Enzyme assays in diseases of erythrocytes

T. A. J. PRANKERD

From the Department of Clinical Haematology, University College Hospital Medical School, London

The human red cell has been the source of a great deal of information in medical science. It has provided evidence of genetic variation in natural populations and of the molecular basis of diseases in the case of abnormal haemoglobin, and it has been a model for studying a number of inherited diseases including those peculiar to the cell itself. It is the latter which is dealt with here.

The erythrocyte is a relatively uncomplicated cell in which metabolic reactions have become highly adapted to function. In addition to its major constituent haemoglobin, it contains a complement of glycolytic enzymes, some residual enzymes from the tricarboxylic acid cycle, and a series of enzymes

forming the pentose phosphate pathway. It is probably true that the limited energy requirements of the cell are provided by glycolysis, whilst a 'reduction potential' which protects against oxidation, especially of haemoglobin, is maintained through the pentose pathway. In the case of glycolysis the energy formed is stored principally in the form of ATP whilst the reduction capacity of the cell exists principally in the form of reduced glutathione. Highly developed in the red cell is an enzyme system for the reduction of methaemoglobin. The interaction of these various metabolic pathways is shown in Figure 1. Abnormal enzyme function, which may be due to a lack of normal enzyme, or the

Fig. Major metabolic pathways in the erythrocyte.

presence of an abnormal enzyme or an unstable one, has been found in both of the principal metabolic pathways and it is often associated with a susceptibility to an increased destruction of the cell itself. When this occurs the disorder forms part of a group of diseases known as the 'congenital nonspherocytic haemolytic anaemias'.

The following glycolytic defects of the red cell have been found and their existence in the main confirmed: hexokinase (EC 2.7.1.1) (Valentine, Oski, Paglia, Baughan, Schneider, and Naiman, 1967); glucose phosphate isomerase (EC 5.3.1.9) (Baughan, Valentine, Paglia, Ways, Simons, and de Marsh, 1967); triose phosphate isomerase (EC 5.3.1.1) (Schneider, Valentine, Hattori, and Heins, 1965); diphosphoglyceromutase (EC 2.7.5.4) (Bowdler and Prankerd, 1964); phosphoglycerate kinase (EC 2.7.2.3) (Kraus, Langston, and Lynch, 1968); and pyruvate kinase (EC 2.7.1.40) (Valentine, Tanaka, and Miwa, 1961).

Of these deficiencies that of pyruvate kinase appears to be much the most common and outnumbers the others by about five to one. It can be detected by a relatively simple enzyme assay in which the pyruvate kinase reaction is coupled with that of lactate dehydrogenase (EC 1.1.1.27) and the rate of conversion of NADH to NAD is measured at 340 nm (Tanaka, Valentine, and Miwa, 1962). It is important to use two concentrations of phosphoenolpyruvate (0.0075 and 0.015 M) as the kinetics of at least two enzyme variants differ, and it should be remembered that the diluted haemolysate is unstable. As leucocyte pyruvate kinase activity is several hundred times that of red cells great care should be taken to remove all white cells while centrifuging and washing the red cells. Removal of most of the reticulocytes at the same time as the leucocytes avoids falsely high enzyme activity in patients with a marked reticulocytosis. Affected patients have 5-20% of normal activity. Screening procedures depending on pH change (Brunetti and Nenci, 1964) resulting from the formation of lactic acid, or the UV fluorescence of NADH (Beulter, 1966), have been described.

The metabolic consequences of this enzyme defect are an increase in 2,3-diphosphoglycerate (Bowdler and Prankerd, 1964), and a fall in cell ATP which may be causally related to the haemolytic instability of the cell. The clinical picture is of a chronic haemolytic anaemia from birth producing a wide range of haemoglobin levels and unremarkable red cell morphology. Reticulocyte counts are high and increase after splenectomy which has only a marginally beneficial effect on the patient. The disease is predominant amongst northern Europeans and only appears in homozygotes. Genetic variants of

pyruvate kinase are being described but few have yet been fully identified (Paglia, Valentine, Baughan, Miller, Reed, and McIntyre, 1967).

