Enzymic Properties of Different Types of Human Erythrocyte Glucose-6-Phosphate Dehydrogenase, with Characterization of Two New Genetic Variants

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Abstract Enzymic properties have been compared in the following five genetic variants of glucose-6-phosphate dehydrogenase from human erythrocytes: the two common variants with normal activity, A and B; the common variant associated with enzyme deficiency, A⁻; and two new rare variants, "Ijebu-Ode" and "Ita-Bale."

The maximal velocity of the enzyme reaction (V_{max}) increases steadily with pH over the entire range explored (from pH 5.5 to 9.5) for all enzyme variants when buffers are used that show no specific ion effects on enzyme activity. Small differences are found among the variants in the pH range 7.5–8.2, where A and B show a "peak and trough," while A⁻, "Ijebu-Ode," and "Ita-Bale" exhibit a plateau.

When the effects of reagents that bind to sulphydryl groups are compared, iodoacetate, bromoacetate, and iodoacetamide are weak inhibitors, while *N*-ethylmaleimide (NEM) and hydroxymercuribenzoate (HMB) are potent inhibitors. The last two reagents have differential inhibitory action on different variants; one of these, "Ijebu-Ode," is strikingly resistant to HMB and totally resistant to NEM (up to 3 mmoles/liter).

The enzyme inactivation as a function of temperature exhibits distinctive profiles for all variants examined.

Both of the new variants described differ significantly from the normal B type in several respects: "Ijebu-Ode" in electrophoretic mobility, thermostability, dependence of V_{max} on pH, and resistance to sulphydryl group reagents; "Ita-Bale" in electrophoretic mobility, Michaelis constant (K_m) for glucose-6-phosphate, and dependence of V_{max} on pH. When these data are compared with those available in the literature, both variants are different from all those previously described. The estimated frequencies of the corresponding genes in western Nigeria are between 0.0005 and 0.0025 for "Ijebu-Ode" and less than 0.0005 for "Ita-Bale".

The A⁻ variant, compared to A, has a distinctly higher K_m for 2-deoxyglucose-6-phosphate and is more inhibited by very low concentrations of HMB. These are the first observed differences in kinetic properties between A and A⁻.

Received for publication 5 February 1968.

INTRODUCTION

An increasing number of genetic variants of glucose-6-phosphate dehydrogenase (glucose-6-phosphate: nicotinamide adenine dinucleotide phosphate [NADP] oxidoreductase, EC 1.1.1.49) is being detected in red cells of human subjects of different populations (1). It is well known that some of the variants are associated with low enzyme activity, whereas others are not. It is not yet clear what factors have allowed three of these variants,[1] A, B, and A⁻, to achieve polymorphic

[1] We have followed the nomenclature recommended in reference 1.

frequencies in various populations, whereas numerous other variants are encountered only sporadically. Some authors have searched for correlations between the frequency of some of the common variants and the incidence of particular diseases, such as malaria (2) or heart disease (3). As an alternative approach, it is possible that a detailed analysis of the enzymic properties of a series of variants might reveal differences which could be of physiological significance in the metabolism of the erythrocyte.

In the present paper, the three common variants, A, B, A^-, and two newly described variants, "Ijebu-Ode" and "Ita-Bale," are compared to each other with respect to their Michaelis constants, (K_m), thermostability, activity/pH dependence, and effect of sulphydryl group reagents.

METHODS

Subjects studied. Enzyme preparations were all from normal male blood donors.

Glucose-6-phosphate dehydrogenase "typing." This was performed by starch-gel electrophoresis in Tris borate–ethylenediaminetetraacetate (EDTA) buffer, pH 8.5, according to the method of Porter, Boyer, Watson-Williams, Adam, Szeinberg, and Siniscalco (4), with the following modifications: (*a*) the concentration of NADP in the gel was 10 μmoles/liter; (*b*) 10 μM NADP was also added to the cathodic buffer reservoir; (*c*) the electric field was 15 v/cm, the current 1.5 mamp/cm, and the duration of the run 5 hr. After the gels were stained, A, B, and A^- variants can be easily recognized by visual inspection. The two new variants to be described were detected by this technique as single bands having an electrophoretic mobility lower than the B variant (see Fig. 1).

Partial purification of enzymes. Hemolysates were processed according to Chung and Langdon (5) through the first ammonium sulfate precipitation. At this point the specific activity was between 0.1 and 0.2 U/mg for the nondeficient variants, and about 0.02 U/mg for A^-. Variants A, B, and A^- were further purified about 50-fold by gel filtration on Bio-Gel P-300 and ion-exchange chromatography on diethylaminoethyl (DEAE)-Sephadex as previously described (6).

Enzyme assays. The assay mixture contained 0.05 M Tris-borate buffer, pH 8.0, 4 mM glucose-6-phosphate (G6P), and 0.3 mM NADP in a final volume of 0.3 ml. Spectrophotometric readings were taken at 30-sec intervals in a 1 cm light path cuvette at 340 nm, at a room temperature of 24 ± 2°C.

