Past-Presidential Address
Major Events in the Evolution of the Oxygen Carriers

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SYNOPSIS. Major events in the history of the blood O2 carriers in multicellular animals include: 1) The origin of the red blood cell hemoglobins, which are both the most primitive and the most advanced O2 carriers. At the molecular level, they are believed to have arisen only once; at the animal level, there is no cogent reason to postulate more than two origins. 2) The origin of the hemerythrins, which occur at about the same phylogenetic level as the primitive red blood cell hemoglobins. They may not have been selected in higher animals because of their temperature sensitivity. The origins of 3) the molluscan hemocyanins and 4) the arthropod hemocyanins. These two events occurred independently, though for similar reasons. Both kinds of hemocyanins offered physiological advantages over the primitive hemoglobins that were important in the context of the more advanced molluscan and arthropod cardiovascular systems. 5) The origins of extracellular heme proteins, which arose independently many times, and probably for as many different reasons. 6) The loss of urea sensitivity, and the acquisition of organic Po4 sensitivity and additional cooperativity and pH dependence by the red blood cell hemoglobins, which occurred well after the origin of the vertebrates.

INTRODUCTION
The evolutionary history of the O2 carriers in animal bloods has often been described as so capricious that, except in vertebrates, these molecules must be unimportant. In fact, this notion ignores a large body of experimental evidence that indicates otherwise, at least for several phyla (Mangum, 1992a, b, c; Truchot, 1992). I believe that it persists because of two critical areas of ignorance, one zoological and the other biochemical.

Many readers of this journal will be more acutely aware than I of our imperfect understanding of the relationships of the animal phyla. As a graduate student, I was greatly influenced by the late G. Evelyn Hutchinson, who once defined the taxonomic category the animal Phylum as a group of organisms that is not very clearly related to any other group. Although facetious, his definition fairly accurately reflects the history of phylogenetic inquiry at that level. Taxonomists have devised subsuming sets until further relationships became unclear, and then they have halted the process and designated the highest set a phylum. If the Hutchinson definition is operationally fairly accurate, then it is the height of absurdity when we turn around and ask ourselves why we do not understand the relationships of the animal phyla! Ignorance has been cleverly built into the process (perhaps to keep ourselves in business?). At present, taxonomists are enthusiastically embracing the techniques of molecular genetics, but the most impressive findings have been made at levels lower than the phylum. The few attempts to elucidate relationships within the animal kingdom (Field et al., 1988; Lake, 1990; Aguinaldo et al., 1997) have yielded such different results that considerable refinement of data collection and analysis, as well as an adequate database, will all be necessary for consensus.

The second area of ignorance is less obvious. It is our imperfect knowledge of the molecular structure of the O2 carriers, especially those found in small or uncommon or unfashionable animals, which often occupy positions of key evolutionary interest. For example, the structures of the O2 carrier...
are unknown in the nemertines and the aplacophoran molluscs, both of which could provide important clues to the phylogeny of higher groups. If the mere existence of high Cu levels were demonstrated in trilobite fossils, the finding would strengthen the hypothesis of arthropod hemocyanin as a conservative, unifying character in a monophyletic phylum.

At present, we agree on the existence of two very coherent classes of blood O₂ carriers, the molluscan hemocyanins (MHcs) and the arthropod hemocyanins (AHcs). Most experts agree that each had a single origin, quite independent of the other, and that they share only a superficial aspect of the active site, viz. gross atomic composition (Markl and Decker, 1992; van Holde et al., 1992).

A third class is very probably coherent, although perhaps only from a molecular and not from a zoological point of view. This class contains the red blood cell hemoglobins (RBC Hbs). They share in exact detail the complex active site and, with a couple of meaningful exceptions (Terwilliger, 1992), they share a common size and the fundamental tertiary structure of the basic polypeptide chain. The uncertainty remains because a number of key representatives are not well known (Mangum, 1992a). In addition, there is an interrupted phyletic line that requires the hypothesis of at least two independent origins in different animal groups, albeit probably from a common molecular ancestor, a tissue heme protein.

A fourth class contains O₂ carriers in which the active site and quaternary structure appear to be common, but too little is known in two of the four phyla in which they occur. Moreover, zoological relationships as presently understood invalidate a hypothesis of a common origin of all four. These are the hemerythrins.

