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Research Article

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Genetic Diversity of the "Mediterranean" Glucose-6-Phosphate Dehydrogenase Deficiency Phenotype

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ABSTRACT Genetic diversity of the "Mediterranean" phenotype of G-6-PD (glucose-6-phosphate dehydrogenase) deficiency was revealed when detailed studies were performed on blood specimens from 79 Greek males with G-6-PD levels 0–10% of normal. Four different mutants were found to be responsible for the severely deficient phenotypes: two mutants, G-6-PD U-M (Union-Markham) and G-6-PD Orchomenos, were distinguishable by electrophoresis, while the other two, G-6-PD Athens-like and G-6-PD Mediterranean, were distinguishable on the basis of their kinetic characteristics. Of the kinetic tests applied, the most useful for differentiating the variants were those measuring utilization rates of the analogue substrates deamino–NADP, 2-deoxyglucose-6-phosphate, and galactose-6-phosphate. Among unrelated males with severe G-6-PD deficiency, the relative frequencies of the four variants were: G-6-PD U-M, 5%; G-6-PD Orchomenos, 7%; G-6-PD Athens-like, 16%; G-6-PD Mediterranean, 72%. Genetic, biochemical, and clinical implications of the findings are discussed.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G-6-PD)[1] has a multimeric structure, each of its identical polypeptide chains having a molecular weight of 40,000–50,000 (1, 2). Over 70 variants, representing mutations at the sex-linked structural locus of this enzyme, have been reported (3). Many of these variants have no clinical effect; they are detected by electrophoresis because of a difference in electrical charge. Others are associated with mild, moderate, or severe reduction of enzymatic activity, which is reflected in altered cellular metabolic behavior, most obvious in the red cell. In these G-6-PD variants, the electrophoretic mobility may be normal, fast, or slow, depending upon the particular amino acid substitution(s) involved. The currently known variants, characterized by electrophoretic and kinetic measurements, have been classified (4, 5) into four categories: variants with (*a*) normal G-6-PD activity in red blood cells; (*b*) mild G-6-PD deficiency; (*c*) severe G-6-PD deficiency; and (*d*) G-6-PD deficiency associated with chronic nonspherocytic hemolytic anemia (CNSHA). Of the variants with reduced enzymatic activity, the most common are G-6-PD A^-, virtually confined to Negroes, and the Mediterranean variant, found in Caucasians and Asiatics (4, 5).

The Mediterranean variant is characterized by very low G-6-PD activity (0–10% of the normal level), electrophoretic mobility indistinguishable from the normal type, G-6-PD B (4, 5), and a particular kinetic behavior pattern, described by Kirkman, Schettini, and Pickard (6). G-6-PD–deficient persons of Mediterranean origin have drastically decreased red cell G-6-PD activity, and are usually considered to have this Mediterranean variant. However, such a designation may not be justified, since structurally different forms of an enzyme can be manifested as identical phenotypes when only assays of enzyme activity are applied. Performance of more complete studies on the enzyme kinetic behavior could uncover differences of potential importance because of their association with both the metabolism of the cell and the clinical phenotype of the carrier.

This study was designed in order to determine whether severe G-6-PD deficiency in Mediterraneans is not a single entity but includes several structurally different mutants. For this purpose, detailed characterization of

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[1] *Abbreviations used in this paper:* ACD, acid-citrate-dextrose; BCB, brilliant cresyl blue; CNSHA, chronic nonspherocytic hemolytic anemia; deamino–NADP, deamino-nicotinamide adenine dinucleotide phosphate; 2-deoxy-G-6-P, 2-deoxyglucose-6-phosphate; G-6-P glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; G-6-PD U-M, glucose-6-phosphate dehydrogenase Union-Markham; k_m, Michaelis constant; NADP, nicotinamide adenine dinucleotide phosphate.

G-6-PD was performed on the blood of Greek males. To avoid ambiguities in the interpretation of the kinetic data, a relatively large number of deficient persons was examined. Since a given G-6-PD variant is expected to have the same kinetic and electrophoretic expression in the hemizygous offspring of heterozygous females, several pairs of G-6-PD deficient brothers were included in the study. The correlation of the findings in these sib-pairs was to provide the evidence for or against genetic origin of any observed diversity.

METHODS

Detection of males with G-6-PD deficiency. The G-6-PD–deficient males were detected during population studies in two areas of Greece (Karditsa and Orchomenos). Initial screening was performed with brilliant cresyl blue (BCB) by the method of Motulsky and Campbell-Kraut (7). In this test, the red cells of males who have less than 10% of normal G-6-PD activity fail to decolorize the BCB within 3 hr. Of the 79 G-6-PD–deficient males chosen for this study, 27 were unrelated, while the other 52 consisted of 26 sib-pairs.

