Defective molecular variants of glucose-6-phosphate dehydrogenase and methaemoglobin reductase

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Unlike any other living cell, the mature erythrocyte is unable to divide and synthesize proteins. It must therefore sustain a relatively long life (about 100 days) without renewing its stock of enzymes. If this stock is genetically altered the red cell will be more likely than any other to suffer. Also the metabolic pathways are greatly simplified in the red cell as its essential metabolites are derived only from anaerobic glycolysis. Therefore it is not surprising that about a dozen genetically determined enzyme defects have already been identified in this cell. Of these two are selected for discussion: glucose-6-phosphate dehydrogenase (G6PD) deficiency, which alters the cell viability, and methaemoglobin reductase deficiency, which impairs its function. In both examples, it can be demonstrated that the basic defect is a structural modification of the enzyme molecule. The phenotypic consequence of the genetic lesion—probably a point mutation in most cases—is the production of an abnormal allele which decreases catalytic capability and/or *in vivo* stability.

Glucose-6-phosphate Dehydrogenase Deficiency

This is not only the most frequent enzyme defect of the red cell but also the most frequent 'inborn error of metabolism', since the total number of individuals affected in the world is about 10^7 . A full account of G6PD deficiency is beyond our scope, and only the major aspects in the field of molecular pathology will be developed here.

HETEROGENEITY OF THE DISEASE

Deficiency of G6PD since its first discovery in 1956 as a cause of primaquine-induced haemolytic anaemia (Carson, Flanagan, Ickes, and Alving, 1956), has appeared to be polymorphic, both clinically and genetically.

Clinically, the condition may remain entirely latent, with overt manifestations of acute haemolysis occurring only in particular circumstances, such as the ingestion of certain drugs or fava beans, or in the

course of certain infections (Beutler, 1971). Less often the defect is responsible for neonatal icterus necessitating exchange transfusion. In rare instances, the defect gives rise to a congenital non-spherocytic haemolytic disease.

The diversity of clinical expression is explained, at least in part, by the extreme diversity of molecular variants.

At the molecular level, G6PD is the enzyme for which the greatest polymorphism has been demonstrated. Until now more than 100 different variants have already been defined. Since G6PD is produced by a single gene located on the X chromosome the defect is fully expressed in hemizygous subjects. On starch gel electrophoresis followed by specific staining the enzyme appears as a single band, and this facilitates the detection of molecular variants. In fact, discrimination between them is now based upon the following minimal criteria: residual activity in red cells and white cells, electrophoretic mobility, affinity for substrate (G6P) and coenzyme (NADP), utilization of analogs (2-deoxy G6P, deamino-NADP), heat stability, and pH curve. A WHO committee has recommended standardized techniques for the characterization of abnormal G6PD variants (World Health Organization, 1967).

At the present time, the 100 variants which have been well characterized can be assigned to five classes (Yoshida, Beutler, and Motulsky, 1971). In table I each class is subdivided into three categories according to their electrophoretic mobility, taking as a normal reference the migration of the common B(+) allele.

The first class, comprising about 20 variants, is the only one which is well characterized clinically and has a shortened red cell life span. However, there are striking dissimilarities between the biochemical characteristics of each variant (Yoshida, 1973). The only common feature is a pronounced instability of the enzyme *in vitro*.

The other variants with enzyme deficiency (classes 2 and 3) require an exogenous stress to induce haemolysis. It should be noted that, as pointed out

Electrophoretic Mobility	Classification of G6PD Variants				
	Class 1: Enzyme Deficiency with CNHD	Class 2: Severe Enzyme Deficiency (RBC activity < 10%)	Class 3: Moderate Enzyme Deficiency (RBC activity: 10-60%)	Class 4: No Enzyme Deficiency	Class 5: Increased Enzyme Activity
Fast	7	12	13	6	1
Normal	13	6	5		
Slow	9	5	10	11	

Table I Heterogeneity of G6PD variants

CNHD = congenital non-spherocytic haemolytic disease

by Beutler (1971), the spectrum of drugs potentially hazardous in these subjects remains ill defined, and the toxicity of a given drug may differ according to the type of G6PD deficiency. In practice, the drugs which should be avoided in any case are the antimalarial derivatives belonging to the 8-aminoquinoline series, the sulphonamides, and the sulphones.