Finally, there is a class of O₂ carriers that experts regard as fairly clearly incoherent, in the sense that its members had half a dozen or more independent origins, which are reflected in quite different quaternary structures. They contain nearly identical active sites, however, and at least some may prove to share enough structural features to warrant a hypothesis of common molecular ancestry though not common animal ancestry. These are the extracellular heme proteins (EC Hbs).

In this essay, my goal is to interpret a flawed classification of molecules in the light of a flawed classification of the animal phyla. In particular, I shall identify what I regard as the significant events in the evolutionary history of these molecules by examining their phylogenetic relationships, respiratory properties and physiological functions. Using a Darwinian approach, I hope to show that the distribution of the O₂ carriers is not as capricious as often supposed.

If the reader wishes to explore many of these subjects further, I unabashedly call attention to the wonderful essays written by the leading investigators in the field, who contributed to a fairly recent, comprehensive volume on the O₂ carriers (Mangum, 1992d). I emphasize that, in my own uncertain phylogenies diagrammed below, the representations are not intended as cladograms. In part because I almost ignore phyla that lack O₂ carriers, the figures may not even represent complete genealogies. They are merely intended to show general phylogenetic affinities, as these relationships appear to me at this writing.

**Origins of the Blood and the RBC Hbs**

The ancestral form of the RBC Hbs, a myoglobin (Mb)-like molecule, is found in the tissues of the simplest animals, such as the turbellarian platyhelminths (Fig. 1). In all likelihood, these molecules preceded the origin of circulating body fluids. RBC Hbs as such are found in some of the simplest circulatory systems. In all likelihood, little time elapsed between the origin of the blood and the origin of RBC Hbs. RBC Hbs persist in seven animal phyla with various degrees of cardiovascular sophistication. The physiological properties of most of these Hbs are fairly similar to one another but different from those of wild type mammalian Hb (HbA). Most RBC Hbs are either noncooperative or almost so, and either pH insensitive or almost so. All but the vertebrate RBC Hbs are insensitive to intracellular organic modulators.
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Much has been said of the higher O\textsubscript{2} affinity of the primitive RBC Hb relative to that of HbA (wild type mammalian Hb) (reviewed by Manwell, 1960; Mangum, 1977); in fact, the difference is small. Under physiological conditions, most of the difference is due to organic P\textsubscript{02} sensitivity and the higher body temperature (Snyder, 1992).

The early workers noted correctly that, in terms of their physiological properties, the primitive RBC Hbs are more like Ms than HbA (reviewed by Manwell, 1960; Mangum, 1977). In view of the design of simple circulatory systems, this is exactly what one would expect. Flow velocities are small, equilibration times are long, and anatomically separated afferent and efferent pathways are often absent. Branchial epithelia, however, are thin. Branchial blood P\textsubscript{02} is believed to be high, and blood P\textsubscript{02} at the tissues approximates P\textsubscript{a0}, classically designated as the "unloading" pressure (Fig. 2). These RBC Hbs work as well as or better than HbA would work under the same conditions. A moderately high O\textsubscript{2} affinity carrier maintains a reasonable O\textsubscript{2} reserve for periodic apnea, and the "venous" reserve (more accurately the open circulatory compartment, the coelom) is actually protected by the noncooperativity. At the branchial end of the system, the noncooperativity does not impair O\textsubscript{2} uptake very much. The primitive RBC Hbs transport between about 20% to more than half of the O\textsubscript{2} consumed, depending on respiratory design and environmental conditions. The other fraction generally enters metabolizing tissues directly from the ambient medium, not from free solution in the blood (Mangum, 1992a).

Primitive RBC Hbs also have a second respiratory function, reminiscent of their ancestral origin. All of the simple animals with RBC Hbs are marine (their freshwater relatives do not osmoregulate strongly enough to protect the fragile RBC), and many are intertidal. When the gills cannot be ventilated at low tide, the animal sustains aerobic metabolism at least in part by drawing on the O\textsubscript{2} store. Primitive circulatory systems have at least one advantage over their more advanced analogues: this advan-

Fig. 1. Two origins of the red blood cell hemoglobin. The groups in which they are found are shown in capital letters and enclosed in boxes.

