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Underwater Life Support Based on Immobilized Oxygen Carriers

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Abstract

One of the primary problems that hinders humans in their efforts to explore and develop the ocean realms is the lack of a ready supply of oxygen. Practical methods have not yet been devised for using the vast amount of oxygen dissolved in ocean waters for human life support in an undersea environment. Fish and other water-breathing animals have solved this problem by utilizing hemoglobin as a molecular oxygen pump. To achieve a similar oxygen extraction capability, we have explored various methods of oxygen extraction that are based on immobilized forms of hemoglobin. Improved methods for immobilizing hemoglobin or other oxygen carrying molecules and a method for extracting the available dissolved oxygen from natural waters and other fluids are described. The techniques that have been developed allow for immobilization of oxygen carriers at high concentration in a state where they are capable of reversible oxygen binding, and also allow for regeneration of the carrier in the event of oxidation of the oxygen-binding site.

Index Entries: Underwater life support, with immobilized O_2 carriers; immobilized oxygen carriers, for underwater life support; oxygen carriers, immobilized; carriers, immobilized oxygen.

Life Support System

In order to discuss the development of oxygen carriers in an immobilized state as important elements in life support systems for maintaining people in underwater

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habitats, it will be useful to consider first the nature of a life support system. Current evidence and theory suggest that the star that we call the Sun developed about 10 billion years ago with a number of discrete planetoids circling it. Recent advances in space technology have led to "on-site" inspection of two nearby planets—Mars and Venus. Neither the Mars nor Venus probes provided any positive evidence for "life." Likewise, Voyager fly-bys of Jupiter and Saturn and some of their satellites have not led to evidence suggesting the existence of life. Approximately 6 billion years ago, on the surface of the planet Earth, "life-forms" appeared in the "primordial soup." Most evidence suggests that the primordial condition was a reducing atmosphere and that the original "life-forms" were synthesized from simple organic chemicals in this anaerobic environment. Some of the driving force for this synthesis is thought to have come from lightning (*1*) and inorganic templates (*2*). An alternative hypothesis has been that the primitive earth was "seeded" with "life-forms" from space (*3*). There is accumulating evidence that the existence of life on the planet Earth has markedly altered Earth's development, both in terms of its atmosphere and the shape and development of its surface (*4*). An incredibly complex series of events has, then, led to the present state of the life support system around our planet. As it presently exists, our planetary life support system provides an oxidizing, aerobic environment. Oxygen is readily available both in its air (20%) and in its water (7 ppm). There is little question that this oxygen has resulted from biological photosynthetic processes (*5*).

Oxygen Transport

As oxygen became a rich chemical in the environment, organisms began to make use of oxygen as an oxidant, evolving what we now call oxidative metabolism. Diffusion of oxygen to the site of the metabolic machinery allowed small and simple organisms to adequately accommodate their metabolic needs. As organisms became more complex, i.e., more structured and larger, systems other than simple diffusion were needed to accommodate their needs for oxygen. In some organisms, complicated pathways and circulatory systems "designed" to minimize diffusional pathlengths evolved. Reversible oxygen carriers evolved in other organisms and apparently offered a better solution to the problem of oxygen delivery to respiring tissues.

Transition metals, normally found in the reduced state in the primordial atmosphere, were probably quickly oxidized as oxygen became a more common molecular species on earth. The "evolutionary ethic" made use of this ready oxidation in terms of "learning" methods to put these oxidizable transition metals in an environment where they could oxygenate rather than oxidize; hence the development of metalloproteins capable of reversible oxygen binding. It is not profitable to speculate on which of the various lineages of proteins that wrap themselves around transition metals and thereby form oxygen carriers are "the best." We have little chance of ever knowing about nature's early and unsuccessful oxygen carriers. At present, we know of three major classes of oxygen carriers that evolved and survived. These are the "molecular pumps" used in the life support systems of all large aerobic organisms.

We refer to the three systems for O_2 transport that exist today as the "Three H's": the hemoglobins, hemerythrins, and hemocyanins. The roles of these metalloproteins are the same: to bind oxygen at the interface between the organism and its environment, either at an air–lung interface or a water–gill interface, and to transfer that bound oxygen to respiring tissues. Given the remarkable diversity of environments, both aerial and aquatic, it is not terribly surprising that these three classes of proteins are in fact functionally flexible; in each class representative proteins can be found that show remarkable similarity. A major aspect of a reversible oxygen carrier's function is its affinity for oxygen. This function is generally measured in terms of the partial pressure of oxygen required to half-saturate the carrier ($P_{1/2}$). If this parameter is plotted as a function of pH for selected hemoglobins, hemerythrins, and hemocyanins, it is impossible to select, *a priori*, which carrier is which, on the basis of its behavior under these conditions. Figure 1 illustrates the great adaptability and flexibility of the 3 H's, and reveals specific oxygen binding characteristics that are adaptive for the organisms that possess them.

Evolution of the 3 H's led to formation of complex multisubunit proteins. The association of subunits (oligomerization) led to a situation where oxygen-binding at one site could influence the reactivity of other sites. The word associated with this kind of interaction is "allostery" and all the 3 H's demonstrate allosteric properties that are critical to their performance. The oxygen-binding sites of the three H's are schematically represented in Fig. 2.