Finally those variants (about 17) which have normal activity are not associated with clinical manifestations. One of them the A(+) variant is very common in Negro populations.

THE COMMON DEFECTIVE VARIANTS (TABLE II)
Among the variants belonging to classes 2 and 3 (table I), there is a striking prevalence of some types in certain ethnic groups and geographical areas.

The most common type in black African populations is the A(-) variant (frequency 10-20%).

Since it is encountered only in ethnic groups where the non-deficient A(+) allele is frequent, one may reasonably assume that it results from a mutation of the G6PD A(+) gene.

The A(+) and the A(-) enzymes have been resolved chromatographically (Luzzatto and Allan, 1965; Yoshida, Stamatiyannopoulos, and Motulsky, 1967). However, the structural difference between those two variants is still unknown. It has been shown that the A(+) allele derives from the B(+) normal allele by a single amino-acid substitution (asparagine→aspartic acid) (Yoshida, 1967).

The main abnormal feature of the A(-) variant is its rapid rate of inactivation *in vivo*, ie, during the process of red cell aging. Its half-life has been estimated to be 13 days instead of 62 days for the normal B(+) enzyme (Piomelli, Corash, Davenport, Miraglia, and Amorosi, 1968). The specific activity of the purified A(-) enzyme is normal

Variant	Population Origin	RBC Enzyme Activity (% of normal)	WBC Enzyme Activity	Electrophoretic Mobility	KmG6P (μmol)	Utilization of Analogs	Heat Stability	pH curve	Comments
B (+)	Widespread	100	Normal	Normal	50-70	Normal	Normal	Normal	Most prevalent non-deficient allele
A (+)	Negro	80-100	Normal	Fast	Normal	Normal	Normal	Normal	Non-deficient
Mediterranean	Greece	< 5	↓	Normal	↓	↑	↓	Abnormal	Widespread, may be heterogeneous
	Italy								
	Middle East								
	India								
A (-)	Negro	10-20	Normal	Fast	Normal	Normal	Normal	Normal	High prevalence (10-20%) in negro populations
Mali	Mali and Senegal	1-5	↓	Normal	↓	↑	↓	Abnormal	Restricted areas in Africa
Athens	Greece	25		Slow	↓	↑	Normal	Abnormal	
Canton	South China	4-24	↓	Fast	↓	↑	↓		
Union	Philippines	< 3		Fast	↓	↑		Abnormal	
Markham	New Guinea	1-10		Fast	↓	↑	↓	Abnormal	
Campbellpur	Pakistan	2-7		Normal	↓	↑	↓	Abnormal	
Mahidol	Thailand	5-20		Normal	Normal	Normal	Normal	Normal	

Table II Common G6PD variants

(Yoshida *et al*, 1967) and the number of active molecules in reticulocytes and young erythrocytes is normal. This provides an explanation for the self-limiting haemolysis observed in subjects having ingested primaquine. For the same reason the cells which can synthesize proteins, such as leucocytes, platelets, or fibroblasts, do not exhibit G6PD deficiency in the hemizygous subjects carrying the A(−) gene. The A(−) enzyme has recently been purified to homogeneity (Babalola, Cancedda, and Luzzatto, 1972) and its intrinsic instability is attributed to an abnormal sensitivity of sulphhydryl groups to oxidation.