Fig. 2. O\textsubscript{2} binding of the RBC Hb of the annelid Glycera dibranchiata (solid line) at 20°C in comparison with that of a hypothetical O\textsubscript{2} carrier with the same O\textsubscript{2} affinity but with the cooperativity of HbA (dashed line). P\textsubscript{co2} is a measured value of coelomic fluid P\textsubscript{02}; P\textsubscript{ao2} is a minimal value for branchial fluid P\textsubscript{02} estimated from branchial thickness, flow velocities and burrow P\textsubscript{02} (Mangum, unpubl. data). A cooperative O\textsubscript{2} carrier would enhance O\textsubscript{2} uptake very little, and it would result in depletion of the O\textsubscript{2} store in the coelom.
Fig. 3. Utilization of the O₂ store in the annelid Glycera dibranchiata (Mangum, 1977). Redrawn from Mangum (1997).

The interrupted phyletic line mentioned above is found within the bivalve molluscs (Fig. 1), in which the most primitive representatives contain a MHc, while RBC Hbs appear de novo in the less primitive blood clams. This example is especially puzzling, because the selective advantages of the bivalve RBC Hbs over the bivalve MHcs are not at all clear.

**TWO ORIGINS OF THE HEMERYTHRINS?**

The hemerythrins (Hrs) appear at about the same phylogenetic level (Fig. 4) as the groups with RBC Hbs. At least as circulating O₂ carriers, the two may have arisen at about the same time, and very likely long before the other O₂ carriers. In the groups in which Hrs occur, non-circulating Mb-like molecules are never found; they are replaced by myohemerythrins (MHrs). No non-circulating molecules that might be ancestral to both Hrs have been identified.

Our understanding of the phylogenetic relationships of Hr-containing animals is especially tentative. I suggest, however, that most viable schemes alternative to the one in Fig. 4 would still permit the hypothesis that sipunculan, brachiopod and priapulid Hrs had a common origin. Nonetheless, there appears to be an undeniable break in the phyletic line between any or all of these groups and the single annelid family with Hrs, viz. the Magelonidae (Fig. 4). To my knowledge, no annelid expert supposes that magelonid polychaetes are especially primitive, so the interruption here appears to be within the Hr line (as shown in Fig. 4) rather than within the RBC Hb line. This is a good example in which our ignorance of molecular structure (a fortunately soluble problem!) impairs our knowledge. Very little is known about the structure of magelonid (or priapulid, for that matter) Hrs, and clearly further progress requires this information.

Hard data on the physiological performance of the Hrs is available for only one of the four phyla in which they occur, the Sipuncula (Mangum, 1992b). Sipunculan Hrs are found in each of two different extracellular compartments, an open coelomic space and a closed system within tentacles that also serve as gills.

Physiologically, coelomic Hrs are almost identical to the RBC Hbs: they are non-cooperative, they have high O₂ affinities, they are pH insensitive, and they are either in-
sensitive to intracellular effectors or so little sensitive that intracellular effectors do not seem to be important (Mangum, 1992b). Also like the RBC Hbs, Hrs are about 50% oxygenated at tissue PO₂ and they store enough O₂ for low tide apnea. In comparison with the primitive RBC Hbs, the Hrs appear to have both advantages and disadvantages.

Within the sipunculans, the Hrs appear to be more adaptable than RBC Hbs. In species with large tentacular surface areas, the respiratory properties of the Hrs in the two compartments differ strikingly. Tentacular Hrs are highly cooperative, their intrinsic O₂ affinity is an order of magnitude lower than that of coelomic Hrs, and the intrinsic O₂ affinity is lowered further by intracellular modulation. Interestingly, the strategy of the modulation differs from that in vertebrates. The effectors of tentacular HrO₂ affinity are Ca²⁺ and Cl⁻, which raise O₂ affinity. So it is the exclusion rather than enrichment (as of the vertebrate organic PO₄s) of effectors within the cell that amplifies the lower O₂ affinity.

In some sipunculan species, the tentacles are ventilated whereas the coelomic compartment is not; inside the animal, the tentacular vessels are bathed by coelomic fluid. The different O₂ affinities permit the system to function in O₂ transfer from the lower affinity, ventilated tentacular compartment to the higher affinity, unventilated coelomic compartment (Fig. 5), just as in the vertebrate fetal-maternal system.

If there are reasons why the Hrs were not selected against in favor of the original RBC Hbs, then why were the RBC Hbs retained, with only minor modifications, throughout much of animal history while the Hrs are confined to a few intermediate phyla? At least sipunculan Hrs have one serious disadvantage for cold-blooded animals, viz. an exaggerated temperature dependence, well within the natural range. Whereas the respiratory role of an annelid RBC Hb is only slightly lower at 10 than 20°C (Mangum, 1992a, b), a sipunculan Hr completely ceases to function at low temperature because its O₂ affinity becomes impossibly high (Mangum and Kondon, 1975). The abrupt change in O₂ uptake is quite clear (Fig. 6).
new O₂ carriers selected? In both cases, though to different degrees and with different details, significant advances had taken place in respiratory and cardiovascular design. Ventilation had become more powerful, and gills with partitioned afferent and efferent pathways had appeared, along with localized muscular pumps that increased blood flow velocity through separate afferent and efferent tubes to and from the tissues.