Screening program for G-6-PD diversity. Blood samples (30–60 ml) were collected in acid-citrate-dextrose (ACD, National Institutes of Health formula B) and shipped refrigerated to Seattle, Wash., where they arrived within 72 hr of sampling. Every shipment included six to eight G-6-PD–deficient samples and one normal control. Specimens were rechecked for G-6-PD activity on arrival; subsequently they were partially purified. Further tests on the partially purified preparations consisted of starch gel electrophoresis in phosphate buffer at pH 7.0, measurement of deamino-nicotinamide adenine dinucleotide phosphate (deamino-NADP) utilization, and measurements of 2-deoxyglucose-6-phosphate (2-deoxy–G-6-P) utilization. The electrophoretic plates were reviewed by two observers, one of whom was not aware of the kinetic fiindings. All kinetic characterizations were performed by one worker, who did not know which samples were obtained from sibs. Subsequently, galactose-6-phosphate utilization, K_m's for nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G-6-P) and pH-dependent G-6-PD activities were studied. In addition to the above procedures, hemolysates from 17 samples were examined by starch gel electrophoresis for G-6-PD pattern.

Experimental procedures. Techniques recommended by the World Health Organization Committee on G-6-PD test standardization were applied (4). Preparation of hemolysates, assay of G-6-PD activity in hemolysates, and partial purification of G-6-PD were carried out as described by Motulsky and Yoshida (5). For electrophoresis, partially purified preparations from normal and enzyme-deficient blood were dialyzed and adjusted to similar G-6-PD activities; separation was performed in starch gels using a phosphate buffer system at pH 7.0, a Tris (tris [hydroxymethyl] aminomethane)-HCl buffer system at pH 8.8, and a Tris-EDTA-borate buffer system at pH 8.6; (for details, see Reference 5). Hemolysates, adjusted to a hemoglobin concentration of 2 g/100 ml for normal samples and 10 g/100 ml for deficient samples were electrophoresed in a phosphate buffer system at pH 7.0. The development of the G-6-PD staining was observed at frequent intervals and the staining solution removed as soon as a faint band appeared in the G-6-PD–deficient samples.

Michaelis constants (K_m's) for G-6-P and NADP were determined in a pH 8.0 buffer containing 10^{-1} M Tris-HCl, and 7×10^{-3} M $MgCl_2$, the G-6-P concentration ranging from 1.5×10^{-5} M to 2×10^{-3} M and the NADP concentration ranging from 1.35×10^{-6} M to 6×10^{-5} M (five to seven different concentrations for each substrate). The relative utilization rate of analogue substrates (2-deoxy G-6-P, galactose-6-P, deamino–NADP) was expressed as a percentage of the rate at which the same amount of the enzyme could utilize G-6-P or NADP; it was determined using the following concentrations of substrates: 10^{-4} M deamino–NADP with 8×10^{-5} M to 10^{-4} M NADP; 10^{-3} M galactose-6-phosphate, with 10^{-3} M G-6-P; 10^{-3} M 2 deoxy G-6-P with 10^{-3} M G-6-P. For measurement of pH-dependent G-6-PD activity (7) dialyzed, partially purified G-6-PD–deficient, and normal control preparations were tested in a 5×10^{-2} M Tris-HCl, 5×10^{-2} M glycine, 5×10^{-2} M $KHPO_4$ buffer, the pH being adjusted at intervals from 5.5 to 10.5 with HCl or NaOH. The substrate concentrations were those used in G-6-PD assay of hemolysates (5) and the enzyme activity at each pH was expressed as percentage of the maximum activity measured.

RESULTS

The findings in the 27 unrelated G-6-PD–deficient males appear in Table I, while those in sib-pairs appear in Table II and III. A summary of the findings is provided in Table IV.

Diversity revealed by electrophoresis

Electrophoretic screening in phosphate buffer, pH 7.0, revealed two G-6-PD variants: in three persons the enzyme moved more rapidly, and in eight, it moved more slowly than normal.

With phosphate buffer at pH 7.0, the migration rate of the slow G-6-PD was 92–94% of G-6-PD B (Fig. 1). In TEB buffer, pH 8.6, and in Tris-HCl buffer, pH 8.8, it migrated 94–96% as far as G-6-PD B. Although the eight examples of this slow variant were detected independently, they were subsequently found to belong to four pairs of brothers. The electrophoretic findings, kinetic data (Table IV), and the genetic evidence indicated that this enzyme was different from the common Mediterranean variant. It was preliminarily called G-6-PD Orchomenos.

