Genetic Variants of Human Erythrocyte Glucose 6-Phosphate Dehydrogenase. II. *In Vitro* and *in Vivo* Function of the A⁻ Variant*

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ABSTRACT: The dependence of reaction rate on the [$NADP^+$]/[NADPH] ratio was determined in the three genetic variants of glucose 6-phosphate dehydrogenase A, B, and A⁻. As expected from previous kinetic data, it was found that at low [$NADP^+$]/[NADPH] ratio (in the likely physiological range), the rate of the enzyme reaction, relative to V_{max}, was higher in A⁻ than in A and B. The rate of intracellular glucose 6-phosphate dehydrogenase activity was determined as a function of methylene blue concentration, by measuring [^{14}C]CO_2 production from [1-^{14}C]glucose in suspensions of erythrocytes having the same three enzyme variants. In the absence of methylene blue all types of erythrocytes behaved alike. With increasing concentrations of the dye, the rate of CO_2 production increased sigmoidally in erythrocytes of type A and B, but hyperbolically in erythrocytes of type A⁻. This behavior mimics that of the corresponding purified enzymes *in vitro*. It is concluded that the previously proposed regulation of glucose 6-phosphate dehydrogenase activity consisting in a $NADP^+$-induced transition of enzyme from a state of low affinity to a state of high affinity for this substrate actually operates in the intact cell. Moreover, the variant A⁻, because it undergoes the transition at a lower $NADP^+$ concentration, operates physiologically at a rate nearer saturation, compared to the other variants. This compensates considerably for the reduction in total enzyme activity characteristic of erythrocytes with the A⁻ type of enzyme, and may explain why their biological properties are almost normal under ordinary environmental conditions.

Deficiency of erythrocyte glucose 6-phosphate dehydrogenase in man is associated with a number of clinical manifestations, the best characterized of which is drug-induced acute hemolytic anemia (reviewed in Tarlov *et al.*, 1962). Although its detailed mechanism is not fully worked out, hemolysis results undoubtedly from the limited capacity of glucose 6-phosphate dehydrogenase deficient red cells to increase NADPH[1] production when challenged by an oxidative agent (such as the drug itself, or a metabolite or by-product thereof). On the other hand, it is not clear why, in the absence of exogenous agents, the metabolism and life span of the same "deficient" cells is paradoxically almost normal (Tarlov *et al.*, 1962).

We have previously demonstrated that the glucose 6-phosphate dehydrogenase of deficient erythrocytes (now referred to as the A⁻ type: WHO, 1967) is not only quantitatively, but also qualitatively different from the normal types, A and B (Luzzatto and Allan, 1965). In the preceding paper (Afolayan and Luzzatto, 1971) it has been further shown that the different structure of A⁻ entails a distinctive pattern of its interaction with the substrate, $NADP^+$, and the reaction product, NADPH. Here we show how the peculiar enzyme kinetics of A⁻ affects the *in vivo* metabolism of glucose 6-phosphate by erythrocytes, and we offer an explanation based on these data for the near-normal metabolism of A⁻ red cells.

Materials and Methods

Reagents, enzyme variants, purification procedures, and *kinetic determinations* have all been described in the previous paper (Afolayan and Luzzatto, 1971).

Determination of CO_2 Production by Intact Erythrocyte Suspensions. The technique used was similar to that used by Sturman (1967) on hemolysates. Blood samples were centrifuged and the RBC washed twice by centrifugation in KRB buffer (pH 7.4). The following additions were then made to 10-ml erlenmeyer flasks: 50% RBC suspension in KRB, 1.3 ml; penicillin (1000 units/ml), 0.03 ml; methylene blue, 0.2 mM, from 0 to 0.20 ml in different flasks; KRB to a final volume of 1.80 ml. After incubation for 10 min at 37°, 0.2 ml of a 0.1 M glucose solution was added, containing 1 μCi of [^{14}C]glucose. Thus, in the final incubation mixture the concentration of glucose was 0.01 M, with a specific radioactivity of about 0.05 μCi/μmole. The concentration of methylene blue varied between 0 and 20 μM. After mixing, 0.6 ml of the mixture was added to the main compartment of each of three small siliconized Warburg flasks, which contained in the center well 0.06 ml of 5 N NaOH and in the side arm 0.2 ml of 50% trichloroacetic acid. The flasks were immediately tightly stoppered, and incubated at 37°. At serial intervals incubation was stopped by tipping trichloroacetic acid from the side arm into the main compartment. After a further 1 hr at 37° the flasks were opened, and 0.04 ml of the NaOH

