Change in Electrophoretic Mobility of Glucose-6-Phosphate Dehydrogenase with Aging of Erythrocytes

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Extract

Electrophoretic properties of glucose-6-phosphate dehydrogenase (G6PD) were studied in mother-infant pairs and in young and old erythrocytes of adults. G6PD isoenzymes of erythrocytes of newborn infants migrated at rates 3–4% faster than the corresponding isoenzymes of maternal erythrocytes. Analysis of hemolysates of young and old erythrocytes of adults revealed that G6PD of young cells also moved about 3% faster than that of old cells. In contrast, active enzyme sedimentation analysis revealed that the G6PD enzyme proteins of old and young erythrocytes of adults, of newborns, and of their mothers sedimented with essentially identical rates ($S_{20,w}$ = 6.8). Migration in gels of different acrylamide concentrations and velocity sedimentation analysis indicated that the G6PD of old and of young erythrocytes was in the same state of aggregation. Extensive equilibration of the hemolysates with nicotinamide-adenine dinucleotide phosphate (NADP) by filtration through Sephadex G-25 induced a clear increase in the migration rate of the G6PD of old cells of adults and of mothers at term to the rate characteristic of the enzyme of neonates and of young cells of adults. Experiments in which NADP was labeled with ^{14}C indicated that, in the process of gel filtration, the NADP content of the aged G6PD was restored. Thus the rate of migration of G6PD in acrylamide gel seems to be a function of the amount of NADP bound.

Speculation

Rates of electrophoretic migration of G6PD of the erythrocytes of infants and of young cells of adults were faster than the rates observed in adult cells; this, at first, was thought to be genetically determined, as with fetal hemoglobin. However, thorough testing proved that the difference was one of charge mediated by NADP, the concentration of which is a function of the age of the erythrocytes. There are numerous examples of aging of enzymes in erythrocytes. It is possible that loss of bound cofactor is a general mechanism for the aging of enzymes.

Introduction

There are many known variants of human G6PD (EC. 1.1.1.49) which are distinguishable on the basis of electrophoretic or enzymatic properties, heat stability, or pH optima [16]. Most of these isoenzymes differ by only 5–10% in their rates of electrophoretic migration. G6PD-*B*, which is the isoenzyme most commonly found in normal individuals, and also the common

Mediterranean variants migrate on starch gel at a rate that has been designated as *100%*. The *Canton* variant migrates 5% faster and *types A* and *A⁻* migrate 10% faster than 100%.

We previously studied an isoenzyme [1] which appeared to be *type A*, but anodal migration was 3–4% faster than that of usual G6PD-*A* of mature erythrocytes. This fast G6PD-*A* was seen in the erythrocytes of two infants, and was especially pronounced in a one-week-old infant. These observations suggest that rapid mobility might be special for the neonate. It was of interest to assess the frequency of this finding and to ascertain whether or not this difference was common to other types of G6PD. This study was initiated in order to answer these questions and to investigate the mechanism of the effect.

Materials and Methods

Venous blood and cord blood samples were collected from 134 pairs of Negro mothers and their infants [20] in tubes containing acid-citrate-dextrose solution (ACD). Of the subjects, 126 had uncomplicated, full term pregnancies, whereas in 8 there were premature deliveries. Samples were chilled in ice and stored in a refrigerator until analysis.

Hemolysates were prepared and examined for the isoenzymes of G6PD by the method previously reported [1], except that electrophoresis was conducted in precision bore tubes with 3 milliamperes per tube and electrophoresis was continued for an additional 50 min after the albumin marker migrated to the bottom of the tube. This procedure brought normal hemoglobin to within 5 mm of the bottom of the tube, and it magnified the differences between the zones of the G6PD isoenzymes.

Erythrocytes of normal adults which had the G6PD-*B* isoenzyme were separated into young and old cells by sedimentation [3] and by differential osmotic lysis [12]. Sedimented cells were washed in saline that contained mercaptoethanol and 14 μM NADP and were lysed in 20 volumes of cold water. Salt solution used for differential osmotic lysis contained mercaptoethanol and 14 μM NADP.

