Oxygen Transport by Hemoglobin

Heimo Mairbäurl[*1] *and Roy E. Weber*[2]

ABSTRACT

Hemoglobin (Hb) constitutes a vital link between ambient O_2 availability and aerobic metabolism by transporting oxygen (O_2) from the respiratory surfaces of the lungs or gills to the O_2-consuming tissues. The amount of O_2 available to tissues depends on the blood-perfusion rate, as well as the arterio-venous difference in blood O_2 contents, which is determined by the respective loading and unloading O_2 tensions and Hb-O_2-affinity. *Short-term* adjustments in tissue oxygen delivery in response to decreased O_2 supply or increased O_2 demand (under exercise, hypoxia at high altitude, cardiovascular disease, and ischemia) are mediated by metabolically induced changes in the red cell levels of allosteric effectors such as protons (H^+), carbon dioxide (CO_2), organic phosphates, and chloride (Cl^-) that modulate Hb-O_2 affinity. The *long-term*, genetically coded adaptations in oxygen transport encountered in animals that permanently are subjected to low environmental O_2 tensions commonly result from changes in the molecular structure of Hb, notably amino acid exchanges that alter Hb's intrinsic O_2 affinity or its sensitivity to allosteric effectors. Structure-function studies of animal Hbs and human Hb mutants illustrate the different strategies for adjusting Hb-O_2 affinity and optimizing tissue oxygen supply. © 2012 American Physiological Society. *Compr Physiol* 2:1463-1489, 2012.

Introduction

Hemoglobin (Hb) is the most ubiquitously occurring oxygen (O_2)-binding protein. Hb or its genes appear to occur in all living organisms (258) and possibly in every cell (182). It also is among the most intensively studied proteins, both in terms of physiological function and molecular structure. Hemoglobin's primary role in vertebrates is to transport molecular oxygen in support of aerobic cellular metabolism. Other globins include muscle myoglobin (Mb), nerve cell neuroglobin (Ngb), and cytoglobin (Cgb) that are found in many tissues and carry out a range of alternative functions (58). The biological significance of Hb is pointedly illustrated by anemia and, in extreme, the Antarctic icefish (*Channichthyidae*), where decreased Hb or its total absence, respectively, is compensated by energy-costly compensatory adaptations including a high cardiac capacity (62, 133). Increasing the Hb concentration per unit volume of blood and its total mass above normal improves performance capacity in humans (203). Also Hb's functional properties affect performance as is illustrated, for example, by the observation that tissue O_2 supply may be enhanced by both increased Hb-O_2 affinity that increases O_2-loading [e.g., in humans and other animals under severe hypoxia (81, 99)], as well as decreased Hb-O_2 affinity that favors the release of bound oxygen from the Hb molecule (146), for example, in the myocardium (30).

The transport of oxygen in blood from the lungs to the tissues requires a carrier molecule such as Hb since the amount of O_2 in physical solution in aqueous media is small compared to tissue O_2 requirement. The solubility coefficient αO_2 for human plasma is 0.03 ml × liter × mmHg. In contrast, human Hb binds 1.34 mL of oxygen per gram of the protein, which increases the oxygen content of blood more than 70-fold. Paul Bert first reported O_2 binding to Hb in 1878 (31). More than a century ago, Bohr (34) demonstrated some characteristic properties of Hb oxygenation that enhance O_2 unloading in the tissues, that is, the sigmoid nature of the O_2 dissociation curve and the Bohr effect (decreased O_2 affinity under acidic conditions), which express, respectively, cooperative (homotropic) interactions between the O_2-binding hemes, and inhibitory (heterotropic) interactions between the hemes and sites for binding protons.

In vertebrates, Hb is packed into red blood cells (RBCs) rather than dissolved in plasma thus avoiding a high oncotic pressure in plasma and a high blood viscosity, which would impair fluid balance and blood flow, respectively. The inclusion of Hb in RBCs moreover provides a cellular microenvironment where the levels of metabolites and other effectors that modulate O_2 binding by Hb can be regulated to optimize O_2 binding in the lung and its release to peripheral tissues. However, Hb is freely dissolved in body fluids of many invertebrate species, where it almost invariably consists of high molecular weight aggregates that are excluded from filtration in the excretory organs.

This review outlines key functional and structural adaptations of Hb and the role of allosteric effectors (substances, whose binding at one site of the molecule affects the

[*]Correspondence to heimo.mairbaeurl@med.uni-heidelberg.de

[1]Medical Clinic VII, Sports Medicine, University of Heidelberg, Germany

[2]Zoophysiology, Department of Bioscience, University of Aarhus, Denmark

Published online, April 2012 (*comprehensivephysiology.com*)

DOI: 10.1002/cphy.c080113

Copyright © American Physiological Society

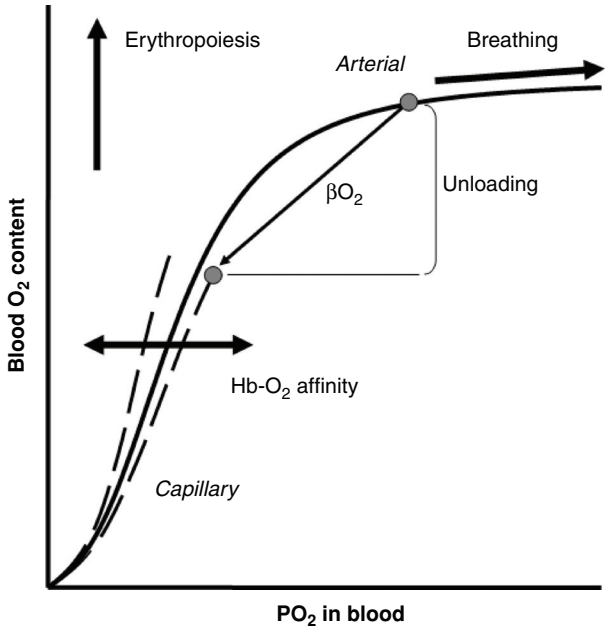

Figure 1 Schematic representation of factors that modify O_2 transport by hemoglobin in blood. Modified, with permission, after Bouverot (44). See text for details.

binding of another substance at a different site) in regulating O_2 transport by blood. Although focusing on human Hbs it also includes comparative aspects that give insight into various strategies that modulate the O_2 transport functions of Hb and increase survival under extreme environmental conditions in other animals.

Hb-O_2 affinity (that is conveniently characterized in terms of P_{50}, the O_2 tension where 50% of the Hb is oxygenated) is the governing factor for binding the O_2 that diffuses from the pulmonary alveoli or other gas exchange surfaces into the blood and its release in peripheral tissues. Thus, Hb-O_2 affinity is an important link between alveolar O_2 tension and tissue oxygen supply. As illustrated in Figure 1 where the upper part of the oxygen-dissociation curve reflects arterial O_2-loading characteristics, ventilation increases alveolar and arterial P_{O_2}. Consequently, arterial SO_2 and O_2 content increase. Erythropoiesis accounts for long-term adjustments of O_2-transport capacity by increasing the total amount of Hb and its concentration in blood. Operating in different time scales augmented ventilation (seconds) and erythropoiesis (days) increase the amount of O_2 loaded during passage of blood through the gas exchange organs. The circulatory system controls blood flow and thus the amount of O_2 delivered to the periphery per unit of time. The Fick equation describes the relation between blood flow and blood O_2 transport:

$$\dot{V}_{O_2} = Q \cdot (CaO_2 - CvO_2)$$

where $\dot{V}_{O_2}$ refers to O_2 consumption, Q is cardiac output, and $(CaO_2 - CvO_2)$ is the arterio-venous O_2-content difference. Thus, for a given cardiac output (blood convection) O_2 transport increases with the O_2-carrying capacity (Hb concentration) and with the O_2 capacitance of the blood (βO_2), which quantifies the amount of O_2 unloaded for a given arterio-venous P_{O_2} difference.

$$\beta O_2 = \frac{(CaO_2 - CvO_2)}{(PaO_2 - PvO_2)}$$

where the arterio-venous O_2 content difference is divided by the difference between arterial and venous O_2-partial pressure, PaO_2 and PvO_2, respectively. The O_2 capacitance is reflected in the steepness (slope and cooperativity) of the operational part of the O_2-dissociation curve. The amount of O_2 delivered varies with the diffusion gradient from the capillaries to the tissue (44,274), which at constant arterial O_2 tension, increases with increasing O_2 consumption by the cells.

Alterations in Hb-O_2 affinity are therefore important means of adjusting both arterial O_2 loading and peripheral O_2 unloading that are of great significance in defending aerobic metabolism when inspired P_{O_2} is decreases and/or O_2 demand increases (e.g., during exercise).

The Hemoglobin Molecule

Vertebrate Hbs are tetrameric molecules with a molecular weight of approximately 64.500 Da. They are composed of two α-like and two β-like chains forming two αβ-pairs. Each subunit contains one O_2-binding heme group. Thus, each Hb molecule binds four molecules of O_2 (Fig. 2). α-like chains are encoded by a cluster of genes located on chromosome 16, whereas β-like chains, are encoded by genes on chromosome 11 (242, 263).

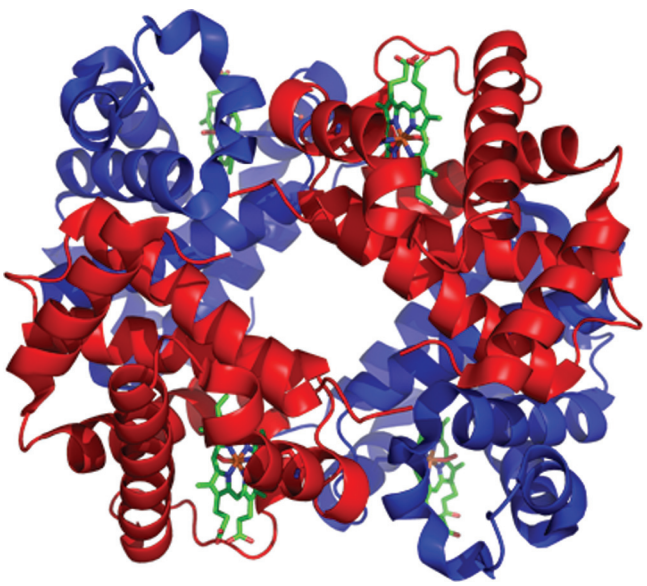

Figure 2 Tetrameric human Hb A, showing two α-chains in red, two β-chains in blue, and the iron-containing heme groups in green. Adapted, with permission, from the Protein Data Bank, 1gzx.

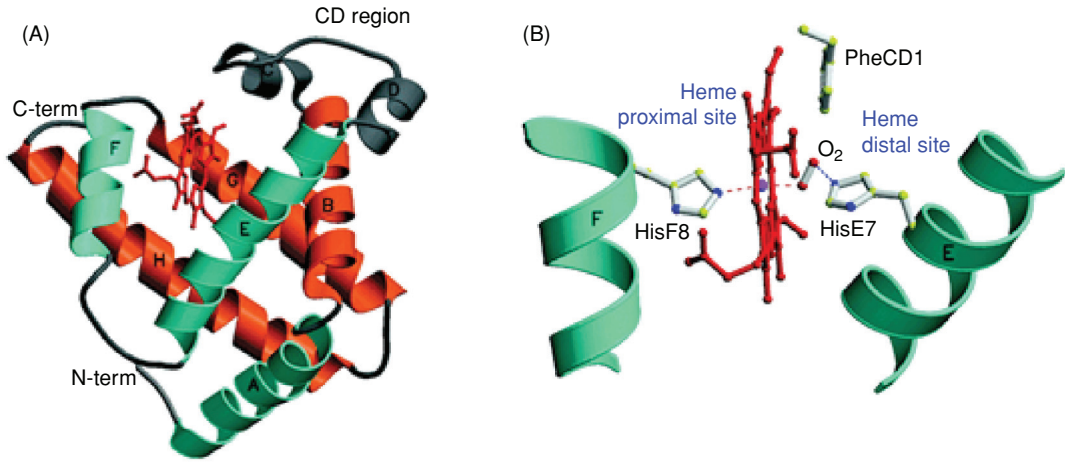

Figure 3 (A) The globin fold typically seen in mammals (a "three-over-three α-helical sandwich" shown in two colors) composed of helices A to H. (B) The heme (in red), the proximal and distal sites defined by E- and F-helices together with key residues PheCD1, HisE7, and HisF8. Red dashed line, the Fe coordination bonds with the proximal HisF8 residue and liganded O_2. The Fe atom is shown in purple, and the hydrogen bond between O_2, and the distal HisE7 residue is indicated in blue. Adapted, with permission, from Pesce et al. (167).

As with most mammals, humans have different Hb-isoforms, whose expression changes in the course of development, that is, embryonic Hbs, fetal Hb F, and adult Hb A.

Modifications of the Hb molecule can also be due to genetic differences caused by point mutations resulting in hemoglobin polymorphs, not all of which are compatible with life. Additionally, posttranslational modifications occur (56), for example, by glycation that leads to the human Hb variants Hb A_1a, Hb A_1b, and Hb A_1c (126), acetylation (191), methylation (227), and nitrosylation (213). Some basic mechanisms underlying multiplicity are described in reference (244).

Primary structure

The amino acid sequence of human Hb A was elucidated by Braunitzer et al. (46). The α- and β-chains contain 141 and 146 amino acid residues, respectively. Identical or analogous residues occupy common positions when the α- and β-chains from different species are aligned. The high sequence homology reflects gene duplication during phylogeny. A comparison of primary structures of more than 20 mammalian Hbs shows that 75 residues of the α-chain and 64 residues of the β-chain are invarient (116), whereas a few are completely invariant among vertebrate Hbs (73). However, of the 45 (27 α-chain and 18 β-chain) amino acid residues that are invariant in Hbs from 32 different vertebrate species, 16 are essential for the allosteric mechanism (85), indicating their fundamental importance.

Three-dimensional structure

The α- and β-chains consist of seven and eight helical segments, respectively, designated A-H. Helices are separated by nonhelical segments marked AB, BC, and so on. A short nonhelical segment called NA forms an N-terminal extension of helix A. Another, HC, follows the last helix at the C-terminal end (163). Amino acid residues are labeled according to their position within a helix and numbered sequentially from the N- to the C-terminus. Thus, His β143(H21) denotes the histidine occupying position 143 in the β-chain and position 21 in helix H, and Val α1(NA1) is the N-terminal valine of the α-chain.

The tertiary structures of the α- and β-chain are akin, both subunits exhibiting the "globin-fold" (163, 164) that characterizes all globins (167).

The heme group, an iron containing protoporphyrin ring, is buried in a hydrophobic pocket that is formed by the E, F, and G helices and the CD corner of each chain (Fig. 3). The heme group is held in place by bonds with Phe α43 and Phe β42 in CD1. The iron is coordinated with the four-pyrrole nitrogen atoms of the heme and covalently bonded with proximal HisF8 (at positions α87 and β92). Compared to pentacoordination of Hb and Mb, Ngb and Cgb are hexacoordinate, in that HisE7 binds to the heme iron at its sixth distal position. The hydrophobic environment protects the heme iron from oxidation.

Hbs of the primitive vertebrates, that is, the hagfish and the lamprey, are monomeric, at least in the oxygenated state. In higher vertebrates the α- and β-subunits assemble to form a spherical molecule made up of two dimers ($\alpha_1\beta_1$, $\alpha_2\beta_2$; Fig. 2), where α- and β-chains are connected by 34 residues located in the G and H helices and in the BC corner. The interdimer contacts ($\alpha_1\beta_2$ and $\alpha_2\beta_1$) are formed by 19 residues in the helices C and G and the FG corner (216); perturbations at this interface leads to loss of cooperativity (170). There are no bonds between β-subunits. The central cavity is lined with polar residues such as Ser and Thr and is filled with water molecules. Its entrance houses positively charged groups of the β-chains forming binding sites for anionic allosteric

effectors such as 2,3-diphosphoglycerate (DPG) and chloride ions (Cl^-) that modulate O_2 binding as discussed later.

Ligands

Ligands of Hb bind either to the central iron atom of the heme group or to amino acid residues constituting the globin subunits. Ferrous iron reversibly binds oxygen, carbon monoxide (CO), nitric oxide (NO), alkylisocyanides, and nitroso-compounds. When the iron atom is oxidized (in ferric form) none of these ligands bind. Hb containing ferric iron is called "metHb" or hemiglobin. The ferric iron atom binds H_2O or hydroxyl anions depending on pH and can reversibly bind other ions such as cyanide, cyanate, thiocyanate, azide, and fluoride.

Nonheme ligands (heterotropic effectors) modulate the binding of O_2 at the hemes and thus affect Hb-O_2 affinity and the Hb's ability to bind and release O_2. The most important effectors are H^+ and CO_2 (that underlie the Bohr effect), Cl^- ions, and organic phosphates that predominantly consist of DPG in mammals, inositol pentaphosphate (IPP) in birds, and ATP in ectothermic vertebrates. RBCs of many fish species additionally contain functionally significant levels of guanosine triphosphate (GTP). Other allosteric effectors that may modulate the oxygen-binding affinity of the ferrous iron in the heme group are lactate ions, CO, NO, and H_2O.

Oxygen binding to Hb

The valence of iron remains unchanged upon O_2 binding and O_2 unloading ("oxygenation" and "deoxygenation," respectively). During oxygenation the molecules shift from the T (tense) to the R (relaxed) structure that represent distinct spatial arrangements of the subunits and distances between the iron atoms, which is mathematically described by an allosteric model (149). The deoxygenated T-state of Hb is constrained by additional salt bridges and hydrogen bonds. O_2 binding causes a change in the spin state of the iron atom and its movement into the plane of the porphyrin ring of the heme group (163). This movement weakens salt bridges between the two αβ-dimers allowing a transition to the R-state (164) that involves a 15° rotation of the two αβ-subunits relative to each other (116, 163, 164). The O_2-bond to iron is stabilized by the "distal" His residue at E7 (His-α58 and His-β63; Fig. 3) (84).

Binding characteristics

The oxygen affinity of Hb in the oxygenated R state is higher than that of Hb in the deoxygenated T state. There is evidence that it even exceeds that of isolated α- or β-monomers (115) due to lack of constraining intersubunit bonds. In contrast to monomeric globins (including myoglobin), vertebrate Hbs show cooperativity, that is, an increase in the O_2 affinity of the remaining subunits when one or more subunits have become oxygenated. This causes the sigmoidal shape of the oxygen-dissociation curve (ODC; Fig. 4). The process can be described by four equilibria:

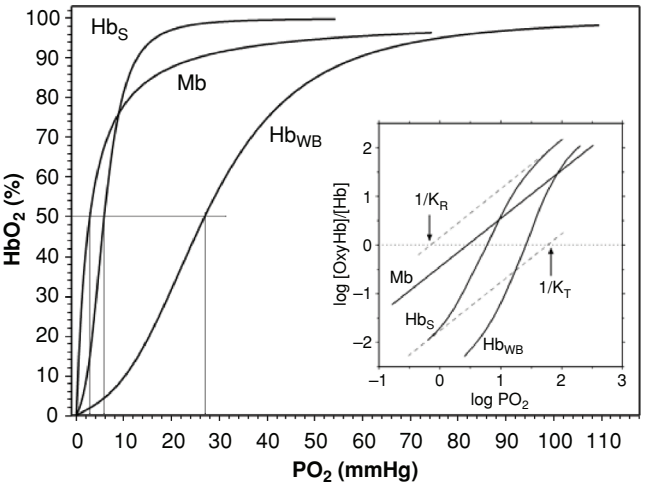

Figure 4 Oxygen dissociation curves (ODCs) for myglobin (Mb), stripped Hb in buffered solution (Hb_S), and intact human RBCs in whole blood (Hb_{WB}). The curve for monomeric Mb is based on $P_{50} = 2.8$ mmHg and $n = 1$ (no cooperativity) and is hyperbolic. The ODC for Hb_s ($P_{50} = 5.8$ mmHg) and Hb_{WB} ($P_{50} = 26.8$ mmHg) are cooperative ($n_{50} \sim 2.5$) and thus sigmoidal. The shift to the right of the ODC of Hb_{WB} relative to the ODC of Hb_S is predominantly due to binding of allosteric effectors, which reduce O_2 affinity. The inset shows Hill plots for Mb, Hb_S, and Hb_{WB} as well as the affinity constants for the T-state (K_T) and the R-state (K_R) of Hb_S. Effectors typically reduce K_T without significantly affecting K_R (252).

$$Hb + 4O_2 \leftrightarrow HbO_2 + 3O_2 \leftrightarrow Hb(O_2)_2 + 2O_2 \leftrightarrow Hb(O_2)_3 + O_2 \leftrightarrow Hb(O_2)_4$$

Precise measurements of the ODC over wide range of O_2 saturations (SO_2) permit determination of the intrinsic O_2 association constants for binding each of the four O_2 molecules (Adair constants A1, A2, A3, and A4) and the association constants in the R- and T-states (K_R and K_T). The oxygen saturation can be calculated using these Adair constants when the oxygen concentration is kept constant during oxygenation

$$SO_2 = \frac{[HbO_2] + 2[Hb(O_2)_2] + 3[Hb(O_2)_3] + 4[Hb(O_2)_4]}{4([Hb] + [HbO_2] + [Hb(O_2)_2] + [Hb(O_2)_3] + [Hb(O_2)_4])} = \frac{A_{1p} + 2A_2p^2 + 3A_3p^3 + 4A_4p^4}{4(1 + A_{1p} + A_2p^2 + A_3p^3 + A_4p^4)}$$

This "Adair equation" (2) expresses SO_2 as a function of the four "Adair" constants and Po_2 (p). For human Hb in intact erythrocytes at 37°C and pH 7.4 the Adair constants were computed as $A1 = 2.57 \times 10^{-2}$, $A2 = 7.80 \times 10^{-4}$, $A3 = 4.4 \times 10^{-6}$, and $A4 = 2.55 \times 10^{-6}$ (189). Slightly different values were reported by Winslow et al. (270). Cooperativity is expressed as the Hill coefficient, n_{max}, which is the maximal slope of the ODC in the Hill-plot, $\log(HbO_2/Hb)$ versus $\log(Po_2)$ (insert Fig. 4). Commonly, $n_{max} = n_{50}$ [the Hill coefficient at 50% SO_2, which for stripped human Hb A is approximately 2.8 to 3.0 (116)].