In the arthropods, however, the advances were accompanied by a respiratory mistake. The gills, along with everything else, were covered up by a gas barrier known as chitin. The relative roles of chitin, branchial and cardiovascular design, and ventilation are not known but, for whatever reason, branchial blood Po₂ fell while blood Po₂ at the tissues rose. A non-cooperative RBC Hb or tentacular Hr would have been a disaster (Fig. 8). A pH insensitive one would not have taken advantage of the pH differences that now existed between the two gas exchange sites, and between hypoxic and normoxic bloods (Truchot, 1992). The resulting AHc is an O₂ carrier that is far more cooperative and pH dependent than even HbA. And, also in the arthropods, the O₂...
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Fig. 9. O₂ binding of Octopus MHc at 22°C, showing the effects of pH on cooperativity as well as O₂ affinity. The solid lines show the combined changes in cooperativity and O₂ affinity, while the dashed lines show the positions of the curves if cooperativity were to remain unchanged from its magnitude at the alternative pH. While the rise in O₂ affinity at high pH greatly enhances O₂ uptake at the gill, the decrease in cooperativity has only a small effect. At the tissues, the drop in O₂ affinity and the rise in cooperativity both enhance O₂ delivery (data from Johansen and co-workers, as summarized by Mangum, 1990; Miller and Mangum, 1989).

carrier is sensitive to a number of allosteric modulators.

Many of the same trends in intrinsic molecular properties occurred independently in the most advanced of animals save the higher vertebrates, the cephalopod molluscs (Mangum, 1992c). In this case the reason was not a gas impermeable exoskeleton but rather extreme metabolic demand and absolute dependence on O₂. In the cephalopods, Bohr shifts are often so great that they are Root shifts. Branchial blood PO₂ is not low, but the large pH dependence of both O₂ affinity and cooperativity has important consequences for both oxygenation at the gill and deoxygenation at the tissues (Fig. 9).

Fairly early on in Hc evolution, a wrinkle occurred in both molluscs and arthropods, once again quite independently. It was the reversal of pH dependence, which took place in some, but not all, gastropod molluscs and some, but not all, chelicerate arthropods. In aquatic and also more primitive gastropods, the reversed Bohr shift does not impair O₂ transport (Mangum, 1992c), because it is largely limited to a non-physiological pH range in aquatic species. In terrestrial pulmonates, it actually enhances O₂ transport during hypoxic dormancy. In the aquatic chelicerate arthropod Limulus, the reversed Bohr shift is both physiological and very important. In normoxic horseshoe crabs, the reversed Bohr shift is not a disadvantage because there is little or no pH difference between the two sites of gas exchange. But, during hypoxia severe enough to require anaerobic metabolism, the resultant acidosis raises instead of lowers O₂ affinity, which is the adaptive response (Fig. 10D).

At low temperature, the reversed Bohr shift of Limulus AHc opposes the effect of intrinsic molecular stability (Fig. 11). O₂ affinity rises due to the direct effect of the decreased temperature (Fig. 11B) but drops due to the direct effect of now higher blood pH (Fig. 11D). Whereas a crustacean AHc can cease functioning below 15°C because temperature and pH have fully cumulative effects, Limulus AHc continues to function at 4°C because temperature and pH have opposite effects.

Crustacean AHcs do not exhibit reversed Bohr shifts. Instead, evolutionary events that occurred only in this group of arthropods (and not in chelicerates) prevent a maladaptive decrease in AHc O₂ affinity during severe hypoxia. These events include the origins of sensitivity to two an-
Fig. 10. Different mechanisms of adaptation of AHc O₂ affinity to hypoxia. Panel A shows the adaptive effect of hyperventilation in a number of decapod crustaceans during acute, moderate hypoxia. Blood PCO₂ drops and pH rises, which raises O₂ affinity of a carrier with a normal Bohr shift. Hyperventilation does not usually occur in the chelicerate *Limulus* (Panel B). In the crustacean *Callinectes sapidus*, chronic sublethal hypoxia (11 da.) acclimation brings about an intrinsic increase in O₂ affinity at the molecular level (Panel A), which does not occur in *Limulus* (Panel B).

