Proc. Natl. Acad. Sci. USA
Vol. 75, No. 4, pp. 1979–1983, April 1978
Medical Sciences

Biochemical mechanisms of glucose-6-phosphate dehydrogenase deficiency

(radioimmunoassay/affinity chromatography)

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Communicated by Rita Levi-Montalcini, January 5, 1978

ABSTRACT **A solid-phase radioimmunoassay for human glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1.49) was developed that allowed the specific activity of this enzyme protein to be measured in lysates from whole erythrocyte populations, in lysates from erythrocytes of different ages, and in purified samples. The enzyme was highly purified from erythrocytes of single donors by a simple procedure of affinity chromatography with insolubilized adenosine 2′,5′-bisphosphate. These techniques were used in an attempt to elucidate the molecular mechanisms leading to deficiency of glucose-6-phosphate dehydrogenase activity in two genetic variants of the enzyme, i.e., the Mediterranean and the Seattle-like variants. The results indicate that the lowered activity of erythrocytes containing the Mediterranean variant of glucose-6-phosphate dehydrogenase is related to an enhanced rate of degradation of a catalytically defective protein synthesized at a nearly normal rate. Synthesis of a normally functioning protein and an increased breakdown of it are involved in the Seattle-like variant of the enzyme**

Although deficiency of glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1.49) is a worldwide genetic disorder, its biochemical basis is uncertain. Quantitation of G6PD in terms of protein content appears to be an essential prerequisite for elucidating the molecular events leading to lowered G6PD activity in the affected cells (1). Some investigators approached this problem with several genetic variants of the enzyme using different techniques (2–7); yet their procedures, although intrinsically valid, did not lead to a direct estimate of the levels of G6PD protein in hemolysates.

Radioimmunoassay of G6PD (8) appeared to be a valuable tool for overcoming these uncertainties. Therefore, a solid-phase radioimmunoassay specific for G6PD was developed whereby G6PD immunologically crossreacting material (CRM) is identifiable with the enzyme protein. Both G6PD activity and protein were determined in several normal and G6PD-deficient erythrocytes. A further issue was the estimate of the specific activity of this enzyme in erythrocyte fractions of different ages, which enabled us to investigate the molecular basis for the decay of G6PD activity throughout the life-span of circulating erythrocytes (9, 10).

Highly purified G6PD variants were obtained from single subjects by refining a procedure of affinity chromatography that had been worked out for the large-scale purification of the enzyme protein from pooled erythrocytes (11, 12). Combination of the two techniques allowed us to measure the specific activity of G6PD in crude hemolysates and to check the experimental results at the level of purified preparations of the enzyme. These procedures were followed in the present study in order to gain an insight into the molecular mechanisms by which deficiency results in two genetic variants of G6PD, i.e., G6PD Mediterranean and G6PD Seattle-like.

MATERIALS AND METHODS

Blood Samples and Enzyme Assays. Blood samples of 26 male subjects were investigated in this study. Out of these, 12, with normal G6PD activity, had the wild-type enzyme (B). Thirteen subjects had a Mediterranean variant because the properties of the purified enzyme (see below) conformed to previous reports on this variant (13), and they were of Sardinian ancestry. Finally, one subject (of Sardinian ancestry, a staff member of the Department of Medicine), had the Seattle-like variant (14), as shown by the electrophoretic and biochemical properties of the purified enzyme.

Blood samples were defibrinated with glass beads and washed with 5 vol of 0.15 M KCl. The packed erythrocytes were hemolyzed with 7 vol of ice-cold H_2O containing 10 μM NADP. G6PD activity in normal and in G6PD-deficient subjects with activities higher than 25% of the normal values was measured according to Glock and McLean (15) under the assay conditions suggested in a World Health Organization technical report (16). When G6PD activity was lower, it was measured in the presence of saturating amounts of Glc 6-*P* and $NADP^+$ only (17).

Protein was determined by the method of Lowry *et al.* (18), with standard solutions of crystalline ovalbumin whose concentration was estimated by absorbance at 280 nm (19).