The following experiments were carried out to characterize further G6PD of young and old cells of adults. Erythrocytes of subjects with the G6PD-*B* isoenzyme were separated into young and old cells by centrifugation [3] and washed in 0.01 M Tris HCl buffer, pH 7.4, that contained 2.5 mM $MgCl_2$, 0.14 M KCl, 0.14 mM 2-mercaptoethanol, and either 2.5, 10, or 15 μM

NADP. Cells were suspended in 9 volumes of the buffer in which they were washed, and they were lysed by freezing and thawing. The resulting lysates were centrifuged 20 min at 24,000 $\times$ g at 4°. To achieve a complete equilibration, 0.4 ml aliquots of clear supernatant were passed through 0.6 $\times$ 11 cm columns of Sephadex G-25 equilibrated with the same buffers which were used for washing and lysing. Aliquots of the filtrates were then subjected to electrophoresis on 8% polyacrylamide gels using Tris-glycine buffer, pH 8.3, that contained 14 μM NADP. In experiments in which the effects of NADP were under study, the Tris-glycine buffer contained 2.5, 10, or 15 μM NADP. Zones of enzyme activity were developed as previously described [1]. Rates of migration of the G6PD of young and old cells were also examined using 6, 8, 10, and 12% acrylamide gels [1].

Binding of NADP by G6PD was determined by filtration of hemolysates through 0.6 $\times$ 11 cm columns of Sephadex G-25 equilibrated with a solution of 2.5 mM $MgCl_2$, 0.14 M KCl, and 0.14 mM 2-mercaptoethanol containing 15 μM NADP (carbonyl)[14]C. The amount of bound NADP-^{14}C was established by measuring the ^{14}C content of the breakthrough component containing the macromolecules and of the subsequent fractions containing small molecules including free NADP-[14]C.

In addition, hemolysates were studied by the method of active enzyme sedimentation analysis [4] in an ultracentrifuge [19] equipped with an ultra violet scanner and a double sector center piece cell [9]. Both sectors of the cell were charged with a buffer solution, pH 8.3, of the following composition: 7.5 mM Tris-HCl, 2.1 mM $MgCl_2$, 10 μM NADP, 0.3 mM glucose-6-phosphate, and 2 M glycerol. Hemolysates were deposited in the sample compartment of one sector and centrifuged at 50,740 rpm. The ultra violet scanner was set at 340 nm and put on the automatic mode of scanning at 8 min intervals.

Results

In nearly every case, G6PD isoenzymes of erythrocytes of newborn infants migrated toward the anode at rates that were 3–4% faster than the corresponding isoenzymes of maternal erythrocytes (Fig. 1). This rapid migration of the "fetal" enzyme was evident in hemolysates of erythrocytes from homozygotes or hemizygotes of G6PD *types A, A⁻,* and *B.* Similar differences were also observed in mother-daughter pairs in which each was heterozygous and had both *A* and *B* isoenzymes (Fig. 1). More rapid migration of the

"fetal" enzyme was independent of sex of the baby and of length of gestation.

Analysis of hemolysates of young and old erythrocytes of adults revealed that G6PD of young, less dense, and osmotically less fragile cells also moved about 3% faster than that of old, more dense, and osmotically more fragile cells (Fig. 2). In contrast, hemolysates of both young and old erythrocytes, fractionated from cord blood, yielded essentially the same profiles as did young cells of adults.