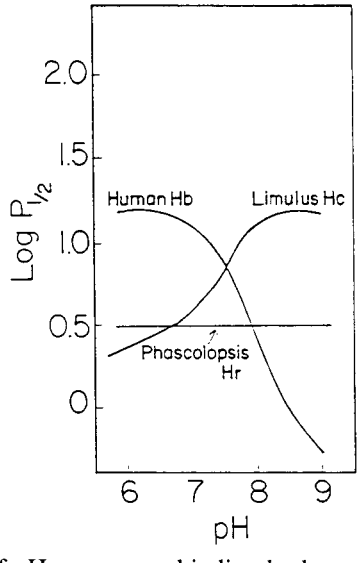

Fig. 1. The effect of pH on oxygen binding by human hemoglobin, horseshoe crab hemocyanin, and sipunculid worm hemerythrin. The pH dependence of the oxygen affinity ($P_{1/2}$) is called the Bohr effect. In human hemoglobin, the positive Bohr effect is of physiological significance in terms of a facilitation of oxygen binding at the lung–air interface and its delivery to the tissues at the capillary–tissue interface. The negative Bohr Effect of horseshoe crab hemocyanin also appears to be adaptive in that the organism frequently must extract oxygen from areas that are simultaneously low in pH and oxygen. The lack of pH dependence in the hemerythrin may represent a situation where oxygen is extracted from the environment and kept on reserve.

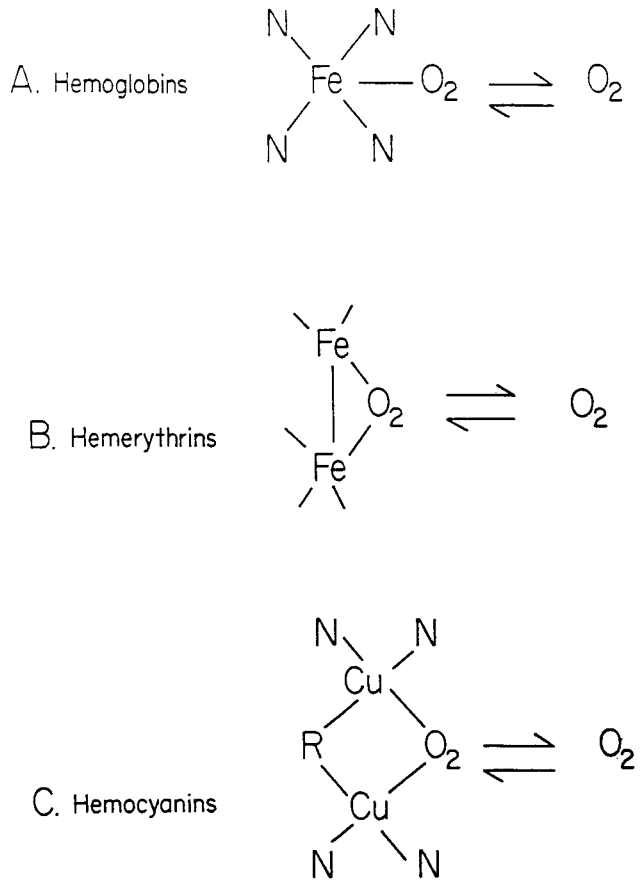

Fig. 2. Schematic representation of the oxygen-binding sites of hemoglobin (A), hemerythrin (B), and hemocyanin (C). Although all three H's have heme- or hemo- in their names, of the three, only hemoglobin possesses heme, iron-protoporphyrin IX.

Underwater Life Support

The sea has always enchanted the human species. The intrinsic beauty of the undersea, as well as the adventure, drama, and value of things found undersea prompts us to intensely explore this frontier. Being air breathers, humans are constrained to make use of their aerial oxygen extraction system. Figure 3 schematically represents how oxygen is extracted from air by humans. In terms of humans going underwater for prolonged periods of time, elaborate systems have been devised for the provision of breathable oxygen. For habitats and other large structures maintaining multiple numbers of humans underwater, air or oxygen must be continuously supplied. The oxygen is typically carried down from the surface in compressed gas bottles or generated by the electrolysis of water. Storage constraints make compressed gas use impractical for long-term maintenance of large numbers of people. In electrolysis, the hydrogen generated along with oxygen can be an undesirable side product. However, many organisms that have oxygen demands

comparable to those of humans are able to live comfortably under water. Porpoises and whales get their oxygen by breathing air. Other underwater inhabitants do it differently: they "breathe" water! Figure 4 is a schematic of oxygen extraction by water-breathing fish. A very important distinction between air breathers and water breathers is that the transfer of oxygen in the latter case is a liquid–liquid one, not involving gases. However, Fig. 4 points to a very interesting feature of the oxygen extraction and delivery systems of some fish. Faced with a problem of either positive or negative buoyancy in the water column, some fish have developed cartesian diver-like systems based on a gas-filled organ, called the swim bladder. Although the precise mechanisms of the regulation and control of gases in the swim bladder is not understood, hemoglobin has been implicated as a proton-driven "molecular pump" for oxygen delivery by this system. The molecular basis for this is that certain fish hemoglobins (root effect hemoglobins) are extremely sensitive to pH and unload their oxygen as the pH is lowered (*6*). Figure 5 illustrates this point.