Fractionation of Erythrocytes of Different Ages. This was performed by centrifugation of whole erythrocyte suspensions on a discontinuous gradient of Stractan II, as described (20). Reticulocyte counts, hemoglobin content, assay of G6PD activity and radioimmunoassay for G6PD were performed on each fraction of the gradient. Reticulocyte counts were made on dry preparations after supravital staining; 1000 cells were counted. Hemoglobin was measured by a routine method. The positions of different samples in the gradient were calculated as the cumulative distribution function (CDF) of hemoglobin concentration in relation to the sample percent of each fraction (10). The cell age of each erythrocyte fraction was calculated on the basis of the individual reticulocyte counts according to a mathematical model (10, 21).

Microscale Purification of G6PD. N^6-(6-Aminohexyl)-adenosine 2′,5′-bisphosphate was synthesized as described (22) and it was coupled to BrCN-activated agarose as reported previously (11). The purification procedure described herein represents a further simplification of the method, which allows large-scale and concurrent isolation of G6PD and of FX, a

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; CRM, immunologically crossreacting material; FX, NADP(H)-binding protein eluted with G6PD from human erythrocyte lysates; $NaDodSO_4$, sodium dodecyl sulfate; P_i/NaCl, phosphate-buffered saline.

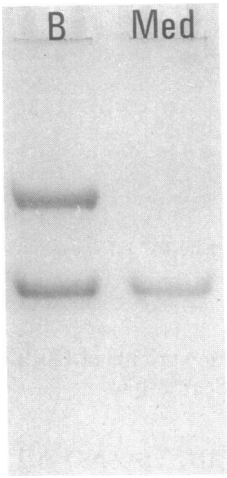

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretic tracings of the purified B and Mediterranean variants of G6PD. Electrophoresis was carried out as described (12). The higher molecular weight protein band is G6PD, while the lower molecular weight (34,000) protein is FX (12).

specific NADP(H)-binding protein (12). The scheme reported here refers to standard purification from 25 ml of packed erythrocytes obtained from single donors having the B or Seattle-like phenotypes of G6PD. All buffers used throughout the procedure contained 1 mM EDTA and 0.2% 2-mercaptoethanol. The freshly prepared hemolysates were made 1 mM in EDTA, 0.2% (vol/vol) in 2-mercaptoethanol, 50 mM in sodium phosphate (pH 6.0), and 25 mM in KOAc (pH 5.8). After adjustment of the pH to 6.0 with 1 M acetic acid, the samples were applied to a small column (7 × 0.64 cm, inner diameter) of adenosine 2′,5′-bisphosphate-agarose that had been equilibrated with 0.1 M KOAc/0.1 M K phosphate, pH 6.0 (buffer A). The flow rate was 1 ml/min, and no G6PD activity was detectable in the effluent. The gel was then sequentially washed with 25 ml of buffer A, with 25 ml of buffer B (0.1 M KOAc/0.1 M K phosphate, pH 7.85), and finally with 160 ml of buffer C (0.1 M K phosphate/0.1 M KCl, pH 7.85). Stepwise elution was carried out with two solutions of 10 ml each of 0.2 mM and 1 mM NADP, respectively, in 80 mM K phosphate/80 mM KCl, pH 7.85, yielding two separate fractions, referred to as fraction I and fraction II, respectively. Average recoveries of G6PD activity were 70–80%; the bulk of the activity was present in fraction I. Both fractions were then concentrated to 0.5–1.0 ml each by dialysis under reduced pressure against 100 vol of 4-fold diluted buffer A.

The starting volume of packed erythrocytes could be decreased to 3 ml, in which case 2 ml of the gel was used and all operations were scaled down accordingly. Fig. 1 illustrates the sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoretic patterns (12) of the purified B and Mediterranean variants.