Allosteric effectors

The major allosteric effectors that modulate Hb-O_2 affinity *in vivo* are organic phosphates, H^+ and CO_2, and Cl- and lactate ions. In the absence of these chemical cofactors Hb exhibits high intrinsic O_2 affinity (Fig. 4, Hb_S) whereby it will not be able to unload and thus transport sufficient quantities of O_2 *in vivo*. The reduction in affinity is achieved by interaction with heterotropic allosteric effectors, which bind at specific sites (amino acid residues in the protein part of the molecule) reducing the O_2 affinity of the ferrous iron atoms in the heme. These effectors commonly bind more strongly to deoxyHb than to the oxygenated form and stabilize the T-state through the formation of additional salt bridges (163, 165). Their concentrations in RBC vary depending on cellular metabolism and changes in the extracellular milieu, leading to adjustments in Hb-O_2 affinity that may be adaptive in optimizing tissue O_2 supply. Increasing the Hb-O_2 affinity improves O_2 loading in the lungs, whereas a decrease improves unloading in the tissues.

The following sections discuss firstly the individual effects of allosteric effectors, thereafter interactive effects of concurring effectors on Hb-O_2 affinity. Point mutations (amino acid substitutions) in the globin part of the molecule that affect ligand binding will be discussed to demonstrate adaptive strategies optimizing Hb-O_2 binding and O_2 transport to tissues. It needs to be pointed out that binding characteristics of modulators of Hb-O_2 affinity have often been determined under very stringent experimental conditions and using strongly diluted Hb-solutions to circumvent problems with spectrophotometric readings. Analysis of experimental data on intact RBCs, although better reflecting the *in vivo* situation, is often complicated by interaction among the modulators under study.

Protons

Protons and CO_2 were the first chemical modifiers of Hb-O_2 affinity that were identified, more than a century ago, when Bohr et al. (35) demonstrated that an increased CO_2 tension (decreased pH) decreases blood-O_2 affinity. The decreased O_2 affinity seen with falling pH between pH 6 and 9 is called the alkaline Bohr effect. Further acidification increases O_2 affinity (acid Bohr effect). It should be borne in mind that the alkaline Bohr effect (influence of pH/CO_2 on O_2 affinity) and the reciprocal Haldane effect (influence of Hb-SO_2 on H^+/CO_2 binding) share the same molecular origin (allosteric interactions between O_2 and H^+/CO_2 binding) (123).

In human Hb A under physiological conditions, the Bohr effect is attributed to deoxygenation-linked proton binding at several residues in α- and β-chains, namely, Val α1 (NA1), His α122 (H5), His β2 (NA2), Lys β82 (EF6), His β143 (H21), and His β146 (HC3) (29, 130, 137, 165, 166). The pK_a values of the implicated residues are lower in oxygenated than in deoxygenated Hb. Recent studies indicate a major involvement of His side chains contributing approximately 90% of the total alkaline Bohr effect observed in human Hb in the presence of 0.1 mol/L chloride (29, 137) thus downgrading the contributions from Val α1 and Lys β82. H^+ binding at His β146 (HC3), a dominant contributor to the alkaline Bohr effect at physiological pH, is favored by the salt bridge between His and Asp FG1β in the T-state that raises its pK from 7.2 in the R-state to 8.1 (131).

Interactions with other allosteric effectors The alkaline Bohr effect is enhanced in the presence of organic phosphates since binding of these anions favors the binding of protons (10). Anion binding is greater in deoxyHb than in HbO_2. Another part of the Bohr effect results from O_2-dependent Cl^- binding, which similarly facilitates the binding of protons (184). Van Beek et al. have shown that the pK of the N-terminal α-amino group of the α-chain increases with increasing Cl^- (236), whereas the N-terminal amino group of the β-chain is independent of Cl^-. Thus, at physiological Cl^- concentration about 25% of the Bohr effect is explained by binding of Cl^-.

In contrast to binding of DPG and Cl^- that is coupled to the uptake of protons (10) binding of CO_2 causes the release of protons from Bohr groups (185). Under physiological conditions the Bohr effect is therefore caused by (i) the direct allosteric effect of protons on O_2 binding and (ii) the interaction between proton binding and binding of Cl^-, organic phosphates, and CO_2. For the alkaline Bohr effect these interactions are summarized in Table 1. The relative contributions of the histidyl residues to the Bohr effect are summarized by Lukin and Ho (137).

Effects on Hb-O_2 affinity Proton binding stabilizes the low-affinity T-state of Hb and thus favors O_2 unloading from hemoglobin and increases P_{O_2} in blood passing through capillaries in acidic regions of the peripheral circulation.

There is no difference between pH effects induced by metabolic and respiratory acidosis, provided there is no change in the degree of carbamate formation (CO_2 binding to Hb), in which case the effect of protons on Hb-O_2 affinity is called the "fixed acid Bohr effect." Respiratory acidosis may include additional effects resulting specifically from increased CO_2 binding to Hb (see Section "Carbon dioxide").

Table 1 Amino acid residues responsible for the alkaline Bohr effect and their dependence on 2 mmol/L 2,3-diphosphoglycerate (DPG) and 100 mmol/L Cl^- (116)

Residue	DPG independent	Cl^- dependent
Val α1	Yes	Yes
His α122	Yes	No
Val β1	Yes	Yes
His β2	Yes	Yes
Lys β82	No	No
His β143	Yes	Yes
His β146	Yes	Yes

Although earlier mathematical analyses of the shape of the ODC using the approach by Wyman (275) indicated that pH affects Hb-O_2 binding uniformly over the entire saturation range (5, 93), Imai and Yonetami (117) demonstrated that the binding constants in the Adair equation are affected differently by pH. Also several other authors found that the Bohr coefficient varies with SO_2 (45,88,103). Braumann et al. (45) showed that the Bohr coefficient deviates only slightly from −0.48 at SO_2 values between 10% and 80% when blood from physically fit individuals was acidified with lactic acid, whereas in unfit individuals, it was approximately −0.32 at 10% SO_2 and increased with increasing saturation, reaching about −0.50. A Bohr coefficient of approximately −0.48 has been proposed for pH correction of P_{50} values when intact red cells are studied (103, 178, 206). However, these Bohr-coefficient values pertain to plasma pH and thus need to be corrected to account for changes in RBC pH. Following Hlastala and Woodson (103) pH correction can be performed using the following equations (206):

$$\frac{\Delta \ln PO_2}{\Delta pH} = \left\{\frac{PO_2}{26.6}\right\}^{0.184} - 2.2$$

Plasma Bohr coefficient (BC):

$$BC_{plasma} = \Delta \log_{10} PO_2 / \Delta pH_{plasma} = -0.48$$

RBC Bohr coefficient:

$$BC_{RBC} = BC_{plasma} \times \Delta pH_{plasma} / \Delta pH_{RBC}$$

The physiological relevance of the Bohr factor and its saturation dependence is that SO_2 as well as pH values change significantly in circulating blood resulting in different Hb-O_2 affinities in different organs. In alveolar capillaries, pH increases due to the release of CO_2 whereas in the periphery it decreases as a consequence of the release of acid metabolites such as CO_2 and lactic acid from the cells. Whereas the pH increase in alveolar capillaries increases O_2 affinity favoring arterial O_2 loading, the (relative) acidosis in capillaries of metabolizing tissues favors O_2 release from Hb and thus O_2 diffusion to the mitochondria in support of aerobic ATP synthesis.

Carbon dioxide

Although Bohr et al. in 1904 (35) demonstrated that increasing CO_2 partial pressure shifted the ODC toward the right, the specific (pH-independent) effect of CO_2 on Hb-oxygen affinity was first demonstrated 25 years later by Henriques (100). It was later confirmed and ascribed to carbamate formation by Rossi-Bernardi et al. (185), who showed that deoxyHb has a higher affinity and binds more CO_2 than HbO_2 at a given pH (185), which favors the removal of CO_2 from CO_2-producing tissues. Direct and indirect measurements as well as studies of Hb variants have shown that the principal sites for carbamate formation are the N-terminal amino groups on the β-chains that have much higher affinity for CO_2 than those of the α-chains (18,95,131,161,165), which are in close proximity of a large number of positively charged groups (8). There seems to be no cooperative or negative interaction between the α- and ß-chain CO_2-binding sites (116). CO_2 binding by Hb can be summarized as

$$CO_2 + HbNH_2 \leftrightarrow HbNHCOOH \leftrightarrow HbNHCOO^- + H^+$$
$$HbNH_3^+ \leftrightarrow HbNH_2 + H^+$$

Interactions with other allosteric effectors The interaction between CO_2 binding and binding of organic phosphates, protons, and Cl^- to Hb complicates a quantitative description of the effect of the CO_2 on the position of the ODC in intact RBCs. Binding of CO_2 results in a release of protons from deoxyHb, which decreases the Bohr effect (20). In deoxyHb, organic phosphates share the same binding sites (the N-terminal α-amino groups of the α-chain) with CO_2, whereby phosphates significantly decrease carbamate formation in deoxyHb (17). Thus, the carbamino reaction will play a lesser role in unloading of O_2 in species whose Hbs show high sensitivity to organic phosphates, and a larger role in felines and ruminants whose Hbs bind organic phosphates with low affinity (20, 22). The significance for gas transport of the negative interaction between CO_2 and DPG binding is illustrated in the Hb of the strictly fossorial Eastern mole *Scalopus aquaticus*, that combines a low intrinsic O_2 affinity with insensitivity to DPG that predictably increases CO_2 binding and transport during burst activity in its hypercapnic burrows (61). There is considerable competitive interaction between CO_2 and Cl^- binding, which also involves common sites (the N-terminal residues), as quantitatively demonstrated in dilute Hb-solutions by Imaizumi et al. [1978; cf. Imai (116)], who showed that increased Cl^- concentration reduced the effect of CO_2 on Hb-O_2 affinity. Interestingly, plots of log(P_{50}) versus log(CO_2) obtained at different Cl^- concentrations converge at a CO_2 level of approximately 14 mmol/L and a P_{50} of approximately 15 mmHg at 25°C (116).

Effects on Hb-O_2 affinity In the absence of organic phosphates and at constant pH (7.4) and Cl^- (100 mmol/L) the effect of CO_2 (given in millimole per liter) on P_{50} of human Hb A in highly dilute solution (116) is

$$\frac{\Delta \log P_{50}}{\Delta \log CO_2} = 1.43$$

In intact RBCs, when pH is decreased solely by increasing the CO_2 tension, about 20% of the Bohr effect is due to carbamate formation of Hb (206). The combined effects of pH and CO_2 may be calculated as

$$\frac{\Delta \ln PO_2}{\Delta pH} = \left\{\frac{PO_2}{26.7}\right\}^{0.184} + 0.003 \times BE - 2.2$$

where BE is the base excess (BE).

These findings are of pertinent physiological significance. Based on the different ionization constants for deoxyHb and HbO_2 (116), oxygenation decreases the affinity of Hb for CO_2 thus inducing its release (the Haldane effect) (66). Since changes in the partial pressure of CO_2 in blood are inversely related to the partial pressure of O_2, binding of each ligand controls binding and release of the other. The CO_2 effect on O_2 binding combines the effects of carbamate formation and CO_2-related pH changes. The lower CO_2 tensions (greater alkalosis) in alveolar capillary blood favors O_2 loading in the lung, whereas the high CO_2 (and the related acidosis) favors O_2 unloading to the tissues. On the other hand, CO_2 released from Hb forms the major driving force for its diffusion from blood into the pulmonary alveolar space together with carbonic anhydrase-catalyzed CO_2 formation from bicarbonate. Conversely, in peripheral blood deoxygenation favors the binding of CO_2 by Hb and its transport to the lung.

Chloride

Although Cl^- binds mainly at the N-terminal α-amino groups of the α- and β-chains of deoxyHb (that also interact, respectively, with Ser 131α and His 82β) (154), the N-terminal α-amino group of the α-chain appears to be solely responsible for the Cl^--induced decrease in Hb-O_2 affinity, based on experiments that involve removal or chemical modification of this residue and on competition between carbamate formation and Cl^- binding (36, 152, 154). Val 1β and His 2β are low affinity binding sites that are not oxygen-linked (65, 118).

Interactions with other allosteric effectors Cl^- binding hinders the reaction of Hb with all major ligands such as protons, CO_2, and organic phosphates (116). As deduced from the effect of the Cl^- concentration on P_{50} it may be assumed that each tetrameric Hb molecule has two independent and equivalent binding sites for Cl^- (3).

Binding of Cl^-, and its interaction with binding of organophosphates (Q) can be described as

$$\begin{aligned} Hb(O_2)_j + Cl^- &\leftrightarrow Hb(O_2)_jCl^- \\ Hb(O_2)_jCl^- + Cl^- &\leftrightarrow Hb(O_2)_j(Cl^-)_2 \\ Hb(O_2)_j + Q &\leftrightarrow Hb(O_2)_jQ \end{aligned}$$

The effect of Cl^- on P_{50} can mathematically be described as (118):

$$P_{50,Cl,Q} = P_{50,0} \times \left\{ \frac{1 + J_4(Cl^-)^2 + R_4(Q)}{1 + J_4(Cl^-)^2 + R_0(Q)} \right\}^{-1/4}$$

where $P_{50,Cl,Q}$ is the P_{50} in presence of Cl^- and organic phosphates, $P_{50,0}$ is that in their absence, and J and R are the intrinsic binding constants for Cl^- and organophosphates, respectively, of the 4 Hb-subunits. The plot of log P_{50} versus log[Cl^-] has a slope of 0.4 indicating about 1.6 Cl binding sites per Hb tetramer. The association constant for Cl^- to deoxyHb is approximately 11 mol/L at 37°C and pH 7.4 (97). Binding increases with decreasing temperature as summarized in reference (116). These results indicate that the relative concentrations of Cl^- and organic phosphates as well as their respective affinities for Hb determine which ligand is bound and illustrate linkage between many reactions. Thus, an independent reaction is impossible particularly in the light of linked changes in the concentrations of the reactants. When DPG is synthesized, the sum of nondiffusible anions inside the RBC increases. In response, protons will be taken up and Cl^- will leave the cells (77, 79).

In dilute, Tris(hydroxymethyl)-aminomethane (TRIS)-buffered Hb solutions the effect of Cl^- on P_{50} can be quantified as $\Delta \log P_{50}/\Delta \log Cl^- = 0.401$ when no other ligand is present (97) and as $\Delta \log P_{50}/\Delta \log Cl^- = 0.289$ in the presence of 1.6 mM CO_2 where P_{50} is given in mmHg and Cl^- in mol/L (116, 118).

The possible effects on Hb-O_2 affinity and their physiological significance are difficult to assess given the complexity of the interactions since, as outlined previously, Cl^- in the RBCs can be expected to change with the alterations in pH and HCO_3^- as the RBCs travel back and forth between the lungs and the peripheral tissues, continuously binding and releasing protons and DPG.

Organic phosphates

DPG [2,3-bisphosphoglycerate (2,3-BPG)] is found in RBCs of mammals and a few species of other vertebrates (175) Benesch and Benesch (26) and Chanutin and Curnish (64) first demonstrated that DPG decreases the Hb-O_2 affinity of human Hb dramatically compared to the effects of other allosteric ligands. Brewer and Eaton reported the direct connection between RBC metabolism and O_2 transport (47). Bird RBCs contain IPP as major allosteric effector, whereas fish, amphibians and reptiles commonly have ATP, that may be accompanied by significant amounts of GTP (in fish) and DPG (in amphibians) (12-14, 98, 216, 252). As expected on the basis of their anionic nature each phosphate is bound by all tetrameric vertebrate Hbs (12, 64) although they may exert different allosteric effects in different taxa. Thus ATP contributes to decreasing O_2 affinity in mammalian RBCs (12).

DPG, which carries four negative charges, binds to the Hb tetramer in the central cavity between the β-chains [Fig. 5; it binds to the amino acid Val β1 of one β chain and to His β2, Lys β82, and His β143 of both chains (6, 7, 165, 181)]. The organic phosphate-to-tetrameric Hb stoichiometry is ~1:1. However, there is evidence for a second DPG binding site in dromedary Hb molecules (4). In human Hb A in solution the apparent binding constants in the presence of approximately 100 mmol/L Cl^- are 5.4×10^4 mol/L and 1000 mol/L for deoxyHb and HbO_2, respectively, at 37°C (118). DPG reduces the binding constants of the first three oxygenation steps without tangibly affecting that of the fourth, which binds to Hb that predominantly has switched to the R-state (232). In decreasing the O_2 affinity of the Hb in the T-state without

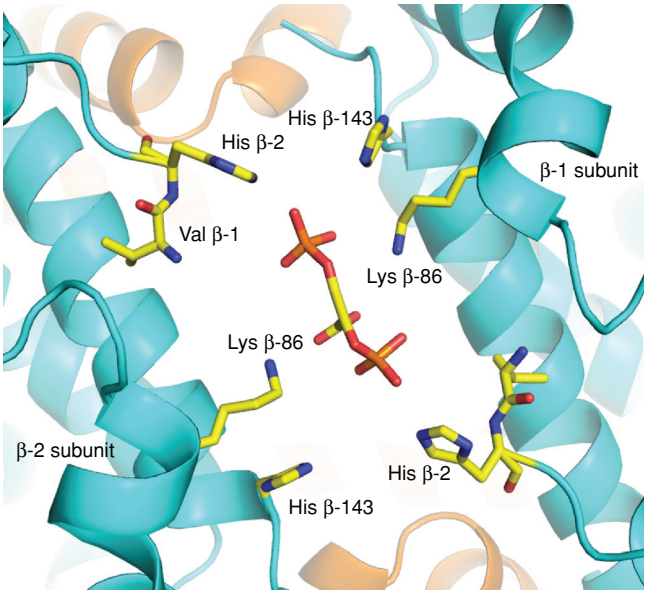

Figure 5 View into the central cavity of the tetrameric Hb molecule, showing the two α-chains (pink, in background) and the seven positively charged amino acid residues of the two β-chains (blue-green) where polyanionic 2,3-diphosphoglycerate (DPG) binds. DPG binding is reduced in fetal Hbs and in camelid Hb where positively charged residues are replaced by neutral ones (His β 143→Ser and His β 2→Asn, respectively). The image is kindly provided by Dr. Jeremy Tame, Yokohama City University, Japan.

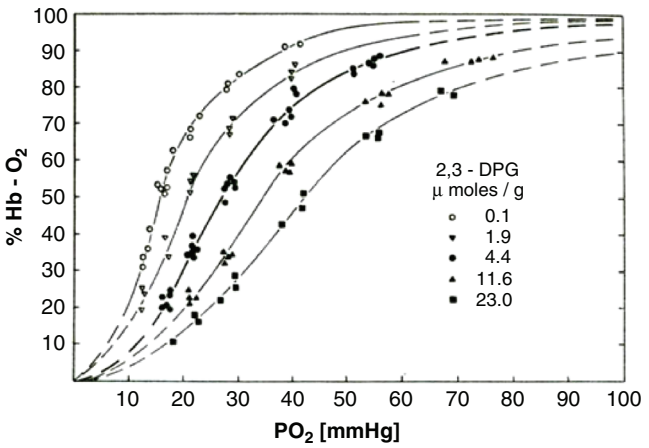

Figure 6 Effect of 2,3-diphosphoglycerate (DPG) on Hb-O_2 affinity. Oxygen-dissociation curves of intact human RBCs after *in vitro* alteration of the intracellular organic phosphate concentration. Conditions: extracellular pH, 7.4; PCO_2, 40 mmHg; temperature, 37°C [adapted, with permission, from Duhm (77)].

significantly altering that in the R-state, DPG increases the Hill coefficient n_{50} of Hb in solution—from approximately 2.5 in stripped Hb to approximately 3.05 in presence of 2 mmol/L DPG (232).

Interactions with other allosteric effectors Given that binding of organic phosphates to Hb inhibits binding of other known ligands (H^+, CO_2, and Cl^-) (116), exclusive DPG binding occurs only in the absence of these. The DPG effect is virtually annihilated in presence of 500 mmol/L Cl^- (116). At intermediate Cl^- concentrations the addition of DPG decreases Hb-O_2 affinity (118). Because of the involvement of the α-amino and imidazole groups in DPG and proton binding, the binding constants for DPG to human Hb are strongly and inversely dependent on pH (28, 237).

In the absence of other allosteric effectors the effect of DPG on P_{50} can be calculated as described (222) from the concentrations of bound and unbound DPG and the association constants for deoxyHb and HbO_2 (118).

Changes in DPG perturb the Donnan equilibrium by changing the intracellular concentration of nondiffusible anions, which affects P_{50} by changing intracellular Cl^- concentration and intracellular pH (77). A quantitative description of these phenomena by Samaja and Winslow (201) shows that at a given red cell pH, P_{50} increases as the molar DPG/Hb ratio increases up to approximately 1.8, whereas higher DPG levels had no effect on P_{50}, but act mainly to decrease intraerythrocytic pH. For intact red cells, Okada et al. (155) suggest the following formula to calculate the magnitude of the DPG-induced change in P_{50}:

$$\Delta \log P_{50} = 0.135 \times \mathrm{DPG} - 0.116$$

where DPG is the molar DPG-to-tetrameric Hb ratio. O_2-binding curves recorded by Duhm (77) following *in vitro* modification of DPG levels in human RBCs are shown in Figure 6.