If fish can extract dissolved oxygen from seawater and turn it into a gas, why can't we? On the basis of this question, investigations, sponsored by the US Office of Naval Research, were begun into some basic structural and functional properties of fish hemoglobins. With an eye toward a practical application of the basic research on fish hemoglobins, it was apparent that if there was ever to be an oxygen extractor based on fish hemoglobins, the hemoglobin would have to be specifically

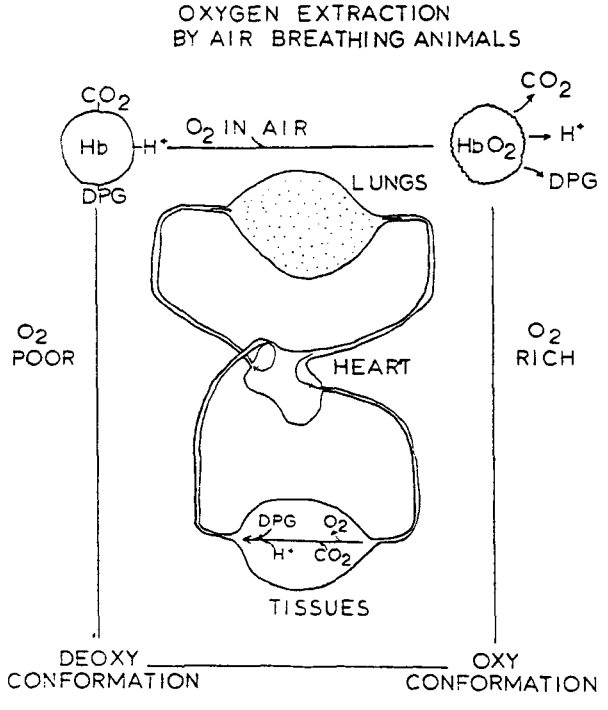

Fig. 3. Schematic of hemoglobin-based oxygen extraction in air-breathing animals. Protein conformational changes and the linkage of oxygen binding and delivery to physiological effectors shows the elegant system controlling the function of the hemoglobin tetramer. DPG stands for 2,3-diphosphoglyceric acid, an effector molecule that, in human red blood cells, exists in equimolar concentration with hemoglobin.

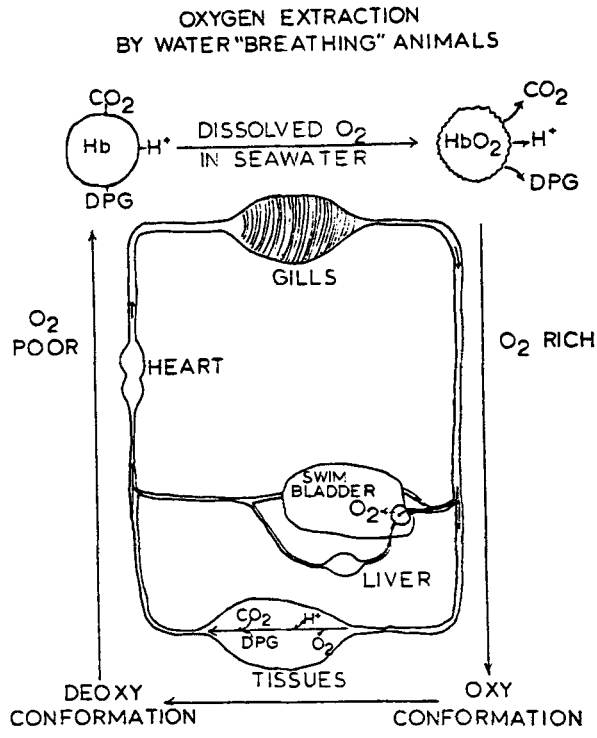

Fig. 4. Schematic of a water-breathing fish. Note the conceptual similarity of metabolic control of oxygen binding and release in this figure and Fig. 2. A notable difference, however, is the fact that some fish put oxygen into their swim bladders via a proton-driven hemoglobin "oxygen pump."

"engineered" to meet the needs of the system. A critical point in our capability to do this was passed on a fall day in 1976 when we learned about a commercial polyurethane that was "cured" with water (Hypol®, W.R. Grace Co.). Biology is really "based" on water and the announcement of a water-curing polymer sounded very exciting; such a polyurethane might be compatible with biochemical systems. Samples were requested and on the morning of their receipt, a blood sample was drawn from one of us (JB). This blood served as the "water" necessary for curing the polyurethane and the first Hemosponge was made. Rather high levels of proteins can be incorporated in Hemosponge and when properly formulated, their stability is remarkable. Some of the original sample still exists (one piece of which has been in the pocket of JB ever since it was made).

Immobilized Oxygen Carriers

The possibility of extracting oxygen from seawater using immobilized fish hemoglobins finally appeared approachable from a practical standpoint. Figure 6 is a schematic representation of how a pH sensitive hemoglobin could be used in an oxygen extraction process. In early stages of the "applied" research, it was decided that an important goal of the project concerned getting hard data on the ana-

lytical aspects of oxygen binding and release. After the initial demonstration that hemoglobin could be immobilized on various supports and cycled through oxy- and deoxy-cycles (as evidenced by the spectral changes of the material), it became necessary to set up various reactors as models of a practical system from which we could obtain quantitative data on uptake and release of oxygen, efficiency of cycling, yield of oxygen per unit volume of reactor, and so on. Accordingly, a system that chemically cycled the hemoglobin between its oxygenated and oxygen-free forms was devised. Figure 7 shows this chemical cycle. Figure 8 illustrates a schematic of the oxygen extraction process, while Fig. 9 shows a schematic of the experimental oxygen recovery apparatus. Typical oxygen loading and unloading curves are shown in Fig. 10a and 10b. During the course of developing these concepts, we simultaneously looked into various types of immobilized systems, small-scale reactors, and analytical methods. Much of our experimental work on immobilization and reactors made use of the "chemical" unloading cycle shown in Fig. 7.