Kinetic constants. For G6P and 2-deoxyglucose-6-phosphate, enzyme velocity measurements were carried out in a mixture similar to that described in the preceding paragraph, but at pH 7.8, with 0.5 mM NADP and variable concentrations of the other substrate. In order to determine the K_m for G6P the reaction velocity was measured at eight different concentrations, ranging from 0.015 to 6 mmoles/liter. For 2-deoxyglucose-6-phosphate the reaction velocity was measured at four different concentrations, ranging from 6 to 48 mmoles/liter. For NADP, enzyme velocity measurements were carried out as above, except that the concentration of G6P was held constant at 5 mmoles/liter, and the concentration of NADP was varied between 8 and 300 μmoles/liter (eight different concentrations). It has been shown for the A variant that under these conditions only the "high affinity" binding constant for NADP is being measured (7). The values of K_m for NADP usually reported in the literature lie close to this constant.

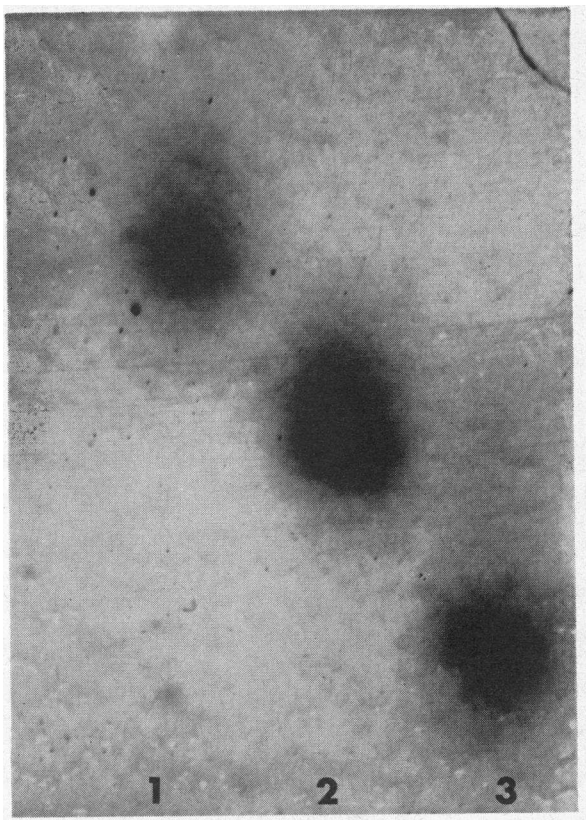

FIGURE 1 Starch-gel electrophoresis of erythrocyte glucose-6-phosphate dehydrogenase type B, "Ijebu-Ode," and "Ita-Bale." The gel is stained for enzyme activity. The origin is at the bottom (not shown). The migration of B was 8 cm. Experimental conditions are described in the Method section. Track 1, B; track 2, "Ijebu-Ode;" track 3, "Ita-Bale."

Maximal velocity (V_{max}) as a function of pH. In order to obtain the true relationship between V_{max} and pH it was necessary to insure that three conditions be satisfied: (*a*) The complete incubation mixture must retain, at each pH and throughout the reaction time, the pH of the buffer used in its preparation. (*b*) The concentrations of each substrate must be saturating the en-

zyme at each pH. (*c*) The ions used in order to adjust the buffer to the desired pH values must not affect the enzyme velocity in any other way than by changing the hydrogen ion concentration.

When the amount of buffer added to the reaction mixture is such as to yield a final concentration of about 0.03 moles/liter, and each of the other components of the reaction mixture has a pH of 7.5, it is found that the pH of the complete reaction mixture deviates from the pH of the buffer. For instance, when the pH of the buffer was 8.8, the pH of the mixture was 8.2; when the pH of the buffer was 9, the pH of the mixture was 8.7. Furthermore, in the course of the reaction acid is produced, and the pH in the first 3 min may drop further by 0.1–0.2 pH units. However, if the final concentration of buffer is about 0.2 moles/liter, and the pH of the G6P solution is preadjusted to the same pH of the buffer, no appreciable pH change is observed. Thus, 14 different solutions of G6P were prepared and adjusted to the desired pH values between 5.5 and 9.5 by addition of 0.5 M boric acid or 1 M Tris as required. In order to measure enzyme velocity, at each pH point the buffer and the G6P solution having that pH were employed. At each point, the pH was checked immediately after preparing the complete reaction mixture and 3 min afterwards. In no case was the deviation from the nominal pH more than 0.05 pH units.

Substrate saturation was checked at the extreme pH values, 5.5 and 9.5. It was found that in excess of G6P (12 mmoles/liter) 95% saturation was reached at 0.33 mM NADP. In excess of NADP (0.5 mmoles/liter) 95% saturation was reached at 10 mM G6P. These substrate concentrations were therefore used throughout the whole pH range.

