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Nitric Oxide, Invertebrates and Hemoglobin¹

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SYNOPSIS. Rich redox chemistry of the diatomic NO gives this molecule the functional flexibility to interact with both metal and non-metal components of biological molecules. This important biological signaling and allosteric control has become evident in such varied applications as brain/nervous system function; immune response; growth and development; behavior; and gas transport. Many of the basic discoveries linking NO to biological systems have arisen from structure-function relationships in hemoglobin. For example, by analogy with hemoglobin, Lou Ignarro, in a now-classic paper on NO, proposed that the activation of soluble guanylate cyclase occurs via a NO-driven planar shift in the enzyme's heme iron (Ignarro *et al.*, 1984). Many other proteins involved in NO biology are heme proteins where NO coordination plays an essential function. In this regard, we may view hemoglobin as a microcosm of NO biology.

Invertebrates provide rich examples in which to explore alternate functions, or even perhaps the original functions, of the globins. Oxygen-carrying proteins could well have evolved from metalloproteins that primarily functioned in nitrogen metabolism rather than reversible oxygen binding. Newly discovered aspects of Hb function relate to the signaling and control processes that nitric oxide shows in biological systems. The comparative approach to these processes has played an important role in their elucidation as well as providing rich, intellectual stimulation to those scientists interested in them.

INTRODUCTION

Current theories maintain that the origin of life on this planet likely occurred under somewhat reducing conditions where there was no free molecular oxygen (Holland, 1984) but redox-active atoms and molecules were probably relatively rich in the early "primordial soup" environment. Methanogenesis, sulfate reduction and nitrogen fixation were the prominent anaerobic pathways competing for a limited supply of abiotic organic carbon. Autotrophic systems, which at first utilized the thermodynamically favorable H₂S and then later the abundant H₂O, eventually began to introduce O₂ into the biosphere where it promptly set about oxidizing reduced minerals long before accumulating as dissolved O₂ in the oceans and ultimately diffusing into

the atmosphere (Schlesinger, 1997). Proteins and other biological molecules involved in the control of oxygen, carbon monoxide, nitric oxide and carbon dioxide are essential components of respiration. The timeline of the biological evolution and development of these molecular benchmarks most certainly does not parallel that of the investigatorial discovery of such molecules. This may be particularly true when we consider proteins that bind nitric oxide. We encourage the reader to discard any bias that may be held solely due to the temporal order in which scientists have elegantly deduced or happily stumbled upon their findings.

Hemoglobin, the "Hydrogen atom" (Brunori, 1999) of molecular biology, is a protein that occurs in all major life forms except viruses and prions. Studies of its properties have blazed the way to our understanding many of the fundamental processes of macromolecular structure and function and are now elucidating fundamental mechanisms for nitric oxide in biological systems. In the coming paragraphs,

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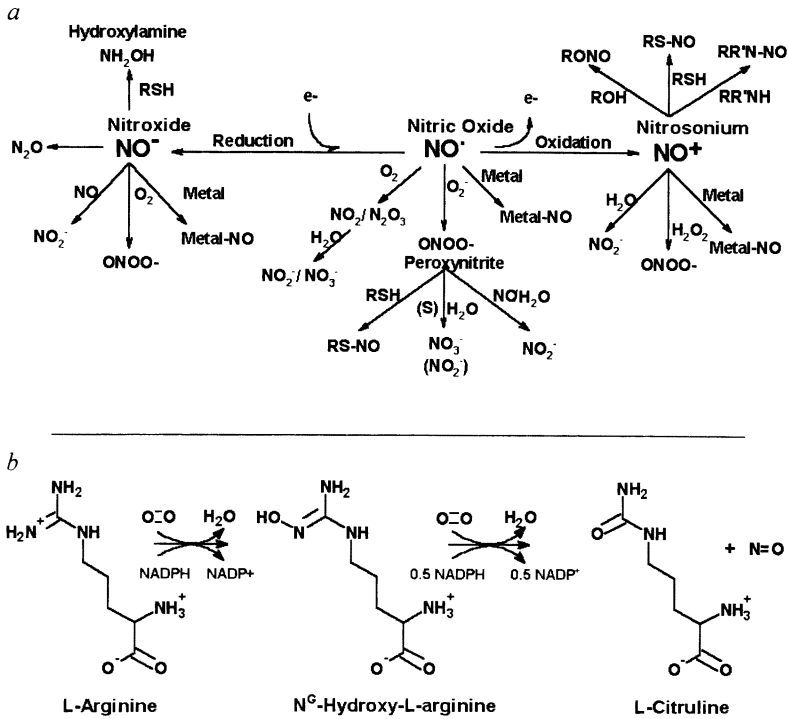


FIG. 1. **a)** Nitrogen monoxide, its redox states and its reactivity with atoms and molecules most often in the context of biology. Oxygen and its redox partner, the superoxide radical anion, form adducts with nitrogen monoxides. Metals, either free in solution or bound to biological macromolecules, are additional targets for NO. Peptidic and proteic thiols form adducts with the three redox states of NO, forming either nitrosothiols or oxidized states of cysteine. Reactive nitrogens and oxygens associated with proteins, nucleic acids and lipids may also interact with nitrogen monoxides producing either further signaling molecules or deleterious adducts which need detoxification (*i.e.*, peroxynitrite). The interplay between the radical form of NO and its anionic and cationic forms positions nitrogen monoxides to act as either anti- or pro-oxidants. **b)** A simplified depiction of the pathway of nitric oxide synthesis by nitric oxide synthases. Note that there are two oxygen-requiring steps in the process that utilizes the cofactor NADPH in the conversion of the substrate arginine first into the reactive intermediate N-hydroxyarginine and then into NO and citrulline. Under conditions of cofactor limitation, particularly tetrahydrobiopterin, the final catalytic product of NOS can be the superoxide radical anion, O₂^{-•}. Under some circumstances, hydrogen peroxide can drive the second half of this reaction.

we will discuss work on human, fish, *Ascaris*, and *E. coli* hemoglobins to illustrate some intriguing NO discoveries.

