Achromatic Regions of Tetrazolium Stained Starch Gels: Inherited Electrophoretic Variation

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STARCH gel electrophoresis of human hemolyzates has been carried out almost daily in our laboratory over the last two years, with subsequent tetrazolium-linked staining of the gels for a number of enzymes, including glucose-6-phosphate dehydrogenase (G6PD) (Shows *et al.*, 1964), 6-phosphogluconate dehydrogenase (6PGD) (Dern *et al.*, 1966), hexokinase (Eaton *et al.*, 1966), and, more recently, phosphoglucomutase and adenylate kinase (Brewer *et al.*, 1967). During this time, hemolyzates from several thousand individuals, from many parts of the world, have been subjected to electrophoresis.

When the starch gel is stained for any of the above enzymes (as well as for many other enzymes) using the phenazine-tetrazolium technique, in addition to the appearance of the blue bands marking the site of the isozymes of the enzyme under investigation, light or achromatic areas in the starch appear. These consist of whitish areas in the normally somewhat bluish background of the gel. The achromatic bands are not restricted to electrophoretic patterns of hemolyzates from humans, but are present in hemolyzate and tissue preparations of all species with which we are familiar. No doubt, the presence of the achromatic bands has been previously noted by dozens of investigators working with various electrophoretic systems.

The pattern of the achromatic bands on a given gel system has been remarkably constant in thousands of human hemolyzates. Recently, however, we detected variation in this pattern in the red cells of an individual and initiated an investigation of his family. The tissue distribution of the enzyme responsible for the achromatic regions has been studied, utilizing autopsy material from the propositus, who died during the course of the study. In addition, we have partially characterized the enzyme or enzyme complex responsible for the achromatic bands.

METHODS

Vertical starch gel electrophoresis was carried out by a method similar to that used by Spencer *et al.* (1964) for studying the enzyme phosphogluco-

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mutase. However, in preliminary experiments, we soon demonstrated that, after electrophoresis of hemolyzates, the development of the bluish background of the gel and the achromatic regions will appear after addition of only phenazine methosulfate and tetrazolium (both phenazine and tetrazolium are required) as long as the gel has some exposure to light. The bluish background and the achromatic regions were not dependent upon the presence of coenzymes or substrates. Therefore, in the experiments reported here, except as noted, only phenazine methosulphate and the tetrazolium dye, MTT, were used in the indicating solution.

The method of preparing hemolyzates for starch gel electrophoresis has been previously described (Shows *et al.*, 1964). Preparation of the tissue extracts for electrophoresis was carried out on fresh autopsy material; tissue was homogenized in small volumes of cold saline by means of a Virtis homogenizer. The capacity of the enzyme to catalyze the "Nadi reaction" (a test for indophenol oxidase) after electrophoresis was tested by incubating the starch gel at 37° C in 10^{-3} M alpha-naphthol and 10^{-3} M N,N-dimethylphenylenediamine in 0.05 M Tris buffer at pH 8.0. Inhibition studies were carried out by incubating the gels with 10^{-3} M concentrations of either cyanide or azide in 0.05 M Tris, pH 8.0, at 37° C for 30 minutes. After incubation with inhibitors, the gels were washed thoroughly with distilled water several times before various indicating solutions were added. Dichloroindophenol oxidase was demonstrated by incubating gels in a 1% agar overlay containing 1.76×10^{-4} M DPNH, 1.4×10^{-4} M 2,6-dichlorophenolindophenol and in a 0.05 M Tris buffer, pH 8.0.

RESULTS

Variant Enzyme in Hemolyzates

The usual achromatic band pattern of human hemolyzates is shown in slots 1 and 3 of Fig. 1. Two bands are usually seen (*a* and *b*), with the leading band the fainter of the two. The variant pattern is shown in slots 2 and 4 of Fig. 1. In the variant pattern, the *a* and *b* bands can usually be seen, but are fainter than normal. In addition, two new, slower migrating bands (*c* and *d*) can be seen. Slots 5 and 6 of Fig. 1 demonstrate the Nadi reaction catalyzed by the normal and variant enzyme respectively. This will be referred to in a later section.