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[1] Abbreviations used are: Hb, hemoglobin; KRB, Krebs–Ringer bicarbonate; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate; $NADPH^+$, reduced nicotinamide–adenine dinucleotide phosphate.

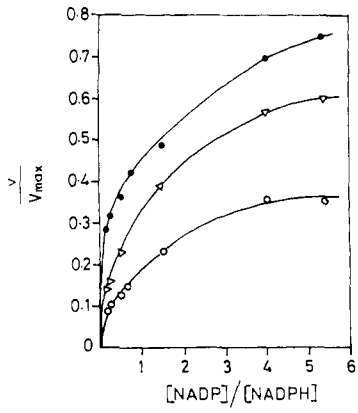

FIGURE 1: Activity of glucose 6-phosphate dehydrogenase variants as a function of the ratio of $NADP^+$ to NADPH in the reaction medium. Experiments were carried out on purified enzyme preparations, as described in Afolayan and Luzzatto (1971). (○—○) A, (▽—▽) B, and (●—●) A^-.

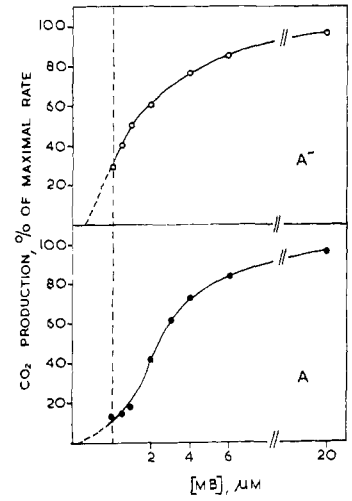

FIGURE 2: Intracellular activity of glucose 6-phosphate dehydrogenase as a function of methylene blue concentration. Experiments were carried out on suspensions of intact erythrocytes (see Materials and Methods). The initial rate of CO_2 production (see Table I) was measured in flasks containing different amounts of methylene blue and is plotted here against methylene blue concentration. Top: red blood cells with enzyme type A. Bottom: red blood cells with enzyme type A^-.

from the center well was applied onto a 20-mm disk of Whatman No. 4 filter paper. After drying, the disks were dropped into glass vials, to which 10 ml of scintillation fluid (0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis[2-(5-phenyloxazolyl)]-benzene in toluene) was added. Counting was done in a Packard Tri-Carp instrument (Model 2004) at an efficiency of about 40%.

Results

Activity of the A^- Variant as a Function of the Concentration of $NADP^+$ and NADPH. The results presented in the previous paper (Afolayan and Luzzatto, 1971) have shown that at low concentrations of $NADP^+$ the reaction velocity (relative to V_{max}) of the A^- glucose 6-phosphate dehydrogenase variant is greater than that of the "normal" variants A or B. Furthermore, A^- was less inhibited by NADPH. Whereas *in vitro* the dependence of reaction rate on concentrations of substrate and product can be studied separately, within the erythrocyte we expect that the total amount of ($NADP^+$ + NADPH) will not vary appreciably over a short time range: rather, any increase in the former will be accompanied by a decrease in the latter, and *vice versa*. We analyzed therefore the reaction rate of A, B, and A^- as a function of the ratio [$NADP^+$]/[NADPH] for fixed values of the sum total ($NADP^+$ + NADPH). It was found again (Figure 1) that A^- behaves quite differently from A and B, in that it displays a higher reaction velocity (relative to V_{max}), especially at low [$NADP^+$]/[NADPH] ratios, and at a total concentration ($NADP^+$ + NADPH) of 25 μM.

