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Volume 10

Editor:
MICHAEL P. TOMBS
Special Professor, University of Nottingham

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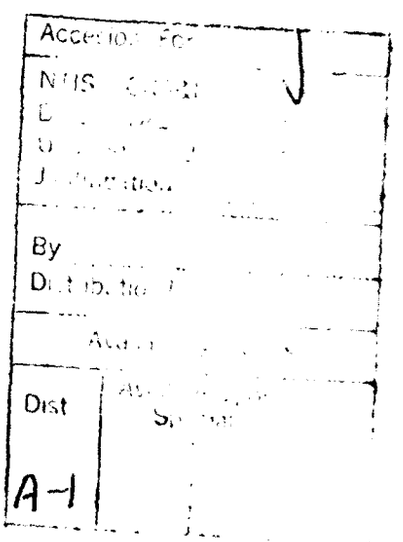

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Blood Substitutes: Engineering the Haemoglobin Molecule

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Introduction

Structural analogues of natural proteins are being designed through techniques in protein engineering to test basic theories of protein structure and function and to create new products for therapeutic or industrial applications. One goal of this relatively new area in biotechnology is to create a clinical substitute for human blood by engineering the haemoglobin molecule.

Blood is a complex fluid containing many different types of cells with distinct functions. A clinical 'blood substitute', as it is defined here, will not replace every function of blood, but will maintain blood pressure by providing fluid volume and will prevent cell death by delivering oxygen. All blood components contribute to total blood volume, but only haemoglobin inside erythrocytes delivers the oxygen necessary to sustain life by reversibly binding dissolved oxygen in the lungs and releasing oxygen in the tissues.

The majority of solutions available now as clinical substitutes for blood are plasma volume expanders that do not provide oxygen transport. One exception is the perfluorocarbon, Fluosol® (Green Cross Corporation), which has been approved by the US Food and Drug Administration (USFDA) for clinical application. Its use, however, has been restricted to coronary angioplasty after it failed to demonstrate efficacy in the treatment of life-threatening anaemia (Gould *et al.*, 1986a). The standard electrolyte solutions, Ringer's lactate and physiological saline, expand blood volume immediately after infusion but then diffuse readily across the endothelium and out of the vasculature. Alternatively, colloidal plasma expanders, such as plasma,

Abbreviations: bis-PL(P)$_4$, bis-pyridoxal tetraphosphate; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; BSE, bovine spongiform encephalitis; cGMP, guanosine 3',5'-cyclic monophosphate; DBBF, 3,5-(dibromosalicyl)fumarate; 2,3-DPG, 2,3-diphosphoglycerate; EDRF, endothelium-derived relaxing factor; GTP, guanosine 5'-triphosphate; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; mRNA, messenger ribonucleic acid; NADPH, nicotinamide adenine dinucleotide phosphate; NFPLP, 2-nor-2-formylpyridoxal 5'-phosphate; P_{50}, O_2 partial pressure at which haemoglobin is 50% saturated; PLP, pyridoxal 5'-phosphate; pO_2, O_2 partial pressure; POE, polyoxyethylene; ROOH, hydroperoxides; ROO$\cdot$, peroxyl radical; USFDA, US Food and Drug Administration.

Dextran-70, Hetastarch, or albumin solutions, exert an oncotic pressure so that vascular volume and blood pressure are maintained, but none of these solutions can support life in the absence of red blood cells. Even when blood volume is preserved, severe loss of red cell mass will be fatal as a result of oxygen deprivation, cell death, and organ failure.

Until an oxygen-carrying resuscitation fluid becomes available, transfusions of red blood cells or whole blood will continue to be the standard method of treatment to conserve oxygen delivery. This clinical necessity demands an organized system to collect blood, either autologously or through donations for storage in blood banks. The blood banking system is currently burdened by several factors: practical limitations in the delivery of blood, the short shelf-life of liquid blood (i.e. 42 days), requirements for type-specific blood products to prevent incompatible transfusions, and growing concerns over viral infection of the blood supply. In some countries, transfusions are now impossible or dangerous because of inadequate storage facilities and the epidemic contamination of blood supplies by the human immunodeficiency virus (HIV).

Approximately 35 transfusion-related deaths that result from errors in blood-group typing or bacterial infection are reported to the USFDA each year (Klein, 1989). Transmission of the hepatitis virus is under-reported and may occur in as many as 5% of the ~13 million units transfused annually (Klein, 1989). But in the last decade, the key driving force in the development of blood substitutes has been the fear of HIV transmission. It has been estimated that about one in 42 000 transfusions of blood screened as HIV antibody-negative results in transmission of the virus (Busch *et al.*, 1990).

To avoid the problems associated with blood transfusions and to meet the need for pre-hospital emergency resuscitation fluids, acellular haemoglobin solutions are being developed as clinical blood substitutes. Haemoglobin has colloid osmotic activity and a high capacity for oxygen transport, it can be isolated from other components of blood and rid of viruses, and can be stored for years without loss of function.

Applications

Blood substitutes are intended for a variety of clinical uses, for all of which their efficacy is unproven. Emergency resuscitation of haemorrhagic shock, short-term support of anaemic patients, angioplasty, isovolemic haemodilution, treatment of vaso-occlusive sickle-cell crises, and administration as an adjunct to autologous blood donations all have been suggested as possible therapeutic uses of these materials. Demonstrations of efficacy await the availability of materials suitable for testing. Non-clinical applications also have been proposed for these high oxygen-capacity solutions for use in tumour therapy and organ preservation.

Early studies with haemoglobin-based blood substitutes

Cell-free haemoglobin solutions were first administered to humans at the end of the nineteenth century. Serious side-effects were not noted with small amounts of haemoglobin, but the solutions were unstable and the experimental results were difficult to interpret (Von Stark, 1898; Sellards and Minot, 1916). In the 1930s, Amberson performed complete exchange transfusions in cats with haemoglobin solutions that were tolerated well (Mulder *et al.*, 1934; Amberson, 1937). Those studies led this group to conduct clinical trials in humans that ended with mixed results (Amberson, Jennings and Rhode, 1949). Some anaemic patients experienced erythropoietic stimulation, but one patient in shock with post-partum haemorrhage developed sepsis and oliguria and died with renal failure. Amberson pointed out that renal failure was not unexpected in this patient, even without administration of haemoglobin. Nevertheless, this result emphasized the possible dangers of complications with haemoglobin solutions. Deleterious side-effects, including oliguria, hypertension, and slowed heart beat continued to be observed during early human studies. It was clear that new methods for the evaluation of haemoglobin structure, function, stability, and purity were required before further progress in haemoglobin-based blood substitutes could be achieved (*for a review, see* Winslow, 1992).

The haemoglobin molecule: structure, function and stability

THE HAEMOGLOBIN MOLECULE

Human haemoglobin is a globular protein of 64 kDa consisting of two similar pairs of α- and β-subunits related symmetrically on a dyad axis. Each subunit has associated with it a ferroprotoporphyrin IX (haem) prosthetic group. The unlike subunits have a strong interaction with one another within an $\alpha\beta$ dimer, and two $\alpha\beta$ dimers combine in a weaker interaction to form the symmetrical $\alpha_2\beta_2$ tetramer (*Figure 1*).

The subunits are primarily helical in tertiary structure: the turns between helices are the only non-helical segments in the molecule. The α-subunits contain 141 amino acids arranged in seven helices: A, B, C, E, F, G and H. The β-subunits contain 146 amino acids and have an extra D helix. The functional importance of the difference in helical content of the α- and β-subunits is not completely understood, but β-tetramers are non-cooperative and unstable. Recently it has been shown by genetic engineering that forcing a D helix into the α-subunits or deleting the D helix from the β-subunits has little effect on O_2 binding (Komiyama *et al.*, 1991).

The interaction between haem and globin is of particular concern in the design of a haemoglobin-based blood substitute. Apoglobin is insoluble in physiological media, and free haem is toxic. Haem is positioned between the E and F helices by hydrophobic and hydrophilic interactions with globin residues (Perutz *et al.*, 1968). The E helix forms a distal pocket over the haem, where ligand binding occurs to the sixth-coordination site of the haem

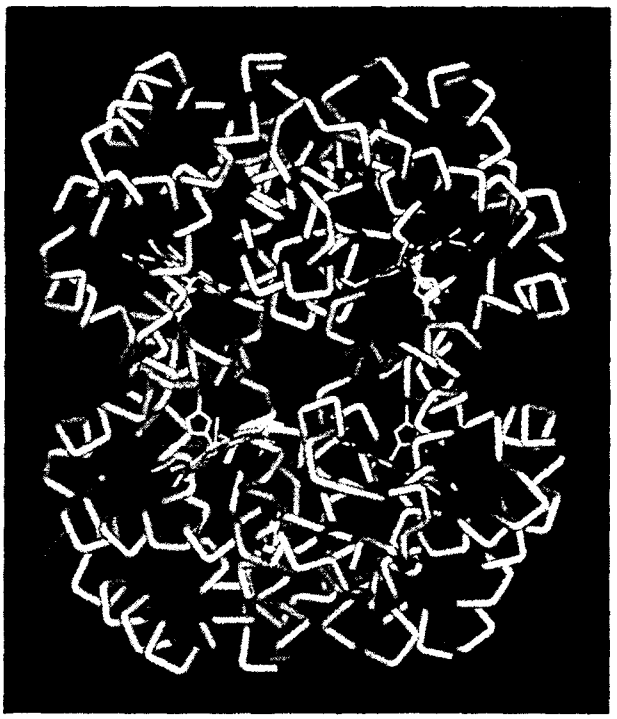


Figure 1. The $\alpha_2\beta_2$ tetramer of haemoglobin shown along the symmetrical dyad axis of the molecule. The α-carbon helices and haem coordinated to proximal histidine (F8) residues are represented (from Lynn Ten Eyck of the Supercomputer Center, University of California, San Diego).

iron atom, and the fifth-coordination site is bound to a histidine residue at the eighth position of the F helix (i.e. the proximal histidine at position F8).

The oxidation state of the haem iron varies from Fe^{2+} in fully reduced ferrous haemoglobin, to Fe^{3+} in ferric haemoglobin (i.e. methaemoglobin), and Fe^{4+} in ferryl haemoglobin. The gaseous ligands, molecular oxygen (O_2), carbon monoxide (CO), and nitric oxide (NO), all bind to ferrous haem, which is the physiologically functional state of haemoglobin. O_2 and CO do not bind to ferric or ferryl haem. NO does bind to ferric haem but with lower affinity than to the ferrous state.

THE DYNAMICS OF THE HAEMOGLOBIN MOLECULE

The multi-subunit molecule undergoes ligand-linked tertiary and quaternary transitions that increase the affinity for ligand as ligand binds. This haem–haem interaction, or positive cooperativity, makes haemoglobin an efficient O_2-carrying molecule, which can be demonstrated in a comparison between the O_2 binding curves of haemoglobin and the single-subunit, non-cooperative, O_2-storage haem protein, myoglobin (*Figure 2*). The low- and high-affinity conformations, which are represented by the two end structures

(i.e. deoxyhaemoglobin and oxyhaemoglobin, respectively) are referred to as the T (for tense) and R (for relaxed) states.

The structural mechanism of the haemoglobin molecule that accounts for the different O_2 affinities in the T and R states was first proposed by Perutz based on the unliganded and fully liganded crystallographic structures (Perutz, 1970). Effects both distal and proximal to the haem were proposed. Later refinement of these two end structures at higher resolution lent further support to Perutz's original proposals (Shaanan, 1983; Fermi *et al.*, 1984). In β-subunits, specific distal residues in the E helix (i.e. β(E7) histidine and β(E11) valine) are positioned such that ligand access to the haem iron atom is blocked in the T state (Fermi *et al.*, 1984). In R-state β-subunits, the E helix is moved across the face of the haem, the β(E7) and β(E11) residues are displaced from their T-state positions, and the distal haem pocket is more open and accessible to ligands (Shaanan, 1983). In α-subunits, the orientation of the proximal histidine in the T-state F helix appears to restrict movement of the haem iron into the plane of the porphyrin (Fermi *et al.*, 1984). In R-state α-subunits, the F helix and proximal histidine are tilted less relative to the haem groups, the iron atom is coplanar with the porphyrin ring, and the sixth-coordination position is more accessible to ligands (Shaanan, 1983). A diagram of the α-subunit haem pocket in oxyhaemoglobin shows the positions of residues E7, E11 and F8 (*Figure 3*).

To describe haemoglobin cooperativity, mathematical models of concerted (Monod, Wyman and Changeux, 1965) or sequential (Koshland, Nemethy and Filmer, 1966) allosteric mechanisms have been formulated. Recent experiments support a functional mechanism in which contributions from both allosteric models are included. Sequential cooperativity occurs during ligand binding within a given quaternary structure, and a concerted quaternary transition occurs after ligands are bound across the dimer interface (Ackers *et al.*, 1992).

Techniques to evaluate haemoglobin structure

Tryptic peptide analysis, isoelectric focusing, gel electrophoresis, molecular-sieve, and ion-exchange chromatography are standard biochemical techniques that are used to determine the molecular size, charge and primary sequence of haemoglobin molecules. Three-dimensional structure is analysed primarily from X-ray diffraction patterns of haemoglobin crystals. New techniques in computer modeling of protein molecules are based on X-ray atomic coordinates and energy-minimization calculations. Moelcular dynamics calculations are used to predict the contributions of individual amino acids and solvent molecules to free energy changes within the haemoglobin molecule. These models require vast computer processing time, however, and only a few picoseconds of simulated haemoglobin dynamics have been accomplished (Gao *et al.*, 1989).

