Characterization of Glucose-6-Phosphate Dehydrogenase Variants. II. G6PD Kephalonia, G6PD Attica, and G6PD "Seattle-like" Found in Greece

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In the course of studies directed toward the mapping of X-linked genes,* four Greek families were encountered that segregated for glucose-6-phosphate dehydrogenase (G6PD) variants different from the normal B type.† In two of these families, the enzyme was electrophoretically slower than B and proved by all techniques employed to be indistinguishable from G6PD Seattle (Kirkman *et al.*, 1965). Three additional cases of electrophoretically slow G6PD variants, resembling G6PD Seattle, were found in 200 unrelated schoolboys from the island of Kephalonia. In the remaining two families, the enzyme was electrophoretically faster than B and appeared, by rate of migration and decreased activity, to be similar to the common African type A−. It was found however, upon more extensive characterization, that both G6PD variants in these two families were different from A− and also different from each other. The two variants have been named G6PD Kephalonia and G6PD Attica.

Although complete hematological studies were not performed, there was no evidence of clinical or hematological abnormalities in any of the subjects with enzyme variants.

METHODS

Preliminary Investigations

Blood samples, consisting of 2–5 ml of whole blood collected in the presence of EDTA,‡ were shipped on ice from Greece to the laboratory in Leiden and usually processed within four to five days from the date of collection. The red cells were

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* The results of these studies, performed in collaboration with the Department of Haematology, Hippocration Hospital, Athens, will be reported elsewhere (Tsevrenis *et al.*, in preparation).

† We have followed the nomenclature recommended in *Bulletin of the World Health Organization*, 1967, 36:319.

‡ The following abbreviations are used in this paper: NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate; G6P = glucose-6-phosphate; Gal6P = galactose-6-phosphate; 2dG6P = 2-deoxyglucose-6-phosphate; DEAE = diethylaminoethyl; CM = carboxymethyl; EDTA = ethylenediaminetetraacetic acid; Tris = *tris*(hydroxymethyl)aminomethane.

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washed and hemolyzed in the usual way. As already described (Lenzerini *et al.*, 1969), dye decoloration tests (Motulsky and Campbell-Kraut, 1961) and cellulose acetate gel electrophoresis, *p*H 7.5 (Rattazzi *et al.*, 1967) were performed on all samples. In cases giving abnormal or uncertain results, the samples were further subjected to starch gel electrophoresis at *p*H 8.6 (Porter *et al.*, 1964), to the quantitative assay of G6PD activity in the red cells (Motulsky *et al.*, 1966), and to the test for cellular localization of G6PD (Gall *et al.*, 1965).

Biochemical Characterization

Blood samples of 50–100 ml collected in ACD were obtained at a later stage from subjects having unusual variants, together with small samples of blood from their relatives. The samples were rechecked by the above-mentioned techniques and then partially purified (Yoshida, 1966). A 60- to 120-fold increase of G6PD specific activity was usually obtained and the preparations showed practically no 6PGD activity. Enzyme preparations were stored at +4° C as precipitates in 60% ammonium sulfate and dialyzed against the appropriate buffers when required.

Samples of blood of G6PD types A and A− from Nigerian donors were obtained from the blood bank of the University College Hospital, Ibadan; a sample of blood with G6PD Seattle was obtained from Dr. H. N. Kirkman, Chapel Hill, North Carolina; samples of normal blood were obtained from the Blood Transfusion Service of the Netherlands Red Cross, Amsterdam. These samples were purified and stored as described above and were used as controls in all experiments.

Michaelis constants (*Km*) for NADP and G6P, relative rate of utilization of the substrate analogues Gal6P and 2dG6P, *p*H dependence of activity, and thermal stability (in absence of $[NH_4]_2SO_4$) were studied as already described (Lenzerini *et al.*, 1969) according to the World Health Organization recommendations (WHO, 1967; see also Beutler *et al.*, 1968).

Thermal inactivation profiles at various NADP concentrations were studied as described by Luzzatto and Allan (1965). The samples were dialyzed and adjusted to the desired concentrations of NADP (10^{-3} M, 10^{-5} M, 10^{-7} M, 10^{-9} M, respectively) in 0.02 M potassium phosphate buffer, *p*H 7.0, containing 0.2 M KCl and 2×10^{-4} M EDTA. After adjustment to similar enzyme activity concentration, aliquots of the enzyme solution were incubated for seven minutes at the desired temperatures, placed on ice and assayed. Temperatures used varied between 25° and 65° C. Transition temperature (T_{tr}), defined as the temperature at which the enzyme retains 50% of the activity it had at 25°, was determined from the thermal inactivation profiles.