Solid-Phase Radioimmunoassay of G6PD. Antiserum to purified human G6PD (type B) was prepared in rabbits, heat-inactivated, and adsorbed as described (8). The immunoadsorbent used throughout all experiments was prepared as follows: 220 mg of purified IgG fraction of the antiserum (dissolved in 36 ml of 0.1 M $NaHCO_3$, pH 9.0) was reacted for 48 hr at 4° with 45 g of BrCN-activated agarose (150 mg of BrCN/1 g of Sepharose 4B).

Pure G6PD (type B) was radioiodinated as described (8). The radioimmunoassay was based upon the capacity of aliquots of purified G6PD samples (Fig. 2) or of crude hemolysates to inhibit the reaction between the above immunoadsorbent and

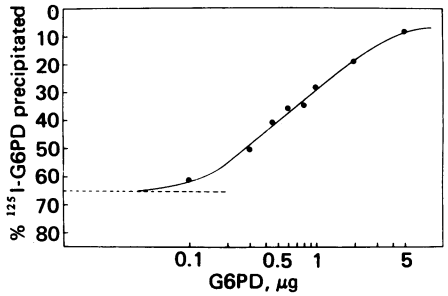

FIG. 2. Solid-phase radioimmunoassay of G6PD. The enzyme protein used as standard antigen was an electrophoretically homogeneous preparation from pooled erythrocytes having the B phenotype (specific activity, 171 units/mg). All samples were run in triplicate. A mixture lacking unlabeled G6PD and containing an excess of the immunoadsorbent (10-fold the usual volume) was run in parallel in order to provide the base-line corresponding to maximal binding of ^{125}I-G6PD. Blank assay tubes (containing Sepharose-bound IgG fraction from antiserum against bovine serum albumin instead of the usual immunoadsorbent) showed that less than 5% of the total radioactivity remained in the precipitate in the absence of the insolubilized antibodies to G6PD. The data in the radioimmunoassay competition profile were corrected by subtracting the radioactivity in the blank controls from the actual radioactivity of each sample. The sensitivity of the assay was approximately 100 ng (at 95% confidence limits, 100 ng was significantly different from 0 ng). The intra-assay coefficient of variation was equal to or less than 9% (n = 10); the inter-assay coefficient of variation was 20.5% (n = 10).

^{125}I-labeled G6PD (^{125}I-G6PD). The amount of immunoadsorbent to be used was chosen on the basis of preliminary experiments and corresponded to the quantity needed to bind 50% of radioiodinated G6PD used in the test (1 μg), under the conditions of the radioimmunoassay. Aliquots (0.5 ml) of the samples to be tested (purified enzyme or freshly prepared hemolysates) were mixed with a constant volume of the immunoadsorbent [diluted 1:4 (vol/vol) with cold phosphate-buffered saline (P_i/NaCl)] in plastic tubes. The mixtures were adjusted to a protein concentration of 25 mg/ml with ovalbumin, when necessary, and to a final concentration of 20 mM Na phosphate (pH 7.0) and 0.15 M NaCl. Most samples were run in triplicate; in several instances, assays of the same samples were repeated in separate experiments. Assay tubes were rotated at 4° for 150 min. At the end of the first incubation, 1 μg of ^{125}I-G6PD (in a solution containing 30 mg of ovalbumin per ml of P_i/NaCl) was added and the mixtures were rotated again at 4° for 150 min. The tubes were then centrifuged at 2500 rpm. Both the supernatants and the pellets (after a washing with 1.0 ml of P_i/NaCl containing 0.5 M NaCl) were measured for radioactivity in a Packard model 5110 γ counter.

RESULTS

Specific Activity of G6PD in Whole Erythrocyte Populations and in Erythrocytes of Different Ages. Table 1 shows levels of catalytic activity and of immunoreactive G6PD in hemolysates from normal individuals and G6PD-deficient subjects. The content of G6PD CRM was lower than estimated by the double-antibody radiommunoassay (8), probably because of the higher background of that procedure with respect to the solid-phase radioimmunoassay. The resulting specific activity in the eight normal subjects reveals a decided similarity to values observed for homogeneous preparations of the B-type enzyme (11, 23–26).