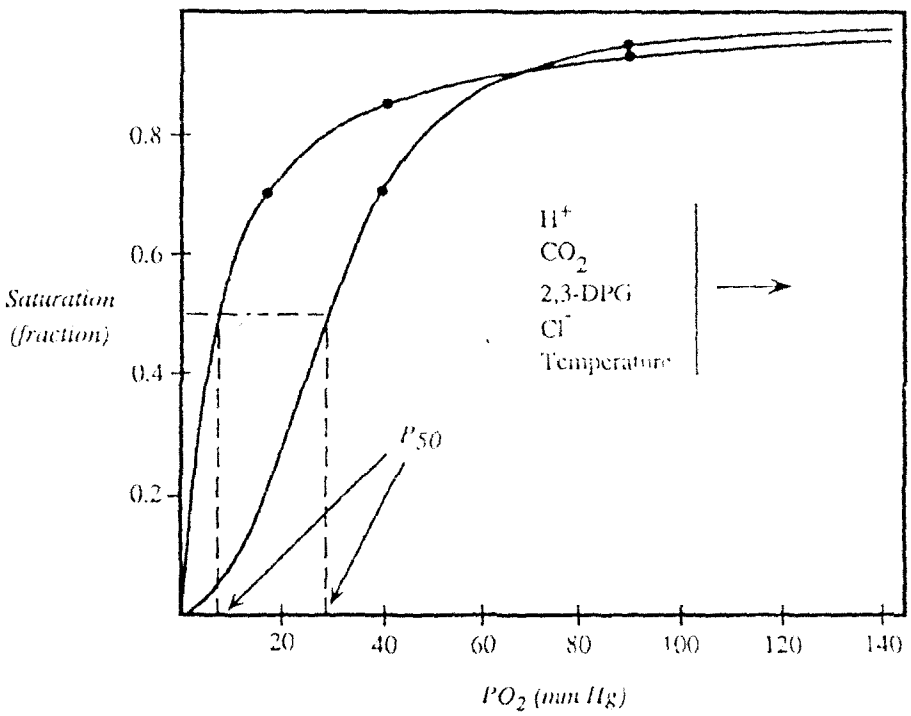


Figure 2. Equilibrium oxygen binding curves of intracellular haemoglobin (right curve) and myoglobin (left curve). P_{50} is the pO_2 at which haemoglobin is half-saturated with oxygen. Effectors that alter the position of the haemoglobin equilibrium are indicated. Note from the points on the graph that although both curves allow saturation of about 95% at normal alveolar oxygen (~90 mm Hg), intracellular haemoglobin releases 23% of its oxygen and myoglobin only 7% of its oxygen at a venous pO_2 of 40 mm Hg. In this example, myoglobin delivers 23% of its bound oxygen at a venous pO_2 of ~16 mm Hg (modified from Winslow, 1992, with permission)

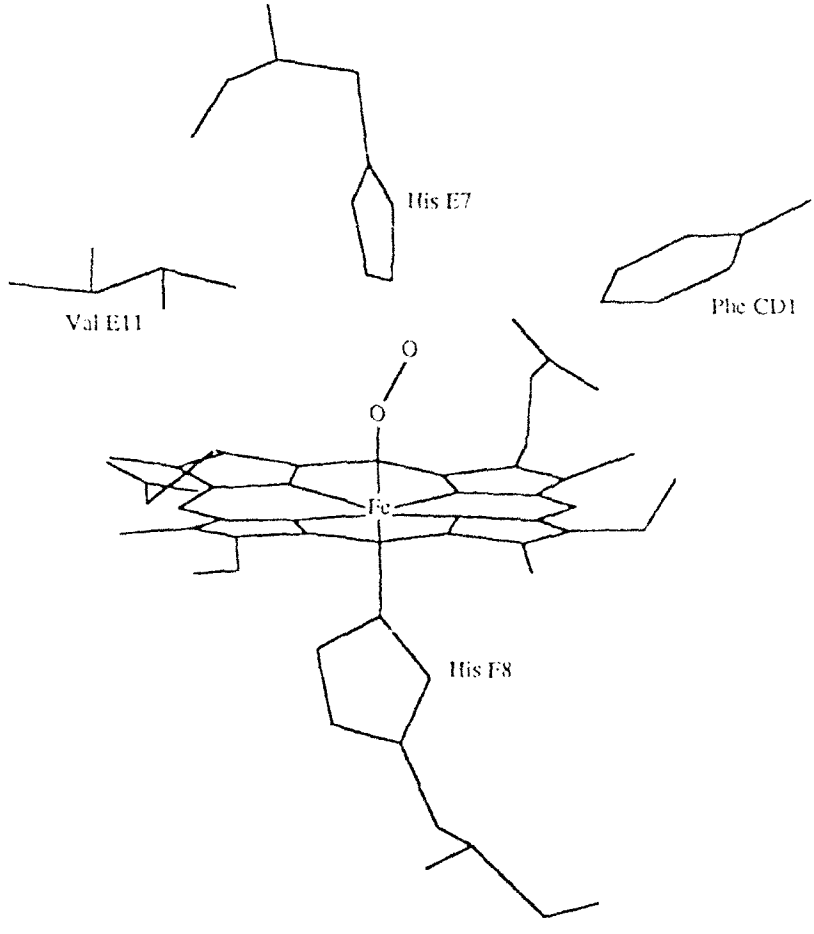
Techniques to evaluate haemoglobin function

EQUILIBRIUM ANALYSIS OF OXYGEN BINDING

Oxygen binding to haemoglobin can be measured by the change in the haemoglobin optical spectrum, usually at a single wavelength, as a function of the partial pressure of O_2 (pO_2) (Gill, 1981; Imai, 1981). The pO_2 at which haemoglobin is 50% saturated is referred to as the P_{50} and provides an empirical measure of O_2 affinity. The P_{50} does not offer any structural or functional information, however, and complete binding curves must be fitted to mathematical models to interpret ligand binding mechanistically.

The allosteric model of Monod, Wyman and Changeux (MWC) is given by

$$Y = \frac{LK_T x(1+K_T x)^3 + K_R x(1+K_R x)^3}{L(1+K_T x)^4 + (1+K_R x)^4} \qquad (1)$$



α subunit

Figure 3. Diagram of the α-subunit haem pocket. The haem ring is shown with bound O_2. Distal residues phenylalanine (Phe) CD1, histidine (His) E7, and valine (Val) E11 and proximal histidine (His) F8 are represented (from Ken Johnson of the Department of Biochemistry and Cell Biology, Rice University).

where L is the allosteric equilibrium constant as the ratio of the concentration of deoxyhaemoglobin in the T and R states (i.e. $[T_0]/[R_0]$), and K_R and K_T are the equilibrium binding constants for the R and T states, respectively (Monod, Wyman and Changeux, 1965). Since L describes the equilibrium between conformational states, this model can be used to derive an allosteric mechanism of haemoglobin cooperativity. However, it is generally found not to be able to fit the haemoglobin O_2 equilibrium curve without systematic deviation, perhaps as a result of sequential cooperativity of O_2 binding within a given quaternary structure (*for example, see* Vandegriff *et al.*, 1989; Ackers *et al.*, 1992).

The Adair equation describes the reaction of four molecules of O_2 binding per haemoglobin tetramer, in which individual equilibrium constants (K_1 to K_4) are determined for each binding step,

$$Y = \frac{a_1x + 2a_2x^2 + 3a_3x^3 + 4a_4x^4}{4(1 + a_1x + a_2x^2 + a_3x^3 + a_4x^4)} \quad (2)$$

where Y is fractional saturation, x is the partial pressure of O_2, and a_1 to a_4 are the Adair constants that are the products of the individual, step-wise binding constants (i.e. $a_1=K_1$, $a_2=K_1K_2$, $a_3=K_1K_2K_3$ and $a_4=K_1K_2K_3K_4$: Adair, 1925). Since the Adair equation is a mass-action calculation, it does not confer a theoretical association between haemoglobin structure and allosteric mechanisms directly, although with certain assumptions, the first and last step-wise binding constants (i.e. K_1 and K_4) can be related, respectively, to allosteric T and R conformations (*for example, see* Nagai *et al.*, 1987). It is difficult, however, to determine all four binding constants with high precision from an equilibrium curve measured at a single wavelength.

The inability to fit O_2 equilibrium curves with a high degree of confidence is partly due to difficulties in measuring the total range of O_2 binding to haemoglobin without requiring certain assumptions. For optical measurements of ligand binding, the primary assumption has been that there is a linear relationship between the fractional degree of haemoglobin saturation and the fractional change in the haemoglobin absorption spectrum. This assumption is probably an oversimplification for experimental observations. It does not account for optical transitions other than those related to O_2 binding, such as methaemoglobin formation or spectral changes due to subunit differences, dimer formation, or the T-to-R conformational transition. As a result, fitting these models to fractional data based on an assumption of linear optical effects will not yield precise values for thermodynamic parameters. To overcome this problem, new rapid-scanning optical techniques are being developed to measure complete spectra of haemoglobin during equilibrium binding reactions, from which all optical transitions above the noise of the measurement can be resolved by singular value decomposition (Ownby and Gill, 1990; Vandegriff *et al.*, 1992).

KINETIC ANALYSIS OF OXYGEN BINDING

Another way to evaluate equilibrium binding constants is from the ratio of kinetic association and dissociation constants (i.e. $K=k'/k$, where k' and k are O_2 association and dissociation rate constants, respectively). Equilibrium binding constants vary through a change in either the rate of ligand association, the rate of ligand dissociation, or both. For example, a significant effect on O_2 reactivity with haemoglobin will not be reflected in an equilibrium constant if the ratio of kinetic constants remains unaltered. Thus, the kinetics of a reaction give dynamic information about mechanisms of ligand binding and can be used to study effects at the reactive haem sites in more detail.

The kinetics of ligand binding to R-state haemoglobin are measured by

ligand displacement and partial photolysis reactions (*for a review, see* Olson, 1981; *for examples, see* Mathews *et al.*, 1989; Vandegriff *et al.*, 1991b). The kinetics of ligand binding to the T state are more difficult to measure because of the high rates of O_2 dissociation and the transition to the R state, but an evaluation of the rates of CO binding to T-state native and site-directed haemoglobin mutants has been accomplished (Mathews *et al.*, 1991).

BINDING OF NON-HAEM LIGANDS

In addition to O_2 delivery, carbon dioxide (CO_2) transport and acid-base balance are important physiological functions of haemoglobin. Between 10 and 20% of the CO_2 produced during respiratory metabolism is transported to the lungs for exhalation bound to haemoglobin as a carbamino compound. The balance of CO_2 is hydrated and transported as the bicarbonate ion (HCO_3^-) in plasma.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (3)$$

The role of protons in this reaction is linked to the Bohr effect of haemoglobin. That is, as O_2 is taken up by haemoglobin in the lungs, protons are released. The decrease in pH drives the CO_2/HCO_3^- equilibrium toward carbonic acid (H_2CO_3), and H_2O and CO_2 are produced in the reaction catalysed by carbonic anhydrase. The opposite reaction occurs in tissues. As O_2 is released from haemoglobin, protons bind to haemoglobin, pH rises, and the equilibrium in *Equation 3* favours hydration of CO_2 and the formation of HCO_3^-.

Binding of non-haem ligands to haemoglobin [e.g. H^+, the chloride ion (Cl^-), CO_2 and 2,3-diphosphoglycerate (2,3-DPG)] are measured by the thermodynamic linkage between O_2 binding at the haem (homotropic binding) and non-haem ligand binding (heterotropic binding; Wyman, 1964). The O_2 equilibrium curve shifts as a function of the concentration of heterotropic ligand (e.g. Bohr effect = $\triangle \log P_{50}/\triangle pH$; *see Figure 2*), and heterotropic effects on individual binding constants are computed by the linkage function.

Stability of the haemoglobin molecule

Oxidation of the haem iron atom to the ferric (Fe^{3+}) or ferryl (Fe^{4+}) states is the first step in the pathway to haemoglobin degradation. The process continues through haem loss or irreversible haemochrome formation (in which the sixth-coordinate haem site binds to an amino acid side-chain in a distorted globin) and ends with protein denaturation and precipitation (*Figure 4*).

Methaemoglobin formation creates at least three problems in the development of haemoglobin-based blood substitutes: (1) methaemoglobin does not bind O_2; (2) the oxidation of ferrous haemoglobin produces activated oxygen as the superoxide anion ($O_2^- \cdot$), which permutates to two other highly reactive oxidative molecules, hydrogen peroxide (H_2O_2) and the hydroxyl radical

(HO·); and (3) methaemoglobin has a greater tendency to release haem (*see below*).

The mechanism of auto-oxidation as originally proposed by Caughey and co-workers invokes an outer sphere mechanism in which an electron is transferred from ferrous haem iron (Fe^{2+}) to O_2 in the haem pocket to produce ferric haem (Fe^{3+}) and O_2^{-}· (Wallace *et al.*, 1982). Thus, deoxyhaemoglobin is oxidized by unliganded O_2, and the rate of methaemoglobin formation will depend on the haemoglobin–O_2 affinity and the ability of nucleophiles, such as Cl^-, to enter the haem pocket. The correlation between O_2 affinity and the rate of auto-oxidation has been verified with site-directed mutant myoglobins (Springer *et al.*, 1989) and with several chemically modified haemoglobins (Macdonald *et al.*, 1991). More recent analyses of the auto-oxidation reaction of myoglobin mutants leads to these three conclusions:

1. Deoxymyoglobin is oxidized by unliganded O_2 in the haem pocket at low O_2 concentrations but by direct dissociation of O_2^{-}· at high O_2 concentrations.
2. The distal histidine of myoglobin inhibits auto-oxidation by stabilizing bound O_2 through a hydrogen bond between the E7 histidine residue and bound O_2. [A hydrogen bond also forms between E7 histidine and bound O_2 in the α-subunits of oxyhaemoglobin (Shaanan, 1983)].
3. The rate of auto-oxidation depends on the volume of the haem pocket and, thus, the accessibility to the haem of proton donors, such as H_2O (Brantley *et al.*, 1992).

Since the haem-pocket structures of myoglobin and haemoglobin subunits are similar, these findings may apply to the mechanism of auto-oxidation of haemoglobin as well.

The interaction between haem and globin is dependent on the oxidation state of the haem iron atom; ferrous haem has a much higher affinity for globin than does ferric haem (Bunn and Jandl, 1968). O_2^{-}· produced during auto-oxidation may contribute to haemoglobin degradation through oxidative damage of globin residues (Winterbourn, McGrath and Carrell, 1976).