NITRIC OXIDE PRODUCTION AND FUNCTION IN BIOLOGICAL SYSTEMS

Nitric oxide, unlike Gertrude Stein's rose, is not just the free radical, diatomic gas that scientists once regarded it as. NO, like oxygen, exists in several redox forms. Nitric oxide, the nitrosonium cation and the nitroxide anion (NO[•], NO⁺ and NO⁻), each have their own characteristic biochemistry (Fig. 1a). In organisms, nitric oxide can be produced from the acidification of nitrite (Benjamin *et al.*, 1994; and see this journal)

or from the amino acid arginine in a multi-electron transfer reaction catalyzed by the enzyme nitric oxide synthase (NOS) (Fig. 1b). The three isoforms of NOS show great sequence identity. All NOS polypeptides have two domains: 1) a heme domain which resembles cytochrome P450 and also binds tetrahydrobiopterin and 2) a reductase domain which resembles cytochrome P450 reductase and binds FAD, FMN and NADPH. Calmodulin binds in an interdomain region and appears to couple the oxygenase and reductase domains. In addition to these cofactor and substrate requirements, NOS activity is oxygen-dependent, utilizing two oxygen molecules in the overall process of

NO synthesis. The overall reaction scheme for NO production is shown in Figure 1b. The functional properties and physical locations of the three forms of NOS are depicted in Figure 2.

HEMOGLOBIN: A MICROCOSM OF NITRIC OXIDE BIOLOGY

Blood, Pure and Eloquent is an interesting book that describes the history of our species' fascination with the crimson fluid that leaks from us when our skin is violated and that was probably the first organ we discovered to be intimately associated with our living state (Wintrobe, 1980). John Edsall's article on the history of hemoglobin studies presents a fascinating account of the development of our knowledge of the globins and, although not stated, shows how the study of these molecules provided researchers with a model for studies of other biological macromolecules (Edsall, 1972). Hemoglobin, the primary intellectual passion of Jeffries Wyman, provided him with a tangible vehicle for the development and exposition of the concept of linked-functions and allosteric interactions (Wyman and Gill, 1990). It is increasingly clear that consideration of the functional linkage between interacting entities adds powerful conceptual clarification to extremely complex biological processes, ranging from simple proteins to nucleic acids to organismic populations and finally to global linkages between organisms and environmental processes.

Linkage between blood, its constituent hemoglobin, and oxygen came in the late 18th century (Bohr *et al.*, 1904). Since that time, reversible oxygen binding by hemoglobins and myoglobins has been their defining functional property. The role of carbon dioxide transport by these proteins became appreciated much later (Bohr *et al.*, 1904) and it was not until the 1970s that the molecular structure and mechanisms of CO₂ transport became known (Perella *et al.*, 1975). Carbon monoxide and nitric oxide as heme ligands have mostly been regarded as molecular probes, aiding in better understanding of structure-function relationships in the globins, but having little physiological significance. An exception to this gen-

erality is Perutz's exposition of the physiological significance of the O₂/CO partition coefficient (Perutz, 1990; Tucker *et al.*, 1978).

The globin fold, discovered by J. C. Kendrew and Max Perutz (Kendrew *et al.*, 1960; Perutz *et al.*, 1960), emerged from the massive data sets that these two giants of molecular biology had acquired from crystals of sperm whale myoglobin and horse hemoglobin respectively. An anecdote *apropos* of this is worth retelling. Kendrew and Perutz made the first molecular models of protein crystallographic studies from a multitude of sheets of clear plastic. Each sheet had drawn on it the electron density corresponding to a particular slice through the protein. At 5 angstrom resolution, individual amino acid side chains were not visible. These men expected to see alpha helices, but had little insight into how they might be folded in three-dimensional space. It was as if they were looking at a scene through a mirror darkly, as the picture of these proteins emerged. What they saw was something very visceral and vermiform. Almost as if they were looking at intestines, of a work, writhing about. This "intestine" or "worm" turned out to have the same shape, more-or-less for myoglobin and the α - and β -chains of hemoglobin! Immediately, it became apparent that not only did these proteins have much amino acid sequence in common, but they had three-dimensional overlap as well! This overlapping structure holds for most hemoglobins and myoglobins whose 3-dimensional structure is referred to as the "globin fold."

Figure 3 shows a representation of the globin fold as exemplified by Sperm Whale myoglobin. The heme, where reversible oxygen binding occurs, is imbedded in a very hydrophobic heme pocket. It is the coordination of the iron by the 4 tetrapyrrole nitrogens and the proximal and distal histidines coupled with this hydrophobic environment that allows for reversible oxygen binding and release. The polypeptide fold also confers necessary functional properties to the active site of the protein, in this case the heme oxygen-binding site. In general, myoglobins are thought of, probably incorrectly, as oxygen storage molecules of mus-

