Oxygen Equilibrium of Brachiopod Lingula Hemerythrin

Abstract. In contrast to hemerythrin from five different species of sipunculid worms, ecardine brachiopod *Lingula* hemerythrin has an oxygen equilibrium which is reversibly altered by *p*H changes, both the oxygen affinity (Bohr effect) and the interactions between oxygen-binding centers being a function of *p*H. The significance of these phenomena is discussed in view of the phylogenetic distribution of hemerythrin and the function of respiratory pigments.

The distribution of the respiratory pigment hemerythrin is phylogenetically interesting. This nonheme iron-containing equivalent of hemoglobin is found only in the phyla Sipunculida, Annelida (one species), Priapulida, and Brachiopoda (*1*). Only the first two of these phyla are related, the priapulids being considered as relatives of the rotifers and nematodes, and the brachiopods being closest to the other lophophorate phyla, the phoronids and ectoprocts (*2*). Consequently, it can be asked how closely the various hemerythrins resemble one another (*3*).

Specimens of the brachiopod *Lingula unguis* were carefully dissected so as to obtain the small amount (¼ ml) of coelomic fluid with a minimum of contamination. The hemerythrin-containing "erythrocytes" were washed repeatedly in isotonic saline, differentially centrifuged each time so as to remove other coelomic cells. Erythrocytes were hemolyzed in 5 to 10 volumes of distilled water; centrifugation and filtration of the hemolyzate resulted in a clear solution of 2 to 4 percent hemerythrin. Concentrated potassium phosphate buffer was added to bring the ionic strength to 0.2 and the *p*H to the desired level (6.5 to 8; the pigment is unstable at *p*H values much above 8).

