Papers and Originals

Clinical Significance of Red-cell Structure and Metabolism*

T. A. J. PRANKERD,† M.D., F.R.C.P.

Brit. med. J., 1965, 2, 1017–1020

The red cell is one of the most highly adapted cells, the sole purpose of which appears to be the efficient transport of oxygen. In the course of evolution a variety of pigments have functioned as transporters of oxygen in different animals. Undoubtedly haemoglobin is the most efficient of these, and its enclosure within the cell increases the oxygen-carrying capacity of blood some fourfold compared with the circulation of equivalent concentrations of haemoglobin in free solution. Thus the enclosure of haemoglobin in circulating red cells confers the obvious benefit of increased mobility on those species possessing them. To achieve the same efficiency of oxygen transport with a solution of haemoglobin would pose serious osmotic problems to the body cells, and without some additional mechanism the free haemoglobin would be filtered away by the renal glomeruli. We have reason, therefore, to be grateful for the existence of our red cells, which in the course of 120 days' life-span travel some 2,000 miles (3,200 km.). Their disappearance from the circulation follows an arithmetical function, and suggests that this is a result of ageing rather than random destruction. If this is so, then we must look to progressive changes in the composition or metabolism of the cell for the cause of its destruction, and though evidence is scanty it looks as if loss of red-cell surface components, such as lipids, and inactivation of certain enzymes are the principal changes accompanying senescence.

Normal Structure

The structure of the red cell is still much debated (Prankerd, 1961a), but it seems to consist of some three layers of material: an inner layer of a rather fibrous protein, elanin; a middle layer of opposed phospholipid molecules linked by cholesterol molecules; and an outer layer of sialic acid, a mucopolysaccharide which determines the cell's electrical charge (Fig. 1). How the membrane structure might be orientated to maintain the curious biconcave shape of this cell remains a mystery. No rigidity of the cell structure can be demonstrated by following the sequence of osmotic swelling of the cell, but there may be some local asymmetry in the structural architecture of the cell wall, or, as recent work suggests, there may be a contractile protein in the membrane which, under the activation of adenosine triphosphate (ATP), can variably control the shape of the membrane (Nakao *et al.*, 1961). The membrane would appear to be traversed by pores about 4 Å wide which allow the permeation of small molecular particles and ions, but not of large ones in excess of about a molecular weight of 100.

Whether haemoglobin is an integral part of the red-cell architectural structure is not certain, but evidence suggests that it is not, as it can be removed by suitable methods of lysis while the red-cell membrane still retains an apparently intact structure. It is an interesting feature of the cell that the membrane pores appear to be stretchable under hypotonic conditions to the extent of releasing the cell contents, yet the "haemolysed" cell can then revert to its original shape if it is returned to an environment of the original tonicity.

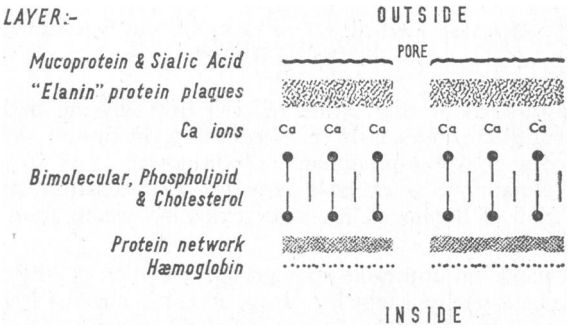

FIG. 1.—Schematic representation of the red-cell membrane.

Normal Metabolism

The red cell may be regarded as "alive" in so far as it actively metabolizes glucose with the liberation of energy (Fig. 2). Other metabolic reactions also occur, some of which have

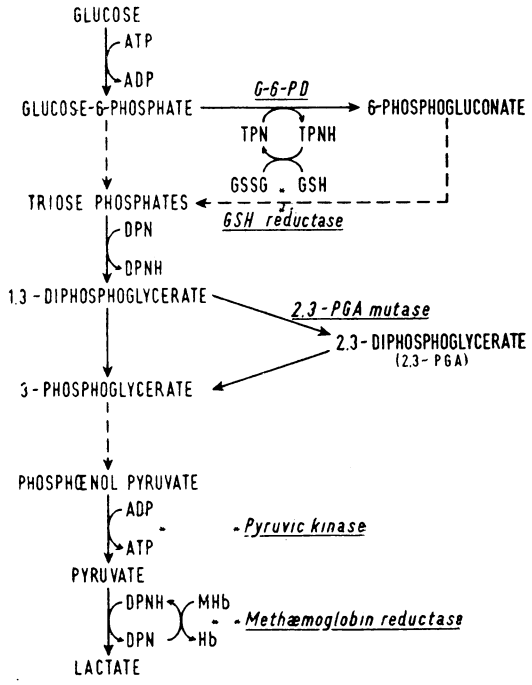

FIG. 2.—Schematic representation of metabolism in the red cell. (Dotted arrows represent abbreviated reactions.)