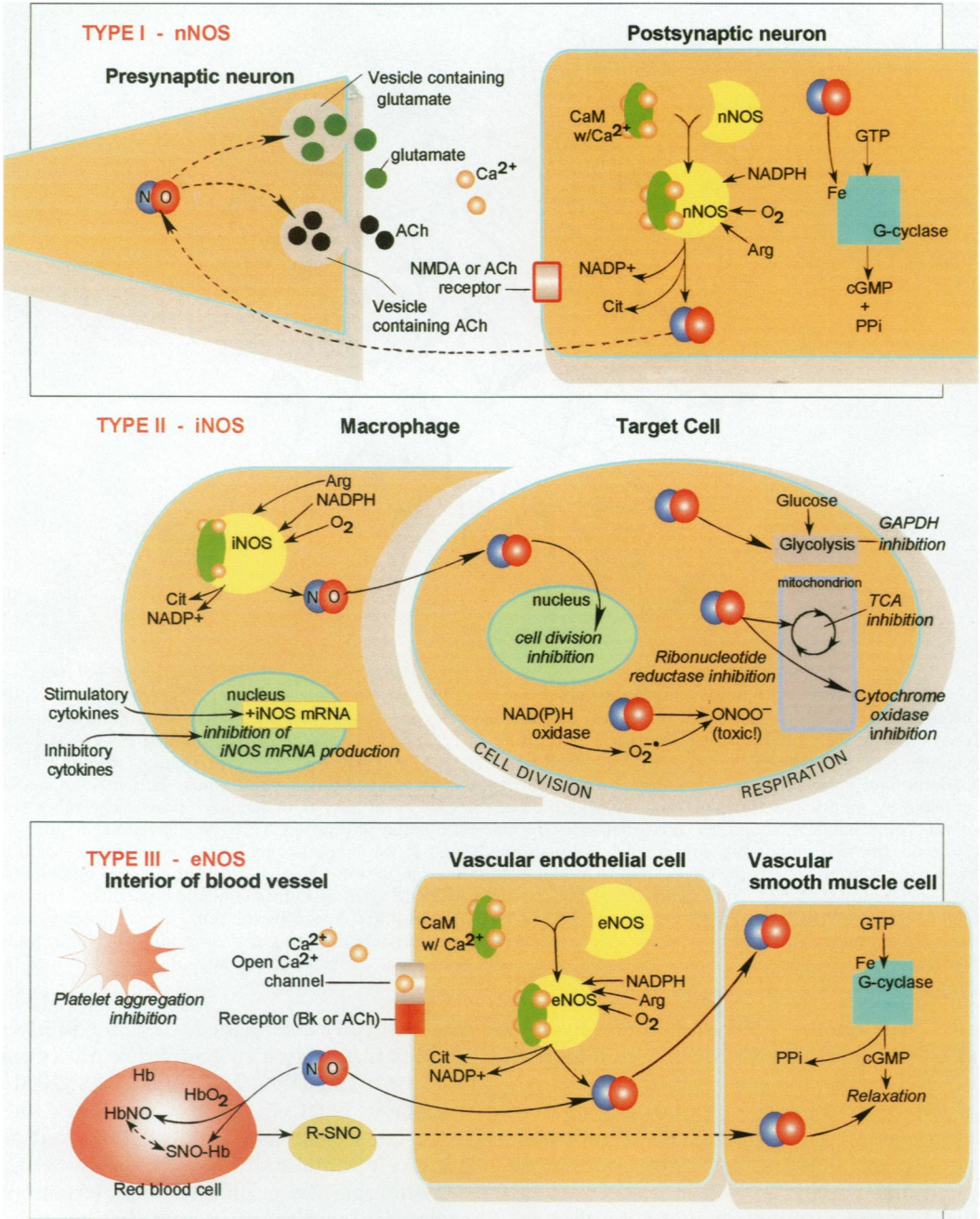


FIG. 2. Artistic representation of the three major isoforms of nitric oxide synthases (redrawn with permission after (Feldman *et al.*, 1993). The top, middle and lowest panels correspond to NOS Types I, II and III, representing neuronal, nNOS, inducible iNOS, and endothelial, eNOS forms. While the drawings may appear authoritative, many details remain unknown, unclear or merely inferred. Solid arrows imply a somewhat comprehensive understanding of a particular linkage while dashed arrows correspond to interactions that are less-well established or still controversial.

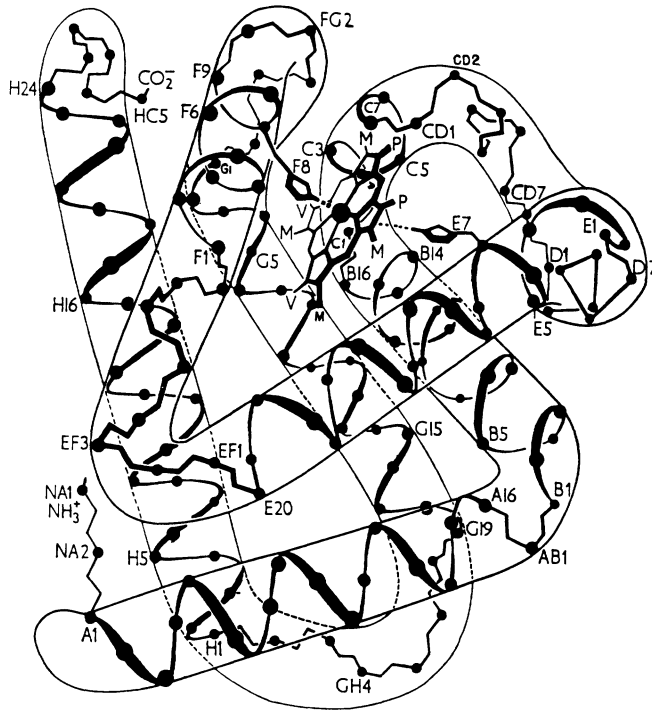


FIG. 3. The "Myoglobin Fold." All hemoglobins and myoglobins thus far studied consist of polypeptides having the general three-dimensional structure depicted here. Even myoglobins (either found in nature or produced recombinantly) which have a third less amino acids than the typical mammalian Mb (the minimyoglobins) have this overall structure. Note that the heme group is noncovalently inserted in the heme pocket and that residues on either side of the heme plane electrostatically ligate the iron. In this representation, residue E7 is a histidine and corresponds to the "distal histidine" that moves away when oxygen binds. This residue can be replaced by others, notably glutamine, in which case, the oxygen affinity is drastically altered. The other side of the heme plane is called the proximal side and the F8 residue is referred to as the "proximal histidine." Cysteine, the -SH containing amino acid, is sometimes found in the heme pocket. Proximal cysteines seem to be involved in NO interactions that are of a signal transduction nature while distal cysteines are involved in catalytic turnover of NO. Hemoglobins are comprised of multiple units of myoglobin folds associated noncovalently by hydrogen bonds, salt bridges and hydrophobic interactions. In red blood cells, hemoglobins usually occur as tetramers. Extracellular hemoglobins found in many invertebrates are giant assemblies of globin folds, forming molecules the size of ribosomes and containing nearly 200 oxygen binding sites.

cle. They bind oxygen in a simple fashion, *i.e.*, without cooperativity, and with high affinity. Usually the oxygen affinity of myoglobins lies in between that of hemoglobin, where oxygen is bound at the organism/environment interface, and mitochondrial cytochrome c oxidase, where oxygen is utilized in the terminal steps of aerobic respiration. The oxygen binding properties of myoglobins are not influenced to a large extent by effector molecules, although there has been some suggestion that they have pH-dependence (Giardina *et al.*, 1996). It is notable that some myoglobins have cysteine, although the functional significance of this reactive residue in myoglobins has not

yet been fully established. Cysteine in myoglobins may play a role in redox protection (Levine *et al.*, 1996; Marcinek *et al.*, 2001). Similarly, reactive cysteinyl residues may represent targets for functionally-linked nitric oxide binding.

