BLOOD SUBSTITUTES

1. Introduction

Artificial blood is herein defined as consisting of red cell substitutes. Red cell substitutes are solutions intended for use in patients whose red cells are either not available or their use is to be avoided for other reasons. Efforts to produce red cell substitutes began when the circulation of the blood was discovered (1), and the current group of potential products have been under development for the last one-half of the twentieth century.

In 1983, the move to develop red cell substitutes intensified when recognition that the acquired immune deficiency syndrome (AIDS) could be transmitted by the blood-borne human immunodeficiency virus (HIV) produced grave concern for the nation's blood supply. Since that time modernized blood bank methods have dramatically reduced the risk of transfused blood. Furthermore, indications for transfusion have been reevaluated, and the use of blood products has become much more efficient. More careful screening of donors, testing of all donated units, and a general awareness in the donor population have all contributed to a decreased risk from transfusion-contracted AIDS.

The idea of red cell substitutes is not new. In Ovid's Metamorphosis the witch Medea restored Jason's aged father, Acson by slitting his throat to let out old blood, replacing it with a magic brew she had concocted (2). Sir Christopher Wren was one of the first to apply the new knowledge about circulation to blood substitutes. In 1656, he infused ale, wine, scammony, and opium into dogs and from these efforts conceived the idea of transfusing blood from one animal to another. Lower actually carried out the first transfusion experiments (3). The early history of blood substitute research has been summarized (4).

2. Historical Blood Substitutes

2.1. Milk. Milk, one of the first materials to be used as a red cell substitute (5,6), was used in cases of Asiatic cholera in 1854. It was suggested that milk could regenerate white blood cells (5). Two patients were given 340 g or more of

cow's milk and did well, but two others died (7). In all, 12 cases of injection of milk into the circulatory system were reported, and it was concluded that using milk in place of blood was a feasible, safe, and legitimate procedure. These results were met with excitement, and it was thought that milk injections would supplant the dangerous transfusion of blood (8). Milk was also shown to support function in isolated, perfused hearts from a variety of mammals (9). However, the transfusion of milk never gained widespread favor and soon disappeared from the literature.

2.2. Normal Saline. In the laboratory, the search for a red cell substitute was directed at understanding the physiologic role of blood and its many components. Some of the early work involved frogs. Salzfrosche-frogs, where the blood was completely washed out and replaced with a pure sodium chloride solution, survived for some hours (10). Urea-frogs and sugar-frogs lived longer; if a small amount of red cells remained, they could survive indefinitely (11). But frogs are simple animals, and a frog's nervous system can be kept alive for some time without any circulation at all. Normal saline is, however, used widely as a plasma volume expander.

2.3. Ringer's Solution. In 1883, the excised ventricle of the frog was found to beat for some hours if supplied with an aqueous solution of sodium, potassium, and calcium salts (12). The concentration of potassium and calcium was critical, whereas the amounts of the anions had little effect on the frog heart. The composition of this saline, coined Ringer's solution, after the English physiologist Sydney Ringer, is given in Table 1. Many years later it was shown to be very close to that of frog plasma.

Ringer's lactate, in which salts of lactic acid (in Europe, salts of acetic acid) are added to Ringer's solution, is probably the most popular crystalloid (ie, noncolloid) solution for intravenous use in humans. The lactate is gradually converted to sodium bicarbonate within the body so that an uncompensated alkalosis is prevented (13). These crystalloid solutions cannot support life without red cells; saline passes rather quickly into the tissue spaces of various organs (14), especially the liver (15).

2.4. Gum-Saline. Gum is a galactoso-gluconic acid having a molecular weight of ~1500. First used in kidney perfusion experiments (16), gum-saline enjoyed great popularity as a plasma expander starting from the end of World War I. The aggregation state of gum depends on concentration, pH, salts, and temperature, and its colloid osmotic pressure (often just "oncotic" pressure, or COP) and viscosity are quite variable. Conditions were identified under which the viscosity would be the same as that of whole blood (17).

Table 1. **The Composition of Ringer's Solution**[a]

	Ringer's solution		Frog plasma
	g/100 mL	mEq/L	mEq/L
NaCl	0.6	102	104
KCl	0.0075	1	2.5
$CaCl_2$	0.01	1.8	1.0
$NaHCO_3$	0.01	1.2	25.4

[a] See Ref. (4).

In early animal studies, gum was found to coat the surface of all blood cells and to promote coagulation. The use of gum-saline became popular in World War 1, but it was soon discovered that if the hematocrit was <25%, gum-saline was not efficacious in hemorrhagic shock. In fact, gum-saline was not as efficacious in treating hemorrhagic shock as was saline alone (14), but it was useful in temporarily stabilizing blood volume (18). Through the 1920s, reports of anaphylaxis and other untoward reactions appeared. Many of these were attributed to impurities and when properly purified, gum-saline was safe for human use. Pharmacologic studies in the 1930s (19) showed that gum was deposited in the liver and spleen and could remain there for many years. Its half-life in the circulation was ~30 h and anaphylaxis occasionally occurred. Success with gum-saline became common in the 1930s, but the need for it decreased with the increased availability of plasma.

2.5. Blood Plasma and Serum. The terms "plasma" and "serum" are frequently confused. Plasma is the liquid component of blood in which cellular elements are suspended. After removal from the body, and after a coagulum formed and was removed, the liquid that remained was serum. Thus, serum contains no coagulation factors or cellular elements.