The G6PD Mediterranean subjects can be divided in two groups: those having normal reticulocyte counts show barely detectable levels of G6PD CRM because of the limited sensi-

Table 1. Specific G6PD activity in hemolysates from normal and G6PD-deficient subjects

Subjects	Activity, units/g Hb (A)	G6PD CRM, μg/g Hb (B)	Specific activity, (A/B) × 1000
Normal (n = 8)	4.1 ± 0.39*	22.4 ± 3.39*	183.3 ± 18.5*
G6PD-deficient Mediterranean†			
1	0.31	ND‡	—
2	0.41	ND‡	—
3	0.84	11.0	76.4
4	1.61	24.9	64.5
Seattle-like	1.0	7.0	142.8

* Mean ± SD.
† The reticulocyte counts of G6PD Mediterranean subjects were 3.2% (*1*), 3.5% (*2*), 11.2% (*3*), and 13% (*4*).
‡ ND, not detectable.

tivity of the radioimmunoassay, which has a threshold of approximately 100 ng (Fig. 2); and those with high reticulocyte counts (recovering from hemolytic episodes) show increased values of catalytic activity and sizeable amounts of G6PD CRM. The specific activity of G6PD in the latter group is consistently lower than that observed in the normal subjects, in agreement with earlier determinations made by a different technique (4, 5).

As far as the G6PD Seattle-like phenotype is concerned, the defect of activity is related to a comparable decrease of G6PD CRM. Thus, the specific activity is only slightly decreased with respect to the average value of normal individuals.

Measurements of G6PD CRM in erythrocyte fractions of different ages appeared to be of interest because G6PD activity has been reported to decline during aging of erythrocytes (9, 10). Fig. 3 demonstrates that a linear correlation exists between values of catalytic activity and levels of G6PD CRM; both measurements show a clearcut decline from the young to the old erythrocyte fractions (reflected by convergence toward the origin of both axes). This correlation, which holds for the normal subjects as well as for the Seattle-like subject, allows an estimate of average G6PD specific activity in both cases of approximately 160 units/mg throughout the lifespan of these erythrocytes, in good agreement with values reported for whole erythrocyte suspensions. Despite this similarity between the controls and the Seattle-like subject, the rate of decline of both activity and immunoreactive G6PD was consistently faster in the mutant than in the control erythrocytes (Table 2).

The decay of G6PD activity during aging was even more pronounced in G6PD Mediterranean subjects (Table 2), confirming the earlier findings of Piomelli *et al.* (10). G6PD CRM was undetectable in old Mediterranean erythrocytes; therefore, the enzyme inactivation during aging appears to be accounted for by removal of immunoreactive protein from the cytoplasm of the affected as well as of the normal cells (Fig. 3).

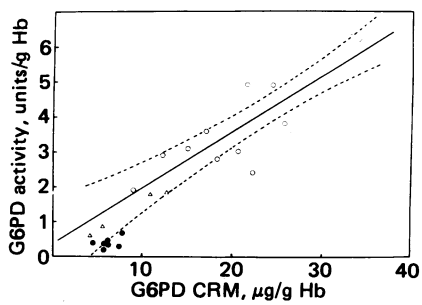

FIG. 3. Correlation between levels of G6PD activity and G6PD CRM in lysates from erythrocytes fractionated according to age (r^2 = 0.80). ○, Normal subjects; ●, G6PD Mediterranean subjects; △, Seattle-like subject. Dashed lines indicate 95% confidence limits of the regression for normal G6PD (B).

Table 2. Specific activity in hemolysates from erythrocyte fractions of different ages

G6PD	Fractions* in density gradient	Activity, units/g Hb (A)	G6PD CRM, μg/g Hb (B)	Specific activity, (A/B) × 1000
B	1	7.81	38.4	203.4
	2	6.20	34.0	182.4
	3	4.93	24.4	202.0
	4	4.22	21.6	195.4
Mediterranean	1	0.33	6.2	53.2
	2	0.18	4.0	45.0
	3	0.13	ND†	—
	4	0.04	ND†	—
Seattle-like	1	1.84	12.7	144.9
	2	1.81	10.9	166.1
	3	0.86	5.6	153.6
	4	0.60	4.2	142.9

* Fractions of increasing age from 1 to 4 (10).
† ND, not detectable.