The intermediate phosphate compound 2,3-diphosphoglycerate (2,3-DPG) is of special interest in the red cell because of its interaction with haemoglobin. Other phosphate compounds such as ADP also interact with haemoglobin producing alterations in its oxygen dissociation, but the major effects seem to result from changes in concentration of 2,3-DPG. An increase in this compound shifts the curve to the right and a decrease shifts it to the left; the degree of shift obtained can produce significant alterations in oxygen delivery to the tissues. This mechanism therefore contributes an internal compensatory mechanism against the anoxia of anaemia (Bellingham and Huehns, 1969) and has now been demonstrated in a number of diseases (Huehns, 1970). The problem of special interest here is the control of 2,3-DPG concentration in conditions other than pyruvate kinase deficiency, where no enzyme defect is present.

Defects involving the reduction system within the red cell include the following: glucose 6-phosphate dehydrogenase (EC 1.1.1.49, G6PD) (Carson, Flanagan, Ickes, and Alving, 1956); phosphogluconate dehydrogenase (EC 1.1.1.43, PGD) (Lausecker, Heidt, Fisher, Hartley, and Lohr, 1966); glutathione reductase (EC 1.6.4.2) (Carson, Brewer, and Ickes, 1961); glutathione peroxidase (EC 1.11.19) (Nechaj, Boles, and Allen, 1968); glutathione deficiency (Chen, Loos, and Prins, 1961).

Amongst this group by far the most common deficiency is of course G6PD which may have an incidence of 20% or more amongst some populations.

Glucose 6-phosphate dehydrogenase catalyses the initial step in the pentose phosphate pathway and thereby results in the generation of NADPH. In the mature red cell, lacking the full enzyme complement of the Krebs cycle, NADPH cannot be formed in any other way. It is important to the cell in maintaining adequate concentrations of reduced glutathione which in turn protects from oxidation the other SH groups of haemoglobin and the cell membrane. Normally about 10% of glucose is utilized in the pentose phosphate pathway.

Glucose 6-phosphate dehydrogenase deficiency is readily detected by assay of the rate of formation of NADPH at 340 nm in a haemolysate with glucose 6-phosphate as the substrate. Once again leucocytes and reticulocytes should be removed first by centrifuging. This method of assay also measures phosphogluconate dehydrogenase and if a separate assay is required then a two-step procedure, with separate substrates, should be employed. A variety of methods have been proposed as screening procedures and make use of such adjuncts as the reduction of

brilliant cresyl blue, methaemoglobin, or a tetrazolium compound (World Health Organization, 1967).

Glucose 6-phosphate dehydrogenase has been studied in considerable detail and some 40 biochemical variants have been described differing in their electrophoretic mobility, kinetic variations, or heat lability. Variants associated with normal enzyme activity (eg, A) are of no clinical significance, whilst others (eg, A —) in which enzyme activity is diminished, lead to haemolysis provoked by drugs (Table); yet others, which result in severe instability of the enzyme protein, are associated with a congenital nonspherocytic type of haemolytic anaemia.

8-Aminoquinolines	Sulfones
Primaquine	Sulfoxone
Pamaquine	Diaminodiphenyl-sulfone
Pentaquine	Thiazosulfone
Chloroquine	
Sulphonamides	Nitrofurans
Sulphanilamide	Nitrofurantoin
Sulphamethoxypyridazine	Furazolidone
Salicylazosulphapyridine	Nitrofurazone
Sulphacetamide	
Acetylphenylhydrazine	Pyrimidone
Acetanilid	Acute bacterial infections
Acetophenetidin	Viral infections
Antipyrine	Diabetic ketosis
Acetylsalicylic acid	Para-aminosalicylic acid
Naphthalene derivatives	Methylene blue
Ascorbic acid	Probenecid
Quinidine	Quinine
Trinitrotoluene	Fava bean

Table Agents reported to cause destruction of red cells deficient in glucose 6-phosphate dehydrogenase

The enzyme appears to be located on the X chromosome and we therefore find homozygous females and hemizygous males most affected by its deficiency. Glucose 6-phosphate dehydrogenase activity declines with the aging of normal red cells, and the variations observed in the severity of the clinical state associated with deficiency of the enzyme are probably determined by a difference in the stability of each particular enzyme variant although in some cases it is possible that there is an actual deficit of enzyme protein (eg, A — individuals). Thus we find grades of clinical affection, the A — type being susceptible only to a limited number of drugs, whilst the mediterranean and south east Asian types are sensitive to the same drugs and also to infection, the effects of the fava bean and haemolytic disease of the newborn; the northern European type is associated with chronic haemolytic anaemia from birth.