DPG metabolism RBCs of most mammals contain high levels of DPG (175) compared to extremely low concentrations (micromolar range) normally found in other cell types. The major determinant of DPG levels is the presence of the enzyme diphosphoglycerate mutase (DPGM), which catalyzes its formation from 1,2-diphosphoglycerate (Fig. 7). It's affinity for 1,3-DPG is about 2 orders of magnitude higher than that of the muscle enzyme (127). Most evidence for this comes from studies of RBCs from patients with hemolytic anemia caused by enzyme defects [summary in (94)]:

1. In RBCs, the formation of DPG is stimulated by a high overall glycolytic activity and a high activity of the enzyme phosphofructokinase, which increases the flow of substrate into the DPGM reaction. Clinical evidence comes from deficiencies in hexokinase (128) and phosphofructokinase (239), two key regulators controlling the overall glycolytic activity, which decrease the concentration of DPG in RBCs.

2. Pyruvate kinase (PK) deficiency causes an accumulation of glycolytic intermediates above the PK reaction (223), which prevents the breakdown of DPG (234) and thus causes an elevation of DPG in RBCs.

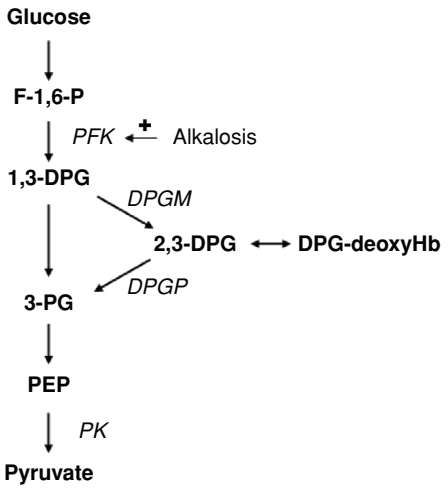

Figure 7 Formation of 2,3-diphosphoglycerate (DPG) in RBC glycolysis. F-1,6-P, fructose-1,6-diphosphate; 1,3-DPG, 1,3-diphosphoglycerate; DPG, 2,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase. Arrows indicate downward reactions of glycolysis only, intermediate steps are not shown.

3. A low activity of DPGM decreases the formation of DPG (234). A deficiency of DPG phosphatase seems not associated with altered DPG levels (221).

4. A major determinant of DPG formation is RBC pH. Alkalosis stimulates several glycolytic enzymes and hence favors the formation of DPG, whereas acidosis inhibits DPG formation (78, 174).

Variation in red cell DPG levels is of direct physiological significance. Alterations in RBC DPG levels potently modulate blood O_2 affinity, adding to contributions from other effectors. The main effect of increased DPG is decreased Hb-O_2 affinity that, together with the coupled increase in cooperativity, favors O_2 unloading in peripheral tissues where the Po_2 is low (201)—as seen in mammals ascending to moderate altitudes (see below). Given that the changes in the concentration of DPG (and of other organic phosphates) in blood are relatively slow, significant differences in DPG between alveolar and peripheral capillary blood cannot be expected to exist.

Interaction between ligands and its effects on Hb-O_2 affinity

The interactive effects of the allosteric, nonheme ligands are complex, and none of these ligands affect oxygen affinity independently. This is important to consider when intact erythrocytes are studied. The ODC of Hb solutions resembles the *in vivo* situation closely in the presence of approximately 100 mmol/L Cl^-, 40 mmHg PCO_2, 1.2 moles DPG per mole Hb tetramer, and at an intracellular pH of 7.2 (106, 116).

The alkaline Bohr effect is decreased by the presence of CO_2 (16, 185) since protons are released when CO_2 binds to deoxyHb. However, the Bohr effect reverts to normal in the additional presence of DPG (and Cl^-), which increases proton binding (16). Table 2 lists possible mutual interactions between ligand and O_2 binding.

Table 2 Interactions between heme and non-heme ligands of Hb

	O_2	H^+	CO_2	DPG	Cl^-
O_2	+	−	−	−	−
H^+		0	−	+	+
CO_2			0	−	−
DPG				0	−
Cl^-					0

The plus and minus signs indicate facilitating and inhibiting interactions, respectively, between the ligand-binding sites. Zero indicates no interaction. Modified after Imai (116).

Most ODCs are determined at a standard pH of 7.4, that is, the pH prevailing in the plasma of arterial blood. However, the pH in the intact RBCs is distinctly lower. DPG and other phosphates produced by cellular metabolism can not permeate the plasma membrane and increase the concentration of intracellular, nondiffusible anions, causing a shift in the Donnan equilibrium and consequently decrease intracellular pH. At a constant extracellular pH of 7.4 the relationship between intracellular pH and the concentration of organic phosphates is described as (77):

$$pH_{RBC} = 7.306 - 0.0083 \times P_{org}(\mu mol/L/g\ RBCs).$$

Duhm (77) accordingly attributed the increase in P_{50} from 15 to 43 mmHg when the DPG concentration in human RBCs increases from of 0.1 to 23 μmol/g Hb to a direct allosteric effect of DPG as well as a DPG-induced change in intracellular pH (79). However, DPG-dependent changes in Cl^- have also to be included since Cl^- moves readily across the RBC membrane via the band 3 protein (59,158,201). From Duhm's (77) data it can be calculated that at an extracellular pH of 7.4, an increase in organic phosphate concentration from 3.8 to 42 μmol/g RBC would decrease Cl^- concentration from approximately 70 to 35 mmol/L cell water. Such a decrease in Cl^- will increase the combined effects of DPG and pH on Hb-oxygen affinity.

Other Hb ligands and modulators of Hb-O_2 affinity

Carbon monoxide (CO) competes with O_2 at the ferrous heme-binding site. Hb has a much higher affinity for CO than for O_2, as evident from the partition constant of approximately 200 for fully ligated HbA under near-physiological conditions (pH 7.35, 100 mmol/L NaCl, 37°C) and saturating DPG/Hb ratio (30, 72). Already some 100 years ago Douglas et al. (75) showed that the presence of CO significantly increased the O_2 affinity of free heme groups (shifted the dissociation curve of the remaining functional hemoglobin in whole blood to the left) and removed its sigmoidal character, resulting in a nearly hyperbolic curve. Accordingly, CO decreased the Hill

coefficient "*n*"—from approximately 2.6 in absence of CO to approximately 1.7 at an FHbCO of 50%—where FHbCO is the percentage of carboxyhemoglobin (156). The decrease in P_{50} with increasing CO is nearly linear up to about 50% CO-Hb (156) and can be described by the equation

$$P_{50} = -3.6 \times \text{FHbCO} + 3.4 \text{ (281)}$$
$$\Delta \log P_{50}/\Delta\%\text{FHbCO} = -0.007 \text{ (156)}$$

Stored blood, which is depleted of DPG, shows a similar relationship but—consistent with the low DPG levels—lower P_{50} values (156). There is no influence of CO on the Bohr effect in the range of 15% to 30% FHbCO and between 20% and 90% SO_2, but higher values of FHbCO seem to increase the Bohr effect (281).

Due to the much higher affinity of Hb for CO than for O_2, small amounts of CO will result in significant CO binding to hemoglobin and thus decrease the amount of O_2 that can be bound. Since CO binding to Hb increases the O_2 affinity of remaining unliganded hemes, the toxicity of CO comes from the reduction of the O_2-binding capacity, similar to anemia, and, in addition, from an increase in the Hb-O_2 affinity resulting in decreased O_2 unloading.

Nitric oxide (NO) is formed by a variety of cell types. It is best known by its function as endothelial-derived relaxing factor (157). NO formed in macrophages fulfills the function of bacterial killing and regulator of cytokine release (238). The action of NO released from cells is modulated by the presence of RBCs where it binds to hemoglobin. Thus, hemoglobin has been suggested as a regulator of NO bioavailability when the NO:Hb ratio is low, that is, at nanomolar concentrations of NO (148). Under these conditions, NO binds to heme iron forming nitrosylhemoglobin (Hb[FeNO]) (90) where both α- and β-chains of Hb are involved (92). In the R-state, NO binds to Cys93 on the β-chain forming *S*-nitrosohemoglobin (Hb(βCysNO); SNO-Hb) and dissociates from there in the T-state (92,213). Release of NO from heme-Fe is much faster in the partially nitrosylated T than in the R-state, where the T-state favors α- over β-nitrosylation [for review see reference (92)]. These reactions were interpreted to provide an oxygen sensing mechanism by hemoglobin, which, at low oxygen, would provide a NO-based vasodilatory response (91).

NO binding to Hb affects Hb-O_2 affinity. Whereas the O_2 affinity of Hb[FeNO] is markedly lower compared to NO-free Hb (278), SNO-Hb has a higher Hb-O_2 affinity (38). NO also oxidizes oxygenated Hb to produce methemoglobin (50). The balance among these three reactions in terms of their effects on Hb-O_2 affinity is difficult to predict. *In vitro* experiments with RBCs indicate that the P_{50} (at pH 7.4) of oxygenated or deoxygenated rabbit blood incubated with NO donors at the rather high NO:Hb molar ratio (1:1) decreases with increased methemoglobin and nitrate levels. Measurements on RBCs from healthy donors and from patients with sickle cell anemia incubated with NO (80 ppm) at low hematocrit revealed that these treatments had no effect on P_{50} values despite methemoglobin formation (107). At higher NO concentrations significant methemoglobin formation and a correlated decrease in P_{50} was observed (107). Thus, changes in Hb-O_2 affinity do not seem to explain the potential beneficial therapeutic effect of NO in sickle disease (107).

Lactate is produced under anaerobic glycolysis, for example, in exercising skeletal muscle cells from where it diffuses into interstitial space and capillary blood. In most instances increased blood lactate is also associated with acidosis. Its uptake by RBCs is mediated by a monocarboxylate transporter (76). Lactate binding to Hb might occur at an α-chain site involving Val α1 and Ser α131 and at several cationic amino acid residues on the β-chain. Since these sites are also involved in Cl^- binding and carbamate formation, interactions between bindings of these substances are likely. Whereas data of Guesnon et al. (96) suggest similar effects of Cl^- and lactate on P_{50} at low anion concentrations (10 and 50 mmol/L) but greater lactate effects at high concentrations, those of Nielsen and Weber (151) indicate no tangible difference between the effects of these two effectors, but demonstrate marked inhibitory interactions between lactate binding and carbamate formation at physiological pH and CO_2 tension. Collectively the results indicate that lactate might weaken the binding of other allosteric effectors of Hb-O_2 binding. *In vitro* experiments indicate that that exposure of human RBCs to varying concentrations of lactate in combination with acidosis decreased the concentration of DPG in the RBCs, and that changes in P_{50} were fully explained by altered pH and DPG, whereas increasing lactate at neutral pH had no effect on DPG and P_{50} (145).

Protein-protein interactions within the RBC may modify Hb-O_2 affinity. The N-terminal cytoplasmic domain of band 3 protein, the RBC membrane anion exchanger, binds to deoxygenated Hb at the same site in the central cavity as organic phosphates; hence, binding of Hb to band 3 decreases Hb-O_2 affinity (241). The cytoplasmic domain of band 3 also reversibly binds glycolytic enzymes (136). Thus, it has been postulated that Hb bound by band 3 might act as an oxygen-dependent regulator of RBC glycolysis and thus of DPG and, subsequently, also of Hb-O_2 affinity (15,136,259). A recent study (67) demonstrates that deoxyHb binds to residues 12-23 of band 3's N-terminal, that are proximal to the binding sites for glycolytic enzymes and the cytoskeletal components band 4,1 and ankyrin, suggesting mechanisms by which the red cell's physical properties may be regulated by the oxygenation state of Hb. Given that red cells contain approximately 225 times more tetrameric Hb molecules than band 3 proteins, the impact of band 3 on red cell O_2 affinity must be negligible. However, band 3 may have impact on NO delivery from Hb, since NO released by Hb is rapidly inactivated, whereby only NO that is produced at the membrane surface is likely to become available for promoting capillary vasodilation (67).

Magnesium (Mg^{2+}) that occurs in RBCs at relatively high concentrations, complexes with the phosphate groups of ATP

and DPG. Since Mg^{2+}-complexed organic phosphates do not bind to Hb, the presence of Mg^{2+} increases O_2 affinity of Hb in the presence of DPG and ATP (57,259). Thus, O_2 affinity of Hb depends on the concentrations of free allosteric effectors rather than their total concentration in the RBCs (143).

Water is a potential effector since Hb's solvation changes upon (de-)oxygenation and thus affects Hb-O_2 affinity. It follows that neutral solutes indirectly affect the allosteric behavior of Hb by varying the chemical potential of water (69). An increase in water activity increases the number of water molecules bound and decreases Hb-O_2 affinity (69, 114). Although the effect of water binding *per se* is quite small compared to that of other effectors, the number of water molecules bound to Hb increases strongly in the presence of chloride and organic phosphates (69, 114). The hydration sites seem not to include Bohr groups. Given that water activity is narrowly regulated in the red cells of vertebrates, the physiological significance of solvation-related changes in red cell O_2 affinity are likely to be negligible in vertebrates.

Temperature Sensitivity of Hb-O_2 Binding

The reaction of O_2 with Hb is exothermic, causing a reduction in O_2 affinity with increasing temperature, which is considered advantageous in enhancing O_2 unloading in warm tissues (exercising muscles) that have an increased O_2 requirement (11). Since the prosthetic hemes groups are the same in all vertebrates the intrinsic enthalpies of heme oxygenation is invariant, and adaptive variation in the temperature sensitivity of P_{50} must be brought about by variations in the enthalpic processes that are coupled to the oxygenation reaction, such as the dissociation of allosteric effectors (protons, chloride ions, and organic phosphates) that is endothermic (249).

The apparent heat of oxygenation ($\Delta H'$) can be measured by direct calorimetry or calculated from the temperature sensitivity of P_{50} at constant pH using the van't Hoff isochore (276):

$$\Delta H' = 2.303\ R\ (\Delta \log P_{50})/[(1/T_1 - 1/T_2)]$$

where R is the gas constant and $\Delta \log P_{50}$ refers to the difference in P_{50} values observed at the two absolute temperatures T_1 and T_2.

As assessed already some 75 years ago, both by calorimetry and indirectly from the van't Hoff isochore (186, 187), the apparent heats of oxygenation approximate −40 and −59 kJ/mol, at pH 6.8 and 9.5, respectively. The higher value at pH 9.5 is consistent with lack of a Bohr effect (endothermic proton dissociation) at this alkalinity. Moreover, it neatly matches the value for the intrinsic heat of O_2 binding (−59 kJ/mol) obtained more recently by calorimetry (9), the value of the intrinsic heats for binding the four O_2 molecules to Hb (∼61 kJ/mol; Imai, 1982), and the value ∼62 kJ/mol) determined for marlin (fish) Hbs in the absence of effectors (250). This correspondence again underscores the invariance of intrinsic heats of heme oxygenation in different vertebrates. Although the shape of the ODC appears invariant with temperature—at least in its middle part (187)—this does not seem to be true for the extreme (high and low) oxygen saturations (188).

The temperature sensitivity of P_{50} of human Hb A has been described by Dill and Forbes (74) for hypothermic patients based on direct calorimetric measurements by Brown and Hill (52) as

$$\Delta \log P_{50}/\Delta T = 0.02$$

at a pH of 7.4 and at constant physiological PCO_2 in human blood [see also (197)]. Rat RBCs show very similar values (141). Severinghaus (205) derived a normogram to correct ODCs for temperature and plasma pH but points out that some variation is due to the fact that intracellular pH may not always strictly follow plasma pH when temperature changes.

Blood is subjected to marked temperature changes as it circulates through the body. Thus, Hb-O_2 affinity varies accordingly. While the core temperature in humans at rest is about 37°C, skin temperature is lower, particularly in cold air or water and can even fall below the freezing point in the absence of insolation. During exercise the core temperature may increase to 39.5°C, and may be even higher in the capillaries of exercising muscles. There the increased temperature will favor O_2 unloading from Hb (see Section "O_2 transport during exercise"). In contrast, under hypothermia O_2 is bound more tightly to Hb and O_2 unloading is impaired. This may have little relevance for the hypothermic patient since the low body temperature also decreases metabolic activity and thus O_2 demand (180).

Interestingly the Hbs of several arctic cold-tolerant mammals (polar bears, whales, and some ruminants including reindeer, musk ox, horse, and cow) exhibit pronounced reductions in the temperature effects that are considered to be adaptations to ensure O_2 unloading in cold appendages and peripheral tissues (68, 70, 249). Compared to human Hbs, the β-chains of bovine and some other ungulate (e.g., horse) Hbs reveal a cluster of basic (His and Lys) residues at positions 8, 76, and 77 that has been suggested to form an "additional" Cl^--binding site, which reduces the overall enthalpy of oxygenation (via increased endothermic dissociation of chloride ions). In ungulate Hbs, characteristic amino acid exchanges found at the N-termini of the β-chains (that drastically reduces DPG sensitivity), contribute to reducing the temperature sensitivity—via greater endothermic contributions from the heats of (T→R) conformational change (176).

A recent study (60) reveal a striking thermal adaptation in the extinct woolly mammoth, whose Hb was resurrected from perma-frozen bones of a 43,000-year-old specimen discovered in Siberia. Remarkably, structural and functional analysis reveal that two of the four amino acid substitutions found in the β-type chain of mammoth compared to that of the closely

related Asian elephant (101Glu→Gln and 12 Thr→Ala) are associated with increased binding of chloride and DPG that thus correlate with the observed reduction in the temperature sensivities of the Hb in the presence of these effectors. These adaptations, which predictably secured adequate O_2 unloading in the cold appendages (analogous to those seen in extant cold-tolerant mammals) illustrate a novel molecular solution to minimize energetically costly heat loss that may have permitted the mammoth to colonize high latitudes when it moved from warm equatorial Africa 1 to 2 million years ago (60).

Measuring Hb-O_2 Affinity

Intact RBCs

Although measurements on whole blood (intact RBCs in their "natural" environment) better reflect the *in vivo* situation, measurements on red cell preparations can provide valuable insight into the specific effect of allosteric ligands. Such studies entail the separation of the RBCs from plasma and white cells and resuspending them in isotonic artificial media. Several methodologies permit variation RBCs' intracellular "milieu". The concentration of organic phosphates inside the cells can be modified by incubation with inosine, iodoacetamide, and deoxy-glucose that deplete the phosphate contents, or with inosine, pyruvate, and orthophosphate that increase their levels [e.g., see reference (77)]. Phosphate compounds can be measured after deproteinization using enzymatic procedures [cf. reference (32)]. The intracellular pH can be modified by altering extracellular pH using buffer systems or by equilibrating red cell suspensions with gases containing different CO_2 tensions. Cellular pH can be measured either directly in freeze-thawed lysates of packed RBCs or calculated from extracellular pH and the Cl^- ratio (87). The latter method can also be used to determine the intracellular Cl^- concentration (105). Intracellular Mg^{2+} can be estimated after permeabilizing the cell membranes with the ionophore A23187 (140, 177). Intracellular ion concentrations can also be calculated from hematocrit values and their concentrations in red cell suspensions (or blood) and extracellular fluid (or plasma) (257). The cell water content calculated from wet-weight to dry-weight ratios (measured after drying packed RBCs to constant weight) permits calculation of the actual concentrations of ligands and Hb inside the RBCs.

Preparation of "stripped" Hb

Measurements on preparations of pure Hb are a prerequisite for analyzing the effects of individual effectors on Hb-O_2 affinity, their binding constants, and the interaction between different effectors. An effective method for removing non-heme ligands from Hb (stripping) is that described by Benesch et al. (27) and Imai and Yonetani (117). Briefly, human red cells are lyzed, cellular debris is removed by centrifugation, and residual organic phosphates are removed by column chromatography. As required, this may be followed by dialysis to adjust the electrolyte composition, and by ultrafiltration to adjust the concentration of the Hb.

Measuring the oxygen-dissociation curve

Hb-O_2 affinities are conveniently quantified by P_{50} (Po_2 at half saturation), which varies inversely with O_2 affinity, and which is interpolated from ODCs obtained by measurement of SO_2 levels in Hb samples equilibrated to varying Po_2. A variety of such methods and their individual advantages and disadvantages have been described [for review see (262)].

Point-by point measurements

Typically samples (of blood, red cell suspensions, or Hb solutions) are equilibrated to different oxygen partial pressures. SO_2 can conveniently be measured by dual-wavelength spectrophotometry based on the difference in absorption spectra of deoxyHb and HbO_2. Alternatively, O_2 contents and thus SO_2 can be derived from increases in Po_2 observed upon the oxidation of equilibrated samples to metHb (e.g., by adding oxidizing agents) (199, 230). In such cases Po_2 changes can be measured with an oxygen electrode such as in a blood gas analyzer.

In the so-called "mixing" method, two samples of blood or of Hb in solution are equilibrated, respectively, with gases lacking oxygen (deoxyHb) and gases with high oxygen partial pressure (oxyHb). As required these gases may contain CO_2 (e.g., at physiological partial pressure levels) to include the effect of carbamate formation. After equilibration, varying proportions of the two samples are mixed, Po_2 is measured, and SO_2 is measured directly or calculated from the mixing ratio with appropriate corrections for the contribution of dissolved oxygen. The same mixes can be used for measurement of intracellular pH.

Continuous measurement

Several procedures involving continuous recording of SO_2 and Po_2 have been described (80, 179, 262, 270). Intact RBCs at low hematocrit or Hb solutions are analysed in cuvettes or in thin layers that permit simultaneous measurement of Po_2 and SO_2. The cuvettes can be fitted with gas-permeable compartments allowing adjustment of the gas composition. The oxygen tensions in the sample can be changed by bubbling or perfusing gases with appropriate oxygen partial pressures. The technique permits the addition of reagents that modify RBC function. Rapid measurements of the ODC are possible in thin-layer samples that may be enclosed between two gas-permeable Teflon membranes (179). After recording SO_2 values at various Po_2's, the absorptions of the fully deoxygenated and fully oxygenated samples can be recorded following equilibration with gases without O_2 and with high Po_2, respectively. Po_2 and SO_2 data can be displayed on an X/Y chart recorder or digitized. Problems might arise from

inhomogeneous mixing and a slow response of the oxygen electrode (which can be corrected mathematically after measuring the response time). Additionally, the accuracy of polarographic O_2 measurements at very low O_2 tensions may be a limiting factor for assessing the O_2 association constant (A1) of the first O_2 molecule binding to the Hb molecule.

