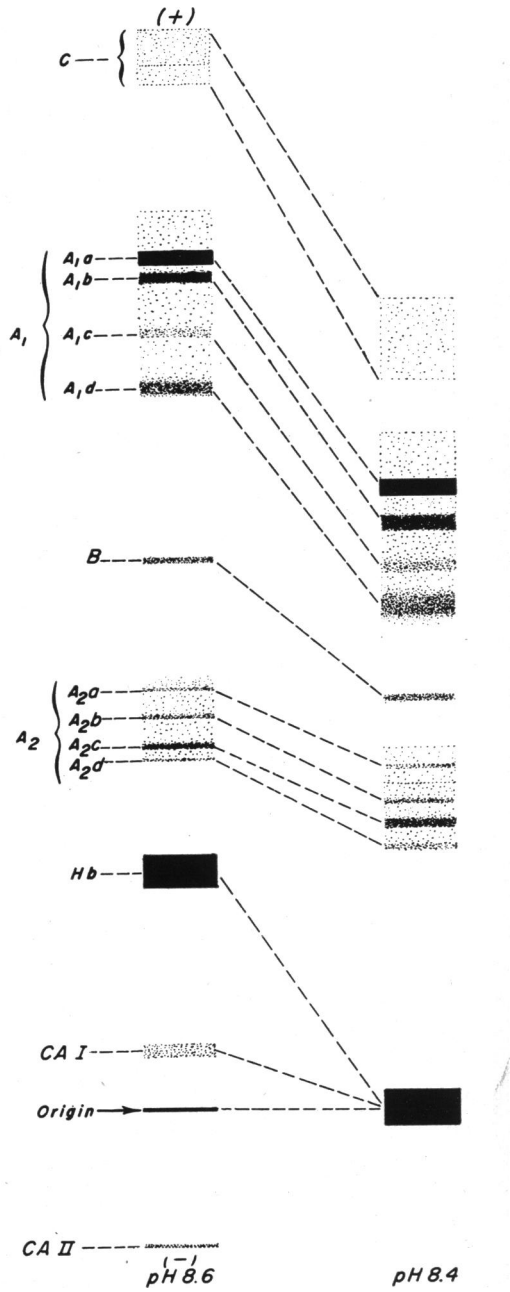

FIG. 1. Diagrams of typical esterase patterns from normal fresh hemolysates after electrophoresis for 18 hours at pH 8.6 and pH 8.4.

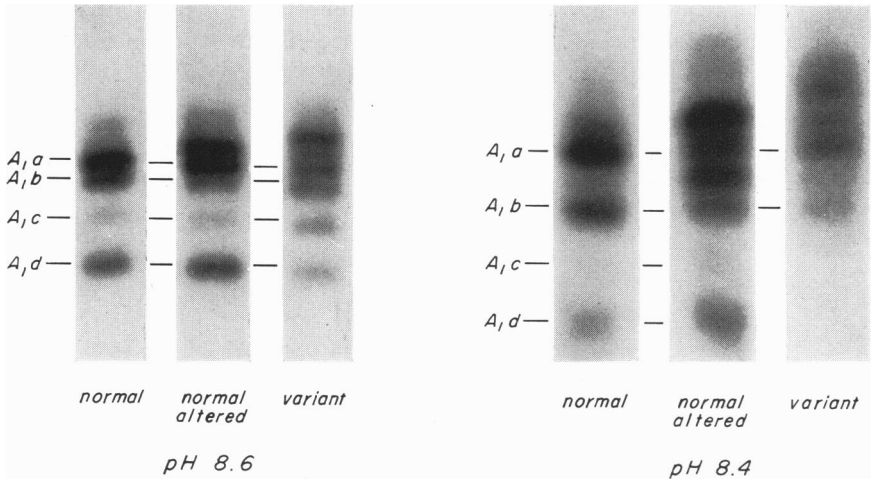

FIG. 2. Comparison of starch gel electrophoresis patterns of A_1 esterases from (left to right) normal hemolysate, same sample frozen and thawed 20 times, and variant sample. The patterns were developed after electrophoresis for 18 hours at 4-5° C. with a gradient of 9 volts/cm. in trays measuring 3 x 140 x 327 mm.; hemolysates inserted 126 mm. from cathodal end. Gels were made up in 0.02 M H_3BO_3 adjusted with NaOH.

though there is a diminution in intensity of the original A_1a and A_1b bands and a gradual increase in the activity of the altered bands, no overall loss of enzyme activity is apparent. A similar effect begins to occur in hemolysates which are incubated at 37 C. for 16 to 20 hours under sterile conditions, or aged at room temperature for two to three days. These alterations can also be induced in corresponding bands of the variant pattern.

The regularity of the alterations acquired in vitro of the A_1a and A_1b bands may indicate depolymerization of these molecules. However, the exposure or masking of polar groups resulting from configurational changes, or loss or addition of non-reactive groups could also explain the results.