As early as 1871, it was noted that frog hearts could be maintained by perfusion using sheep and rabbit serum (20). This solution was superior to 0.6% aqueous NaCl (21). Over ensuing years it was recognized that serum exerts a colloid osmotic pressure, contains bicarbonate, and may ensure capillary integrity. After dismissing a physiological role for plasma lipids, it was eventually agreed that albumin added to a balanced salt solution was superior to salt solution alone in maintaining the frog heart (22).

In the first one-half of' the twentieth century, much work was devoted to the study of plasma and serum as blood substitutes. One problem in this field was the recognition of toxic substances (23). Reports were published of intravascular coagulation and vasotonins that appeared mysteriously after the infusion of serum or plasma. It was suggested that this activity could be reduced by heating the serum or by filtering it before use. Platelets, insulin, and also adenosine triphosphate (ATP) were implicated. Once the red cell surface antigens were elucidated, these effects were attributed to immune reactions, and the use of serum from donors of blood group AB markedly reduced the vasoconstrictor activity.

World War II ushered in the modern era of blood fractionation. It was shown that plasma could be administered directly to humans (24,25). Although cases of serum sickness frequently occurred 5–7 days after the infusion, the procedure could be life-saving in eases of hemorrhagic shock (26) (see, FRACTIONATION, BLOOD).

2.6. Albumin. Investigation into the safety of bovine plasma for clinical use was undertaken in the early 1940s in anticipation of wartime need (27). Modern methods of protein chemistry, including electrophoresis and ultracentrifugation, have shown that many of the human adverse reactions to blood plasma were caused by the globulin fraction, and that albumin was generally safe for parenteral use. Human albumin is now used extensively as a plasma expander in many clinical settings.

2.7. Starches. The use of nonanimal sources of colloidal plasma expanders arose from the earlier use of gum, but with more controlled and predictable

chemistry. In the United States, the commonly used starch derivative is Hetastarch, a plant-derived waxy starch composed mainly of amylopectin. Hydroxyethyl ether groups are introduced into the glucose units of the starch, and the resultant material is hydrolyzed to yield a product with a molecular weight suitable for use as a plasma volume expander. For Hetastarch, the molecular substitution is ~0.75, which means there are ~75 hydroxyethyl groups for every 100 glucose units. The molecular weight ranges from 450 to 800 kDa. Recently, the U.S. FDA approved a novel formulation of Hetastarch. Its commercial name is Hextend, which combines Hetastarch with salts, including calcium, glucose, and lactate. Clinical trials with Hextend showed a significant improvement of the major concern with starches in general, prolonged, or increased bleeding (28,29).

2.8. Perfluorocarbons. In 1966, Clark and Gollan demonstrated that a laboratory mouse could survive total immersion in a perfiuorochemical (PFC) solution (30). This material, similar to commercial Teflon, is almost completely inert and is insoluble in water. A water-soluble emulsion was prepared that could be mixed with blood (31), and in 1968 (32) the blood volume in rats was completely replaced with an emulsion of perfluorotributylamine [311-89-71], $C_{12}F_{27}N$. The animals survived in an atmosphere of 90–100% O_2 and went on to long-term recovery. However, the O_2 content of the perfluorochemicals has a linear dependence on the partial pressure of oxygen (pO_2), as shown in Figure 1. The very high pO_2 required to transport physiologic amounts of O_2 and the propensity of the perfluorocarbon to be taken up by the reticuloendothelial cells were considered to be severe limitations to the development of clinically useful perfluorocarbon blood substitutes (33).

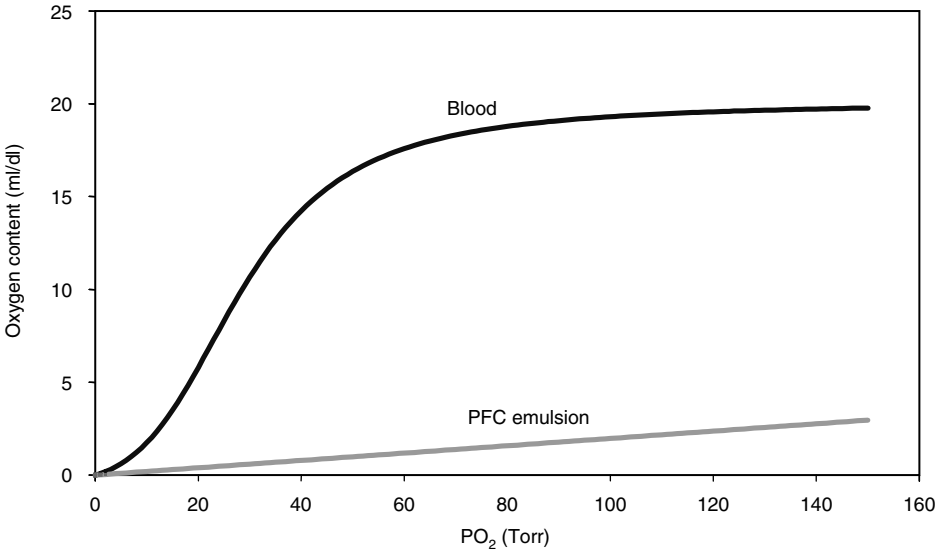

Fig. 1. Comparison of the oxygen capacity of isooncotic blood (14 g/dL) and a PFC emulsion. Note that the tetermeric structure of hemoglobin and its cooperativity lead to nearly complete saturation at the arterial oxygen partial pressure of ~100 mmHg.

One perfluorocarbon emulsion, Fluosol-DA, a 20% by weight emulsion, was licensed for use in coronary angioplasty. However, due to poor market acceptance and limited efficacy, it was subsequently withdrawn from the market by its manufacturer. An emulsion (oxygent) with a greater amount of perfluorocarbon (and, hence, O_2 capacity) was tested extensively, but also discontinued because of poor results in clinical trials (34).