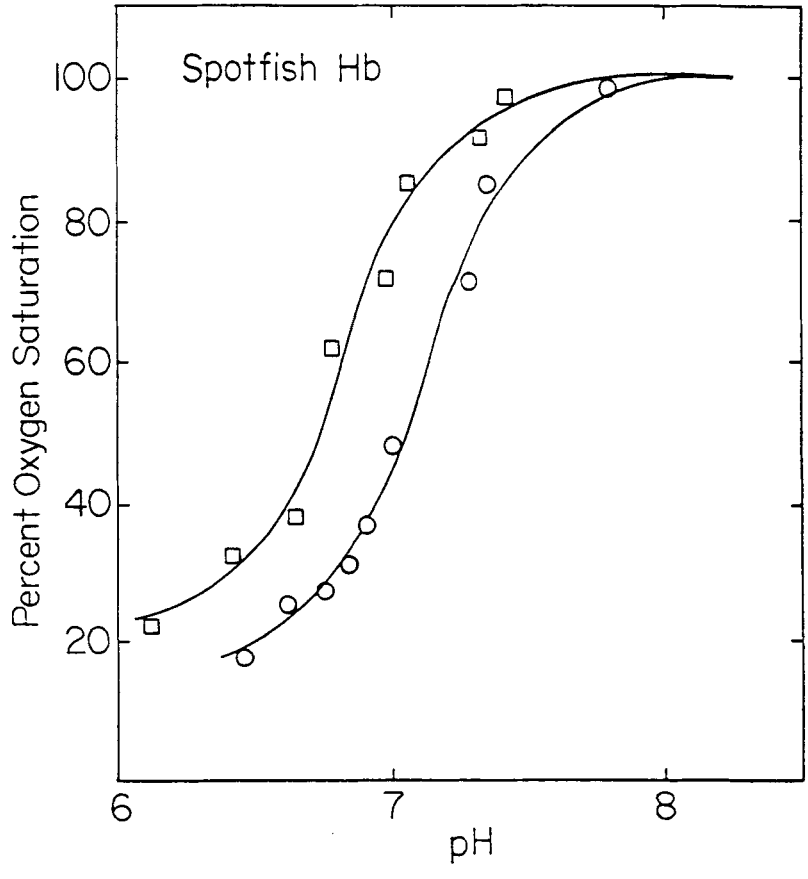

Fig. 5. A representation of the unloading of oxygen by spot fish hemoglobin as the pH drops. The transition from fully oxygenated to deoxygenated is not only modulated by pH but also by metabolic effectors such as CO_2, DPG (or other polyphosphate effectors such as adenosine triphosphate or inositol pentaphosphate). Squares correspond to data obtained in 0.05*M* bis-Tris or Tris buffers, and circles correspond to data obtained in the same buffers, but with DPG added (100-fold excess over hemoglobin).

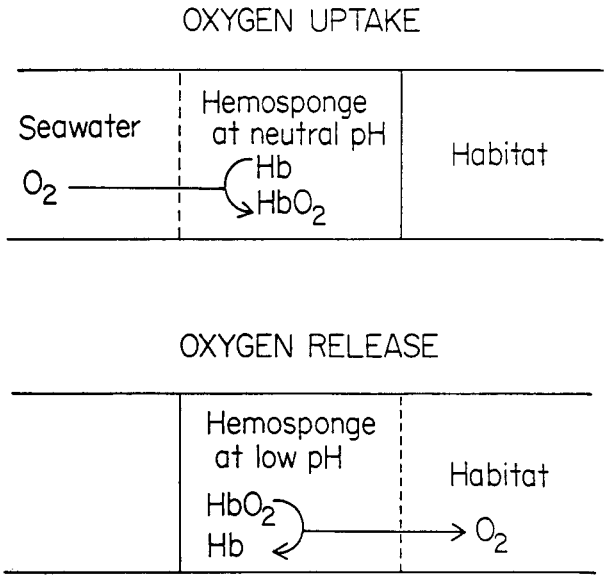

Fig. 6. Idealized oxygen extraction system using fish hemoglobin bound in a Biosponge matrix. Oxygen is extracted at seawater pH (around 8) where the fish hemoglobin will become fully saturated with oxygen. The system is cycled by lowering the pH and allowing the fish hemoglobin to release bound oxygen. Refilling the reactor with normal seawater leads to a rise in the pH and subsequent binding of dissolved oxygen.

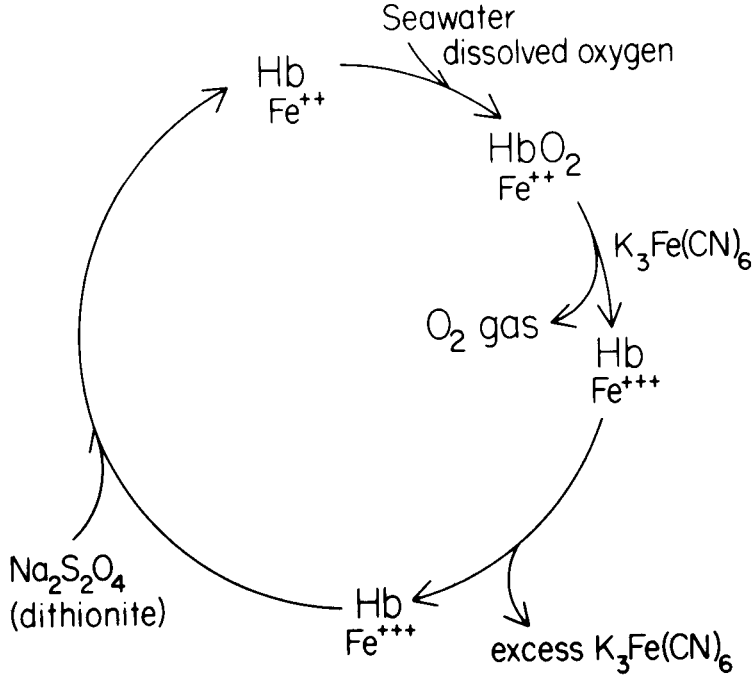

Fig. 7. Chemical oxygenation cycle used in test systems with immobilized hemoglobin. Starting with deoxygenated hemoglobin, oxygen from seawater is bound. The bound oxygen is removed by oxidation of the ferrous ions to ferric ions by potassium ferricyanide. Completion of the cycle, and a return of the ferric ion to ferrous, deoxy, is accomplished by treatment with the reductant sodium dithionite.