Work with yeast glucose-6-phosphate dehydrogenase has shown that several anions activate and several cations inhibit the enzyme (8, 9). Tris and boric acid were found to have no such effect. Tris-borate buffer was therefore chosen for our experiments, and used in the pH range from 7.0 to 8.5. With this buffer the activity of the erythrocyte enzyme was the same when the buffer concentration was varied over a 31-fold range (from 8 to 250 mM Tris and from 9 to 280 mM borate at a constant pH of 8.1). For the pH points below 7.0, citric acid was added to the Tris-borate buffer. In order to insure that citrate had no effect on the enzyme activity, a portion of a solution of Tris-borate, pH 7.0, was brought to pH 5.5 with citric acid and then back to 7.0 with Tris. The reaction velocity was then measured in the remaining portion of the Tris-borate buffer and in the Tris-borate-citrate buffer, both at pH 7.0, and found to be the same. For the pH points above 8.5, triethylamine was added to the Tris-borate buffer, and a similar experiment was performed in order to insure that this base had no effect on enzyme activity.

In summary, the buffers used were as follows: pH 7.0–8.5: Tris-borate, prepared by adding 1M Tris to 0.5 M boric acid until the desired pH is obtained; pH 5.5–6.5: Tris-borate-citrate, prepared by adjusting the pH of a Tris-borate solution (pH 7.0, see above) with 0.5 M citric acid; pH 8.8–9.5: Tris-borate-triethylamine, prepared by adjusting the pH of a Tris-borate solution (pH 8.5, see above) with 0.2 M triethylamine.

Thermostability studies. The rate of enzyme inactivation at specified temperatures was determined as described by Kirkman, Rosenthal, Simon, Carson, and Brinson (10). The temperature dependence of inactivation ("melting curve") was studied as previously described (6).

Effect of sulphydryl group reagents. Fresh neutralized solutions of hydroxymercuribenzoate (HMB), *N*-ethylmaleimide (NEM), iodoacetic acid, bromoacetic acid, and iodoacetamide were added directly to the reaction mixtures, unless otherwise stated, to the final concentrations indicated in the Results section.

Reagents. Starch was from Connaught Lab., Toronto, Canada; G6P and NADP were from Boehringer, Mannheim, West Germany; nitro-blue tetrazolium was from Sigma Chemical Co., St. Louis, Mo.; phenazine methosulfate was from Light, Colnbrook, England; DEAE-cellulose was from H. Reeve Angel & Co., Clifton, N. J.; DEAE-Sephadex was from Pharmacia, Uppsala, Sweden; Bio-Gel P-300 was from Bio-Rad, Richmond, Calif.; HMB was from Calbiochem, Los Angeles, Calif.; NEM, iodoacetic acid, bromoacetic acid, and iodoacetamide were from Fluka, Buchs, Switzerland.

RESULTS

Identification of two new variants. The two variants, electrophoretically slower than B (see Fig. 1), were detected while normal male blood donors were screened. They are provisionally designated as "Ijebu-Ode" and "Ita-Bale," from the respective places of origin of the two donors, according to the World Health Organization recommendations on nomenclature (1). The "Ita-Bale" enzyme type was also found in one male sib and, together with type B, in the mother of the propositus. No relatives of the "Ijebu-Ode" donor were available for study. The electrophoretic abnormality was consistently observed when this donor was tested on three successive occasions over a 2 yr interval. The enzyme activity of crude hemolysates from the donors of both new variants was within the normal range. The amount of blood available for enzyme purification was 300 ml for "Ijebu-Ode" and 420 ml for "Ita-Bale."

Kinetic constants. In the experimental conditions described in the Methods section the K_m for G6P for all enzyme variants was found to fall between 26 and 90 μmoles/liter, and for 2-deoxyglucose-6-phosphate between 16 and 105 mmoles/liter (see Table I). On repeated determinations on different preparations of A, B, and A⁻ the ex-

TABLE I

*Michaelis Constants (K_m) of Glucose-6-Phosphate (G6P) Dehydrogenase Variants**

Preparation	K_m for G6P	K_m for 2-deoxy-glucose-6-phosphate	K_m for NADP
	μmoles/liter	*mmoles/liter*	*μmoles/liter*
A	50	16	13
A	32	27	20
B	40	53	14
B	48	41	12
A⁻	26	91	8
A⁻	40	105	14
"Ijebu-Ode"	60	—	24
"Ita-Bale"	91	25	11

NADP, nicotinamide adenine dinucleotide phosphate.
* Experimental conditions are described in the Methods section.

perimental variability was found to be ± 30%. Therefore, the deviation from normal of the K_m for G6P of "Ijebu-Ode" may not be significant, but the K_m of "Ita-Bale" is definitely increased. In the case of 2-deoxyglucose-6-phosphate it was found that A⁻ has a higher K_m than all the other variants, which all show values not differing appreciably among them. The K_m values for NADP fall within a narrow range for all variants examined. It should be pointed out that all kinetic constants were measured in the absence of Mg^{++}.

