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Invited Review

The phylogenetic odyssey of the erythrocyte. I Hemoglobin: the universal respiratory pigment

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Summary. Hemoglobin is a molecular entity that is capable of reversibly binding and releasing oxygen in either extra- or intracellular milieus. It is present in scattered invertebrates in physical solution or in cellular sites while in vertebrates it is universally located in circulating erythrocytes. These cells serve as the vehicle for and otherwise foster the optimum utilization hemoglobin. Hemoglobin's variable sphere of respiratory activities can be viewed as reflecting the specific requirements for each organism in which it is observed. Once these concepts have been established and the advantages and limitations of its cytologic packaging recognized, the study of the erythrocyte as expressed in its dimensions, colligative aspects, geometry, internal morphology and pathologic variations can be approached in a purposeful manner.

Key words: Hemoglobin, Erythrocyte, Comparative hematology, Respiratory pigments

Introduction

The erythrocyte, by far the most numerous cell in the circulating blood, is the major carrier of oxygen in man and all other vertebrates. This cell is uniquely adapted for this function by its high content of an iron-bearing, oxygen binding protein, hemoglobin. An understanding of the red cell's functional and pathologic activities in man (as well as in other vertebrates) can be approached by first assessing the phylogenetic expression and variation in activity of its respiratory pigment. This establishes a basis for an enhanced, continued study and deeper understanding of this cell. Hemoglobin is central to the biology of the erythrocyte.

Discussion

The hemoglobin molecule is truly ancient and has been extant for millenia. It has a long evolutionary history and in fact can be viewed as a molecular clock. It has been used to date the separation of the vertebrates and invertebrates some 1000 million years ago. The emergence of a vertebrate hemoglobin molecule with differing alpha and beta globin chains occurred 500-600 million years later. Hemoglobin has persisted since those primordial events and has become established as the single, dominant, essentially universal respiratory pigment of the vertebrates. By definition respiratory pigments, of which hemoglobin is the prime representative, are inherently pigmented metallo-proteins having the unique ability of reversible binding with oxygen. Although other phylogenetically transitory respiratory molecules have appeared in the invertebrates (e.g. hemocyanin, chlorocruorin) none have approached the importance of hemoglobin. It has become increasingly apparent that hemoglobin enjoys a remarkably broad biological distribution. It is identifiable in some bacteria, occasional molds, some single celled animals (e.g. the ciliated protozoans *Paramecium caudatum* and *Tetrahymena pyriformis*), scattered invertebrates, essentially all vertebrates and most surprisingly in the plant kingdom. This molecule has been identified in the nitrogen fixing nodules on the roots of some legumunous plants as soy bean plants and *alfalfa* as well as in the non-nodulating plant *Trema tomentosa.* Indeed, recent isolation of the hemoglobin gene from plant root cells has suggested that hemoglobin genes, inherited from an ancestor common to plants and animals, might be present in all plants. All aerobic organisms are capable of synthesizing catalysts such as the heme-containing cytochromes. Consequently they can be viewed as potential carriers of the heme moiety of hemoglobin. The limiting factor in the appearance of hemoglobin apparently lies in the evolved ability to synthesize the highly specific proteins (globins) which when linked with

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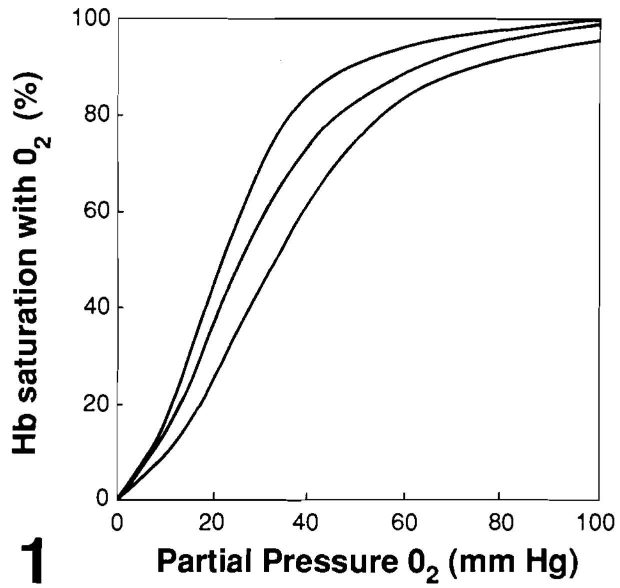