Fig. 3 shows that the points recorded for the G6PD Mediterranean subjects are displaced to the lower right as compared with those observed for the controls and the Seattle-like subject, although they appear to be somewhat scattered (which reflects proximity to the threshold of the catalytic activity assay and of the radioimmunoassay). Such distinctive clustering indicates a lower specific activity of the G6PD Mediterranean variant with respect to the G6PD B and the G6PD Seattle-like types, yet this conclusion is restricted to young erythrocytes.

Purification of G6PD Variants from Single Individuals. Table 3 shows the values of specific activity determined on several purified G6PD preparations from single donors having the B, Mediterranean, and Seattle-like enzyme types. With all subjects tested there was agreement between the values obtained in the purified enzyme samples (like those shown in Fig. 1) and those in the corresponding hemolysates.

With the Mediterranean variant, the amount of G6PD protein obtained from the purification procedure is so small as to escape clearcut identification on the $NaDodSO_4$ slab gel electrophoretic tracings (Fig. 1). This finding appears to reflect an inherently reduced number of G6PD molecules in the affected erythrocyte as compared with the normal one. In fact, the percent recoveries of catalytic activities were similar in the Mediterranean variant and in normal subjects, thus excluding inactivation of the mutant enzyme protein during purification.

A result that emerged by combining the radioimmunoassay technique and the microscale purification procedure was the low specific activity of the purified G6PD Mediterranean, irrespective of the proportion of reticulocytes in the starting erythrocyte populations (Table 3). Accordingly, the finding of such reduced catalytic efficiency of the Mediterranean variant applies to young and to old erythrocytes as well, overcoming the failure to estimate directly G6PD CRM in lysates from old erythrocytes.

Specificity of Solid-Phase Radioimmunoassay. The similarity in specific activities of the purified and crude G6PD preparations seemed to rule out any immunologically cross-reactive protein possibly affecting the specificity of the ra-

Table 3. Specific activity of purified G6PD from single individuals (normal and G6PD-deficient)

G6PD phenotype	Clinical remarks	% recovery of activity	Specific activity, units/G6PD CRM
B			
1	—	71	146
2	—	97	185
3	—	92	202
4	—	58	215
5	—	76	189
6	—	51	202
Mediterranean			
1	—	20	92
2	—	72	82
3	Hemolytic crisis (11.2% reticulocytes)	56	65
4	Hemolytic crisis (13% reticulocytes)	45	70
5	—	80	88
6	—	91	74
7	Hemolytic crisis (12.2% reticulocytes)	71	52
8	Hemolytic crisis (14% reticulocytes)	83	58
9	—	95	71
Seattle-like	—	64	143

dioimmunoassay technique. Particularly, FX had been shown not to interfere with the immunological assay of G6PD (8). Nevertheless, search for CRM was undertaken on electropherograms of G6PD samples purified from single donors (Fig. 4). Both fractions I and II were examined in order to facilitate detection of immunoreactive protein bands. This experiment showed absence of G6PD CRM throughout the gel except in the zone of migration of catalytically active G6PD, where the peak of immunoreactive material accounted for 60–70% of the amount electrophoresed. Such a finding provides clear evidence for the structural identification of G6PD CRM with active enzyme protein under the conditions of the solid-phase radioimmunoassay described herein. Similar results were obtained with purified preparations of the G6PD Seattle-like and Mediterranean variants.