The Mediterranean variant, or B(−), is the commonest defective G6PD found in white populations. It is highly predominant in the Mediterranean basin (especially among Sardinians, Greeks, Sephardic Jews, and Arabs), in the Middle East, and even in more eastern Asiatic populations (northern India, Indonesia) (Kirkman, Kidson, and Kennedy, 1968). Some subjects with the Mediterranean variant, males and even female carriers, suffer from favism, a very severe acute haemolysis produced by the ingestion of fava beans. However, if G6PD deficiency is found invariably in all favic subjects, only a few G6PD-deficient individuals are sensitive to fava beans (Beutler, 1971). It has been postulated that an additional genetic defect, possibly autosomal, is required to produce favism (Stamatoyannopoulos, Fraser, Motulsky, Fessas, Akrivakis, and Papayan-nopoulou, 1966). Actually the problem of favism still remains unsolved (see discussion in Beutler, 1971). Another clinical problem in the Mediterranean variant category is that some subjects have been reported to suffer from congenital non-spherocytic haemolytic disease. This raises the question of a different enzyme mutation mimicking the Mediterranean variant or that of an additional inherited trait (Beutler, Mathai, and Smith, 1968).

Biochemically, the Mediterranean variant is characterized by an almost total lack of enzyme activity in the red cells regardless of their age. The enzyme deficiency is also expressed in other cells such as leucocytes and platelets. Contrasting with the A(−) variant all the parameters, except the electrophoretic mobility, are abnormal (see table II). This variant appears to be extremely unstable *in vivo* since the activity disappears during the maturation of reticulocytes (Piomelli *et al*, 1968). Furthermore, by immunological means, it has been demonstrated that the specific activity of the Mediterranean variant enzyme molecule is drastically reduced (Yoshida, Stamatoyannopoulos, and Motulsky, 1968; Rosa, Alexandre, Kaplan, and Dreyfus, 1970). The precise nature of the molecular lesion is still unknown.

Between the two most common and widespread variants, A(−) and Mediterranean, and the numerous rare defective variants identified in single families, there are several abnormal variants which are found to be common only in certain geographical areas (table II). This was found in the Negro populations of Mali and Senegal (Mali variant) (Kahn, Boivin, and Lagneau, 1973), in Greece (Athens variant) (Stamatoyannopoulos, Voigtlander, Kotsakis, and Akrivakis, 1971), in South China (Canton variant) (Chan, Todd, and Lai, 1972), in the Philippines (Union variant) (Yoshida, Baur, and Motulsky, 1970), in New Guinea (Markham variant) (Kirkman *et al*, 1968), in Pakistan (Camp-lellpur variant) (cited in Yoshida *et al*, 1971), and in Thailand (Mahidol variant) (Panich and Sungnate, 1973).

Noteworthy is the fact that in some areas where G6PD deficiency is frequent, 'polymorphic' variants may coexist with rare—'non-polymorphic'—variants. Careful population studies have thus revealed a considerable degree of genetic heterogeneity at the G6PD locus. Such a genetic diversity was found in subjects with the so-called 'Mediterranean' phenotype in Greece (Stamatoyannopoulos *et al*, 1971), in different populations of Africa (Luzzatto and Afolayan, 1968; Reys, Manso, and Stamatoyannopoulos, 1970; Kahn *et al*, 1973), and also in Asia (Kirkman *et al*, 1968; Chan *et al*, 1972; Panich and Sungnate, 1973). These findings imply a very high mutation rate at the G6PD locus. In this respect G6PD has been considered to be a 'permissive' enzyme, tolerating a great many amino acid substitutions (Stamatoyannopoulos, Kotsakis, Voigtlander, Akrivakis, and Motulsky, 1970). It would be interesting to test this hypothesis by comparing the primary structure of G6PD along the phylogenetic tree, since we know examples of proteins, eg, cytochromes, which are very stable from the evolutionary point of view and are therefore considered to possess very stringent structural requirements for their function or stability. However such a study cannot be undertaken in the near future since the primary structure of human G6PD is still not established.

Also the attractive hypothesis of an intracistronic recombination between two already mutated genes has been put forward to explain the evolving of new rare G6PD variants in populations already characterized by a high polymorphism at the G6PD locus (Beutler, 1971; Kahn *et al*, 1973).

FUTURE PROSPECTS

A considerable amount of data have been accumulated from the study of G6PD deficiency, some of which have permitted dramatic accomplishments

in the field of molecular biochemistry as well as molecular genetics but many questions remain unanswered.