Haemoglobin inside versus outside the red blood cell

Certain manipulations of the haemoglobin molecule are required before it can be useful as a cell-free, haemoglobin-based blood substitute. Important functional differences between haemoglobin packaged in its native environment inside the red blood cell compared with haemoglobin in a cell-free solution define these requirements:

1. Vertebrate haemoglobin is at very high concentration inside the erythrocyte (e.g. ~20 mM in haem inside the human red cell), which favours the tetrameric form of haemoglobin. Diluted outside the red blood cell, haemoglobin tetramers tend to dissociate into dimers. Dimeric half-

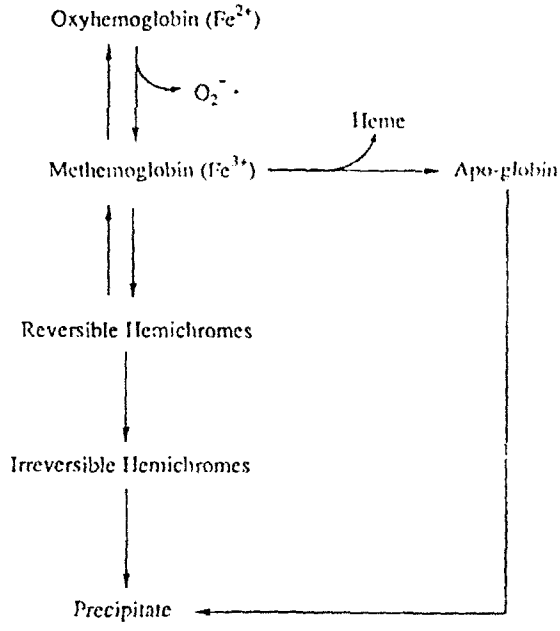


Figure 4. Haemoglobin degradation pathway. Auto-oxidation of oxyhaemoglobin yields methaemoglobin and $O_2 \cdot$. Methaemoglobin can either lose its haem to produce haem-depleted apoglobin or be converted to reversible hemichromes, followed by conversion to irreversible hemichromes. Both irreversible hemichromes and apoglobin subunits will precipitate as denatured protein.

molecules are not cooperative and are small enough to be filtered through the kidneys.

2. An enzymatic reducing system maintains methaemoglobin at <2–3% of total haemoglobin inside the erythrocyte, which conserves maximal O_2 delivery. In the absence of a reducing system, the percentage of methaemoglobin will increase to a new equilibrium redox level that will depend on the other redox molecules in solution with haemoglobin.
3. Inside the red blood cell, a by-product of glycolytic metabolism, 2,3-DPG, binds to haemoglobin as an effector molecule, reduces the O_2 affinity of haemoglobin, and enhances O_2 release in tissues. In the absence of 2,3-DPG, only 2–5% of O_2 bound to haemoglobin is released at the usual mixed-venous pO_2 of 40 mm Hg, compared with ~20% in the presence of 2,3-DPG.

PRINCIPLES OF OXYGEN TRANSPORT

Isolating haemoglobin from the red blood cell also will have significant effects on the physiological properties of blood flow and O_2 transport. In higher animals, O_2 transport from the air to mitochondria is controlled by two physico-chemical events: (1) diffusion of O_2 to and from haemoglobin inside erythrocytes, and (2) chemical reaction of O_2 with haemoglobin.

In vivo, O_2 diffuses from air in the alveolar sacs of the lungs, through

alveolar epithelial and capillary endothelial membranes, through mixed and unstirred plasma layers, to its site of chemical reaction with haemoglobin inside erythrocytes. The chemical reaction between O_2 and haemoglobin to form oxyhaemoglobin removes O_2 from solution inside the red cell and creates a steep gradient for O_2 flux in the lungs. When O_2 is released in tissues, it diffuses down its concentration gradient, the height of which is determined by the unbound concentration of O_2 in red blood cells because the O_2 concentration at its site of reduction at cytochrome oxidase is close to zero.

Oxygen diffusion to cell-free haemoglobin will be under different physical constraints. Instead of a steep and focused O_2 gradient leading from the red blood cell and through the diffusional barrier of the plasma layer surrounding the red cell, dilute cell-free haemoglobin will be exposed directly to the entire surface area of a capillary vessel wall.

According to Fick's law of diffusion, O_2 flux (k_{flux}) is given by the product of the O_2 diffusing capacity (D_{O_2}) and the O_2 concentration gradient. This relationship as it is applied to O_2 transport is based on the hypothesis that O_2 uptake can be limited by diffusion, and the rate of diffusion is proportional to the O_2 gradient. The unbound concentration of O_2 in solution is usually measured as pO_2 and the gradient as a difference in partial pressures.

Applying Fick's law to oxygenation in the lungs, the O_2 gradient will be equal to the difference between the pO_2 in the alveolar gas spaces ($pO_{2(lung)} \approx 100$ mm Hg) and the mean pulmonary capillary pO_2 ($pO_{2(capillary)}$), which, in a healthy lung with normal blood flow, will be a function of the position (i.e. P_{50}) and shape (i.e. cooperativity) of the haemoglobin–oxygen equilibrium curve.

$$k_{flux} = D_{O_2} \cdot (pO_{2(lung)} - pO_{2(capillary)}) \quad (4)$$

For deoxygenation, k_{flux} will be determined primarily by the mean muscle capillary pO_2 ($pO_{2(capillary)}$), since the pO_2 of respiring tissues at maximal work is close to zero.

$$k_{flux} = D_{O_2} \cdot (pO_{2(capillary)} - pO_{2(mitochondria)}) \approx D_{O_2} \cdot pO_{(capillary)} \quad (5)$$

As a result, for normal blood flow and O_2 affinity, the O_2 gradient will be greater in the lungs than in the muscles [i.e. $(\sim 100 \text{ mm Hg} - pO_{2(capillary)}) > (pO_{2(capillary)} - 0)$]. Thus, O_2 efflux in peripheral tissues will be more dependent on the chemical reaction properties of haemoglobin and capillary transit times than on tissue O_2 demand.

Experimental results are consistent with this theory. The maximal rate of O_2 uptake ($\dot{V}_{O_{2max}}$) in muscle *in situ* is linearly related to mean capillary pO_2, as reflected by muscle venous O_2 tension (Hogan *et al.*, 1989). Rates of O_2 uptake and release by red blood cells *in vitro* or by haemoglobin solutions in artificial capillaries are limited by diffusion of O_2 through the red blood cells and solvent layers (Vandegriff and Olson, 1984a, b; Lemon *et al.*, 1987). As a result of diffusional limitations, the apparent rate of O_2 uptake by human red blood cells *in vitro* is approximately 10-fold lower than the rate of O_2 association to cell-free human haemoglobin, and the apparent rate of red cell

deoxygenation is approximately six-fold lower than the rate of O_2 dissociation from haemoglobin (Vandegriff and Olson, 1984a, b). If changes in the haemoglobin molecule lower the chemical reaction rates with O_2 by more than five- to ten-fold, then rates of O_2 transport could become limited kinetically by chemical reaction constants.

Chemical modifications of the haemoglobin molecule

Original designs for a cell-free, haemoglobin-based blood substitute were based on methods to modify the haemoglobin molecule to prevent tetramer dissociation and to lower O_2 affinity. But until methods are developed to restrict oxidative reactions, the benefits of reduced O_2 affinity and the liability of higher rates of auto-oxidation must be balanced. Ultimately, the overall stability of the modified protein may determine its final success as a clinical product.

Several chemically derived haemoglobin structures have been produced that meet either one or both of these original goals. Dissociation of the tetramer can be prevented by introducing intramolecular cross-linkers. Alternatively, polymeric or conjugated haemoglobin structures can be prepared to produce molecules larger than the tetramer. The products from these more extensive reactions are difficult to characterize, however, and are less reproducible than those produced by site-specific, intramolecular modifications.

The appropriate attributes for a haemoglobin-based blood substitute have not been defined experimentally or theoretically. Human blood offers an obvious model for O_2 binding, although specific applications might call for unique characteristics, and haemoglobin derivatives could be developed with a wide range of O_2 binding properties. Also undefined is the optimal molecular size for a haemoglobin-based blood substitute. Molecules larger than a tetramer may be required to slow extravasation, but the relative benefits of increased viscosity versus decreased rates of leakage have not been evaluated.

Targets for chemical modification on the haemoglobin molecule are areas of the protein where native effectors bind. These sites include the terminal α-amino groups of the subunits, where H^+, Cl^-, and CO_2 bind, groups involved in the 2,3-DPG binding site in the β-subunit pocket, and ε-amino groups of lysine residues in the central cavity of the molecule, which may form another Cl^- binding site.

Some examples of the types of reactions that are used to modify haemoglobin are presented below. This list should not be considered as comprehensive.

MODIFICATIONS TO LOWER OXYGEN AFFINITY OR TO STABILIZE THE TETRAMER: PYRIDOXAL DERIVATIVES

Pyridoxal 5'-phosphate (PLP), a derivative of the vitamin pyridoxal, is similar in size and charge to the 2,3-DPG molecule. In the reaction with haemoglobin, a Schiff's base forms between the aldehyde of pyridoxal and an amino

group on the protein. The imine can be reduced with borohydride to form a covalent linkage as a secondary amine (Benesch and Benesch, 1981; *see Figure 5*). In the presence of O_2, the α-subunit amino-terminal valines are pyridoxalated, and a high-affinity haemoglobin variant is produced. In the reaction with deoxyhaemoglobin, a single covalent bond is formed between PLP and one β-subunit at the 2,3-DPG binding site; O_2 affinity is reduced, but tetramer dissociation is not prevented.

Two dialdehyde derivatives of pyridoxal have been synthesized to prevent tetramer dissociation. These are 2-nor-2-formylpyridoxal 5'-phosphate (NFPLP) and bis-pyridoxal tetraphosphate (bis-PL(P)$_4$) (Benesch and Benesch, 1981; Benesch and Kwong, 1988; *see Figure 6*). Both of these compounds react with deoxyhaemoglobin to form divalent bonds between β_1(NA1) valine and the ε-amino group of the β_2(EF6) lysine residue (Benesch *et al.*, 1975; Keipert *et al.*, 1989). The β_1(NA1)–β_2(EF6) cross-linked haemoglobins have reduced O_2 affinity and do not dissociate.

CARBOXYMETHYLATION

Haemoglobin function is modulated *in vivo* by Cl^- and CO_2 binding reversibly to the amino termini. Manning and his colleagues have mimicked this reaction by reductive carboxymethylation of haemoglobin using glyoxylate as an analogue of CO_2 (Fantl *et al.*, 1987; *see Figure 7*). The extent of the reaction and distribution of products depend on whether the reaction is carried out under oxygenated or deoxygenated conditions. Undesirable modifications at surface lysine groups occur from the reaction with oxyhaemoglobin. Carboxymethylation of deoxyhaemoglobin produces a higher yield of modified amino-terminal groups and creates a product with reduced O_2 affinity. However, since these compounds are not covalently cross-linked, tetramer dissociation does occur.

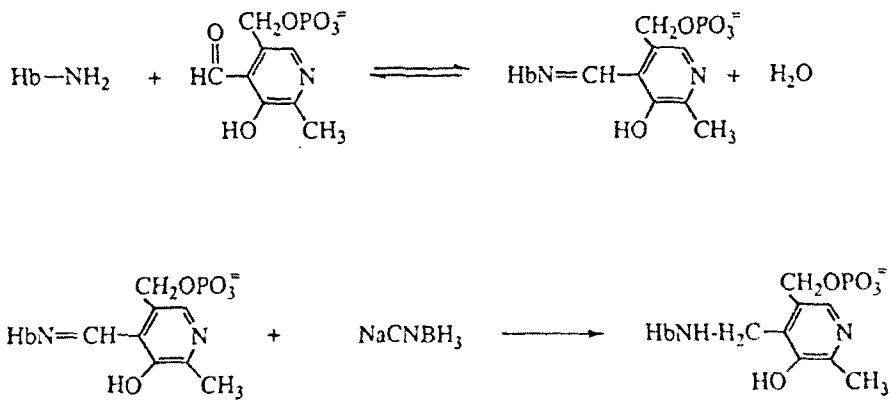
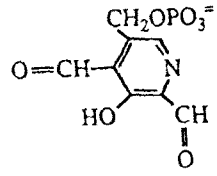


Figure 5. The reaction of pyridoxal 5'-phosphate with an amino group on haemoglobin. The imine adduct can be reduced with sodium cyanoborohydride to yield the covalently linked pyridoxalated haemoglobin derivative in the form of a secondary amine.

NFPLP



bis-PL(P)$_4$

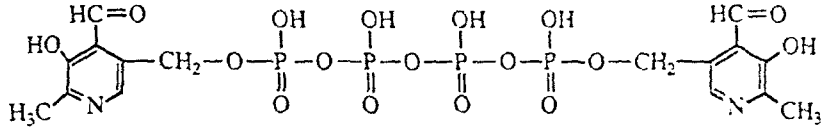


Figure 6. Structures of the pyridoxal compounds 2-nor-2-formylpyridoxal 5'-phosphate (NFPLP) and bis-pyridoxal tetraphosphate (bis-PL(P)$_4$).

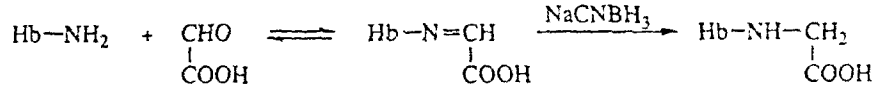


Figure 7. Carboxymethylation reaction. Haemoglobin is reacted with glyoxylate to form a Schiff's base that is reduced with sodium cyanoborohydride to form the stable secondary amine.

DIBROMINATED SALICYLATE DERIVATIVES

Mono- and disalicylates are acylating agents that can react with lysine residues of haemoglobin. Bromination of salicylate groups enhances their reactivity as leaving groups and, in combination with a small carbon molecule such as succinate or fumarate, the reaction forms a carbon bridge covalently cross-linked between lysine ϵ-amino groups. 3,5-(Dibromosalicyl)fumarate (DBBF) was first reacted with oxyhaemoglobin to create a high-O_2 affinity derivative to treat sickle-cell crisis (Walder *et al.*, 1979). It was determined later that this reaction produces two haemoglobin derivatives. Eighty per cent of the reaction product is cross-linked between β_1 and β_2(EF6) lysine residues. The remaining 20% is thought to be cross-linked between β_1(EF6) and β_2(HC1) lysine residues, and only the minor component exhibits increased O_2 affinity; the O_2 affinity of the major component is unaltered (Shibayama *et al.*, 1991).

The reaction of DBBF with deoxyhaemoglobin cross-links haemoglobin between α_1(G6) and α_2(G6) lysine residues in the central cavity of the tetramer to produce a stable, cooperative, low-affinity haemoglobin derivative (Walder, Chatterjee and Arnone, 1982; Chatterjee *et al.*, 1986; *see Figure 8*). Because the functional properties of α_1(G6)–α_2(G6) cross-linked haemoglobin are similar to those of blood, and because tetramer dissociation is prevented by the divalent cross-link, this modified haemoglobin derivative is a primary candidate for a blood substitute product.