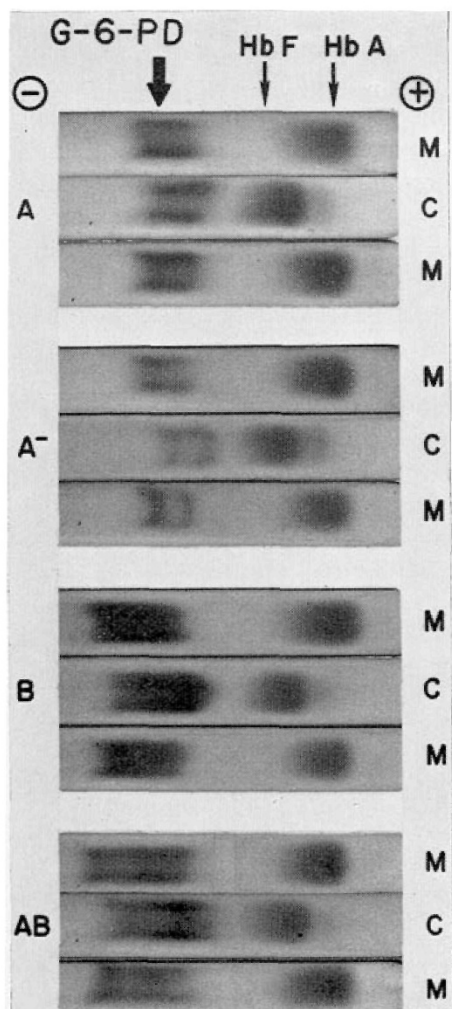When hemolysates of young and old erythrocytes were electrophoresed on 6, 8, 10, and 12% gels for 180

Fig. 2. Electropherograms of G6PD-*A* isoenzymes of young and old erythrocytes of an adult. *Gel 1:* light erythrocytes; *gel 2:* heavy erythrocytes (these cells were treated like those in Fig. 1); *gel 3:* erythrocytes lysed in 0.06 M NaCl; and *gel 4:* erythrocytes lysed in 0.08 M NaCl. Both salt solutions contained 14 μM NADP. Electrophoresis was conducted at 4° with 3 milliamperes per tube.

Fig. 3. Effect of acrylamide concentration on the migration of the G6PD of young and old erythrocytes of adults. *Gels 1, 3, 5,* and *7:* light erythrocytes; *gels 2, 4, 6,* and *8:* heavy erythrocytes. Electrophoresis was conducted at 4° for 180 min with 3 milliamperes per tube.