A method that circumvents the use O_2 electrodes is a modification of the thin-layer dynamic diffusion chamber method first described by Sick and Gersonde (207), which moreover is suitable for analyzing ultrasmall ($\sim$ 3 μL) samples. In this procedure absorption (SO_2) is recorded continuously while the sample is equilibrated to mixtures of air, CO_2 (optional), and inert filler gas (N_2) prepared by cascaded gas mixing pumps that give stepwise increased O_2 tensions (260) so that the Po_2 value at each step can be calculated from the mixing ratios. This method permits precise assessment of O_2 equilibria at extreme saturations (near 1% and 99%), and thus of the association equilibrium constants of the oxygenated and oxygenated states of Hb (K_T and K_R) and of the four Adair constants (243, 254).

In vivo

In general only a discrete number of points of the *in vivo* ODC need to be determined to assess Hb-O_2 affinity. Measurements can be performed *ex vivo* on samples drawn from different blood vessels (arterial and venous) for measurement of Po_2 and SO_2 using a blood gas analyzer and co-oximeter, or *in vivo* by inserting catheters with Po_2 and SO_2 sensing tips into different blood vessels (51). This results in a multitude of points that resemble the *in vivo* ODC since conditions (pH, CO_2) are different in individual samples [see, e.g., reference (139)]. Another approach to obtain several points of the ODC is to vary alveolar Po_2 with a gas-mixing device and to measure Po_2 and SO_2 transcutaneously by oximetry. Derivation of the P_{50} value is also possible from measurement of Po_2 and SO_2 in a single blood sample assuming a constant Hill coefficient (143, 266), or by use of a reference ODC (1). With these methods, P_{50} values can be obtained with an accuracy of approximately ±1 mmHg when SO_2 is in the range of 20% to 80%, where the Hill plot is essentially linear. SO_2 of arterial samples from normoxic individuals is too high for extrapolation of P_{50} from a single point but venous blood samples can be used (139).

Modeling the oxygen-dissociation curve and obtaining "standard" P_{50} values

The classical mathematical description of the ODC comes from the pioneering work of Adair (2). Another model often used to estimate the binding constants is Monod-Changeux-Wyman's two-state model (149). A key problem for these applications is the need for precise measurements at extreme SO_2 (below 1.5% and above 98.5%) as done by Kernohan and Roughton (129), Roughton et al. (188), Roughton and Severinghaus (189). Errors introduced by incomplete saturation or desaturation when equilibrating samples in the absence of oxygen or in pure oxygen can be minimized by extrapolating the absorbance values at zero and full saturation from data in the fitting procedure [cf. reference (83)].

Hill's plots of ODCs [log (SO_2/(100-SO_2)) vs. log(Po_2)] are almost linear in the middle portions (20%-80% SO_2) permitting ready determination of the P_{50} values and the cooperativity coefficients (102):

$$\log\left(\frac{y}{(1-y)}\right) = \log(k) + n \times \log(PO_2)$$

where y is the fractional O_2 saturation, k is the dissociation constant, and n is the maximal cooperativity.

Severinghaus (206) published a model of best fit of the standard ODC with an average SO_2 error of 0.26% in the physiological Po_2 range for a P_{50} value of 26.86 mmHg and plasma pH = 7.4 at 37°C, based on a modification of the Hill plot, where (S) is fractional saturation:

$$S = 100 \times \left(\left(\left(PO_2^3 + 150 \times PO_2\right)^{-1} \times 23400\right) + 1\right)^{-1}$$

$$\ln PO_{2,st} = 0.385 \times \ln(S^{-1} - 1) + 3.32 - (72 \times S)^{-1} - 0.17 \times S^6$$

From an experimentally determined $P_{50,obs}$ the entire curve can be calculated by multiplying the $Po_{2,st}$ values obtained from the last-mentioned formula with the ratio of observed to standard P_{50}:

$$PO_{2,actual} = PO_{2,std} \times \frac{P_{50,obs}}{26.86}$$

To correct P_{50} values measured under standardized conditions for different *in vivo* conditions of plasma pH, temperature (T), BE, and DPG, Okada et al. (155) published the following modification of the model proposed by Roughton and Severinghaus (189):

$$\Delta \log_{50} = 0.48 \times (7.4 - pH_{plasma}) + 0.024 \times (T - 37) + 0.0013 \times BE + 0.135 \times DPG - 0.116$$

where P_{50} is in mmHg, T in °C, BE in mEq/L, and DPG is the molar ratio of DPG to Hb. This equation estimates P_{50} and oxygen saturation values with accuracies of ±2.5% and ±5%, respectively. Others equations and nomograms to estimate changes in P_{50} values upon changes in DPG, CO_2, and pH have been reported (198, 269).

Optimizing O_2 Loading and Unloading by Altering Hb-O_2 Affinity

Most mammalian Hbs show a high intrinsic Hb-O_2 affinity in the absence of allosteric effectors and a high sensitivity to organic phosphates and other cellular effectors that thus

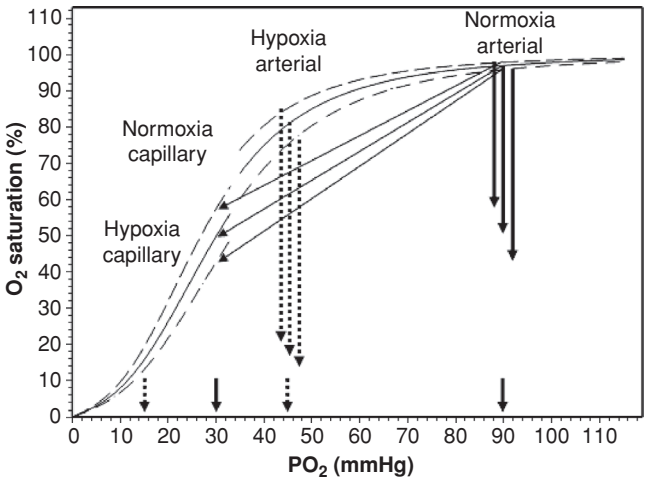

Figure 8 Effects of increased and decreased Hb-O_2 affinity on arterial loading and capillary unloading of oxygen. Oxygen-dissociation curves (ODCs) refer to arterial (three curves at $P_{O_2} > 35$ mmHg) and capillary blood ($P_{O_2} < 33$ mmHg). The continuous line represents a normal ODC with a "standard" P_{50} of 26.8 mmHg for arterial blood [calculated according to reference (206)]. The leftward- and rightward-shifted ODCs are calculated from pH shifts of ±0.1 resulting in P_{50} values of 24 and 30 mmHg, respectively. The ODCs for capillary blood are right-shifted relative to those of arterial blood due to the more acidic environment in the capillary ($\Delta pH = -0.1$). A Bohr coefficient of −0.48 was used for calculations. Long vertical arrows indicate the percent O_2 unloaded from Hb assuming arterial $P_{O_2} = 90$ and 45 mmHg and capillary $P_{O_2} = 30$ and 15 mmHg in normoxia and hypoxia, respectively, as indicated by the short continuous (normoxia) and broken (hypoxia) arrows on the P_{O_2} axis. The slopes of the diagonal arrows (that only are drawn for the normoxic condition) indicate the capacitance "β" that is the driving force for O_2 unloading. The resulting values for arterial and capillary SO_2 and the effects on unloading of O_2 from Hb are summarized in Table 3. See text for further details.

play important roles in adjusting O_2 binding and unloading to physiological needs. With this background, several models were developed to calculate P_{50} values for optimizing the arterio-venous oxygen content difference at a given arterial P_{O_2} (231, 264). The effects are summarized in Figure 8 and Table 3.

Table 3 Effects of increased and decreased Hb-O_2 affinity on arterial (art) and capillary (cap) SO_2 and on O_2 unloading from Hb

$P_{50,art}$	24		26.8		30	
$P_{O_2,art}$	90	45	90	45	90	45
$SO_{2,art}$	97.8	85.2	96.9	80.7	95.8	75.2
$P_{50,cap}$	26.8		30		33.5	
$P_{O_2,cap}$	30	15	30	15	30	15
$SO_{2,cap}$	57.4	19.4	49.9	15.4	42.2	13.1
ΔSO_2	40.4	65.8	47	65.3	53.6	62.1

P_{50} and P_{O_2} values are in mmHg, SO_2 values in percent (%). The difference in SO_2 between arterial and capillary blood (ΔSO_2) are derived from calculated oxygen dissociation curves (details in text and in legend to Fig. 6).

Increased Hb-O_2 affinity increases arterial O_2-loading A high Hb-O_2 affinity favors the binding of O_2 to Hb, which is of importance in blood passing through lungs or gills where O_2 is transferred from the ambient air or water to the blood. As indicated in Figure 1, a high O_2 content in blood leaving the gas exchange organs allows a larger amount of O_2 to be delivered to the O_2 consuming cells. A high Hb-O_2 affinity is of particular importance for O_2 loading in a hypoxic environment (196), where it supplements other adjustments such as increases in ventilation and lung/gill blood flow that augment O_2 uptake at the gas exchange surfaces. There are two basic strategies for increasing SO_2 at a given P_{O_2}:

1. Decreasing the concentration of allosteric effectors in the RBCs. A fast increase in Hb-O_2 affinity is achieved by hyperventilation that decreases CO_2 and H^+ under environmental hypoxia. This adjustment occurs within seconds to minutes and is readily reversible. A somewhat slower response (minutes to hours) is a decrease in DPG or other organic phosphates, which may result from changes in the activities of enzymes involved in their formation or breakdown.

2. Decreased number of binding sites for allosteric effectors. This is typically a genetic adaptation, which is found in animals living permanently in a low-oxygen environment. Their Hb molecules have fewer binding sites for the effectors (organic phosphates, protons, or Cl^-) that reduce Hb-O_2 affinity. These adaptations may go hand in hand with low activities of the enzymes responsible for the synthesis of allosteric effectors. This is aptly illustrated in bovine RBCs that have low DPG levels and where the loss of specific amino acid residues implicated in DPG binding—compared to human Hb His-2β is deleted and Val 1β is replaced by Met—correlates with low DPG sensitivity (165).

Decreased Hb-O_2 affinity improves tissue O_2 supply A low Hb-O_2 affinity favors the unloading of O_2 at high P_{O_2}, which increases the gradient for diffusion of oxygen to the cells. It is of particular significance when the O_2 demand is increased such as during exercise. However, a strongly right-shifted ODC will compromise arterial O_2 loading. Thus, a low Hb-O_2 affinity is specifically beneficial in blood of peripheral capillaries. The implicated mechanisms are basically opposite to those that increase O_2 loading.

1. Increase in the red cell concentration of allosteric effectors: fast adjustments occur during the passage of blood through peripheral capillaries where the end products of cell metabolism, H^+ and CO_2, are taken up by red cells. Thus, particularly at the venous end of capillaries Hb-O_2 affinity is decreased relative to arterial blood. An increased temperature in working skeletal muscle contributes to the decreased Hb-O_2 affinity. A slower mechanism, which most likely affects RBCs in the entire vasculature, is an

increase in the concentration of allosteric affecters such as organic phosphates. These mechanisms appear of particular importance with Hbs that have high intrinsic Hb-O_2 affinity such as fetal hemoglobin (Hb F).

2. Hbs with a high sensitivity to allosteric effectors will bind less oxygen at a given Po_2 for a given set of intraerythrocytic conditions.

Advantage or disadvantage of increased and decreased Hb-O_2 affinity Figure 8 and Table 3 compare the effects of left- and right-shifted ODCs on arterial O_2 loading and capillary unloading for human blood. The curves were calculated for pH changes of ±0.1 pH units (see legends for detail). As illustrated, an increase and decrease in Hb-O_2 affinity resulting from a P_{50} change of ± 3 mmHg only slightly increases and decreases, respectively, SO_2 (by ~ 1%) in arterial blood in normoxia ($Po_{2,art}$ = 90 mmHg). However, in hypoxia ($Po_{2,art}$ = 45 mmHg corresponding to an arterial Po_2 found at an altitude of ~ 4500 m), the increased Hb-O_2 affinity increases arterial SO_2 by approximately 4.5%, whereas a decrease in affinity, associated with an increase in P_{50} by 3 mmHg, decreases it by approximately 5.5%.

For unloading O_2 from Hb the conditions prevailing in the capillary (that may be assumed to be more acidic than in the arteries) need to be considered. The ODCs were thus also calculated for pH 7.3 with superimposed left and right shifts corresponding to a change in pH of ±0.1 units. To demonstrate the effect of these changes on O_2 unloading, capillary Po_2 values of 30 mmHg and 15 mmHg were assumed for normoxia and hypoxia, respectively. These values of Po_2 also indicate low and increased oxygen demand, respectively. Calculations based on these curves (Fig. 8) are summarized in Table 3. In normoxia (arterial Po_2 90 mmHg), a rightward shift of the ODC decreases SO_2 in capillary blood from approximately 57% (the value for the left-shifted curve) to approximately 42%. In hypoxia, capillary SO_2 decreases from approximately 20% in the blood with the highest Hb-O_2 affinity to approximately 13% at the lowest Hb-O_2 affinity.

The proportion of O_2 unloaded can be derived from the difference between arterial and venous SO_2. Table 3 shows that in normoxia, unloading of O_2 from Hb increases from approximately 40% to 53% and thus is improved when the P_{50} value increases from the lowest to the highest value. In hypoxia, unloading is approximately 66% for the most left-shifted curve. However, unloading decreases as P_{50} increases due to decreased arterial SO_2.

Species differences

Although exhibiting common quaternary and tertiary structures, the Hbs of vertebrate animals differ considerably in primary structure and thus in their subunit interactions and ability to bind allosteric ligands such as protons, Cl^-, and organic phosphates. Also, the type of organic phosphate encountered in the RBCs varies from species to species and with development. Thus, a wide range of Hb-O_2 affinities is encountered both intrinsically (in stripped Hb) and in whole blood. Representative P_{50} values for stripped Hbs and intact red cells together with the major organic phosphates encountered in selected vertebrates are shown in Table 4. In the following, some illustrative examples will be described.

Table 4 Diversity of Hb-O_2 affinity in animals

	P_{50}, mmHg			
	Hb solution	RBCs	Major organic phosphate(s)	Reference
Humans	5.8	26.8	DPG	(226)
Mouse	13.0	41.0	DPG, ATP	(169)
Camel	12.0	26.9	DPG	(19)
Llama	14.8	25.2	DPG	(19)
Ostrich	7.8	24.5	ATP, ITP, IPP	(119)
Chicken	7.4	48.3	IPP	(21)
Greylag goose	6.0	39.5	IPP	(168)
Bar headed goose	4.6	29.7	IPP	(168)
Trout	13.4	22.0	ATP	(260)
Carp	0.6	7.0	GTP, ATP	(257)

P_{50} values of purified Hb in solution and in intact RBCs and the major organic phosphates affecting Hb-O_2 affinity in selected species. The reader is referred to the original publications for specific conditions (pH, temperature, etc.) of measuring P_{50} of the Hbs in solution and in the RBCs in the different species.

Mammals exhibit a wide range of blood P_{50} values that vary from about 20 to 50 mmHg at pH 7.4 and 37°C (Fig. 9). Schmidt-Nielsen and Larimer (204) reported an inverse relation between P_{50} and body mass that has generally been confirmed (55, 172). This relation indicates that a low Hb-O_2 affinity appears favorable in animals with a high specific metabolic rate, whereas a high affinity favors tissue oxygen supply in animals with a large body mass, which moreover seems to make them more hypoxia-tolerant (224) than the small animals.

In normoxia, arterial oxygen loading in small animals such as rat or mouse is somewhat compromised by the low Hb-O_2 affinity whereas it will be favored by the high affinity in large animals. However, the same percentage of unloading of O_2 from Hb results in a considerably higher capillary Po_2 in the small than in the large animals (224, 231). Since the Po_2 gradient from capillary blood to the cells is the driving force for oxygen diffusion to the mitochondria, increased O_2 unloading at high Po_2 favors a high metabolic rate.

Fish are of pertinent comparative interest. Inhabiting a much less favorable respiratory medium (water is ~ 800 times more dense than air and contains ~ 35 times less O_2 when aerated), fish are exposed to large and rapid natural variations in oxygen availability. Also, temperatures range from about −2°C to high values found in tropical water and locally in the vicinity of deep-sea hydrothermal vents. Additionally, some species, like the tuna and some sharks have core temperatures that well exceed those in the ambient water and gills, whereby the circulating blood is exposed to rapidly changing temperature. Since the physical solubility of O_2 decreases

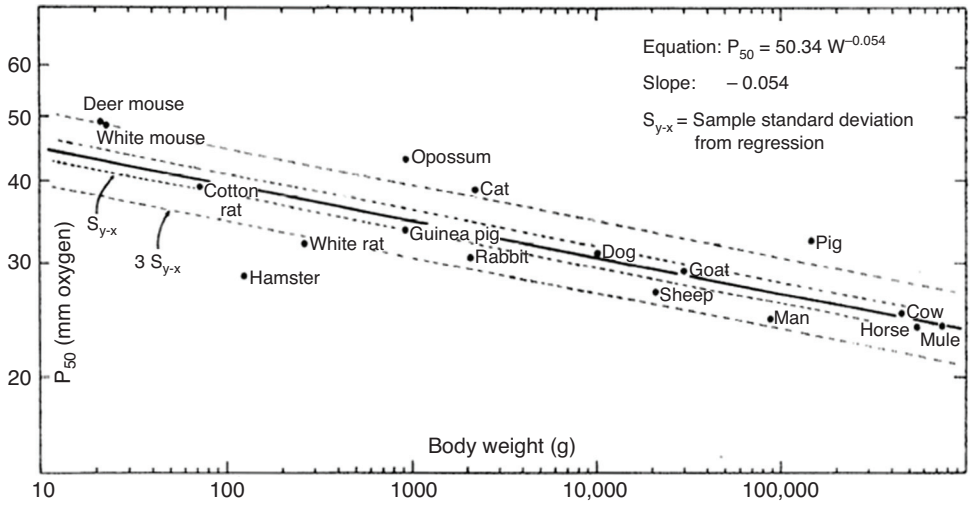

Figure 9 Blood P_{50} values of mammalian Hbs as a function of body weight. The P_{50} values of blood samples of mammals ranging in body mass from 21 g to 635 kg were determined at 37°C and at PCO_2 = 40 mmHg [adapted, with permission, from Schmidt-Nielsen and Larimer (204)].

with increasing temperature, warmer waters have lower contents of dissolved O_2. O_2-content also varies with depth and the content of planktonic and other organisms that consume oxygen.

Fish Hbs exhibit a striking variation in molecular adaptations to optimize tissue oxygen supply in the face of large and independent variations in Po_2 in the gills and tissues. Fish use different red cell phosphates (invariably ATP, in some species also GTP, and in isolated cases also IPP and DPG) (233, 247). Typically, GTP exerts a greater effect on Hb-O_2 affinity than ATP at the same phosphate:Hb concentration ratio. The large variation in molecular and functional properties of the Hbs is manifested both interspecifically (when comparing Hbs from different species) and intraspecifically, that is, within the same species and even the same individual animals that commonly possess multiple isoHbs (247). Generally, active species living in well-aerated waters, like trout, have low blood O_2 affinities associated with low intrinsic O_2 affinities of the stripped Hbs, whereas hypoxia-tolerant species like carp exhibit high O_2 affinities in blood and in stripped Hb preparations (see Table 4). Exposure to hypoxic (O_2-poor) water increases O_2 affinity through decreases in red cell ATP and/or GTP levels [cf. reference (247)]. Apart from this direct allosteric effect, the lowered RBC phosphate concentrations increase O_2 affinity indirectly via perturbations of the Donnan distribution of protons across the RBC membranes that increase intracellular pH (271). In some species of fish (e.g., trout), O_2 affinity of the Hb in the RBCs may also increase via adrenergic stimulation of the Na/proton exchanger in the RBC membranes that alkalinizes the cells (43, 153).

On the basis of isoHbs composition, fish can be divided into two categories, (i) the majority of species that, like other classes of vertebrates, only expresses electrophoretically anodic Hbs (e.g., carp) and (ii) species that in addition to anodic Hbs also express cathodic Hbs (catfish, eels, and salmonids like trout). The cathodic Hbs usually exhibit high O_2 affinities and low Bohr effects and likely assume increased functional importance under conditions of low O_2. In salmonids the cathodic isoHbs moreover lack organophosphate sensitivity. The reduced Bohr effect and phosphate sensitivity in these isoHbs correlate neatly with the replacement of specific charged amino acid residues that bind protons and ATP (in the anodic components) by neutral residues (247). In contrast to the anodic isoHbs, the cathodic fish Hbs commonly lack pronounced Bohr and Root effects (i.e., reductions in the O_2 affinities and O_2 carrying capacities, respectively, with decreasing pH) (247).

O_2 binding by Hbs of the heterothermic fish like tuna and the porbeagle shark that have warm swimming muscles shows special adaptations. Their Hb-O_2 affinities show drastically reduced (or reversed) temperature sensitivities, that may curb excessive O_2 unloading from blood entering warm tissues, and reduce the risk of O_2 diffusion from arterial to venous blood in the countercurrent heat-exchanging "rete" that underpin endothermy (132, 162). In tuna and the porbeagle shark, the exothermic nature of Hb oxygenation (that underlies the "normal" temperature effect) is masked by massive, oxygenation-linked, endothermic release of protons (250). For fast swimming swordfish that maintain warm eyes and brains to increase the temporal resolution of visual stimuli in deep, dim-lit waters, the temperature sensitivity of Hb oxygenation at physiological pH conditions is annihilated by oxygenation-linked dissociation of ATP and protons (250).