2.9. Cell-Free Hemoglobin. Hemoglobin seems to be the logical choice for a red cell substitute because of its high capacity to carry oxygen (Fig. 1) and its oncotic properties. Probably the first treatment of anemic patients with hemoglobin solution occurred in 1898 (35). Although the results were encouraging, stable solutions could not be prepared and the studies were not pursued further. Better preparations were reported in 1916 (36) when very small amounts of hemoglobin were administered in an effort to discover its renal threshold. No untoward reactions were reported in 33 subjects.

After these reports, there were many attempts to administer hemoglobin solutions to humans. Some patients did well, but others demonstrated hypertension, bradycardia, oliguria, and even anaphylaxis. These untoward effects were not correlated with specific biochemical properties of the solutions themselves.

2.10. Modified Hemoglobin. Whereas interest in hemoglobin-based red cell substitutes remained extremely high, particularly in wartime, difficult problems impaired progress. First, hemoglobin in dilute solution is rapidly cleared by the kidney, as the tetrameric protein dissociates into smaller dimers. Second, dilute hemoglobin has a very high affinity for oxygen, and it was feared that little of the bound oxygen would be released in tissue capillary beds. Third, even exceedingly small amounts of stromal, ie, cell membrane, contaminants, or endotoxin in hemoglobin solutions, appeared to be toxic.

The rapid disappearance of hemoglobin from the circulation was solved when it was discovered that cross-linking with bis(*N*-maleimidomethyl) ether (BME) prolonged plasma retention (37,38). It was concluded that cross-linking reduced the tendency to form dimers, and therefore the hemoglobin was not filtered by the kidney. Accordingly, most of the hemoglobin was found in various tissues, but not in the urine.

The problem of the high oxygen affinity of cell-free human hemoglobin was solved when reagents were discovered that could bind to the 2,3-diphosphoglycerate (2,3-DPG) binding site and thus reduce the affinity for oxygen (39). This discovery led to a variety of hemoglobin modifications that not only reduce oxygen affinity but also stabilize the hemoglobin tetrameric structure so that vascular retention was prolonged. The most widely used of these agents is pyridoxal 5′-phosphate [54-47-7] (PLP), $C_8H_{10}NO_6P$ (39).

In the l960s, it was believed that contradictory toxicity reports could be explained by contamination of the solutions with foreign materials. Novel ways to remove stroma from red cell hemolysates were studied, and the phrase stroma-free hemoglobin (SFH) was coined (40). These included filtration methods that could be applied to large volumes of hemolysate, making possible physiologic studies in large animals. As a result, it was generally accepted that the toxic effects of hemoglobin could be prevented by rigorous purification.

Several pure hemoglobin solutions were later produced on a large scale for experimental use. A procedure was described for crystallization of hemoglobin

and the product was evaluated in a series of animal trials (41–44). A 6-g/dL hemoglobin solution that had a p50 of (18–20 Torr) was used in studies of tissue distribution (45) as was a similar solution of stroma-free hemoglobin. This hemoglobin was used for many basic studies of O_2 transport (46) as well as for a clinical trial in humans (47). The term p50 corresponds to the partial pressure of oxygen at which 50% of the oxygen binding sites are filled.

The polymerization of proteins using the tissue fixative glutaraldehyde [111-30-8]. The compound $C_5H_8O_2$ was described in 1973 (48). Soon, a process for polymerizing hemoglobin with the agent was patented (49). This material demonstrated a markedly prolonged intravascular retention time. Although the reaction is extremely difficult to control, products for infusion have been developed (45,50,51). For example, PLP-polyhemoglobin, obtained by reaction with PLP followed by polymerization with glutaraldehyde, was the first modified hemoglobin to be used in published human trials (52). Now, many preparations of modified hemoglobin have been tested in animals and humans. It appears that most are efficacious in transporting oxygen, and the nature of the specific modification affects biological properties such as plasma retention time, oxygen affinity, and colloid osmotic pressure, more than the toxicity.

Many other variations of modified hemoglobin have been studied, including those stabilized using various cross-linkers. Some products are derived from hemoglobin conjugated to synthetic materials such as dextran [9004-54-0] or poly(ethylene glycol) [25322-63-3]. Sources other than human outdated blood also have been investigated, including cow and recombinant hemoglobins produced in bacteria, yeast, and even transgenic mammals.

2.11. Encapsulated Hemoglobin. Because hemoglobin is normally packaged inside a membrane, encapsulated hemoglobin is thought to be the ultimate red cell substitute. The use of microencapsulated hemoglobin as artificial red blood cells was reported in 1957 (53). Since that time, dramatic results have been reported in the complete exchange transfusion of laboratory animals (54), but progress toward development of an artificial red cell for human use has been slow. The reason is because of reticuloendothelial and macrophage stimulation problems (55). Other problems include maintaining sterility and endotoxin contamination.

2.12. Synthetic Heme. Synthetic compounds that bind or chelate O_2 have been produced. These are commercially attractive because manufacture and licensure might be developed as a drug, rather than as a biological product. Synthetic heme can be used to transfuse animals (56). Although synthetic O_2 carriers would avoid any potential limitations of hemoglobin as raw material, the synthetic procedures are very tedious, and the possibility of scale up seems remote.