Activity of Different Variants of Glucose 6-Phosphate Dehydrogenase in the Intact Erythrocyte. The ideal way to assess whether the kinetics of the A^- variants with respect to $NADP^+$ and NADPH is relevant to its physiological function, would be to repeat, *within the intact cells*, the experiments described hitherto. If we refer to Figure 1, this means that we should be able to vary *ad libitum* the [$NADP^+$]/[NADPH] ratio in the erythrocyte, and then measure for different values of this ratio the activity, v, of glucose 6-phosphate dehydrogenase. Whereas this seems technically impracticable at the moment, we can measure some entity *proportional* to v, as a function of some variable *proportional* to [$NADP^+$]/[NADPH]. We have chosen, as proportional to v, the amount of [^{14}C]CO_2 liberated from [1-^{14}C]glucose (see Bloom *et al.*, 1953); and as a variable proportional to [$NADP^+$]/[NADPH] the concentration of methylene blue in the medium, which is known to affect this ratio (see Szeinberg and Marks, 1961).

We first determined CO_2 production in the absence and presence of methylene blue, *i.e.*, at low and high [$NADP^+$]/[NADPH] ratios, respectively. The results (Table I) agree with those of previous investigators (Brin and Yonemoto, 1958; Szeinberg and Marks, 1961; Fornaini *et al.*, 1962) in showing that methylene blue stimulates CO_2 production about tenfold in normal cells, but at best threefold in glucose 6-phosphate dehydrogenase deficient (A^-) cells. However, it is noteworthy that in the absence of methylene blue, there is no significant difference between the various types of cells (in agreement with Szeinberg and Marks, 1961). We next determined the full dependence of CO_2 production from methylene blue concentration. Representative experiments for erythrocytes of type A and A^- are shown in Figure 2. Here the "intracellular saturation curves" mimic those obtained with the purified enzyme preparations, in that A is sigmoid shaped (see Luzzatto, 1967), while A^- is near hyperbolic (see Afolayan and Luzzatto, 1971).

Discussion

Regulation of Glucose 6-Phosphate Dehydrogenase in Erythrocytes. The activity of this enzyme is known to be affected by a variety of metabolites (such as adenine nucleotides: Bonsignore *et al.*, 1963; steroid hormones: Marks and Banks, 1960; long-chain fatty acids: Wieland *et al.*, 1965; $NADP^+$ and NADPH: Luzzatto, 1967; see review by Pontremoli and Grazi, 1969), and it is not easy to establish which ones are most significant physiologically. However, since the main function of glucose 6-phosphate dehydrogenase in the mature erythrocyte is to produce NADPH, one can enunciate a number of properties which are to be expected of a control system for efficient regulation of its activity. (1) NADPH production should increase whenever excessive oxidation to $NADP^+$ has taken place. (2) NADPH production should

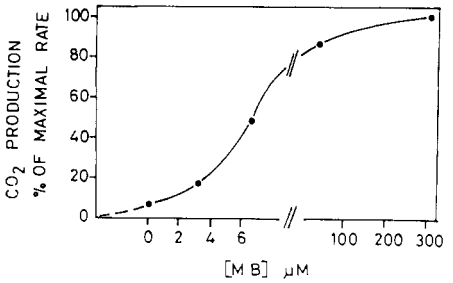

FIGURE 3: Intracellular activity of glucose 6-phosphate dehydrogenase as a function of methylene blue concentration. The initial slopes of the curves of Figure 2 of Bonsignore *et al.* (1963) are plotted against methylene blue concentration. Experiments were carried out on red blood cells with enzyme type B.

decrease whenever its level is restored to normal. (3) These effects should occur in the intact cells. (4) If the structure of the enzyme is altered in a way that will affect its interaction with the regulating system, the erythrocytes containing the altered enzyme should exhibit changes in some of their physiological responses.