The Root effect that appears to occur exclusively in fish Hbs, is an extreme Bohr effect that is characterized by anticooperativity resulting in a reduction of the O_2-carrying capacity of blood at low pH. Following local acidification it causes "pumping" of O_2 against a concentration gradient from

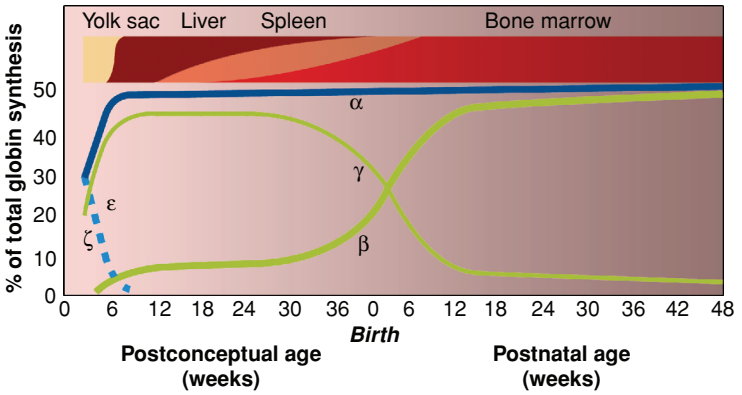

Figure 10 Change in the expression of human globin genes during embryonic, fetal, and postnatal development [modified, with permission, After Wood (272) and Schechter (202)]. The figure shows that the embryonic Hbs Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$), and Portland ($\zeta_2\gamma_2$) are predominantly expressed within the first 6 weeks of intrauterine life, that fetal Hb ($\alpha_2\gamma_2$) predominates from a about 3 weeks after conception until about 3 weeks after birth, and that adult Hb ($\alpha_2\beta_2$) increases strongly around birth.

blood into the swim bladder and retinal tissue. It thus plays a role not only in the control of buoyancy but also in supplying O_2 to the poorly vascularized retina.

The Root effect is absent in cathodic Hbs (159) but is observed in a broad spectrum of anodic Hbs (160). As with the Bohr effect, the Root effect increases in the presence of organic phosphates, GTP exerting a greater effect than ATP (160, 251).

The molecular mechanisms causing the Root effect vary greatly among fish as evident from the variation in mutations encountered in fish Hbs (37, 277). In *Leiostomus xanthurus* (spot, a bony fish), where it is associated with an accumulation of positive charges at the interface between the two β-chains, protonation of His 147β (HC3) and the βN-terminus destabilizes the R-state and promotes an acid-triggered switch from the R- to the T-state and a concomitant release of O_2 (37, 150, 277).

Birds. Most adult birds express only one major isoHb, Hb A, which is functionally similar to human Hb A. Some species, particularly those that are hypoxia (high-altitude) tolerant also express the isoHb D, which has the same β-chains as HbA but different α-chains. Specific amino acid exchanges increasing the Hb's intrinsic affinity for O_2 in high-altitude-tolerant bird species have recently been reviewed (248). O_2 affinity is modulated by organic phosphates, mainly IPP, that bind in the same cavity as does DPG in human Hb A. However, bird Hbs are characterized by two additional insertions of basic residues that favor phosphate binding. These neutralize negative charges rather than being directly involved in phosphate binding (165). As an exception amongst birds, ostrich red cells contain high levels of inositol tetraphosphate (ITP) (120) that predictably has a lower allosteric effect on Hb. It may contribute to a higher O_2 affinity in this large bird (248), thus "scaling" blood O_2 affinity with body mass as seen in mammals (cf. Fig. 7).

O_2 transport during ontogenetic development and ageing

Humans

Lacking direct access to atmospheric air, human embryos and fetuses are dependent on maternal circulation for oxygen, and accordingly show specific adaptations for maternal-fetal O_2 transfer that include higher O_2 affinity and higher O_2-carrying capacity in fetal than in adult human blood. While all mammals appear to express embryonic Hbs during early intrauterine life, many species also express fetal Hbs before switching to adult HbA around birth (245). This isoHb differentiation or Hb switching may not be solely under the control of the genes, which regulate embryonic and fetal growth, but may be modulated by Po_2.

As shown in Figure 10, human embryonic Hbs that occur in early gestation (< 3-month pregnancy) comprises of specific embryonic α- and β-like chains called ξ, and ε, respectively [summary in references (263, 272)]. In humans, these chains form three tetrameric embryonic Hbs, Gower I, Gower II, and Portland, composed of $\zeta_2\varepsilon_2$, $\alpha_2\varepsilon_2$, and $\zeta_2\gamma_2$, respectively (48, 108), that later in gestation are replaced by fetal Hb F composed of $\alpha_2\gamma_2$. Hb Portland has a higher O_2 affinity than Hb A, and a small Bohr effect (229), which tallies with the presence of acetylated serine at ζ1 (i.e., loss of a proton-binding site compared to the α-like chain). Moreover, the presence of γ-chains found in fetal Hb F (instead of adult type β-chains) predicts a reduced DPG effect in this Hb (Fig. 11). In the course of the first 3 months of pregnancy RBCs containing fetal Hb F replace those with embryonic Hbs (Fig. 8). About 3 months after birth, about 90% of the RBCs containing Hb F is replaced with new ones containing Hb A. The expression of these isoforms is independent of the site of erythropoiesis, which switches from yolk sac,

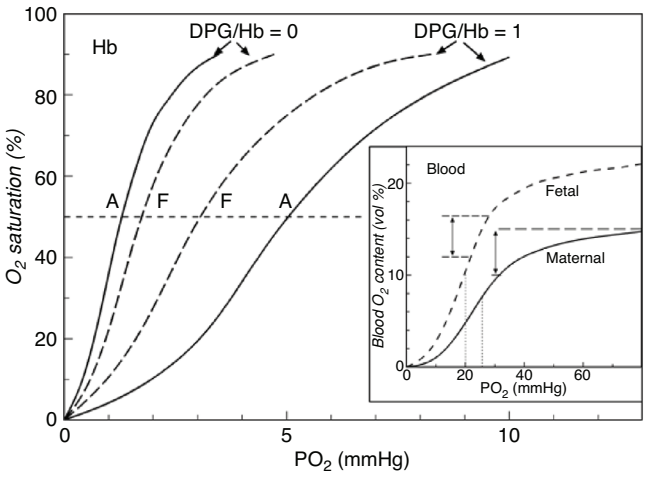

Figure 11 Oxygen-dissociation curves (ODCs) of stripped human adult and fetal Hbs (A and F, respectively) in the absence (DPG/Hb = 0) and presence (DPG/Hb = 1) of equimolar concentrations of 2,3 diphosphoglycerate at 20°C and pH 7.2 (the approximate intracellular pH value). Inset: ODCs for maternal (continuous curve) and fetal (broken curve) human blood at 37°C and pH 7.4, illustrating arteriovenous content differences (double arrows) and higher O_2 affinity and O_2-carrying capacity in fetal blood [adapted, with permission, from (225, 245)].

liver, spleen to bone marrow (Fig. 10) in the transition from embryonic to adult life (272, 273). In normal human adults, the major Hb is Hb A (~ 97%); minor components are Hb A_2 (2%-3%) and Hb F (< 1%).

IsoHbs expressed during development commonly have a higher Hb-O_2 affinity than adult Hbs. Human fetal Hb (Hb F), shows a lower intrinsic O_2 affinity than adult Hb A. However, resulting from amino acid exchange (e.g., His-143β→Ser) that removes two binding sites for 2,3-DPG (86), Hb F has a higher O_2 affinity than Hb A in the presence of DPG (as in intact RBCs, see Fig. 11). In Gower II Hb-O_2 affinity is increased by a specific exchange (His-77β→Asn-77ε) that deletes a Cl^--binding site (219, 279).

Placental O_2 transfer is favored by various mechanisms involving local changes in pH and CO_2 and differences between fetal and adult Hb-O_2 affinities that change as the blood passes in opposite directions through countercurrent fetal and maternal blood capillaries in the placenta. Thus the Hb-O_2 affinity of maternal Hb is decreased by CO_2 and acidification due to the uptake of acid metabolites from the fetal circulation as blood passes through placental capillaries. All together, these effects decrease the Hb-O_2 affinity of maternal blood that favors O_2 unloading. The higher Hb-O_2 affinity in fetal than adult blood is moreover increased by the removal of acid and CO_2 from fetal blood across the placenta, which further increases O_2 binding. Maternal-fetal O_2 transfer is additionally facilitated by the lower Po_2 in fetal than in maternal placental blood.

Adult humans reveal a gender-related difference in Hb-O_2 affinity with higher P_{50} values in women than in men (109, 228). Hb-O_2 affinity in women moreover depends on age and state of maturity (113). Before puberty, P_{50} and DPG values are the same in males and females. However, with puberty P_{50} increases significantly (by about 2 mmHg) in women but not in men. P_{50} decreases again after menopause, the changes correlating with changes in DPG (113). In the age group above 55 years there is no difference between men and women (112). Hb-O_2 affinity is not related to age in men (112). This blood O_2 affinity change implies improved release of O_2 from blood in women during the child-bearing age that will increase the maternal-to-fetal O_2 transfer, although it might compromise O_2 loading in the lungs of the mother.

Other mammals

Higher O_2 affinities in fetal and embryonic stages than in adults are similarly encountered in other mammals. Again, the differences result from lower levels of allosteric effectors (mainly DPG) in RBCs in the developmental stages and/or expression of specific fetal and/or embryonic Hbs with high intrinsic O_2 affinity (245). Three major strategies exist [illustrated in Fig. 3 in reference (246)]: (i) expression of distinct fetal and adult Hbs (Hb F and Hb A), where the former has higher intrinsic O_2 affinity and both Hbs exhibit low sensitivity to DPG (33, 209) (as found in ungulates), (ii) markedly lower concentration of DPG in fetal than in adult red cells, while Hbs in both stages exhibit similar O_2 affinities and high sensitivies to DPG (as in small rodents, rabbits, dogs, and seals), and (iii) expression of specific fetal and adult Hbs, where the former shows lower DPG sensitivity—as in humans (Fig. 11), where lower DPG levels in fetal RBCs contribute to higher O_2 affinity in fetal than in adult blood despite Hb F having a lower intrinsic O_2 affinity than Hb A.

DPG levels during development often correlate with activities of specific enzymes. Examples are small rodents, rabbits, and dogs where very low concentrations of DPG in the fetal RBCs are due to a high activity of PK (122). In rats the activity of the enzyme diphosphoglyceromutase is very low in fetal RBCs (121).

Variations in the O_2-binding properties of embryonic Hbs are largely determined by variations in their molecular structures, and thus intrinsic O_2 affinities (49). Structure-function relationships of embryonic Hbs systems have been traced in a few mammals (246). During embryonic development, pigs that lack fetal Hbs, express Gower I and II (as in man) as well as Heide I and II ($\zeta_2\theta_2$ and $\alpha_2\theta_2$) and Hb A. Hbs Gower I and Heide I that have the highest O_2 affinities and lowest Bohr effects are most abundant in the earliest stages of development, and are successively replaced by Gower II and Heide II, and then by Hb A, (256). Their O_2-binding properties reflect a progressive decrease in the O_2-affinity difference between maternal and embryonic blood in parallel with increases in O_2 tensions in the developmental stages associated with increasing placental development (246). This aptly illustrates how Hb's functional properties may complement morphological adaptations in gas exchange organs. Of the three embryonic mouse isoHbs investigated, $\chi_2\varepsilon_2^Y$, $\alpha_2\varepsilon_2^Y$, and $\alpha_2\varepsilon_2^Z$, the

first mentioned lacks a Bohr effect and cooperativity and may function as a short-term O_2 store (261).

O_2 transport at high altitude

Under hypoxia as occurs at high altitude, the reduced inspiratory P_{O_2} will predictably decrease arterial S_{O_2}. Thus compensatory mechanisms are required to ensure adequate pulmonary O_2 uptake. These involve ventilatory, cardiac, and circulatory, as well as metabolic adjustments (44).

In species permanently subjected to high-altitude hypoxia, the adaptive mechanisms are generally genetically coded, whereas sojourners at high altitude depend on fast and readily reversible adjustments (104). One means of adjustment is an increase in HIF-2α and erythropoietin-dependent erythropoiesis that increases red cell mass, which, at least in humans living in the Andes, can cause polycythemia [summary in reference (265)].

As applies to other stress situations associated with decreased O_2 availability, the adjustments in Hb-O_2 transport to hypoxia at high altitude basically involve two strategies (165, 216, 246, 248): (i) modifications in the structure of the Hb molecule (including isoHb switching) that modulate intrinsic Hb-O_2 affinity or its sensitivity to allosteric effectors and (ii) changes in the concentration of allosteric effectors.

The basic strategies encountered in vertebrate animals that have recently been reviewed [see references (216, 246, 248)] will now be briefly discussed and illustrated with a few representative examples.

Altered structure of Hb

Birds Adult birds flying at high altitude, such as Rüppell's griffon (*Gyps rueppellii*), the bar headed goose (*Anser indicus*), and the "Andean goose" (*Chloephaga melanoptera,* which really is a duck), express isoHbs whose high intrinsic oxygen affinities correlated with specific structural modifications (amino acid exchanges) in the α- and/or β-chains. In common with many other avian species, their RBCs not only contain Hb A but also Hb D, which has different α-chains (101). Uniquely, Rüppell's griffon that holds the high-altitude record (soaring at 11,300 m) expresses four isoHbs (Hb A, Hb A', Hb D, and Hb D') with differentiated intrinsic oxygen affinities (253). One of the most striking examples of molecular adaptation is the bar headed goose that flies from Qinghai lake in China across the Himalayans to breed in the Gulf of Bengal. Its blood O_2 affinity is considerably higher ($P_{50} \sim 29.7$ mmHg at 37°C) than that of its close relative, the greylag goose (*Anser anser*; $P_{50} \sim 39.5$ mmHg). The difference correlates with the replacement of only four amino acid residues of which only one (Pro α119→Ala) is unique amongst bird Hbs. Moreover, this exchange deletes a contact between 119α and 55β that stabilizes the T-structure and thus predictably increases Hb-O_2 affinity (165). This is supported by the finding that Hb from the Andean goose that also has a high blood O_2 affinity shows an exchange (Leu β55 → Ser) that deletes the same contact.

The hypothesis that loss of this single contact underlies the ability of the barheaded goose to fly at altitudes 3000 m higher than the greylag and other geese, was confirmed by site-directed mutagenesis showing that the introduction of Ala α119 and Ser β55 (the key amino acid exchanges found in the barheaded goose and the Andean goose, respectively) into human Hb each markedly increased its O_2 affinity (125, 255). The lack of a salt bridge between His β146 and Asp β94 moreover decreases the Cl^--independent Bohr effect (89, 135, 183).

The previously described mechanisms increase O_2 binding by Hb in the lungs of these high-flying birds. The question arises how these highly specialized RBCs manage to fulfill the function of releasing the tightly bound O_2 to the O_2-consuming tissues. Compared to human, bird Hbs have more positively charged residues at the phosphate-binding site than human Hb (165). Also chicken Hb binds organic phosphate in the T- as well as the R-state (54), although IPP exerts a relatively greater effect on Hb in the deoxy (T) state (135). At the low P_{O_2} in the hypoxic environment, the amount of O_2 unloaded from Hbs with left-shifted ODCs can be expected to equal that unloaded with the right-shifted curve in a lowland (normoxic) animal. The latter operates in the rather flat, upper part of the ODC, where a large change in P_{O_2} causes only a small change in S_{O_2}. In contrast, in the high-flying goose, arterial (loading) and as well as capillary (unloading) O_2 tensions fall on the steep portion of the ODCs, resulting in greater unloading over a small difference in P_{O_2}.

Mammals Illustrative examples are high-altitude tolerant camelids and deer mice. *Camelids* living at different altitudes are a prime example of divergent evolution of Hb with different O_2 affinities. Stripped (phosphate-free) Hbs of camelids living at high altitudes in the Andes (alpaca, guanaco, llama, and *vicuña*) have a slightly lower intrinsic Hb-O_2 affinity than the Hb of their low land relative, the camel (19). However, in intact RBCs, Hb-O_2 affinity is much higher in the high-altitude camelids. This is due to substitution of His β2 → Asn, which eliminates two binding sites for DPG and thus decreases the affinity for DPG binding (19). Hb-O_2 affinity is even higher in the guanaco (168), which correlates with an Ala α130 → Thr substitution, which predictably decreases Cl^- binding (171).

Deer mice (*Peromyscus maniculatus*) found at altitudes of up to 3800 m in the Rocky Mountains segregate into natural high-altitude and low-altitude populations (210, 211) with different aerobic performance capacities (216). In this species subtle changes in the geometry of the heme pocket (215-217) underlie allelic variation in Hb-O_2 affinity encountered in different populations (211). Specifically, two of the three genes that express α-globins segregate the same two alternative protein alleles. They differ from one another by substitutions of 5 amino acid residues spanning the E-helix. Thus, compared to a high-affinity allele that predominates in high-altitude

populations (211) and has Pro, Gly, Ala, Gly, and Gly at α-chain positions 50, 57, 60, 64, and 71, the low-affinity allele that predominates at low altitudes has His, Ala, Gly, Asp, and Ser at the same positions (215-217). One of these 5 substitutions (Asp→Gly at position 64) predictably alters the orientation of the E-helix that includes the distal histidine residue (Fig. 3) with affinity-enhancing effects (215). This is illustrated in a human Hb variant (Hb Guangzhu-Hangzhou) that also has Gly at this position (280).

Altered concentration of allosteric effectors

Humans inhabit widely different altitudes ranging from approximately 400 below sea level, to approximately 4500 m above sea level, for example, in the Andean, Tibet, and East African plateaus, and show large differences in arterial SO_2 (23). Unlike other vertebrates described previously, humans, neither those living permanently at different altitudes nor sojourners temporarily exposed to high altitude, show altitude-related differences in Hb structure. Thus, the intrinsic Hb-O_2 affinity remains unchanged, although blood P_{50} values differ significantly. Measured under "standard" conditions (pH 7.4, $PCO_2 = 40$ mmHg, 37°C), the P_{50} values in human blood increase with increasing altitude, which mainly reflects the effect of increased DPG.

As discussed previously, the *in vivo* blood P_{50} values may decrease rapidly as a result of hyperventilation that decreases PCO_2 and raises pH. Depending on the degree of renal compensation, plasma pH in humans may remain unchanged [at moderate altitude (111,146)] or increase (at high and extreme altitudes). There might be a negative BE (42), and DPG is increased [summarized in reference (139)]. The latter perturbates the difference between RBC and plasma pH (42, 77). These parameters vary depending on the altitude and duration of stay, which complicates analysis of their effects on Hb-O_2 affinity. Summarized, the results from literature indicate that at moderate altitudes Hb-O_2 affinity might be unchanged (42) or slightly decreased as a consequence of a mild increase in DPG (111, 146).

Ascending to higher altitudes results in a more pronounced increase in DPG (193). But since alkalosis is not fully compensated and CO_2 is decreased, P_{50} values decrease compared to those at moderate altitude, approaching normal values at altitudes between approximately 4500 m (138, 147) and approximately 5400 m (195). Upon ascending to even higher altitudes the effects of alkalosis and decreased CO_2 on Hb-O_2-affinity dominate over that resulting from the increase in DPG, even causing a pronounced leftward shift of the ODC at extreme altitudes [estimated $P_{50} \sim 19.4$ mmHg; (268)]. There seems to be no differences in these responses between sojourners and high-altitude natives (200, 267).

The major mechanism increasing DPG at high altitude is hyperventilation-induced alkalosis, which stimulates overall RBC glycolysis. Another influencing factor is red cell age since reticulocytes and young RBCs have increased DPG (147, 193) and since erythropoiesis is stimulated at high altitude (82, 147, 192, 208).

The pH- and CO_2-related changes in Hb-O_2 affinity refer to arterial blood. The slight increase in P_{50} at moderate altitude only moderately impairs arterial oxygen loading, whereas the decrease in P_{50} at higher and extreme altitudes clearly improves O_2 loading at low inspired Po_2. Thus, survival in hypoxia seems to be favored by a high Hb-O_2 affinity as also has been demonstrated in patients that have Hbs with high O_2 affinity (134) and rats with experimentally increased blood-O_2 affinity (81). This is further supported by functional data obtained from individuals with an increased Hb-O_2 affinity ($P_{50} \sim 17$ mmHg), who show smaller increases in erythropoietin and lower resting heart rates at high altitude than controls (99).

Changes in Hb-O_2 affinity in peripheral capillaries under hypoxia are difficult to assess. Here the higher Bohr effect attending the increase in DPG and the slightly increased temperature in the tissue capillaries relative to the lung might now weaken the effect of alkalosis on Hb-O_2 affinity, thus favoring O_2 unloading (194). This effect is enhanced by the higher cooperativity when DPG is increased, which shifts the Po_2 values of arterial O_2 loading and capillary O_2 unloading into the steep portion of the ODC (Fig. 8, Table 3) thus improving unloading at a given difference in Po_2 (224).

O_2 transport during exercise

Besides respiratory and cardio-vascular adjustments the increased demand for oxygen associated with exercise can be met by increased O_2 unloading, which is favored by a decrease in Hb-O_2 affinity as blood passes through the capillaries of the exercising muscles. On the other hand, O_2 loading of Hb in the lung should not be compromised. The differences in pH, metabolite concentrations, and temperature between central venous blood and blood in the capillaries of exercising muscle described below seem to reflect an optimal compromise between these conflicting requirements for Hb function.

During exercise the increased O_2 demand of working muscle decreases Po_2 in the capillaries supplying active muscle fibers. The increased metabolic activity causes an accumulation of end products of aerobic and anaerobic metabolism (protons, CO_2, and lactate), and temperature increases. Taken together, all effects favor the unloading of O_2 from Hb. Due to admixture of blood coming from tissues with a low metabolic activity the changes are less pronounced in central venous blood than in muscular capillaries. As blood passes through the lung, the CO_2 content of blood decreases due to alveolar gas exchange causing an alkalinization relative to muscle capillary blood. DPG seems not to be affected acutely during exercise (145). Therefore, ODCs differ markedly in different organs during exercise, and there certainly is not "the one ODC," not even within a single RBC as it travels back and

forth between lung and working muscle (Fig. 9). The changes are outlined below [see also reference (139)].