* Based on an inaugural lecture given at University College Hospital Medical School on 2 December 1964.

† Professor of Clinical Haematology, University College Hospital Medical School, London.

special importance in relation to the cell's function, while others appear to be remnants of the cell's previous nucleated activities. The breakdown of glucose to lactate anaerobically is accompanied by the release of energy and the formation of an important intermediate, 2,3-diphosphoglycerate. The energy released from glycolysis is stored in the form of adenosine triphosphate, but the significance of the large quantities of the phosphate ester, 2,3-diphosphoglycerate, is obscure. Ten per cent. of the glucose utilized by the red cell is oxidized by way of the pentose phosphate pathway, the principal clinical importance of this pathway being the provision of a high concentration of reduced glutathione (GSH). Glutathione can

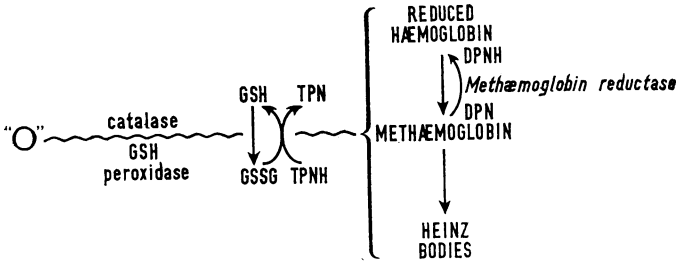

FIG. 3.—Reactions involved in the protection of haemoglobin from irreversible oxidation.

be synthesized by the mature red cell from glycine, and it is maintained predominantly in a reduced form by the reactions involving glucose-6-phosphate dehydrogenase. As is shown later, glutathione is available as a defence mechanism for the protection of haemoglobin against oxidative denaturation (Fig. 3).

Haemoglobin undergoes spontaneous oxidation to methaemoglobin at a rate of about 2% daily, and it is obvious from the point of view of cellular function that this must be prevented, or the cell would be useless to the body after half its life-span. There exists in the cell an effective system for reducing methaemoglobin. This utilizes the reduced form of diphosphopyridine nucleotide (DPNH), which is made available during glycolysis, and by its combined activity with the enzyme methaemoglobin reductase reduction is brought about. This enzyme will also react with the reduced form of triphosphopyridine nucleotide (TPNH), made available in the glucose-6-phosphate dehydrogenase reaction, but this is probably not a reaction sequence in the normal physiological state of the red cell. Apart from these reactions glycolysis also provides energy for the maintenance of the electrochemical gradients of sodium and potassium in the cell and perhaps other unknown reactions. It appears that none of the structural components of the cell is synthesized, as it has not been possible to demonstrate either protein or lipid synthesis, though cell cholesterol seems to exist in an equilibrium with that in the plasma.

Haemoglobin is an unstable molecule, and indeed its vital function of surrendering oxygen depends on this instability. Apart from being readily oxidized to methaemoglobin a variety of agents react with it, and may result in denaturation of its globin component. Such reactions would be disastrous to the efficient functioning of the red cell, and there are various protective compounds in the cell which help to prevent this event; one of these involves reduced glutathione, the SH groups of which are more reactive than those of haemoglobin (Jandl *et al.*, 1960) (Fig. 3). In addition to protecting haemoglobin reduced glutathione may also afford protection to the unsaturated bonds and sulphydryl groups of the membrane lipids and to the reduced form of diphosphopyridine nucleotide. The active reduction of methaemoglobin by its reducing enzyme is also an important factor in preventing further breakdown of haemoglobin, since the formation of methaemoglobin appears to be one of the first reactions in the chain of oxidative events which culminate in the formation of Heinz bodies.

Abnormal Structure

Only scanty information is as yet available about the structural abnormalities of the red cell, though a variety of morphological changes have long been recognized. The poikilocytosis and bizarre red-cell forms seen in pernicious anaemia, the myeloproliferative states, and certain toxic anaemias must be associated with relevant changes in chemical structure, but these await elucidation. Such changes are, however, invariably associated with diminished red-cell survival, and probably account for the haemolytic process which accompanies these disorders.