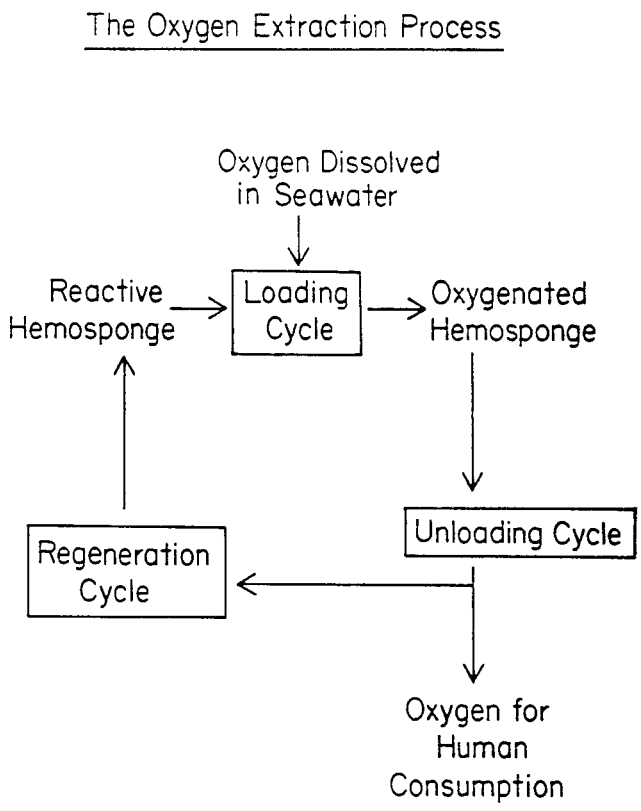

Fig. 8. Schematic diagram of an oxygen extraction process based on an immobilized oxygen carrier, in this instance, Hemosponge.

One factor in the design of any life support system, laboratory or shipboard, is the fact that seawater, although in equilibrium with air, contains only about 6–8 μg/mL (6–9 ppm). One person requires about 600 L O_2/d, or an average of about 0.6 g/min. Making reasonable assumptions of efficiency, simple calculations suggest that it is necessary to extract the oxygen from about 4000–5000 gal seawater/min for a complement of 150 men. Viewed from this aspect, it is easy to see that a practical system must provide for the rapid processing of huge volumes of water. If the reactor size is to be kept reasonable, space velocity through the reactor must be high, and both the loading and unloading cycles must be short. The theoretical maximum amount of oxygen bound to hemoglobin is 1.9 mg O_2/g hemoglobin. The actual amount of oxygen recovered from each cycle will depend on the efficiency of both loading (extent of saturation) and unloading (completeness of removal). It also depends on the extent to which the hemoglobin retains its functional properties after immobilization. Assuming an overall efficiency of 50% and that the reactor is in the uptake mode for 40% of the time (which allows 60% of the operating time for turnaround and unloading), with a 5-min cycle time one can calculate that approximtely 190 kg of hemoglobin must be immobilized in a reactor built to supply 150 people with breathable oxygen. It is interesting to compare this amount of hemoglobin with the amount of hemoglobin used by 150 people to extract their needed metabolic oxygen *from air*. Each 70 kg person has about 0.9 kg

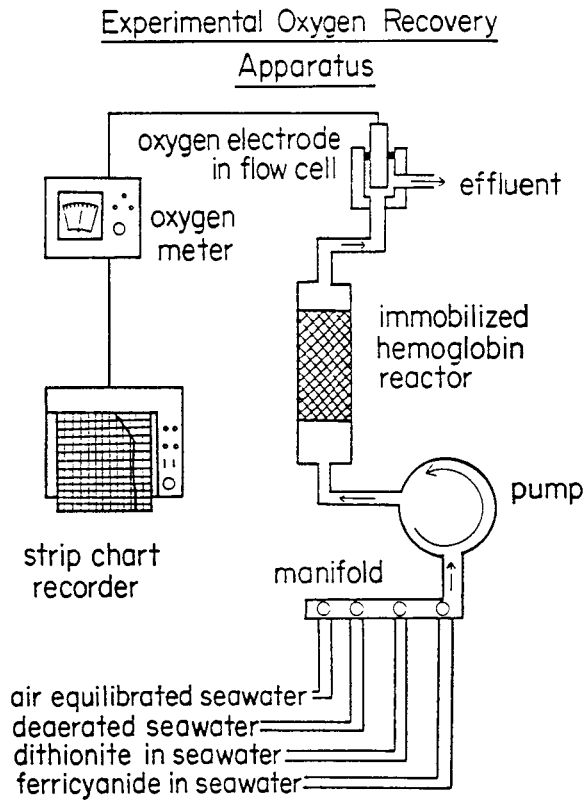

Fig. 9. Diagram of experimental oxygen recovery apparatus with immobilized hemoglobin in the oxygen extraction reactor.

hemoglobin. Hence the 190 kg of reactor-bound hemoglobin compares favorably to the 135 kg of hemoglobin surging through the circulatory systems of 150 people!

