

## The Search for Blood Substitutes by Mary L. Nucci and Abraham Abuchowski

Before reading this article, review pages 204-221 in *Lehninger Principles of Biochemistry*, 3e.

In Chapter 7 of *Lehninger Principles of Biochemistry*, 3e, our discussion of protein function introduces two of the most important functions of human blood: oxygen transport and immunity. Oxygen transport relies on red blood cells and their cargo of the oxygen-binding protein hemoglobin. Immunity is conferred by white blood cells and the antibodies and related proteins they synthesize. These are by no means the only life-sustaining roles of blood. As this article describes, blood is a pH buffer; it contributes to osmotic balance; it clots when tissue is damaged; and it transports a wide range of nutrients and molecules in addition to oxygen.

With blood donations declining and the risk of HIV and hepatitis infection still a factor in any discussion of the blood supply, the effort to find a safe and storage-stable alternative to whole blood has been renewed. Finding a blood substitute to replace all of the major functions of blood is probably well beyond the capacity of current technology. Current efforts focus on the replacement of the oxygen transport function of blood for use in emergencies or during surgery, when blood loss can be immediately life threatening.

As the authors relate, there are two major types of blood substitute currently in clinical trials. One is based on a class of compounds called perfluorocarbons. These are short molecules (usually 8-10 carbons) highly substituted with fluorine. They are not very soluble in water, but when combined with the right emulsifying agents they form a suspension that remains in liquid form in an aqueous solution. Although intended to replace the function of hemoglobin, these molecules do not bind oxygen like hemoglobin, but instead greatly enhance the solubility of oxygen in solution. The amount of oxygen present becomes a simple linear function of the partial pressure of the surrounding air. When these compounds are injected into a patient, oxygen is dissolved in much higher than normal levels in the lungs and delivered to tissues where the oxygen partial pressure is lower. Problems associated with this technology are well described in the article.

The alternative approach is to use hemoglobin itself, but without the red blood cells. The problems associated with the use of solutions of free hemoglobin nicely complement the discussion of the structure and function of this important protein in Chapter 7.

### FURTHER READING

- Alayash, A.I. (1999) Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? *Nature Biotechnol.* 17, 545-549.
- Clark, L.C. & Gollan, R. (1966) Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. *Science* 152, 1755-1756.
- Remy, B., Deby-Dupont, G., & Lamy, M. (1999) Red blood cell substitutes: fluorocarbon emulsions and hemoglobin solutions. *Br. Med. Bull.* 55, 277-298.

### QUESTIONS

1. The authors state that 19th century trials using hemoglobin as a blood substitute resulted in kidney damage and the death of the canine subjects. Part of the problem was the dissociation of the hemoglobin tetramer into dimers. From what you know about the structure of hemoglobin, which types of subunits ( $\alpha$  or  $\beta$ ) were present in the dimers?
2. The amount of oxygen dissolved in a perfluorocarbon (PFC) solution increases as a linear function of the partial pressure of oxygen in the surrounding atmosphere. How does this property tell you that the oxygen is not binding to anything in the solution? (How would the amount of oxygen in solution respond to oxygen partial pressure if an oxygen-binding protein were present?)
3. Hemoglobin in solution can be made into a useful oxygen carrier if it is modified and/or crosslinked. The types of modifications are not specified in the article. Which conformation of hemoglobin, T or R, would be required for hemoglobin to function well in the bloodstream?
4. The PFCs used as blood substitutes tend to evaporate as gas quite rapidly. Why is this property useful?
5. What chemical properties (besides oxygen solubility) must a PFC solution possess to function as a safe blood substitute?

## *The threat of global shortages of blood and fears about contamination have hastened attempts to find life-sustaining alternatives. Several of these compounds look promising*

In the mid-1980s blood became a bad word. Reports that HIV—the human immunodeficiency virus, which causes AIDS—was being transmitted through transfusions led to general panic among the public and to the institution of new massive screening procedures for U.S. blood banks. In some places, including France, certain physicians maintained that the blood supply was safe—when, tragically, it was not. As a consequence, fear has not abated. Although the chance of contracting HIV through a transfusion is between one in 450,000 and one in a million, the perception remains that most blood is tainted.