Vertebrate hemoglobins are tetrameric molecules comprised of two distinct polypeptide chains. Adult human hemoglobin, for example, consists of two α -chains and two β -chains, each having the characteristic myoglobin fold (see Fig. 3). The tetramer is the functional unit of hemoglobin and its constituent chains are in communication with one another. This communication allows for physiologically relevant functional

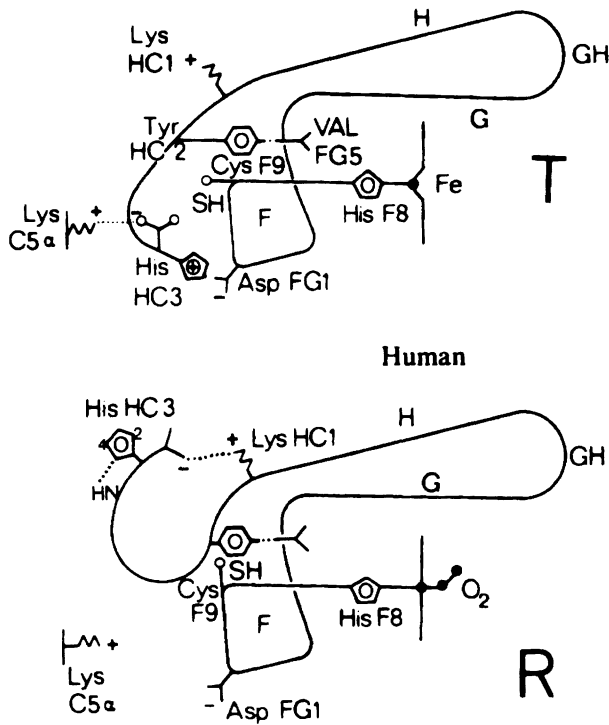


FIG. 4. Reproduced from the classic 1972 “stereochemical model” paper of Perutz (Perutz, 1972) proposing how hemoglobin changes conformation in carrying out its allosteric functions. The out-of-plane iron atom in deoxy hemoglobin is pulled into plane when oxygen binds. This movement appears to be the stereochemical trigger that leads to more global conformational changes, *i.e.*, the transformation from the T-state to the R-state of the hemoglobin molecule. These changes, when communicated among subunits of a tetramer, lead to the major homotropic allosteric effect in hemoglobin, cooperativity, or heme-heme interaction. Heterotropic allosteric effects occur when third-party effector groups, ranging from protons to polyphosphates, bind differentially to either the R- or T-state of the molecule. Thermodynamics demands that when binding between the two states is differential, the higher-affinity form is stabilized. Hence, if protons (remember that higher proton concentration is associated with higher pH) bind more strongly to the T-state, then lowering the pH will lower oxygen affinity. It is precisely this effect that was discovered by physiologists in the early 1900s and is now referred to as the Bohr Effect. See the review of the history of hemoglobin research by Edsall (Edsall, 1972). Note that while it was not known to play any particular role in function, the nearly invariant cysteine, F9, on the proximal side of the heme is depicted in two conformationally-dependent positions. This is particularly relevant to NO-hemoglobin interactions.

linkages to be established—linkages that facilitate oxygen loading and unloading. The concept of functional linkage, allostery, is basic to all biological systems and can be reviewed in the excellent book on the topic by Wyman and Gill (Wyman and Gill, 1990). The major allosteric properties of hemoglobins are depicted in Figure 4. How are these properties manifest? That is to say, what is the relationship between the position of specific amino acids in three-dimensional space to the reversible binding of oxygen and these linked functions?

After many, many years of intense work,

biochemists, specializing in the area of structure-function relationships, have elucidated the functional roles of all of the amino acids that have functional linkage in hemoglobins and myoglobins. The “Stereochemical Model for Hemoglobin Function” was published by Perutz in 1972 and it remains accurate to a great extent today (Perutz, 1972). Figure 5 is reproduced from the same Perutz paper and shows a cartoon of many essential features of his stereochemical description of allosteric effects in hemoglobin. While Perutz did not describe the residue in terms of any functional effects,