Experiments with polyurethane/hemoglobin sponges showed them not to be ideal for the large scale required to maintain scores of people supported with oxygen. Flow rates, loading of hemoglobin, and the yield of oxygen on cycling were all too low for a "properly" sized system. Various alternative immobilization methods were examined. One of the most promising was the nonfoaming polyurethane gel (nonfoaming Hypol®, W.R. Grace Co.). This polyurethane gel incorporated hemoglobin in good yield. Material with 50–100 mg hemoglobin/mL of gel was made by simply reacting hemoglobin solutions with the prepolymer. For use in the reactor, the resulting gel was ground, sieved to appropriate size, and packed. Analytical testing in small closed-system reactors showed that the hemoglobin was operating at 60–80% efficiency. In the reactor system, we obtained most of our analytical data by monitoring the oxygen content of the effluent stream and integrating in the appropriate fashion to measure the oxygen uptake, or oxygen yield. The uptake efficiency remained good with space velocities of about 1/min (flow rate = 1 column volume/min).

Having a good O_2-absorbing system in hand, we started looking for useful unloading systems. We were constrained by the fact that unloading had to be rapid and could not require extensive chemical treatment. After consideration of a wide

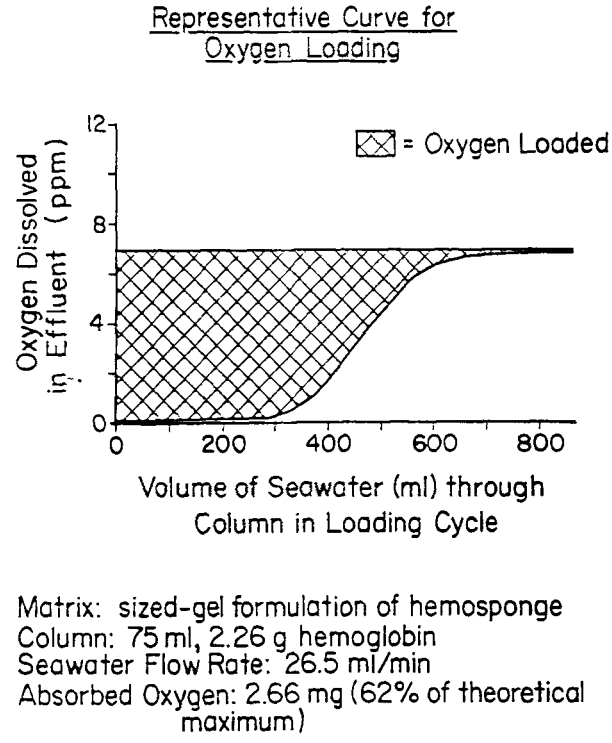

Fig. 10A. Strip-chart recorder output showing the oxygen electrode response to seawater effluent from the oxygen recovery apparatus shown in Fig. 9. This curve demonstrates the loading of oxygen on a column of immobilized deoxygenated hemoglobin.

variety of alternatives, the most practical way to unload the oxygen-rich absorber appeared to be by lowering the partial pressure of O_2 surrounding the absorber and allowing the O_2 to come off in gaseous form. In the laboratory, this was most simply done by passing a stream of nitrogen through a drained absorber bed. This method of cycling gave yields of less than 50% of the oxygen calculated to be in the hemoglobin-containing gel and the release was slower than needed to make the system practical. To better understand these results we determined the oxygen dissociation curve for the immobilized hemoglobin by using a Hemoscan Analyzer (American Instrument Co.).* This instrument measures the spectral changes of hemoglobin immobilized in finely ground gel that are brought about by changes in oxygen pressure. As expected, we found a relatively high oxygen affinity for the immobilized hemoglobin. The $P_{1/2}$ (O_2 pressure required for half-saturation) values were in the range of 0.3–3.0 mm Hg, compared to about 5–10 mm Hg for hemoglobin in solution and 20–30 mm Hg for hemoglobin in the human red blood cell. This finding offered an explanation for the relatively low yields and slow removal of oxygen from the sized gel particles. A low oxygen-affinity absorber appeared to be a necessity.

During the course of developing alternate supports, it appeared that porous glass or ceramic particles would be good ones. After experimentation with various

*The Hemoscan Analyzer is used to determine the absolute oxygen affinity of blood or hemoglobin (see refs. 7, 8).

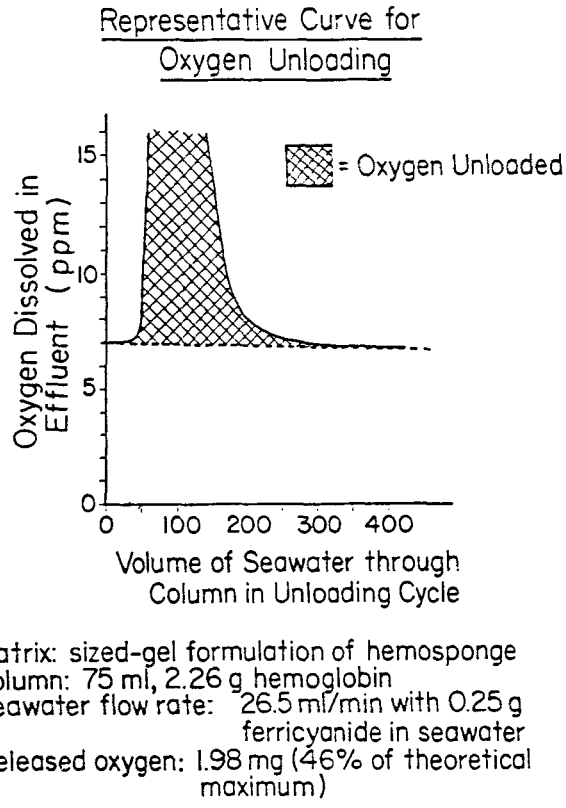

Fig. 10B. Strip-chart recorder output showing the oxygen electrode response to seawater effluent from the oxygen recovery apparatus shown in Fig. 9. This curve demonstrates the unloading of oxygen from immobilized hemoglobin after addition of potassium ferricyanide.

controlled-pore glasses and ceramics, we settled on a porous silica for further work.† This porous silica was available in commerical quantities and was supplied with an aminopropyl surface produced by silanization. It was treated with succinic anhydride to give a carboxyl derivative. Hemoglobin was coupled to the support with a water-soluble carbodiimide. Approximately 50–100 mg of hemoglobin could be bound per gram of silica by this technique. The bound hemoglobin was typically about 75–80% active. Oxygen uptake with the hemoglobin immobilized on silica was more efficient than with gel support, but unloading was still slow and incomplete. Again, we found that the bound hemoglobin had a high affinity for O_2 ($P_{1/2}$ of about 3 mm Hg). Again, the high oxygen affinity appeared to adversely influence the unloading cycle.