Family Study

The pedigree of the kindred with the variant is shown in Fig. 2. The variant segregates as if due to a single gene, and the affected individuals are presumably heterozygous for the mutant gene. Because II-4, the mother of III-7, did not have the rare mutant gene, it can be assumed that transmission occurred through the deceased father of III-7; the gene is therefore autosomal. We have designated this variant as "Morenci" after the town of origin. The propositus of the family had a clinical diagnosis of multiple myeloma, but autopsy failed to substantiate this diagnosis. He died of arterio-

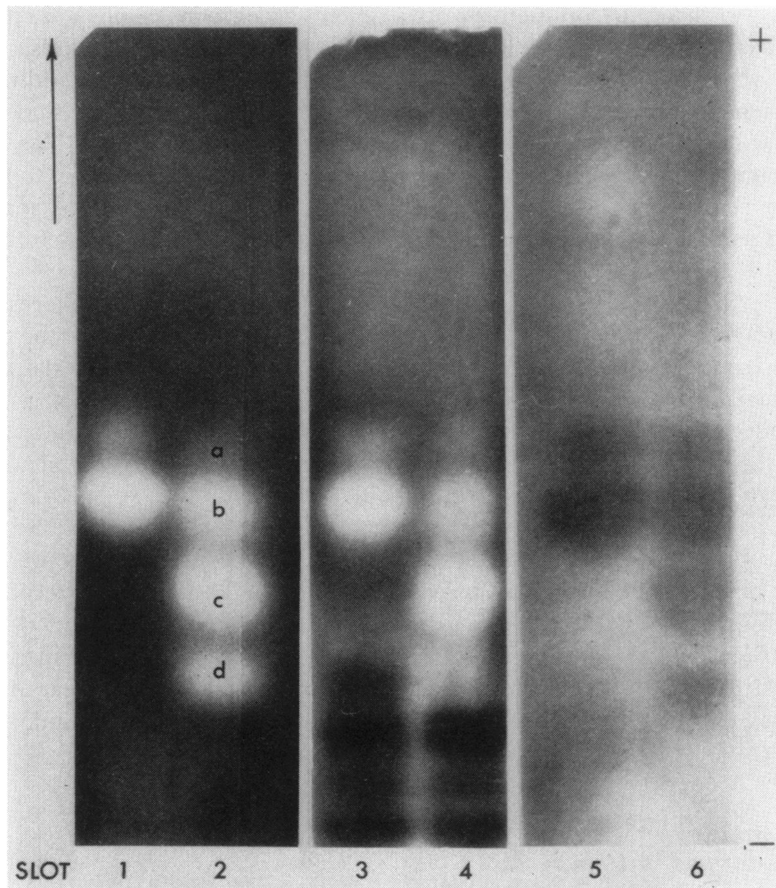

FIG. 1. Starch gel electrophoretic patterns of hemolyzates from a normal individual (slots 1, 3, and 5) and from the propositus of the family with the electrophoretic variation in achromatic bands (slots 2, 4, and 6). The gels were stained as follows: slots 1 and 2, achromatic band technique; slots 3 and 4, phosphoglucomutase technique (the dark bands are phosphoglucomutase); slots 5 and 6, Nadi technique (see text).

sclerotic cardiovascular disease at 82 years of age. Other carriers of the enzyme variant are in good health.

Tissue Studies

The propositus died during the course of these investigations, and tissue samples were obtained from the heart, liver, kidneys, skeletal muscle, skin, and intestines. As a control, tissue was obtained from these same organs during the autopsy of an individual who had been killed in an automobile accident. Tissue extracts were prepared from all six tissues of the propositus and of the control and subjected to starch gel electrophoresis. Two banding regions were seen in the nonerythrocytic tissues, one identical to the red cell bands (region A in Fig. 3) and one nearer the origin (region B in Fig. 3). The propositus and the control showed similar patterns in region B.

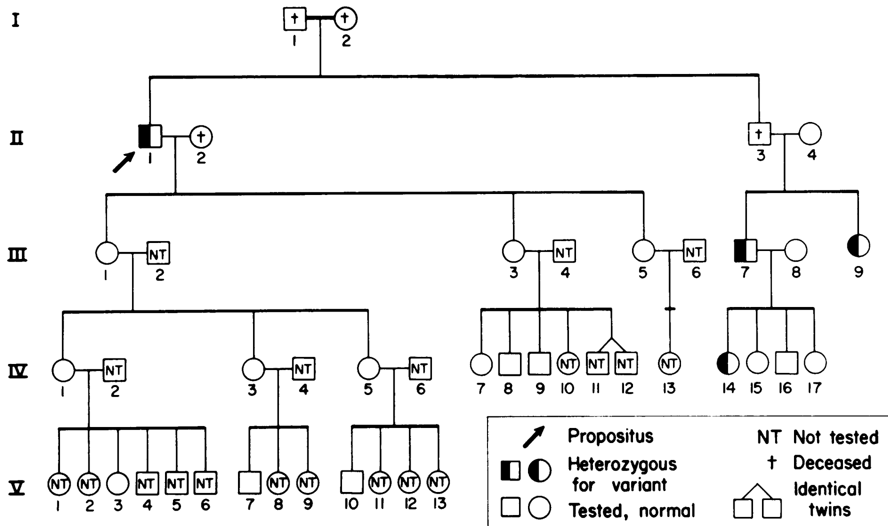

FIG. 2. Pedigree of the family with the electrophoretic variant of achromatic bands.