The chromatographic behavior of some of the variants was studied by the method of Luzzatto and Allan (1965) on DEAE Sephadex A50, lots 7886, 6702 (new type, bead form) and To-6961 (old type) and also by the method of Yoshida (1966) on CM Sephadex C50, lots 8666, 6702 (new type, bead form) and To-5545 (old type). Satisfactory results were obtained with DEAE Sephadex lot To-6961 equilibrated with 0.005 M phosphate buffer, *p*H 6.9, containing 0.05 M KCl; 10^{-4} M EDTA; and 2×10^{-6} M NADP. The column size was 0.8×60 cm; elution was by a linear gradient obtained by mixing 100 ml of buffer with 100 ml of 0.45 M KCl in buffer. Flow

rate was about 4 ml/hour; 2 ml fractions were collected. Chloride concentration was checked in the fractions by titration with silver nitrate.

Sucrose gradient centrifugation was performed on some of the samples (Martin and Ames, 1961).

RESULTS

Electrophoretically Slow Variants

Low erythrocyte G6PD activity with low electrophoretic mobility (about 90% compared to the B type) was observed in five cases. Three of the variants, found among 200 unrelated schoolboys of the island of Kephalonia, could not be further characterized (Fig. 1). Of the other two, one (ATH3) was found during the screening of 30 unrelated blood donors of the Blood Transfusion Service, Hippocration Hospi-

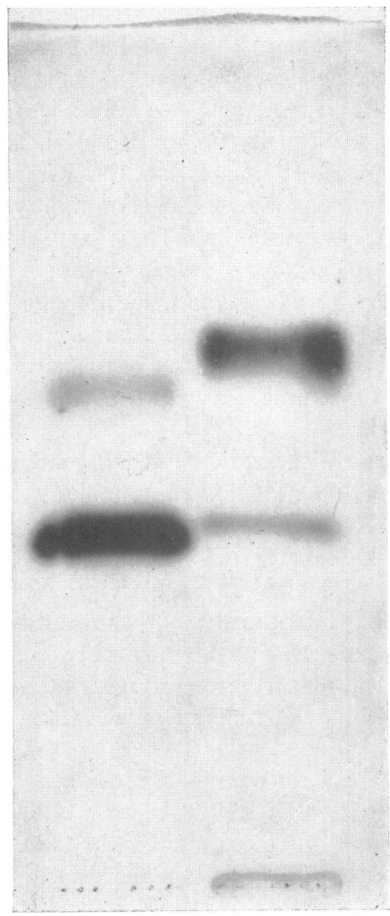

FIG. 1.—Cellulose acetate gel electrophoresis, *p*H 7.5, of one of the unidentified (possibly "Seattle-like") G6PD variants. Left: G6PD variant; Right: G6PD B. The samples are crude hemolyzates, the lower band in the figure being hemoglobin. Migration is toward the anode (top).

tal, Athens; the other (HHA2) was found among patients with Factor VIII deficiency. These two variants were fully investigated.

The pedigrees of family ATH3 and family HHA2 are shown in Figure 2. In both cases, the transmission of the defect is compatible with X-linked inheritance; in family HHA2, the G6PD variant was found to segregate in coupling with hemophilia A.

The red-cell enzyme activity of the affected males is about 25% of normal in the two families and that of the heterozygous females is variable, as expected (see Table 1).

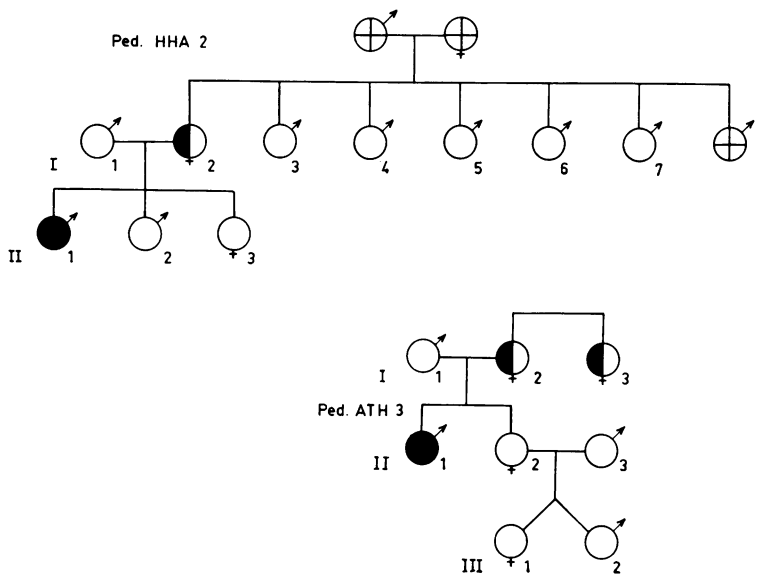

FIG. 2.—Families HHA2 and ATH3. Black = G6PD variants; crossed circles = dead. Subject II-1 of family HHA2 and his dead maternal uncle were hemophilic. Subjects II-3 and III-2 of family ATH3 were not tested.