The monosalicylate fumarate reacts with a single β(EF6) lysine residue in

the β-pocket to produce a haemoglobin derivative with increased affinity for O_2 between pH 6·5 and 7·2 and decreased affinity for O_2 above pH 7·2. Although the modified haemoglobin can still dissociate because it is not divalently cross-linked, the tetramer appears to be stabilized by electrostatic or hydrophobic interactions (Bucci *et al.*, 1989).

DERIVATIVES OF BENZENE ISOTHIOCYANATE

Benzene isothiocyanates (e.g. 2-, 3- and 4-isothiocyanatobenzenesulphonic acids) react with the four amino termini of haemoglobin to produce a covalent attachment through a 'thiourea' type bond (Currell *et al.*, 1981). Reactions with isothiocyanates carrying a negative charge produce haemoglobins with markedly reduced O_2 affinity over a wide pH range, whereas reactions with uncharged isothiocyanates, such as 4-isothiocyanatobenzenesulphonamide, produce a slight increase in O_2 affinity (Currell *et al.*, 1982). Since the monofunctional reagents do not form a divalent cross-link, the most promising derivative from this class of compounds for clinical application is 2,5-diisothiocyanatobenzenesulphonate (*Figure 9*). This compound cross-links the two α(NA1) valine amino termini of deoxyhaemoglobin to form a stable haemoglobin derivative with increased O_2 affinity (Manning *et al.*, 1991).

HAEMOGLOBINS MODIFIED WITH BI- AND TRIFUNCTIONAL ACYL METHYL PHOSPHATE REAGENTS

The reactions of bi- and trifunctional acyl methyl phosphate reagents with human haemoglobin form non-cross-linked, intra- and intersubunit single and doubly cross-linked amide derivatives with a range of O_2 affinities that bracket those of native human haemoglobin. The reactions take place at the amino groups of β(NA1) valine, β(EF6) lysine, and β(HC1) lysine residues. In the presence of 2,3-DPG, the smaller bifunctional reagents react with the amino groups of α(NA1) valine, α(G6) lysine, and α(HC1) lysine residues (Jones *et al.*, 1991).

DERIVATIVES OF CLOFIBRATE

Several synthetic agents that are derived from clofibrate and that are similar to bezafibrate have been prepared which react reversibly with deoxyhaemoglobin in the central cavity of the α-subunits (Lalezari *et al.*, 1990; Wireko, Kellogg and Abraham, 1991). The strongest interactions involve α(G6) lysine, α(HC3) arginine, and β(G10) asparagine residues. These compounds are potent effectors of haemoglobin function and exhibit a wide range of allosteric activity. Their potency is determined by how tightly they bind to haemoglobin and is related to the substitution of chlorine for hydrogen atoms (i.e. increasing the hydrophobicity of the compounds enhances their reactivity with haemoglobin) (Lalezari *et al.*, 1990). Since these compounds bind to haemoglobin reversibly, they are not being considered as haemoglobin-based

blood substitute derivatives, but they do provide useful models for studying the activity of allosteric effectors.

MODIFICATIONS TO INCRLASE THE SIZE OF THE MOLECULE: POLYMERIZATION OF HAEMOGLOBIN

Increasing the size of the haemoglobin molecule so that it is larger than that of the tetramer may provide certain advantages in the design of a blood substitute. Larger molecules have lower osmotic activity at higher haem concentrations. However, the benefits of polymers may be negated by their higher viscosity and greater instability.

Reactions of haemoglobin with aldehydes, such as glutaraldehyde and glycolaldehyde (*Figure 10*), create intra- and intermolecularly cross-linked polymers. Glutaraldehyde is an inherently toxic dialdehyde that reacts primarily with α- and ϵ-amino groups of proteins but may also react with the phenolic ring of tyrosine, the imidazole group of histidine, and the sulphhydryl group of cysteine (Habeeb and Hiramoto, 1968). These reactions produce multiple cross-links, which can distort the protein structure, and, thus, do not allow the preparation of stable, reproducible derivatives. Glutaraldehyde-polymerized haemoglobin solutions are heterogeneous, unstable, and more prone to auto-oxidization (Hsia, 1989; Marini *et al.*, 1990). Polymerization reduces cooperativity and, for most reactions, increases O_2 affinity. As a result, polymerization reactions with human

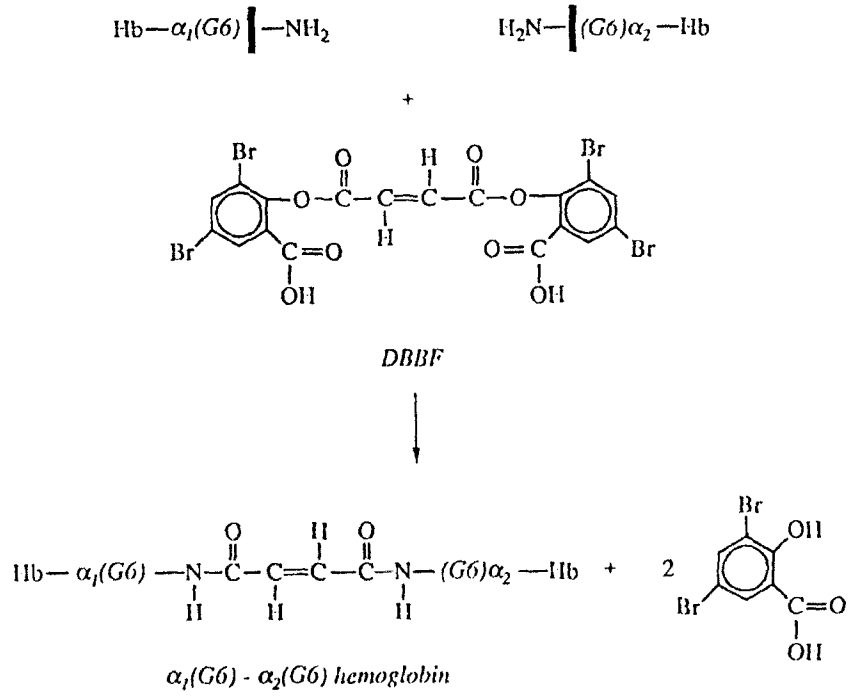


Figure 8. Acylation reaction of α(G6) lysine residues of deoxyhaemoglobin with 3,5-(dibromosalicyl)fumarate to form covalently α_1(G6)–α_2(G6) cross-linked haemoglobin.

Diisothiocyanatobenzenesulfonic acid

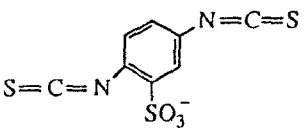
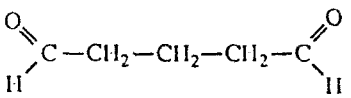


Figure 9. Structure of 2,5-diisothiocyanatobenzenesulphonic acid.

Glutaraldehyde



Glycolaldehyde

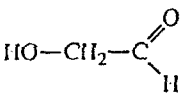


Figure 10. Structures of the polymerizing agents, glutaraldehyde and glycolaldehyde.

haemoglobin are usually preceded by a reaction to lower O_2 affinity, such as pyridoxalation with either PLP or NFPLP (Gould *et al.*, 1980; De Venuto and Zegna, 1983; Berbers *et al.*, 1991).

Electron paramagnetic resonance and Mössbauer spectroscopic studies of a glutaraldehyde cross-linked haemoglobin showed that the O–O bond length of bound O_2 is stretched and that the steric constraints of the proximal histidine Fe–N bond and of the distal pocket are decreased (Chevalier *et al.*, 1990). Thus, the haem pocket of glutaraldehyde-polymerized haemoglobin appears to be more open, and these changes in the haem environment might explain the increased O_2 affinity and higher rates of auto-oxidation.

Bovine haemoglobin, which naturally exhibits a lower O_2 affinity in the presence of the chloride anion, is another choice for polymerization (Feola *et al.*, 1983; Gawryl, Clark and Rausch, 1991), and the reaction of human deoxyhaemoglobin with a ring-opened raffinose produces low-affinity, non-cooperative polymers (Hsia, 1991).

Glycolaldehyde is a monoaldehyde that has been used as a milder polymerizing reagent to covalently cross-link haemoglobin. It forms a Schiff's base with amino groups and then takes part in an Amadori rearrangement to produce a new aldehyde function that is capable of cross-linking. Manning's group found that the molecular-size distribution of glycolaldehyde-polymerized, carboxymethylated haemoglobin solutions depended on the initial haemoglobin concentration and that the final O_2 affinity depended on whether the reactions were carried out under oxygenated or deoxygenated

conditions. Reduced O_2 affinity was achieved by cross-linking carboxymethylated deoxyhaemoglobin (Manning and Manning, 1988).

Glycolaldehyde also has been used in combination with NFPLP to prepare polymers of human haemoglobin that have a reproducible distribution of molecular weights from 64 to 128–512 kDa (MacDonald and Pepper, 1991). MacDonald and Pepper have outlined a new procedure to simplify the reaction process by deoxygenating haemoglobin during the polymerization reaction and following polymerization with the NFPLP reaction. Sodium borohydride is then added both to stop polymerization and to stabilize bound NFPLP.

CONJUGATION OF HAEMOGLOBIN TO INERT MOLECULES

The molecular size of haemoglobin also can be increased by conjugation to large, inert molecules. PLP-modified haemoglobin conjugated to the activated diester of polyoxyethylene (POE) produces a high molecular-weight derivative with reduced O_2 affinity. The product consists of three components of one, two or three haemoglobin tetramers conjugated to POE. Each tetramer is conjugated to six molecules of POE and has an average molecular weight of 90 000 (Yabuki *et al.*, 1991).

Haemoglobin conjugated to dextran produces derivatives with unaltered functional properties, which then can be reacted with inositol hexaphosphate to lower O_2 affinity (Wong, 1988). Alternatively, haemoglobins conjugated to dextran polymers having benzene tri-, tetra- or hexacarboxylates covalently bound have increased molecular size and reduced O_2 affinity (Dellacherie *et al.*, 1992; Sacco, Prouchayret and Dellacherie *et al.*, 1992).

Functional properties of chemically modified haemoglobins

OXYGEN BINDING REACTIONS

Three examples of site-specifically, chemically modified haemoglobins that have reduced O_2 affinity are (1) haemoglobin modified at the amino termini with 4-isothiocyanatobenzenesulphonic acid (Bellelli *et al.*, 1986); (2) β_1(NA1)–β_2(EF6) cross-linked haemoglobin produced from the reaction with NFPLP (Bellelli *et al.*, 1987); and (3) α_1(G6)–α_2(G6) cross-linked haemoglobin produced from the reaction with DBBF (Vandegriff *et al.*, 1989; *see Figure 11*). It is interesting that the functional effects of all three modifications are similar even though the structural modifications are at different locations on the protein. The O_2 affinities of all three are reduced as the result of a perturbed R-state structure (i.e. their high-affinity conformations do not bind O_2 as tightly as native R-state haemoglobin). Kinetic measurements show that O_2 dissociates faster from the R states of both β_1(NA1)–β_2(EF6) cross-linked and α_1(G6)–α_2(G6) cross-linked haemoglobins (Bellelli *et al.*, 1987; Vandegriff *et al.*, 1991b). The O_2 binding kinetics of the individual subunits of α_1(G6)–α_2(G6) cross-linked haemoglobin have been resolved and

show that O_2 dissociates faster from both α- and β-subunits and associates more slowly only to α-subunits (Vandegriff *et al.*, 1991b).

These results were used to formulate a mechanism for the structural effects of cross-linking between the G helices of α-subunits. It was postulated that the rigid fumarate cross-link between the α-subunit G helices in α_1(G6)–α_2(G6) cross-linked haemoglobin restricts the movement of the proximal histidine and iron atom toward the plane of the haem ring during the T-to-R transition (Vandegriff *et al.*, 1989). This mechanism is consistent with two other experimental observations. Resonance Raman spectroscopy demonstrated that the proximal bond in R-state α_1(G6)–α_2(G6) cross-linked haemoglobin was more 'T-state like' (Larsen *et al.*, 1990), and laser-photolysis spectral measurements of the same haemoglobin derivative showed that the transition from R to T states was faster (Vandegriff *et al.*, 1991b).

HETEROTROPIC LIGAND BINDING REACTIONS

Since heterotropic reactions are O_2-linked, most haemoglobin modifications that alter O_2 affinity also alter heterotropic reactions. The Bohr effects are reduced for haemoglobin modified at the amino termini with benzene isothiocyanates (Bellelli *et al.*, 1986), for PLP-glutaraldehyde polymerized haemoglobin (Sehgal *et al.*, 1988), for bis-PL(P)$_4$-modified β_1(NA1)–β_2(EF6) cross-linked haemoglobin (Keipert *et al.*, 1989), and for α_1(G6)–α_2(G6) cross-linked haemoglobin (Vandegriff *et al.*, 1989). Modifications with the bezafibrate derivatives, L35 and L345, increase the alkaline Bohr effect (Lalezari *et al.*, 1990). The Cl^- and CO_2 effects of glycolaldehyde cross-linked, carboxymethylated haemoglobin and α_1(G6)–α_2(G6) cross-linked haemoglobin are reduced but not eliminated (Manning and Manning, 1988; Vandegriff *et al.*, 1989, 1991a).

These effects depend on the site of modification. Modifications to mimic CO_2 or Cl^- at the amino termini or modifications in the 2,3-DPG binding pocket are designed to imitate permanently those heterotropic effectors and usually result in altering binding of the native effectors. If the sites of modification are removed from the known heterotropic binding sites, cause and effect may not be as obvious. For example, cross-linking subunits between α(G6) lysine residues reduces the H^+, Cl^- and CO_2 effects by ~50% (Vandegriff *et al.*, 1989, 1991a). It was suggested recently that the α(G6) residues in the internal, central cavity of the haemoglobin tetramer constitute a direct Cl^- binding site (Ueno and Manning, 1992), but it is not clear how this might relate to H^+ and CO_2 binding. The α-subunit amino termini are involved in all three of these heterotropic interactions, but the amino-terminal groups are ~10 Å away from site of cross-linking in the central cavity. Long-range electrostatic interactions may be occurring, which, if common in modified proteins, will make these effects difficult to predict.