Family study

Following the discovery of the above described esterase variant, repeat blood samples from the twins as well as samples from their parents and two sisters were obtained. The normal pattern was found in the father and one sister, while the atypical pattern appeared in the mother and the other sister. There is no known consanguinity between the parents.

The pedigree obtained from the investigation of the maternal relatives is shown in Fig. 3. The genetic determinant for the atypical esterase pattern is present in four males and four females in four segregating sibships in three generations, which fits the hypothesis of a single gene effect. The altered allele has been transmitted to offspring of both sexes by affected males and affected females, consistent with an autosomal pattern of inheritance. After correction for ascertainment, the ratio of "normal" to "atypical" is 6:5, which fits well with an expected 1:1 ratio.

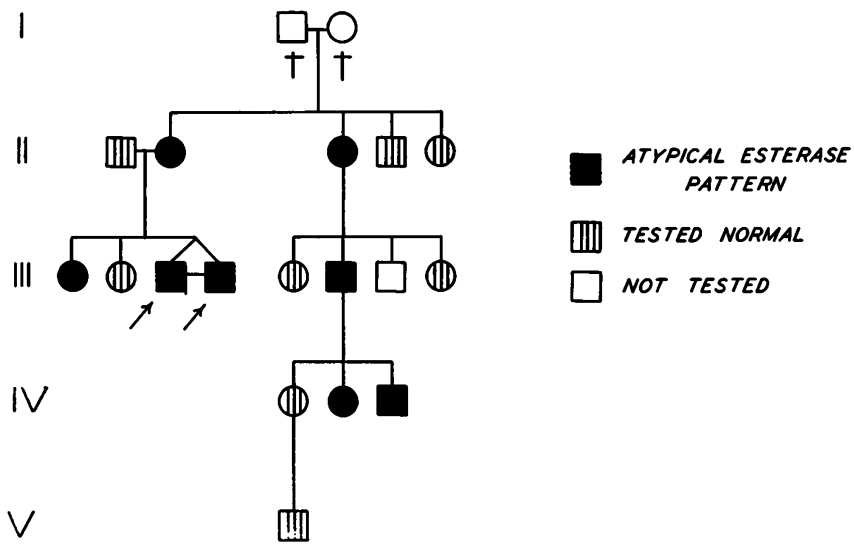

FIG. 3. Pedigree showing segregation of the variant A_1 esterases.

DISCUSSION

Analysis of the genetic alteration of multiple enzyme forms with similar chemical properties (isozymes) has the unique advantage of studying phenotypic effects at the level of the primary gene product. The chemical and genetic behavior of the A_1 complex of acetylesterases described in the present study indicate that some aspect of the structure or regulation of each of these forms may be under the control of a single genetic locus. Single gene control of esterase isozymes has been demonstrated in the Protozoan *Tetrahymena pyriformis* by Allen (1961), and in alkaline phosphatase isozymes of *Escherichia coli* by Bach, Singer, Levinthal and Sizer (1961).

The mechanism underlying the variant observed in the present study is obscure. Until more is known about the molecular structure of these enzymes, it is difficult to determine whether the alterations are qualitative or quantitative. One might speculate, however, that if the four forms of the A_1 complex represent structurally related molecules with an identical polypeptide chain, then a change in the primary structure of this chain could be reflected in an electrophoretically detectable alteration of one-half of the molecules formed in the heterozygote. Thus, if we assume that the genetically altered enzyme for each of the forms has a total charge equal to the unaltered enzyme immediately anodal to it (e.g., A_1d-altered $= A_1c$), then one-half of the altered molecules (with the exception of the most anodal variant band) would be superimposed over one-half of the original unaltered molecules. This would then produce the pattern observed in the variant. That these alterations represent quantitative variations at the regulatory level cannot, of course, be excluded.

The origin of the mutation is not apparent. Since there are two affected individuals in generation II, then one of the parents (generation I) possessed the gene for the atypical pattern. Both of these ancestors were of French Canadian origin, and there was a history of American Indian admixture in the female.

Medical histories obtained from various members of the family produced no evidence for correlation between the atypical phenotype and specific pathological conditions. Individual III-1 in Fig. 3 has far advanced multiple sclerosis with onset at age 30, while II-2 and II-3 suffered severe cerebrovascular accidents resulting in hemiplegia at ages 67 and 68, respectively. Both propositi and the other three individuals with the atypical pattern were seemingly in good health. No history of drug sensitivities could be established. Because of the history of cerebrovascular disease and multiple sclerosis in the family under study, four other patients with these diagnoses were investigated with negative results.

Examination in our laboratory of hemolysates from almost 900 individuals (80 per cent Caucasian, 20 per cent American Negro) has failed to reveal another example of the genetic variant described here. Of particular interest, however, has been the discovery by Shaw, Syner and Tashian (1962) of a genetically determined variant of one form (CA I) of carbonic anhydrase.

SUMMARY

An acetylesterase variant from human red blood cells which is transmitted as if due to a single autosomal gene difference was observed in eight closely related individuals.

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