This reputation, ill deserved as it may be, is one of the two major problems facing blood banks today. The second dilemma concerns supply. In the U.S., a country where someone requires a transfusion every three seconds or so, the number of blood donors continues to fall: no more than 5 percent of the population now gives blood. At the same time, the group of people who most often require transfusions, the elderly, is growing. Although estimates vary, it appears that every year the world needs 7.5 million more liters of blood; by 2030, experts anticipate there will be an annual shortfall of four million units (a unit is 500 milliliters) in the U.S.

For these reasons and others, the race is on to find blood substitutes. Although researchers have been investigating the possibilities since the 1950s, efforts redoubled after the Food and Drug Administration, the National Institutes of Health and the Department

of Defense held large conferences in the 1980s on the need to develop such compounds. Scientists at several institutions and six companies have already developed substitutes. But although there is great progress and hope, the challenges remain enormous. A decade into the campaign, no perfect solution is visible on the horizon. It is, after all, the essence of life that these investigators, ourselves among them, are trying to understand and manufacture.

### **The Heart of the Matter**

Blood is as complex as the challenge of finding a substitute suggests. It is made up of blood cells, salts and other substances, such as proteins and vitamins, that are suspended in plasma. The three kinds of blood cells—red cells, white cells and platelets—comprise about 45 percent of the volume of blood: normally, a cubic centimeter of human blood contains between 4.5 million and 5.5 million red cells, between 7,000 and 12,000 white cells, and between 150,000 and 400,000 platelets.

This complex of cells and compounds performs myriad tasks: blood transports nutrients, hormones and waste products; it defends the body against infection; and its ability to clot prevents blood loss. Yet by far the most familiar function of blood is its role in respiration and its capture and release of oxygen and carbon dioxide. The protein hemoglobin—250 million molecules of which can be found inside each red blood cell—is the key to this process.

The most common protein in blood, hemoglobin is found in most vertebrates and has been conserved through evolution—that is, it looks remarkably similar in different species and is always composed of four polypeptide chains. In humans, hemoglobin consists of two identical alpha and two identical beta chains, each one about 140 amino acids long. An alpha binds strongly to a beta, creating a dimer; the two alpha-beta dimers

then bind weakly to each other, creating a tetramer.

Each of these polypeptide chains contains a heme unit, which, in turn, contains a molecule of iron. These iron atoms are the binding sites for oxygen; thus, each molecule of hemoglobin can bind four molecules of oxygen. Hemoglobin picks up oxygen in the lungs and transports it throughout the body. The more oxygen the hemoglobin molecule binds, the more adept it becomes at picking up the gas. This is because grabbing a molecule of oxygen changes the shape of the hemoglobin molecule; this change in configuration literally opens hemoglobin to more oxygen, until the four iron molecules are filled. Once the oxygen is released from the hemoglobin in various parts of the body, the red blood cells pick up carbon dioxide—a waste product of cellular respiration—which travels through the blood to the lungs, where it is released and, ultimately, exhaled.

Hemoglobin can also pick up other gases. For instance, researchers recently discovered that hemoglobin can transport nitric oxide. Nitric oxide has an important role in, among many other functions, maintaining blood pressure [see “Biological Roles of Nitric Oxide,” by Solomon H. Snyder and David S. Bredt; *SCIENTIFIC AMERICAN*, May 1992]. Hemoglobin thereby serves as a vital shuttle, carrying gases that are essential both to the body and to its own smooth functioning.

### **Lifeblood**

Obviously, severe blood loss threatens many important processes. If people lose 30 to 40 percent of their blood, their bodies can compensate by quickly producing red blood cells, by moving blood away from nonessential organs and by shunting fluid into circulation in order to restore blood volume. But, depending on the age and health of the individual, once a person loses more

**TRANSFUSION** must occur once a person has lost more than 40 percent of his or her blood. In the U.S. alone, a transfusion takes place about every three seconds—and some 12 million units of blood are used every year. As the population ages, the need for transfusions is increasing; by 2030, there will be an estimated shortage of four million units of blood.

than 40 percent of his or her blood, a transfusion is generally needed.