3. Hemoglobin Modifications

3.1. Reactivity. Hemoglobin can exist in either of two structural conformations, corresponding to the oxy (R, relaxed) or deoxy (T, tense) states. The key differences between these two structures are that the constrained T state has a much lower oxygen affinity than the R state and the T state has a lower tendency

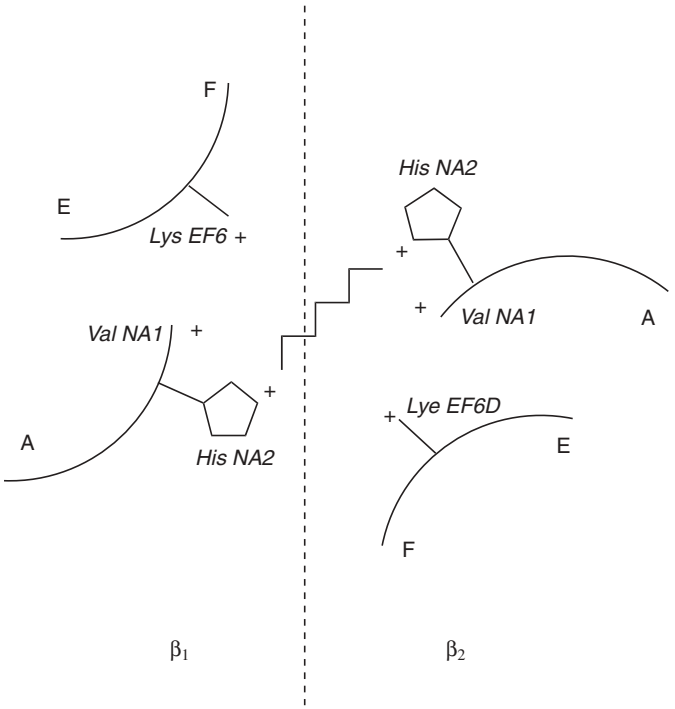

Fig. 2. Reaction of 2,3-DPG and deoxyhemoglobin. The molecule fits into the central cavity of hemoglobin and forms salt bridges with valine NA1(1)β, histidines NA2(2)β, H121(143)β, and lysine EF6(82)β. A, E, and F refer to specific hemoglobin helices and NA is the sequence from the amino-terminals to the A helix.

to dissociate into subunits that can be filtered in the kidneys. Therefore, stabilization of the *T* conformation would be expected to both reduce renal filtration and maintain oxygen affinity similar to that of red cells. The transition between the *T* and *R* states of hemoglobin is also deeply involved in the critical for the Bohr (pH) effect and cooperativity. Therefore stabilization of either of the two structures should diminish these effects, which might have important physiologic consequences.

Stabilization of the *T* conformation under normal conditions is illustrated by the reaction of 2,3-diphosphoglycerate, (2,3-DPG) (Fig. 2). The negative charges on this polyphosphate form electrostatic, reversible interactions with eight positive charges on hemoglobin: two α-amino groups of valine NA1(l)β, two ε-amino groups of lysine EF6(82)β and four histidines, NA2(2)β and HC3(l43)β. In the *R*, state the dimensions of the pocket change enough so that 2,3-DPG does not fit as well, and it drops out. Thus 2,3-DPG preferentially stabilizes the *T* conformation and has an overall effect of reducing oxygen affinity and increasing cooperativity. Analogues of 2,3-DPG, used to modify hemoglobin by forming permanent covalent bonds, are variously effective, depending on molecular dimensions and charge. Some of the compounds bind to only one of the reactive amino groups in the 2,3-DPG pocket; others react with all four.

Table 2. **Classes of Hemoglobin Modification**[a]

Class	Examples
amino-terminal modification	carbamylation
	carboxymethylation
	pyridoxylation
	acetaldehyde
Lys EF6(82)β modification	mono(3,5-dibromosalicyl)fumarate
Val NA1(1)β-Lys EF6(82)β cross-link	2-nor-2-formylpyridoxal 5′-phosphate (NFPLP)
	bis(pyridoxal) tetraphosphate (bis-PL)P_4
Lys G6(99)α_1-Lys G6(99)α_2 cross-link	bis(3,5-dibromosalicyl)fumarate
2,3-DPG analogue	pyridoxal 5′-phosphate
surface, multisite	glutaraldehyde
	polyaldehydes
	ring-open dials
	diimidate esters
conjugated hemoglobin	dextran, starch aldehydes
	poly(ethylene glycol)

[a] See Ref. (4).

In addition to the 2,3-DPG pocket, human hemoglobin contains 40 reactive lysines, ie, ε-amino groups, two α-chain N-terminal α-amino groups, and two sulfhydryl groups, ie, cysteine F9(93)β. Most of the lysines are on the surface of the molecule, but some are internal, such as lysine G6(99)α. Thus the groups can be accessed by various cross-linkers and polymerizing agents, especially aldehydes. Although the lysine groups provide many potential sites for modification, their large number also means that such reactions are difficult to control.

All of the reactions considered to be useful in the production of hemoglobin-based blood substitutes use chemical modification at one or more of the sites discussed above. Table 2 lists the different types of modifications with examples of the most common reactions for each. Differences in the reactions are determined by the dimensions and reactivity of the cross-linking reagents. Because the function of hemoglobin in binding and releasing oxygen is intricately connected to the transition between *T* and *R* conformations, it is not surprising that the p50 yields are highly variable. Even small differences among structures of the reagents can yield products having very different properties. In addition, the conditions of the reaction are very important, not only in regard to the state of ligation, ie, oxygen saturation, but also in regard to the presence of agents or molecules that block or compete for certain reactive sites.

A further complication of these reactions is that many nonhemoglobin proteins also contain reactive groups that may also be co-modified. These molecules, if present at the time of reaction, could affect the properties of the final solution. For this reason, derivatives prepared for studies of the hemoglobin molecule per se, must start with highly purified stroma-free hemoglobin.