The mobility of the fast G-6-PD was 102–104% that of G-6-PD B. Its electrophoretic pattern and the kinetic properties (Table IV) were similar in some respects to those of G-6-PD Markham (8) as well as G-6-PD Union (9). These variants are characterized by electrophoretic migration rates faster than G-6-PD B, by severe reduction of red cell enzyme activity, and by distinctly higher than normal rates of 2-deoxy–G-6-P and galactose-6-P utilization. Direct comparisons of these two variants with that detected in our study could not be done. Therefore, this Greek G-6-PD was tentatively called G-6-PD Union-Markham (G-6-PD U-M). Like G-6-PD Markham, G-6-PD UM was found to be un-

TABLE I

G-6-PD Characterization in 27 Unrelated Greek Males with Severe G-6-PD Deficiency

Case no.	G-6-PD activity*	K_m ($\times 10^{-6}$) for		Utilization of			Assigned G-6-PD variant
		G-6-P	NADP	2-deoxy-G-6-P§	Deamino-NADP‖	Galactose-6-P§	
1	4	7.8	2.0	225.0	375	114.5	U–M
2	0	—	1.6	200.0	421	—	"
3	1	18.5	3.7	11.9	153	20.5	"Athens-like"
4	5	18.4	3.7	15.7	164	23.3	"
5	7	13.1	3.5	10.0	151	18.2	"
6	3	17.6	2.9	11.0	173	20.0	"
7	2	—	—	14.8	139	—	"
8	0	12.6	2.3	58.0	343	49.0	Mediterranean
9	0	10.7	1.9	49.0	303	—	"
10	0	9.5	1.6	60.0	312	40.7	"
11	0	11.5	2.1	60.8	331	48.2	"
12	0	11.5	2.4	53.1	303	43.3	"
13	3	11.6	2.0	49.3	271	44.4	"
14	2	12.6	2.4	51.3	326	47.1	"
15	2	10.1	1.8	53.2	300	39.3	"
16	1	10.9	1.7	44.4	311	—	"
17	3	10.4	2.1	51.8	333	—	"
18	3	11.5	2.5	54.8	341	38.7	"
19	0	11.8	2.1	52.3	339	40.5	"
20	0	12.4	1.9	42.4	339	41.6	"
21	2	10.2	2.1	48.7	295	33.7	"
22	6	12.3	2.2	51.5	314	34.7	"
23	3	—	—	57.5	280	—	"
24	6	—	—	42.5	315	—	"
25	6	—	—	44.0	306	—	"
26	0	9.6	2.0	50.5	312	36.9	"
27	3	10.4	1.8	52.2	304	41.3	"

*, ‖, § as in footnote of Table IV.

stable. Partially purified preparations were almost devoid of activity after storage at 4°C for 2–3 wk. Furthermore, although the dialyzed, partially purified preparations of G-6-PD U-M were adjusted before electrophoresis to activities similar to the activity of G-6-PD B controls, the variant was always faintly stained, probably because of loss in catalytic activity during electrophoresis.

Diversity revealed by kinetic techniques

Utilization of deamino–NADP. Utilization of deamino NADP for the electrophoretically distinguishable G-6-PD U-M and Orchomenos as well as for the remaining 68 cases are plotted in Fig. 2. Differences in deamino–NADP utilization rates separated the enzyme in the 68 cases with similar electrophoretic mobility into two nonoverlapping groups. In one group of 56 cases, the deamino–NADP utilization ranged from 240 to 385% (mean = 312.86 ±26.7) and in the other group of 12 cases the range was 115–175% (mean = 152.67 ±11.8); the difference between the 2 groups was statistically significant ($P < 0.0001$).

To test whether this difference in kinetic behavior was genetically determined, the correlation of deamino-NADP utilization values in sibships was examined. Of the total of 26 pairs of brothers included in this study, 22 pairs (Table II) had mothers who were heterozygous for G-6-PD deficiency, and thus, the brothers in each pair had the same G-6-PD deficiency gene. Four of these sib-pairs had G-6-PD Orchomenos. The correlation of utilization values in the remaining 18 pairs is shown in Fig. 3. In 15 pairs, the deamino–NADP utilization value was high; in 3 pairs it was low. On the basis of the bimodality in the distribution of measurements and the correlation of the findings in sibs, the 12 males with deamino–NADP utilization values between 115 and 175% (Fig. 2) were considered to possess a G-6-PD mutant different from the more common Mediterranean type. Since similar values of deamino–NADP utilization have been described in G-6-PD Athens (reference 10 and unpublished data), the enzyme in these 12 individuals was preliminarily called "Athens-like" G-6-PD.