Hemoglobin from varied species and various chemical modifications of human hemoglobin were tried in order to find a lower oxygen affinity absorber. Sheep hemoglobin, known for its particularly low oxygen affinity outside the red blood cell (*9*), also gave a lower oxygen affinity when immobilized. The immobilized sheep hemoglobin gave improved oxygen unloading. This hemoglobin, though, was found to be quite unstable, rapidly changing to the oxidized (ferric) derivative.

†Kindly supplied by H. Weetall of Corning Glass Works, Corning, New York.

Modification of human hemoglobin with isothiocyanatobenzene sulfonic acid (*10*) resulted in an immobilized hemoglobin with lowered O_2 affinity. This technique showed some promise, but subsequent tests gave relatively low yields of O_2 release. The "engineering" of oxygen affinity was shown to be possible, but an "ideal" absorber was still elusive.

Considering the practical limits on oxygen tension and our ability to manipulate it, we might ask "What would the characteristics of the ideal absorber be?" If one accepts 5 and 150 mm Hg partial pressures of oxygen, as the practical limits of the reactor's oxygen partial pressure, an absorber with a $P_{1/2}$ of 27.5 mm Hg would be close to ideal. Under equilibrium conditions, it would cycle between 15% and 85% O_2 saturation, even if no cooperativity in oxygen binding existed. This is illustrated in Fig. 11. Of course, if the immobilized hemoglobin retained cooperativity, the yields would be increased, as also shown in Fig. 11. The successful development of a practical oxygen unloading system then appeared to depend on preparation of a relatively low oxygen-affinity form of immobilized hemoglobin.

Benesch et al. (*11*) demonstrated that a pyridoxal derivative of hemoglobin can be fixed covalently as a "bound diphosphoglycerate" analog and have lowered hemoglobin oxygen affinity. The reduction in affinity, though, is not to the "ideal" level described above. A survey of the literature on mutant human hemoglobins was also carried out, with the idea that these genetic changes could provide us with leads to appropriate chemical modifications. The survey showed that most mutant forms had higher oxygen affinity, although some modifications involving the binding site for DPG were lower in affinity than normal hemoglobin in the absence of allosteric effectors (*12*). No dramatically lower oxygen affinity forms that were attainable by chemical modification were suggested by this survey.

It appeared from the literature (*13*) that cobalt hemoglobin (coboglobin) has a low oxygen affinity and would therefore fit the "ideal" requirements. Coboglobin is a modified form of hemoglobin in which the iron of the heme is substituted by cobalt. We therefore prepared cobalt hemoglobin and immobilized it on silica by our standard procedures. In general, the preparation of cobalt hemoglobin by the literature methods proceeded well. One major difficulty in using coboglobin as the basis for an oxygen absorber is in the lack of analytical methods for determining the oxidation state of the cobalt atom. With normal hemoglobin, one can spectrophotometrically determine the oxy-, deoxy-, and ferric- (met) states. With coboglobin, the oxy- and met- states are practically indistinguishable by optical spectrophotometry. In addition, the reduction of the nonbinding met-form to functional coboglobin proceeds very slowly upon the addition of dithionite, in contrast to the situation with hemoglobin.

In spite of these problems, we succeeded in preparing coboglobin in fair yield and in functional form. Prior to immobilization, the coboglobin had a low affinity for oxygen ($P_{1/2}$ about 80–100 mm Hg). Immobilization of the coboglobin on silica by our standard method was uneventful (more than 50 mg/g substrate) and the product was functional. The oxygen affinity of the immobilized coboglobin was determined to be in the desired range, having a $P_{1/2}$ of approximately 40 mm Hg.

In small fluidized-bed reactors, both oxygen uptake and release by coboglobin was good. The slope of the oxygen uptake curve was somewhat lower than for the