3.2. Amino-Terminal Modification. *Carbamylation.* Modification of the amino-terminal groups of hemoglobin (Fig. 3) by the carbamylation reaction using isocyanic acid [75-13-8] was used to show that valine NA1(1)a is one of the residues involved in the alkaline Bohr effect, and that the sickling of cells containing hemoglobin S could be inhibited specifically (57). It was also used to

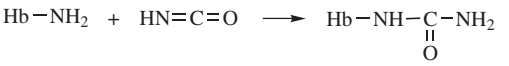

Fig. 3. Carbamylation of the α-amino groups of hemoglobin.

show the site of binding of carbon dioxide. The products of these reactions have increased oxygen affinity if the α-chains are carbamylated and decreased affinity if the β-chains are carbamylated. The carbamylation reaction held great promise in the control of sickling, but orally administered sodium cyanate was toxic.

Carboxymethylation. Other modifications of the amino-terminal groups of hemoglobin have also been studied. It was reasoned that a covalent adduct at the amino-terminal amino group might reduce oxygen affinity by lowering or neutralizing the net positive charge in the 2,3-DPG pocket (58). Carboxymethylation using glyoxylic acid [298-12-4] followed by reduction with sodium borohydride [16940-66-2], $NaBH_4$, resulted in a product that demonstrated lowered oxygen affinity and nearly intact Bohr and carbon dioxide effects (59). X-Ray and nuclear magnetic resonance (nmr) studies confirmed that the introduced group occupies nearly the same position as the naturally occurring carbamino group, ie, a carbon dioxide adduct (60).

Acetaldehyde. Acteldehyde [75-07-0], C_2H_4O, has a slightly different reaction mechanism with hemoglobin (61). Although this reagent reacts with surface lysines under some conditions, the principal products are derivatives of the amino-terminal groups, both of the α and β chains. These products are not reduced with sodium borohydride, and therefore do not involve an intermediate Schiff's base. Instead, a stable cyclic imidazolidinone derivative is formed.

Pyridoxal Derivatives. Various aldehydes of pyridoxal react with hemoglobin at sites that can be somewhat controlled by the state of oxygenation (62). It is thereby possible to prepare derivatives having a wide range of functional properties. The reaction of PLP with hemoglobin first involves the formation of a Schiff's base between the amino groups of hemoglobin and the aldehyde(s) of the pyridoxal compound, followed by reduction of the Schiff's base with sodium borohydride, to yield a covalently linked pyridoxyl derivative in the form of a secondary amine. This reaction has been used widely to reduce the oxygen affinity of the final product (62).

Pure diPLP-hemoglobin, in which both β-chain amino termini are modified, was isolated by column chromatography. The structure was confirmed by X-ray diffraction (63) and peptide analysis (64). An electrostatic interaction of the 5′-phosphate with the 2,3-DPG binding site, lysine EF6(82)β, was shown, so that this modification closely mimics the action of 2,3-DPG in stabilizing the deoxy conformation. The oxygen affinity of' the derivative was found to be about one-half of that of unmodified hemoglobin under similar conditions, but a degree of cooperativity was preserved. Equilibrium and kinetic ligand-binding studies on this derivative (65) showed a perturbed *R* state.

The reaction of hemoglobin with PLP was scaled up (66,67) to batches of 20 L yielding 70–80% modified hemoglobin. Methemoglobin was <10%, and the material was apparently unchanged after infusion into baboons. This solution was effective in resuscitation from hemorrhagic shock (68,69), but the plasma retention was thought to be too short and colloid osmotic pressure (COP) too

high to be a definitive red cell substitute (70–72). A major problem with the large-scale preparation of pyridoxylated hemoglobin was the heterogeneity of reaction products, probably representing modifications at either or both of the α- and β-amino-terminal residues as well as surface lysines.

3.3. Lysine EF6(82)β Modification. In this reaction, sometimes called a "pseudolink" (73) hemoglobin reacts with the monofunctional reagent, mono-(3,5-dibromosalicyl)fumarate, in oxygenated conditions. The product is specifically acylated at lysine EF6(82)β, in about 70% yield. Although cooperativity is reduced somewhat. ie, to a Hill coefficient of 2.0, the p50 under physiologic conditions is ~25 Torr, and carbon dioxide binding is intact, because the sites for carbon dioxide binding are unaffected. It is of particular interest that the tetramer–dimer dissociation is retarded, possibly by stabilization at the β–β interface (74). The resulting plasma retention half-time in the rat is also prolonged by about fourfold for this acylated material, as compared to unmodified hemoglobin.

3.4. Valine NA1(1)β-Lysine EF6(82)β Cross-link. *2-Nor-2-Formylpyridoxal 5′-Phosphate.* 2-Nor-2-formylpyridoxal 5′-phosphate, CHNOP, (NFPLP), is of special interest because it contains two reactive aldehyde groups and reacts as shown in Figure 4 at two sites: at the amino-terminal group of one β-chain and at lysine 82 of the other (63,75). Thus in one modification reaction, this reagent both reduces the oxygen affinity of native hemoglobin and prevents its dissociation into αβ-dimers.