Utilization of 2-deoxyglucose-6-phosphate. The findings for persons with G-6-PD U-M, G-6-PD Orchomenos, Athens-like G-6-PD, and for the remaining 56 G-6-PD–deficient individuals are given in Fig. 4. Since in this latter group of cases no further diversity was detected with the methods used, the males of this group were considered to possess the Mediterranean variant of G-6-PD deficiency. Mean values of 2-deoxy–G-6-P utilization appear in Table IV; the differences between the Athens-like, Mediterranean, and Orchomenos variants

TABLE II

G-6-PD Characterization in 22 Pairs of G-6-PD–Deficient Brothers Having Gd^+/Gd^- Heterozygous Mothers

Sibship and case	G-6-PD activity*	K_m ($\times 10^{-6}$) for		Utilization of			Assigned G-6-PD variant
		G-6-P	NADP	2-deoxy–G-6-P§	Deamino-NADP‖	Galactose-6-P§	
1a	0	10.6	2.4	111.0	386	59.4	Orchomenos
b	0	9.2	2.0	103.8	350	62.8	"
2a	4	11.1	2.2	91.2	306	58.3	"
b	7	10.7	2.3	103.9	334	50.0	"
3a	0	10.4	1.9	97.0	303	64.2	"
b	4	12.9	2.2	98.3	364	54.8	"
4a	0	11.5	2.1	117.0	393	62.0	"
b	0	10.5	1.9	116.5	366	55.0	"
5a	7	17.5	3.2	16.4	150	22.5	"Athens-like"
b	4	16.9	3.1	21.7	167	20.7	"
6a	5	16.1	3.1	21.6	152	—	"
b	6	—	—	16.1	156	—	"
7a	10	16.5	3.4	17.4	144	22.0	"
b	10	18.3	3.6	11.0	153	24.2	"
8a	10	12.9	2.4	58.6	347	38.9	Mediterranean
b	2	10.2	1.9	47.8	340	43.8	"
9a	0	10.3	2.2	51.0	338	45.6	"
b	2	11.9	2.1	45.0	280	45.0	"
10a	2	12.4	2.2	54.8	277	39.4	"
b	0	11.8	2.3	59.2	327	43.6	"
11a	6	12.6	2.1	58.9	380	50.8	"
b	2	11.4	2.0	63.7	331	47.3	"
12a	8	12.6	2.7	48.0	370	53.0	"
b	0	11.8	2.3	58.1	298	48.8	"
13a	2	10.4	2.1	48.4	319	38.5	"
b	6	12.1	2.1	53.9	286	43.3	"
14a	—	11.2	2.0	55.6	361	42.2	"
b	5	12.4	2.1	42.0	293	33.3	"
15a	6	12.1	2.1	39.0	253	36.4	"
b	—	12.5	2.1	51.5	276	31.0	"
16a	2	—	1.5	51.7	333	—	"
b	0	—	—	54.0	319	—	"
17a	2	—	—	53.0	282	—	"
b	0	—	—	41.9	351	—	"
18a	0	—	—	42.3	312	—	"
b	0	—	—	55.3	336	—	"
19a	0	12.5	1.6	49.2	300	—	"
b	8	—	—	38.2	312	—	"
20a	0	—	2.1	51.8	319	—	"
b	0	—	—	48.3	297	—	"
21a	4	—	—	47.8	335	—	"
b	5	—	—	45.7	286	—	"
22a	10	—	—	49.2	314	—	"
b	2	—	—	53.1	280	—	"

*, ‖, § as in footnote of Table IV.

TABLE III

G-6-PD Characterization in Four Pairs of G-6-PD–Deficient Brothers Having Severely G-6-PD–Deficient Mothers

Sibship and case	G-6-PD activity*	K_m ($\times 10^{-6}$) for		Utilization of			Assigned G-6-PD variant
		G-6-P	NADP	2-deoxy-G-6-P§	Deamino-NADP‖	Galactose-6-P§	
23a	7	13.1	2.3	50.0	291	48.2	Mediterranean
b	5	11.5	2.1	44.0	310	37.1	"
24a	3	11.0	2.4	37.0	295	43.2	"
b	4	9.5	1.7	52.8	262	37.8	"
25a	0	9.3	1.7	52.8	305	50.0	"
b	3	6.2	1.4	176.0	396	111.7	U–M
26a	0	11.4	1.7	51.0	327	42.8	Mediterranean
b	4	17.9	3.1	17.4	130	26.6	"Athens-like"

*, ‖, § as in footnote of Table IV.

are statistically significant ($P < 0.0001$). That the observed differences are genetically determined is indicated by the correlation of findings in pairs of brothers whose mothers are heterozygous for G-6-PD deficiency (Fig. 5).

Utilization of galactose-6-phosphate. This measurement was performed in 55 individuals; the findings are given in Table IV. There is a clear-cut discrimination of values between Athens-like G-6-PD and Mediterranean G-6-PD ($P < 0.0001$). The values in G-6-PD Orchomenos are significantly higher than in Mediterranean G-6-PD ($P < 0.0001$), although their distributions overlap.