Since H^+, Cl^- or CO_2 have important *in vivo* functions, concerns were raised that modifications of heterotropic binding reactions could have significant physiological consequences. However, no imbalances in acid–base status

were observed in swine exchange-transfused with α_1(G6)–α_2(G6) cross-linked haemoglobin (Hess, Wade and Winslow, 1991; Hess, Macdonald and Winslow, 1992). Either these are insignificant effects *in vivo*, or physiological compensatory mechanisms are adequate to moderate acid–base fluctuations.

Stability of chemically modified haemoglobins

The stability of a modified protein depends on the effects of modification on the folded and unfolded states and the ability of the protein to compensate for change (e.g. protein flexibility can minimize instability). Covalently cross-linking proteins can stabilize their folded structures by decreasing the conformational entropy of the unfolded polypeptide. However, cross-linkers that decrease the flexibility of the folded structure are less stabilizing (Alber, 1989). Both α_1(G6)–α_2(G6) cross-linked and β_1(EF6)–β_2(EF6) cross-linked haemoglobins have improved thermal stabilities (Yang and Olsen, 1991), suggesting that the conformational entropy of the unfolded, cross-linked haemoglobins is decreased relative to that of the unfolded, native polypeptide.

The stability of haemoglobin can be evaluated by its rates of auto-oxidation and precipitation. The rates of auto-oxidation of monofunctionally, bifunctionally and intermolecularly cross-linked haemoglobins are highly correlated to their O_2 affinities (*Figure 12*). Rates of menadione-induced oxidation and precipitation of bifunctionally and intermolecularly cross-linked human haemoglobins and native bovine haemoglobin are markedly lower than rates for monofunctional derivatives or for unmodified human haemoglobin (Macdonald *et al.*, 1991). Haemoglobins that have greater stability naturally, such as bovine or human foetal haemoglobin, may provide paradigms for future designs of haemoglobin-based blood substitutes.

Rates of haem exchange between methaemoglobin and high-affinity haem binding proteins provide another experimental evaluation of haemoglobin stability. Cross-linking haemoglobin with bifunctional PLP derivatives was shown to reduce the rate of haem exchange with human serum albumin (Benesch and Kwong, 1990). Preliminary experiments with glutaraldehyde polymerized haemoglobins showed multiphasic haem-exchange reactions with high sample-to-sample variation (K.D. Vandegriff and Y.C. Le Tellier, unpublished results). These results are probably related to the heterogeneity of polymerized solutions and suggest that some polymerized haemoglobins contain a mixture of stabilized and unstabilized subunits.

Starting materials for haemoglobin modification reactions

Outdated human blood is the major source of haemoglobin used for modification reactions, but its availability is diminishing (Klein, 1989). Bovine haemoglobin is a practical choice as a starting material for a blood substitute. Its supply is not threatened, its O_2 affinity is modulated by Cl^-, which is a normal component of blood plasma, and it is inherently more stable in solution. The disadvantages of using bovine blood are concerns over

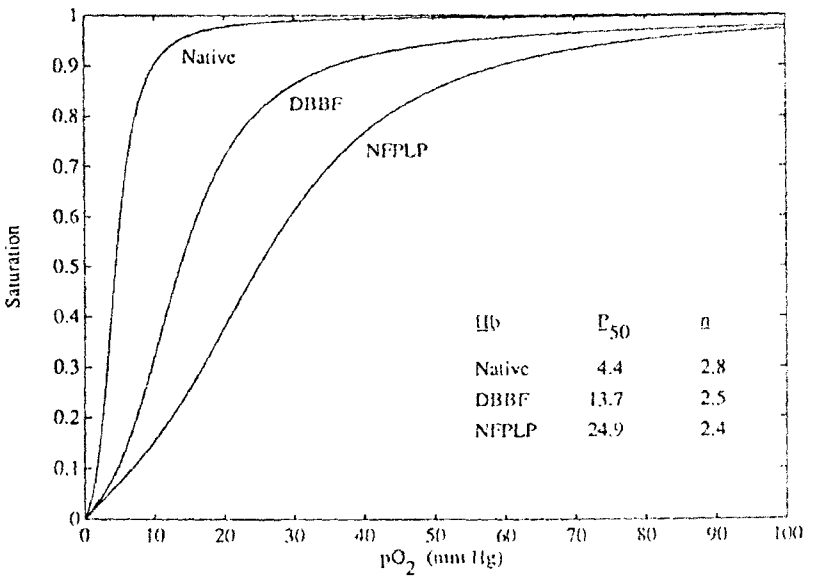


Figure 1. Oxygen equilibrium binding curves of unmodified human (Native), α_1(G6)–α_2(G6) cross-linked (DBBF), and β_1(NA1)–β_2(EF6) cross-linked (NFPLP) haemoglobins (Hb). Measurements were made using the automatic method of Imai (Imai, 1981), as adapted by Vandegriff (Vandegriff *et al.*, 1989). The reactions were carried out in bis-Tris buffer, 0·1 M Cl^- at pH 7·4 and 25°C. P_{50} is the pO_2 at which haemoglobin is half-saturated with oxygen. The Hill coefficient, n, is the index of cooperativity.

transmission of the bovine spongiform encephalitis (BSE) virus and the possibility of antigenic reactions from infusions of large amounts of bovine haemoglobin into humans. Expression of human haemoglobin in recombinant organisms may eventually provide a source of material, but large-scale expression systems are not available yet. Recombinant haemoglobins would eliminate risks of viral transmission, and if site-specific mutant haemoglobins could be designed as final products, ultrapure haemoglobins could be isolated directly from hosts without the need for further chemical processing (*see below*).

Large-scale process and purification of haemoglobin solutions

Potentially dangerous contaminants of haemoglobin solutions include free phospholipids from red cell membrane fragments and endotoxin from bacterial cell walls. Endotoxin causes vascular damage by activating inflammatory responses and stimulates procoagulant activity by induction of tissue factor in macrophages or endothelial cells (Davie, Fujikawa and Kisiel, 1991). Free phospholipids also are involved in inflammation and coagulation pathways and provide a source for free radical damage. Elimination of red cell stromal contaminants from haemoglobin solutions by gentle lysis, centrifugation and

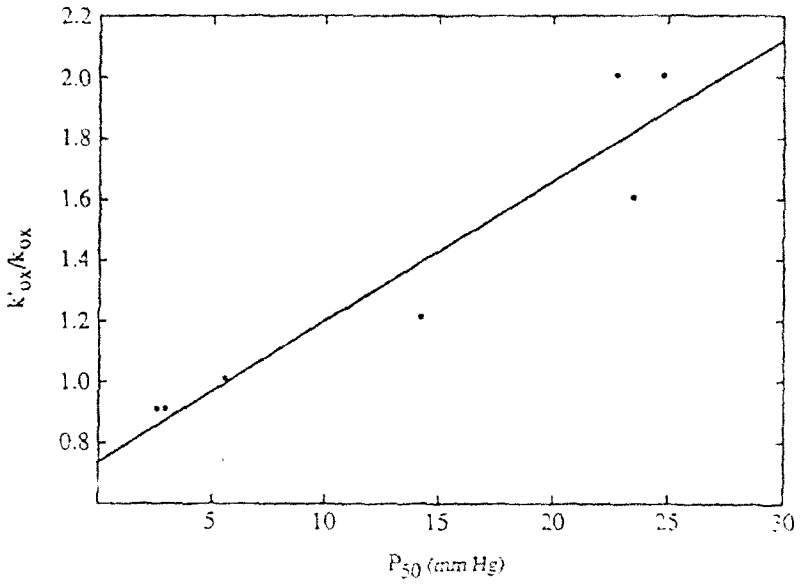


Figure 12. Rates of auto-oxidation of chemically modified haemoglobins relative to the rate of auto-oxidation of haemoglobin A_0 (k'_{ox}/k_{ox}) as a function of P_{50}. (Note that for haemoglobin A_0, $k'_{ox}/k_{ox} = 1$.) The reaction conditions are given in *Figure 11*. The haemoglobin derivatives for these measurements, in increasing order of P_{50}, were oxyhaemoglobin polymerized by ring-opened raffinose, oxyhaemoglobin cross-linked with DBBF, haemoglobin A_0, deoxyhaemoglobin cross-linked with DBBF, haemoglobin reacted with 4-isothiocyanatobenzenesulphonic acid, deoxyhaemoglobin cross-linked with ring-opened raffinose, and haemoglobin cross-linked with NFPLP (Macdonald *et al.*, 1991).

filtration was a major breakthrough in the development of haemoglobin-based blood substitutes (Rabiner *et al.*, 1967). Complete elimination of endotoxin from haemoglobin solutions is more difficult but can be achieved on large scales by employing stringent precautions.

Because commercial products are usually proprietary, the US Army Medical Research and Development Command designed and built a haemoglobin production facility to provide large quantities of well-characterized haemoglobin solutions for scientific research but not for human application. The US Army facility produces 20–30 l of 7–11 g dl^{-1} pyrogen-free, stroma-free haemoglobin from each run, starting with ~80 units of outdated red blood cells. Typical batches have <2% methaemoglobin, undetectable levels of residual red cell membrane components, inorganic phosphate, or, as determined from the kinetic turbidimetric *Limulus* amoebocyte lysate assay, endotoxin (Chapman *et al.*, 1992). To isolate the major adult human haemoglobin fraction (haemoglobin A_0) from minor haemoglobin components and other red cell proteins, additional purification of the haemoglobin solutions has been achieved by ion-exchange high performance liquid chromatography (HPLC) (Christensen *et al.*, 1988).

For the large-scale preparation of α_1(G6)–α_2(G6) cross-linked haemoglo-

bin in the US Army facility, the haemoglobin solution is deoxygenated by circulation through a hollow-fibre gas-exchange filter with a counter-current flow of nitrogen gas outside the fibres. (A faster method of large-scale deoxygenation has been described in which haemoglobin is circulated against a 1% (w/v) sodium dithionite solution in hydrophobic hollow fibres; MacDonald and Pepper, 1991). Deoxyhaemoglobin is transferred to a 70 l bioreactor at 37°C, and DBBF is added. During the reaction, the degree of cross-linki monitored by reverse-phase or molecular-sieve HPLC. When the reaction is completed, the temperature in the bioreactor is raised to 76°C for 90 min. The heating step has two purposes: (1) it facilitates purification of the cross-linked product by precipitating non-cross-linked haemoglobin; and (2) it inactivates viruses (Farmer *et al.*, 1992). The process typically produces 15–20 l of 6–9 g dl^{-1} haemoglobin with <3% methaemoglobin. The haemoglobin is stable indefinitely during storage at –80°C but oxidizes significantly when stored at –20°C in the absence of salts (Moore *et al.*, 1992).

Chemical techniques are being developed for large-scale reduction of methaemoglobin solutions. Ferric haemoglobin can be reduced electrochemically at a platinum electrode using flavin mononucleotide as the catalyst (Durliat and Comtat, 1987) or through photoactivation of flavin mononucleotide as the catalyst with DL-methionine as an electron donor (Everse, 1993). Another method to reduce large biological molecules uses platinum embedded in a high surface area, polymeric catalyst with H_2 as the reductant (Chao *et al.*, 1988). This technique has been adapted to regenerate functional, reduced haemoglobin from solutions containing ~50% methaemoglobin (McGown *et al.*, 1992).

Haemoglobin solutions can become unstable during large-scale processing procedures. Polymers of denatured haemoglobin have been shown to form when solutions are exposed to a large surface area at the gas–liquid interface (Přistoupil and Mařík, 1990). Particles in the μm size range have also been observed in the solutions produced at the US Army's haemoglobin production facility. These particles were assayed and found to be positive for protein and negative for phospholipid. They appeared as fibrous polymers by electron microscopy, and had an optical spectrum similar to that of hemichrome. Processing temperature was found to be the greatest factor; particle formation was reduced markedly when heat exchangers were used to lower the processing temperature from 18°C to 4–8°C (R.M. Winslow and K.W. Chapman, unpublished results).

Genetic modification of the haemoglobin molecule

Genetically engineering the haemoglobin molecule by DNA recombinant technology and site-directed mutagenesis of globin genes has been accomplished for the expression of pure, native or modified haemoglobins in prokaryotic (i.e. *Escherichia coli*) and eukaryotic (i.e. *Saccharomyces cerevisiae*) host cells and in transgenic animals.

EXPRESSION SYSTEMS

Escherichia coli

Nagai and his colleagues first synthesized human β-globin in *E. coli* as a cleavable fusion protein from the expression vector pLcIIFXβ (Nagai and Thøgersen, 1984). The β-globin gene was fused to a short coding sequence of the bacteriophage λ cII gene and a tetrapeptide of prothrombin to allow specific cleavage by blood coagulation factor X_a. The advantages of this expression system are that (1) translation of messenger RNA (mRNA) is efficient because it is initiated at the highly expressed λ cII gene and (2) authentic β-globin is obtained because the initiation methionine codon that is usually expressed in eukaryotic proteins in *E. coli* is eliminated after cleavage of the fusion gene product.

To produce functional haemoglobin tetramers, β-globin was expressed in *E. coli* upon heat induction as a fusion protein, the fusion protein inclusion bodies were solubilized in 8 M urea, chromatographically purified, digested with factor X_a, and the apo-β-globin was folded and reconstituted *in vitro* with exogenous cyanohaemin and native α-subunits. The reconstituted haemoglobin tetramers exhibited slightly increased O_2 affinity and reduced cooperativity (Nagai, Perutz and Poyart, 1985).

A similar fusion protein expression vector for α-globin (pLcIIFXα) was found not to express at high levels. To obtain acceptable yields of α-globin, the α-globin gene was inserted after an 18 codon β-globin sequence to produce a chimeric protein (i.e. λ cII, an FX_a recognition site, the amino-terminal portion of β-globin, another FX_a site, and α-globin). The cIIFXβα inclusion bodies were solubilized in 6 M guanidine hydrochloride buffer, transferred to 8 M urea, chromatographically purified, and cleaved with factor X_a. The α-globin product did not fold in the presence of β-globin, and the apo-α-globin had to be diluted in acidic buffer with cyanohaemin before native β-subunits were added (Tame *et al.*, 1991).

Another plasmid (pJK05) that is similar to that of Nagai and Thøgersen was prepared by fusing the β-globin gene and the factor X_a recognition sequence to the influenza virus NS1 gene (Fronticelli, O'Donnell and Brinigar, 1991). Expression was induced chemically with nalidixic acid, and the resulting fusion protein was solubilized in 50 mM NaOH, purified by dialysis, digested with factor X_a, for 48–72 h, and folded in the presence of cyanohaemin and native α-subunits. This method of expression produced a high yield of β-globin without requiring extensive purification. Like the recombinant β-globin haemoglobin tetramers produced from Nagai's expression system, the reconstituted haemoglobin was found to be functionally similar to native human haemoglobin in P_{50} and alkaline Bohr effect, but cooperativity was not fully recovered.