Transfusions have a long, somewhat murky history. Various fluids have been tried throughout the centuries, including ale, urine, opium, plant resins, milk and sheep's blood. In 1667 Jean-Baptiste Denis, a physician to Louis XIV, performed the first documented, successful human-to-human blood transfusion. The procedure was banned in France—as well as in Rome and England shortly thereafter—when the wife of one of Denis's transfusion patients sued him. It turned out that the man had died not as a result of transfusion but rather because his wife had poisoned him with arsenic. Nevertheless, the reputation of transfusions had been tarnished. Per-

haps for good reason, because those that were performed resulted as frequently in death as they did in prolonged life.

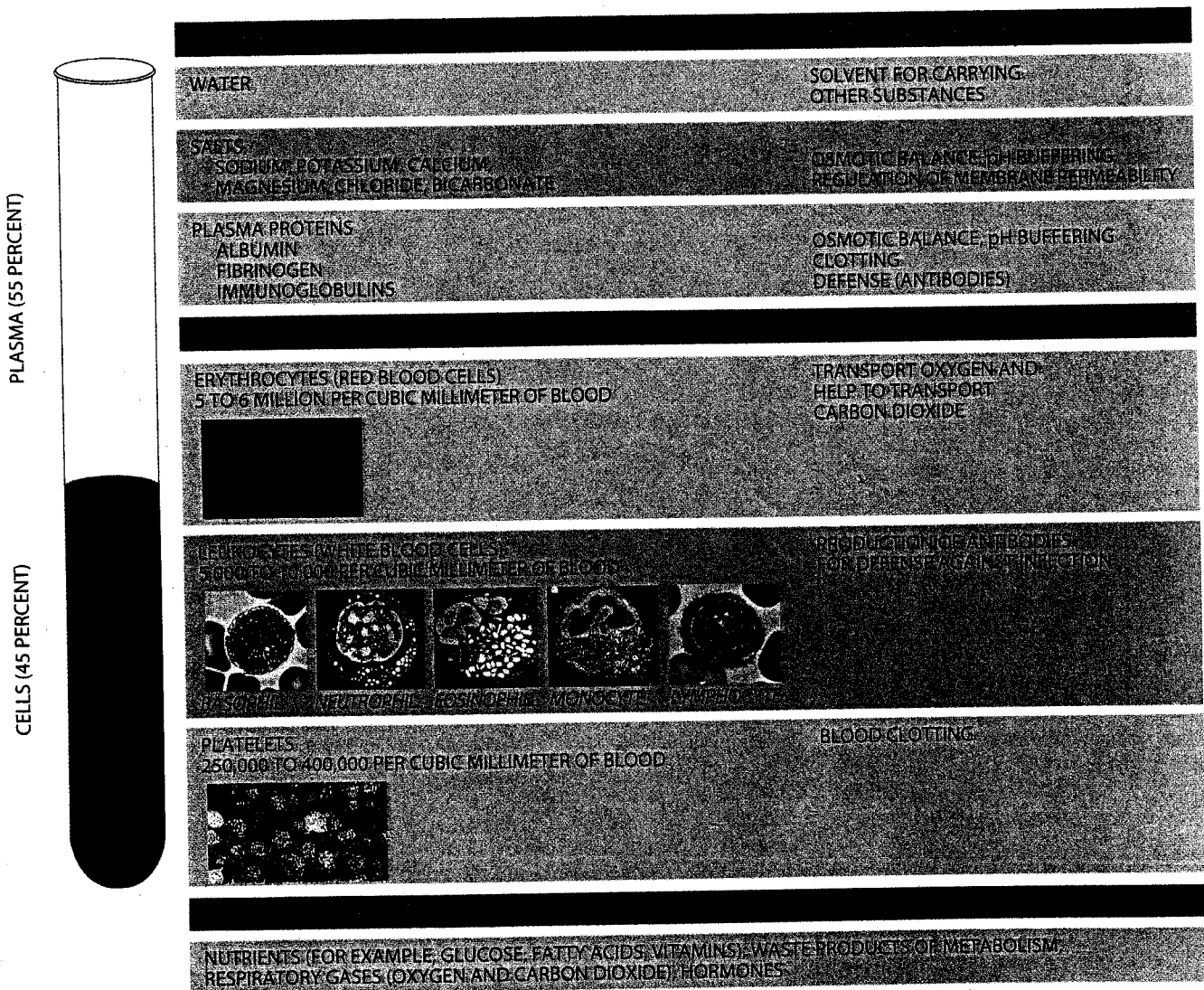
Medical interest in the procedure did not really reawaken until the early 1900s, when Austrian-American pathologist Karl Landsteiner discovered the ABO blood group system and greatly improved the success of transfusions. Landsteiner found that two sugars—he called them A and B—can adorn the surface of red blood cells and that each individual has some combination, or lack, of these two sugars. Today physicians know that there are four such combinations and, hence, blood types. If these types are mixed during a transfusion, antibodies found in the bloodstream of the patient react against the sugars,