The regulation of glucose 6-phosphate dehydrogenase by $NADP^+$-mediated transition of the enzyme from low to high affinity for $NADP^+$ and by NADPH inhibition (Afolayan and Luzzatto, 1971) meets requirements 1 and 2. The *in vivo* relevance of these effects (property 3) depends critically on the intracellular concentration of $NADP^+$ and NADPH, which is rather accurately known (Gross *et al.*, 1966; Cartier, 1968), but also on the possible complicating action of other intracellular substances, which are not completely known. Therefore, rather than attempting to mimic the *in vivo* situation, it is preferable to test whether the $NADP^+$ saturation kinetics can be reproduced in the intact cell. This was found to be the case for erythrocytes with the enzyme variant A (compare Figure 2a with Figure 1 of Luzzatto, 1967), or with the enzyme variant B (see Figure 3, drawn by replotting previous data of Bonsignore *et al.*, 1963).

As for property 4, we find that in experimental conditions in which the ratio of $NADP^+$ to NADPH is varied in intact erythrocytes, their behavior is quite different according to whether they have the A^- variant, or one of the other common variants of glucose 6-phosphate dehydrogenase (Figure 2 and Table I). Thus, a genetically determined change in the interaction of the enzyme with $NADP^+$ and NADPH directly affects its behavior inside the cell. Like in many other systems, largely worked out in bacteria, we can perhaps consider this as the most crucial evidence that the proposed regulation mechanism operates physiologically.

TABLE I: Activity of the Pentose Phosphate Pathway in Genetically Different Erythrocytes.[a]

Type of Glc-6-P-dehydrogenase	CO_2 Produced (μmoles/g of Hb hr)[b] No MB[c]	+MB (20 μM)	Stimulation Factor
B	0.24	2.27	9.5
B	0.33	4.36	13.4
A	0.19	2.42	12.6
A	0.24	2.33	9.7
A^-	0.33	0.90	2.7
A^-	0.25	0.74	3.0

[a] Techniques as described under Materials and Methods. [b] $[^{14}C]CO_2$ produced was measured at intervals of up to 3 hr, and was always linear for at least 2 hr. Values shown represent initial rates. [c] MB = methylene blue.

TABLE II: Absolute Activity of Glucose 6-Phosphate Dehydrogenase Variants as a Function of $NADP^+$ and NADPH.[a]

		μmoles of NADPH Produced/min per 10^{10} RBC		
	% $NADP^+$[b]	B	A	A^-
1	10	0.08	0.04	0.11
2	20	0.26	0.08	0.18
3	40	0.55	0.46	0.20
4	60	1.01	0.80	0.21
5	80	1.37	1.22	0.24
	V_{max}[c]	2.4	2.1	0.35

[a] The values listed for variants B and A^- are obtained from the data of Figures 1 and 2 of Afolayan and Luzzatto (1971), making use of visual interpolation wherever necessary. The values for variant A are similarly obtained from the data of Luzzatto (1967). [b] $NADP^+$ as percentage of the total amount of ($NADP^+$ + NADPH), which has been arbitrarily chosen at 50 μM for the present calculations (see Cartier, 1968). [c] Values for variants B and A are the average of determinations on two groups of 100 blood donors each (L. Luzzatto and F. A. Fasuan, unpublished results). The finding of a slightly higher activity for B agrees with the report by Nance (1967). The value for A^- is taken as being about 15% of that of B (WHO, 1967). The figure of 2.4 for V_{max} of enzyme type B is equivalent to 140 megamolecules of NADPH produced per minute per red cell at 26°, in good agreement with the value of 200 megamolecules obtained by Bishop (1965) at 37°.