A system for production of soluble recombinant human haemoglobin in *E. coli* has been described in which α- and β-globins were co-expressed as non-fused proteins from a single operon (Hoffman *et al.*, 1990). This technique improves the efficiency of eukaryotic mRNA translation in *E. coli*

and is based on the two-cistron expression system, which separates the Shine–Dalgarno ribosome binding-site sequence (the first cistron) from the 5′ translational start codon of the gene to be expressed (the second cistron) (Schoner *et al.*, 1984). Efficient translation occurs by optimizing the distance between the Shine–Dalgarno sequence and the translational start codon of the gene by placing a short coding sequence in front of the gene to be expressed. Hoffman and co-workers employed this technique to produce a plasmid (pDLIII-13e) with an operon under regulation of the P_{tac} promotor that consisted of two pairs of cistrons coupled through a translational coupling sequence, with each pair containing the ribosome binding sequence and either the α- or β-globin gene (Hoffman *et al.*, 1990).

E. coli cells were transformed with the pDLIII-13e plasmid, and α- and β-globins were co-expressed after induction with isopropyl β-D-thiogalactopyranoside. The globins were produced in the soluble cytoplasmic fraction of the cells, where they reconstituted with endogenous haem and folded *in vivo*. The recombinant polypeptides retained the translational initiation methionine residues, however, so that the primary structures of the recombinant globin chains were longer than native human globins by one residue. The final product contained ~25% methaemoglobin, ~25% globin that did not contain a full complement of haem (i.e. <4 haem per tetramer), and ~50% recombinant haemoglobin with a normal haem-to-globin ratio. The recombinant haemoglobin showed slightly altered functional properties, which could have resulted from the extra methionyl residue at the amino termini of the protein. The O_2 affinity was increased, cooperativity was reduced, and heterotropic interactions were diminished (Hoffman *et al.*, 1990).

A similar vector has been designed by the same group to express human globins with correct polypeptide lengths but with native, terminal valine residues replaced by methionine. The vector is equivalent to pDLIII-13e except that the codons for the amino-terminal valines are deleted. This recombinant haemoglobin (α(NA1) valine → methionine and β(NA1) valine → methionine) has slightly reduced O_2 affinity and cooperativity compared with native haemoglobin A_0 (Komiyama *et al.*, 1991; Looker *et al.*, 1992).

SACCHAROMYCES CEREVISIAE

Saccharomyces cerevisiae offers a good expression system for human globins because the proteins can be produced *in vivo* as soluble, folded, haem-containing tetramers with the correct amino-terminal sequence. (Eukaryotic methionyl aminopeptidases efficiently remove initiating methionine residues.)

Wagenbach and co-workers constructed a synthetic hybrid promoter (pGGAP) and cloned separate transcription units into vector pGS389 to co-express α- and β-globins in *S. cerevisiae*. Globin synthesis was induced by galactose, and equal amounts of α- and β-globins appeared after 8 h that constituted 3–5% of the total cellular protein. The functional properties of the wild-type recombinant human haemoglobin were similar to those of

native human haemoglobin with only a slight reduction in cooperativity (Wagenbach *et al.*, 1991).

Ogden and colleagues have designed two *S. cerevisiae* expression systems, one in which yeast was transformed with single plasmids carrying genes for both α- and β-globins and the other in which yeast was co-transformed with two plasmids carrying either α- or β-globin expression cassettes. Recombinant haemoglobin was produced at ≥95% purity, with correct molecular mass as determined by electrospray mass spectrometry, and normal O_2 and CO binding properties (Ogden *et al.*, 1992).

TRANSGENIC ANIMALS

To express human haemoglobin in transgenic mice, human α- and β-globin genes have been linked downstream to sequences for the deoxyribonuclease I super-hypersensitive site (i.e. the β-globin locus control region) that controls normal, erythroid-specific β-globin gene expression (Grosveld *et al.*, 1987; Behringer *et al.*, 1989; Hanscombe *et al.*, 1989). α- and β-globin gene constructs were co-injected into fertilized eggs to establish transgenic mouse lines for high-level expression of human globin mRNA. Mice have a small blood volume, however, and do not provide a suitable host for the production of large volumes of blood products.

Pigs are an ideal species for transgenic production of human haemoglobin. Pig and human haemoglobins are similar enough, so that swine should be able to function well with the human protein. For example, 2,3-DPG is an effector of both haemoglobins. Transgenic swine have been produced using a construct of the human β-globin locus control region, two α genes, and a single β gene injected into day-old fertilized eggs (Swanson *et al.*, 1992). In that experiment, three transgenic pigs were produced from 112 births from 909 ova that were transferred into 19 recipients, giving an overall transgenic yield (i.e. number of transgenic animals to number of eggs injected) of only 0·4%. The transgenic pigs were not anaemic and grew at a rate similar to that of non-transgenic pigs from the same litter. The highest yield of human haemoglobin was 9% of total cellular haemoglobin, which included human and pig haemoglobins and a hybrid of human α- and pig β-subunits. (Human β and pig α hybrids did not form; human α-globin was expressed at higher yields than β-globin.) Human haemoglobin was fractionated from the other haemoglobins and pig proteins to >99% purity by ion-exchange chromatography.

Haemoglobin design by recombinant DNA engineering

NATURAL MUTANTS AS MODELS FOR HAEMOGLOBIN DESIGN

Some natural haemoglobin mutants can be used as models for the design of haemoglobin-based blood substitutes by site-directed mutagenesis. For example, haemoglobin Presbyterian, with the point mutation β(G10) asparagine → lysine, has reduced O_2 affinity, a high level of cooperativity, an enhanced

Bohr effect, and normal tetramer stability (Moo-Penn *et al.*, 1978). It is not known how this mutation lowers O_2 affinity, but it may occur through disruption of hydrogen bonds and electrostatic repulsions that favour the oxyhaemoglobin structure. The native asparagine residue at β(G10) is hydrogen bonded through water molecules to α(G10) histidine and to other residues in the internal cavity. Alternatively, the introduction of a lysine residue at β(G10) may create a new Cl^- binding site (Ueno and Manning, 1992).

Haemoglobin Chico (β(E10) lysine → threonine) is another natural mutant with reduced O_2 affinity (Shih *et al.*, 1987). Haemoglobin Chico maintains high cooperativity and normal anion and Bohr effects below pH 8·0, and the O_2 affinities of both the R and T states are reduced. Its X-ray crystal structure reveals a hydrogen bond either directly between β(E10) threonine and the distal β(E7) histidine or through a bridging water molecule (Bonaventura *et al.*, 1991). The mutation may introduce an additional steric hindrance to ligand binding or alter the water structure and electron density in the β-haem pockets and, thus, lower reactivity with ligands. The disadvantage of the Chico mutation is that the tetramer is destabilized, and a second mutation would be required to prevent dissociation.

Natural haemoglobins that form polymers provide genetic models for producing larger haemoglobin molecules. Turtle, frog and some mouse haemoglobins polymerize by the formation of intermolecular disulphide bridges (Riggs, Sullivan and Agee, 1964; Riggs, 1965). Three polymeric, natural human haemoglobin mutants also have been described: haemoglobin Pôrto Alegre (β(A6) serine → cysteine) (Tondo *et al.*, 1974), haemoglobin Mississippi (β(CD3) serine → cysteine) (Adams *et al.*, 1987), and haemoglobin Ta-Li (β(EF7) glycine → cysteine) (Blackwell, Liu and Wang, 1971). Each of these human mutants has a residue on the surface of the β-subunit that is replaced by a cysteine residue capable of forming intermolecular disulphide bonds. Like the chemically polymerized haemoglobins, these mutants exhibit increased O_2 affinity, reduced haem–haem interaction, and mild instability. It is interesting that haemoglobin Ranier (β(HC2) tyrosine → cysteine) does not polymerize and instead forms an intra-subunit disulphide bond with the sulphydryl group of β(F9) cysteine that renders the molecule more resistant to alkaline denaturation (Hayashi *et al.*, 1971).

SITE-SPECIFICALLY DESIGNED MUTATIONS

Novel, site-specific recombinant haemoglobins produced by Nagai's expression system demonstrate the influence of the size and polarity of distal pocket residues E7 and E11 on ligand binding (*see Figure 3*). Although kinetic measurements reveal significant increases in rate constants of O_2 association and dissociation for R-state α(E11) valine → alanine mutants (Mathews *et al.*, 1989), no overall effect on O_2 affinity has been observed for any mutation of the α-subunit E11 residue (Tame *et al.*, 1991). The only mutations of valine E11 that alter O_2 affinity are on the β-subunits. β(E11) valine → alanine increases the affinity of the T state, and β(E11) valine → isoleucine reduces

the affinity of both T and R states (Nagai *et al.*, 1987). Kinetic evaluation of the low R-state affinity of the β(E11) valine → isoleucine mutant reveals slower O_2 association (Mathews *et al.*, 1989). The X-ray crystallographic structure of this mutant shows that the δ-CH_3 group of the E11 isoleucine side-chain lies directly over the β-haem iron and probably restricts O_2 access to the haem (Nagai *et al.*, 1987).

Mutations at the E11 residues have greater overall effect on T-state CO binding kinetics, probably by altering steric constraints in the T-state distal pocket (Mathews *et al.*, 1991). The effect of α(E11) mutations on T-state CO binding rates are in contrast to the lack of effect of these mutations on O_2 affinities and emphasizes the difference between O_2 and CO as haemoglobin ligands (Mathews *et al.*, 1991; Tame *et al.*, 1991).

Another engineered mutation at β(E11) (valine → threonine) alters the polarity of the distal pocket without steric perturbation (i.e. threonine and valine are isosteric but have different charges). This mutant exhibits a biphasic O_2 equilibrium curve with low cooperativity, an effect that has been interpreted to reflect reduced O_2 affinity at the β haem and increased O_2 affinity at the α haem (Fronticelli *et al.*, 1991b). β(E11) threonine might inhibit the movement of O_2 to its binding site at the β haem by stabilizing a water molecule bound non-covalently in the distal pocket, and communication between subunits is disabled through some unresolved mechanism.

Site-specifically designed recombinant haemoglobin Presbyterian has been expressed in *Saccharomyces cerevisiae* and *Escherichia coli* (Wagenbach *et al.*, 1991; Looker *et al.*, 1992). For expression in *E. coli*, the Presbyterian mutation was combined with the (NA1) valine → methionine construct to produce a mutant haemoglobin with correct polypeptide length, lowered O_2 affinity, and slightly reduced cooperativity. But it could still dissociate into dimers.

To prevent tetramer dissociation, another genetic construct was designed by the same group with duplicated α-globin genes fused in tandem by the insertion of a glycine codon between the carboxyl terminus of α_1- and the amino terminus of α_2-globin genes (Looker *et al.*, 1992). α_1(HC3)–α_2(NA1) cross-linked haemoglobin has increased O_2 affinity and reduced cooperativity and, therefore, was combined with the Presbyterian and terminal valine → methionine mutations to produce the low-affinity, recombinant haemoglobin product rHb1·1 (Looker *et al.*, 1992). The function of rHb1·1 is similar to that of chemically α_1(G6)–α_2(G6) cross-linked haemoglobin in both equilibrium and kinetic O_2 binding (Looker *et al.*, 1992; A.J. Mathews, personal communication), and thus, rHb1·1 provides the first recombinant haemoglobin candidate for a blood substitute product.

Fronticelli has formulated a new design for recombinant haemoglobins based on heterotropic ligand binding reactions (Fronticelli, 1990). Her idea is to prepare a human haemoglobin variant in which O_2 affinity is regulated by normal components of blood plasma, a design that is based on Cl^- modulation of bovine haemoglobin. One recombinant human haemoglobin, β(NA1) valine → methionine and β(NA2) histidine → deleted, was prepared with β-subunit amino termini that are identical to those of bovine haemoglobin

(Fronticelli *et al.*, 1991a). The Cl^- effect was stronger for this mutant than for native human haemoglobin but still weaker than for native bovine haemoglobin.

As shown by these examples, studies on the effects of size and charge of pertinent haemoglobin residues are providing a data base for future designs. However, the primary advantage of site-specific mutagenesis may ultimately arise from modifications that improve protein stability. Electrostatic interactions, hydrogen bonds, hydrophobic forces, and van der Waal's contacts all contribute to stability, but not every protein residue contributes to stability to the same degree. Generally, only a small portion of residues are critical, and the net consequences of specific mutations on protein structure and stability are not necessarily predictable (Alber, 1989).

As an example, it is not clear how the expression of recombinant mutant globins affects haemoglobin folding and assembly. Circular dichroism studies of semi-synthetic haemoglobin containing recombinant β-globins suggest that the decrease in cooperativity may be linked to disorder in the haem environment (Fronticelli, O'Donnell and Brinigar, 1991). Genetically fusing polypeptides, such as in rHb1·1, may alter the stability of the molecule in solution either beneficially by stabilizing the tetramer or non-beneficially by breaking the dyad symmetry of the molecule. A comparison of the X-ray crystallographic structure of deoxygenated rHb1·1 with native human deoxyhaemoglobin by difference Fourier analysis shows that the cross-linked termini are more constrained and that there are substantial structural changes around the B and G helices from the Presbyterian mutation (Looker *et al.*, 1992). There have been no reports on the stability of rHb1·1, however, and a correlation between these site-specific structural effects and overall molecular stability awaits studies on auto-oxidation, precipitation, and haem exchange.

Large-scale expression and production of recombinant haemoglobins

Problems in large-scale production of recombinant haemoglobins can arise from the lack of technological experience in mass producing recombinant proteins, inadequate expression yields, or difficulties associated with the purification of the recombinant protein product from other cellular proteins, genetic material, or contaminants (e.g. endotoxin from *Escherichia coli*). Compared with other recombinant proteins that are being manufactured for therapeutic use, much higher levels of cost-effective haemoglobin synthesis will be required to market a blood substitute product (Ogden, 1992).

Low expression levels of recombinant globins may be attributed to several factors. Mutant mRNAs could be transcribed, processed, or transported less efficiently or could be unstable or degraded more rapidly. Translation could be less efficient, or even if mutant polypeptide globin chains were synthesized at normal levels, structural instabilities could result from impaired haem binding, decreased solubility, or the inability to assemble normally with other subunits to form functional tetramers. These possibilities are still difficult to detect because the pathway of haem–globin interaction and folding intermediates are not fully characterized.