The electrophoretic mobility of the two variants on cellulose acetate gel (*p*H 7.5) as well as on starch gel (*p*H 8.6) was about 90% compared with the normal (G6PD B) enzyme, and identical with that of G6PD Seattle. The relative mobility did not change after partial purification. The starch gel electrophoretic patterns of G6PD Seattle, of these two Greek variants, and of G6PD "Seattle-like" (Sardinia) (Lenzerini *et al.*, 1969) are shown in Figure 3. Similar results were obtained on cellulose acetate gel at *p*H 7.5.

Some kinetic parameters of these two Greek variants and of G6PD Seattle are listed in Table 2. It can be seen that all three enzymes have low *Km* values for NADP and G6P and increased rates of utilization of substrate analogues. Thermal stability at high NADP concentrations was found to be normal. The *p*H activity curve is slightly but significantly biphasic and similar for the three samples, as shown in Figure 4.

TABLE 1

RED-CELL G6PD ACTIVITY AND ELECTROPHORETIC MOBILITY IN HHA2 AND ATH3

Key to Pedigree	Dye Decol. Test (Minutes)	Red-Cell Activity (IU/g Hb)*	Electrophoretic Mobility† (%)	G6PD Cellular Local. Test‡
HHA2:				
I-1, ♂	40	.....	100	.....
2, ♀	50	3.82	90, 100	8
3, ♂	45	4.72	100	0
4, ♂	40	.....	100	.....
5, ♂	40	.....	100	.....
6, ♂	40	7.21	100	0
7, ♂	40	5.08	100	.....
II-1, ♂	105	1.57	90 ...	31
2, ♂	40	.....	100	.....
3, ♀	45	6.38	100	0
ATH3:				
I-1, ♂	40	5.88	100	.....
2, ♀	65	4.03	90, 100	.....
3, ♀	70	.....	90, 100	.....
II-1, ♂	115	1.33	90 ...	.....
2, ♀	50	5.00	100	.....
III-1, ♀	35	.....	100	.....
Normal values:		SE SD		
35 males	30–50	6.32, ±0.16, ±0.93	100	0–5
54 females	30–50	5.99, ±0.19, ±1.44	100	0–5

* One international unit (IU) = the quantity of G6PD which reduces one micromole of NADP in one minute.
† On starch gel pH 8.6 and cellulose acetate gel pH 7.5. G6PD B = 100.
‡ Percentage of eluted cells.

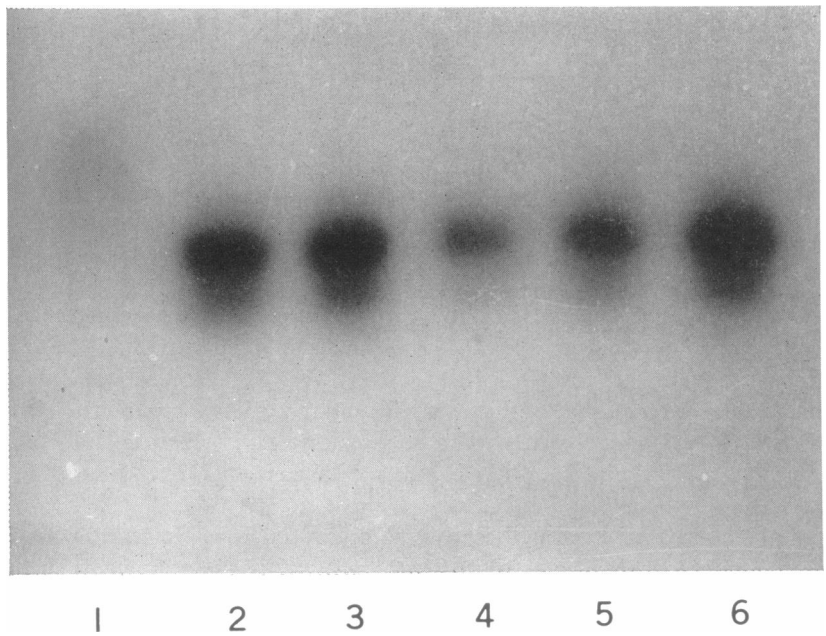

FIG. 3.—Starch gel electrophoresis at pH 8.6 of the slow-moving G6PD variants. *1* = G6PD B (faint); *2* = variant HHA2; *3* = variant ATH3; *4, 5* = G6PD "Seattle-like" Sardinia; *6* = G6PD Seattle.