Right hand panels show effects on O₂ affinity of acidosis and the metabolic end products resulting from anaerobic metabolism during severe hypoxia. In crustaceans (Panel C), the allosteric actions of metabolites counteract the otherwise maladaptive effects of the acidosis on an O₂ carrier with a normal Bohr shift. In *Limulus* (Panel D), the AHc is not specifically sensitive to anaerobic metabolites but the acidosis has the same net effect, viz. an increase in O₂ affinity, as that of hyperventilation and acclimation in crustaceans. *Limulus* is more tolerant of severe hypoxia.

Aerobic metabolites, *viz.* L-lactate and urate, which allosterically raise O₂ affinity (Truchot, 1992). L-lactate, although responsible for much of the acidosis, at the same time brings about an increase in AHc O₂ affinity (Fig. 10C). Urate has a similar effect (Morris et al., 1985), as does the inorganic cofactor Ca²⁺, which is believed to be etched from the exoskeleton (deFur et al., 1990). Finally, in at least one species, acclimation to chronic sublethal hypoxia brings about an intrinsic increase in AHc O₂ affinity (Fig. 10A), via a shift in the ratio of two oligomers in the blood (Mangum, 1994).

Identification of the ancestors of both Hcs has been difficult. When both MHcs and AHcs arose, they were extruded into the bloodstream; not only are there no BBCs (blue blood cells), there are no myoHcs. Why not? The minimum functional unit (of either AHc or MHc) with the necessary respiratory properties is at least a 450 kDa molecule. As pointed out to me by G. K. Snyder (personal communication), BBCs containing a high concentration of 450 kDa molecules would not be very deformable and, thus, would be highly viscous. The AHc monomer, analogous to monomeric Mb, is at least a 75 kDa particle that would both store much less O₂ than a 15 kDa Mb and would facilitate diffusion at an imperceptible rate. The analogous MHc monomers are about six times larger. Neither would work very well in non-circulating tissue.

**MULTIPLE ORIGINS OF THE EXTRACELLULAR HEME PROTEINS**

The next set of events, in order of discussion not chronology, was the origin of
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Effect of temperature  Effect of pH  Cumulative effects

Normal Bohr Shift

\[ \text{Effect of temperature} \quad \text{Effect of pH} \quad \text{Cumulative effects} \]

\[ \begin{align*}
A & \quad 5^\circ C \quad 25^\circ C \\
B & \quad 5^\circ C \quad 25^\circ C \\
C & \quad 7.8 \quad 7.5 \\
D & \quad 7.5 \quad 7.8 \\
E & \quad = \\
F & \quad = \\
\end{align*} \]

PO₂

FIG. 11. AHc O₂ transport at low temperature with normal and reversed Bohr shifts. In both cases, low temperature raises O₂ affinity (Panels A and B). But the effects of a thermally induced rise in pH are opposite, depending on the direction of the Bohr shift (Panels C and D). Thus the effects of temperature and pH are fully cumulative with a normal Bohr shift (Panel E), and counteracting with a reversed Bohr shift (Panel F). O₂ uptake is less sensitive to low temperature in Limulus than in Callinectes (Mauro and Mangum, 1982; Mangum and Ricci, 1989), as is locomotor behavior.

the other extracellular O₂ carriers, in this case using that complex active site known as Fe²⁺ protoporphyrin or heme. These events occurred on at least half a dozen, and probably more, occasions and also probably for at least half a dozen different reasons (Fig. 12). As mentioned above, one reason is the invasion of freshwater. This event pertains to the osmotic fragility of the RBC (nemertines). It also involves the advantage of a much higher O₂ affinity in often hypoxic fresh waters (planorbid gastropods, insects and planktonic crustaceans) than found among the MHcs or AHcs. I should mention poorly studied examples such as the repeated appearance of EC Hbs in various groups of crustaceans in which either the primitive O₂ carrier is an AHc (copepods, amphipods) or the primitive O₂ carrier is unknown (ostracods) or, primitively, no O₂ carrier is believed to exist (cirripedes).