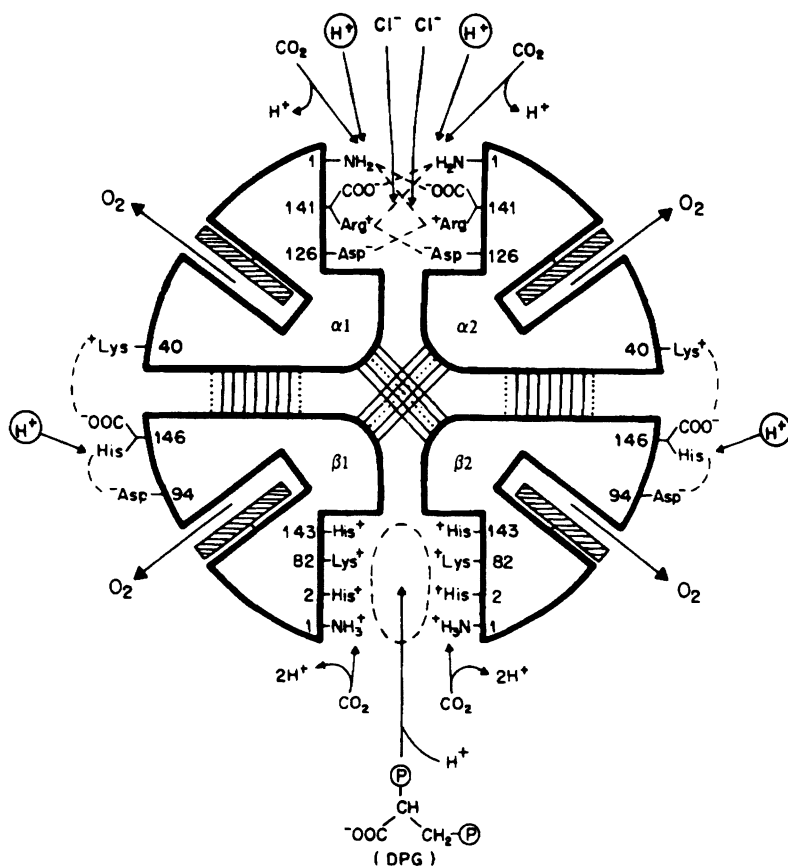


FIG. 5. Major linked-functions or allosteric interactions of tetrameric hemoglobin (Perutz, 1987). During arterial-venous transit, the blood cells are plastically deformed in the capillaries and come into intimate contact with the vessels, a process that is thought to facilitate mass transport into and out of the RBCs. Not shown are the transport processes that must occur between many of these effectors and the red blood cell as it courses through the body. Newly discovered interactions between NO species reacting with heme iron and thiol groups give a more complete picture of hemoglobin function *in vivo*.

note the -SH group, which is shown in both the T and R conformation states of hemoglobin. Based on the chemically reactive properties of cysteine, the nearly invariant (evolutionarily) β -93 was thoroughly investigated for participation in some, any, allosteric effect in hemoglobin. Following numerous experiments, it appeared that this reactive amino acid played no functional role. At least this was the case until we began to investigate the interactions between Hb and various redox states of nitric oxide.

In a 1996 *Nature* paper, a dynamic NO cycle that exists in mammalian blood circulation was described (Jia *et al.*, 1996). The *in vitro* study showed that the β -93 cysteinyl residue of hemoglobin (invariant

in all mammalian and most other known vertebrate hemoglobins) is S-nitrosylated in a conformationally-sensitive manner, forming S-nitrosohemoglobin (SNO-hemoglobin). Figure 6 shows the time course of SNO hemoglobin synthesis and the relative high reactivity of oxyhemoglobin compared with deoxyhemoglobin. The unexpected discovery of this cycle has stimulated much interest in the field as well as in the popular press (Blakeslee, 1996, 1997; Perutz, 1996). *In vivo*, this phenomenon is manifest with high levels of SNO-hemoglobin in the left heart (300–400 nM) and very low levels of SNO-hemoglobin in the right heart (10–30 nM). Interestingly, the concentration differences of nitrosyl-hemoglobin (HbNO) mir-

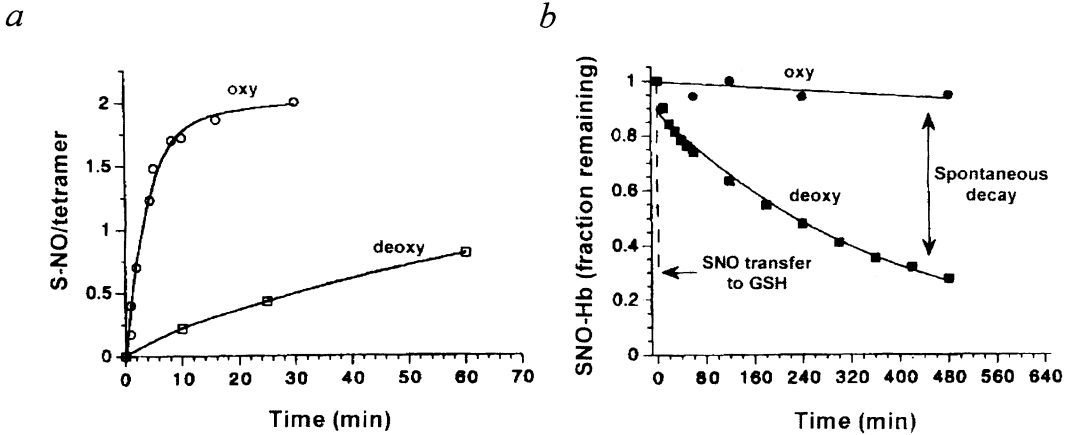


FIG. 6. Rates of SNO Hemoglobin formation and decay as a function of oxygen ligation at the heme (from *Nature* with permission; Jia *et al.*, 1996). **a)** S-nitrosylation of oxy (“fast” reacting) and deoxy (“slow” reacting) hemoglobin by transnitrosylation with S-nitrosocysteine, a molecule which behaves like Endothelium Derived Relaxing Factor. Two SNO/tetramer is the maximum that can be bound at the β -3 groups of hemoglobin. **b)** Decay of oxy- and deoxy-SNO hemoglobin. Note that the rates of decay are a mirror-image of the oxy formation rates. Addition of low molecular thiols greatly increases the intrinsic spontaneous rates. These thiols are believed to mediate transport of NO groups into and out of red blood cells.

rered those of SNO-Hb, *i.e.*, were low in arterial and high in venous blood instead of *vice versa*. A new picture of hemoglobin function began to appear—one in which ni-

tric oxide interacts with hemoglobin to effect the transport and delivery of oxygen to tissues (Fig. 7).