Fig. 1. Electropherograms of G6PD isoenzymes obtained from hemolysates of erythrocytes of mother-infant pairs. The designations *A, A⁻, B,* and *AB* on the left of the profiles denote the G6PD variants. The letters *M* and *C* on the right designate maternal and cord erythrocytes, respectively. Cells were washed in saline containing 2-mercaptoethanol and 14 μM NADP, lysed in water, and electrophoresed at 4° with 3 milliamperes per tube.
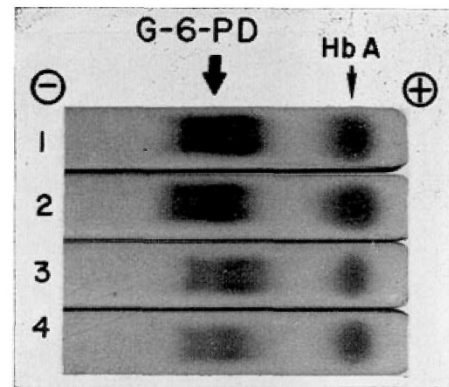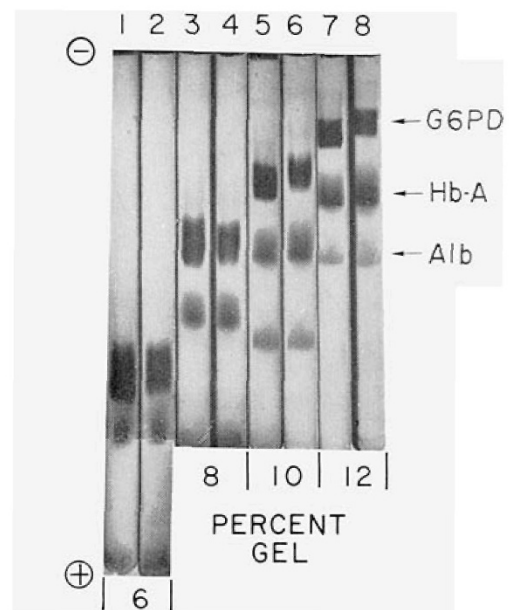
min, the migration of albumin, hemoglobin, and G6PD decreased almost linearly with increase in concentration of the gel (Fig. 3). Albumin and hemoglobin migrated at parallel rates while G6PD did not. In Figure 4, the rate of migration relative to albumin (R_m) was calculated and shown for the experiment illustrated in Figure 3. The R_m of hemoglobin was 0.72 in 6% gel and 0.73 in 12% gel. However, the R_m of the
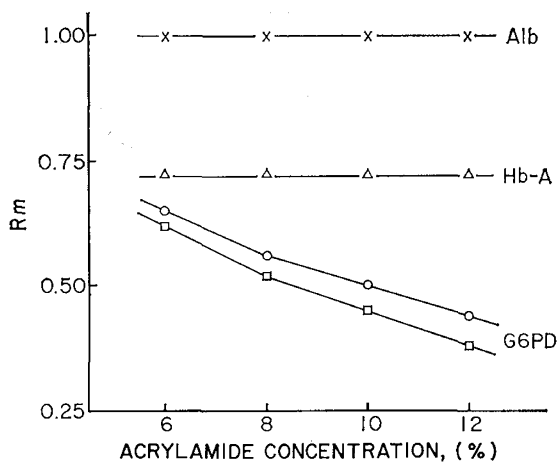
Fig. 1. Relative migration of the G6PD of young and old erythrocytes of adults in gels of different concentrations. -○-○-: light cells; -□-□-: heavy cells.
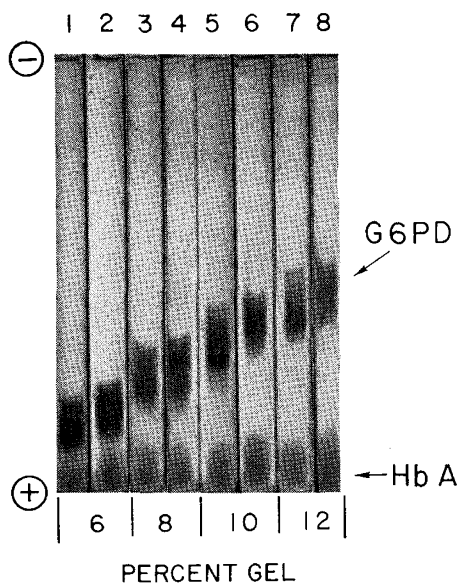

Fig. 5. Effect of acrylamide concentration on electrophoretic migration of the G6PD of young and old erythrocytes of adults. *Gels 1, 3, 5,* and *7:* light erythrocytes; *gels 2, 4, 6,* and *8:* heavy erythrocytes. Electrophoresis was conducted at 4° with 3 milliamperes per tube.

G6PD of young cells was 0.65 and 0.44 in 6 and 12% gels, whereas the R_m of the G6PD of old cells was 0.62 and 0.38, respectively.

In experiments in which hemolysates were electrophoresed until hemoglobin migrated to the bottom of the tubes, rates of migration were considered relative to hemoglobin. In 6% gel the R_m of the front of the zone of G6PD from young cells was 0.90 and that of the back of the zone was 0.80, whereas in 12% gel

these values were 0.66 and 0.51 respectively (Figs. 5 and 6). The R_m values to the front and back of the zones of G6PD from old cells were 0.88 and 0.77, respectively in 6% gels and 0.62 and 0.48 in 12% gel. Thus, in 12% gel the R_m values of G6PD of old cells decreased 1.5% more than did those of G6PD of young cells.