The formation of target cells is often seen when there is a disorder of haemoglobin synthesis, as in iron deficiency, thalassaemia, and other haemoglobinopathies. In general, target cells appear to have a decreased survival when transfused into compatible recipients (Prankerd, 1961b), indicating that some structural or chemical change in the cell is involved in maintaining its viability. It is interesting that the target cells found in obstructive jaundice are also less viable. Whether a defect in the protein or lipid constituents of the cell is associated with target formation is not clear.

Spherocytes are well recognized abnormal forms of the red cell, and may occur in the inherited disease hereditary spherocytosis, or in response to the action of certain antibodies and drugs. They may also be produced artificially either by heating cells or by exposing them to lysolecithin. In such cells the volume is usually reduced, the diameter decreased, and the thickness increased. In the inherited form of the disease sphering appears to be a progressive process throughout the life of the red cell, the process being accentuated by passage through the spleen (Motulsky *et al.*, 1958). Spherocytes, however produced, have a markedly diminished life-span, their site of destruction being almost entirely in the spleen (Harris *et al.*, 1957). The chemical and structural basis for this change of shape in the inherited disease has received much attention. By virtue of their thickness these cells can swell further only by a limited extent before lysis, and they therefore show increased osmotic fragility. During prolonged incubation for 24 hours they undergo greater lysis than normal cells, which may be partly corrected by the addition of extra glucose to the cell suspension (Selwyn and Dacie, 1954). Investigations of the metabolism of the inherited spherocyte have produced conflicting results, and are considered later, while abnormalities of the lipid structure of these cells have also been claimed and disputed (Allison *et al.*, 1960; de Gier *et al.*, 1961). In spite of these conflicting findings, studies of cell survival suggest that other obscure factors besides cell shape are involved in diminishing the survival of these cells (Prankerd, 1960).

One of the problems associated with analyses of red cells in haemolytic conditions is that the cell population under study is of a younger age distribution than normal, and differences in red-cell age are associated with structural changes (Prankerd, 1961a). Thus it may prove difficult to evaluate the finding of abnormal red-cell lipids in certain haemolytic states, and this has proved particularly so with the erythrocyte of paroxysmal nocturnal haemoglobinuria (van Deenen and de Gier, 1964).

Acanthocytosis is a rare condition in which an inherited lack of β-lipoprotein is associated with a haemolytic anaemia and degeneration of certain tracts of the central nervous system. The red cells in this disease appear surrounded by spiky excrescences, and analysis of the cells has shown that the ratio of lecithin to sphingomyelin in their membrane is reversed (Ways *et al.*, 1961), though the total phospholipid content is normal.

Abnormal Metabolism

Abnormal metabolism can be considered under two main categories—those defects which affect the energy potential in the cell and those which affect its reduction potential (Table I). The red cell needs energy for maintaining the functions already

described, and there is little doubt that there is a critical level of energy stored in the cell, presumably in the form of ATP, which is directly related to red-cell survival. Energy in the red cell is derived from the anaerobic degradation of glucose and stored predominantly in chemical form. The reduction potential of the cell is a different concept, and embodies the capacity of the cell to protect haemoglobin against oxidation. The mechanisms involved in this protection have been discussed.

TABLE I.—*Specific Metabolic Abnormalities of the Red Cell Associated With Haemolytic Disease*

Involving Energy Potential	Involving Reduction Potential
Pyruvic kinase deficiency 2,3-Diphosphoglycerate deficiency Triose phosphate isomerase deficiency	Glucose-6-phosphate dehydrogenase deficiency Glutathione reductase deficiency Reduced glutathione deficiency

1. Disorders of Glycolysis

The first suggestions that disorders of glycolysis in red cells might be related to haemolysis were made by Selwyn and Dacie (1954) with respect to certain inherited haemolytic disorders. Selwyn and Dacie studied the amount of haemolysis in blood after sterile incubation for 24 hours with and without the addition of extra glucose. By these criteria they distinguished two groups of disorders which they called types I and II. In type I haemolysis was only slightly increased at 24 and 48 hours, but was reduced to near normal by the addition of extra glucose at the onset of incubation, while in type II haemolysis at 24 and 48 hours was increased, but not significantly reduced by the addition of glucose. Hereditary spherocytosis behaves as a type I disorder in that the amount of haemolysis is reduced by glucose to about the same extent as it is with normal cells. Hereditary elliptocytosis usually behaves similarly, as do the non-spherocytic haemolytic anaemias associated with glucose-6-phosphate dehydrogenase deficiency, reduced glutathione deficiency, and 2,3-diphosphoglycerate deficiency also. Type II disorders appear to consist predominantly of pyruvic kinase deficiency (Tanaka *et al.*, 1962), most of the cases showing similar biochemical characteristics (de Gruchy *et al.*, 1960).