Although it was not discussed in the pa-

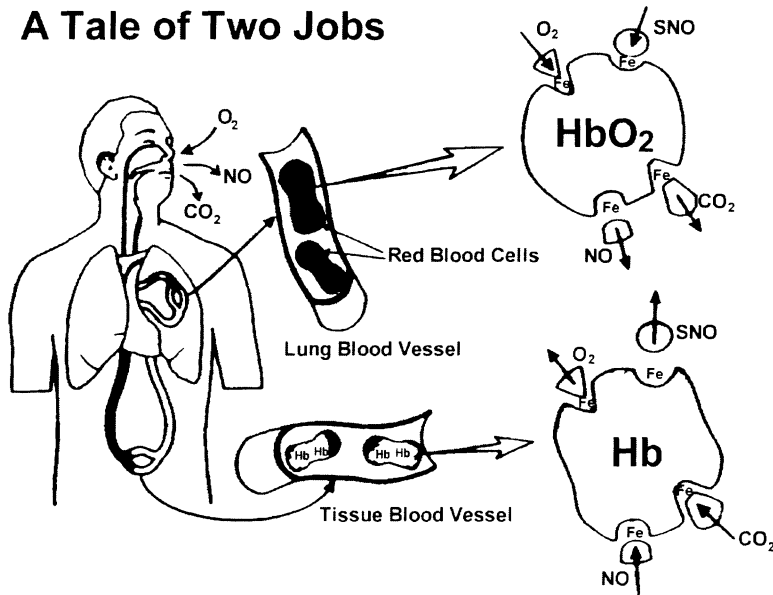


FIG. 7. The “new” picture of mammalian oxygen transport and metabolism. This cartoon was drawn by Duke University Medical Center artists to convey to news media the simplicity of what might otherwise seem a complex interaction between RBCs, oxygen, CO_2 and nitric oxide. The existence of this cycle was immediately apparent when concentrations of SNO hemoglobin were found to be strikingly different in the right (venous) and left (arterial) hearts (Jia *et al.*, 1996).

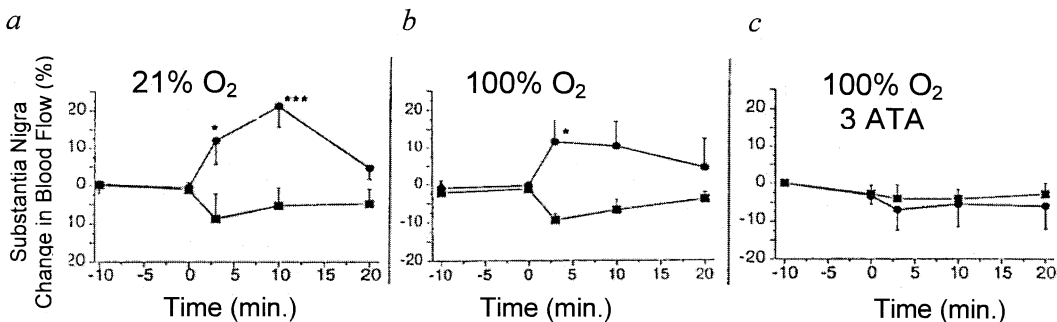


Fig. 8. The effect of oxygen tension on hemoglobin and SNO-mediated effects on cerebral blood flow in the Rat *Substantia nigra* (redrawn with permission from *Science*; Stamler *et al.*, 1997). **a**) In rats breathing 21% oxygen, hemoglobin alone has a slight effect in decreasing blood flow, while SNO hemoglobin increases blood flow substantially. **b**) In 100% oxygen, SNO hemoglobin has an effect that is attenuated relative to 21% oxygen condition. **c**) In 100% oxygen at 3 atmospheres hyperbaria, the effect of SNO hemoglobin is not distinguishable from that of unmodified hemoglobin. The physiologically important R to T transition that facilitates oxygen delivery to tissues and is associated with delivery of NO-related vasodilation is progressively lost as oxygen tension is decreased. At 3 atmospheres of oxygen, hemoglobin remains fully oxygenated in both arterial and venous parts of the circulation. Under these conditions, no differential delivery of NO activity by SNO hemoglobin is seen.

per, this was one of the first indications that the heme pocket and β -93 cysteine could communicate with one another via NO. In this cycle, hemoglobin is S-nitrosylated in the lung when red blood cells are oxygenated and the NO group is released during arterial-venous transit. *In vitro*, SNO-Hb is a vasodilator that is potentiated by low molecular weight thiols like glutathione. Both SNO-Hb and SNO-Hb-loaded red blood cells have a profound hypotensive effect *in vivo*. Within the red cell, the vasoactivity intrinsic to SNO-Hb is transduced by erythrocytic transport of low molecular weight S-nitrosothiols. The allosteric effects of oxygen on SNO-Hb are mirrored by electronic effects. Oxidation of SNO-oxyHb to SNO-metHb markedly increases its vasoactivity. Hence, SNO-Hb appears to participate in the control of blood pressure and efficient delivery of oxygen to tissues. In *Science* the following year, we showed that the physiological oxygen gradient was essential for the binding and delivery of vasoactive nitric oxide groups (Stamler *et al.*, 1997). *In vitro*, the vasoactivity of SNO-Hb is dependent on oxygen concentration in that the allosteric transition from oxy (R-state) to deoxy (T-state) increases NO-related vasoactivity. *In vivo*, rats were exposed to a wide range of oxygen concentrations and the effect of SNO-Hb on cerebral blood flow was

monitored. SNO-Hb and HbNO levels were not different in rats under hyperbaric conditions, where arterial and venous hemoglobin do not undergo oxy-deoxy transitions (Fig. 8). Thus, it is reasonable to assume that intraerythrocytic hemoglobin senses the physiological gradient and exploits the conformationally-dependent differences in β 93 SNO to bring local blood flow in line with oxygen requirements. In further support of this hypothesis, we proposed 3-dimensional structures for deoxy (T-State) and oxy (R-State) SNO-Hb that provide a plausible explanation for the conformational-dependent nature of the activity of SNO-Hb.