Why is G6PD deficiency so common in some areas? Undoubtedly it confers a selective advantage to the carriers of the trait. It has been suggested (Motulsky, 1964) and later proven (Luzzatto, Usanga, and Reddy, 1969) that it protects against malaria. A greater resistance against the Plasmodium infestation has also been found in males carrying the non-deficient A(+) variant (Bienzie, Ayeni, Lucas, and Luzzatto, 1972). According to these authors the high frequency of the deficient A(-) gene would be maintained mainly by the protection against malaria of the heterozygous females carrying the A(-)/B(+) genotype. However, the problem is not entirely settled and the malaria theory may not apply to all categories of G6PD deficiency.

At the molecular level, the biochemist has to face one fact. In contrast to the haemoglobin molecule, whose anatomy is now perfectly understood and in which amino acid substitutions can be located precisely, our knowledge of the normal G6PD molecule and its variants is at an embryonic stage. Complete purification of the B(+) normal enzyme, the A(+) common variant and the Hektoen variant has been achieved, and after fingerprinting a different single amino acid substitution was found in the two latter variants (Yoshida, 1967; Yoshida, 1970). A considerable amount of blood was necessary to achieve these results. Recently micro-scale techniques of purification have been developed which might possibly be applied to the leucocytes from a single donor (Kahn and Dreyfus, 1974).

By using the refined micro-scale (for one nanomole of protein) peptide mapping method described by Watanabe and Yoshida (1971), one can expect soon to reach the level of molecular pathology. Until

this is achieved it will become more and more difficult to distinguish by conventional means the new variants from those already described.

Immunological methods already permit the determination of specific activity of the enzyme and therefore the estimation of the total number of G6PD molecules—active together with inactive—per cell, as given by the residual activity vs specific activity ratio (table III).

A novel approach to the study *in vivo* of the functional capacity of normal and abnormal G6PDs results from the elegant experiments of Yoshida (1973). When studied *in vitro* in a medium mimicking the environment which prevails in the red cell, in particular in the presence of ATP and NADPH which are strong competitive inhibitors of the enzyme, G6PD is normally found to work at 0.1% of its maximal potential activity. Measuring the activity of variants in artificially optimal conditions therefore appears almost meaningless. Some variants associated with the severe clinical syndrome of congenital non-spherocytic haemolytic disease have a surprisingly high residual enzyme activity when measured in these conditions. Instead, by studying the enzyme under simulated physiological conditions and taking into account the K_i for NADPH, Yoshida (1973) could demonstrate that these G6PDs must be actually completely offset within the red cell. This is due to the kinetic abnormalities peculiar to these variants which exhibit both a decreased affinity for NADP and an increased sensitivity to the inhibitory effect of NADPH and ATP.

Methaemoglobin Reductase Deficiency

The enzymatic maintenance of haemoglobin (Hb) in its functional ferrous state is promoted by a flavoprotein which requires NADH as a hydrogen

G6PD Variant	Residual Activity in RBCs (% of normal)	Specific Activity Catalytic Activity = $\frac{\text{Antigenic Activity}}{\text{Antigenic Activity}}$ (% of normal)	Number of G6PD Molecules per cell Residual Activity = $\frac{\text{Specific Activity}}{\text{Specific Activity}}$
A (-) (Yoshida <i>et al</i> , 1967)	20	100	Decreased
Athens (Stamatoyannopoulos <i>et al</i> , 1967)	25	100	Decreased
Union (Yoshida <i>et al</i> , 1970)	3	10	Decreased
Mediterranean (Yoshida <i>et al</i> , 1968)	Very low	30	Decreased
Port Royal (Kaplan <i>et al</i> , 1971)	50	50	Normal
Hektoen (Dern <i>et al</i> , 1969)	500	100	Increased

Table III Level of G6PD molecules in G6PD-deficient RBCs

donor. Since *in vitro* this enzyme reduces actively artificial (xenobiotic) electron acceptors, such as 2,6-dichlorophenol indophenol, it belongs to the ill defined category of diaphorases (E.C. 1.6.99).