Similar analysis of hemolysate of unfractionated cells on gels of different concentrations yielded G6PD zones that were broader, more diffuse, and, as a rule, lacked clearly defined bonds. In reference to hemoglobin, the R_m to the front of the G6PD zone in 6% gel was approximately 0.90 and to the back 0.77, while in 12% gel these values were 0.66 and 0.48. Thus the G6PD zone represented essentially a composite of the enzyme proteins found in fractionated cells.

Molecular size was calculated from the ratio of migration of proteins in gels of different concentrations by the technique of Zwaan [18], using albumin and hemoglobin as references. The molecular size of the G6PD of young cells of adults as calculated in this way was between 118,000 and 119,000, and that of old cells, 121,000–122,000. In contrast, active enzyme sedimenta-
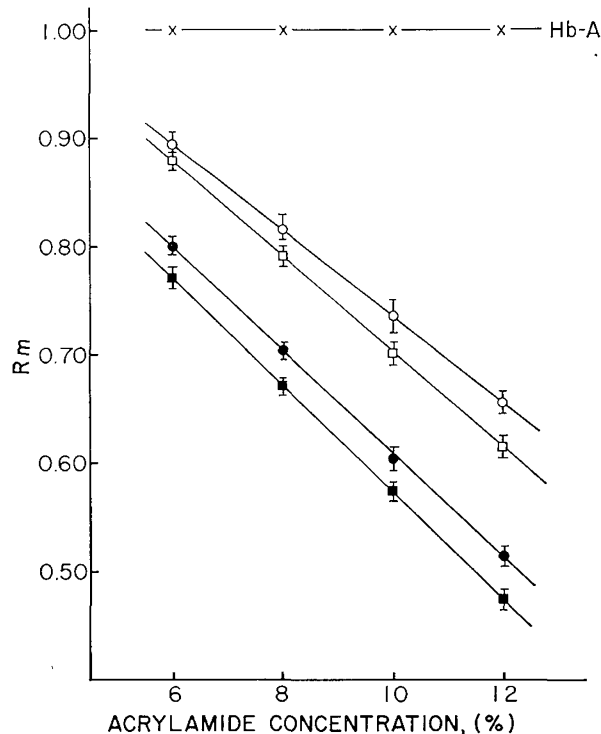

Fig. 6. Relative migration of the G6PD of young and old erythrocytes in gels of different concentrations. -○-○-: front, and -●-●-: back of the G6PD zone of old cells; -□-□-: front, and -■-■-: back of the G6PD zone of young cells.
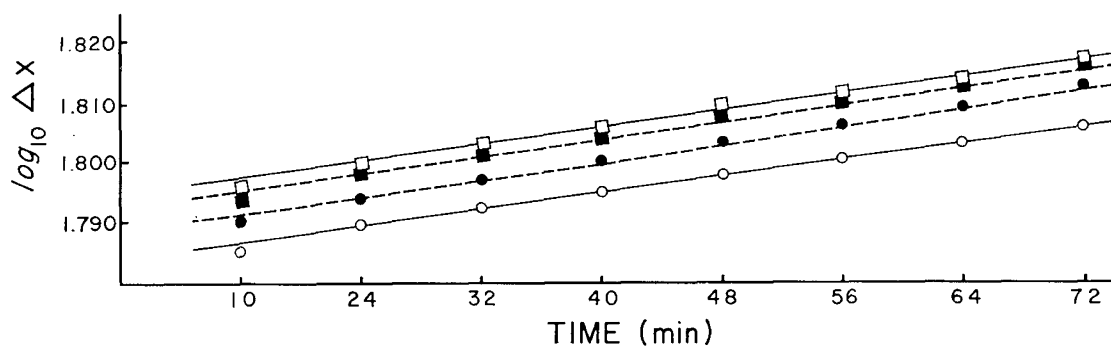
Fig. 7. Rates of sedimentation of G6PD observed by active enzyme sedimentation analysis. -O-O-: young, and -□-□-: old erythrocytes of adults; -●-●-: cord blood erythrocytes; -■-■-: maternal erythrocytes.

tion analysis revealed that the G6PD enzyme proteins of 1:20 hemolysates of old and young erythrocytes of adults, of newborn babies, and of their mothers sedimented with essentially identical rates (Fig. 7). The average sedimentation coefficients $S_{20,w}$ for the active enzyme proteins were calculated to be 6.8, which would imply a molecular weight of about 120,000. This was in agreement with the values obtained by other investigators for the purified enzyme protein with Schlieren optics [5] as well as by active enzyme sedimentation analysis [17]. Since the precision of active enzyme sedimentation analysis is about 1%, the molecular weight of G6PD could vary by ± 1,200 [4].