Arterial O_2 loading

During exercise, the blood entering the lung is a mixture of blood coming from working muscle, which is acidified, has a high lactate concentration, high PCO_2, and an elevated temperature, and blood coming from inactive tissues, where these changes are less pronounced. Changes vary with exercise intensity, the active muscle mass, and the distribution of the blood to active and inactive tissues. These conditions are adverse for O_2 loading since they impart a low Hb-O_2 affinity, but improve as CO_2 diffuses into the alveolar space as blood passes through alveolar capillaries, causing alkalinization and decreasing CO_2 binding by Hb, both of which increase Hb-O_2 affinity. There is no difference in temperature between alveolar space and alveolar capillary blood. Sun and colleagues reported blood gas levels in systemic arterial and pulmonary artery blood at rest and during submaximal and maximal exercise, where maximal work loads of approximately 300 W were accomplished (218). These measurements indicate P_{50} values of approximately 29 and 26 mmHg for blood in the pulmonary arterial and venous portion of alveolar capillaries, respectively, at rest (Fig. 12; Pa,r; Pv,r) when applying the approximations reported by Roughton and Severinghaus (189) and the values of pH, P_{CO2}, and temperature given in (Fig. 12). In contrast, during maximal exercise the P_{50} increases to approximately 41 mmHg at the pulmonary arterial portion of the capillary and decreases to approximately 34 mmHg at the end of the alveolar capillary. These P_{50} values indicate a shift to the left of the ODC as blood passes through the alveolar capillaries. The exercise-induced right shift of the ODC decreases arterial SO_2 at the venous end of alveolar capillaries from approximately 97.5% at rest (Fig. 12, point Pv,r) to approximately 95% during maximal exercise (Fig. 12; point Pv,e) at P_{O_2} values of 105 and 99 mmHg, respectively, measured by Sun et al. (218). This small difference in SO_2 estimated from ODCs indicates that altered P_{50} values during exercise barely compromises arterial O_2 loading of Hb. However, an increased DPG in trained individuals might widen this gap (142). Thus, the release of CO_2 from Hb into the alveolar space helps maintaining a high systemic arterial SO_2 during exercise; without this change arterial SO_2 might decrease to approximately 92%. A further decrease in SO_2 might occur as a consequence of a diffusion limitation of O_2 associated with the shortened contact time of RBCs in alveolar capillaries when cardiac output reaches very high values in well-trained athletes (71). In the latter, changes in pH, PCO_2 and temperature might also be more pronounced than in the moderately fit individuals studied by Sun et al. (218), which might shift the ODC somewhat further to the right increasing the contribution in a somewhat larger contribution of altered Hb-O_2 affinity to the exercise-induced decrease in systemic arterial SO_2.

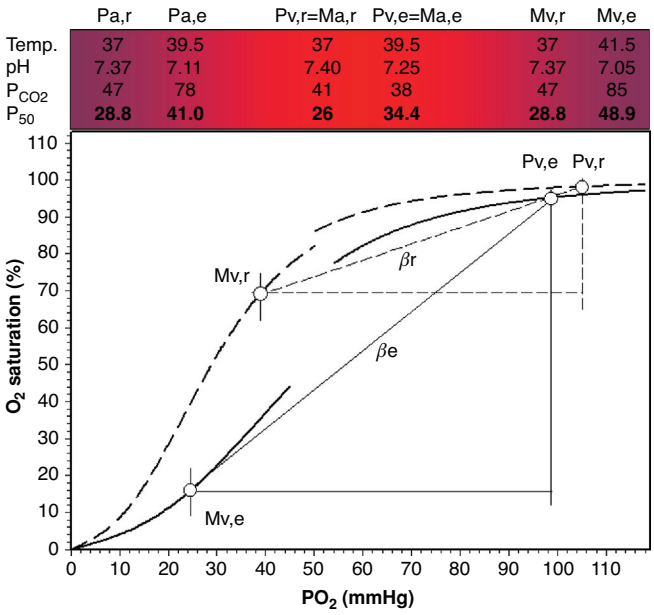

Figure 12 **Effects of exercise on Hb-O_2 affinity** O_2-dissociation curves (ODC) and their shifts are calculated on arterial pH = 7.4 and temperature of 37°C at rest, and the changes of blood gases induced by exercise indicated in the table as reported by Sun et al. (218) using the formulas reported by Severinghaus (206). Acid-base and temperature differences between arterial and capillary blood cause a rest increase P_{50}-values from 26 mmHg in arterial blood to 30 mmHg in capillary blood. During exercise capillary blood P_{50}-value increases ~49 mmHg. The difference in SO_2 at the pulmonary venous PO_2 at rest (Pv,r) and during exercise (Pv,e) and SO_2 in venous blood leaving the exercising muscle at rest (Mv,r) and during exercise (Mv,e) is 28% at rest but 79% during exercise indicating a 2.8-fold increase in the amount of O_2 unloaded from Hb. Pa, Pv, and Ma, Mv indicate pulmonary and muscular arterial and venous blood, respectively, and the indices "r" and "e" denote to rest and exercise. Temp. is the temperature in the respective blood in °C, pH is the plasma pH (likely changes in intraerythrocytic pH are difficult to estimate and are thus not accounted for). PCO_2 is the CO_2 partial pressure (mmHg).

O_2 unloading to exercising muscle

As blood perfuses the capillaries supplying exercising muscles its pH decreases as a consequence of the uptake of acid metabolites. Also PCO_2 increases as a result of aerobic metabolism and the temperature may increase to 41°C. From the values indicated in Figure 12. P_{50} values at the arterial portion of muscle capillaries are approximately 26 mmHg at rest and 34 mmHg during exercise. These values increase to approximately 29 and 49 mmHg, respectively, at the venous end of capillaries of working muscles. This enormous rightward shift of the ODC increases unloading of O_2 considerably (30). Since trained individuals have higher Bohr coefficients at low SO_2 the resulting arterio-to-venous O_2 difference might even be greater (41,45). The increased Bohr coefficient is probably the result of increased DPG due to a decreased average RBC age in trained individuals (142). The amount of O_2 delivered to the working muscle is further increased by elevated blood flow to working muscles and by a much greater extraction due to increased aerobic metabolism that decreases muscle

capillary Po_2. The ODCs shown in Figure 12 indicate that the improved unloading easily outscores the decrease in systemic arterial O_2 content during exercise.

Exercise in hypoxia

Maximal workloads decrease with increasing altitude due to the decreased ambient Po_2 (63,173,220). Thus, the changes in metabolites, CO_2, temperature, and thus Hb-O_2 affinity can be expected to be less pronounced during exercise at high altitude than at low altitude. CO_2 will be lower at high than at low altitude because of hypoxia-induced hyperventilation and decreased aerobic metabolism. On the other hand, renal compensation of respiratory alkalosis leads to a decrease in the concentration of bicarbonate, an important buffer during exercise. Therefore, a more pronounced acidification of blood has been observed at a given blood lactate concentration at high than at low altitude (63). This stronger acidification and the increased DPG should favor O_2 unloading, compensating for the increased Hb-O_2 affinity in arterial blood.

The small rightward shift of the ODC at moderate altitude (42, 111) potentially impairs arterial oxygen loading. On the other hand, the decreased Hb-O_2 affinity might increase unloading and thus oxygenation of the working muscle, which has been interpreted as a beneficial effect given the decreased heart rate observed during exercise at an altitude of 2300 m (146,231). Samaja et al. (195) calculated that a rightward shift of the ODC might be beneficial for tissue oxygenation up to altitudes of about 5400 m above sea level.

Hematological Disorders

Variant Hbs and O_2 transport

In addition to the six normal isoHbs, more than 1000 variant Hbs have been described in humans (http://globin.bx.psu.edu/cgi-bin/hbvar/counter). Although amino acid substitutions encountered commonly are functionally neutral and without significant clinical consequences, several mutations alter Hb function and/or cause increased morbidity and even mortality (263). Neonatal screening for hemoglobinopathies is often performed on a universal basis. Screening is undertaken to detect possible genetic risks to the fetus of sickle cell disease, thalassemias, and other important variants such as Hb C, Hb D^{Punjab}, Hb E, Hb Lepore, and Hb O^{Arab}, and to provide proper clinical management of hemoglobinopathies. Tests include detailed amino acid and DNA sequencing, electrophoresis, and measurement of Hb instability and O_2 affinity. More than 100 Hb variants with altered Hb-O_2 affinity (25, 56) had been described. Their detection is of importance since they can cause erythrocytosis and anemia (214, 240) and since knowledge of O_2 affinities can improve diagnosis (190). Low-affinity Hbs are often associated with reduced Hb concentration, such as observed with Hb Seattle ($P_{50} \sim 40.5$ mmHg), where the anemia might be due to feedback involving increased O_2 unloading, improved tissue oxygenation and, subsequently, a decreased erythropoietin production (212).

Some special cases of hemoglobinopathies with altered Hb-O_2 affinity are noteworthy. The lowest blood P_{50} value (approximately 11 mmHg) was found for Hb Syracuse, which also has a drastically decreased cooperativity (124), as also was found for Hb Rainer. An advantage of its low cooperativity might be an improved unloading of O_2 at Po_2 values that exceed the P_{50}, which, together with the increased arterial SO_2 optimizes tissue O_2 supply (24). The record high P_{50} of approximately 70 mmHg was found in patients with Hb Kansas, where it results in a significantly decreased arterial SO_2 (39). All here quoted P_{50} values refer to pH 7.4 and 37°C.

Anemia and O_2 transport

In many hematological disorders, enzymatic defects affect DPG (234). The disorders subsequently change red cell Cl^- and pH, whereby many of these diseases are associated with altered Hb-O_2 affinity. The observation that Hb-O_2 affinity is decreased in most anemias (40, 110, 228, 235) has been interpreted as an adaptive mechanism to improve tissue oxygenation when the O_2-transport capacity is low. In most cases, it has been associated with an increase in DPG (40, 228). One exception is renal anemia, where acidosis, uremia, and an increased average RBC age resulting from impaired erythropoiesis cause a low concentration of DPG (110, 144). Stimulating erythropoiesis with recombinant erythropoietin increases P_{50} values and DPG in uremic patients (53).

Summary and Conclusion

Tissue oxygen supply depends on a variety of mechanisms that include ventilation of the respiratory surfaces, diffusion of oxygen from the respiratory medium (air or water) into blood, the circulation and regional distribution of blood flow, and the diffusion of O_2 from the capillaries to the mitochondria of the respiring cells. Continuous adjustments of O_2 affinity secure O_2 loading and unloading—in the face of independent variations in O_2 tensions at both implicated sites—extending the tolerance to environmental factors (O_2 availability, temperature, etc.). All of the large variety of mechanisms adjusting Hb-O_2 affinity affect the basic, reversible reaction of O_2 with ferrous iron atoms. These adaptations depend variously on RBC metabolic processes that modify the levels of heterotropic effectors [organic phosphates, chloride, protons (pH), and CO_2] and are basic to rapid and often localized changes in Hb-O_2 affinity. In contrast, the long-term adjustments (e.g., in red cell metabolism or Hb structure) that are encountered in species or developmental stages that are continuously exposed to decreased ambient O_2 availability are commonly gene-based and involve changes in Hb structure that impacts the Hb's intrinsic O_2 affinity and its ability to bind effectors. The changes in Hb structure commonly result from substitutions of only a few of the 287 amino acid residues

found in each dimer (half of the Hb molecule), indicating convergent selection of relatively few basic adaptive mechanisms in different stages and species. As documented by the large range of hemoglobinopathies encountered in humans, some mutations have deleterious side effects.

References

1. Aberman A, Cavanilles JM, Weil MH, Shubin H. Blood P_{50} calculated from single measurements of pH, PO_2, and SO_2. *J Appl Physiol* 38: 171-176, 1975.
2. Adair GS. The hemoglobin system. VI. The oxygen dissociation curve of hemoglobin. *J Biol Chem* 63: 529-545, 1925.
3. Amiconi G, Antonini E, Brunori M, Wyman J, Zolla L. Interaction of hemoglobin with salts. Effects on the functional properties of human hemoglobin. *J Mol Biol* 152: 111-129, 1981.
4. Amiconi G, Bertollini A, Bellelli A, Coletta M, Condo SG, Brunori M. Evidence for two oxygen-linked binding sites for polyanions in dromedary hemoglobin. *Eur J Biochem* 150: 387-393, 1985.
5. Antonini E, Schuster TD, Brunori M, Wyman J. The kinetics of the Bohr effect in the reaction of human hemoglobin with carbon monoxide. *J Biol Chem* 240: 2262-2264, 1965.
6. Arnone A. X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin. *Nature* 237: 146-149, 1972.
7. Arnone A. Mechanism of action of hemoglobin. *Annu Rev Med* 25: 123-130, 1974a.
8. Arnone A. X-ray studies of the interaction of CO_2 with human deoxyhaemoglobin. *Nature* 247: 143-145, 1974b.
9. Atha DH, Ackers GK. Calorimetric determination of the heat of oxygenation of human hemoglobin as a function of pH and the extent of reaction. *Biochem* 13(11): 2376-2382, 1974.
10. Bailey JE, Beetlestone JG, Irvine DH. Reactivity differences between hemoglobins. XVII. The variability of the Bohr effect between species and the effect of 2,3-diphosphoglycerate on the Bohr effect. *J Chem Soc A* 5: 756-762, 1970.
11. Barcroft J, King WO. The effect of temperature on the dissociation curve of blood. *J Physiol* 39: 374-384, 1909.
12. Bartlett GR. Phosphate compounds in rat erythrocytes and reticulocytes. *Biochem Biophys Res Commun* 70/4: 1055-1062, 1976.
13. Bartlett GR. Phosphate compounds in red cells of reptiles, amphibians and fish. *Comp Biochem Physiol A Comp Physiol* 55: 211-214, 1976.
14. Bartlett GR, Borgese TA. Phosphate compounds in red cells of the chicken and duck embryo and hatchling. *Comp Biochem Physiol A Comp Physiol* 55: 207-210, 1976.
15. Barvitenko NN, Adragna NC, Weber RE. Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 15: 1-18, 2005.
16. Bauer C. Antagonistic influence of CO_2 and 2,3-diphosphoglycerate on the Bohr effect of human haemoglobin. *Life Sci* 8: 1041-1046, 1969.
17. Bauer C. Reduction of the carbon dioxide affinity of human haemoglobin solutions by 2,3-diphosphoglycerate. *Respir Physiol* 10: 10-19, 1970.
18. Bauer C, Baumann R, Engels U, Pacyna B. The carbon dioxide affinity of various human hemoglobins. *J Biol Chem* 250: 2173-2176, 1975.
19. Bauer C, Rollema HS, Till HW, Braunitzer G. Phosphate binding by llama and camel hemoglobin. *J Comp Physiol* 136: 67-70, 1980.
20. Bauman R, Bauer C, Haller EA. Oxygen-linked CO_2 transport in sheep blood. *Am J Physiol* 229: 334-339, 1975.
21. Baumann FH, Baumann R. A comparative study of the respiratory properties of bird blood. *Respir Physiol* 31: 333-343, 1977.
22. Baumann R, Haller EA. Cat haemoglobins A and B: Differences in the interaction with Cl minus, phosphate and CO_2. *Biochem Biophys Res Commun* 65: 220-227, 1975.
23. Beall CM. Andean, Tibetan, and Ethiopian patterns of adaptation to high-altitude hypoxia. *Integr Comp Biol* 46: 18-24, 2006.
24. Bellingham AJ. The physiological significance of the Hill parameter 'n'. *Scand J Haematol* 9: 552-556, 1972.
25. Bellingham AJ. Haemoglobins with altered oxygen affinity. *Br Med Bull* 32: 234-238, 1976.
26. Benesch R, Benesch RE. The effect of organic phosphates from human erythrocytes on the allosteric properties of hemoglobin. *Biochem Biophys Res Commun* 26: 162-167, 1967.
27. Benesch R, Benesch RE, Yu CI. Reciprocal binding of oxygen and diphosphoglycerate by human hemoglobin. *Proc Natl Acad Sci U S A* 59: 526-532, 1968.
28. Benesch RE, Benesch R, Yu CI. The oxygenation of hemoglobin in the presence of 2,3-diphosphoglycerate. Effect of temperature, pH, ionic strength, and hemoglobin concentration. *Biochem* 8: 2567-2571, 1969.
29. Berenbrink M. Evolution of vertebrate haemoglobins: Histidine side chains, specific buffer value and Bohr effect. *Respir Physiol Neurobiol* 154: 165-184, 2006.
30. Berlin G, Challoner KE, Woodson RD. Low-O_2 affinity erythrocytes improve performance of ischemic myocardium. *J Appl Physiol* 92: 1267-1276, 2002.
31. Bert P. *La Pression Barometrique*. Paris: Masson, 1878.
32. Beutler E. *Red Cell Metabolism: A Manual of Biochemical Methods*. New York: Grune & Stratton, 1975.
33. Blunt MH, Kitchens JL, Mayson SM, Huisman TH. Red cell 2,3-diphosphoglycerate and oxygen affinity in newborn goats and sheep. *Proc Soc Exp Biol Med* 138: 800-803, 1971.
34. Bohr C. Die Sauerstoffaufnahme des genuinen Blutfarbstoffs und des aus dem Blute dargestellten Hämoglobins. *Zbl Physiol* 17: 688-691, 1903.
35. Bohr C, Hasselbalch K, Krogh A. Über einen in biologischer Beziehung wichtigen Einfluß, den die Kohlensäurespannung des Blutes auf dessen Sauerstoffspannung ausübt. *Scand Arch Physiol* 16: 402-412, 1904.
36. Bonaventura C, Bonaventura J. Competition in oxygen-linked anion binding to normal and variant human hemoglobins. *Hemoglobin* 4: 275-289, 1980.
37. Bonaventura C, Crumbliss AL, Weber RE. New insights into the proton-dependent oxygen affinity of Root effect haemoglobins. *Acta Physiol Scand* 182: 245-258, 2004.
38. Bonaventura C, Ferruzzi G, Tesh S, Stevens RD. Effects of S-nitrosation on oxygen binding by normal and sickle cell hemoglobin. *J Biol Chem* 274: 24742-24748, 1999.
39. Bonaventura J, Riggs A. Hemoglobin Kansas, a human hemoglobin with a neutral amino acid substitution and an abnormal oxygen equilibrium. *J Biol Chem* 243: 980-991, 1968.
40. Böning D, Enciso G. Hemoglobin-oxygen affinity in anemia. *Blut* 54: 361-368, 1987.
41. Böning D, Schweigart U, Tibes U, Hemmer B. Influences of exercise and endurance training on the oxygen dissociation curve of blood under in vivo and in vitro conditions. *Eur J Appl Physiol* 34: 1-10, 1975.
42. Böning D, Trost F, Braumann KM, Bitter H, Bender K, Mühlen A, Schweigart U. Altitude acclimatization in skiing lowlanders. *Int J Sports Med* 1: 191-198, 1980.
43. Borgese F, Garcia-Romeu F, Motais R. Catecholamine-induced transport systems in trout erythrocyte: Na^+/H^+ countertransport or NaCl cotransport? *J Gen Physiol* 87: 551-566, 1986.
44. Bouverot P. *Adaptation to Altitude Hypoxia in Vertebrates*. Berlin: Springer Verlag, 1985.
45. Braumann KM, Böning D, Trost F. Bohr effect and slope of the oxygen dissociation curve after physical training. *J Appl Physiol* 52: 1524-1529, 1982.
46. Braunitzer G, Hilse K, Rudloff V, Hilschmann N. The hemoglobins. *Adv Protein Chem* 19: 1-71, 1964.
47. Brewer GJ, Eaton JW. Erythrocyte metabolism: Interactions with oxygen transport. *Science* 171: 1205-1211, 1971.
48. Brittain T. Molecular aspects of embryonic hemoglobin function. *Mol Aspects Med* 23: 293-342, 2002.
49. Brittain T, Wells RMG. Oxygen transport in early mammalian development: Molecular physiology of embryonic hemoglobins. In: Johnson MH, editor. *Development in Mammals*, Vol. 5. Amsterdam: Elsevier Science Publishers, 1983, p. 135-154.
50. Brooks J. The action of nitrite on haemoglobin in the absence of oxygen. *Proc Roy Soc B* 123: 368-382, 1937.
51. Brown EG, Krouskop RW, McDonnell FE, Monge CC, Winslow RM. A technique to continuously measure arteriovenous oxygen content and P_{50} in vivo. *J Appl Physiol* 58/4: 1383-1389, 1985.
52. Brown WEL, Hill AV. The oxygen dissociation curve of blood, and its thermodynamical basis. *Proc Roy Soc B* 94: 297-334, 1923.
53. Brunet P, Berland Y, Merzouk T, Vanuxem D, Badier M, Klinkmann H, Crevat A. Effect of recombinant human erythropoietin treatment in uremic patients on oxygen affinity of hemoglobin. *Nephron* 66: 147-152, 1994.
54. Brygier J, de Bruin SH, Van Hoof MK, Rollema HS. The interaction of organic phosphates with human and chicken hemoglobin. *Eur J Biochem* 60: 379-383, 1975.
55. Bunn HF. Regulation of hemoglobin function in mammals. *Am Zool* 20: 199-211, 1980.
56. Bunn HF, Forget BG, Ranney HM. *Human Hemoglobins*. Philadelphia: Saunders, 1977.
57. Bunn HF, Ransil BJ, Chao A. The interaction between erythrocyte organic phosphates, magnesium ion, and hemoglobin. *J Biol Chem* 246/17: 5273-5279, 1971.
58. Burmester T, Haberkamp M, Mitz S, Roesner A, Schmidt M, Ebner B, Gerlach F, Fuchs C, Hankeln T. Neuroglobin and cytoglobin: Genes, proteins and evolution. *Iubmb Life* 56: 703-707, 2004.
59. Cabantchik ZI. Erythrocyte membrane transport. *Novartis Found Symp* 226: 6-16, 1999.