TABLE 2

KINETIC PARAMETERS OF KEPHALONIA, ATHENS, AND "SEATTLE-LIKE" VARIANTS

SUBJECTS EXAMINED	MICHAELIS CONSTANTS (μM)*		SUBSTRATE UTILIZATION†		pH ACTIVITY CURVE	THERMAL STABILITY‡
	NADP	G6P	2dG6P	Gal6P		
G6PD Kephalonia:						
HHA1: I-2	3.8±0.2	34.2±1.4	2.1	1.5	Truncate	Normal
G6PD Attica:						
HHA29: I-2	4.7±0.2	40.7±1.8	1.8	1.4	Truncate	Normal
G6PD A−, Ib. 10235	4.2±0.2	49.7±1.5	2.8	1.7	Truncate	Normal
G6PD Seattle-like:						
HHA2: II-1	2.7±0.2	22.4±2.0	6.0	5.4	Slightly biphasic	Normal
ATH3: II-1	2.4±0.1	20.4±1.0	7.3	5.8	Slightly biphasic	Normal
G6PD Seattle, Ki	2.1±0.1	22.9±3.7	6.1	5.4	Slightly biphasic	Normal
Controls:						
G6PD B, HHA23-3	3.8±0.3	42.7±2.3	2.6	2.2	Truncate	Normal
G6PD A, Ib. 6015	3.2±0.5	48.2±3.3	2.5	2.0	Truncate	Normal
15 Caucasian males	3.3/ 4.5	34.8/63.6	1.5/2.9	1.3/2.8	Truncate	Normal

* Regression coefficient of V (rate) versus V/S (rate/substrate concentr.), ± SD of the regression coefficient.
† Percentage of rate of utilization of G6P.
‡ Fixed temperature, high concentration of NADP (see Methods).

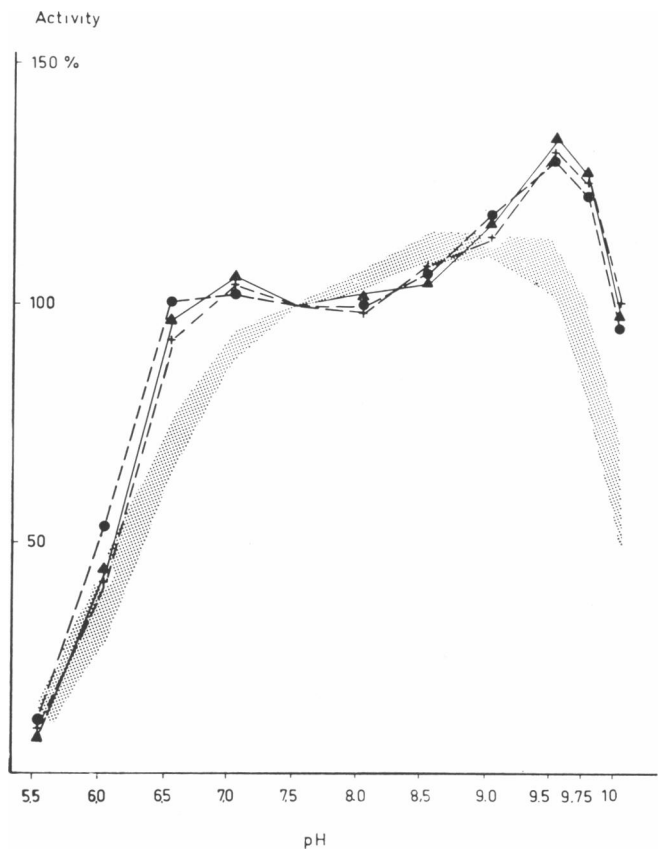

FIG. 4.—The *p*H-dependent activity curve of the electrophoretically slow G6PD "Seattle-like" variants. Crosses = variant HHA2; circles = variant ATH3; triangles = G6PD Seattle; shaded area gives the normal range (15 subjects). Activity is expressed as percentage of the activity at *p*H 7.5.

Electrophoretically Fast Variants

Low erythrocyte G6PD activity with high electrophoretic mobility (about 110% compared with the B type) was observed among members of two hemophilic families from the island of Kephalonia (HHA1) and from Attica (HHA29). As in the case of family HHA2, these families were ascertained through hemophilia A patients (their pedigrees are shown in Fig. 5). In both pedigrees, the transmission of the defect is compatible with X-linked inheritance; there are no cases of males carrying both hemophilia and G6PD deficiency. The variant in HHA1 must have been transmitted from one branch to the other of the pedigree through the brother of I-1, 2, since both I-4 and I-6 have electrophoretically and quantitatively normal G6PD.