which are called antigens, on the surface of the donor's red blood cells. This reaction causes tiny clots, hemolysis (when hemoglobin leaks out of red blood cells) and, subsequently, death.

Matching must therefore be precise. Type A can be given to a person with A or AB blood; type B to someone with B or AB blood; and AB can be given only to another AB type. Type O, which has neither A nor B antigens, can be given to anyone—making those with O universal donors—but type O individuals can accept only O. Finally, AB types are universal acceptors: they can receive A, B, AB or O.

Blood typing must also account for the Rh groups. Working with rhesus monkeys in the 1940s, researchers discov-

## THE COMPOSITION OF BLOOD



SOURCE: *Biology*, by Neil Campbell © 1990

**MANY TASKS** performed by blood account for the fluid's complexity. Made of almost equal parts plasma and cells, blood or-

chestrates the transport of nutrients and chemical messengers, while regulating respiration, clotting and immune function.

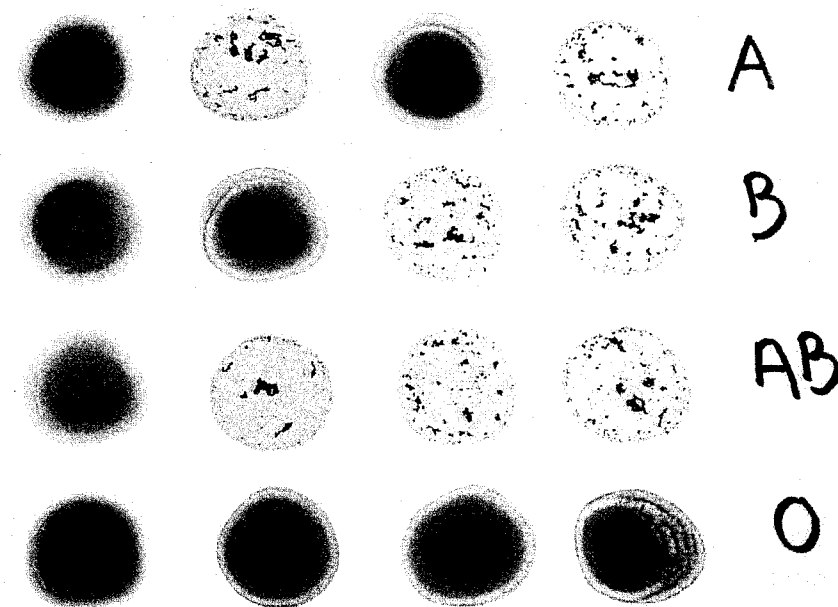
ered that blood can have an Rh antigen, in which case it is called Rh+. Absent that antigen, the blood is labeled Rh-. Transfusions must give Rh+ blood only to Rh+ patients; Rh- blood, however, can be given to both Rh+ and Rh- individuals.