Patterns of G6PD CRM during Aging of Normal and Affected Erythrocytes. As illustrated in Table 2, a characteristic decline of G6PD activity in the transition from young to old erythrocytes is observed; this decline is more pronounced in the Seattle-like type and still more evident in the Mediterranean type of deficiency. From plots of the experimental data by the method of Piomelli *et al.* (10, 21), it is possible to extrapolate the levels of catalytic activity (or of any parameter related to it) to a 100% pure population of reticulocytes. The corresponding estimates for the three reticulocyte groups (in units/g of Hb) were 11.5 (B), 11.1 (Seattle-like), and 3.3 (Mediterranean). The specific activities of the three G6PD types tested are constant throughout the life-span of the erythrocytes at values averaging 160 (B), 154 (Seattle-like), and 61 (Mediterranean) (Fig. 3 and Table 3), Accordingly, on the basis of these specific activities, the experimentally determined decay of catalytic activity during aging could be converted to the corresponding levels of G6PD protein (Fig. 5, straight lines). The resulting patterns provide further evidence for distinctive rates of removal of the three G6PD types examined.

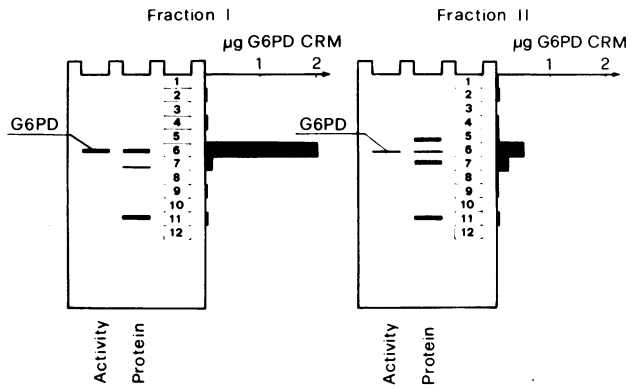

FIG. 4. Search for G6PD CRM in a purified G6PD preparation from a normal subject. G6PD was purified from 25 ml of packed erythrocytes. Each of the two fractions obtained (fraction I and fraction II) was electrophoresed in triplicate on gel slabs containing 7.5% polyacrylamide. The buffer, which was continuously recycled by a peristaltic pump, was 25 mM Tris/190 mM glycine, containing 10 μm NADP, pH 8.6. A current of 25–30 mA, corresponding to 100–120 V, was applied and electrophoresis was at 4° for 3 hr. Two lanes were cut off and stained for G6PD activity and protein, respectively (27). The third parallel lane of each sample was cut perpendicularly to the direction of migration into 12 slices of 5 mm each. These were soaked overnight at 4° with 0.75 ml of a solution containing 30 mg of ovalbumin per ml of P_i/NaCl. Aliquots (0.5 ml) of these subfractions were then checked by radioimmunoassay.

DISCUSSION

Since all known G6PD variants are amenable to structural mutations, it can be postulated that the abnormality in G6PD structure results in a deficiency of catalytic activity by one of the following mechanisms (28); (*a*) lowered catalytic efficiency, (*b*) decreased synthesis, or (*c*) accelerated breakdown. Elucidation of the site(s) involved in the expression of G6PD defect in different genetic variants should provide a sound basis for an understanding of the relevant pathology.

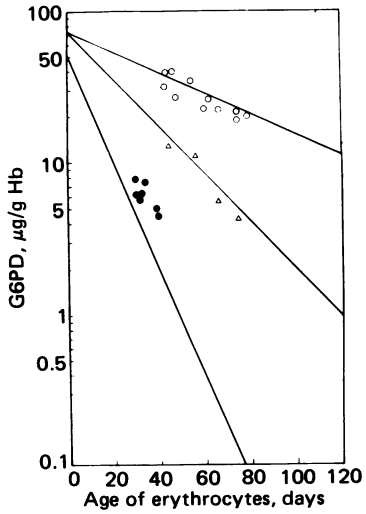

FIG. 5. Decay of G6PD *in vivo*. The straight lines represent the best fit estimates of several erythrocyte fractionations (10 normal and 11 G6PD Mediterranean subjects); they were obtained by dividing experimental values of catalytic activity in cells of different ages by the corresponding specific activities (see text). The points refer to levels of G6PD CRM directly determined by radioimmunoassay (taken from experiments shown in Fig. 3): all CRM values (including those obtained in the young Mediterranean erythrocytes) fall within the 95% confidence limits of the calculated decay of G6PD protein (straight lines). ○, G6PD B; ●, G6PD Mediterranean; △, G6PD Seattle-like.