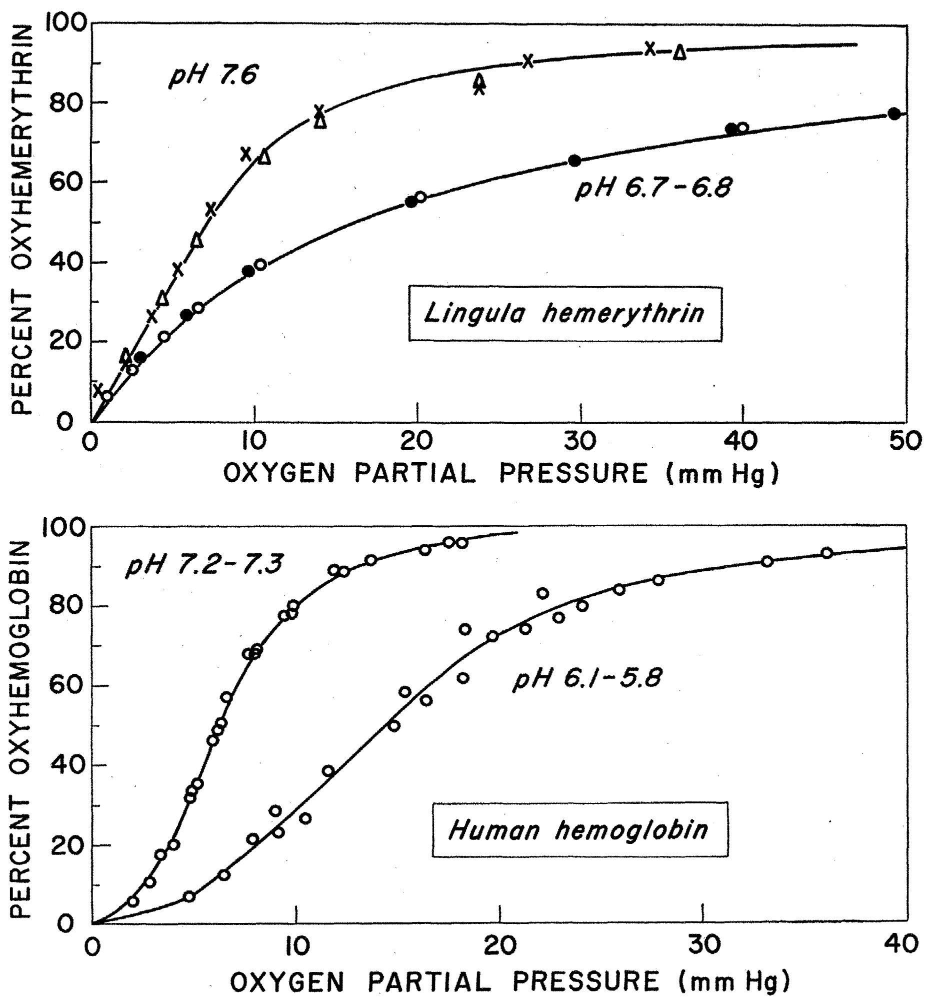

Fig. 1. Oxygen equilibrium curves for *Lingula* hemerythrin (top) and human hemoglobin (Hb A) (bottom). Temperature, 22° to 24°C; potassium phosphate buffer, ionic strength approximately 0.2; hemerythrin concentrations, 2 to 3 percent; hemoglobin concentrations, 7 to 15 percent. Reversibility of Bohr effect of hemerythrin is demonstrated by bringing sample (O), originally at *p*H 6.77, to *p*H 7.63 (△) and back again to *p*H 6.74 (●); control experiment is a second sample whose oxygen equilibrium is determined at *p*H 7.65 (X).

Oxygen equilibria were determined with a Beckman model DU spectrophotometer as described in other hemerythrin studies (*4, 5*). To insure that any effects of *p*H change on the oxygen equilibrium represent reversible changes characteristic of the Bohr effect and not irreversible changes resulting from mild denaturation of the protein, it was customary to bring the sample to a different *p*H by the addition of dilute HCl or K_3PO_4 after the first oxygen equilibrium experiment, to determine again the oxygen equilibrium, then to return to the original *p*H, and follow this with a third oxygen equilibrium determination. Most experiments were performed at room temperature (22 to 24°C), although a few were done at 9°C with essentially similar results, except, of course, for a higher O_2 affinity.

Data on the oxygen equilibrium of *Lingula* hemerythrin are presented in Fig. 1 at two different *p*H's; for purposes of comparison, data are also presented on human adult hemoglobin at two different pH's (*6, 7*). It can be seen that *Lingula* hemerythrin possesses a "normal" Bohr effect at physiological *p*H's that is approximately two-thirds of that for human hemoglobin. Coelomic hemerythrins of the sipunculids *Sipunculus nudus* (*8*), *Golfingia gouldii* (*9*), and *Phascolosoma agassizii* (*4*) have no Bohr effect. I have found (*5*) that the sipunculids *Dendrostomum zostericolum* and *Siphonosoma ingens* have biochemically distinct hemerythrins in the coelom and in the separate tentacular main contractile vessel circulation. None of these pigments possess a Bohr effect, although the oxygen affinities of coelomic and vascular hemerythrins can be very different. Besides a Bohr effect it can be seen that at the more alkaline *p*H's *Lingula* hemerythrin has distinct interactions between oxygen-binding centers — $n = 1.7$ to 1.8 (*10*); at the more acid *p*H's n approaches 1.0. Like the Bohr effect this is a completely reversible phenomenon. Reversible changes in the magnitude of n as a function of *p*H have been observed in teleost fish (*11*) and turtle (*12*) hemoglobins, the latter behaving in a manner identical with *Lingula* hemerythrin. For the various sipunculid hemerythrins some have small oxygen-affine center interactions (*Golfingia*, *Sipunculus*, and *Dendrostomum*) and others have perfectly hyperbolic oxygen equilibrium curves (*Siphonosoma* and *Phascolosoma*); in no case are the interactions *p*H dependent. Hence, *Lingula* hemerythrin differs significantly from sipunculid hemerythrins in this respect also. Unfortunately no data are available on the oxygen equilibria of priapulid or polychaete *Magelona* hemerythrins. The data on *Lingula* hemerythrin oxygen equilibria indicate that a single proton-binding group whose *p*K changes from 7.9 to 6.5 upon oxygenation controls both the oxygen affinity (that is, determines the Bohr effect) and the strength of the interaction between the oxygen-binding sites [the theoretical considerations and calculations for human hemoglobins are in press (*7*)]. This proton-binding group is most likely an imdiazole group of a histidine residue that is not present in such a critical position in sipunculid hemerythrins.

