ELECTROPHORETIC BEHAVIOUR OF HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE DURING PURIFICATION

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Extensive work on the properties of glucose 6-phosphate dehydrogenase (G6PD) from human erythrocytes has been performed in several laboratories (Marks, Szeinberg and Banks, 1961; Kirkman and Hendrickson, 1962; Chung and Langdon, 1963; Luzzatto and Allan, 1965; Yoshida, 1966); interest in this area of research is underscored by the occurrence of several variants of the red cell enzyme, with wide variations in different ethnic groups. Yoshida (1966) and Cohen P. (personal communication) succeeded in obtaining G6PD in pure, homogeneous form, as judged by criteria of ultracentrifugal and electrophoretic analyses; on the other hand, starch and cellulose acetate gels both yield single-banded electrophoretic patterns of G6PD activity with crude hemolysates (Marks, Szeinberg and Banks, 1961; Bonsignore, Fornaini, Leoncini and Fantoni, 1966). Results reported in this paper describe some marked differences in electrophoretic patterns on polyacrylamide gel of the normal enzyme which are observed during the purification procedure; although only a single broad band can be detected in crude hemolysates, partial removal of hemoglobin results in the appearance of five activity bands, whose number shows a progressive decrease with the degree of purification of the enzyme.

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MATERIALS AND METHODS

G6PD and 6-phosphogluconic dehydrogenase (6PgD) activities were assayed according to Kornberg and Horecker (1955). The unit of activity was defined as the amount catalyzing an absorbancy increase of 1.0/min at 340 mμ. G6PD was partially purified according to Marks, Szeinberg and Banks (1961) from the erythrocytes drawn from single donors exhibiting the B^+ electrophoretic form of the enzyme. For some experiments 10 ml of the first ammonium sulfate fraction (between 35% and 55% saturation) was absorbed on DEAE-cellulose (dry weight cellulose / mg of protein = 80) and washed with 200 volumes of $5x10^{-3}$M phosphate buffer, pH 7.0, containing $2x10^{-6}$M NADP, $1x10^{-3}$M ε-aminocaproic acid and $5x10^{-4}$M EDTA (buffer A); after three washings with buffer A containing $5x10^{-2}$M, $1x10^{-1}$M, $1.2x10^{-1}$M KCl respectively, G6PD activity was eluted by increasing the KCl concentration to 0.4 M (Fraction I); further G6PD activity was recovered with buffer A containing $6x10^{-1}$M KCl, $1.2x10^{-5}$M NADP and β-mercaptoethanol 0.1% (v/v) (Fraction II). Fractions I and II were then made 80% saturated with solid ammonium sulfate and each precipitate collected with 1 ml of 0.25M glycyl-glycine, pH 7.5. By the latter procedure an overall purification ranging between 2,000 and 4,000 was obtained for both G6PD fractions I and II, with a 20%-30% yield. Before electrophoretic analysis the enzyme fractions were dialysed three hours against 1000 volumes of 0.015M KCl, buffered to pH 7.5 with $NaHCO_3$, containing $2x10^{-6}$M NADP and $5x10^{-4}$M EDTA, or against 1000 volumes of 0.01 M Tris-HCl, pH 8.4, containing $2x10^{-6}$M NADP and $5x10^{-4}$M EDTA.

Disc electrophoresis was performed at 2° in glass tubes on vertical gel columns (57x5 mm) composed of 8.5% acrylamide, 0.23% N,N-Methylene-bisacrylamide, $1.3x10^{-4}$M potassium ferricyanide, 0.066% (v/v) N,N, N', N'-tetramethylethylenediamine, 0.06% ammonium persulfate, 0.43 M Tris and 0.07 N HCl; the upper gel was eliminated, after it was established that this caused no differences in the electrophoretic patterns. Samples of the enzyme solution (0.015-0.15 units) in $2.9x10^{-4}$M sucrose were applied to gel columns beneath the buffer (0.01M Tris-HCl, pH 8.4, containing $2x10^{-6}$ M NADP and $5x10^{-4}$M EDTA). A current of 8 mA per gel column, corresponding to 210-240 volts, was applied, for 75'-90': longer runs resulted in partial

denaturation of the enzyme. Specific staining for G6PD (6-PgD) activity was performed at 37° in 0.1M Tris-HCl, pH 8.6, containing $2x10^{-3}$M G6P (6-PgA), $5x10^{-4}$M NADP, phenazine methosulfate (80 µg per ml) and P-nitro blue tetrazolium (150 µg per ml).