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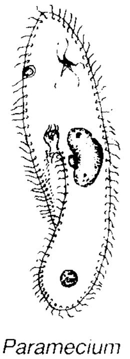
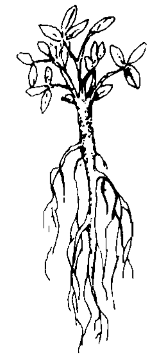
Fig. 1. Typical sigmoid hemoglobin oxygen dissociation curve (human hemoglobin). The Bohr effect is also illustrated. Note the progressive shift of the dissociation curve to the right with a decrease in pH. After VanSlyke and Peters.

heme establish the constraints of its remarkable property of reversible binding and release of oxygen.

The simplest molecular configuration of hemoglobin consists of one heme prosthetic group (a porphyrin ring complexed with a single atom of iron) attached to one globin chain approximately 150 amino acids in length. The molecular weight is approximately 17,000 daltons. Hemoglobins of this order can be found in the free coelomocytes of some annelids (multisegmented worms), the larva of the insect *Chironomous* and in some sea cucumbers. The molecular weights of the primitive (invertebrate) hemoglobins that exist free in solution conversely, tend to be markedly greater and frequently approach three million daltons as a result of a high degree of polymerization. Their large molecular dimensions serve to prevent them from diffusing out of the plasma into the tissues. Most intracellular invertebrate hemoglobins are monomers or dimers of the basic molecular model although tetramers and larger forms can be represented. The characteristic tetrameric molecule of vertebrates (including man) most likely arose by the joining of two dimers. The vertebrates, with the exception of the jawless fish (Agnatha, cyclostomes), all have a more complex, tetrameric hemoglobin containing two pairs of dissimilar (alpha and beta) globin chains; it has an average molecular weight of 68,000. It has been concluded that the original primitive vertebrate hemoglobin consisted of four heme units and four identical polypeptide chains; it evolved by gene duplication into a tetramer composed of two alpha and beta protein units. This developmental process is believed to have occurred after the evolution of the jawless fish (e.g. lamprey and hagfish) which do not demonstrate heterogeneous hemoglobins composed of pairs of differing globin chains but before the cartilaginous fish (sharks) and all other vertebrates which do manifest this progression.

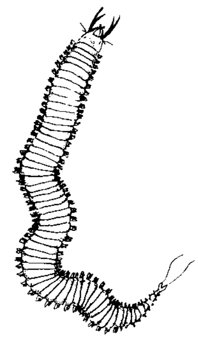


Paramecium caudatum, a protozoan

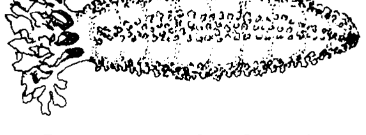


Alfalfa, a legumunous plant

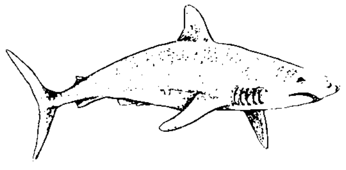
The evolutionary ascendance to polymeric hemoglobins can be appreciated when viewed in light of the benefits that are obtainable by polymeric as opposed to monomeric forms. The oxygenation of one heme in an oligomeric hemoglobin molecule, for example, subsequently permits an adjacent fellow heme to bind oxygen with greater facility. This alteration in binding affinity, termed heme-heme interaction or cooperativity, is due to small changes in the three dimensional structure of the molecule. An extension of this enhancement is the observation that all hemoglobin molecules in a solution or within an erythrocyte become equally oxygenated when exposed to a given volume of oxygen; e.g. if a solution of human (tetrameric) hemoglobin is 50% saturated with oxygen all of the molecules will have two oxygenated ferrous atoms. This characteristic also leads to the establishment of the well recognized sigmoid hemoglobin oxygen dissociation curve which is ideally suited for taking up and release of oxygen under different conditions (Fig. 1). Monomeric hemoglobins, in contradistinction, usually have hyperbolic dissociation curves. The heme moiety of the molecule can be considered evolutionarily stable and responsible for the reversible binding of oxygen while the globin is structurally variable and through this variation determines the conditions under which oxygen binding and release will be favored (CO_2, temperature, oxygen tension, organic phosphate and inorganic ion levels etc.). Hemoglobin molecules can therefore be viewed as «tailor made» for specific species and temporal requisites. Man himself demonstrates at least four different hemoglobins during his existence, the Gower I and II hemoglobins of early embryonic life, fetal and adult hemoglobins. The chicken develops multiple hemoglobins