K_m for G-6-P and NADP. K_m for G-6-P was measured in 60 individuals and for NADP in 63 individuals. Distribution of measurements in G-6-PD Orchomenos and G-6-PD U-M were within the range characteristic of Mediterranean G-6-PD. In G-6-PD Athens-like K_m's for NADP and G-6-P were significantly higher ($P < 0.0001$) than in Mediterranean G-6-PD and within the range reported for G-6-PD Athens (10).

pH-dependent G-6-PD activity. G-6-PD activity as a function of pH was measured in 11 persons with the Mediterranean variant, 2 with G-6-PD Orchomenos and 2 with G-6-PD U-M. The mean G-6-PD activities per pH unit are plotted in Fig. 6. The pH-dependent ac-

TABLE IV

Characterization of Four G-6-PD Variants Found among 79 Greek Males with Severe G-6-PD Deficiency

	G-6-PD B	G-6-PD Mediterranean	G-6-PD "Athens-like"	G-6-PD Orchomenos	G-6-PD U-M
G-6-PD activity*	100	0–10	1–10	0–7	0–4
Electrophoretic migration‡	100	98–99¶	98–99¶	92–94	102–104
K_m ($\times 10^{-6}$) for					
G-6-P	47.3 ±4.0	11.41 ±1.05	17.08 ±1.62	10.86 ±1.06	7.0 ±1.13
NADP	3.8 ±0.7	2.06 ±0.26	3.33 ±0.29	2.11 ±0.19	1.67 ±0.30
Utilization of					
2-deoxy–G-6-P§	4.5 ±1.0	50.34 ±6.07	15.42 ±3.91	104.8 ±9.36	200.3 ±24.5
deamino-NADP‖	58.4 ±3.1	312.86 ±26.7	152.67 ±11.8	350.25 ±33.8	397.33 ±23.1
galactose-6-P§	7.1 ±1.6	42.2 ±5.3	22.0 ±2.5	58.3 ±4.8	113.0 ±1.98
pH dependent G-6-PD activity					
pH curve	Truncate	Biphasic	—	Biphasic	Biphasic
pH peaks	9.0	6.5, 9.5	—	6.0, 9.5	6.0, 9.5

* Per cent of mean G-6-PD activity in RBC of normals.
‡ Per cent of the rate of G-6-PD B migration.
§ Expressed as per cent of the rate of glucose-6-phosphate utilization.
‖ Expressed as per cent of the rate of NADP utilization.
¶ Slightly slower than G-6-PD B.

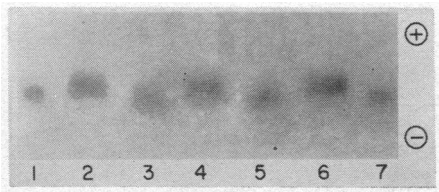

FIGURE 1 Horizontal starch gel electrophoresis of partially purified G-6-PD preparations. Phosphate buffer 0.05 M at pH 7.0. 1, 3, 5, and 7, G-6-PD Orchomenos. 2, 4, and 6 G-6-PD B.

tivity was biphasic and the configuration of the curves characteristic for each variant (Fig. 6).

Heterogeneity within families

Four sib-pairs (Table III) had mothers whose RBC G-6-PD activity was compatible with homozygosity for G-6-PD deficiency. In two pairs, both sibs had the Mediterranean variant. In the third and fourth pairs, one brother had the Mediterranean variant while the other had Athens-like G-6-PD and G-6-PD U-M, respectively. The discordant findings in these two sib-pairs suggested that their mothers were heterozygotes for two types of G-6-PD deficiency. Genetic studies of the maternal families were not possible.

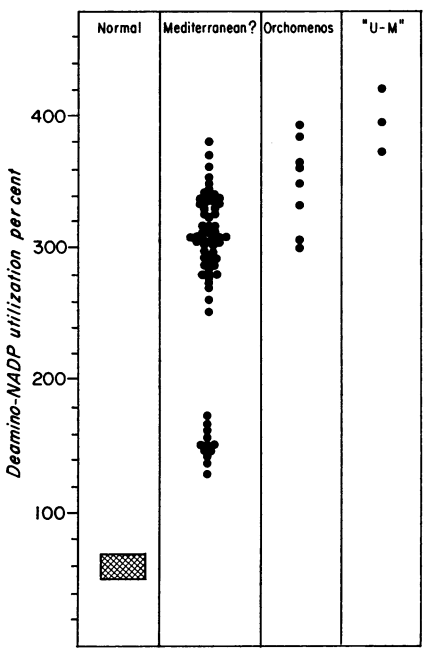

FIGURE 2 The distribution of measurements of the rate of deamino-NADP utilization (expressed as percentage of the rate of NADP utilization) in 79 Greek males with severe G-6-PD deficiency in the red cells. The values in cases with G-6-PD Orchomenos and G-6-PD U-M are plotted separately. The values in the remaining 68 cases display bimodal distribution without overlap.