Heat inactivation. (see Fig. 2) The rate of inactivation of "Ijebu-Ode" and "Ita-Bale" was measured at 37°C and compared with that of the B variant at the same temperature. "Ita-Bale" had a minimally increased thermolability, "Ijebu-Ode," a markedly increased thermolability. The time at which 50% of the activity was lost was 85 min for B, 72 min. for "Ita-Bale" and 30 min for "Ijebu-Ode." The time course of inactivation of a mixture of "Ita-Bale" and B was, as expected, intermediate between those of the two separate enzymes. In the case of "Ijebu-Ode," however, the mixture was less labile than expected from the results with the two separate enzymes, presumably because of protection of "Ijebu-Ode" by some unknown impurity in the preparation of B.

The dependence of enzyme inactivation on temperature and NADP concentration was also studied as previously described (6) (see Fig. 2). "Ijebu-Ode" shows a marked "shift to the left" in inactivation profiles, especially at low NADP concentrations. The values of the transition temperature (the temperature at which 50% of the activity is lost) are given for purposes of comparison for all five variants in Table II. It is seen that the range of transition temperatures for all variants is about 2°C in 10^{-3}M NADP, but it increases as the concentration of NADP is decreased, up to 14.5°C in 10^{-9}M NADP.

Maximal velocity as a function of pH. For all variants examined, there is an over-all steady increase of V_{max} with pH over the entire range explored from 5.5 to 9.5 (Fig. 3). However, whereas A⁻, "Ijebu-Ode," and "Ita-Bale" have a plateau in the pH region between 7.5 and 8.2, A

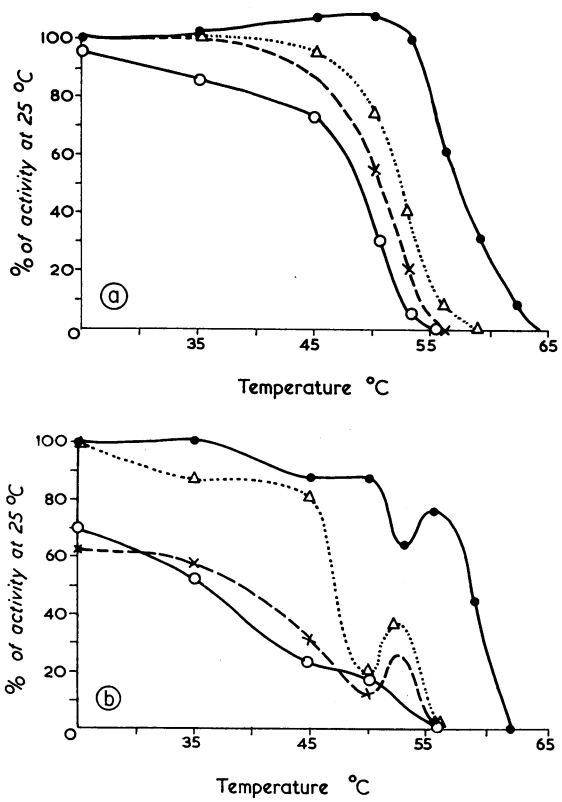

FIGURE 2 Heat inactivation of glucose-6-phosphate dehydrogenase variants at various nicotinamide adenine dinucleotide phosphate (NADP) concentrations. Aliquots of each enzyme preparation were heated for 7 min at different temperatures in 5 mM phosphate buffer, pH 6.9, 50 mM KCl, 0.1 mM ethylenediaminetetraacetate (EDTA), and NADP at the specified concentrations. After chilling on ice assays were carried out at room temperature. (*a*) "Ita-Bale;" (*b*) "Ijebu-Ode." ●——●, 10^{-3} M NADP; △ · · · △, 10^{-5} M NADP; × – – – ×, 10^{-7} M NADP; ○——○, 10^{-9} M NADP.

TABLE II

*Transition Temperature of Enzyme Inactivation as a Function of NADP Concentration**

Enzyme type	NADP concentration			
	10^{-3} M	10^{-5} M	10^{-7} M	10^{-9} M
	Transition temperature			
	°C			
A	59	53.5	51.1	50.5
A⁻	59	52.5	48	47.5
B	58.5	51.5	44	43
"Ijebu-Ode"	58	47.5	37	36
"Ita-Bale"	57	52	48	50

NADP, nicotinamide adenine dinucleotide phosphate.

* Transition temperature is defined as the temperature at which 50% of the enzyme is inactivated under the conditions of the experiment. For technique see the Methods section and legend to Fig. 2. Data for A and A⁻ are from reference 6, for B from Rattazzi, Lenzerini, and Luzzatto (unpublished), and for "Ijebu-Ode" and "Ita-Bale" from this paper.

and B lack this plateau and have a "peak and trough" in the same region. The activity/pH curves obtained by the technique of Kirkman et al. (10) are shown for the two new variants in Fig. 4. Here "Ita-Bale" conforms to the "truncate" pattern known for the three common variants A, B, and A⁻ (11), whereas "Ijebu-Ode" shows a "biphasic" pattern, slightly different from that observed with Canton (12) and with the Mediterranean variant (13).