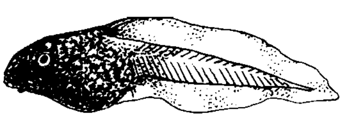
Representative polychaete annelid (multisegmented bristly, marine worm)



Representative Sea Cucumber, a holothurian during its lifetime (hemoglobins P, E, D, A and H) while fetal and adult hemoglobins are commonplace among mammalians. The maturation of a tadpole to a frog is similarly associated with a molecular change of its hemoglobin. Some fish concomitantly manifest two types of hemoglobin, one highly sensitive to acid pH and one that is acid-insensitive. Certain representatives of the annelids and molluscs (bivalves or clams) that have erythrocytes demonstrate the simultaneous existence of mono- and polymeric intracellular hemoglobins. The arcid clam *Noetia ponderosa* (a colloquial «blood clam») possesses erythrocytes with two dimeric hemoglobins with own oxygen-binding properties. It can be assumed that each molecular version in an organism reflects certain characteristics and biochemical biases (not always apparent) that are desired at a given period of life or under certain activity. The oxygen dissociation curves of the hemoglobin in various animals sometimes reveal these preferred features. For example, the llama which inhabits the terrain of the Andes mountains has a dissociation curve that reveals a marked avidity (loading capacity) for oxygen at the lowest of oxygen concentrations. This is of obvious benefit to an animal that must capture its required oxygen in a rarefied atmosphere. Another useful feature of most hemoglobins is that at lower pH levels their dissociation curve shifts to the right thereby facilitating the release of oxygen. This favorable characteristic is manifested in venous blood which has increased acidity due to its higher levels of carbon dioxide. This phenomenon is known as the Bohr effect (Fig. 1). The hemoglobins of small mammals demonstrate a greater Bohr shift than those of the larger species, i.e. the incremental volume of oxygen released per unit increase in acidity will be proportionately greater in smaller versus larger animals. The Bohr effect is consequently progressively greater in elephants, cattle, hogs, man, rabbits and mice. This is seen as a reflection of and in physiologic concert with the metabolic rate which increases as the size of an animal diminishes. The result thereby achieved is an improved availability of oxygen for subjects with the greatest need. The Bohr effect is not manifested by monomeric hemeproteins. It is typically absent or not prominent in annelid erythrocytic or free hemoglobin and is likewise not expressed in some submammalian vertebrates as frog tadpoles and certain salamanders, e.g. *Cryptobranchus alleganiensis*, the hellbender.

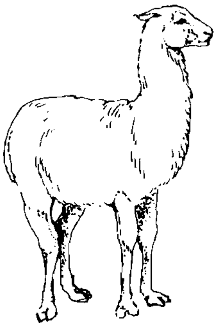


Mako or Sharp Nose Makerel Shark, *Isurus oxyrinchus*



Tadpole of Common Frog, *Rana temporaria*

The benefits and/or disadvantages of a given molecular configuration of hemoglobin in an animal are interpreted as the net result of its two major functions, the loading and unloading of oxygen. The effects of ambient organismal conditions upon hemoglobin at the oxygen-rich side of the cycle (interface with air or water at the gills, lungs, etc.) and the physiologic circumstances at the oxygen-poor (tissue) side have to be both taken into balanced consideration. Thus, fish with both acid sensitive and acid independent hemoglobins (e.g. rainbow trout, *Salmo irideus*,) are said to benefit from their bimolecular profile during extreme swimming effort at which time significant amounts of lactic acid are likely to be accumulated. The resultant drop in blood pH would prevent an acid sensitive (Bohr effect) hemoglobin from combining with any oxygen and lead to asphyxia. The presence of an acid independent hemoglobin however, insures that some hemoglobin can bind with oxygen even during maximal activity.