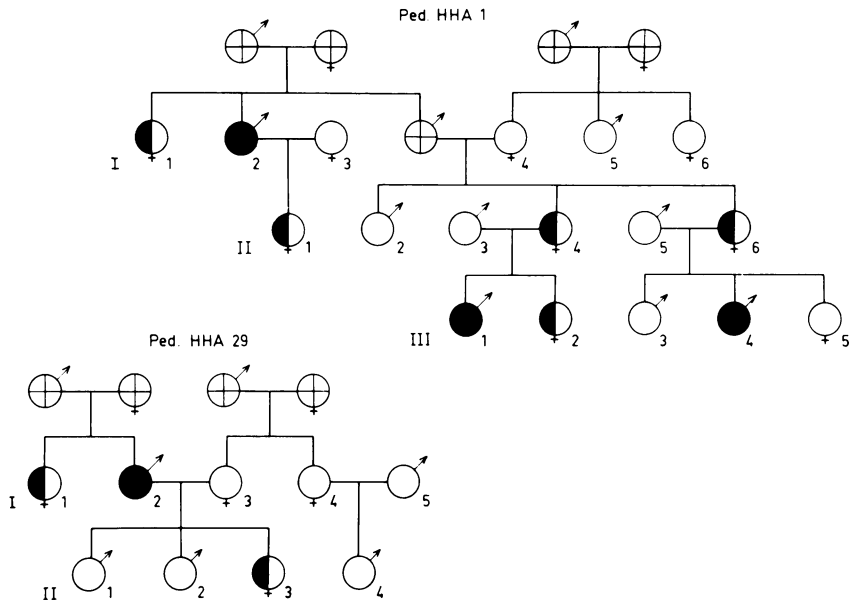

FIG. 5.—Families HHA1 and HHA29. Black = G6PD variants; crossed circles = dead

It can be seen from Table 3 that the red-cell enzyme activity is different for the two variants, being about 20% of normal in the affected males of HHA1 (I-2; III-1, 4) and about 50% of normal in the only affected male (I-2) of HHA29.

The electrophoretic mobility on cellulose acetate gel, *p*H 7.5 (see Fig. 6), is identical with that of type A in the case of HHA1, and intermediate between A and B in the case of HHA29. The relative mobilities of the enzymes were the same in crude hemolyzates and partially purified preparations.

All heterozygous females showed two bands of activity, well separated from each other in the case of HHA1, less so in the case of HHA29. The slower-moving band had always the mobility of B. The relative staining intensity of the normal and the variant G6PD bands varied slightly from case to case. On starch gel electrophoresis at *p*H 8.6 (not shown), no clear-cut difference was evident between both variants and G6PD A, all showing the same migration rate of about 110% of normal.

TABLE 3

RED-CELL G6PD ACTIVITY AND ELECTROPHORETIC MOBILITY IN HHA1 AND HHA29

Key to Pedigree	Dye Decol. Test (Minutes)	Red-Cell Activ. (IU/g Hb)*	Electrophoretic Mobility† (%)	G6PD Cellular Local. Test‡
HHA1:				
I-1, ♀	60	...	100, 110	...
2, ♂	145	1.45	... 110	77
3, ♀	40	...	...	...
4, ♀	35	5.98	100 ...	...
5, ♂	35	4.40	100 ...	...
6, ♀	40	...	100 ...	...
II-1, ♀	65	...	100, 110	...
2, ♂	35	5.03	100 ...	...
3, ♂	55	...	100 ...	...
4, ♀	75	3.04	100, 110	47
5, ♂	35	4.49	100 ...	...
6, ♀	125	1.86	100, 110	...
III-1, ♂	140	1.47	... 110	...
2, ♀	60	5.11	100, 110	30
3, ♂	35	5.51	100 ...	...
4, ♂	125	1.17	... 110	...
5, ♀	45	6.08	100 ...	0
HHA29:				
I-1, ♀	75	3.82	100, 105	56
2, ♂	155	3.08	... 105	69
3, ♀	45	...	100 ...	...
4, ♀	45	...	100 ...	...
II-1, ♂	45	5.50	100 ...	...
2, ♂	45	...	100 ...	...
3, ♀	45	4.77	100, 105	...

NOTE.—For normal values, see Table 1.

* One IU = the quantity of G6PD which reduces one micromole of NADP in one minute.

† On cellulose acetate gel *p*H 7.5. G6PD B = 100.

‡ Percentage of eluted cells.