A number of haemolytic anaemias are now known which are characterized by defective glycolysis. Suspicion has centred on hereditary spherocytosis for some years, but no clearly defined abnormality has been demonstrated. Prankerd *et al.* (1954) originally thought that intracellular phosphorylation was abnormal, but the changes they observed, though found also by other workers, have not been universally confirmed (Zirpursky *et al.*, 1962 ; Keitt, 1965), and it is likely that the abnormalities were non-specific effects of cell damage. In this respect Gomperts (unpublished) has recently found that changes in lactate concentration profoundly affect the concentrations of intracellular phosphate esters. Recent work by Jacob and Jandl (1964) has suggested that ATP turnover is increased in these cells possibly as a result of an increased permeability to potassium which creates an increased demand for intracellular energy.

Pyruvic kinase deficiency leads directly to impairment of ATP synthesis, and low ATP levels are found in affected cells (Tanaka *et al.*, 1962 ; Bowdler and Prankerd, 1964). The disorder is inherited as a recessive character, and results in a haemolytic anaemia dating from birth and predominantly unresponsive to splenectomy. There is no specific morphological defect of the red cells, but they do show a pronounced tendency to crenate in smears.

Two further defects in glycolysis have been described ; one probably involves 2,3-diphosphoglyceromutase (Bowdler and Prankerd, 1964), the enzyme converting the 1,3 to the 2,3-diphosphate ester. In a family studied with this disorder its transmission appeared to occur as a dominant character resulting in a moderately severe haemolytic anaemia from birth. A rather similar abnormality was described by Lelong *et al.* (1961). Another glycolytic defect has been described recently in association with a congenital haemolytic anaemia, involving the enzyme triose phosphate isomerase (Schneider *et al.*, 1965).

2. Disorders of Reduction Potential

If we regard the reduction potential of the red cells as its latent ability to protect haemoglobin against oxidation, then several defects in the system occur which result in shortened red-cell survival. The best known of these is glucose-6-phosphate dehydrogenase deficiency (G6-PD) (Beutler, 1959 ; Kellermeyer *et al.*, 1962).

A considerable amount of information has now accumulated about this defect. The original description of this disorder in American negroes focused on a haemolytic disorder, inherited as a sex-linked incomplete dominant character, in which haemolysis occurred predominantly in males after exposure to the antimalarial primaquine. This disorder had previously been demonstrated to be associated with the formation of Heinz bodies in the red cells and with a low level of red-cell glutathione. In addition, it had also been shown that the glutathione of an affected individual's red cells was less stable than normal in the presence of acetylphenylhydrazine. Thus it appeared that the enzyme defect slowed the reaction pathway of glucose oxidation to pentose phosphates ; as a result of this the red cell was less efficient in reducing oxidized glutathione, and the fall in reduced glutathione that resulted on exposure of the cell to potential oxidizing agents failed to protect the cell haemoglobin from complex oxidative denaturation reactions with the incriminated compound. The denatured haemoglobin accumulated in the cell in the form of inclusion bodies and in some way, as yet unknown, led to the increased cell destruction observed. Though this seems a rational sequence of events, evidence suggests that it is much more complicated than at first appears.

A large number of different reagents have now been incriminated in this type of haemolytic disorder (Table II). In addition, it is now known that the type and degree of enzyme defect varies in different races. For instance, while the enzyme defect in affected negroes appears to result in enzyme concentrations of about 10% of normal with limited but fairly widespread susceptibility to the agents listed in Table II, in Southern Caucasians the enzyme defect appears to be more severe and the susceptibility to the listed agents more widespread. In Northern Caucasians complete deficiency of the enzyme may be found associated with severe haemolysis and anaemia from birth in the absence of any of the offending agents in Table II (Shahidi and Diamond, 1959 ; Bowdler and Prankerd, 1964). Though haemolysis appears to be episodic in negroes and Southern Caucasians there is in fact a shortened red-cell survival between episodes, but this is only mild and does not lead

TABLE II.—*Chemical Agents Reported to Cause Destruction of Red Cells Deficient in Glucose-6-phosphate Dehydrogenase*