Electrophoreses on starch and cellulose acetate gels were performed according to Bonsignore, Fornaini, Leoncini, Fantoni and Segni (1966) and Rattazzi, Bernini, Fiorelli and Mannucci (1967), respectively, in 0.01 M Tris-HCl, pH 8,4, containing $2x10^{-6}$M NADP and $5x10^{-4}$M EDTA.

RESULTS AND DISCUSSION

Fig. 1 shows the electrophoretic behaviour of G6PD activity in fractions during purification of the enzyme preparation (left side). With 0.03 units of G6PD on each gel column, hemolysate (no. 1) gives one band only (width of the stain is due to overlapping of hemoglobin, which is present in large amounts); purification results in resolution of active G6PD into five different bands (no. 2); the major G6PD component lies constantly in the most anodic band. None among these bands could be attributed to 6-PgD activity, since substitution of 6-PgA for G6P resulted in complete lack of staining. Experiment no. 4 refers to a 2,200-fold purified enzyme (according to the procedure of Marks, Szeinberg and Banks) and no. 3 to that part of the enzyme activity which is usually lost during calcium phosphate gel absorption: after normal elution from gel, the residual G6PD activity was recovered by bringing the eluting phosphate buffer, pH 7.6, to 0.3 M and precipitating with ammonium sulfate at 70% saturation. Two intermediate bands which were lost during the purification procedure were recovered from the gel. The right side of Fig. 1 depicts the electrophoretic patterns of Fraction I and II, which were obtained from DEAE-cellulose as reported above, starting from the same hemolysate: thus, the purification procedure leads to loss of three of the five bands which were present at the first step.

In Fig. 2 a microdensitometric tracing of the electropherogram of a 110-fold purified AS Fraction I is reported. Usually at the first step of purification disc electrophoresis was found to resolve G6PD activity into five bands; in other cases only three bands appeared, corresponding

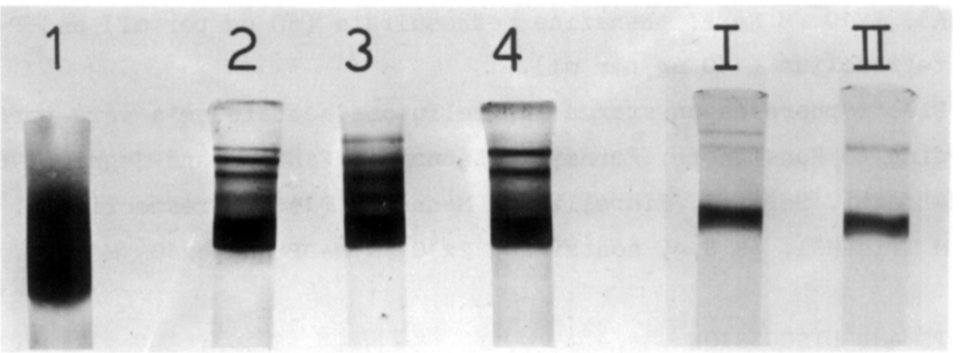

Fig. 1 - Disc gel electrophoresis of erythrocyte G6PD at different steps of purification.

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to peaks 1,3 and 4 of fig. 2, and in one experiment two bands (1 and 3) were observed. However in these cases, a greater concentration of hemoglobin was present, possibly interfering with the electrophoretic run.

Since it has been reported that disc electrophoresis may artifactually resolve a homogeneous protein into multiple bands (Brewer, 1967), we have verified in several ways that this is not the case for electrophoresis of

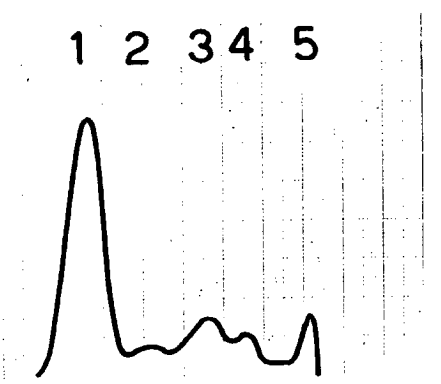

Fig. 2 - Microdensitometric tracing of a G6PD electropherogram.

erythrocyte G6PD. First, disc gel electrophoresis was performed on gel columns lacking potassium ferricyanide and from which ammonium persulfate had previously been removed by prewashing the gel for 90' at 8 mA per tube, according to Mitchell (1967)*; moreover Tris-HCl was employed at the same concentration (0.01 M) and pH (8.4) both inside the gel and as the running buffer. In this procedure, the electrophoretic patterns were identical with those obtained under the usual conditions.