Unprecedented scales of *E. coli* fermentation are planned for the production of haemoglobin, for which massive volumes of water and methods of waste-water disposal that meet environmental protection laws will be needed (*Somatogen Prospectus*, 1991). Yeast fermentation technologies may allow more efficient large-scale processing for three reasons: (1) less water is needed for yeast fermentation; (2) the brewery indusry already has a significant amount of experience with the large-scale fermentation of yeast; and (3) perhaps most importantly, yeast does not contain endogenous endotoxin (Ogden *et al.*, 1992).

Production of human haemoglobin in transgenic animals has a natural advantage in that large-scale fermentations are not required. Instead, herds of transgenic livestock could be established. Pigs offer a particularly promising host system because they reach sexual maturity early, have a short gestation period, and produce large litters. A typical 100 kg animal will yield 6–8 l of blood. Before this method of production can provide a profitable alternative, however, the current low yields of transgenic swine must be improved, which most likely will be accomplished through manipulations of globin-expression regulatory sequences. Purification methods will follow well-described techniques for isolating haemoglobin from human blood, but complications can arise in purifying human haemoglobin from native animal haemoglobin and haemoglobin hybrids on a large scale that may require the development of new, sophisticated technologies.

The potential benefits of recombinant products overshadow these problems in large-scale production. Site-specific mutagenesis permits protein engineering by rational design. Every amino acid becomes a candidate for modification based on steric, electrostatic, hydrogen bonding, and hydrophobic effects. In the future, it may be possible to design haemoglobin molecules of various sizes with a range of affinities for ligands that are stable to oxidation or denaturation.

Toxicity and metabolism of acellular haemoglobin solutions

At this time, the major obstacle in the development of haemoglobin-based blood substitutes lies not in finding appropriate methods to modify haemoglobin, but rather in determining whether cell-free haemoglobin may be inherently toxic. The clinical safety of either chemically or genetically derived modified haemoglobins has not been demonstrated adequately. Areas under research include renal dysfunction, systemic and pulmonary vasoconstriction, macrophage activation, arachidonate metabolism, peroxidative tissue damage, and toxic synergy between haemoglobin and endotoxin.

RENAL INJURY

Renal function is degraded during hypovolaemic shock by reduced blood flow to the kidneys. A combination of hypovolaemia and filtration of haemoglobin solutions can intensify renal damage through tubular obstruction by precipitated haemoglobin in the lumen or tubular damage from iron or haem

release, by further reduction in blood flow due to vasoconstrictive effects, or by changing the glomerular filtration rate through hydrostatic forces from the oncotic pressure of haemoglobin.

Haemoglobinuria can be prevented or reduced markedly by cross-linking, polymerizing, or conjugating haemoglobin solutions to produce larger molecules. Glomerular filtration of haemoglobin and tubular obstruction did not occur after infusions of glutaraldehyde-polymerized haemoglobin (Friedman *et al.*, 1984). Acute elevations in plasma creatinine levels that were evident after infusions of unmodified human haemoglobin solutions in swine did not occur after infusions of α_1(G6)–α_2(G6) cross-linked haemoglobin (Hess, Macdonald and Winslow, 1992), and kidney function was found to be normal in dogs after replacement of 30% of their blood volume with rHb1·1 (Looker *et al.*, 1992).

Haemoglobin-induced nephrotoxicity is inversely proportional to the purity of the haemoglobin solution (Rabiner *et al.*, 1967). Residual red cell stroma can cause renal failure (Birndorf, Lopas and Robboy, 1971), and free haem and iron have been implicated in mechanisms of renal damage (Braun *et al.*, 1970; Paller, 1988). Whether haem or iron in intact haemoglobin can produce these same effects is unclear, mainly because of the difficulty in complete elimination of micro-quantities of these moieties from concentrated solutions of haemoglobin.

VASOCONSTRICTION AND INFLAMMATORY RESPONSES

Nitric oxide has been identified recently both as a messenger for intracellular signal transduction by its action with soluble guanylate cyclase and as an effector molecule in immunological reactions (*for a review, see* Moncada, Palmer and Higgs, 1991). Nitric oxide-mediated mechanisms have been identified in smooth muscle vascular relaxation (Gruetter *et al.*, 1979), inhibition of platelet aggregation (Radomski, Palmer and Moncada, 1987; Durante *et al.*, 1992), penile erection (Rajfer *et al.*, 1992), gastrointestinal adaptive relaxations (Desai, Sessa and Vane, 1991), neuronal transmission (Garthwaite *et al.*, 1989; Bredt and Snyder, 1989), retrograde transmission for long-term memory storage (Schuman and Madison, 1991), cytotoxicity of activated macrophages (Marletta *et al.*, 1988), and neutrophil adhesion to endothelial cells (Kubes, Suzuki and Granger, 1991).

The very high affinity of haemoglobin for NO as a haem ligand may explain some of the unanticipated physiological reactivities of cell-free haemoglobin solutions. Alternatively, changes in vascular flow patterns or oxygen delivery that result when red cell mass is diminished and replaced by a low-viscosity, acellular haemoglobin solution may affect NO production.

Endothelial cells are required for the regulation of vascular tone (Furchgott and Zawadzki, 1980), and the diffusible endothelium-derived relaxing factor (EDRF) has been identified as NO (Palmer, Ferrige and Moncada, 1987). Nitric oxide regulates the basal tone of the cardiovascular system by responding to changes in blood pressure and flow and mediates the interaction between the endothelium and platelets (Moncada, Palmer and Higgs, 1991).

Nitric oxide has a short biological half-life (~6 s). It is inactivated by reaction with dissolved O_2 to yield nitrite

$$2NO + O_2 \rightarrow 2NO_2^- \quad (6)$$

or with the superoxide anion to yield the peroxynitrite anion,

$$NO + O_2^{-}\cdot \rightarrow {}^-OONO \quad (7)$$

which can rearrange to form nitrate (NO_3^-) (*for a review, see* Ignarro, 1990) or decompose to the potent oxidants HO· and NO_2· upon protonation (Beckman *et al.*, 1990).

Nitric oxide is synthesized in endothelial cells from the terminal guanidino nitrogen atom of L-arginine in a reaction that is mediated by calmodulin and catalysed by NO synthase, a constitutive, Ca^{2+}-dependent dioxygenase. It diffuses from its site of synthesis to underlying smooth muscle cells where it activates guanylate cyclase by binding as a haem ligand. Vasodilation is initiated by an increase in intracellular levels of guanosine 3′,5′-cyclic monophosphate (cGMP), which promotes a sequence of protein phosphorylation reactions that are associated with smooth muscle relaxation (Moncada, Palmer and Higgs, 1991). A schematic diagram of the synthesis and possible actions of endothelium-derived NO are shown in *Figure 13*. Haemoglobin can compete with guanylate cyclase for binding NO and inhibit activation of the enzyme (Murad *et al.*, 1978). In isolated aortic rings, haemoglobin inhibits vasodilation when endothelial cells are intact, but not when the endothelial cells are removed (Buga *et al.*, 1991).

Interference with vasodilation by loss of NO may explain systemic and pulmonary hypertensive effects that have been reported after infusions of cell-free haemoglobin solutions. Increased blood pressure has been observed post-infusion in rats (Pryzbelski *et al.*, 1991), rabbits (White *et al.*, 1986a), swine (Hess, Macdonald and Winslow, 1992), baboons (Moss *et al.*, 1976), humans (Amberson, Jennings and Rhode, 1949; Savitsky *et al.*, 1978), and in isolated renal (Lieberthal *et al.*, 1989) and cornary (Vogel *et al.*, 1986; Macdonald *et al.*, 1990) systems. In human trials in which blood pressure was evaluated, hypertension was reported ~50% of the time (Winslow, 1992). Resuscitation with α_1(G6)–α_2(G6) cross-linked haemoglobin was followed by a 10-point rise in mean arterial pressure in a hydrated, haemorrhaged swine model (Burhop *et al.*, 1991) and an increase of ~40 mm Hg in mean arterial pressure in a dehydrated, haemorrhaged swine model (Hess, Macdonald and Winslow, 1992).

Application of the Fick equation to overall O_2 transport gives

$$\dot{V}_{O_2} = \dot{Q} \cdot (A - \bar{v})O_2 \quad (8)$$

where $\dot{V}_{O_2}$ is the rate of O_2 uptake, $(A - \bar{v})O_2$ is the difference in arterial–venous O_2 content, and $\dot{Q}$ is cardiac output, which is a function of O_2 demand and vascular resistance to flow. The increased vascular resistance in the dehydrated swine model caused cardiac output to decrease, and as a result, the added benefit of the O_2-carrying capacity of haemoglobin as a resuscitation fluid was eliminated. Thus, total O_2 delivery was not improved

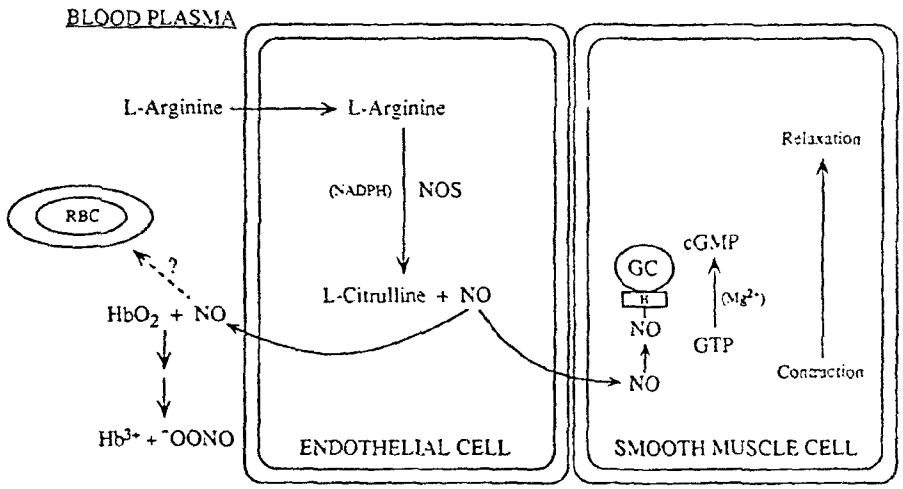


Figure 13. Schematic diagram of the biosynthesis of NO and possible action in endothelial cells, smooth muscles, and blood plasma in the presence of cell-free haemoglobin. Extracellular L-arginine is transported into endothelial cells and converted to L-citrulline and NO in a reaction catalysed by nitric oxide synthase (NOS), with NADPH as a cofactor. Nitric oxide diffuses out of the endothelial cell into adjacent smooth muscle cells where it activates guanylate cyclase (GC) by binding to the haem moiety (H) of the enzyme. Guanosine 5'-triphosphate (GTP) is converted to cGMP in the reaction, with Mg^{2+} as a cofactor. As levels of intracellular cGMP accumulate, the smooth muscle cells relax. Alternatively, NO diffuses out the endothelial cell into the blood plasma in the vessel lumen, where it can bind to cell-free oxyhaemoglobin. Nitric oxide will displace O_2 from oxyhaemoglobin (HbO_2) to form the final reaction products of methaemoglobin (Hb^{3+}) and the peroxynitrite anion (^-OONO). The intermediate products of this reaction are probably $Hb^{3+} + O_2^{-\cdot} + NO$. Nitric oxide diffusion and reaction with haemoglobin inside the red blood cell (RBC) may also occur, in which case, the intracellular methaemoglobin reductase system would maintain Hb^{3+} produced by reaction of HbO_2 with NO at low levels.

with haemoglobin over either albumin or Ringer's lactate because of hypertensive effects (Hess, Macdonald and Winslow, 1992).

Endothelium-derived NO modulates acute inflammatory responses by inhibiting platelet aggregation (Radomski, Palmer and Moncada, 1987) and neutrophil adhesion to endothelial cell walls (Kubes, Suzuki and Granger, 1991). Vasoconstrictor substances are released upon platelet aggregation, and neutrophil adhesion to the endothelium damages vessel walls. Impairment of NO function by haemoglobin will exacerbate the tendency to vasospasm and cause damaged endothelial cells to become more predisposed to inflammatory responses. Local ischaemia can result either from a shut-down in circulatory flow as interstitial pressure increases because of oedema or from occlusion of blood vessels from neutrophil plugging of capillaries (McCord, 1986).

If inhibition of EDRF activity depends on direct exposure of haemoglobin to endothelial cells or extravasation, the size of the haemoglobin molecule may become a critical factor. Larger molecules will not be extravasated as readily as haemoglobin tetramers. Alternatively, since red blood cells do not

appear to alter or disrupt vascular tone, haemoglobin solutions could be encapsulated in artificial membranes to protect endothelial cell function. The integrity of the endothelial cell layer and the geometry of the vessel walls may influence NO reactivity with cell-free haemoglobin. Principles used to evaluate O_2 diffusion (*see above*) might also apply to interactions between haemoglobin and NO.

The blood–brain barrier is probably secure against leakage of cell-free haemoglobin into neuronal tissue. For example, infusions of α_1(G6)–α_2(G6) cross-linked haemoglobin showed no neurological effects in rats (Przybelski *et al.*, 1990; Bauman, Przybelski and Bounds, 1991). However, in the case of head trauma, the appearance of haemoglobin in the brain could have severe neuropathological consequences if neurotransmission is disrupted.

Nitric oxide also acts as an immunological factor that is synthesized by a Ca^{2+}-dependent, inducible enzyme in activated macrophages (Marletta *et al.*, 1988; Moncada, Palmer and Higgs, 1991). Nitric oxide has been implicated in a number of cytotoxic reactions as a host defence mechanism (McCall and Vallance, 1991). The activated oxygen species, $O_2^{-}\cdot$, reacts with NO to produce cytotoxic oxidants (*see Equation 7*). $O_2^{-}\cdot$ is also known to inactivate EDRF (Gryglewski, Palmer and Moncada, 1986) and to increase capillary permeability (McCord, 1986). Thus, increased production of $O_2^{-}\cdot$ from auto-oxidation of haemoglobin could have several effects that are interrelated with NO action and immune function. It can yield cytotoxic mediators, destroy endothelium-derived NO, and trigger inflammation of tissues. All of these scenarios are consistent with the white cell activation and endothelial cell damage that was observed in haemoglobin-infused rabbits (White *et al.*, 1986a).