However, with respect to region A, extracts of all six tissues of the propositus demonstrated the same variant pattern as were present in his red cells, that is, bands *a, b, c,* and *d.* The control had only bands *a* and *b* in region A in each tissue studied, similar to the pattern observed in normal hemolyzates. Figure 3 shows the pattern of the liver preparation of the control compared with the liver, heart, and kidney preparations of the propositus. Patterns from the skeletal muscle, intestine, and skin of the propositus also showed the variant pattern, but the bands were too weak to photograph well.

Partial Characterization of the Enzyme Causing the Achromatic Region

A number of studies have been carried out in an effort to identify the protein responsible for the achromatic regions.

Nadi reaction. We investigated the possibility that this protein might be part of the cytochrome system. Histochemically, cytochrome activity has been localized by means of the Nadi reaction, the products of the reaction resulting in a blue color (Yonetani, 1963). After electrophoresis of hemolyzates from the propositus and from a normal control, the gel was sliced horizontally, the top half was exposed to the ingredients of the Nadi reaction (Fig. 1, slots 5 and 6), and the bottom half to the usual stain for achromatic bands. The two types of activity occurred in identical locations on the gel, with both the normal and the variant enzyme, demonstrating that the protein causing the achromatic regions catalyzes the Nadi reaction.

Dichloroindophenol oxidase activity. Those enzymes of the cytochrome system which catalyze the Nadi reaction are referred to as indophenol oxidases. The red cell enzyme was tested for this catalytic activity by exposing the top half of a horizontally sliced gel to an indicating solution containing

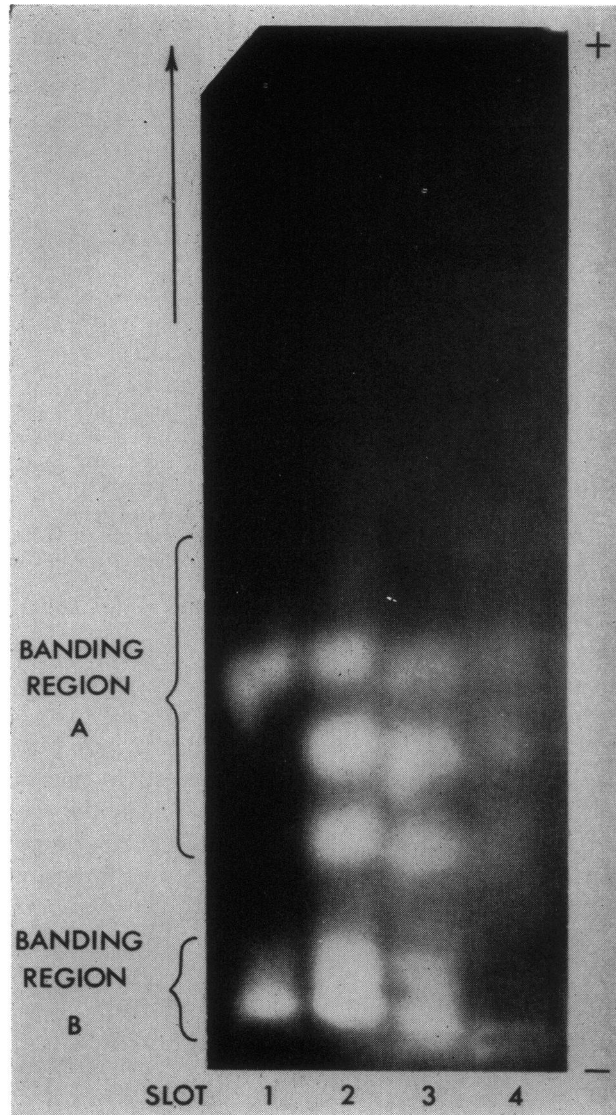

FIG. 3. Starch gel electrophoretic patterns of various tissues, stained by the achromatic band technique. Slot 1, liver preparation from a control. Slots 2, 3, and 4 are liver, heart, and kidney preparations, respectively, from the propositus. Skeletal muscle, small intestine, and skin preparations from the propositus showed bands similar to those seen in slots 2, 3, and 4, but the bands were too weak to photograph well. Band *a*, the leading band, does not show up well in the photograph but was present in all patterns.

dichlorophenolindophenol and the bottom half to the indicating solution for achromatic regions. Blue bands indicating oxidation of dichlorophenolindophenol occurred in the same sites of the gel as the achromatic regions. This was true of both the normal and variant enzymes.