Inhibition by sulphydryl group reagents. Among thiol-alkylating agents, iodoacetic acid, bromoacetic acid, and iodoacetamide all have a mild, time-dependent inhibitory action on the enzymes studied. For example, after 3 hr of preincubation with iodoacetamide, the inhibition is 15–30% at 10 mmoles/liter and 5–15% at 2 mmoles/liter. With these three reagents no significant differences were observed among the five variants in either the time course or the extent

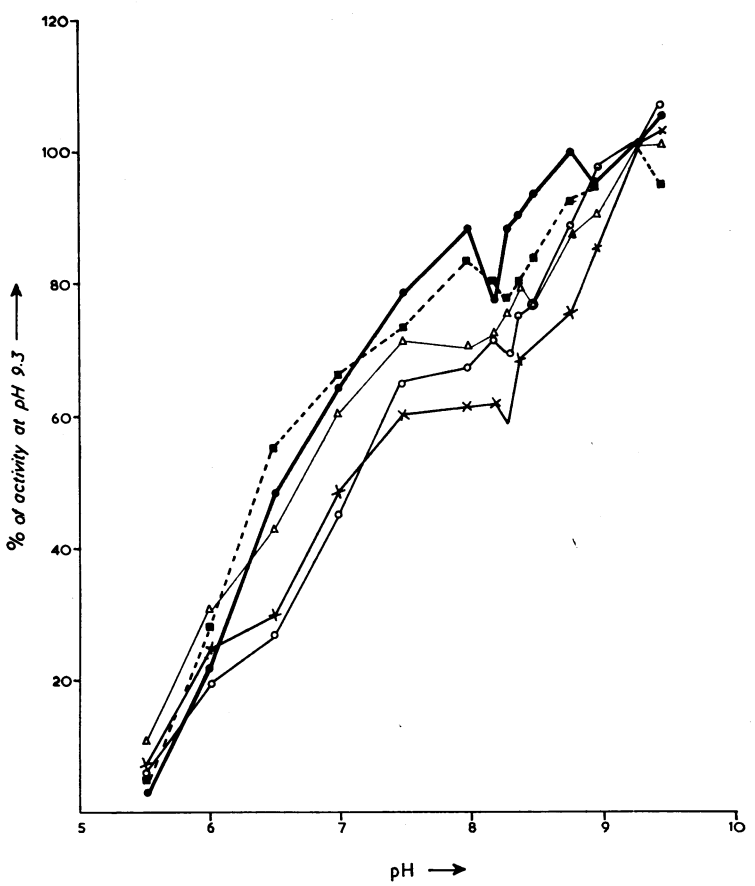

FIGURE 3 Dependence of V_{max} from pH for glucose-6-phosphate dehydrogenase variants. For buffers and substrate concentrations employed see Methods section. ●——●, A; △——△, A⁻; ■ · · · ■, B; ×——×, "Ita-Bale;" ○——○, "Ijebu-Ode."

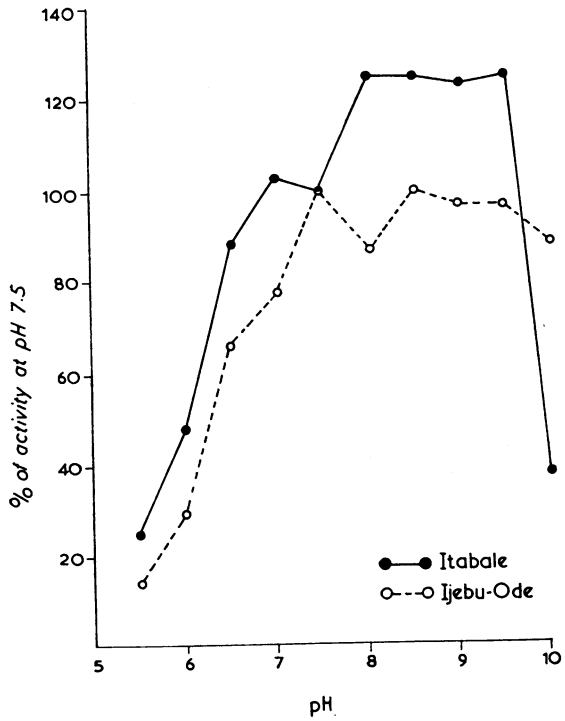

FIGURE 4 Activity/pH curves in tris-glycine-phosphate buffer, adjusted to desired pH with NaOH or HCl, according to Kirkman, Rosenthal, Simon, Carson, and Brinson (10).

of inhibition. On the other hand, NEM is a more powerful inhibitor. Here the action is instantaneous, and a striking difference is found between the inhibition profiles for "Ijebu-Ode" and for the other four variants (Fig. 5).