Subsequently, the group of Arthur Arnone published a structure derived from X-ray crystallography of liganded (R-State) SNO-hemoglobin (Chan *et al.*, 1998) confirming the one proposed in the Stamler *et al. Science* paper. While the work of Chan *et al.* focussed on crystallography, these experiments shed light on important chemical matters that relate to NO movement within the hemoglobin tetramer. The crystals of carbonmonoxy HbA were exposed anaerobically to NO. The resultant crystals became SNO-nitrosyl-HbA. The NO must have first replaced the CO bound at the heme and then reacted with the β -93 cysteine (potentially by intramolecular trans-

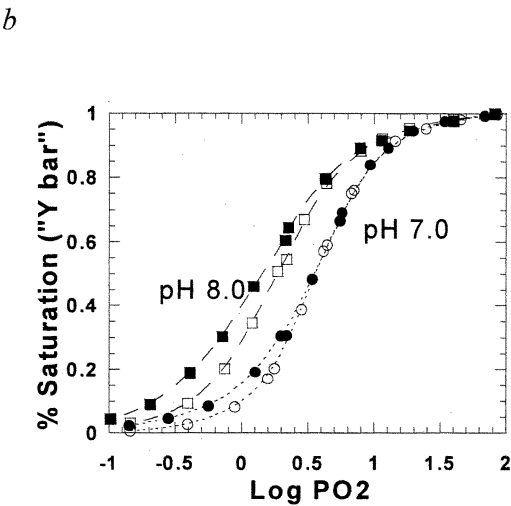
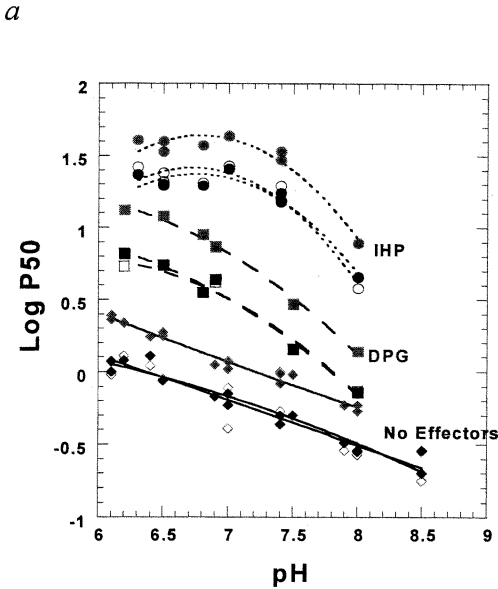


FIG. 9. a) The effect of S-nitrosylation on the pH-dependence of oxygen binding by normal and sickle cell hemoglobin. 15 μ M hemoglobins were in 0.05M HEPES buffers at 20°. Inositol hexaphosphoric acid (IHP) (grey circles, HbA; open circles, SNO-HbA; black circles, SNO-Hb Sickle Cell) and diphosphoglyceric acid (DPG) (grey squares, HbA; open squares, SNO-HbA; black squares, SNO-Hb Sickle Cell) allosteric effectors were 10 fold over tetramer concentration, hence, 150 μ M. (Experiments using no effectors: grey diamonds, HbA; open diamonds, SNO-HbA; black diamonds, SNO-Hb Sickle Cell.) SNO-Hb shows a higher oxygen affinity than Hb. The oxygen-linked groups responsible for the Bohr Effect are not altered by S-nitrosylation. b) Oxygen equilibrium curves for

fer). Later, it was shown that human hemoglobin's interaction with NO can be described in terms of a dynamic interplay between binding at the hemes and at the β -93 cysteine (Gow and Stamler, 1998) when NO and oxygen are presented to the hemoglobin molecule at concentrations that are physiologically relevant.

The discovery of SNO-Hb has clearly stimulated the field of hemoglobin research. The SNO-hemoglobin cycle has been shown to occur in fetal circulation (Funai *et al.*, 1997). SNO-hemoglobin has also been shown to be stable enough to survive the rigors of electrospray mass spectroscopy (Ferranti *et al.*, 1997) and unpublished observations (JB). Platelet aggregation is inhibited both by cell-free and erythrocytic SNO-Hb (Pawloski *et al.*, 1998). The field is not, however, without controversy. Yonetani *et al.* (Yonetani *et al.*, 1998) have studied hemoglobin-NO interactions where there is no observed NO binding at the β -93 cysteine and have proposed that NO bound to alpha subunits acts as a negative allosteric effector affecting oxygen and carbon dioxide transport. Recently, the oxygen equilibrium of SNO-hemoglobin A was published (Patel *et al.*, 1999). They show that the oxygen affinity of SNO-Hb is higher than Hb and, based on simulations, conclude that the kinetics of SNO formation and decay are too slow to account for the mechanism we have proposed. A number of publications have now appeared on the oxygen-binding properties of SNO hemoglobin (Bonaventura *et al.*, 1999; McMahon *et al.*, 2000; Patel *et al.*, 1999). In Beaufort, we have also studied these equilibria with Sickle Cell and normal human hemoglobin in the presence and absence of anionic effectors. Our data (Fig. 9) show increased

←

Spot hemoglobin (open symbols) and SNO-Spot Hb (closed symbols) at pH 7 (circles) and pH 8 (squares). S-nitrosylation shifts the lower part of the oxygen binding curves toward higher oxygen affinity while having little or no effect on the higher levels. This effect is just like that observed with HbA suggesting S-nitrosylation stabilizes the oxygenated derivative of SNO hemoglobins rather than destabilizing the deoxy form of the protein.

oxygen affinity for SNO-A and SNO-S hemoglobins arising from a destabilization of the T-state of the molecule by the introduction of the NO group at β -93. The R-state is little-altered by the presence of SNO. The X-ray data (Chan *et al.*, 1998) as well as molecular modeling experiments (Stamler *et al.*, 1997) support this interpretation. These studies show that pharmacologically-relevant amounts of SNO HbA are synthesized by either transnitrosylation or NO-transfer between heme and β -93 sulfur in times that are sufficiently short that the role of the nitrosylated species in vasoactivity is clear.

So how universal is the SNO hemoglobin effect? To reiterate, the β -93 position is a highly conserved one and is cysteine in all mammals, reptiles and birds, as well as many other vertebrates. Our unpublished experiments suggest that the NO-reactivity of this residue in several of these hemoglobins show the same sort of conformational sensitivity in reaction as does human hemoglobin.