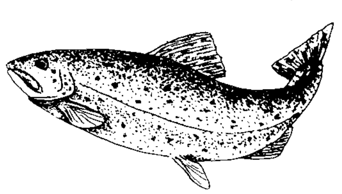


Llama, *Llama glama*

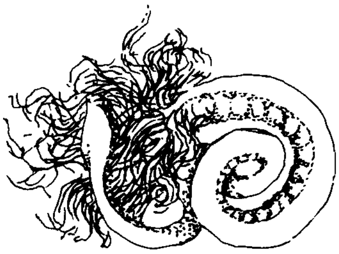
There is no smooth evolutionary expression of hemoglobin as it progresses from the free, extracellular to the cellular milieu or from invertebrate to vertebrate species. The blood or hemolymph of invertebrates is characterized by respiratory pigments (frequently other than hemoglobin) existing free in the plasma in contrast to the vertebrates which have specialized, circulating, hemoglobin-containing cells (erythrocytes) in their vasculature. It is possible and sometimes conceptually convenient, however, to identify lower forms, so called «species of hemoglobin transition». In such cases this molecule is observed in physical solution, stored in fixed, non-motile cells or exists simultaneously both in cells and in the free state. Such models often yield unanticipated insight into the modes of harnessing hemoglobin's respiratory capability. In addition, because of the absence of a predictable, stepwise pattern in the phylogenetic expression of hemoglobin and its cytophores it is possible to identify «early or delayed» appearances of circulating erythrocytes on either side of the boundary between the vertebrates and invertebrates.

Mouse, *Mus musculus*

Numerous polychaete annelids (multisegmented, bristly marine worms) such as the bloodworm *Glycera dibranchiata* have hemoglobin in their free coelomic cells. It is usually of modest molecular weight and may be mono-, di- or polymeric. This evolutionary cellular

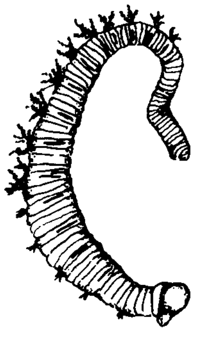


Rainbow Trout, *Salmo irrideus*



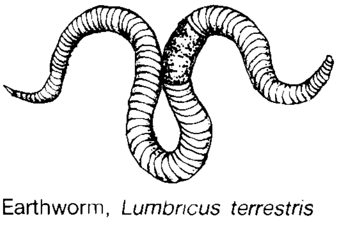
Thelepus crispus, an annelidan worm

advance approaches the limits seen in invertebrates. For this reason two genera in the annelid phylum, *Thalassema* and *Magelona*, both of which demonstrate free cells containing a respiratory pigment, are sometimes cited as filling the evolutionary gap between dissolved, free and cellular packaged respiratory pigments. The organism *Magelona papillicornis* is additionally unique in that it has true erythroplastids (denucleated erythrocytes), its pigment however is hemerythrin which does contain iron but not a porphyrin ring. *Telepus crispus* and *Pista pacifica*, also marine annelids, simultaneously demonstrate both extracellular vascular and intracellular coelomocytic hemoglobins. The lugworm (*Arenicola marina*) offers a demonstration of an invertebrate's modified use of its plasma-dissolved hemoglobin. This intertidal polychaete possesses hemoglobin sufficient to sustain its respiratory needs for perhaps several minutes. Although the organismal mechanism is not clear, it is proposed that the hemoglobin offers the worm some oxygen support perhaps during ventilatory pauses or perhaps as it switches to anaerobic metabolism during the intertidal period. It is at this time, in between the oceanic tides, that the sea worm's mud burrow is deprived of its usual supply of fresh oxygen-laden sea water and consequently the worm is obliged to make respiratory adjustments. A land-dwelling, minimally bristled (oligochaete) annelid representing species with free, mega-scale hemoglobins is the common earthworm (*Lumbricus terrestris*). Its hemoglobin polymer is comprised of about 144 hemes and has a molecular weight of three million.