More than 23 million units of blood are transfused each year, the American Association of Blood Banks reports. The risk of dying from a blood transfusion is about one in 100,000—as compared with a two-in-100,000 chance of dying in a car accident or a one-in-10,000 chance of dying from influenza. This risk includes the possibility of blood-typing errors as well as infection from bacteria and viruses. As noted earlier, transfusions have contained HIV. As of June 1997, 8,450 people had developed AIDS as the result of contaminated blood, according to the Centers for Disease Control and Prevention; this number does not reflect actual infection, because AIDS symptoms take years to develop. Transfusions can also introduce various forms of the hepatitis virus. A recent study in the *New England Journal of Medicine* states that a person has a one-in-63,000 chance of contracting hepatitis B and a one-in-103,000 chance for hepatitis C.

### The Two Paths

A successful blood substitute has to meet a minimum, but hefty, set of requirements. It has to be nontoxic, free from disease and easily transportable; it cannot elicit an immune response; and it has to work for all blood types. The compound also has to remain in circulation until the body is able to restore its own blood, and then it should be excreted without side effects. Because storage is so difficult and expensive—blood must be kept at four degrees Celsius, and, even then, it stays fresh for a maximum of 42 days—a good substitute should have a long shelf life. And, if it were to be ideal, the mimic would perform blood's many tasks.

Blood substitutes on or coming on the market seek to meet all these requirements except the last; they focus only on reproducing blood's most basic duty—that of transporting oxygen. (They differ from what are called volume expanders—such as saline, plasma or dextrose solution—which have been developed simply to increase blood volume, not to restore any of its other functions.) Over the years, two major approaches to developing blood substitutes have emerged:



**BLOOD TYPE** is defined by the presence or absence of antigens—A, B and Rh—on the surface of red blood cells. Type A blood is so called because it carries antigen A, type B carries only B, type AB has both and type O has neither. In addition, blood is termed Rh+ if it has the Rh antigen; otherwise it is Rh-. Blood must be carefully matched so that a transfusion does not provoke a fatal immune reaction in the patient.

one is based on chemicals; the other relies on hemoglobin.

Proponents of the chemical-based solution rely on synthetic oxygen-carrying compounds, known as perfluorocarbons (PFCs)—compounds similar to Teflon. Advocates of the hemoglobin-based strategy argue that blood's ability to capture and transport gases can be reproduced only by using the real thing. (Other research is ongoing but has not yet yielded any products that are in clinical trials, including the production of hemoglobin in transgenic animals, the alteration of the surface of red blood cells to produce universal donor red cells, the freeze-drying of red blood cells and the encapsulation of hemoglobin into liposomes—or so-called neo red cells.)

PFCs can dissolve large quantities of gases such as oxygen and carbon dioxide. They were made famous in the 1960s, when Leland Clark of the University of Alabama showed that a mouse immersed in PFC liquid could breathe relatively normally. A more recent example figured in the 1989 movie *The Abyss*, in which a character survives a deep-ocean dive by "breathing" an oxygen-carrying liquid. Because they are inert and will not dissolve in plasma, PFCs being developed today must be emulsified with an agent that permits them to form particles that can then disperse in blood.