NFPLP has been studied extensively. Because hemoglobin dimerization is prevented, NFPLP is not eliminated in the urine (76–78) and the plasma retention of the modified material is at least three times that of either unmodified hemoglobin or pyridoxylated hemoglobin (79). Tissue distribution and elimination have been documented in detail (80–82). Accumulation of this modified

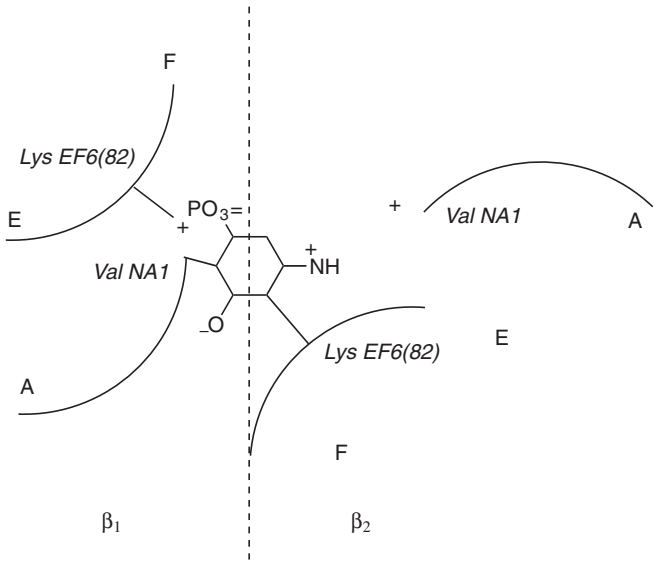

Fig. 4. Reaction of NFPLP and hemoglobin. A, E, and F refer to helices; NA is the sequence from the amino-terminals to segment A.

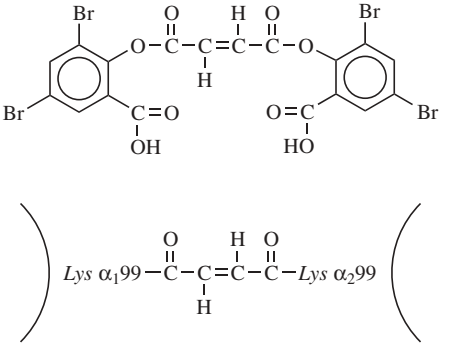

Fig. 5. Structures of (**a**) bis DBBF and (**b**) the αα-hemoglobin cross-link (4).

hemoglobin derivative in the kidney is much reduced as compared to unmodified hemoglobin (83). The oxygen affinity of the derivative under physiologic conditions is ∼47 Torr, with cooperativity retained. When used to perfuse isolated organs, the derivative supports a higher tissue oxygen tension in both the rabbit heart (84) and rat liver (85).

Although the 60–80% yield of the NFPLP product may have been satisfactory for commercialization (86), the main drawback was difficulty in preparation of the reagent itself.

Bis(pyridoxal) tetraphosphate. A second class of bifunctional reagents, described in 1988, involves two pyridoxal groups linked by phosphates of different lengths (87) (Fig. 5). As shown in Table 3, the yield of intramolecularly cross-linked hemoglobin increases dramatically with increasing length of the phosphate backbone. It is believed that the site of reaction of (bis-PL)P_4 is between the amino-terminal amino group of one β-chain and the lysine 82 of the other β-chain, as for NFLP (87). However, the distance between these two residues is only 1.1 nm, and the reagent is much longer. Therefore it is concluded that the cross-linker must fold back upon itself to form a stacked pyridine ring conformation.

Further study of (bis-PL)P_4 modified hemoglobin (88) showed its P50 to be 31 Torr (pH 7.4, pCO_2 40 Torr, 37°C), with a Bohr effect about one-half of that of unmodified hemoglobin. Its plasma retention is prolonged threefold in the rat,

Table 3. **The Effect of Polyphosphate Backbone Size on the Resulting Hemoglobin Polymer Size**[a]

Compound	R	% Tetramer
(bis-PL)P_2	0	16
(bis-PL)P_3	orthophosphate	18
(bis-PL)P_4	pyrophosphate	68
CH_2-(bis-PL)P_4	methylene diphosphate	53
fructose (bis-PL)P_4	fructose 1,6-diphosphate	41
PLP-DPG-PLP	2,3-diphosphoglycerate	70

[a] Refer to Figure 5.

and there was no apparent toxicity in screening studies. An attractive feature of (bis-PL)P_4 is that its synthesis is much simpler than that of NFPLP–hemoglobin (89).

Other 2,3-Diphosphoglycerate Pocket Cross-Linkers. The reactivity of the valine NA1(l)α and lysine EF6(82)β residues in the 2,3-DPG pocket shown by NFPLP and (bis-PL)P_4 stimulated the search for other reagents that react similarly but have potentially greater efficiency and ease of scaleup. The systematic study of four different dicarboxylic acid derivatives, cross-linked in both oxygenated and deoxygenated conditions, has been reported (90). Each of these derivatives presents problems in purification, and proof of the sites of reaction is tedious.

3.5. Lysine G6(99)α_1-Lysine G6(99)α_2 Cross-Link. A class of bifunctional reagents that cross-link human hemoglobin internally to preserve the native dimensions of the molecule has been very useful in the production of a very well characterized product for research purposes (91–94). The derivatives increased the oxygen affinity of native hemoglobin and were thought to have potential in preventing sickling in patients having sickle-cell anemia. When oxyhemoglobin was cross-linked using his(3,5-dihromosalicyl)fumarate [71337-53-6], (DBBF) $C_{18}H_8Br_4O_6$, the reaction site was shown to be between lysine EF6(82)β_1 and lysine EF6(82)β_2(93). However, when cross-linking was carried out in deoxyhemoglobin, the α-chains were modified (95,96) (Fig. 5).