The haemolytic anaemia affecting all except north Caucasians is intermittent and related to the administration of some offending drug (Table). In

negroes the haemolysis is self-limiting but this may not be so in Mediterraneans. Diagnosis can be made by enzyme assay but it should be realized that this may be normal soon after a haemolytic episode because the surviving cells are all young, and the diagnosis may then have to await the development of a remission or depend on family studies.

Reduced glutathione is probably involved in the protection of both haemoglobin and the red cell membrane against oxidative damage; two enzymes, catalase (EC 1.11.1.6), and glutathione peroxidase, which are both present in the red cell, are involved in the elimination of peroxides. A deficiency of reduced glutathione in the red cell tends to result in the production of Heinz bodies in the presence of oxidative agents *in vitro*, but these may not be apparent during a haemolytic episode *in vivo*. Although a deficiency of reduced glutathione occurs as a result of G6PD deficiency, it also may be due to a deficiency of glutathione synthase resulting from deficiencies of glutathione reductase and phosphogluconate dehydrogenase. Unlike the majority of cases of G6PD deficiency these other disorders appear to be associated with moderately severe forms of congenital nonspherocytic haemolytic anaemia in which drug exacerbation is not always apparent. Deficiency of catalase or glutathione peroxidase has no adverse effect on red cell survival unless both deficiencies are present in the same individual.

The other important enzyme with pathological associations in the red cell is methaemoglobin reductase. This enzyme is responsible for the reduction of methaemoglobin which forms in the cell spontaneously, and without a suitable reducing system the red cell would soon become functionless. The deficiency state results in the accumulation of methaemoglobin and the presence of persistent cyanosis and moderate erythrocytosis. The activity of this enzyme in reducing methaemoglobin is maintained by glycolysis through a redox coenzyme system involving NADH. The system appears to be geared to the phosphorylation of glyceraldehyde 3-phosphate (Fig. 1).

This deficiency can be differentiated from the presence of unstable haemoglobin (Hb-M) by haemoglobin electrophoresis and by assay of the enzyme. The assay depends on the reduction of an indophenol dye to a colourless form by the NADH-methaemoglobin reductase complex (Scott, 1960). The substrate used in this assay is not methaemoglobin because the reaction time is very much shorter with the dye. Differentiation from acquired methaemoglobinemia is not difficult as this is not lifelong and disappears rapidly after the offending agent has been removed. The deficiency is inherited as an autosomal character presenting in homozygotes. It

is probably a heterogeneous disorder and different species of abnormal enzymes have been detected by starch gel electrophoresis (West, Gomperts, Huehns, Kessel, and Ashby, 1967). There is also clinical heterogeneity, some patients having mental deficiency which does not obviously correlate with the degree of methaemoglobinaemia. All patients appear to lose their cyanosis when treated with methylene blue.

Many other enzyme deficiencies can be demonstrated in red cells and in some of them the deficiency is part of a generally distributed cellular deficiency. Deficiencies of catalase, galactose-phosphate uridyl transferase (EC 2.7.7.10), argininosuccinate synthetase (EC 4.3.2.1) are examples. In these states the functional activity of the red cell does not appear to be jeopardized although diminished red cell survival may occur in untreated galactosaemia.

Acetylcholinesterase (EC 3.1.1.7) is of interest in that its activity is reduced in the red cells of patients with paroxysmal nocturnal haemoglobinuria and pernicious anaemia, but the exact significance of this finding is not clear (Auditore and Hartmann, 1959). An inherited deficiency has been described but without any haemolytic condition (Jones, 1962), and the enzyme can be inhibited by drugs without resulting in haemolysis (Metz, Stevens, van Rensburg, and Hart, 1961).

From a genetic point of view other red cell enzymes have been of interest in the assessment of the extent or character of genetically determined enzyme variations. Widespread polymorphism has been demonstrated in population studies of acid phosphatase (EC 3.1.3.2), carbonic anhydrase (EC 4.2.1.1), and adenylate kinase (EC 2.7.4.3). It is interesting that some polymorphic variations detected by electrophoresis are associated with altered enzyme activity (Harris, 1966). Up to date no attempts have been made to correlate these quantitative differences with any change in red cell function but this work is now occupying our attention.

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