Its genetically determined deficiency gives rise in the homozygous state to recessive congenital methaemoglobinaemia (Scott and Griffith, 1959). This is a rare disease characterized by permanent cyanosis due to the presence in the red cells of a certain amount of methaemoglobin (ranging from 10 to 40% of total Hb). Contrary to the haemoglobins M the protein moiety of this methaemoglobin is quite normal. The consequence of the enzyme defect is therefore an impaired oxygen-transport capacity of the red cell without modification of its viability.

The lack of metHb reductase can be established by assaying the enzyme as an NADH-diaphorase (Scott and McGraw, 1962) or as an NADH-ferrocyanide-methaemoglobin reductase (Hegesh, Calmanovici, and Avron, 1968). Experiments with intact red cells show that they have a reduced capacity to reduce metHb (Kaplan, 1966).

Usually the clinical condition is well tolerated, and the aesthetic disadvantage of cyanosis is easily controlled by continuous administration of ascorbic acid or methylene blue. The mechanism of the spectacular action of methylene blue—which may be administered orally or intravenously—is explained by the intervention of an NADPH-dependent diaphorase (Sass, Caruso, and Axelrod, 1969). The latter enzyme, whose physiological role remains obscure, is an entirely different protein. This could be established from genetic, electrophoretic, and immunological evidence (Kaplan and Beutler, 1967; Sass *et al.*, 1969; Hsieh and Jaffé, 1971; Leroux and Kaplan, 1972).

In a small but significant proportion of cases (about 10%), the methaemoglobinaemic syndrome is associated with a very severe neurological disorder consisting of mental retardation and progressive athetosis causing death at an early age (Fialkow, Browder, Sparkes, and Motulsky, 1965; Heusden, Willems, Lambotte, Hainaut, Chapelle, and Malchair, 1971; Jaffé and Hsieh, 1971). Until recently the relationship between methaemoglobinaemia and the nervous system involvement was poorly understood.

Molecular Variants of Methaemoglobin Reductase

The introduction of electrophoretic methods for characterizing the enzyme (West, Gomperts, Huehns, Kessel, and Ashby, 1967; Kaplan and Beutler, 1967) enabled identification of several abnormal defective alleles (Bloom and Zarkowski,

1969; Hsieh and Jaffé, 1971; West, 1972; Leroux and Kaplan, 1974; Kaplan *et al.*, unpublished), as well as rare alleles with normal activity (Hopkinson, Corney, Cook, Robson, and Harris, 1970) (table IV).

At least eight different defective alleles have been described on the basis of their electrophoretic properties (table V). This indicates that here again, as in most enzymopathies (Dreyfus, 1972), the genetic defect of metHb reductase deficiency involves a structural gene and is heterogeneous.

Not all cases of metHb reductase deficiency are characterized by an electrophoretically abnormal allele. In our own collection of 12 unrelated patients, there are respectively four cases with an abnormal electrophoretic pattern (California variant, Beni-Messous variant, Grenoble variant, and La Tronche variant), four cases with traces of an electrophoretically normal enzyme, and four cases without a detectable enzyme after electrophoresis.

In contrast to G6PD deficiency, parameters other than electrophoresis are still not routinely studied on the mutated metHb reductases. In the fast-moving Puerto-Rican variant (Schwartz, Paresse, Ross, Di Pillo, and Rizek, 1972) a marked enzyme thermal-instability was found together with abnormal kinetic parameters. We also found in one patient with an electrophoretically normal enzyme that the reductase was markedly thermally unstable, but it had normal kinetic characteristics (Leroux and Kaplan, 1974). An abnormal affinity for NADH has been reported for the slow-moving 'ma' variant (West, 1972).

We performed immunological studies in four patients, searching for non-functional antigenically reacting enzyme ('cross-reacting material') (Leroux and Kaplan, 1974). This was done with the double-step antibody consumption test (Dreyfus, 1972), exploring the removal of specific anti-metHb

Alleles with Normal Activity	Defective Alleles
DIA ¹ (common allele)	DIA ^{**}
DIA ³	Princeton†
DIA ^{3*}	California†
DIA ^{**}	Boston slow†
DIA ^{**}	Duarte†
Boston fast	Puerto-Rico†
	Beni-Messous†
	Grenoble†
	'ma' [‡]
	La Tronche†

Table IV Electrophoretically abnormal alleles of red cell NADH-diaphorase

*Only observed in heterozygotes

†Observed in homozygotes with congenital methaemoglobinaemia

‡Observed in a double-heterozygote with congenital methaemoglobinaemia.