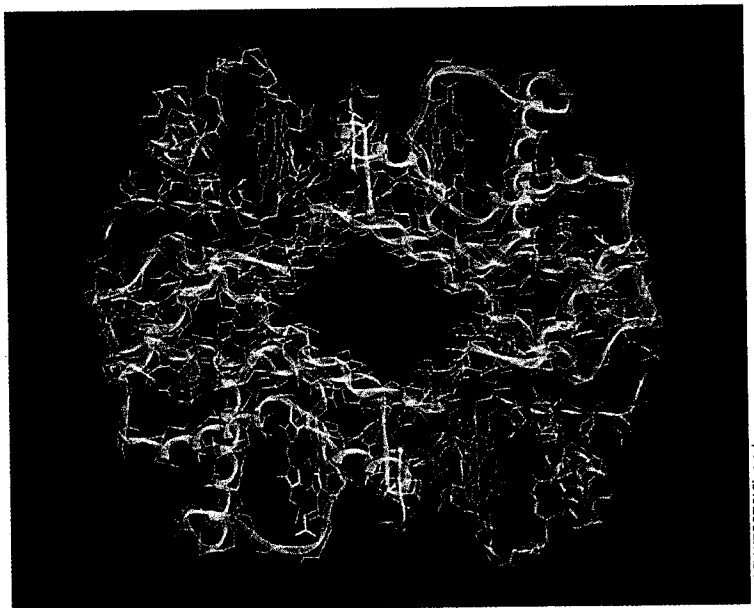
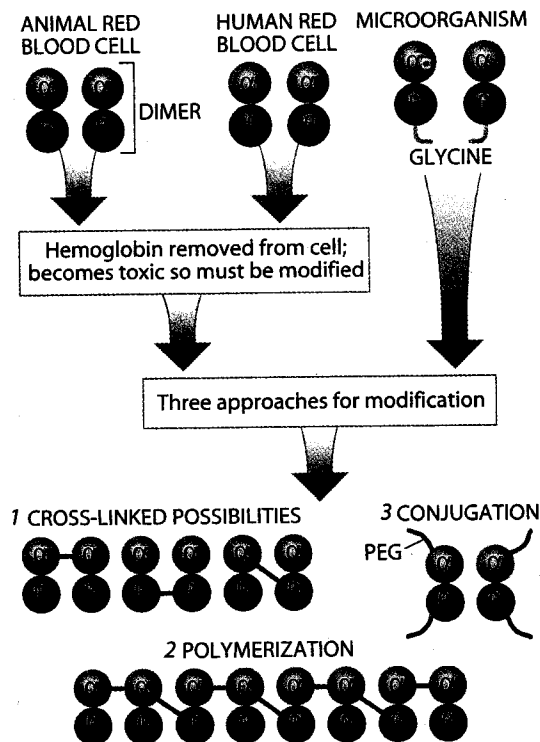
Unlike hemoglobin—which actively grabs and frees the oxygen—perfluorocarbons deliver gas passively. Oxygen from the lungs moves directly to the PFCs that are floating in the plasma, bypassing the red blood cells; the PFCs then travel to the rest of the body, where they diffuse out in the capillaries, exchanging oxygen for carbon dioxide.

One benefit of PFCs is that the amount of oxygen they can pick up is directly proportional to the amount of oxygen breathed in. So a patient can be given gas with a higher partial pressure of oxygen than is found in room air, and the PFCs can absorb and transport more of it. PFCs can also transfer gases quickly, because the gases do not have to diffuse across the membrane of a red blood cell. Hemoglobin molecules, on the other hand, can carry only four molecules of oxygen at any one time—regardless of the amount of oxygen available—and the gas must move across the blood cell membrane.

PFCs are cleared from the circulation by the reticuloendothelial system, which stores the droplets in the spleen and liver until they are exhaled as vapor by the lungs. The droplets are cleared within four to 12 hours of injection of PFCs into the body, but little is known about the long-term effects of PFC retention.

When PFCs were first used as blood substitutes in mice in the 1960s, they

## SOURCES OF HEMOGLOBIN



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**HEMOGLOBIN-BASED SUBSTITUTES** take one of three forms. Hemoglobin (above) gathered from humans or animals—or from organisms genetically engineered to produce it—can be toxic when not housed in a red blood cell. So researchers cross-link (1), polymerize (2) or attach polyethylene glycol, or PEG (3), to render the hemoglobin safe. Any of these configurations can be used to make blood substitutes.

had a serious drawback: they were not well excreted and would accumulate in body tissues. In the 1980s, however, a new version of PFC entered clinical development. This compound, Fluosol-DA (made by Green Cross Corporation in Osaka, Japan), was approved in the U.S. for use in select patients, including some who refuse blood transfusions because of religious beliefs. But storage complications, side effects and low efficacy prevented it from achieving widespread use.