Studies of the effect of NADP revealed that addition of NADP to the buffer used for washing and lysing erythrocytes, as well as to the gels and electrophoretic buffer, did not alter the rates of migration of the G6PD of the various erythrocytes. However, extensive equilibration of hemolysates with NADP by filtration through Sephadex G-25 induced a clear increase in rate of migration (Fig. 8). After such equilibrations with NADP, migration of G6PD of old cells of adults and of mothers at delivery was accelerated by about 3%. Whereas 2.5 μM NADP had little effect on the rate of migration of the "old" G6PD enzyme, similar equilibration with 10–15 μM NADP concentration reversed the effects of aging and restored rates of migration to the level characteristic of the enzyme of young cells of adults and of total cell populations of newborn babies (Fig. 9).

When binding of NADP to G6PD was studied using NADP-^{14}C, the following data were obtained. In the breakthrough or macromolecular component of the Sephadex column, there were 1100–1200 dpm when young cells were studied. In contrast, the breakthrough component from the elution of old cells contained 1800–1900 dpm.
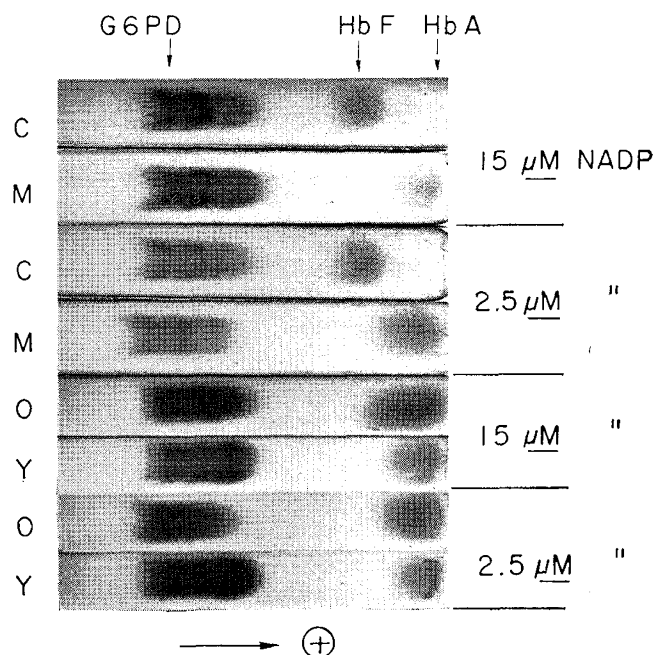

Fig. 8. Effect of equilibration with NADP by filtration through Sephadex G-25 on the migration of G6PD. Gels *C* and *M* contained hemolysates of cord blood and maternal erythrocytes; gels *Y* and *O* contained hemolysates of young (light) and old (heavy) erythrocytes of adults.

Discussion

This study showed that the rates of migration of G6PD of newborn infants, as observed when hemolysates were electrophoresed on polyacrylamide gel, were faster than those of their mothers. This was true for G6PD types *A*, *A⁻*, and *B*, and for the heterozygote with both *A* and *B* isoenzymes, regardless of whether the neonate was male or female, full term or premature.