60. Campbell KL, Roberts JE, Watson LN, Stetefeld J, Sloan AM, Signore AV, Howatt JW, Tame JR, Rohland N, Shen TJ, Austin JJ, Hofreiter M, Ho C, Weber RE, Cooper A. Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. *Nat Genet* 42: 536-540, 2010.
61. Campbell KL, Storz JF, Signore AV, Moriyama H, Catania KC, Payson AP, Bonaventura J, Stetefeld J, Weber RE. Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. *BMC Evol Biol* 10: 214, 2010.
62. Carroll SB. *The Making of the Fittest*. New York: Norton & Company, 2007.
63. Cerretelli P, DiPrampero PE. Aerobic and anaerobic metabolism during exercise at altitude. In: Rivolier J, Cerretelli P, Foray J, Segantini P, editors. High Altitude Deterioration. Basel: Karger, 1985, p. 1-19.
64. Chanutin A, Curnish RR. Effects of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. *Arch Biochem Biophys* 121: 96-102, 1967.
65. Chiancone E, Norne JE, Forsen S, Antonini E, Wyman J. Nuclear magnetic resonance quadrupole relaxation studies of chloride binding to human oxy- and deoxyhaemoglobin. *J Molecular Biology* 70: 675-688, 1972.
66. Christiansen J, Douglas CG, Haldane JS. The absorption and dissociation of carbon dioxide by human blood. *J Physiol* 48: 244-271, 1914.
67. Chu H, Breite A, Ciraolo P, Franco RS, Low PS. Characterization of the deoxyhemoglobin binding site on human erythrocyte band 3: Implications for O_2 regulation of erythrocyte properties. *Blood* 111: 932-938, 2008.
68. Coletta M, Clementi ME, Ascenzi P, Petruzzelli R, Condo SG, Giardina B. A comparative study of the temperature dependence of the oxygen-binding properties of mammalian hemoglobins. *Eur J Biochem* 204: 1155-1157, 1992.
69. Colombo MF, Seixas FAV. Novel allosteric conformation of human Hb revealed by the hydration and anion effects on O_2 binding. *Biochem* 38: 11741-11748, 1999.
70. De Rosa MC, Castagnola M, Bertonati C, Galtieri A, Giardina B. From the Arctic to fetal life: Physiological importance and structural basis of an 'additional' chloride-binding site in haemoglobin. *Biochem J* 380: 889-896, 2004.
71. Dempsey JA, Wagner PD. Exercise-induced arterial hypoxemia. *J Appl Physiol* 87: 1997-2006, 1999.
72. Di Cera E, Doyle ML, Morgan MS, De Cristofaro R, Landolfi R, Bizzi B, Castagnola M, Gill SJ. Carbon monoxide and oxygen binding to human hemoglobin F0. *Biochem* 28: 2631-2638, 1989.
73. Dickerson RE, Geis I. *Hemoglobin: Structure, Function, Evolution, and Pathology*. Menlo Park: Cummings Publishing, 1983.
74. Dill DB, Forbes WH. Respiratory and metabolic effects of hypothermia. *Am J Physiol* 132: 685-697, 1941.
75. Douglas CB, Haldane JS, Haldane JBS. The laws of combination of hemoglobin with carbon monoxide and oxygen. *J Physiol (London)* 44: 275-304, 1912.
76. Dubinsky WP, Racker E. The mechanism of lactate transport in human erythrocytes. *J Membrane Biol* 44: 25-36, 1978.
77. Duhm J. Effects of 2,3-Diphosphoglycerate and other organic phosphate compounds on oxygen affinity and intracellular pH of human erythrocytes. *Pflügers Arch Eur J Physiol* 326: 341-356, 1971a.
78. Duhm J. On the mechanisms of the hypoxia-induced increase of 2,3-diphosphoglycerate in erythrocytes. *Pflügers Arch Eur J Physiol* 326: 254-269, 1971b.
79. Duhm J. Dual effect of 2,3-diphosphoglycerate on the Bohr effects of human blood. *Pflügers Arch Eur J Physiol* 363: 55-60, 1976.
80. Duvelleroy MA, Buckles RG, Rosenkaimer S, Tung C, Laver MB. An oxyhemoglobin dissociation analyzer. *J Appl Physiol* 28: 227-233, 1970.
81. Eaton JW, Skelton TD, Berger E. Survival at extreme altitude: Protective effect of increased hemoglobin-oxygen affinity. *Science* 183: 743-744, 1974.
82. Eckardt KU, Boutellier U, Kurtz A, Schopen M, Koller EA, Bauer C. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 66: 1785-1788, 1989.
83. Fago A, Bendixen E, Malte H, Weber RE. The anodic hemoglobin of *Anguilla anguilla*. Molecular basis for allosteric effects in a Root-effect hemoglobin. *J Biol Chem* 272: 15628-15635, 1997.
84. Fermi G. Three-dimensional Fourier synthesis of human deoxyhaemoglobin at 2-5 A resolution: Refinement of the atomic model. *J Mol Biol* 97: 237-256, 1975.
85. Fermi G, Perutz MF. *Atlas of Molecular Structures in Biology. 2. Hemoglobin and Myoglobin*. Oxford: Clarendon Press, 1981.
86. Frier JA, Perutz MF. Structure of human foetal deoxyhaemoglobin. *J Mol Biol* 112: 97-112, 1977.
87. Funder J, Wieth JO. Chloride and hydrogen ion distribution between human red cells and plasma. *Acta Physiol Scand* 68: 234-245, 1966.
88. Garby L, Robert M, Zaar B. Proton- and carbamino-linked oxygen affinity of normal human blood. *Acta Physiol Scand* 84: 482-492, 1972.
89. Giardina B, Mosca D, De Rosa MC. The Bohr effect of haemoglobin in vertebrates: An example of molecular adaptation to different physiological requirements. *Acta Physiol Scand* 182: 229-244, 2004.
90. Gibson WH, Roughton FJ. The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin. *J Physiol* 136: 507-524, 1957.
91. Gladwin MT, Kim-Shapiro DB. The functional nitrite reductase activity of the heme-globins. *Blood* 112: 2636-2647, 2008.
92. Gow AJ, Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 391: 169-173, 1998.
93. Gray RD. The kinetics of the alkaline Bohr effect of human hemoglobin. *J Biol Chem* 245: 2914-2921, 1970.
94. Grimes AJ. *Human Red Cell Metabolism*. Oxford: Blackwell Science, 1980.
95. Gros G, Rollema HS, Forster RE. The carbamate equilibrium of alpha- and epsilon-amino groups of human hemoglobin at 37 degrees C. *J Biol Chem* 256: 5471-5480, 1981.
96. Guesnon P, Poyart C, Bursaux E, Bohn B. The binding of lactate and chloride ions to human adult hemoglobin. *Respir Physiol* 38: 115-129, 1979.
97. Haire RN, Hedlund BE. Thermodynamic aspects of the linkage between binding of chloride and oxygen to human hemoglobin. *Proc Natl Acad Sci U S A* 74: 4135-4138, 1977.
98. Hazard ES, Hutchison VH. Distribution of acid-soluble phosphates in the erythrocytes of selected species of amphibians. *Comp Biochem Physiol A Physiol* A73: 111-124, 1982.
99. Hebbel RP, Eaton JW, Kronenberg RS, Zanjani ED, Moore LG, Berger EM. Human Llamas. Adaptation to altitude in subjects with high hemoglobin oxygen affinity. *J Clin Invest* 62: 593-600, 1978.
100. Henriques OM. Über Carboxyhämoglobin. *Ergeb Physiol Biol Chem Exp Pharmakol* 28: 625-689, 1929.
101. Hiebl I, Weber RE, Schneeganss D, Kosters J, Braunitzer G. High-altitude respiration of birds. Structural adaptations in the major and minor hemoglobin components of adult Ruppell's Griffon (*Gypus rueppellii*, Aegypiinae): A new molecular pattern for hypoxic tolerance. *Biol Chem Hoppe Seyler* 369: 217-232, 1988.
102. Hill AV. The possible effects of aggregation of the molecules of hemoglobin on its dissociation curve. *J Physiol (London)* 40: iv-vii, 1910.
103. Hlastala MP, Woodson RD. Saturation dependency of the Bohr effect: Interactions among H^+, CO_2, and DPG. *J Appl Physiol* 38(6): 1126-1131, 1975.
104. Hochachka PW. Defense strategies against hypoxia and hypothermia. *Science* 231: 234-241, 1986.
105. Hoffman JF, Laris PC. Determination of membrane potentials in human and amphiuma red blood cells by means of a fluorescent probe. *J Physiol (London)* 239: 519-552, 1974.
106. Horvath SM, Malenfant A, Rossi F, Rossi-Bernardi L. The oxygen affinity of concentrated human hemoglobin solutions and human blood. *Am J Hematol* 2: 343-354, 1977.
107. Hrinczenko BW, Alayash AI, Wink DA, Gladwin MT, Rodgers GP, Schechter AN. Effect of nitric oxide and nitric oxide donors on red blood cell oxygen transport. *Br J Haematol* 110: 412-419, 2000.
108. Huehns ER, Faroqui AM. Oxygen dissociation properties of human embryonic red cells. *Nature* 254: 335-337, 1975.
109. Humpeler E, Amor H. Sex differences in the oxygen affinity of hemoglobin. *Pflügers Arch Eur J Physiol* 343: 151-156, 1973.
110. Humpeler E, Amor H, Braunsteiner H. Unterschiedliche Sauerstoffaffinität des Hämoglobins bei Anämien unterschiedlicher Ätiologie. *Blut* 29: 382-390, 1974.
111. Humpeler E, Inama K, Deetjen P. Improvement of tissue oxygenation during a 20 days-stay at moderate altitude in connection with mild exercise. *Klin Wochenschr* 57: 267-272, 1979.
112. Humpeler E, Vogel S. Oxygen affinity of hemoglobin in post-menopausal women. *Pflügers Arch Eur J Physiol* 372: 287-288, 1977.
113. Humpeler E, Vogel S, Schobersberger W, Mairbäurl H. Red cell oxygen transport in relation to sex and age. *Mechan Aging Develop* 47: 229-239, 1989.
114. Hundahl C, Fago A, Malte H, Weber RE. Allosteric effect of water in fish and human hemoglobins. *J Biol Chem* 278: 42769-42773, 2003.
115. Imai K. Analyses of oxygen equilibria of native and chemically modified human adult hemoglobins on the basis of Adair's stepwise oxygenation theory and the allosteric model of Monod, Wyman, and Changeux. *Biochem* 12: 798-807, 1973.
116. Imai K. *Allosteric Effects in Haemoglobin*. Cambridge: Cambridge University Press, 1982.
117. Imai K, Yonetani T. pH dependence of the Adair constants of human hemoglobin. Nonuniform contribution of successive oxygen bindings to the alkaline Bohr effect. *J Biol Chem* 250: 2227-2231, 1975.
118. Imaizumi K, Imai K, Tyuma I. The linkage between the four step binding of oxygen and the binding of heterotropic anionic ligands in hemoglobin. *J Biochem* 86: 1829-1840, 1979.

119. Isaacks R, Harkness D, Sampsell R, Adler J, Roth S, Kim C, Goldman P. Studies on avian erythrocyte metabolism. Inositol tetrakisphosphate: The major phosphate compound in the erythrocytes of the ostrich (Struthio camelus camelus). *Eur J Biochem* 77: 567-574, 1977.
120. Isaacks RE, Harkness DR, Goldman PH, Adler JL, Kim CY. Studies on avian erythrocyte metabolism. VII. Effect of inositol pentaphosphate and other organic phosphates on oxygen affinity of the embryonic and adult-type hemoglobins of the turkey embryo. *Hemoglobin* 1: 577-593, 1977.
121. Jelkmann W, Bauer C. High pyruvate kinase activity causes low concentration of 2,3-diphosphoglycerate in fetal rabbit red cells. *Pflügers Arch Eur J Physiol* 375: 189-195, 1978.
122. Jelkmann W, Bauer C. 2,3-DPG levels in relation to red cell enzyme activities in rat fetuses and hypoxic newborns. *Pflügers Arch Eur J Physiol* 389: 61-68, 1980.
123. Jensen FB. Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O_2 and CO_2 transport. *Acta Physiol Scand* 182: 215-227, 2004.
124. Jensen M, Oski FA, Nathan DG, Bunn HF. Hemoglobin Syracuse (alpha2beta2-143(H21)His leads to Pro), a new high-affinity variant detected by special electrophoretic methods. Observations on the autooxidation of normal and variant hemoglobins. *J Clin Invest* 55: 469-477, 1975.
125. Jessen TH, Weber RE, Fermi G, Tame J, Braunitzer G. Adaptation of bird hemoglobins to high altitudes: Demonstration of molecular mechanism by protein engineering. *Proc Natl Acad Sci U S A* 88: 6519-6522, 1991.
126. John WG. Glycated haemoglobin analysis. *Ann Clin Biochem* 34(Pt 1): 17-31, 1997.
127. Joyce BK, Grisolia S. The purification and properties of muscle diphosphoglycerate mutase. *J Biol Chem* 234: 1330-1334, 1959.
128. Keitt AS. Hemolytic anemia with impaired hexokinase activity. *J Clin Invest* 48: 1997-2007, 1969.
129. Kernohan JC, Roughton FJ. The precise determination of the bottom of the oxyhaemoglobin dissociation curve of human blood. In: Rorth M, Astrup P, editors. Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status. Copenhagen: Munksgaard, 1972, p. 54-64.
130. Kilmartin JV, Imai K, Jones RT, Faruqui AR, Fogg J, Baldwin JM. Role of Bohr group salt bridges in cooperativity in hemoglobin. *Biochim Biophys Acta* 534: 15-25, 1978.
131. Kilmartin JV, Rossi-Bernardi L. Interaction of hemoglobin with hydrogen ions, carbon dioxide, and organic phosphates. *Physiol Rev* 53: 836-890, 1973.
132. Larsen C, Malte H, Weber RE. ATP-induced reverse temperature effect in isohemoglobins from the endothermic porbeagle shark (*Lamna pasus*). *J Biol Chem* 278: 30741-30747, 2003.
133. Ledingham IM. Factors influencing oxygen availability. *J Clin Pathol Suppl (R Coll Pathol)* 11: 1-6, 1977.
134. Lee YW, Ki CS, Kim HJ, Lee ST, Kim CK, Shin HB, Hong DS, Lee YK. High oxygen-affinity hemoglobin variant associated with high-level venous oxygen saturation. *Clin Chem Lab Med* 46: 417-418, 2008.
135. Liang Y, Hua Z, Liang X, Xu Q, Lu G. The crystal structure of bar-headed goose hemoglobin in deoxy form: The allosteric mechanism of a hemoglobin species with high oxygen affinity. *J Mol Biol* 313: 123-137, 2001.
136. Low PS, Rathinavelu P, Harrison ML. Regulation of glycolysis via reversible enzyme binding to the membrane protein, band-3. *J Biol Chem* 268: 14627-14631, 1993.
137. Lukin JA, Ho C. The structure-function relationship of hemoglobin in solution at atomic resolution. *Chem Revs* 104: 1219-1230, 2004.
138. Mairbäurl H. Red blood cell function at high altitude. *Ann Sports Med* 4(4): 189-195, 1988.
139. Mairbäurl H. Red blood cell function in hypoxia at altitude and exercise. *Int J Sports Med* 15(2): 51-63, 1994.
140. Mairbäurl H, Hoffman JF. Internal magnesium, 2,3-diphosphoglycerate, and the regulation of the steady-state volume of human red blood cells by the Na/K/2Cl cotransport system. *J Gen Physiol* 99: 721-746, 1992.
141. Mairbäurl H, Humpeler E. Diminution of the temperature effect on the oxygen affinity of hemoglobin after prolonged hypothermia. *Pflügers Arch Eur J Physiol* 383: 209-213, 1980.
142. Mairbäurl H, Humpeler E, Schwaberger G, Pessenhofer H. Training dependent changes of red cell density and erythrocytic oxygen transport. *J Appl Physiol* 55(5): 1403-1407, 1983.
143. Mairbäurl H, Oelz O, Bärtsch P. Interactions between Hb, Mg, DPG, ATP, and Cl determine the change in Hb-O_2-affinity at high altitude. *J Appl Physiol* 74(1): 40-48, 1993.
144. Mairbäurl H, Schobersberger W, Hasibeder W, Knapp E, Hopferwieser T, Dittrich P. Increase in Hb-O_2-affinity at moderate altitude (2000 m) in patients on maintenance hemodialysis. *Clin Nephrol* 31(4): 198-203, 1989.
145. Mairbäurl H, Schobersberger W, Hasibeder W, Schwaberger G, Gaesser G, Tanaka KR. Regulation of red cell 2,3-DPG and Hb-O_2-affinity during acute exercise. *Eur J Appl Physiol* 55: 174-180, 1986.
146. Mairbäurl H, Schobersberger W, Humpeler E, Hasibeder W, Fischer W, Raas E. Beneficial effects of exercising at moderate altitude on red cell oxygen transport and on exercise performance. *Pflügers Arch Eur J Physiol* 406: 594-599, 1986.
147. Mairbäurl H, Schobersberger W, Oelz O, Bärtsch P, Eckardt KU, Bauer C. Unchanged in-vivo P_{50} at high altitude despite decreased red cell age and elevated 2,3-DPG. *J Appl Physiol* 68(3): 1186-1194, 1990.
148. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, Gow AJ, Pawloski JR, Watke P, Singel DJ, Piantadosi CA, Stamler JS. Nitric oxide in the human respiratory cycle. *Nat Med* 8: 711-717, 2002.
149. Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: A plausible model. *J Mol Biol* 12: 88-118, 1965.
150. Mylvaganam SE, Bonaventura C, Bonaventura J, Getzoff ED. Structural basis for the Root effect in haemoglobin. *Nat Struct Biol* 3: 275-283, 1996.
151. Nielsen MS, Weber RE. Antagonistic interaction between oxygenation-linked lactate and CO_2 binding to human hemoglobin. *Comp Biochem Physiol A Mol Integr Physiol* 146: 429-434, 2007.
152. Nigen AM, Manning JM, Alben JO. Oxygen-linked binding sites for inorganic anions to hemoglobin. *J Biol Chem* 255: 5525-5529, 1980.
153. Nikinmaa M. Membrane transport and control of hemoglobin-oxygen affinity in nucleated erythrocytes. *Physiol Rev* 72: 301-321, 1992.
154. O'Donnell S, Mandaro R, Schuster TM, Arnone A. X-ray diffraction and solution studies of specifically carbamylated human hemoglobin A. Evidence for the location of a proton- and oxygen-linked chloride binding site at valine 1 alpha. *J Biol Chem* 254: 12204-12208, 1979.
155. Okada Y, Tyuma I, Sugimoto T. Evaluation of Severinghaus' equation and its modification for 2, 3-DPG. *Jap J Physiol* 27: 135-144, 1977.
156. Okada Y, Tyuma I, Ueda Y, Sugimoto T. Effect of carbon monoxide on equilibrium between oxygen and hemoglobin. *Am J Physiol* 230: 471-475, 1976.
157. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524-526, 1987.
158. Passow H. Anion transport across the red blood cell membrane and the protein in band 3. *Acta Biol Med Ger* 36: 817-821, 1977.
159. Pelster B, Weber RE. Influence of organic phosphates on the Root effect of multiple fish haemoglobins. *J Exp Biol* 149: 425-437, 1990.
160. Pelster B, Weber RE. The physiology of the Root effect. *Adv Comp Environm Physiol* 8: 51-77, 1991.
161. Perrella M, Kilmartin JV, Fogg J, Rossi-Bernardi L. Identification of the high and low affinity CO_2-binding sites of human haemoglobin. *Nature* 256: 759-761, 1975.
162. Perry SF, Daxboeck C, Emmett B, Hochachka PW, Brill RW. Effects of temperature change on acid-base regulation in skipjack tuna (Katsuwonus pelamis) blood. *Comp Biochem Physiol A Comp Physiol* 81: 49-53, 1985.
163. Perutz MF. Stereochemistry of cooperative effects in haemoglobin. *Nature* 228: 726-739, 1970.
164. Perutz MF. Regulation of oxygen affinity of hemoglobin: Influence of structure of the globin on the heme iron. *Annu Rev Biochem* 48: 327-386, 1979.
165. Perutz MF. Species adaptation in a protein molecule. *Mol Biol Evol* 1: 1-28, 1983.
166. Perutz MF, Muirhead H, Mazzarella L, Crowther RA, Greer J, Kilmartin JV. Identification of residues responsible for the alkaline Bohr effect in haemoglobin. *Nature* 222: 1240-1243, 1969.
167. Pesce A, Bolognesi M, Bocedi A, Ascenzi P, Dewilde S, Moens L, Hankeln T, Burmester T. Neuroglobin and cytoglobin. Fresh blood for the vertebrate globin family. *Embo Rep* 3: 1146-1151, 2002.
168. Petschow D, Würdinger I, Baumann R, Duhm J, Braunitzer G, Bauer C. Causes of high blood O_2 affinity of animals living at high altitude. *J Appl Physiol* 42: 139-143, 1977.
169. Petschow R, Petschow D, Bartels R, Baumann R, Bartels H. Regulation of oxygen affinity in blood of fetal, newborn and adult mouse. *Respir Physiol* 35: 271-282, 1978.
170. Pettigrew DW, Romeo PH, Tsapis A, Thillet J, Smith ML, Turner BW, Ackers GK. Probing the energetics of proteins through structural perturbation: Sites of regulatory energy in human hemoglobin. *Proc Natl Acad Sci U S A* 79: 1849-1853, 1982.
171. Piccinini M, Kleinschmidt T, Jurgens KD, Braunitzer G. Primary structure and oxygen-binding properties of the hemoglobin from guanaco (Lama guanacoe, Tylopoda). *Biol Chem Hoppe Seyler* 371: 641-648, 1990.
172. Poyart C, Wajcman H, Kister J. Molecular adaptation of hemoglobin function in mammals. *Respir Physiol* 90: 3-17, 1992.
173. Pugh LG, Gill MB, Lahiri S, Milledge JS, Ward MP, West JB. Muscular exercise at great altitudes. *J Appl Physiol* 19: 431-440, 1964.