This hemoglobin derivative was proposed to be developed as a blood substitute (96,97) because a single modification could achieve the dual goals of reduced oxygen affinity and restricted tetramer–dimer dissociation. The product, called αα-Hb by the U.S. Army and DCLHb by Baxter, was formulated in Ringer's lactate. The p50 under physiologic conditions was approximately that of human blood (98), Hill's parameter was 2.2, and the Bohr effect was reduced (99). Plasma retention was increased, and the product appeared to be less heterogeneous than some of the other derivatives under study. An interesting property of αα-Hb is its thermal stability, which was exploited to achieve both a partial purification of the crude reaction mixture after cross-linking and inactivation of viruses in the final product (100,101). Its production was scaled up by Baxter Healthcare, under contract to the U.S. Army, but later abandoned because of its propensity to cause vasoconstriction in animals and humans (102).

3.6. Surface, Multisite Reagents. Surface modification of hemoglobin with multifunctional aldehydes has been one of the most popular modifications because it results in large aggregates of molecules with potentially prolonged intravascular retention time. An inherent problem is that the extent of polymerization may be both nonspecific and difficult to control. Glutaraldehyde is a prime example of such a reagent (Fig. 6).

Glutaraldehyde. Polymerization of pyridoxylated human hemoglobin using glutaraldehyde was first reported in 1980 (103). In the years that followed,

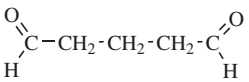

Fig. 6. Glutaraldehyde.

Table 4. **Properties of a Typical Pyridoxylated Glutaraldehyde-Polymerized Hyman Hemoglobin**[a]

hemoglobin concentration, g/dL	12–14
methemoglobin (%)	<5
molecular weight (kDa)	64–400
mean molecular mass (kDa)	150
p50 (Torr)	~18
n (Hill parameter)	1.5–2.2
Bohr factor (Δp50/ΔpH)	−0.12 to −0.25
colloid osmotic pressure, mmHg	~25
viscosity (cPs)	~2
endotoxin (EU/mL)	<0.6
rabbit pyrogen test	Pass

[a] see Ref. 4.

this polyhemoglobin was studied intensively. A research team at Michael Reese Hospital in Chicago commercialized a glutaraldehyde-polymerized human hemoglobin product that has been tested extensively in humans (104).

The production process begins with pyridoxylated hemoglobin (14–16 g/dL) that is then polymerized using a 12.5% solution of glutaraldehyde. When the COP of the reaction mixture reaches normal values of 20–25 mmHg, the reaction is quenched by the addition of an amino acid such as 1.3 *M* lysine. The resulting product has a distribution of molecular masses from 120 to 600 kDa, a p50 of 2.1 ~16 Torr, a Bohr effect reduced by one-half, and a Hill coefficient of 1.7. The viscosity is ~2 cP, compared to 4 cPs for human blood, and the solution has no effect on coagulation, as measured by the prothrombin and partial thromboplastin times. Some of the properties of glutaraldehyde hemoglobin are shown in Table 4. Physiologic studies with a glutaraldehyde derivative with a molecular weight of 124 kDa (51) showed that the product transports oxygen as expected and that the reduced p50 did not diminish its usefulness (105). The plasma half-life in baboons was up to 46 h, compared with ~6 h for PLP–hemoglobin (70).

Reexamination of the products of the glutaraldehyde reaction of pyridoxylated hemoglobin revealed extreme heterogeneity (106–108) and showed that the products are unstable on storage at 4°C. Rearrangements of polymeric species occur so that it is difficult to prepare a predictably modified species. This heterogeneity and instability are regarded as serious drawbacks to the product because reactions with plasma proteins in vivo would he impossible to predict and toxicity would be difficult to understand. Concern has been raised (108) that the low molecular weight material might be preferentially lost through the kidneys, leaving the inherently less stable polymers with the less favorable oxygen-transport properties.

Glutaraldehyde treatment of hemoglobin has the effect of making the tetrametric structure of the molecule more rigid. Indeed, it seems that the more highly modified the polymerized hemoglobin molecules are, the more rigid they become, as reflected by increasing oxygen affinity and decreasing cooperativity. Studies using the very sensitive Mossbaur technique (109) have shown that glutaraldehyde-treated hemoglobin has an increased rate of autooxidation and increased thermal stability. These properties could be explained by a weakening of the hemoglobin linkage.

Toxicology studies with glutaraldehyde products are of great concern because glutaraldehyde can leach out of prosthetic devices (110,111). Glutaraldehyde is also used as a tissue fixative, and even small amounts have been found to have cytotoxic activity (112).

Other Polyaldehydes. Other dialdehyde reagents can be prepared by oxidizing the ring structures of sugars or nucleotides (113). These reagents can react with hemoglobin at any of its amino groups, and therefore form a variety of modifications including intramolecular and intermolecular links. One example of this type of modification involves opening the ring of inositol tetraphosphate. Another example, involves the opening of the pyridine ring of ATP (114) to form modified ATP–hemoglobin. This latter product was reported to have an elevated p50 and normal cooperativity.

Optimization of the ATP–hemoglobin reaction conditions produced a derivative with a reduced oxygen affinity. Five fractions from a reaction mixture, when isolated, were found to have P50 values ranging from 8 to 38 Torr. Most fractions have little cooperativity (115). These results are consistent with those found with other polyfunctional reagents that react on the surface of hemoglobin.

Diimidate Esters. Diimidate esters bifunctional reagents have been used in cross-linking a variety of proteins including hemoglobin. In a typical reaction, a lysyl ε-amino group reacts with the ester. The reagent is specific for surface ε-amino groups and forms polymers of varying size. One of the advantages of the reaction is that it replaces the $-NH_3^+$ group with an $=NH_2^+$ group, so the overall charge is unchanged. One reported product (116) had 30 of the 44 surface lysyl residues modified and had a molecular mass ranging from 68 to 600 kDa. Intravascular retention time was increased about fourfold in rabbits.