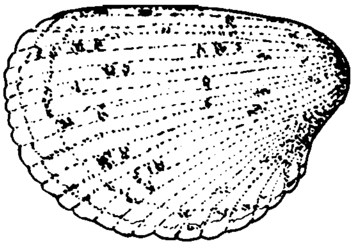


Lugworm, *Arenicola marina*

The members of the phylum Mollusca seldom have erythrocytic or free hemolymphic hemoglobins. Nevertheless, a few representatives do manifest this respiratory feature. The major members of this taxonomic group include the gastropods (snails), bivalves (oysters, cockles, and clams) and cephalopods (squids and octopi). The erythrocellular hemoglobins when present are found exclusively in the bivalves while extracellular hemoglobins are observed in bivalves and gastropods. Consistent with the concept that the distribution of hemoglobin is scattered and not predictably represented in the invertebrates, the cephalopods considered to be the most advanced molluscs as well as the zenith of the evolution of invertebrates nevertheless fail to demonstrate erythrocytic and extracellular hemoglobins. The molluscan erythrocytic hemoglobins not surprisingly are typically small (mono- to oligomeric), as seen in the cockle *Anadara ovalis* (the blood ark). Conversely, the *Planorbis corneus* (a European fresh water snail, the great ramshorn) exhibits an intravascular hemoglobin with a molecular weight in excess of one million daltons. Perhaps the most prodigious invertebrate or vertebrate hemoglobin is that observed in the clam *Cardita borealis*. Its molecular weight is in the order of twelve million, the molecule is composed of forty subunits each containing thirteen hemes.

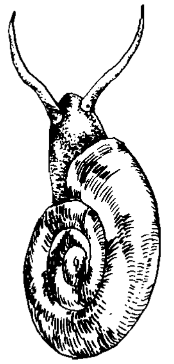


Earthworm, *Lumbricus terrestris*

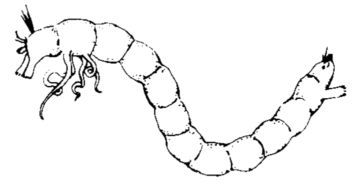


«Blood Ark», *Anadara ovalis*

Of the approximate one million recognized species of insects, the vast majority show no trace of hemoglobin or other respiratory pigments; their tissues become oxygenated via the spiracles and tracheae which conduct external air into the interior of the organism. However, some exceptions do exist such as the larval Chrironomidae a family of two winged mosquito-like insects, midges) which demonstrate hemoglobin in their blood, most of which is degraded by adulthood. They are often called bloodworms due to their hemoglobin-derived red color. Certain fat body cells in a few species of insects also contain hemoglobin (e.g. the hemipterans or true bugs *Anisops* and *Buenoa*). Two long ventral bands of large hemoglobin-containing cells are seen along each side of their abdomen. Since they are situated near the spiracles and receive numerous tracheae their location has apparent functional implications. Members of genus *Buenoa*, the «backswimmers», exhibit a dark red abdomen (unusual for insects) because of these cells. This aquatic insect seldom comes to the surface of the water and just swims submerged. The fat body cells are thought to function as a store of oxygen as opposed to circulating free or erythrocellular hemoglobin subserving continuous oxygen transport. The bot fly larva *(Gastrophilus intestinalis)* also stores hemoglobin in specialized fat cells. Hemoglobin is found in solution in the blood plasma of

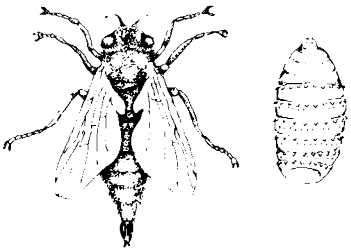


Planorbis corneus, a fresh water snail

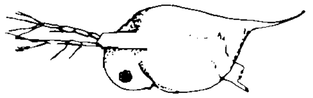


Chironomous tetans larva, a «bloodworm» numerous entomostracan crustaceans (a class of simple, small, aquatic arthropods with a chitinous exoskeleton, e.g. *Daphia magna*, a water flea and *Chirocephalus*. An interesting respiratory phenomenon observed in the former species is the stimulation of the synthesis of hemoglobin in the absence of oxygen.