HMB, a mercaptide-forming reagent, had already been found to be a potent inhibitor of red cell glucose-6-phosphate dehydrogenase (14, 15). The inhibition is manifest within seconds after the addition of the reagent to the reaction mixture. The effect of relatively high concentration of HMB on enzyme activity again sharply differentiates between "Ijebu-Ode," the most resistant variant,[2] and the other four that have been examined (Fig. 6). At much lower concentrations a finer analysis is possible; this analysis was carried out for the A and A⁻ variants (Fig. 7) on more

[2] The high resistance of "Ijebu-Ode" to these inhibitors was not due to protection by a protein impurity present in the enzyme preparation, as shown by an experiment in which the inhibition observed with a mixture of "Ijebu-Ode" and B was the arithmetical mean between the inhibition effects with the two separate enzymes.

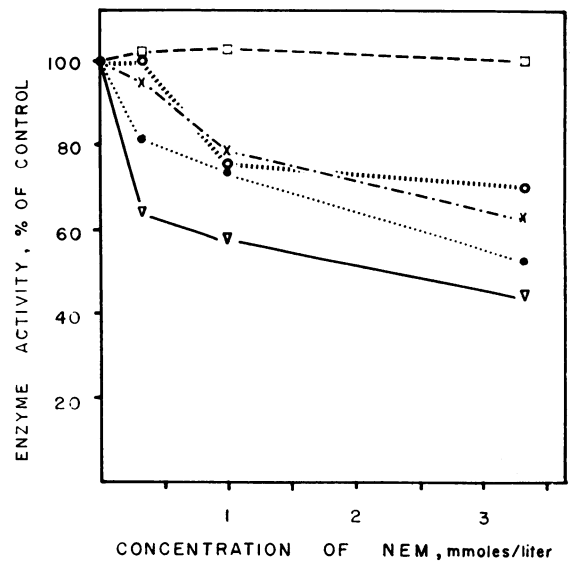

FIGURE 5 Inhibition of glucose-6-phosphate dehydrogenase variants by *N*-ethylmaleimide (NEM). Experimental conditions are the same as described under "Methods" section, except that buffer was 20 mM Tris-HCl, pH 8.6, containing 20 mM $MgSO_4$. The rate of the uninhibited reaction for all variants was 0.08 ΔA_{340}/min. ×–·–·–×, A; ▽——▽, A⁻; ● · · · ●, B; □– – –□, "Ijebu-Ode;" ○ · · · ○, "Ita-Bale."

highly purified preparations (30 and 2.5 U/mg, respectively). It is found that A⁻ is significantly more susceptible to HMB inhibition than A.

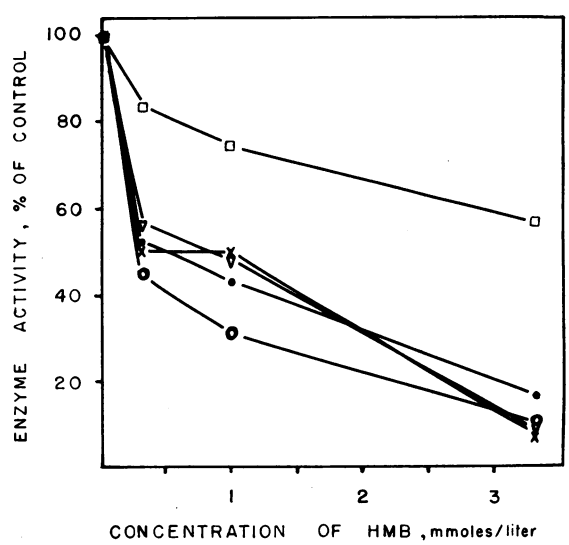

FIGURE 6 Inhibition of glucose-6-phosphate dehydrogenase variants by hydroxymercuribenzoate (HMB). Conditions and identity of variants are the same as from Fig. 5.

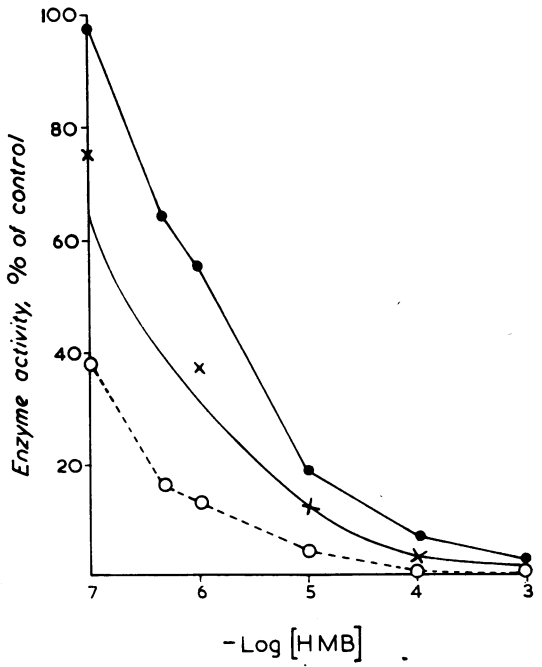

FIGURE 7 Inhibition of glucose-6-phosphate dehydrogenase variants A and A⁻ by low concentration of HMB. Here the enzyme preparations were first incubated for 15 min at 22°C with various concentrations of HMB in 5 mM phosphate buffer, pH 6.9, 50 mM KCl, 0.1 mM EDTA, and 1 μM NADP. Enzyme activity was then measured in the usual assay system. HMB concentrations shown on abscissa are the concentrations in the assay mixture. Concentrations during preincubation were 2.3-fold higher at each point. ●——●, A; ○- - -○, A⁻; ×, mixture of A and A⁻, experimental points. The middle full line indicates the calculated inhibition curve for the mixture if there is no interaction between the two preparations.