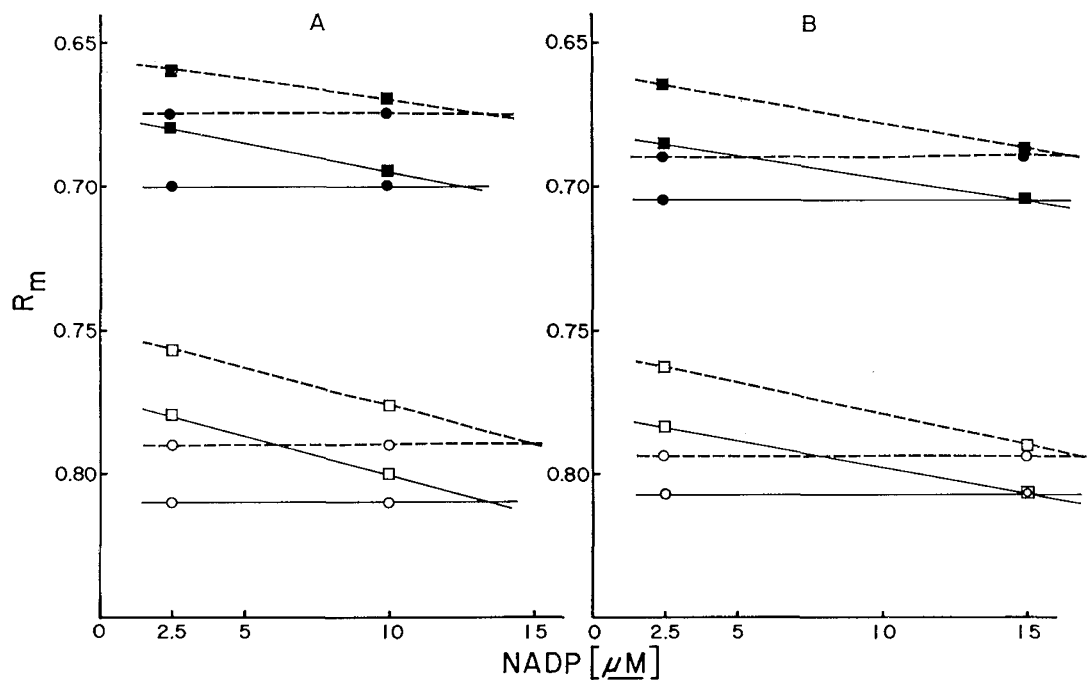These experiments at first seemed to suggest the ex-

Fig. 9. Effect of equilibration with NADP by filtration through Sephadex G-25 on the rate of migration of G6PD. *A*: erythrocytes of mature individuals; *B*: erythrocytes of a neonate and his mother. -O-O-: front, and -●-●-: back of the G6PD zone of young and cord blood erythrocytes; -□-□-: front, and -■-■-: back of the G6PD zone of old and maternal erythrocytes; ———: 20 μl and — — — —: 40 μl aliquots of 1:20 hemolysates.

istence of a fetal form of G6PD. Such a difference between the protein of infants and that of adults could represent a genetically determined change, as in the case of fetal hemoglobin. An embryonic G6PD variant was reported by Nadler [13] to be present in amniotic cells at 10-wk gestation. Nevertheless, the faster migrating enzyme of the neonate appears not to represent a specific fetal isoenzyme. It seems more likely that the faster migration of this G6PD is a function of the age of the erythrocytes. Similarly, the quantitative enzymatic activity of G6PD of neonates was usually elevated, and it paralleled the activity found in young cells of mature individuals. On this basis, it has generally been accepted that the erythrocyte population of the neonate consists mainly of young cells [14]. The similarity between the electrophoretic migration of the G6PD of young cells of adults and of neonates, and the observed difference between young and old cells of adults on one hand, and between the neonate and his mother on the other hand, support this view.