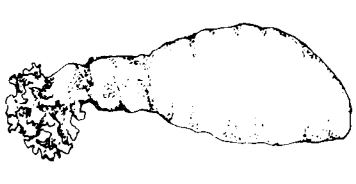
A small number of echinoderms (marine invertebrates with a «spiny skin», e.g. sea urchins, starfish) utilize hemoglobin, a fact recognized by the early hematologists such as Jolly and Foettinger. The brick-red colored sea cucumber *Cucumaria miniata* has a modest number of hemoglobiniferous cells in its coelomic fluid. Other members of the class Holothuroidea (sea cucumbers) with erythrocytes are *Molpadia arenicola*, *Caudina retetzii* and *Thyone*. These species are mud dwellers and hence likely to experience oxygen-poor conditions. Their hemoglobin serving as an oxygen store has obvious benefits. Finally, the brittle star *(Phiactis virens)* is an example of an echinoderm that has nucleated hemoglobinized cells in a water vascular system. There are, needless to say, many other species of invertebrates that synthesize and employ hemoglobin as a respiratory pigment. Those discussed here are presented as illustrations of hemoglobin's broad distribution and adaptation to various organismal requirements.



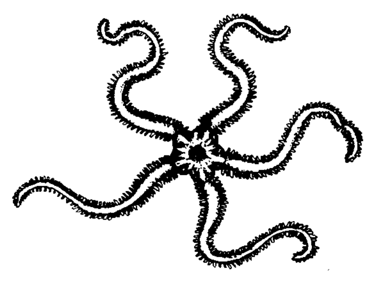
Bot Fly and larva (bot or maggot), *Gastrophilus intestinalis*