DISCUSSION

pH Dependence of normal B enzyme. The findings on the relation between enzyme activity and pH are somewhat unexpected, since the curves obtained (Fig. 3) differ considerably from those previously reported (11, 16). There are probably two reasons for the discrepancy: (*a*) the choice of buffers which do not affect enzyme activity through the action of ions other than H^+, and (*b*) the choice of substrate concentrations sufficient to reach maximal velocity even at extreme pH values (see Methods section). Thus, the data in Fig. 3 are true plots of V_{max} vs. pH. Kirkman, Schettini, and Pickard (13) have already discussed how nonsaturating substrate concentrations influence the enzyme activity pH curves. The inhibitory action of Na^+ (described for the yeast enzyme [9]) is also likely to have affected substantially the shape of previously reported curves in the alkaline range. If this ion is replaced by a noninhibitory organic cation such as triethylamine, we find that V_{max} increases almost linearly with pH over the entire range tested, suggesting that negative charges on one or both of the substrates strongly favor the activity of the enzyme. An attempt to interpret the plots of V_{max} vs. pH in terms of ionization of specific groups in the enzyme molecule would be premature at this stage but may become feasible when more complete data are obtained on the pH dependence of K_m (experiments in progress).

Identity and frequency of new variants. If the two new variants here described are compared with a comprehensive table of previously described ones (1), it appears certain for "Ijebu-Ode" and likely for "Ita-Bale" that they differ from all of them. "Ijebu-Ode" resembles "Baltimore-Austin" in some respects, but its thermostability (Fig. 2 and Table II), its activity/pH curve (Fig. 4), and its behavior with respect to sulphydryl group reagents are very distinctive (Figs. 5 and 6), although not all of these properties have been similarly tested for Baltimore-Austin. "Ita-Bale" resembles Ibadan-Austin but has normal activity in the crude lysate and an abnormally high K_m for glucose-6-phosphate (Table I), resembling in this respect the Barbieri (17) and Oklahoma (18) variants (though it is clearly distinct from them by other criteria). For both "Ijebu-Ode" and "Ita-Bale" the curves of V_{max} vs. pH (Fig. 3) are distinctive.

In the course of an electrophoretic analysis of hemolysates from approximately 2000 unrelated male subjects, the "Ita-Bale" variant was encountered only once, whereas a band having the electrophoretic mobility of "Ijebu-Ode" was observed five times. Since only one of the five has been fully characterized, we do not know whether the others were biochemically identical to "Ijebu-Ode" or not. Therefore we can only give upper estimates for the gene frequencies in the western Nigerian population, and these are less than 0.0005 for "Ita-Bale" and between 0.0005 and 0.0025 for "Ijebu-Ode;" both of these are below polymorphic frequencies. All the subjects with such electrophoretically slow-moving variants of red

cell glucose-6-phosphate dehydrogenase were clinically and hematologically normal.

Kinetic differences between A and A⁻. It has been shown previously in this laboratory that the A and A⁻ variants of glucose-6-phosphate dehydrogenase have different heat inactivation profiles and can be resolved by column chromatography on DEAE-Sephadex (6). Confirmatory results have been obtained by Yoshida, Stamatoyannopoulos, and Motulsky (19). Nevertheless, no difference in kinetic properties has previously been detected between the two variants. For example, we have found, in agreement with previous investigators (20, 21), that the Michaelis constants for G6P and NADP respectively are very similar. On the other hand, the Michaelis constants for 2-deoxyglucose-6-phosphate are different (Table I). This difference has probably been made apparent by the assay conditions employed in this work. For instance, we have chosen a buffer, Tris-borate, which does not per se affect the activity of the enzyme (see Methods section), and we have omitted from the reaction mixture Mg^{2+}, which inhibits the binding of substrates with both the yeast enzyme (22) and the human red cell variants A and B (Luzzatto, unpublished data).

A further distinction in kinetic properties between A and A⁻ was revealed by the sulphydryl group reagents: A⁻ is much more sensitive than A to inhibition by HMB (Fig. 7). The HMB concentration giving 50% inhibition was less than 0.1 μmoles/liter for A⁻ and about 2 μmoles/liter for A. Thus, these two variants are distinct not only in their physicochemical properties (6, 19, 23) and in their rate of inactivation in the red cells in vivo (19, 24), but also in their enzymic properties. The findings with HMB suggest that the structural abnormality of A⁻ may lie at or near a cysteine residue.