174. Rapoport S. The regulation of glycolysis in mammalian erythrocytes. *Essays Biochem* 4: 69-103, 1968.
175. Rapoport S, Guest GM. Distribution of acid soluble phosphorus in the blood of various vertebrates. *J Biol Chem* 131: 269-282, 1941.
176. Razynska A, Fronticelli C, Di Cera E, Gryczynski Z, Bucci E. Effect of temperature on oxygen affinity and anion binding of bovine hemoglobin. *Biophys Chem* 38: 111-115, 1990.
177. Reed PW. Effects of the divalent cation ionophore A23187 on potassium permeability of rat erythrocytes. *J Biol Chem* 251: 3489-3494, 1976.
178. Reeves RB. Temperature and acid-base balance effects on oxygen transport by human blood. *Respir Physiol* 33: 99-102, 1978.
179. Reeves RB. A rapid micro method for obtaining oxygen equilibrium curves on whole blood. *Respir Physiol* 42: 299-315, 1980.
180. Reeves RB. The role of blood oxygen affinity in oxygen delivery to cold tissues. In: Sutton JR, Houston CS, Coates G, editors. Hypoxia and Cold. New York: Praeger, 1987, p. 89-99.
181. Richard V, Dodson GG, Mauguen Y. Human deoxyhaemoglobin-2,3-diphosphoglycerate complex low-salt structure at 2·5 Å resolution. *J Mol Biol* 233: 270-274, 1993.
182. Riggs AF, Gorr TA. A globin in every cell? *Proc Natl Acad Sci U S A* 103: 2469-2470, 2006.
183. Rollema HS, Bauer C. The interaction of inositol pentaphosphate with the hemoglobins of highland and lowland geese. *J Biol Chem* 254: 12038-12043, 1979.
184. Rollema HS, de Bruin SH, Janssen LHM, van Os GAJ. The effect of potassium chloride on the Bohr effect of human hemoglobin. *J Biol Chem* 250/4: 1333-1339, 1975.
185. Rossi-Bernardi L, Roughton FJ. The specific influence of carbon dioxide and carbamate compounds on the buffer power and Bohr effects in human haemoglobin solutions. *J Physiol* 189: 1-29, 1967.
186. Roughton FJ. Thermochemistry of the oxygen-haemoglobin reaction: Direct measurements of the heat of reaction under various conditions. *Biochem J* 29: 2604-2621, 1935.
187. Roughton FJ, Adair GS, Barcroft J, Goldschmidt G, Herkel W, Hill RM, Keys AB, Ray GB. The thermochemistry of the oxygen-haemoglobin reaction: Comparison of the heat as measured directly on purified haemoglobin with that calculated indirectly by the Van't Hoff Isochore. *Biochem J* 30: 2117-2133, 1936.
188. Roughton FJ, Otis AB, Lyster RL. The determination of the individual equilibrium constants of the four intermediate reactions between oxygen and sheep haemoglobin. *Proc R Soc Lond B Biol Sci* 144: 29-54, 1955.
189. Roughton FJ, Severinghaus JW. Accurate determination of O_2 dissociation curve of human blood above 98.7 percent saturation with data on O_2 solubility in unmodified human blood from 0 degrees to 37 degrees C. *J Appl Physiol* 35: 861-869, 1973.
190. Rumi E, Passamonti F, Pagano L, Ammirabile M, Arcaini L, Elena C, Flagiello A, Tedesco R, Vercellati C, Marcello AP, Pietra D, Moratti R, Cazzola M, Lazzarino M. Blood p50 evaluation enhances diagnostic definition of isolated erythrocytosis. *J Intern Med* 265: 266-274, 2009.
191. Salak J, VodrazkA Z. Acetylation of human haemoglobin and globin. I. Reactivity and accessibility of the functional groups. *Biochim Biophys Acta* 65: 115-120, 1962.
192. Samaja M. Hypoxia-dependent protein expression: Erythropoietin. *High Alt Med Biol* 2: 155-163, 2001.
193. Samaja M, Brenna L, Allibardi S, Cerretelli P. Human red blood cell aging at 5,050-m altitude: A role during adaptation to hypoxia. *J Appl Physiol* 75: 1696-1701, 1993.
194. Samaja M, Crespi T, Guazzi M, Vandegriff KD. Oxygen transport in blood at high altitude: Role of the hemoglobin-oxygen affinity and impact of the phenomena related to hemoglobin allosterism and red cell function. *Eur J Appl Physiol* 90: 351-359, 2003.
195. Samaja M, DiPrampero PE, Cerretelli P. Simulation of oxygen delivery to tissues: The role of the hemoglobin oxygen equilibrium curve at altitude. *Int J Clin Monit Comp* 2: 95-99, 1985.
196. Samaja M, Mariani C, Prestini A, Cerretelli P. Acid-base balance and O_2 transport at high altitude. *Acta Physiol Scand* 159: 249-256, 1997.
197. Samaja M, Melotti D, Rovida E, Rossi-Bernardi L. Effect of temperature on the p50 value for human blood. *Clin Chem* 29: 110-114, 1983.
198. Samaja M, Mosca A, Luzzana M, Rossi-Bernardi L, Winslow RM. Equations and nomogram for the relationship of human blood p50 to 2,3-diphosphoglycerate, CO_2, and H^+. *Clin Chem* 27(11): 1856-1861, 1981.
199. Samaja M, Rovida E. A new method to measure the hemoglobin oxygen saturation by the oxygen electrode. *J Biochem Biophys Meth* 7: 143-152, 1983.
200. Samaja M, Veicsteinas A, Cerretelli P. Oxygen affinity of blood in altitude sherpas. *J Appl Physiol* 47: 337-341, 1979.
201. Samaja M, Winslow RM. The separate effects of H^+ and 2,3-DPG on the oxygen equilibrium curve of human blood. *Br J Haematol* 41: 373-381, 1979.
202. Schechter AN. Hemoglobin research and the origins of molecular medicine. *Blood* 112: 3927-3938, 2008.
203. Schmidt W, Prommer N. Impact of alterations in total hemoglobin mass on VO2max. *Exerc Sport Sci Rev* 38: 68-75, 2010.
204. Schmidt-Nielsen K, Larimer JL. Oxygen dissociation curves of mammalian blood in relation to body size. *Am J Physiol* 195: 424-428, 1958.
205. Severinghaus JW. Oxyhemoglobin dissociation curve correction for temperature and pH variation in human blood. *J Appl Physiol* 12: 485-486, 1958.
206. Severinghaus JW. Simple, accurate equations for human blood O_2 dissociation computations. *J Appl Physiol* 46(3): 599-602, 1979.
207. Sick H, Gersonde K. Method for continuous registration of O_2-binding curves of hemoproteins by means of a diffusion chamber. *Analyt Biochem* 32: 362-376, 1969.
208. Siri WE, Van Dyke DC, Winchell HS, Pollycove M, Parker HG, Cleveland AS. Early erythropoietin, blood, and physiological responses to severe hypoxia in man. *J Appl Physiol* 21: 73-80, 1966.
209. Smith RC, Garbutt GJ, Isaacks RE, Harkness DR. Oxygen binding of fetal and adult bovine hemoglobin in the presence of organic phosphates and uric acid riboside. *Hemoglobin* 3: 47-55, 1979.
210. Snyder LR. Low P_{50} in deer mice native to high altitude. *J Appl Physiol* 58: 193-199, 1985.
211. Snyder LR, Born S, Lechner AJ. Blood oxygen affinity in high- and low-altitude populations of the deer mouse. *Respir Physiol* 48: 89-105, 1982.
212. Stamatoyannopoulos G, Parer JT, Finch CA. Physiologic implications of a hemoglobin with decreased oxygen affinity (hemoglobin seattle). *N Engl J Med* 281: 916-919, 1969.
213. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 276: 2034-2037, 1997.
214. Stephens AD. Polycythaemia and high affinity haemoglobins. *Br J Haematol* 36: 153-159, 1977.
215. Storz JF. Hemoglobin function and physiological adaptation in high-altitude mammals. *J Mammalogy* 88: 24-31, 2007.
216. Storz JF, Moriyama H. Mechanisms of hemoglobin adaptation to high altitude hypoxia. *High Alt Med Biol* 9: 148-157, 2008.
217. Storz JF, Sabatino SJ, Hoffmann FG, Gering EJ, Moriyama H, Ferrand N, Monteiro B, Nachman MW. The molecular basis of high-altitude adaptation in deer mice. *PLoS Genet* 3: e45, 2007.
218. Sun XG, Hansen JE, Ting H, Chuang ML, Stringer WW, Adame D, Wasserman K. Comparison of exercise cardiac output by the Fick principle using oxygen and carbon dioxide. *Chest* 118: 631-640, 2000.
219. Sutherland-Smith AJ, Baker HM, Hofmann OM, Brittain T, Baker EN. Crystal structure of a human embryonic haemoglobin: The carbonmonoxy form of gower II (alpha2 epsilon2) haemoglobin at 2.9 A resolution. *J Mol Biol* 280: 475-484, 1998.
220. Sutton JR, Reeves JT, Wagner PD, GROVES BM, Cymerman A, Malconian MK, Rock PB, Young PM, Walter SD, Houston CS. Operation Everest II: Oxygen transport during exercise at extreme simulated altitude. *J Appl Physiol* 64: 1309-1321, 1988.
221. Syllm-Rapoport I, Jacobasch G, Roigas H, Rapoport S. [2,3-PGase deficiency as a possible cause of increased ATP-content]. *Folia Haematol Int Mag Klin Morphol Blutforsch* 83: 363-365, 1965.
222. Szabo A, Karplus M. Analysis of the interaction of organic phosphates with hemoglobin. *Biochem* 15: 2869-2877, 1976.
223. Tanaka KR, Paglia DE. Pyruvate kinase deficiency. *Semin Hematol* 8: 367-396, 1971.
224. Tenney SM. Functional significance of differences in mammalian hemoglobin affinity for oxygen. In: Sutton JR, Houston CS, Coates G, editors. Hypoxia and the Brain. Burlington: Queen City Printers, 1995, p. 57-68.
225. Tomita S. Modulation of the oxygen equilibria of human fetal and adult hemoglobins by 2,3-diphosphoglyceric acid. *J Biol Chem* 256: 9495-9500, 1981.
226. Torelli G, Celentano F, Cortili G, D'Angelo E, Cazzaniga A, Radford EP. Hemoglobin-oxygen equilibrium at different hemoglobin and 2,3-diphosphoglycerate concentrations. *Physiol Chem Physics* 9/1: 21-38, 1977.
227. Tornqvist M, Osterman-Golkar S, Kautiainen A, Naslund M, Calleman CJ, Ehrenberg L. Methylations in human hemoglobin. *Mutat Res* 204: 521-529, 1988.
228. Torrance J, Jacobs P, Restrepo A, Eschbach J, Lenfant C, Finch CA. Intraerythrocytic adaptation to anemia. *N Engl J Med* 283: 165-169, 1970.
229. Tuchinda S, Nagai K, Lehmann H. Oxygen dissociation curve of haemoglobin Portland. *FEBS Lett* 49(3): 390-391, 1975.
230. Tucker VA. Method for oxygen content and dissociation curves on microliter blood samples. *J Appl Physiol* 23: 410-414, 1967.

231. Turek Z, Kreuzer F, Hoofd LJC. Advantage or disadvantage of a decrease of blood oxygen affinity for tissue oxygen supply at hypoxia. *Pflügers Arch Eur J Physiol* 342: 185-197, 1973.
232. Tyuma I, Shimizu K, Imai K. Effect of 2,3-Diphosphoglycerate on the cooperativity in oxygen binding of human adult hemoglobin. *Biochem Biophys Res Commun* 43: 423-428, 1971.
233. Val AL. Organic phosphates in the red blood cells of fish. *Comp Biochem Physiol A Mol Integr Physiol* 125: 417-435, 2000.
234. Valentine WN, Tanaka KR, Paglia DE. Hemolytic anemias and erythrocyte enzymopathies. *Ann Intern Med* 103: 245-257, 1985.
235. Valeri CR, Fortier NL. Red-cell 2,3-diphosphoglycerate and creatine levels in patients with red-cell mass deficits or with cardiopulmonary insufficiency. *N Engl J Med* 281: 1452-1455, 1969.
236. van Beek GG, Zuiderweg ER, de Bruin SH. The binding of chloride ions to ligated and unligated human hemoglobin and its influence on the Bohr effect. *Eur J Biochem* 99: 379-383, 1979.
237. Van Beek GGM, de Bruin SH. The pH dependence of the binding of d-glycerate 2,3-bisphosphate to doexyhemoglobin and oxyhemoglobin. *Eur J Biochem* 100: 497-502, 1979.
238. Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, Willoughby DA. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci U S A* 91: 2046-2050, 1994.
239. Vora S, Davidson M, Seaman C, Miranda AF, Noble NA, Tanaka KR, Frenkel EP, DiMauro S. Heterogeneity of the molecular lesions in inherited phosphofructokinase deficiency. *J Clin Invest* 72: 1995-2006, 1983.
240. Wajcman H, Galacteros F. Hemoglobins with high oxygen affinity leading to erythrocytosis. New variants and new concepts. *Hemoglobin* 29: 91-106, 2005.
241. Walder JA, Chatterjee R, Steck TL, Low PS, Musso GF, Kaiser ET, Rogers PH, Arnone A. The interaction of hemoglobin with the cytoplasmic domain of band 3 of the human erythrocyte membrane. *J Biol Chem* 259: 10238-10246, 1984.
242. Weatherall DJ, Clegg JB, Wood WG, Pasvol G. Human haemoglobin genetics. *Ciba Found Symp* 147-186, 1979.
243. Weber RE. Cationic control of O_2 affinity in lugworm erythrocruorin. *Nature* 292: 386-387, 1981.
244. Weber RE. Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In: Truchot J-P, Lahlou B, editors. *Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects*. Basel: Karger, 1990, p. 58-75.
245. Weber RE. Hemoglobin-based O_2 transfer in viviparous animals. *Isr J Zool* 40: 541-550, 1994.
246. Weber RE. Hemoglobin adaptations to hypoxia and altitude - the phylogenetic perspective. In: Sutton JR, Houston CS, Coates G, editors. Hypoxia and the Brain. Burlington: Queen City Printers, 1995, p. 31-44.
247. Weber RE. Adaptations for oxygen transport: Lessons from fish hemoglobins. In: DiPrisco G, Giardina B, Weber RE, editors. Hemoglobin Function in Vertebrates. Molecular Adaptation in Extreme and Temperate Environments. Milano: Springer, 2000, p. 23-37.
248. Weber RE. High-altitude adaptations in vertebrate hemoglobins. *Respir Physiol Neurobiol* 158: 132-142, 2007.
249. Weber RE, Campbell KL. Temperature dependence of haemoglobin-oxygen affinity in heterothermic vertebrates: Mechanisms and biological significance. *Acta Physiologica* 202: 549-562, 2011.
250. Weber RE, Campbell KL, Fago A, Malte H, Jensen FB. ATP-induced temperature independence of hemoglobin-O_2 affinity in heterothermic billfish. *J Exp Biol* 213: 1579-1585, 2010.
251. Weber RE, deWilde JAM. Multiple haemoglobins in plaice and flounder and their functional properties. *Comp Biochem Physiol* 54B: 433-437, 1976.
252. Weber RE, Fago A. Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respir Physiol Neurobiol* 144: 141-159, 2004.
253. Weber RE, Hiebl I, Braunitzer G. High altitude and hemoglobin function in the vultures Gyps rueppellii and Aegypius monachus. *Biol Chem Hoppe Seyler* 369: 233-240, 1988.
254. Weber RE, Jensen FB, Cox RP. Analysis of teleost hemoglobin by Adair and Monod-Wyman-Changeux models. Effects of nucleoside triphosphates and pH on oxygenation of tench hemoglobin. *J Comp Physiol* 157B: 145-152, 1987.
255. Weber RE, Jessen TH, Malte H, Tame J. Mutant hemoglobins (alpha 119-Ala and beta 55-Ser): Functions related to high-altitude respiration in geese. *J Appl Physiol* 75: 2646-2655, 1993.
256. Weber RE, Kleinschmidt T, Braunitzer G. Embryonic pig hemoglobins Gower I (zeta 2 epsilon 2), Gower II (alpha 2 epsilon 2), Heide I (zeta 2 theta 2) and Heide II (alpha 2 theta 2): Oxygen-binding functions related to structure and embryonic oxygen supply. *Respir Physiol* 69: 347-357, 1987.
257. Weber RE, Lykkeboe G. Respiratory adaptations in carp blood. Influences of hypoxia, red cell organic phosphates, divalent cations and CO_2 on hemoglobin-oxygen affinity. *J Comp Physiol* 128: 127-137, 1978.
258. Weber RE, Vinogradov SN. Nonvertebrate hemoglobins: Functions and molecular adaptations. *Physiol Rev* 81: 569-628, 2001.
259. Weber RE, Voelter W. 'Novel' factors that regulate oxygen binding in vertebrate hemoglobins. *Micron* 35: 45-46, 2004.
260. Weber RE, Wood SC, Lomholt JP. Temperature acclimation and oxygen-binding properties of blood and multiple haemoglobins of rainbow trout. *J Exp Biol* 65: 333-345, 1976.
261. Wells RMG. Haemoglobin-oxygen affinity in developing embryonic erythroid cells of the mouse. *J Comp Physiol* 129: 333-338, 1979.
262. Wells RMG, Weber RE. The measurement of oxygen affinity in blood and haemoglobin solutions. In: Bridges CR, Butler PJ, editors. Techniques in Comparative Respiratory Physiology: An Experimental Approach, Society for Experimental Biology Seminar Series. Cambridge: Cambridge University Press, 1989, p. 279-303.
263. Wild BJ, Bain BJ. Detection and quantitation of normal and variant haemoglobins: An analytical review. *Ann Clin Biochem* 41: 355-369, 2004.
264. Willford DC, Hill EP, Moores WY. Theoretical analysis of optimal P_{50}. *J Appl Physiol* 52: 1043-1048, 1982.
265. Winslow RM, Monge C. *Hypoxia, Polycythemia, and Chronic Mountain Sickness*. Baltimore: Johns Hopkins University Press, 1987.
266. Winslow RM, Monge CC, Brown EG, Klein HG, Sarnquist F, Winslow NJ, McKneally SS. Effects of hemodilution on O_2 transport in high altitude polycythemia. *J Appl Physiol* 59: 1495-1502, 1985.
267. Winslow RM, Monge CC, Statham NJ, Gibson CG, Charache S, Whittembury J, Moran O, Berger RL. Variability of oxygen affinity of blood: Human subjects native to high altitude. *J Appl Physiol* 51(6): 1411-1416, 1981.
268. Winslow RM, Samaja M, West JB. Red cell function at extreme altitude on Mount Everest. *J Appl Physiol* 56(1): 109-116, 1984.
269. Winslow RM, Samaja M, Winslow NJ, Rossi-Bernardi L, Shrager RI. Simulation of continuous blood O_2 equilibrium curve over physiological pH, DPG, and PCO_2 range. *J Appl Physiol* 529: 524-529, 1983.
270. Winslow RM, Swenberg ML, Berger RL, Shrager RI, Luzzana M, Samaja M, Rossi-Bernardi L. Oxygen equilibrium curve of normal human blood and its evaluation by Adair's equation. *J Biol Chem* 252/7: 2331-2337, 1977.
271. Wood SC, Johansen K. Blood oxygen transport and acid-base balance in eels during hypoxia. *Am J Physiol* 225: 849-851, 1973.
272. Wood WG. Haemoglobin synthesis during human fetal development. *Br Med Bull* 32: 282-287, 1976.
273. Wood WG, Weatherall DJ. Haemoglobin synthesis during human foetal development. *Nature* 244: 162-165, 1973.
274. Woodson RD. Evidence that changes in blood oxygen affinity modulate oxygen delivery: Implications for control of tissue PO_2 gradients. *Adv Exp Med Biol* 222: 309-313, 1988.
275. Wyman J, Jr. Heme proteins. *Adv Protein Chem* 4: 407-531, 1948.
276. Wyman J, Jr. Linked functions and reciprocal effects in hemoglobin: A second look. *Adv Protein Chem* 19: 223-286, 1964.
277. Yokoyama T, Chong KT, Miyazaki G, Morimoto H, Shih DT, Unzai S, Tame JR, Park SY. Novel mechanisms of pH sensitivity in tuna hemoglobin: A structural explanation of the Root effect. *J Biol Chem* 279: 28632-28640, 2004.
278. Yonetani T, Tsuneshige A, Zhou Y, Chen X. Electron paramagnetic resonance and oxygen binding studies of alpha-Nitrosyl hemoglobin. A novel oxygen carrier having no-assisted allosteric functions. *J Biol Chem* 273: 20323-20333, 1998.
279. Zheng T, Zhu Q, Brittain T. Origin of the suppression of chloride ion sensitivity in human embryonic hemoglobin Gower II. *Iubmb Life* 48: 435-437, 1999.
280. Zhou ZQ, Chen LC, Chen PF, Zhang KQ, Wang YH. Hemoglobin Hangzhou alpha 64(E13)Asp—-Gly. A new variant found in China. *Hemoglobin* 11: 31-33, 1987.
281. Zwart A, Kwant G, Oeseburg B, Zijlstra WG. Human whole-blood oxygen affinity: Effect of carbon monoxide. *J Appl Physiol* 57: 14-20, 1984.