The next set of PFC-based substitutes, designed to overcome these problems, is currently in clinical development. One called Oxygent (made by Alliance Pharmaceutical Corporation in San Diego) has a shelf life of two years if it is refrigerated. Other, newer PFCs deliver up to four times as much oxygen as their earlier versions did. Yet increasing the oxygen-carrying capacity of blood can lead to the accumulation of oxygen in tissues, which, in turn, can cause damage. Clinical studies of PFC-based substitutes are under way, and researchers are trying to find ways to reduce side effects.

By far the greatest number of researchers working on blood substitutes have focused on manipulating the structure of hemoglobin. Apparently, the compound was tested as a blood substitute as early as 1868, when an experi-

menter injected dogs with hemoglobin. The results were not promising. The dogs became ill and suffered severe kidney damage, and their blood's ability to transport oxygen decreased. It became apparent that when the dog's hemoglobin was naked—that is, it lacked the envelope of the red blood cell—it became unstable. It broke down into its dimers, passed into the kidneys, where it caused damage, and was finally excreted in just a few hours. The subunits were so small that they could not be filtered by the renal system or returned to the body.

### Looking to Hemoglobin

The same problems can arise with human hemoglobin. To be effective, hemoglobin must contain a compound known as 2,3-diphosphoglycerate (2,3-DPG), which is present only in red blood cells. Without 2,3-DPG, hemoglobin binds oxygen in the lungs but will not release it elsewhere in the body. Without 2,3-DPG and other elements of red blood cells, hemoglobin is also prone to auto-oxidation—during which the iron atoms change state and irreversibly lose their ability to bind gas molecules.

In 1969 scientists discovered that they could reverse this process by chemically modifying unbound hemoglobin. At this point, it again became possible to

consider using the compound as a blood substitute. Researchers have subsequently found several modifications effective: cross-linking alpha-alpha, beta-beta or alpha-beta chains; polymerization of hemoglobin molecules; or conjugation with a polymer called polyethylene glycol (PEG), a compound found in some foods and cosmetics. Because modification usually increases the size of the hemoglobin molecules, renal damage can be avoided, and the substitute does not leave the body so quickly.

Thus far, five products are being tested in people in the U.S. Several of these are made from donated blood that is too old to use. PolyHeme, manufactured by Northfield Laboratories in Evanston, Ill., is a polymerized human hemoglobin that is being evaluated as a replacement for blood during surgery. This compound is prepared by pyridoxylation, which involves reshaping of the hemoglobin molecule to improve its oxygen-carrying capabilities, and then polymerization to increase its size. HemAssist, made by Baxter International in Deerfield, Ill., is also prepared from outdated human blood. This form of cross-linked hemoglobin is being tested during cardiac surgery as well as in people suffering hemorrhagic shock and in trauma patients.

Other products use different approach-

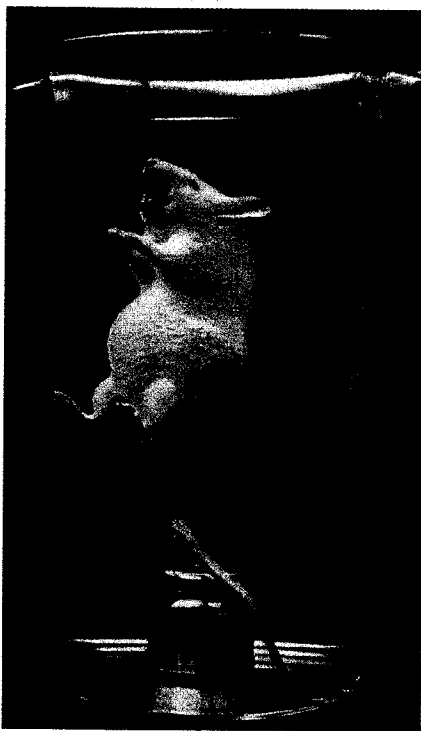
es. Optro, by Somatogen in Boulder, Colo., cross-links human hemoglobin subunits and is produced recombinantly in *Escherichia coli*. And Hemopure, by Biopure in Cambridge, Mass., is made of polymerized hemoglobin from cow's blood. This substitute is being studied for use in trauma, surgery and sickle cell anemia.