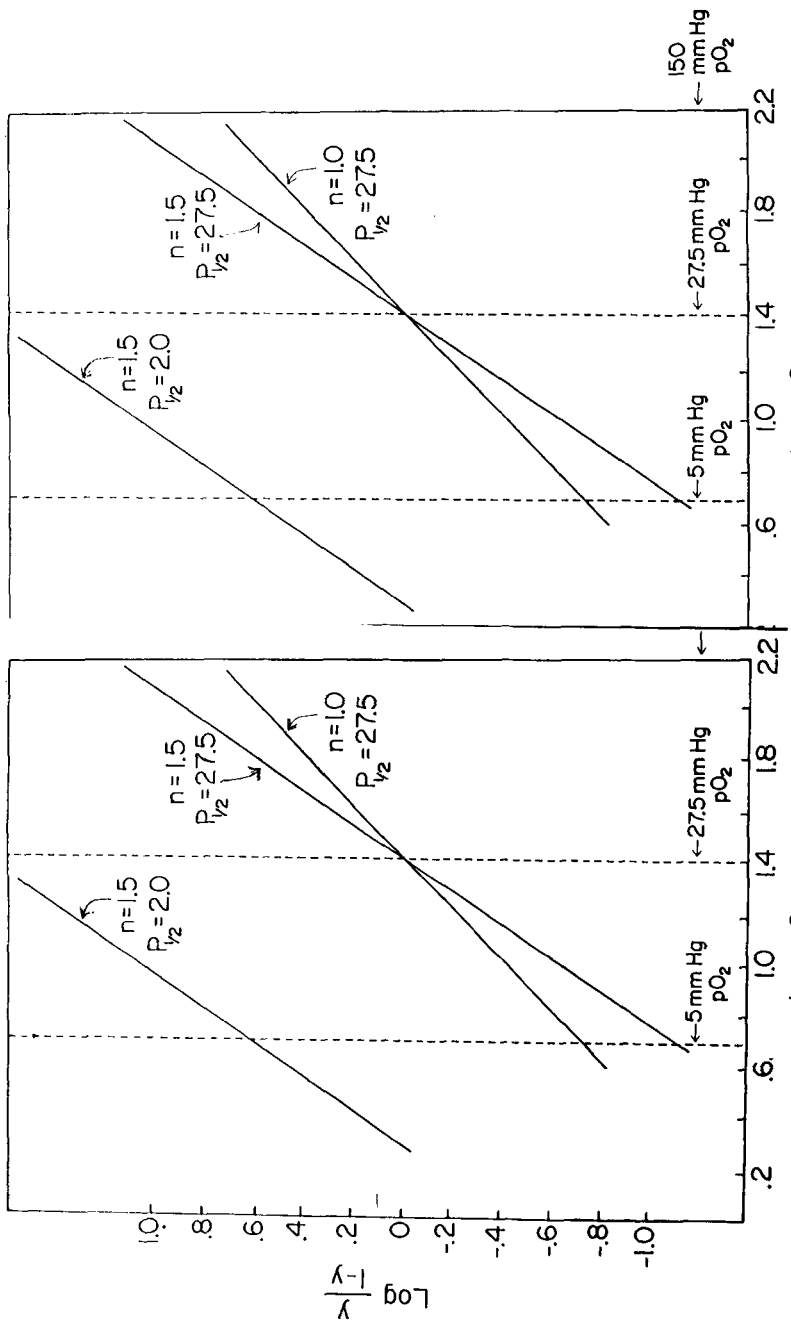

Fig. 11. Theoretical oxygen recovery of oxygen from reversible oxygen binders. Oxygen binding by high affinity hemoglobin ($P_{1/2}$ = 2.0 mm Hg, n = 1.5) compared to oxygen affinity hemoglobin ($P_{1/2}$ = 27.5 n = 1.5). Note the importance of both parameters in terms of oxygen gradient that would lead to significant loading and unloading of oxygen. The quantity y stands for % oxygenation of the binder. Values of log $y/1-y$ of 1.0 and -1.0 correspond to 90% and 10% oxygenation, respectively; n is a measure of the cooperativity of the hemoglobin.

higher oxygen affinity hemoglobin preparations, a result of the lower oxygen affinity. The oxygen uptakes, however, were satisfactory, and the reactor could be quickly loaded to a desired level. Using a nitrogen purge system for the unloading of O_2 from coboglobin, we obtained reasonable values for the percent oxygen yield from these systems, indicating that more than 50% of the oxygen carrier present was functional. The most encouraging feature of these studies was the fact that oxygen unloading seemed to be complete in 2 min, in contrast to the rather incomplete unloading in 20 min that we found for high oxygen-affinity hemoglobins. Given the fact that the overall yield is diminished by the nonfunctional coboglobin in the reactor and by the incomplete oxygen saturation in the uptake cycle (because of the lowered oxygen affinity of coboglobin), the yields obtained were excellent!

It appears that the system developed, using coboglobin immobilized on a ceramic support, loaded with oxygen in a fluidized bed and unloaded by decreasing the oxygen partial pressure, meets many of the requirements for an effective oxygen-producing system for a relatively large number of people. It appears possible that a synthetic heme or heme analog (*14*) can be developed that will meet the system's requirements. These compounds, vastly simpler than hemoglobin, could be bound on ceramic or polymeric supports.

If oxygen can be extracted from seawater, it is not unreasonable to consider its extraction from other fluids. Commercial extraction of oxygen from liquified air for example, is an important commercial process. Although fractional distillation of liquified air is currently the most widely used method, selective membranes have successfully been used to enrich gases. Along these lines, immobilized oxygen carriers might be of value in the selective removal of oxygen from air streams. Any such stream, depleted of oxygen, would become more "noble." Conversely, an air-processing reactor might be used in oxygen-enrichment of air for internal combustion engines. Not only might air be separated into pure oxygen and essentially pure nitrogen, but fluids might also be similarly treated. Such treatment of oxygen-labile liquids might be of considerable industrial importance. Depending upon the chemical nature of the liquid (pH, polarity, etc.), a suitable oxygen reactor could be based on either hemoglobin, hemerythrin, hemocyanin, or some synthesized oxygen carrier. Immobilized oxygen carriers might be used to supply oxygen to immobilized cells in microbial cell-based reactors or to oxygen-requiring reactions. They might also serve as blood substitutes. Many of these applications are described in a recently issued patent (*15*).

In conclusion, the 3 H's, hemoglobin, hemerythrin, and hemocyanin, evolved to carry out very important tasks in aerobic organisms. By virtue of great advances made in the field of immobilization of biochemical species, we may now be in a position to expand the role of these oxygen carriers to do a great deal more in terms of isolation, delivery, and utilization of oxygen.

Acknowledgment

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