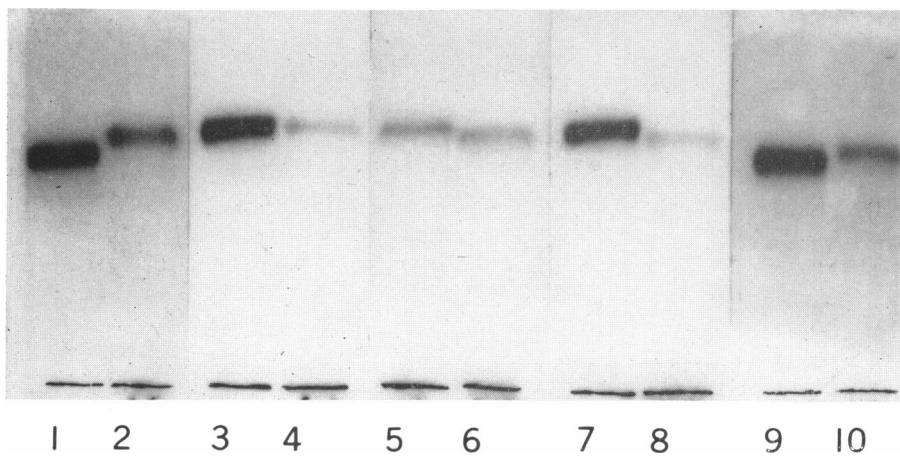

FIG. 6.—Cellulose acetate gel electrophoresis, *p*H 7.5, of the fast-moving G6PD variants. *1, 9* = G6PD B; *3, 7* = G6PD A; *2, 4, 5* = variant HHA1 (G6PD Kephalonia); *6, 8, 10* = variant HHA29 (G6PD Attica). Partially purified samples; migration toward the anode (top).

The results of the biochemical characterization on the partially purified enzymes of male subjects HHA1:I-2 and HHA29:I-2 are shown in Table 2. No significant differences were detected between the two variants and G6PD A and A−. Michaelis constants for NADP and G6P were also determined on smaller samples obtained from HHA1:III-1 and III-4 and were found to be similar to those of I-2. No effects of collection technique, shipping, or aging of the samples could be detected by using as a control a similarly handled blood sample from a normal subject, HHA23-3.

The thermal stability of the "fast-moving" Greek variants was found to be normal when studied by incubating the samples at 48.5° C in the presence of 2×10^{-5} M NADP as recommended by the WHO technical report (WHO, 1967). However, the study of thermal denaturation profiles showed the two variants to behave differently from G6PD A− (see Fig. 7). The transition temperatures of HHA1 and

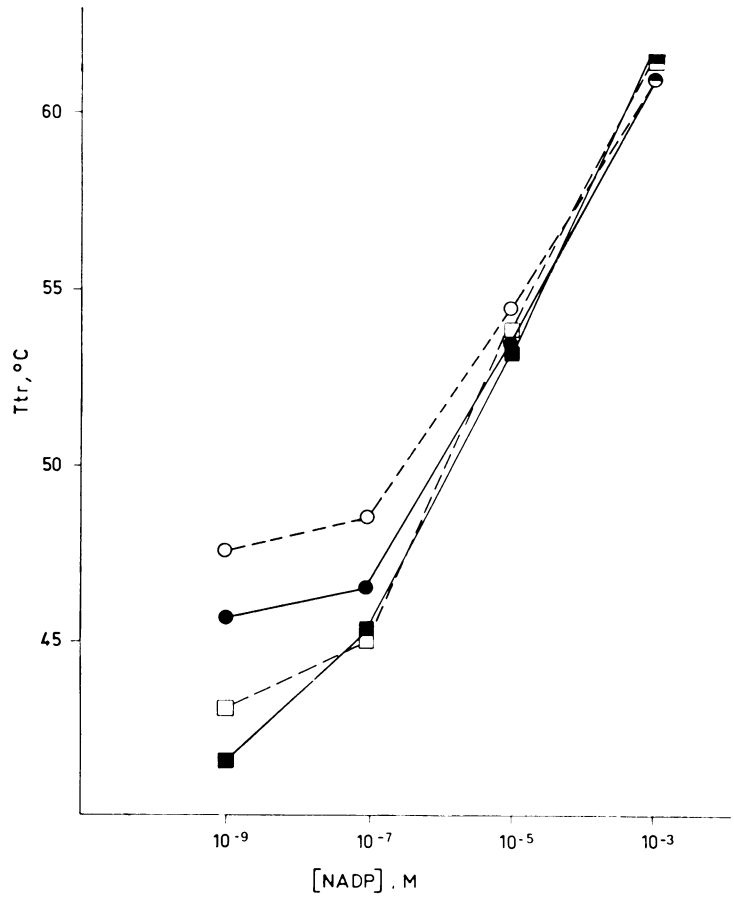

FIG. 7.—Transition temperatures (T_{tr}, see text for definition) of G6PD variants as a function of NADP concentration. Empty circles = G6PD A; black circles = G6PD A−; empty squares = variant HHA29 (G6PD Attica); black squares = variant HHA1 (G6PD Kephalonia).

HHA29 enzymes are, at low NADP concentrations, considerably lower than those of G6PD A−, but not very different from one another.