An important parameter that is directly related to the catalytic potential of the enzyme and whose assessment would accordingly check mechanism *a* is the specific activity of G6PD variants. Estimates of this property throughout the lifespan of the affected erythrocytes should also provide information on the *in vivo* lability of G6PD (mechanism *c*). Former approaches to defining the G6PD specific activity in some genetic variants yielded controversial results; thus, for instance, neutralization experiments of lysates from whole erythrocyte populations by antiserum against G6PD suggested that the specific activity of the mutant Mediterranean enzyme is one-third that of the B enzyme (4). On the other hand, Kahn *et al.* (7), using electroimmunodiffusion with specific staining of the immunoprecipitate peaks for G6PD activity, were unable to detect any G6PD CRM in G6PD Mediterranean erythrocytes.

The need of quantitating the amount of G6PD protein in order to estimate its specific activity prompted us to develop two different yet complementary approaches: a single-step affinity chromatography technique yielding highly purified G6PD preparations from single donors, and a specific solid-phase radioimmunoassay for G6PD protein. Application of the two procedures to the study of G6PD Mediterranean and G6PD Seattle-like showed that both variant enzymes undergo a faster degradation than the B type (accounted for by a progressively decreasing number of uniformly active enzyme molecules during aging of the erythrocyte). While the Seattle-like variant has normal catalytic function and the related defect is only sustained by a lowered amount of enzyme protein, both an impaired catalytic efficiency and a reduced quantity of G6PD (because of its accelerated breakdown) account for the defect of activity in the Mediterranean type. The conclusion of an inherently lowered specific activity of G6PD Mediterranean rests on the reasonable assumption that the corresponding structural mutation does not enhance the immunological reactivity of the protein with the antibody against the B enzyme.

If a reduced synthesis were responsible for the deficiency of G6PD activity, then extrapolation to an ideally homogeneous population of reticulocytes would be expected to yield significantly lower levels of G6PD protein compared with the B type. Inspection of Fig. 5 reveals that this is not the case with the Seattle-like and Mediterranean variants. On the contrary, the average content of G6PD protein in the "pure" reticulocyte (10) is even higher than predictable on the basis of the enhanced breakdown of the two "deficient" variants. While such discrepancy requires further investigations, the present findings appear not to favor the view of a decreased synthesis of both variants examined.

The increased *in vivo* lability of mutant over wild-type proteins is documented in several cell systems, including reticulocytes (reviewed in ref. 29), although the paths of protein degradation within the erythrocytes are unknown. As far as G6PD is concerned, in only one case has the molecular basis of the intrinsic instability of a "deficient" variant been postulated: thus, the native form of the A^- variant has been shown to be spontaneously converted, probably by formation of disulfide bonds, to another species which appears to be more susceptible to inactivation (30). The results reported in this paper demonstrate that aging of erythrocytes is characterized by a progressive loss of active molecules of G6PD which is distinctively greater in the two "deficient" types examined. Defining the cellular mechanisms by which G6PD undergoes *in vivo* degradation represents a crucial step for further investigations.

We thank Dr. L. Lenzerini, Ospedale Civile S. Francesco, Nuoro, Italy, for his interest and for supplying several blood samples from Sardinian subjects. We are indebted to Prof. S. Pontremoli for critically reading the manuscript and to Mrs. S. Galiano and M. Frascio for skillful technical assistance. This work was supported by Research Grant 6/77.00631.86/115.6414 from the Consiglio Nazionale delle Ricerche, Rome, and by C.N.R. Research Grant 40/75.01007.65, under the cooperative agreement between Italy and the United States.

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