Endotoxin induces synthesis of large amounts of NO in endothelial cells and provokes vasodilation, vascular damage and extreme hypotension (McCall and Vallance, 1992), and combinations of endotoxin and haemoglobin have been shown to enhance toxic effects in rabbits (White *et al.*, 1986b). Opposing effects of endotoxin and haemoglobin would certainly confuse vascular tone regulation and perhaps potentiate inflammatory responses.

Vaso- and bronchoconstriction also can result from the production of thromboxanes, which are metabolites of arachidonic acid derived from membrane phospholipids. These vasoactive compounds are released by phagocytic macrophages after stimulation from foreign particles in the blood (Grisham, 1985), and thus, large polymers of haemoglobin might be able to stimulate macrophage activation. Increased levels of thromboxanes were measured in rabbits infused with polymerized bovine haemoglobin. However, these effects were attributed primarily to contaminants in the haemoglobin solution (i.e. phospholipids, polymerized haemoglobin degradation products, and/or endotoxin) rather than directly to the haemoglobin polymers (Feola *et al.*, 1988). Direct measurements of macrophage stimulation showed that most toxicities occurred with bovine haemoglobin solutions that were contaminated with stromal phospholipids or endotoxins. Pure haemoglobin was less reactive, and haemoglobin that was cross-linked site-specifically with DBBF

was less reactive than glutaraldehyde-polymerized haemoglobin (Simoni *et al.*, 1990).

OXIDATIVE STRESS

Activated O_2 species ($O_2^{-}\cdot$, H_2O_2, $HO\cdot$) are implicated in tissue re-perfusion injury and cellular membrane damage (Halliwell and Gutteridge, 1984; McCord, 1986). Oxygen reduction is catalysed by transition metals such as iron (*for a review, see* Cadenas, 1989).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-}\cdot \quad (9)$$

The $O_2^{-}\cdot$ decays through disproportionation to H_2O_2,

$$2O_2^{-}\cdot + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (10)$$

and H_2O_2 decomposes to the reactive hydroxy radical, HO , which is a potent initiator of tissue damage by lipid peroxidation (Pryor, 1986).

Evidence that $HO\cdot$ formation is inhibited in the presence of a chelator of free iron, desferrioxamine, suggests that iron is an intermediate catalyst in the Fenton reaction (Gutteridge, Richmond and Halliwell, 1979).

$$Fe^{3+} + O_2^{-}\cdot \rightarrow Fe^{2+} + O_2 \quad (11)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + HO\cdot \qquad \text{(Fenton reaction)} \quad (12)$$

The net balance of reactions 11 and 12 is referred to as the Haber–Weiss reaction.

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + HO^{-} + HO\cdot \quad \text{(Haber–Weiss reaction)} \quad (13)$$

H_2O_2 reacts with haemoglobin and causes iron to be released, which then stimulates lipid peroxidation (Gutteridge, 1986). In the absence of red blood cell antioxidant defences (e.g. catalase, superoxide dismutase, and the glutathione peroxidase redox system), haemoglobin could add to oxidative stress by release of free iron (Faasen *et al.*, 1988), in which case iron chelators may be required to prevent cellular damage (Hedlund, 1991). Free haem is another catalyst of peroxidative mechanisms (Vincent, 1989) and is particularly destructive because of its ability to partition into membranes (Rose *et al.*, 1985).

H_2O_2 also can act as a cytotoxic mediator by reaction with oxyhaemoglobin (Fe^{2+}) or methaemoglobin (Fe^{3+}) to produce ferrylhaemoglobin (Fe^{4+}), which is a highly reactive, intermediate oxidative species similar to compound II of peroxidase. The reaction with oxyhaemoglobin occurs through a comproportionation of ferrylhaemoglobin-OH and oxyhaemoglobin to yield methaemoglobin and appears to be catalysed by an intermolecular electron transfer between globin tyrosine residues (Giulivi and Davies, 1990). Ferryl-haemoglobin, or its ferryl-hydroxo complex (Fe^{4+}–OH), can promote a chain

reaction of lipid peroxidation by reaction with hydroperoxides (ROOH) to form methaemoglobin and the peroxyl radical (ROO·) (Cadenas, 1989).

$$ROOH + Fe^{4+} - OH \rightarrow ROO\cdot + Fe^{3+} + H_2O \quad (14)$$

Even at quantitatively low levels, the ferryl-haem complex may be a significant factor in mechanisms of lipid peroxidation and destruction of biological tissues (Dix *et al.*, 1985).

The mechanism for a ferryl intermediate in tissue and haemoglobin destruction gains further support from experiments showing that myoglobin is transformed to a transiently activated oxidase in the presence of H_2O_2 (Osawa and Korzekwa, 1991). A ferrylmyoglobin radical is postulated to be the precursor of the oxidase form of myoglobin, a form that is generated when the protein radical reacts with the haem moiety to form a globin-bound haem product. The oxidase activity of this species leads to further production of H_2O_2 and finally protein destruction and haem release. Analogous transformations of haemoglobin-based blood substitutes could have pathological significance similar to that seen in ischaemia re-perfusion injury, in which xanthine dehydrogenase is converted to an oxidase through the action of a cytosolic protease.

METABOLISM

Intact red blood cells are metabolized by the reticuloendothelial system (i.e. the liver, spleen and bone marrow) after their removal from the circulation. Globin is digested, protoporphyrin is cleaved, and iron is recycled. Free haemoglobin in the plasma dissociates to dimers, which bind to haptoglobin and are taken up in the liver. Oxidized dimers can release their haem to albumin or haemopexin, and free iron is bound to transferrin. When haptoglobin becomes saturated, dimers filter through the glomerulus in the kidney and are reabsorbed by proximal tubule cells. (If reabsorption is exceeded, haemoglobinuria occurs.)

Acellular haemoglobin-based solutions may follow some but not all of these metabolic pathways. Cross-linked haemoglobins are retained longer in the circulation than native haemoglobins because they do not dissociate into excretable dimers (Bunn and Jandl, 1967; Keipert, Verosky and Triner, 1988) or bind to haptoglobin (Panter *et al.*, 1991). Plasma retention times of modified haemoglobins are size (i.e. dimeric, tetramer, polymerized or conjugated), dose, and species dependent (Matsushita *et al.*, 1988; Hess *et al.*, 1989; Berbers *et al.*, 1991; Hsia *et al.*, 1992).

The circulatory half-life of haemoglobin solutions depends on the redistribution of the molecule between blood and lymph and its rate of catabolism. $\alpha_1(G6)$–$\alpha_2(G6)$ cross-linked haemoglobin has been shown to diffuse across the endothelial–cell barrier twice as fast as albumin, and half of its circulating mass recirculates between interstitial and lymphatic spaces (Hess *et al.*, 1990). The clinically optimal time for circulation of cell-free haemoglobin solutions will depend on the application of the product. Circulatory times should be

long enough to preserve O_2 delivery to prevent hypoxia, but haemoglobin should be metabolized and excreted in as short a time as possible to prevent untoward toxic effects.

The distribution of haemoglobin metabolic products in organ systems depends on the size of the molecules. NFPLP cross-linked haemoglobin distributes to the liver, spleen, lymph and kidney but is not found in urine or the brain (Bleeker *et al.*, 1989; Keipert *et al.*, 1992). A general rule is that higher molecular weight species distribute to the liver, and lower molecular species or degradation products distribute to the kidneys (Bleeker *et al.*, 1989; Keipert *et al.*, 1992). The physiological consequence of this profile is as yet undetermined.

Modern clinical trials with haemoglobin solutions

The recognition of endotoxin contamination of haemoglobin solutions and demonstrations that rigorous purification of haemoglobin could prevent toxicities ushered in the modern era of clinical studies with haemoglobin-based blood substitutes (Rabiner *et al.*, 1967).

Savitsky and his colleagues at the Warner–Lambert Company conducted the first modern human clinical trials with a highly purified, unmodified acellular haemoglobin solution (Savitsky *et al.*, 1978). The haemoglobin solution was administered in a small dose (0·25 g kg^{-1}) to eight healthy volunteers. Seven volunteers experienced increased blood pressure and slowed heart rate, two experienced abdominal and back pains, and all eight demonstrated reduced creatinine clearance as a manifestation of oliguria. Savitsky's conclusion was that administration of unmodified haemoglobin solutions prompted renal vasoconstriction.

In the early 1980s, the first modified haemoglobin product, which was pyridoxalated to increase P_{50}, was developed for clinical trials. A small clinical trial was begun at the Biotest Serum Institut of Germany with three healthy volunteers, but the trial was terminated after two volunteers developed reversible renal failure (Siler, Jereski and Baker, 1990).

In response to the results from the Warner–Lambert and Biotest trials, further clinical evaluation of haemoglobin solutions did not occur until the mechanism of renal toxicity was identified. In the middle of the 1980s it was shown that kidney tubular obstruction could be prevented by increasing the molecular weight of haemoglobin (Friedman *et al.*, 1984). Soon afterwards, Moss and his colleagues at the University of Chicago carried out studies with pyridoxalated, polymerized human haemoglobin solutions that had been shown to transport O_2 and to support life in primates (Gould *et al.*, 1986b). In 1987, they obtained permission from the USFDA to conduct Phase I safety trials in humans with a polymerized haemoglobin solution produced at Northfield Laboratories. In the initial trial, six healthy volunteers received a dose of 0·26 g kg^{-1}. One of the six patients developed bronchospasm, but the reaction was attributed to the predisposition of the patient to an anaphylactic response. The other five volunteers showed no untoward symptoms, and

these data, which were presented at an international meeting in 1987, revealed no renal toxicities (Moss *et al.*, 1989). The Northfield trial continued with post-operative patients until two patients experienced dyspnoea and severe chest and back pains, and the tests were stopped (Siler, Jereski and Baker, 1990).

Another polymerized haemoglobin product using bovine haemoglobin as starting material is being developed at the Biopure Corporation. The bovine haemoglobin is cross-linked with a limited amount of glutaraldehyde and has a significant proportion of non-cross-linked haemoglobin (Gawryl, Clark and Rausch, 1991). The formulation for the Biopure product was licensed to a firm in the Cayman Islands, and the product was used in a human trial in Guatemala in 1990 that was monitored by the Pan American Health Organization. Ten healthy volunteers received 0·25 g kg^{-1} of the haemoglobin solution. They reported no ill effects, and creatinine clearance remained normal (Garcia-Gallont, Herrera-Llerandi and Lopez Herrera-Llerandi, 1991).

The Biopure Corporation formed a partnership with the Upjohn Company and obtained USFDA approval to begin Phase I clinical trials with their product in 1991. The trials were begun in March of 1991 but were halted soon after due to 'medical events' (*Wall Street Journal*, 1991). No further information about this trial has been released.

Another bovine haemoglobin product that was polymerized with a ring-opened adenosine triphosphate and produced at the Istituto Sierovaccinogeno Italiano was administered in 1991 at the Institut de Recherche en Sciences de la Sante in Zaire to nine children with sickle cell anaemia who were experiencing painful crises. The children received volumes of the haemoglobin solution at 20–30% of their estimated blood volume. At an international meeting in 1991, Feola reported that the children experienced no adverse side-effects and that the solutions were effective in the treatment of the crises (Luhruma *et al.*, 1991). The trial was criticized at that meeting, however, because it was not sanctioned by regulatory agencies and no Phase I safety trials or control experiments were conducted.

As of this writing, Somatogen, Inc. has gained USFDA approval to conduct Phase I safety trials with the first recombinant haemoglobin product (rHb1.1). Likewise, Baxter Healthcare Corp. has begun Phase I trials with the chemically modified α_1(G6)-α_2(G6) cross-linked human haemoglobin. Early Phase I results reported from Somatogen, Inc. have indicated observations of fever and 'flu-like' symptoms. Early results from the Baxter Healthcare Corp. trial have been reported as 'uneventful' with 'mild hypertension'. More details on these clinical trials will be disclosed at the Fifth International Symposium on Blood Substitutes in March, 1993.

Conclusions

Requirements for a blood substitute centre around the need for O_2-carrying resuscitation fluids. Two key limitations that exclude unmodified cell-free haemoglobin solutions from use as a blood substitute product (i.e. high O_2

affinity and tetramer dissociation) have been remedied by chemical and genetic modifications of the haemoglobin molecule. Although methaemoglobin formation does not appear to limit the use of cell-free haemoglobin *in vivo*, oxidative reactions and protein instability may be important factors in the final success of these solutions. Activated O_2 species initiate biological peroxidative reactions with proteins and lipids, and the inverse correlation between the rate of haemoglobin auto-oxidation and O_2 affinity dictates a trade-off between desirable O_2 releasing properties and unwanted production of oxidized species. The creation of a haemoglobin derivative with acceptable *in vivo* O_2 binding properties but with reduced oxidative potential has become an important goal in site-specific protein design.

Undefined toxic mechanisms of cell-free haemoglobin solutions remain. Whether these are due to low-level contaminants in the solutions or to inherent properties of the haemoglobin molecule is still unresolved. The newly defined roles of NO as a signal transducing molecule in cardiovascular control and immune function raise serious concerns about *in vivo* applications of haemoglobin solutions. Haemoglobin interference with these pathways, either by binding NO as a haem ligand or by destruction of NO through oxidation reactions, may lead to disturbances in cell–cell communication or enhanced cytotoxicities. Shock, dehydration, endothelial cell damage, and immune reactions are all critical determinants in resuscitation that are interrelated with physiological mechanisms of NO. Thus, a clear understanding of the diffusion and chemical properties of NO has become another priority in blood substitutes research. If interference is the result of direct binding of NO to cell-free haemoglobin solutions, it is highly unlikely that haemoglobin could be modified to prevent NO binding without a detrimental effect on O_2 binding.

The not so subtle differences between haemoglobin inside the red blood cell and free in the circulation have provided interesting challenges to protein engineers for several decades, and it appears that they probably will continue to do so for some time. In the meantime, it might be possible to reduce oxidative damage of cell-free haemoglobin solutions by co-administration of antioxidants or to alleviate increased vascular resistance by antihypertensive drug therapy. Although it is possible that increased blood pressure may be beneficial in the treatment of hypovolaemic or endotoxic shock, it is hard to imagine how increasing the cardiac work of O_2 transport will lead to clinical benefit. In any case, these are not long-term solutions, and careful, scientific studies are needed to define the exact characteristics and discreet mechanisms of cell-free haemoglobin *in vivo*.

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Author's note

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