Relative frequencies of the variants

Some information was derived regarding the relative frequency of the four mutants among Greeks with G-6-PD deficiency. Among the males studied, there were 57 G-6-PD deficiency genes (27 in the unrelated persons; 22 in the 22 sib-pairs with Gd^+/Gd^- heterozygous mothers and eight in the four pairs with homozygous or doubly heterozygous mothers). The relative frequencies of the four variants are shown in Table V.

DISCUSSION

The possibility that the Mediterranean type of G-6-PD deficiency is a composite of several mutants was first raised by Kirkman, Doxiadis, Valaes, Tassopoulos, and Brinson (11) who noticed diverse kinetic behavior in the G-6-PD of 16 Greek males who were severely deficient in that enzyme. In the absence of family data, no definitive genetic interpretations of these observations could be made, but the authors indicated that if the observed diversity were due to genetic heterogeneity, at least three G-6-PD variants would be required to account for their findings. In the present study, four different G-6-PD variants were detected. Ambiguity of the data was not encountered because the large number of examined persons lent high statistical significance to the differences in kinetic measurements. Furthermore, the study of sibs rather than unselected individuals provided clear-cut evidence that the phenotypes we observed were, in fact, genetically determined. It is apparent that sib-pairs should be included in any study in which the genetic diversity of a protein is investigated with kinetic or electrophoretic techniques.

Recent population studies have revealed considerable heterogeneity at the G-6-PD locus: 1 out of 450–600 individuals without G-6-PD deficiency has an uncommon electrophoretic G-6-PD variant (12, 13). This fre-

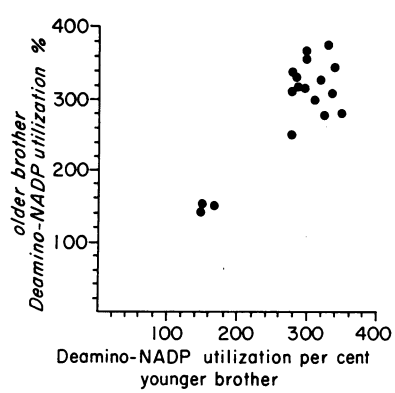

FIGURE 3 Comparison of deamino NADP utilization measurements in pairs of brothers having Gd^+/Gd^- heterozygous mothers. The values of 18 sib-pairs included in the "Mediterranean?" column of Fig. 2 are plotted.

quency of the "nonpolymorphic" G-6-PD variants has been considered as an indication that the structure of the G-6-PD molecule is such that amino acid substitutions are permitted which would not be tolerated in several other proteins. Of the variants observed in this study, none can be placed in the category of the rare nonpolymorphic mutants, since their frequencies ranged from 1% in the case of G-6-PD U-M to as much as 13% in the case of Mediterranean variant (Table V). These findings suggest that a number of different Gd locus mutations associated with deficiency of the enzyme may be favored by selection and may coexist in the same population. It remains to be seen whether our findings are characteristic only of the Greek population or whether a similar molecular diversity exists among the other Mediterranean ethnic groups with high frequencies of G-6-PD deficiency.

The kinetic and electrophoretic studies of G-6-PD have so far been useful in detecting and discriminating between variants, when comparisons of kinetic and electrophoretic behavior are made under standard conditions (4, 5). Even in the case of variants with small differences in electrophoretic migration or kinetic constants, these techniques have been adequate when the comparisons were made in the same, rather than in separate, laboratories. Conclusions about differences or similarities between variants based on reported properties are possible only when they are significantly larger than the errors inherent in the experimental procedures. However, with over 70 different G-6-PD's already reported (3), meaningful comparisons are now probably beyond the power of the differentiation techniques. It is thus not possible for us to conclude that the three "new" variants described in this study (G-6-PD U-M, Athens-like, Orchomenos) are really new, since one or more of them could represent previously recognized mutants. For example, G-6-PD U-M could be identical with either G-6-PD Union (8) or G-6-PD Markham (9). Its electropho-