8-Aminoquinoline	Primaquine Pamaquin Pentaquine
Sulphonamides	Sulphanilamide Sulphamethoxypyridazine Salicylazosulphapyridine Sulphacetamide
Sulphones	Sulfoxone Diaminodiphenylsulphone Thiazosulphone
Nitrofurans	Nitrofurantoin Furazolidone Nitrofurazone
Acetylphenylhydrazine Acetanilide Antipyrine Aminopyrine Acetylsalicylic acid Para-aminosalicylic acid Phenacetin	Naphthalene derivatives Methylene blue Ascorbic acid Probenecid Quinidine Trinitrotoluene Fava bean

to anaemia. In addition to these differences there appear to be basic differences in the type of enzyme defect in different races. Thus Northern Caucasians appear to have a molecularly different protein with less enzyme activity than that of normals, while in Southern Caucasians and negroes what appears to be a normal enzyme, molecularly, is present in diminished amounts within the cell (Kirkman and Crowell, 1963). There are many other peculiarities to this group of disorders which cannot be discussed here, but one interesting feature is the self-limiting nature of the haemolytic reaction to the agents in Table II shown by negroes and Southern Caucasians. This seems to be a result of the fact that glucose-6-phosphate dehydrogenase activity decreases with red-cell age and that a certain critical deficiency of the enzyme is required before the usual therapeutic concentration of the offending drug is effective in leading to haemoglobin breakdown.

A number of other defects involving glutathione have also been described in association with the formation of Heinz bodies and haemolytic anaemia. In one, haemolytic anaemia, associated with severe lack (90%) of glutathione in the red cell and therefore a deficiency of the reduced form, occurred in a family as a recessive character (Oort *et al.*, 1961). In these individuals the red-cell glucose-6-phosphate dehydrogenase activity was normal. A further disorder has been described in association with haemolytic anaemia in which there was a deficiency of the enzyme glutathione reductase (Löhr and Waller, 1962). This deficiency led to a reduction of reduced glutathione in the red cells and glutathione instability. Glucose-6-phosphate dehydrogenase activity was normal, and there was no reduction in the total cell glutathione.

Abnormalities of the Haemoglobin Molecule

It may or may not be correct to regard haemoglobin as an integral part of red-cell structure, but there are certain disorders in which the occurrence of Heinz bodies within the cells results in haemolysis and suggests that a change in molecular configuration of haemoglobin disorganizes the physical state of the cell membrane. Some of the abnormalities of the haemoglobin molecule which result in haemolytic anaemia are now very familiar, but apart from the case of Hb S, which results in characteristic distortion of the red-cell shape, the mechanism through which the abnormal haemoglobin results in haemolysis is not always clear.

Recently, certain haemolytic syndromes with fairly well defined clinical features have been found to be associated with abnormalities of the haemoglobin molecule which may or may not be obvious from the conventional methods of analysis. These disorders take the form of haemolytic anaemias dating from birth and often associated with the passage of dark urine. The peripheral blood often shows macrocytosis, and in some instances hypochromia. Heinz bodies may be present in fresh cells or only after prolonged incubation; they are also seen after incubation with acetylphenylhydrazine. The appearance of dark urine is usually intermittent and due to the excretion of a mesobilifuchsin. The haemolytic disorder seems to be due to the inheritance of an unstable haemoglobin which may undergo spontaneous denaturation. This instability may be sufficient to produce abnormal electrophoretic mobility as in the case of Hb Zurich, without pretreatment of the cells (Frick *et al.*, 1962; Bachmann and Marti, 1962). In other instances incubation of the cells at normal or acid *p*H may be required to alter the physical behaviour (Dacie *et al.*, 1964; Huehns and Shooter, 1965). These disorders, like that due to the occurrence of Hb H, may be aggravated if the patient is exposed to some of the drugs in Table II. Most of these disorders show normal osmotic fragility, but autohaemolysis may be in keeping with either type of conventional pattern.

It is interesting how our current views on haemolytic anaemia are developing. Apart from haemolysis due to extracorporeal factors it is becoming increasingly important to look carefully at the metabolic functioning and chemical constitution of the red cell in order to find the cause of a haemolytic process. Such investigations are becoming more and more rewarding, but there are still many problems to be examined and explained.

Summary

A rational approach to certain haemolytic anaemias, particularly those which are inherited, is through the structure and metabolism of the red cell, and a number of abnormalities of this nature have now been described which account to a variable extent for the haemolysis in these diseases. The structural changes involved are likely to be associated mainly with the lipid composition of the cell, although an abnormal membrane protein remains a possibility. Metabolic defects involve either a fall in the energy or reduction potential of the cell and changes of each type have been described. Finally there have been further developments in the field of abnormal haemoglobins in which chemically unstable proteins have been found, some of which undergo spontaneous denaturation.

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