An even sharper differentiation of the two variants from G6PD A− and from one another could be obtained by ion-exchange chromatography on DEAE Sephadex. Under the experimental conditions employed, G6PD A− was eluted at 0.17 M KCl, whereas the variant enzymes of subjects HHA29:I-2 and HHA1:I-2 were eluted at higher concentrations of KCl, that is, 0.30 M and 0.42 M, respectively (Fig. 8). As mentioned under Methods, these results could only be obtained when DEAE Sephadex of the old type was used: with CM Sephadex or DEAE Sephadex in bead form, G6PD B, A, A− and the Greek variants were all eluted at approximately the same concentration of KCl (about 0.15 M) with slight variations from lot to lot of resin.

The sedimentation velocity in a sucrose gradient of variant HHA1 was not different from that of G6PD A.

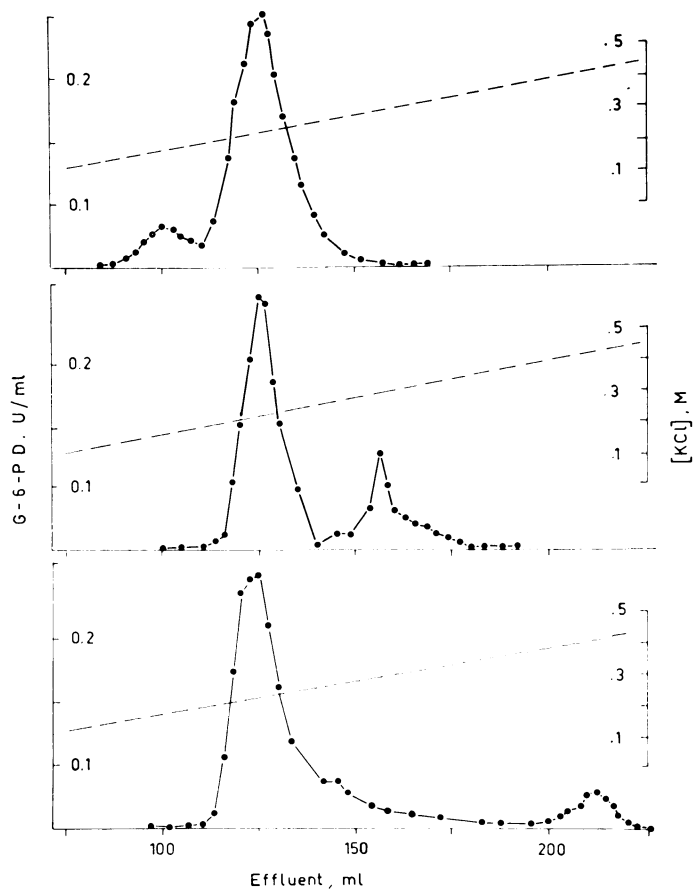

FIG. 8.—Elution diagrams of ion-exchange chromatography on DEAE Sephadex A50. Top = mixture of G6PD A and G6PD A−; middle = mixture of G6PD A and variant HHA29 (G6PD Attica); bottom = mixture of G6PD A and variant HHA1 (G6PD Kephalonia).

DISCUSSION AND CONCLUSIONS

Judging from the techniques employed, the slow-moving G6PD variants described in this paper (HHA2 and ATH3) have the same characteristics as G6PD Seattle (Kirkman *et al.*, 1965). In analogy with the name proposed for the variants encountered in Sardinia (Lenzerini *et al.*, 1969), we refer to them as G6PD "Seattle-like" (Greece). They resemble G6PD Athens (Stamatoyannopoulos *et al.*, 1967) but differ from it in electrophoretic mobility (starch gel, Tris-EDTA-borate buffer, *p*H 8.6) and in the rate of 2dG6P utilization. They are also different from G6PD Kerala (Azevedo *et al.*, 1968) which has a higher red-cell activity, a lower *Km* for NADP, and can be distinguished from G6PD Seattle in starch gel electrophoresis at *p*H 8.6 (Tris-EDTA-borate buffer). If the three examples of electrophoretically slow, partially deficient G6PD found among the 200 schoolboys in Kephalonia are identical with the "Seattle-like" (Greece) type, the gene frequency for this variant would be of the order of 0.015. Variants of G6PD with lower red-cell activity and lower electrophoretic mobility have been found in Greece by other authors (Stamatoyannopoulos *et al.*, 1967), but no details on their characterization are given. Clearly, only an analysis of the primary structure could decide whether these enzyme types are identical with each other and with G6PD Seattle. A relatively simple method for the purification and peptide mapping of G6PD from small amounts of blood is currently being developed with this aim in mind (Rattazzi, 1969).