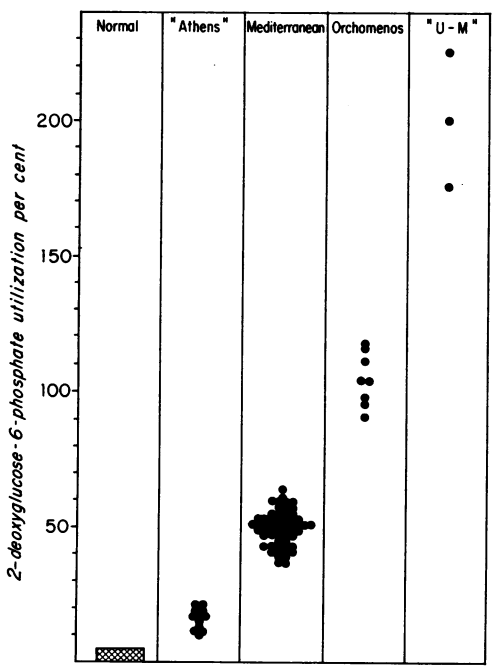

FIGURE 4 The distribution of measurements of the rate of 2-deoxy-glucose-6-phosphate utilization (expressed as percentage of the rate of glucose-6-phosphate utilization) in 79 Greek males with severe G-6-PD deficiency in the red cells. The differences in mean values between G-6-PD Athens-like, G-6-PD Mediterranean, and G-6-PD Orchomenos (Table IV) are highly significant ($P < 0.0001$).

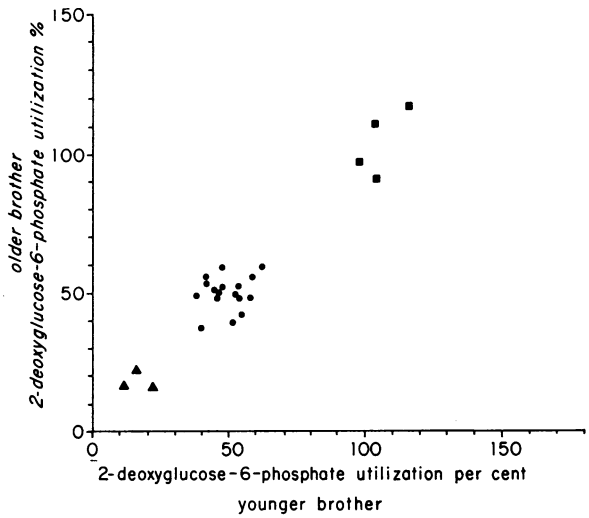

FIGURE 5 Comparison of 2-deoxy-glucose-6-phosphate utilization measurements in 22 pairs of brothers having Gd^+/Gd^- heterozygous mothers. (■): G-6-PD Orchomenos; (●): G-6-PD Mediterranean; (▲): G-6-PD Athens-like.

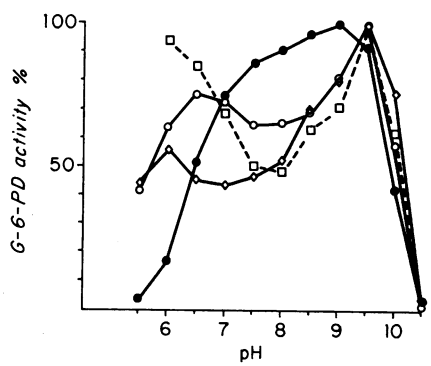

FIGURE 6. pH-dependent G-6-PD activity. (●) G-6-PD B; (○) G-6-PD Mediterranean; (◇) G-6-PD Orchomenos; (□) G-6-PD U-M. G-6-PD activity at individual pH point is expressed as percentage of the higher measured activity.

TABLE V

Relative and Population Frequencies of the Four G-6-P Deficiency Mutants

Class of individuals	No. of cases	No. of genes	Gd Mediterranean	Gd Athens-like	Gd Orchomenos	Gd U-M
Unrelated	27	27	20	5	—	2
Sib-pairs (heterozygous mother)	44	22	15	3	4	—
Sib-pairs (homozygous or double heterozygous mother)	8	8	6	1	—	1
Totals	79	57	41	9	4	3
Relative frequency of variants, % (Gd^- = 100%)			71.9	15.8	7.0	5.3
Population frequency of variants, % (Gd^- = 18%)			12.94	2.85	1.26	0.95

retic migration and substrate utilization properties are very similar to those reported for these two enzymes, but proof of identity is impossible in the absence of direct comparisons.

The kinetic profile of G-6-PD Athens-like is identical with that of G-6-PD Athens (10), but the two enzymes differ in the degree of G-6-PD deficiency. In individuals with G-6-PD Athens, G-6-PD activity is moderately reduced, while in G-6-PD Athens-like, the deficiency is severe. It is possible that variations in erythrocyte G-6-PD activity are sometimes secondary to other causes and thus not directly related to a structural change in the enzyme molecule. On the other hand, different mutations may cause the same alteration in kinetic characteristics but different degrees of G-6-PD instability or rate of synthesis. Without comparative structural analyses, one cannot speculate usefully about whether or not the Athens and Athens-like G-6-PD deficiency mutants are identical.