The fact that *Lingula* hemerythrin has a fairly low oxygen affinity, a somewhat sigmoid oxygen equilibrium curve, and a Bohr effect is of interest from the standpoint of respiratory pigment function, for observations on the living animal indicate that frequently the pigment must serve in oxygen storage as well as in oxygen transport. As the coelomic fluid is circulated into the translucent peduncle, it is possible to obtain a measure of the degree of oxygenation of the blood by the intensity of color in the peduncle of a particular animal. It was found that 20 to 30 percent of the time the blood was colorless; that is, the hemerythrin was completely deoxygenated. Hence, apparently during periods when respiratory and feeding activity are stopped the oxyhemerythrin in the coelom gives up the oxygen and becomes completely deoxygenated.

Conceivably such an arrangement of oxygen storage might be significant in an intertidal animal such as *Lingula,* a form that withdraws quickly into its coral sand or mud burrow when disturbed. However, these properties of low oxygen affinity, oxygen-binding center interactions, and Bohr effect are exactly what Wald (*13*) has postulated as representing the epitome of the evolution of respiratory pigments—blood gas transport pigments that are correlated with high metabolic rate and activity—for example, in mammals and cephalopods. Figure 1 indicates the similarity between human hemoglobin and *Lingula* hemerythrin in so far as oxygen equilibrium is concerned. Therefore, that *Lingula,* a genus of animals essentially unchanged since Precambrian times, can evolve the same properties of the oxygen equilibrium as are found in squid hemocyanins and mammalian hemoglobins adds further doubt to the validity of Wald's (*13*) speculations. The hagfish *Polistotrema stouti* (Lockington), a representative of the most primitive group of living vertebrates, has a hemoglobin that possesses all three of Wald's "primitive" properties—high oxygen affinity, no heme-heme interactions, and no Bohr effect. However, as I have pointed out (*1, 14*), this does not necessarily prove Wald's theory. The properties of hagfish hemoglobin are very likely the result of selection pressure favoring the development of a hemoglobin with high oxygen affinity and no Bohr effect, for the combination of mud-dwelling and almost endoparasitic habits of the myxinoids must frequently expose hagfish to situations of high carbon dioxide and low oxygen concentrations. By way of constrast, hemoglobin from the equally primitive lampreys displays a low oxygen affinity, very large Bohr effect, and, under certain conditions, slight heme-heme interactions (*15*). Lampreys remain outside the bodies of their hosts and, as adults, are very sensitive to low oxygen or high carbon dioxide concentrations. At present it is more suitable to explain the properties of hagfish and lamprey hemoglobins as adaptations to particular environments and habits rather than as indicating that any particular set of biochemical characteristics of respiratory pigments is "primitive."

Similarly, whereas *Lingula* hemerythrin possesses none of Wald's three oxygen equilibrium properties of a "primitive" respiratory pigment, various sipunculid hemerythrins, except for *Dendrostomum zostericolum* vascular hemerythrin, which has a low oxygen affinity (*5*), have all three of the basic properties. One cannot regard sipunculids as phylogenetically any more or less primitive than brachiopods; in addition, it is impossible to decide whether the occurrence of hemerythrin in both phyla represents relationship or convergence, though *Lingula* hemerythrin is quite different from all known sipunculid hemerythrins in its oxygen equilibrium properties. Accordingly, just as for hemoglobin, it is apparent that for hemerythrin there is no reason to assume any particular set of oxygen equilibrium properties is "primitive"; in addition, the set of oxygen equilibrium properties that is observed for a particular pigment can be often correlated with the ecology and physiology of the particular animal (*16*).