Our research, conducted at Enzon in Piscataway, N.J., also centers on bovine hemoglobin. Such hemoglobin is cheap, readily available and, unlike human hemoglobin, does not require the presence of 2,3-DPG to deliver oxygen. By combining bovine hemoglobin with PEG, we have been able to stabilize the molecule, make it larger and increase the time it can spend in the body.

PEG-hemoglobin is currently being evaluated as a way to enhance the radiation treatment of certain solid tumors. Many such tumors have low levels of oxygen at the core, and because radiation therapy requires oxygen to be effective, they resist radiation. Using PEG-hemoglobin, however, some physicians have been able to get oxygen to the tumor. The substitute is also being used for the treatment of stroke and ischemia: because the free hemoglobin is smaller than a red blood cell, it can travel into blocked blood vessels, delivering oxygen to oxygen-starved cells.

### Safety in Substitutes

Despite the evident progress, efforts to find a substitute for blood continue to be plagued by lack of success. For every advance, there is a retreat. Because of the volume that would be administered to each patient, researchers must address safety concerns that do not arise for therapeutics administered in smaller amounts. Most drugs are dispensed in milligrams; hemoglobin-based substitutes will be given in amounts as great as 50 to 100 grams. This is because blood substitutes are also used for the purpose of restoring blood volume



COURTESY OF SYNTHETIC BLOOD INTERNATIONAL, INC.

**LIQUID-BREATHING MOUSE** is able to survive because of the presence of perfluorocarbons. These compounds can dissolve large quantities of oxygen—thereby allowing this mouse to respire, as Leland Clark showed in the 1960s in this famous photograph. Perfluorocarbons are used in one form of blood substitute.

in addition to oxygen-carrying capacity.

Further, the long-term effects of these substitutes are not known. All those being tested have shown some short-term toxicity—including hypertension, renal shutdown and damage, increased heartbeat, and gastrointestinal pain. Because most blood substitutes will be administered in life-threatening situations, researchers will have to prove that short-term benefits outweigh long-term risks—as well as risks arising from chronic use.

Each form of blood substitute also faces its own hurdles. PFC-based compounds must address the problems of retention, toxicity, a short circulating life and the dangers of delivering too much oxygen. Blood substitutes using outdated human blood are faced with the problem of finding enough source material. Genetically engineered, recombinant hemoglobin will have to be produced in staggering amounts—53,000 kilograms annually—to meet just 10 percent of the U.S. demand; such pro-

duction requires massive, costly facilities. Finally, bovine-based substitutes must address the danger of transmission of bovine spongiform encephalopathy and perhaps other not yet identified diseases. Manufacturers can avoid using cows that have been fed the animal by-products blamed for the spread of mad-cow disease, but they must ensure that no other disease-causing agents are present and that there are no adverse immune responses for humans receiving a cow-based product.

Despite these challenges, blood substitutes in clinical trials will be available in the near future. Once approved by the Food and Drug Administration, the products will face the ultimate test: the consumer. What will the consumer be willing to pay for such a product? Or, more important, what will health care providers be willing to pay? Considering the enormous expense of development, these products will undoubtedly cost two to five times more than blood; hospitals currently pay between \$60 and \$85 for one unit of blood, and handling costs often raise that to as much as \$240 per unit. The blood substitute market is estimated to be \$5 billion a year—and that is in the U.S. alone.

Issues of cost aside, however, blood substitutes could significantly improve health care. Forestalling blood shortages and alleviating fears of contamination would be only two of the benefits. Physicians could use substitutes for organ preservation, in the treatment of both anemia and sickle cell anemia and during angioplasty, as well as other procedures. The possibilities are endless. ■

### The Authors

MARY L. NUCCI and ABRAHAM ABUCHOWSKI have collaborated on blood substitute research since the late 1980s. Nucci has served as an immunologist at Enzon, a company Abuchowski helped to found. The two recently left Enzon to start New Paradigm Consulting, a medical and scientific consulting group based in New Jersey. In addition to his work on blood substitutes, Abuchowski, who established the Biotechnology Council of New Jersey in 1994, has conducted research on childhood leukemia and has long been devoted to development of drugs for rare conditions.

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