It was possible to test this regulation mechanism in erythrocytes thanks to some of the biochemical peculiarities of these cells. Thus, $NADP^+$ concentration can be varied *ad libitum* by using methylene blue, because in the red cell no enzyme other than glucose 6-phosphate dehydrogenase (and 6-phosphogluconate dehydrogenase which depends on the former for supply of substrate) can reduce $NADP^+$. CO_2 production can be used as a measure of glucose 6-phosphate dehydrogenase activity because in the red cell no other enzyme system liberates CO_2. On the basis of the results thus obtained, this is one of the few cases in which an *in vitro* sigmoid saturation curve can be reproduced *in vivo:* just like an oxygen dissociation curve of purified hemoglobin is similar to one carried out on whole blood.

Physiology of Erythrocytes Containing the A^- Variant. In the preceding paper (Afolayan and Luzzatto, 1971) we have shown that a structural change in the A^- variant affects drastically its interactions with $NADP^+$ and NADPH. The data presented here indicate that these altered interactions in turn affect drastically the physiology of the cell.

Under normal conditions the rate of operation of the pentose phosphate pathway is limited by both the concentration of $NADP^+$ and that of glucose 6-phosphate dehydrogenase (Roigas *et al.*, 1970). The intracellular activity of the

enzyme under physiological conditions can be estimated in two different ways. (a) CO_2 production is measured and is related to maximal CO_2 production (Table I). (b) The enzyme velocity is calculated by combining the data on V_{max} with the previously measured (Afolayan and Luzzatto, 1971) relative rates at various concentrations of $NADP^+$ and NADPH (Table II). By method a, we find that in normal cells glucose 6-phosphate dehydrogenase operates at about one-tenth of the maximal rate in A and B cells, but about one-third of the maximal rate in A^- cells. By method b, we find that the enzyme operates at less than half-maximal rate in A and B cells, but at two-thirds the maximal rate in A^- cells. Although qualitatively both methods agree, method a is likely to reflect more accurately the *in vivo* situation, since it is based on findings in intact erythrocytes.[2] In addition, if $NADP^+$ had been overestimated by Cartier (1968), due to artefactual oxidation before or during extraction, the difference between the two estimates would be reduced. We conclude that, in absolute terms and under basal conditions, there is relatively little difference in glucose 6-phosphate dehydrogenase activity between "normal" and "deficient" erythrocytes (see, *e.g.*, lines 1 and 2 in Table II), because the enzyme of type A or B will be largely in state I, but the enzyme of type A^- will be largely in state II (Afolayan and Luzzatto, 1971). This is confirmed by the finding of comparable CO_2 production in the absence of methylene blue (Table I). Thus, under basal conditions A^- cells are not truly deficient in glucose 6-phosphate dehydrogenase activity. This probably explains the lack of clinical or hematological manifestations in subjects with this variety of red cells.

The position is drastically different, of course, under conditions of oxidative stress. In our experiments, this consisted in methylene blue; *in vivo*, a variety of chemical agents could be responsible (see WHO, 1967). The immediate chemical response by the erythrocyte will be conversion of NADPH into $NADP^+$ with consequent increase in the latter. Now, only glucose 6-phosphate dehydrogenase will be rate limiting (Roigas *et al.*, 1970) and the behavior of A and B cells will differ drastically from that of A^- cells. The former can step up about tenfold the rate of NADPH production, but the latter are already working at near saturation. The remaining NADPH will be rapidly exhausted, and eventually irreversible structural damage to the cell will take place, by the way of intermediary steps which still remain to be elucidated.

[2] Method b can hardly be expected to show exact quantitative agreement with method a, since intracellular substances other than $NADP^+$ and NADPH (for instance, metabolites; or divalent ions: R. Cancedda and A. De Flora, personal communication) probably affect the reaction rate of glucose 6-phosphate dehydrogenase. In addition, the bulk of our *in vitro* experiments were carried out, for convenience, at 27°. However, we have repeated at 37° on variants A and A^- kinetic experiments similar to those reported here in detail. We found that A^- still had binding constants for NADP about five times lower than A, and a binding constant for NADPH about five times higher than A. Thus, the physiological implications are the same as those inferred from the experiments at the lower temperature.

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