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References and Notes

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2. L. H. Hyman, *The Invertebrates: Smaller Coelomate Groups* (McGraw-Hill, New York, 1959).
3. I express my appreciation to Mrs. N. Banner (University of Hawaii) for sending living specimens of the brachiopod *Lingula*; these were easily kept alive in salt-water aquaria for months.
4. C. Manwell, *Science* **127**, 592 (1958).
5. ———, *Comp. Biochem. and Physiol.*, in press.
6. These human hemoglobins consist of samples of Hb A (normal adult), dialyzed against cationic and anionic ion-exchange resins for purposes of comparison with various abnormal and fetal hemoglobins.
7. ———, *J. Biol. Chem.*, in press.
8. M. Florkin, *Arch. intern. physiol. et biochem.* **36**, 247 (1933).
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10. The oxygen equilibrium of respiratory pigments, like the equilibrium of proteins with some dyes or ions, can under many conditions be quite accurately represented by the Hill approximation

$$y = 100\ (p/p_{50})^n/[1 + (p/p_{50})^n]$$

where p_{50} is the oxygen partial pressure at which the pigment is 50 percent oxygenated and n is a measure of the interaction between oxygen-binding sites on the same molecule. If $n = 1.00$, the sites are totally independent; $n > 1$ indicates positive interactions between oxygen-binding sites. If $n < 1$, the interactions are negative (hindering) and the above equation fits the data poorly; slight deviations from the Hill approximation are seen when for a multiheme hemoglobin oxygenation of each site (heme) progressively increases the affinity of the remaining unoxygenated sites.
11. R. W. Root, L. Irving, E. C. Black, *J. Cellular Comp. Physiol.* **13**, 303 (1939).
12. C. Manwell, unpublished experiments.
13. G. Wald, in *Modern Trends in Physiology and Biochemistry*, E. S. G. Baron, Ed. (Academic Press, New York, 1952), pp. 337–376; ——— and D. W. Allen, *J. Gen. Physiol.* **40**, 593 (1957).
14. C. Manwell, *Biol. Bull.* **115**, 227 (1958).
15. Data on hemoglobin of adult lamprey *Petromyzon marinus* may be found in G. Wald, and A. Riggs, *J. Gen. Physiol.* **35**, 45 (1951). I have determined oxygen equilibria of hemoglobin from adult and larval lampreys of the species *Petromyzon marinus* and *Ichthyomyzon unicuspis* (unpublished). Although alkaline denaturation, solubility, and electrophoretic mobility studies indicate that adult and ammocoete hemoglobins are different proteins, both pigments display similar very large Bohr effects; the oxygen affinity of the ammocoete pigment is higher than that of the adult. Oxygen equilibria of blood and crude hemolyzates indicate small but significant heme-heme interactions ($1.2 < n < 1.5$); careful purification by fractional precipitation more readily eliminates this phenomenon from adult than from larval hemoglobins. The presence of heme-heme interactions is probably associated with only some of the hemoglobin molecules, as "salting out" and electrophoretic investigations indicate heterogeneity—at least three components in ammocoete hemoglobins and two components in adult hemoglobins. At more acid *p*H's adult lamprey hemoglobin does not show heme-heme interactions ($n = 1.00$). Similarly, Wald and Riggs observed $n = 1.2$ for the more alkaline samples and $n = 1.00$ for the most acid of three oxygen equilibrium curves of lamprey hemoglobin. Hence, there is no doubt that under certain conditions heme-heme interactions are shown by at least some of the molecules in a cyclostome hemoglobin preparation.
16. The wide range of internal oxygen tensions at which oxygen transport occurs in annelid worms is quite nicely correlated with the varied properties of the oxygen equilibrium curves; see, for example, C. Manwell, *J. Cellular Comp. Physiol.* **53**, 61 (1959), or *Comp. Biochem. and Physiol.*, in press.

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