Artifacts were also excluded in the following way: two samples of Fraction I were electrophoresed in parallel for 90' and one only stained for G6PD, yielding bands 1-5 (Fig. 3, C); from the other unstained gel column, five zones were cut out, corresponding to each single band, and re-electrophoresed with the usual conditions on five different gel columns.

Fig. 3 shows that each zone yields a single band, which is identically situated and stained as the original one: thus, the polyacrylamide gel

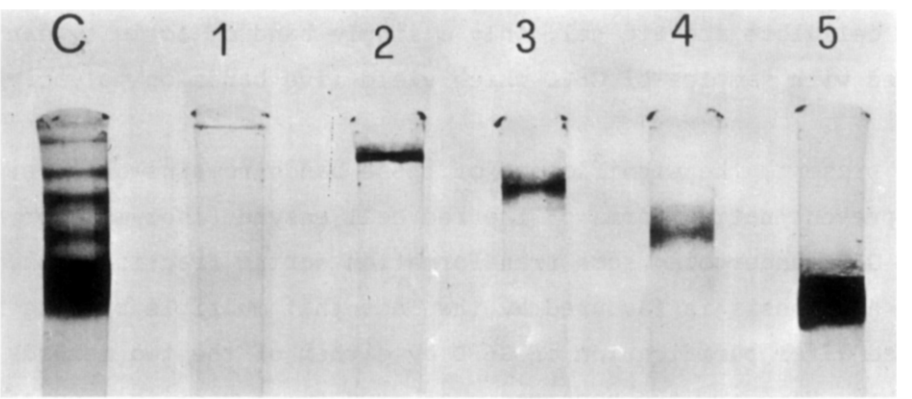

Fig. 3 - Electrophoretic patterns of single forms of G6PD.

was shown not to lead to any dissociation of electrophoretically homogeneous forms of G6PD. Moreover commercial yeast G6PD (Zwischen-

* We are indebted to Dr. Calissano for making this modification of the electrophoretic procedure available before publication.

ferment, from Boehringer), which was calculated, on the basis of specific activity, to be 50% pure, showed a single band on disc electrophoresis.

Since difficulties have been encountered in elution of enzyme activity from the individual zones of gel column (grinding with a Potter-Elvehjem homogenizer yielded 30% only of the activity from band no. 1 and less from the other bands), we are looking for other procedures in which the G6PD forms may be separated and investigated. First attempts by chromatography on DEAE-cellulose and by gel-filtration on Sephadex G-100 and G-200, even in the presence of ammonium persulfate, were unsuccessful; by these methods, a single peak of G6PD was always recovered.

The multiple banding of human erythrocyte G6PD may have escaped previous recognition because such analyses are usually performed with crude hemolysates. Yoshida (1966) found only one band for the crystalline, homogeneous enzyme possibly because of a progressive loss of the residual forms during purification (see fig. 1). When other procedures are employed, such as electrophoresis on starch gel or cellulose acetate gel, only a single band of activity can be observed with samples of G6PD which yield five bands on polyacrylamide gel.

At present, the significance of these bands remains obscure; they may represent native forms of the red cell enzyme (isozymes) or be due to G6PD undergoing some transformation during fractionation. The former hypothesis is favoured by the fact that multiple banding is observed after purification of G6PD by either of the two methods described. However, the occurrence of structural modification affecting the enzyme molecule during removal of hemoglobin, cannot be excluded. The possibility must be considered that multiple banding of G6PD reflects an equilibrium between a polymeric form and subunits having different size and electrophoretic features. Apart from the genetic variants of G6PD, the only known polymorphism of the red cell enzyme is related to dissociation of the normally active form into sub-active subunits. The molecular weight of erythrocyte G6PD (both

the polymeric form and the subunits) as well as the number of subunits forming the active enzyme, has given rise to much controversy: thus, a dimer-monomer reaction was first reported (Kirkman and Hendrickson, 1962; Chung and Langdon, 1963), but the tetramers have also been detected (Mangiarotti, Garrè and Acquarone, umpublished); on the other hand, Yoshida found the crystalline enzyme to yield some patterns for a hexamer (m.w.=240,000) undergoing dissociation into trimers (m.w.= 120,000) and subsequently into monomers (m.w.=40,000). Yoshida postulated the occurrence of five molecular forms of G6PD of different size (with interconversion between hexamers and trimers) and catalytic activities; the relationship between these hypothetical forms and the five bands we have observed is at present under investigation.

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