Inhibition studies. After electrophoresis of hemolyzates, gels were sliced in half horizontally, and the top half was incubated with cyanide in a Tris buffer

for 30 minutes, while the bottom half was incubated in buffer alone. After thorough washing, both halves were stained for achromatic regions, and both showed activity. However, in similar experiments, cyanide was found to inhibit the Nadi reaction. Similar experiments with azide produced similar results. The reaction producing the achromatic regions was not prevented by azide, while the Nadi reaction was blocked by azide.

Electrophoresis of commercial cytochrome products. Cytochrome reductase, cytochrome *c*, and acid-modified cytochrome *c* were subjected to starch gel electrophoresis and the gels stained for the achromatic regions. Acid-modified cytochrome *c*, but not the other two products, demonstrated achromatic regions quite similar in appearance to those obtained from hemolyzates or tissues. Prior to staining, a faint pinkish area appeared in the gel, probably due to the heme content of the cytochrome. The achromatic region appeared at this site after staining.

Exclusion of various enzyme activities. The enzyme was shown not to have appreciable activity of the following types by staining starch gels by appropriate methods and finding no activity: hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, acid phosphatase, adenylate kinase, phosphoglucomutase, diaphorase, and catalase.

DISCUSSION

The bluish background that develops in starch gels incubated with phenazine methosulfate and tetrazolium dyes in the presence of light is probably due to reduction of tetrazolium, catalyzed by light. After electrophoresis of hemolyzates or tissues, the achromatic bands which appear in this bluish background seem to be due to a protein or proteins with certain catalytic properties. Among these properties is the capacity to oxidize, or to maintain in the oxidized form, tetrazolium dyes in the presence of phenazine and light. This protein appears to be determined by an autosomal gene in man, as shown by the study of the family of the individual with a genetically determined variant of the protein. In this family, the male, II-3, almost certainly transmitted the gene to his male offspring, III-7.

The enzyme appears to be distributed throughout most tissues in the human. This conclusion is warranted by the finding of the variant pattern in the propositus in all six tissues studied. Nonerythrocytic tissues appeared to have an additional protein or proteins capable of producing achromatic regions (region B of Fig. 3). The two regions of activity appear to be determined by separate genetic loci; genetically determined variation in the bands of region A did not produce variation in the bands of region B.

The enzyme responsible for the achromatic bands of region A has not been precisely identified but is at least partially characterized by the studies reported here. Operationally, the enzyme may be called an indophenol oxidase, a term referring to intracellular enzymes which catalyze the Nadi reaction, as this enzyme does (Fig. 1).

According to Yonetani (1963), enzymes of this general class would be considered soluble oxidases, capable of catalyzing aerobic oxidation of various intracellular substances, including reduced cytochrome *c*. These enzymes are

usually sensitive to cyanide, to azide, and sometimes to carbon monoxide. The enzyme under study fits these criteria in that it is sensitive to azide and cyanide with respect to the Nadi reaction but is not sensitive with respect to the reaction causing the achromatic regions, or at least it is not detectably sensitive under the conditions of the experiment.

The existence of dichloroindophenol oxidase activity in the enzyme under study was shown more directly by incubating the gel in the presence of dichloroindophenol and DPNH. In this system, the regions analogous to the achromatic areas were stained blue, consistent with oxidation of the dichlorophenolindophenol.

In preliminary experiments, the approximate size of the molecule producing the achromatic regions has been determined using Sephadex gel filtration. The molecule came through a Sephadex G100 column just slightly after hemoglobin, indicating that it is a slightly smaller molecule than hemoglobin, which has a molecular weight of about 66,000.

It is of interest that cytochrome *c* (type 12, acid modified from horse heart, 95% pure, Sigma Chemical Co.) shares the property of being able to produce achromatic regions in starch gels under the conditions that the indophenol oxidase under study here does. It has been established that acid modified cytochrome *c* is capable of oxygen transfer to substrate, unlike unmodified cytochrome *c* (Paléus and Paul, 1963). It appears, then, that the reaction producing achromatic regions can be catalyzed by more than one protein, perhaps several proteins, all of which share the characteristic of being able to catalyze the transfer of electrons from reduced tetrazolium to oxygen.

SUMMARY

A variant form of the oxidase of human tissues which oxidizes tetrazolium dyes in the presence of phenazine and light has been observed in three generations of a family. This electrophoretically variant form appears to be transmitted as an autosomal dominant trait.

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