My favorite example of de novo origin is the appearance of extracellular Hbs (and chlorocruorin, a slight variant thereof; see Terwilliger, 1992) in the higher annelids, in which the event is closely related to the origin of the cardiovascular system. Except in the anachronistic magelonids, the O₂ carrier in the annelid cardiovascular system is always an EC Hb, while RBC Hbs are located in the more primitive, open coelom. This arrangement is another example of O₂ transfer from a lower affinity compartment (usually the cardiovascular system, which is ventilated at the gills) to a higher affinity compartment (the coelom, which may become so packed with reproductive material that it is essentially a tissue) in the adult animal. The annelid EC Hbs are the most genetically adaptable of the O₂ carriers. In fairly closely related species, O₂ affinity varies by two orders of magnitude, cooperativity varies from none to three times that of HbA, and pH dependence from none to twice that of HbA. All of this variation
is intrinsic; it occurs in the absence of sensitivity to organic modulators. The evolution of these molecules produced a quaternary structure so complicated that its details still defy full understanding, despite extensive investigation by a number of able groups (Terwilliger, 1992). And, it was accompanied by an increase in molecular mass to 3–4 MDa.

Since an annelid may retain RBC Hbs in the open, coelomic circulation while supplementing them with an EC Hb in the closed cardiovascular tubes, there must be something about RBCs that is disadvantageous in this simple cardiovascular system. I have suggested that it is RBC viscosity (Mangum, 1976). The annelid cardiovascular system lacks a microcirculation in deep tissue. It consists almost entirely of large bore tubes, too big for the reduction in viscosity at the capillaries (the Fahraeus-Lindqvist phenomenon) that confers an advantage to RBCs in the vertebrate system. In the annelids, a cardiovascular blood with RBCs would have a higher viscosity than an equimolar Hb solution, an especially great problem for the fragile and not very powerful annelid heart. RBCs were abandoned.

**ORIGIN OF ORGANIC PO₄ SENSITIVITY OF RBC HBS**

A recent event in the scenario is the transition of the simple RBC Hbs from the non-cooperative or weakly so, pH independent or weakly dependent, modulator-insensitive O₂ carriers to the higher vertebrate Hbs with their complex respiratory properties which
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FIG. 13. Origin of organic $P_O_4$ sensitivity of the RBC Hbs. The group in which this event occurred is shown in capital letters and enclosed in a box.

we are all familiar. This event appears to have begun well after the origin of the vertebrates, viz. within the elasmobranchs (Fig. 13). Like the agnathan RBC Hbs, batoid RBC Hbs are insensitive to organic $P_O_4$s. The selachians are the most primitive group known with organic $P_O_4$-sensitive Hbs (Weber et al., 1983; Scholnick and Mangum, 1992). Neither the mechanistic nor the adaptive reasons for this distinction are yet clear. One might suppose that organic $P_O_4$ modulation requires a binding site of sufficient size between the two beta chains, or a cooperative tetramer. This site is absent in agnathans, and unstable in many elasmobranchs, in which the RBC Hbs are dimeric in the oxygenated state. There must be additional reasons, however, because the RBC Hb of the cow nose stingray *Rhinoptera* is a stable tetramer, and yet it is insensitive to ATP (Scholnick and Mangum, 1992).

It has been suggested that the elasmobranch RBC Hbs are uniquely insensitive to urea, which denatures HbA, to protect them from their own intracellular osmolytes (Bonaventura et al., 1974). Coelacanth RBC Hbs are also insensitive while teleost Hbs are not (Mangum, 1991; Scholnick and Mangum, 1992). Urea insensitivity appears to have been inherited by the elasmobranchs rather than specifically evolved by them, however. A variety of RBC Hbs in simpler animals such as annelids and molluscs share urea insensitivity with elasmobranchs even though they are incapable of synthesizing it and do not use it as an osmolyte (Scholnick and Mangum, 1992).

FIG. 14. Loss of urea sensitivity and enhancement of cooperativity, pH dependence and $P_O_4$ sensitivity of the RBC Hbs. The group in which these events occurred is shown in capital letters and enclosed in a box.

**Loss of Urea Sensitivity and Enhancement of Cooperativity, pH Dependence and $P_O_4$ Sensitivity**

The completion of the transition to the familiar vertebrate RBC Hbs occurred in the teleosts, in which pH dependence and cooperativity were enhanced, and intrinsic...
modification of the molecule produced a somewhat wider array of O₂ affinities (Fig. 14). Following this event, few major changes occurred.

In closing, I unabashedly acknowledge that I have chosen to address questions that can be elucidated by examining the distribution of the O₂ carriers in living, breathing animals, and I have chosen to ignore many more elusive questions. But I hope that I have shown that this approach can be fruitful.

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