The third variant, G-6-PD Orchomenos, appears electrophoretically and kinetically different from the other mutants which are associated with severe G-6-PD deficiency but conclusive proof requires comparison of peptide maps and amino acid analyses of isolated peptides.

When the aberrant G-6-PD could not be classified as Athens-like, U-M, or Orchomenos, the individual was considered to have the Mediterranean variant. This designation seemed reasonable, because there was no apparent bimodality in the values obtained from kinetic measurements and also because in analysis of variance of these measurements in sib-pairs, the intrafamilial similarities did not differ statistically from the interfamilial similarities. It is true that the rates of utilization of 2-deoxy–G-6-P and galactose-6-P were higher among the subjects considered to have the Mediterranean variant than those assigned to the Mediterranean variant by Kirkman et al. (6). However, this disparity probably reflects slight differences in substrate concentrations and also differences in the batches of the reagents used, since higher values were also obtained with our normal controls. Although the Mediterranean variant has been described as electrophoretically indistinguishable from G-6-PD B (4–6), we found its migration to be slightly slower than the normal enzyme, particularly when partially purified G-6-PD preparations were electrophoresed. With hemolysates, the slight retardation was observed only when the normal control hemolysate was diluted and the development of enzyme staining was interrupted when the Mediterranean G-6-PD zone first became visible.

The males included in this study were chosen on the basis of a single criterion, i.e., the results of a screening test for G-6-PD deficiency. Hematologic and clinical observations were not done, and thus the clinical implications of these variants remain unknown. However, there is no a priori reason to assume that all the variants comprising severe G-6-PD deficiency in inhabitants of the Mediterranean area have the innocuous hematological phenotype of the Mediterranean mutant or that they have a similar degree of predisposition to favism. This acute hemolytic syndrome does not occur at random among G-6-PD–deficient individuals, but rather has a familial predisposition even in areas where the frequency of G-6-PD deficiency is high (14, 15). Family studies have indicated that a second genetic factor may act synergistically with G-6-PD deficiency in predisposing the individual to acute hemolysis after *Vicia faba* ingestion (14). The postulated second genetic factor might represent a polymorphism of the enzyme(s) metabolizing the hemolytic agent in *Vicia faba;* or a polymorphism of intraerythrocytic enzymes regenerating NADH, NADPH, or GSH; or even a polymorphism involving hemoglobin stability. In this view, favism appears to be a multifactorial disease in which the occurrence of G-6-PD de-

ficiency alone or the "second genetic factor" alone is insufficient to trigger an attack; the combination, however, of G-6-PD deficiency and of the postulated second metabolic defect, make the red cell susceptible to acute destruction when fava beans containing the noxious agent are ingested. Differences also exist in the susceptibility of the G-6-PD deficiency carriers to neonatal hyperbilirubinemia. As in the case of favism, severe jaundice does not occur at random among the G-6-PD–deficient neonates but has a familial predisposition (16) which may also indicate that other genetic factors may act synergistically with G-6-PD deficiency in enhancing hyperbilirubinemia in the newborn.

A part of the familial disposition to favism could be attributed to an increased susceptibility to hemolysis among carriers of certain G-6-PD deficiency mutants. If, for example, the severity of the hemolytic crisis in favism is also dependent on the dose of the unknown hemolytic substance(s) in *Vicia faba,* G-6-PD mutants with higher lability (G-6-PD U-M) or relatively normal K_m's for G-6-P and NADP (Athens-like G-6-PD) might be associated with hemolysis even with minimal ingestion of *Vicia faba.* A higher frequency of favism in families or in areas with such variants would then be expected. G-6-PD characterization and subtyping in Mediterranean persons selected for acute drug-induced hemolysis or favism could resolve this question. In G-6-PD–deficient children who exhibited severe jaundice at birth, the G-6-PD kinetics have been studied by Kirkman et al. (11). Of the eight *propositi* examined, five had the kinetic characteristics of the usual Mediterranean type, whereas the kinetics in the three others could fit with the diagnosis of Athens-like G-6-PD, if the differences in 2-deoxy–G-6-P values between the study by Kirkman et al. and the present investigation are taken into consideration. A similar degree of diversity was, however, present in the G-6-PD–deficient controls, who did not have a history of severe neonatal jaundice. On the basis of these findings, Kirkman et al. concluded that G-6-PD kinetics in persons with severe neonatal jaundice and in G-6-PD–deficient males without a history of hemolysis are similar. These findings, however, do not exclude the possibility of a higher predisposition to neonatal jaundice among the carriers of certain aberrant G-6-PD's.

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