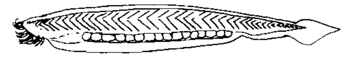
Water Flea, *Daphnia* species



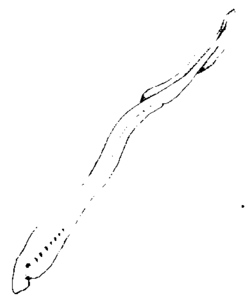
Cucumaria miniata, a Sea Cucumber



Representative Brittle Star, an echinoderm



Amphioxus, a lancelet, *Branchiostoma lanceolatum*



Lamprey, *Petromyzon marinus*

The vertebrates, with rare exceptions, are characterized by a universal possession of specialized, hemoglobin-bearing erythrocytes. These cells are independent, mobile, deformable and passive-flowing. In all but the mammals they can be expected to be oval in configuration, biconvex, and permanently nucleated. The amphioxus, *(Branchiostoma lanceolatum)*, a primitive, semitransparent fish-like animal that is recognized as an archetypal chordate (a cephalochordate) i.e. a species more taxonomically advanced than the invertebrates but still not attaining the developmental stature of a vertebrate, has blood devoid of erythrocytes, hemoglobin and other respiratory pigments. Other primitive chordates (e.g. some urochordates) have alternative non-hemoglobinous pigments in their circulating cells. The jawless fish (lamprey, *Petromyzon marinus* and hagfish, *Myxine glutinosa* are the most primitive vertebrates to demonstrate red cells. These recognizably, poorly developed agnathic species also possess the most primitive hemoglobins observed in vertebrates. They reveal their incomplete evolution by a retention of monomeric hemoglobins. On the other hand the physiologic properties of their hemoglobins (especially the lamprey) do show some degree of molecular cooperativity; the lamprey hemoglobin also has a Bohr effect while the hagfish does not. In some remarkable circumstances it is possible to identify a well developed vertebrate that has evolved without the development of erythrocytes or dissolved pigment in its plasma. This well-documented status is observed in certain antarctic ice fish («bloodless fish»). Such fish (e.g. *Chaenocephalus aceratus*) inhabit the Antarctic ocean, are described as sluggish, attain reasonably large dimensions (60 cm length and 1kg weight), and have colorless blood. All oxygen that is available to the fish is derived via a high level of cutaneous respiration and from physically dissolved gas in the plasma. Since the ambient sea water has a temperature less than 2° C and because lower temperatures increase the solubility of gasses in liquids, it becomes apparent why this species could be a biological possibility. Of interest is the reported presence of small numbers of colorless «erythrocytes» in this species. Though devoid of hemoglobin they are said to have the enzymes related to carbon dioxide-bicarbonate transport, another function of the red cell. Support for the concept of temperature-assisted aqueous solubilization of oxygen is seen in another endemic Antarctic species, the Nototheniidae. These teleostine (bony) polar fish have low hemoglobin levels, about 3-4 gm/dl. This value is apparently within the physiologic limits required for the organism due to the significant level of oxygen dissolved in the blood. The larva of the eel (*Anguilla anguilla*) is another example of a vertebrate that is adapted to exist without the benefit of erythrocytes or dissolved respiratory pigments. Rare instances have been recorded in which the African clawed toad *(Xenopus laevis)*, which normally has an erythrocyte count appropriate for an amphibian, was found to have no erythrocytes in its blood. Whether this relates to a particular metabolic or pathologic state or is possible because of the toad's cutaneous respiration has not been established.

The evolution of oxygen transport initiating with simple physical aqueous solution, advancing to utilization of an extracellular protein carrier (hemoglobin) and terminating in erythrocytic microenvironmentalization of this respiratory pigment yielded ever increasingly efficient mechanisms of oxygenation required by complex, high energy dependent organisms. The development of the circulating red cell permits that attainment of higher concentrations of hemoglobin in the blood than would likely be physiologically feasible if it were in free solution. Considering the fact that man's hemoglobin level is approximately 15g/100ml of blood, the absence of its erythrocellular vehicle would impose a formidable solute load upon the plasma, far greater than the aggregate of all other plasma proteins. Sea water, the original milieu, has an oxygenic carrying capacity of approximately 0.5cc/100ml while the addition of mammalian hemoglobin to plasma increases this volume forty fold. It is noteworthy that hemoglobin's oxygen binding capability is greater in water than in the mammalian erythrocyte because of the latter's ionic and organic phosphate constituents (e.g. diphosphoglycerate, adenosine triphosphate). This diminution is recognized as a shift of the hemoglobin dissociation curve to the right, i.e. a decrease in the oxygen affinity of the hemoglobin from the maximum. The reduction from the theoretical potential can be viewed as a trade-off for the physiologic functions performed by these other hematogenous components.

The most important advantage of enclosing hemoglobin in circulating cells is the opportunity to have different chemical environments in the plasma and within the erythrocyte. The intracellular pH of the erythrocyte is lower than that of plasma and both media have their own individual molecular composition. Cellular packaging of hemoglobin allows its respiratory functions to be promoted and shelters it from irreversible oxidation in the plasma by the development of appropriate protective intracellular enzymes. Its biological half-life is thus extended by avoiding degradation in the plasma and also by preventing its loss through phagocytic and renal tubular activity. Mammalian hemoglobin free in the plasma in the concentration of 50mg/100ml solution, for example, has a half-life of only about 40 minutes. The osmoregulatory problem of the addition of a large number of hemoglobin molecules to the plasma is eliminated by cellular sequestration as is the necessity for ponderous, less efficient molecules so designed to inhibit their diffusion out of this medium into the interstitial fluid. Finally, evolutionary modifications of the early erythrocellular geometry and internal organization as the cell ascended through aquatic, amphibian and mammalian phylogeny have enhanced the red cell's respiratory capabilities even further. These considerations will be the subject of subsequent discussion.

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