It appears that the active form of adult human G6PD is made up of two subunits, one of which is electrophoretically faster than the other. We have postulated that these subunits conjugate, and, when submitted to polyacrylamide gel disc electrophoresis, they

yield three bands [1]. Three bands are observed on electrophoresis of G6PD-*A*, -*A* ⁻, and -*B* isoenzymes; in the G6PD-*AB* heterozygote, six bands are seen. The three main variables which govern the electrophoretic migration of proteins in polyacrylamide gel are molecular size, charge, and configuration of the protein molecules. In the case of an enzyme protein, irreversible binding of a substrate, a product, or a conezyme; loss or modification of a functional group; change in configuration resulting in a change in the radius of the protein molecule; or intracellular degradation of the enzyme protein could alter its electrophoretic properties.

Migration in gels of different acrylamide concentrations and velocity sedimentation analysis indicated that the G6PD of old and young erythrocytes was in the same state of aggregation, that it had a molecular weight of approximately 120,000 and possibly represented a dimer. However, the enzyme of old cells appeared to differ from that of young cells with respect to its charge. Acceleration of the migration of "old" G6PD by equilibration with NADP supports this possibility.

It was recently suggested that G6PD dimers with an average sedimentation coefficient of $S_{20,w} = 6.74$ had 3

sites for binding NADP, 1 "catalytic" and 2 "structural" [2]. The dissociation constants Ks_1 and Ks_2 for the catalytic and the 2 structural sites were found to be 45 and 13 μM NADP, respectively [10], and, in another study, 20 and 13 μM NADP, respectively [6]. Since the G6PD proteins of old and of young cells appear to be in the same state of aggregation, and depletion at structural sites leads to disaggregation of the enzyme, it is unlikely that the structural sites of the "old" enzyme are severely depleted of coenzyme. It is more likely that catalytic sites (Ks_1 = 20–45 μM NADP) are insufficiently charged. It has been found that the activity of G6PD in young cells is considerably greater than that of old cells in the adult [11]. The several-fold decrease in G6PD activity in old cells is consistent with the possibility that there are changes with age in the NADP content of catalytic sites. According to our data, the change which governs the migration of active G6PD is achieved at 14 μM NADP concentration. It is concluded that the differences in migration between the enzymes of the neonate and his mother or between the enzymes of young and old cells of the adult result from an *in vivo* effect of aging which alters the amounts of NADP bound at the catalytic sites. This was in fact confirmed by the demonstration of increased binding of radioactively labeled NADP by the G6PD of old erythrocytes. Differences in the amounts of bound NADP would be expected to produce differences in charge and to result therefore in differences in mobility as well as in enzymatic activity. This is consistent with the effects observed. The effect of *in vitro* aging on the behavior of enzymes has been studied in lysates which were several days old. For example, the activity of adenosine deaminase decreases as hemolysates are aged *in vitro*, but its electrophoretic migration in starch gel increases [15]. These changes were found to be due to the state of oxidation of sulfhydryl groups of the enzyme. A similar effect of aging was also observed for nucleoside phosphorylase [8]. These effects have not been examined in young or old cells *in vivo*. The alteration in the binding of NADP could represent a change in the conformation of the protein during aging. Certainly it was not possible to increase the amount of NADP bound and the electrophoretic mobility of the protein by mixing the enzyme with NADP. These changes took place only when the two were processed together through a Sephadex column, which could have altered conformation during the process to make sites available for binding to NADP. These conclusions are supported by the observation of

Fornaini *et al.* [7] that the affinity of old G6PD for NADP is decreased.

Summary

G6PD isoenzymes of erythrocytes of neonates and of young erythrocytes of adults migrated in acrylamide gel 3–4% faster than the corresponding isoenzymes of maternal erythrocytes or of old erythrocytes of adults. Thorough equilibration of hemolysates of old cells of adults or of cells of mothers at term with NADP induced them to migrate at the same rates as the G6PD of erythrocytes of neonates and of young erythrocytes of adults. Inasmuch as the G6PD of all these cells was in the same state of aggregation, the faster migration of young cells appears to be due to a difference in charge mediated by the amount of NADP bound. This age-dependent change could reflect a change in configuration.

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20. Parents of patients were informed about the planned studies in every detail, and consent was obtained prior to the studies.

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