Comparison of the five variants examined. A number of properties of erythrocyte glucose-6-phosphate dehydrogenase, such as electrophoretic mobility, Michaelis constants, etc., have been well established over the past few years as useful for the characterization of its genetic variants (25). From the data above reported, it appears that three of the techniques employed in the present work can be used successfully for the same purpose and are sometimes complementary to previous ones, in the sense that they may detect differences between otherwise indistinguishable variants: (*a*) the transition temperature for the thermal inactivation, as a function of the concentration of NADP, which shows a different pattern for each of the five variants (Table II); (*b*) The inhibition by sulphydryl group reagents (high resistance to HMB and NEM [Figs 5 and 6] represents the most distinctive feature of the "Ijebu-Ode" variant); (*c*) The pH dependence of enzyme activity, under conditions where a true V_{max} is determined (see Methods section). We note for instance that while by the "standard" technique (Fig. 4) the activity/pH curve for "Ijebu-Ode" does not differ significantly from that of the normal B type, the curve of V_{max} vs. pH (Fig. 3) for the same enzyme has a plateau between pH 7.5 and 8.2, which is not found with the B type. (By contrast, "Ita-Bale" has pH curves which are clearly different from B by both techniques.)

In addition to helping in the characterization and classification of enzyme variants, a comparison of their properties can serve a more important purpose. It can contribute to our understanding of how the enzyme operates and possibly of what might be the selective advantage, if any, of particular variants. Genetic evidence is consistent with the idea that all structural variants of human erythrocyte glucose-6-phosphate dehydrogenase described thus far are controlled by allelic genes at a single X-linked locus (2, 26). Of, course, it is conceivable that a subunit of the enzyme molecule might be under the control of another (autosomal) gene, but if this is so, no polymorphism at this second hypothetical locus has yet been discovered. In addition, there is no convincing evidence for the existence of a separate nonstructural gene controlling the rate of synthesis of the enzyme, mutated in subjects with deficiency of glucose-6-phosphate dehydrogenase. Thus, all characterized variants with enzyme deficiency have revealed one or more distinctive properties, indicating the presence of a structural abnormality in each case. This applies to the common Mediterranean variant (13, 27), to the common A⁻ variant (6), and to the common Chinese variant (12), as well as to some of the rare variants (10, 18, 28, 29). Regarding the nature of the structural abnormality, a single amino acid substitution is, of course, the most likely and attractive possibility.

This has been recently proven by Yoshida (30) for the difference between the common A and B variants and is likely to be true for most variants, except possibly for those with "complete" deficiency (31). If most variants turn out to represent single amino acid replacements, further study will offer the possibility to investigate, for a human enzyme, the dependence of enzymic properties from specific and localized changes in its primary structure.

Until more structural data become available, we can only try the reverse approach, namely to infer from comparative data on the known variants some features of special regions of the enzyme molecule. For instance, it was already clear from previous data (1) that in a number of variants a relatively high rate of activity with 2-deoxyglucose-6-phosphate is associated with a low K_m for G6P, suggesting that in the binding of substrate an increase in affinity parallels a decrease in specificity. Further, the action of HMB and NEM suggests that either sulphydryl groups are important as such for the activity of the enzyme, or that the attachment of these reagents to the enzyme molecule interferes with activity, by steric hindrance of substrate binding, for instance.[3] Whereas enzyme inhibition per se does not enable us to choose between these two alternatives, the fact that "Ijebu-Ode" is much less inhibited than the other variants (Figs. 5 and 6) favors the second one. Indeed, if a cysteine residue, essential for activity, were lacking by mutation in "Ijebu-Ode," the activity of this enzyme should be reduced; it is not. On the other hand, if in this variant a cysteine residue is lacking that is not essential for activity, but which in the other variants serves as an attachment site for HMB and NEM, then "Ijebu-Ode" would be expected to resist the effect of these reagents. Finally, the differences in thermostability, already observed by several investigators, (21, 11, 27), can be made sharp enough when inactivation profiles are obtained at low NADP concentrations to enable us to visualize a critical region in the enzyme molecule where the secondary-tertiary structure of the polypeptide chain is strongly stabilized by NADP. If mutation causes a change in that region, either the binding of NADP or the stability of the structure may be affected, and this will result in "melting out" of the structure at subnormal temperature. From the data in Table II (especially the last column) it appears that different mutations cause different degrees of destablization of the hypothetical critical region of the molecule.

[3] The finding by Chung and Langdon (15) that preincubation with high concentration of NADP protects the enzyme from HMB inhibition appears compatible with either interpretation.

ACKNOWLEDGMENTS

We thank Dr. H. McFarlane and Professor G. M. Edington for encouragement in the course of the work and Mr. V. C. N. Okoye and Mr. A. Usifor for technical assistance. Dr. David Schlessinger and Dr. M. J. Schlesinger offered valuable advice in revising the manuscript. This work received financial support from the World Health Organization.

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