Variants	Origin	Relative Mobility (percentage of normal)	Residual Enzyme Activity in RBCs (percentage of normal)
California (Kaplan and Beutler, 1967)	European (undefined)	133	25
ma (West <i>et al.</i> , 1967; West, 1972)	?	slow	10
Boston slow (Bloom and Zarkowsky, 1969)	Irish	90	35
Duarte (Hsieh and Jaffe, 1971)	English-Polish	108	12
Puerto Rico (Hsieh and Jaffe, 1971)	Puerto-Rican	117	6-23
Beni-Messous (Kaplan <i>et al.</i> , unpublished)	Algerian	109	0
Grenoble (Kaplan <i>et al.</i> , unpublished)	Gipsy	115	1
La Tronche (Kaplan <i>et al.</i> , unpublished)	French	110	1

Table V Electrophoretic variants of NADH-diaphorase (metHb-reductase) in homozygous congenital methaemoglobinaemia

reductase antibody by a given amount of enzyme activity. As shown in table VI, in all cases some cross-reactive material could be detected, corresponding to an enzyme with a reduced specific activity.

The Problem of Congenital Methaemoglobinaemia Associated with Neurological Involvement

We have recently demonstrated, on the basis of electrophoretic and immunological studies, that the NADH-diaphorase which promotes metHb reduction in the red cell is also present in cells or tissues which do not contain haemoglobin, such as leucocytes, platelets, brain, muscle, liver, and placenta (Leroux and Kaplan, 1972). This finding prompted us to investigate systematically the enzyme level and properties of 'metHb reductase' in the leucocytes of subjects suffering from enzymopenic methaemoglobinaemia. In three subjects without neurological disease the enzyme defect was found to be restricted to the erythrocytes, while their leucocytes showed little or no abnormality. In contrast, in three individuals with neurological disease, the enzyme

defect was found both in the erythrocytes and in the leucocytes (Kaplan, Hanzlickova-Leroux and Dreyfus, 1970; Leroux and Kaplan, 1974). We therefore conclude that the severe form of congenital methaemoglobinaemia associated with a neurological disorder represents a systemic disease due to a generalized deficiency of NADH-diaphorase. In contrast, in the common form of methaemoglobinaemia the enzyme deficiency is only expressed in the erythrocytes. Such variability in the extension of an enzyme defect has already been discussed *à propos* of G6PD deficiency. It may receive the same explanations, ie, differential lability *in vivo* and/or functional disability of the abnormal alleles. This points further to the genetic heterogeneity of 'metHb reductase' deficiency.

Last but not least, the question of the metabolic role of the so-called metHb reductase in cells which do not contain haemoglobin should be considered. It was recently postulated that this enzyme is actually a soluble form of cytochrome b_5 reductase in the red cells (Hultquist and Passon, 1971; Passon and Hultquist, 1972). We could substantiate this theory by finding a parallel decrease of cytochrome

Variant	Antibody Consumption (percentage of normal)	Residual Enzyme Activity in RBCs (percentage of normal)
Beni-Messous variant	11	0
Case Alc...	7	0
Case Fri...	25	10
Grenoble variant	6	1
La Tronche variant	Young RBCs 21 Old RBCs 7	Young RBCs 8 Old RBCs 1

Table VI Cross-reacting material in methaemoglobin reductase deficiency¹

¹Personal results

b_5 reductase activity and metHb reductase activity in the leucocytes of two patients with severe neurological disease (Leroux and Kaplan, 1974).

The basic defect in enzymopenic congenital methaemoglobinaemia might be in fact cytochrome b_5 reductase deficiency. When restricted to the erythrocytes it would give rise to the benign form; when major and widespread in all tissues, including brain, it would produce the severe form with neurological involvement.

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