Both the electrophoretically fast variants here described are distinct from all those previously reported. Variant HHA29 is very close to G6PD Canton (McCurdy *et al.*, 1966) and G6PD Markham (Kirkman *et al.*, 1968) but differs from them in red-cell activity, in the utilization of substrate analogues, and in thermostability. Variant HHA1 mimics almost all properties of the common African A— type, including marked deficiency in enzyme activity, and could not be distinguished from A— by "conventional" methods but was resolved from it by ion-exchange chromatography in columns or thin layer (see Luzzatto and Okoye, 1967) as well as by analysis of thermal inactivation profiles. In compliance with the recommendation of a WHO Study Group (WHO, 1967), we propose the names G6PD Attica for the HHA29 variant and G6PD Kephalonia for the HHA1 variant, from the places of origin of the two corresponding families. These two new variants must presently be regarded as rare ones; however, the occurrence of electrophoretically fast G6PD variants in Greece, associated with partial deficiency, has been mentioned by others (Stamatoyannopoulos *et al.*, 1967). When further details on their characterization are available, it will be possible to make some estimate of the relevant gene frequencies.

It is noteworthy that four different G6PD variants are now known, A, A—, Kephalonia, and Attica, which exhibit identical electrophoretic mobility on starch gel (G6PD Attica is slightly slower on cellulose acetate gel). This may indicate that the corresponding amino acid replacements do not involve any charge difference, an occurrence well documented, for example, in the case of the multiple G or D hemoglobin variants (see Dayhoff and Eck, 1968). All four variants can be resolved by the same chromatographic technique (Luzzatto and Allan, 1965) employing DEAE

Sephadex, from which they are eluted at characteristic and widely different KCl concentrations, ranging from 0.17 M for A— to 0.42 M for Kephalonia.

Among hemoglobins, those with uncharged substitutions are less likely to have different physicochemical properties than those with charged substitutions (J. Beetlestone, personal communication). Similarly, in the case of the four G6PD variants having the mobility of A, nearly all biochemical properties tested have proven identical. However, it is likely that a more refined analysis could reveal distinctive kinetic behavior, for instance in the response to inhibitors, or in the interactions with NADP and NADPH (see Luzzatto and Afolayan, 1968, and results to be published). In this connection, we should point out that no definitive analysis has yet been done, for Kephalonia and Attica, of the NADP saturation function at very low NADP concentration. We cannot state whether it is sigmoid shaped, as in the case of G6PD A (Luzzatto, 1967) or hyperbolic. Experiments on this point are in progress.

The identification of these two new variants has confirmed the usefulness and versatility of thermal denaturation profiles and of DEAE Sephadex chromatography for the detection of subtle structural differences among enzyme variants. It must be recognized that, because of the great sensitivity of these techniques, the results they yield may be critically influenced by changes in purification procedure, by storage conditions, by the presence of other "stabilizing" proteins, of minute amounts of salts, etc. It was found, for instance, that freezing at −20° C and subsequent thawing of dialyzed enzyme preparations could alter considerably the thermal denaturation profiles at low NADP concentrations (L. Lenzerini and M. C. Rattazzi, unpublished observations). Although no systematic study of the phenomenon was attempted, it was clear that meaningful results could only be obtained when controls and samples to be investigated were collected at the same time and processed in the same way.

As for ion-exchange chromatography, the new type of CM Sephadex in bead form does not give the same results as the old type of resin, no longer commercially available (A. Yoshida, personal communication). The same was found to be true for DEAE Sephadex in bead form, and repeated attempts to modify the techniques described to suit the new types of resin were unsuccessful (M. C. Rattazzi, unpublished data). Examples of a similar influence of the physical form of the ion-exchanger upon its selectivity have been reported by others (Peeters and Blaton, 1966).

SUMMARY

A number of variants of glucose-6-phosphate dehydrogenase electrophoretically different from the normal B type were found in unrelated Greek families. All of them were associated with incomplete enzyme deficiency.

The first variant, which we have named G6PD Kephalonia, although similar to G6PD A— in electrophoretic mobility, red-cell activity, and most of its biochemical and functional characteristics, was found to differ from G6PD A— by its thermal denaturation profile at low NADP concentrations and by its chromatographic behavior on DEAE Sephadex A50.

The second variant, which we have named G6PD Attica, was found to differ from G6PD A− and G6PD Kephalonia by its higher red-cell activity, slightly lower electrophoretic mobility on cellulose acetate gel (*p*H 7.5), and also by its thermal denaturation profile and chromatographic behavior on DEAE Sephadex.

Two other variants, for which the provisional name G6PD "Seattle-like" (Greece) is proposed, were undistinguishable from G6PD Seattle in all the characteristics studied.

Three more variants were found in 200 random samples; they were not fully characterized, but resembled G6PD Seattle in their electrophoretic mobility and red-cell activity. It is suggested that the frequency of G6PD "Seattle-like" might be appreciable in Greece and that further studies are needed to assess the homogeneity of this type of G6PD variants.

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