What about hemoglobins without β -93 cysteine? Do they break the paradigm of the SNO-hemoglobin respiratory cycle? The Spot fish, *Leiostomus xanthurus*, has a Root Effect hemoglobin where strategically positioned charged residues make the oxygen affinity supersensitive to pH facilitating oxygen secretion into the swimbladder and to the retina (Bonaventura *et al.*, 1976). The X-ray structure of its carbon monoxide-ligated derivative has been determined and the molecular mechanism for the Root Effect has been proposed (Mylvaganam *et al.*, 1996). Spot hemoglobin has a single cysteine at position 133 of the alpha chain, but no cysteines in the β -chain (MS in preparation). The discovery of the identity of this residue came from nitrosocysteine transnitrosylation experiments we did with this hemoglobin (unpublished observations). Electrospray ionization mass spectroscopy of S-nitrosylated Spot Hb gave the expected 29 mass unit heavier α -chain mass. Our studies with the Spot hemoglobin also showed that the rates of nitrosylation were dependent on the state of hemoglobin oxygen ligation. OxyHb reacted with SNO-Cys more rapidly than deoxy. In striking contrast with human

hemoglobin, however, we observed that, particularly in deoxy samples of Spot hemoglobin, S-nitrosylation was associated with the appearance of nitrosyl hemoglobin (NO bound to the heme) suggesting an interaction as well as communication between the heme pocket and α 133 cysteine. UV/Vis spectroscopy and SNO quantification are also consistent with oxygen-dependent NO group transfer in this hemoglobin. In a one-off experiment with freshly caught fish, we sampled *in vivo* oxygenated and deoxygenated blood for SNO- and nitrosyl-hemoglobin content. We obtained results that were suggestive of the differences reported for mammals in *Nature* (Jia *et al.*, 1996). Initial oxygen-binding experiments with SNO-Spot hemoglobin show an effect similar to that seen with human hemoglobin. A destabilization of the T-state shifts the lower part of the oxygen-binding curve to the left while there is little or no effect on the R-state affinity. Therefore, the SNO-Spot hemoglobin has a higher oxygen affinity than its non-nitrosylated form.

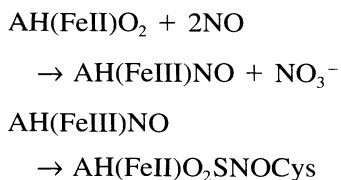
We can conclude that the conformationally sensitive cysteine in Hb need not be in the β -93 position to react with NO. We have speculated that if the β -93 cysteine is not conserved in a particular organism, selective pressure might lead to the introduction of one elsewhere.

However, our ongoing testing of this hypothesis has revealed a few fish and some invertebrate Hbs that are resistant to SNO formation (unpublished data, JB and VPL). Some of these organisms have multiple hemoglobins. They may have no cysteines what-so-ever, cysteines rendered non-reactive by their position, or they may have such a low concentration that S-nitrosylation is undetectable with the methods used. We need to investigate the SNO phenomenon with more sensitive assays for *in-vitro* experiments as well as explore *in-vivo* relevance and amino acid sequences. The SNO-cycle we've so clearly and routinely demonstrated in the laboratory may still be found to be physiologically necessary.

Ascaris lumbricoides is a parasitic nematode that currently infects at least a billion human beings and countless other mammals. Decades ago, physiologists were puz-

zled by the existence of a hemoglobin of remarkably high oxygen affinity in its perienteric fluid. The oxygen affinity, deduced to be a thousandth of a millimeter of mercury, was so high that it was difficult to imagine it functioning as either a transport or a storage protein. The enigma of this hemoglobin's function has recently been solved. Oxygen is a toxic molecule for this worm whose mitochondria are modified for anaerobic metabolism (Blaxter, 1993). O₂ is consumed in an NADPH-dependent deoxygenase activity utilizing nitric oxide which is present in the host's intestine (Imai, 1999; Minning *et al.*, 1999)!

Ascaris hemoglobin, unlike tetrameric vertebrate hemoglobins, occurs free in solution and is a multiglobin domain molecule having eight 175 kDa subunits. While the globin domains of *Ascaris* hemoglobin (AH) possess the typical globin-fold, key residues in the heme pocket are present which allow for catalytic activity to occur. First, the distal histidine characteristic of many hemoglobins, is replaced by a glutamic acid. This change dramatically increases the oxygen affinity of the heme iron. Secondly, a cysteinyl residue is positioned in the distal pocket, close to the heme. It is clear from the X-ray structure (Yang *et al.*, 1995) that both oxygen and nitric oxide can come into the distal side of the heme and interact with both the heme iron and the thiol of cysteine E15. The oxygen consumption is believed to occur in a multi-step process in which electrons are provided by the cofactor NADPH. The major steps thought to be involved are shown below.



(NO⁺ leaves the heme to E15 Cys leaving FeII which binds another O₂)

From nematodes we go to *E. coli* and *Salmonella* where the molecular function of these bacteria's inducible flavohemoglobins have been found to be NO and SNO me-

tabolizers in response to nitrosative stress. The *E. coli* flavohemoglobins are functioning as dioxygenases, while the *Salmonella* HMP functions independently from oxygen (Crawford and Goldberg, 1998; Hausladen *et al.*, 1998).

In light of the invertebrate and bacterial hemoglobin discoveries, we may say "Hemoglobin **also** binds oxygen!" Hemoglobins and myoglobins in many modern organisms clearly function in oxygen transport and storage. We do not, of course, fully know the evolutionary details of primitive globins. There is evidence these molecules arose early in the development of life on this planet. We hypothesize that one primary functional benefit conferred in their evolution was not reversible oxygen binding, but rather oxygen detoxification. The signal molecule nitric oxide served to respond to nitrosative stress that came about as emerging plants began to alter the atmosphere, "polluting" it with O₂. Hence, before these proteins were functioning to transport oxygen, they were functioning to protect from the devastating effects of oxygen combined with NO, H₂S and other molecules that were not toxic until O₂ arrived on the scene.

CONCLUSION

The interaction of nitric oxide with biological systems has been pervasive throughout the evolutionary history of life on this planet. It is the redox properties of NO that poise it for polyvalent function. The uncharged free radical, the nitroxide anion, and the nitrosonium cation all participate in signaling and control. We speculate that NO is not the only signal of this nature in biological systems. This type of signaling may have also evolved with other simple atoms, radicals and molecules during the evolution of life. Other simple signal systems may be there, just "before our eyes." To see them, though, we must view conventional wisdom critically. It is up to us to maintain open minds and to let creativity guide our work.

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