Zero-Link Polymerization. Zero-link polymerization is a variety of polymerization developed by researchers at the University of Maryland (117). This product is called "zero-linked" hemoglobin (ZL-HbBv) because the chemistry involves direct coupling hemoglobin molecules together without using polymerizing agents such as glutaraldehyde or other bifunctional agents (Fig. 7). The key property of ZL-HbBv appears to be its very large molecular size, which results in reduced extravasation compared to native hemoglobin. The developers of ZL-HbBv believe that this reduced extravasation avoids NO binding that would cause vasoconstriction. This was based on the theory that hemoglobin in interstitial space more effectively binds NO than intravascular hemoglobin.

3.7. Conjugated Hemoglobin. An alternative approach to prolongation of the plasma retention is to conjugate hemoglobin to a larger molecule, which was first done by coupling hemoglobin to dextran (118–120). The coupling reaction is carried out using a lysate of human red cells and bromodextran, molecular weight 20 kDa. The product was shown to support life in the absence of red cells in dogs and cats (121), and it did not appear to be immunogenic (122). Because the oxygen affinity of dextran-hemoglobin was essentially that of hemoglobin, it was modified further by covalently linking an analogue of inositol hexaphosphate (123,124). This new derivative had a p50 of 55 Torr, compared to 23 Torr) for dextran-hemoglobin, and the oxygenation curve showed cooperativity. These modifications were demonstrated to reduce renal toxicity of unpurified hemoglobin.

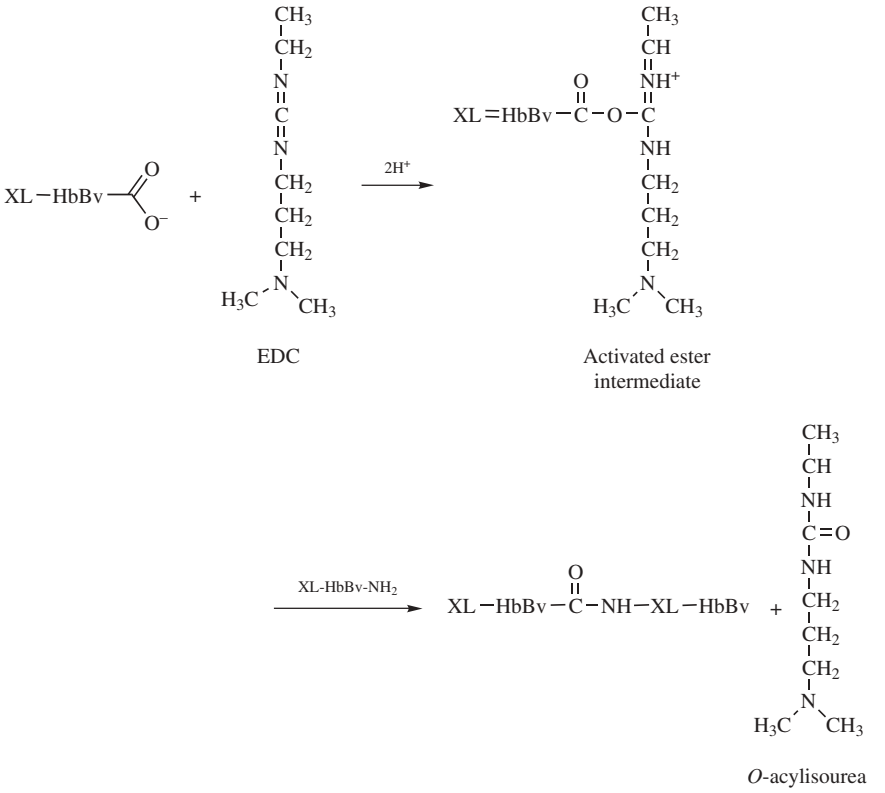

Fig. 7. Production of ZL-HbBv. Deoxygenated bovine hemoglobin is first internally cross-linked with bis(3,5-dibormosalicyl)-adipoate to form XL-HbBv. These molecules are then polymerized at free carboxyl groups after activation with 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC)(117).

Hemoglobin also can be conjugated to other synthetic polymers such as inulin [9005-80-5] (125), poly(vinylpyrrolidinone) [9003-39-81], $(C_6H_9NO)_x$ (126), and poly(ethylene glycol) [25322-68-3], $(C_2H_4O_n)H_2O$) (127,128). One of the most studied of these conjugates is pyridoxylated hemoglobin- polyoxyethylene (PHP) (127). PHP has a molecular mass of ~90 kDa and a p50 of ~22 Torr, compared to 15 Torr for hemoglobin under the same conditions, but is has reduced cooperativity (129). The plasma retention half-life in dogs is ~36 h, and apparently causes no renal, hepatic or coagulation toxicity. Histologic examination of the lungs and spleen of transfused dogs at 2 weeks and later showed no abnormalities (130).

A new conjugated hemoglobin product is based on a novel view of the physiology governing microvascular blood flow. According to this theory, microvascular vasoconstriction in the case of cell-free hemoglobin does not result entirely from NO scavenging, but from oversupply of O_2 to regulatory arterioles because of facilitated diffusion of hemoglobin in the plasma space (131). The product MP4, controls facilitated diffusion by its large molecular size and high O_2 affinity. It is produced by novel attachment of six strands of maleimide-poly- (ethylene glycol) (5 kDa) to human hemoglobin in a site-specific

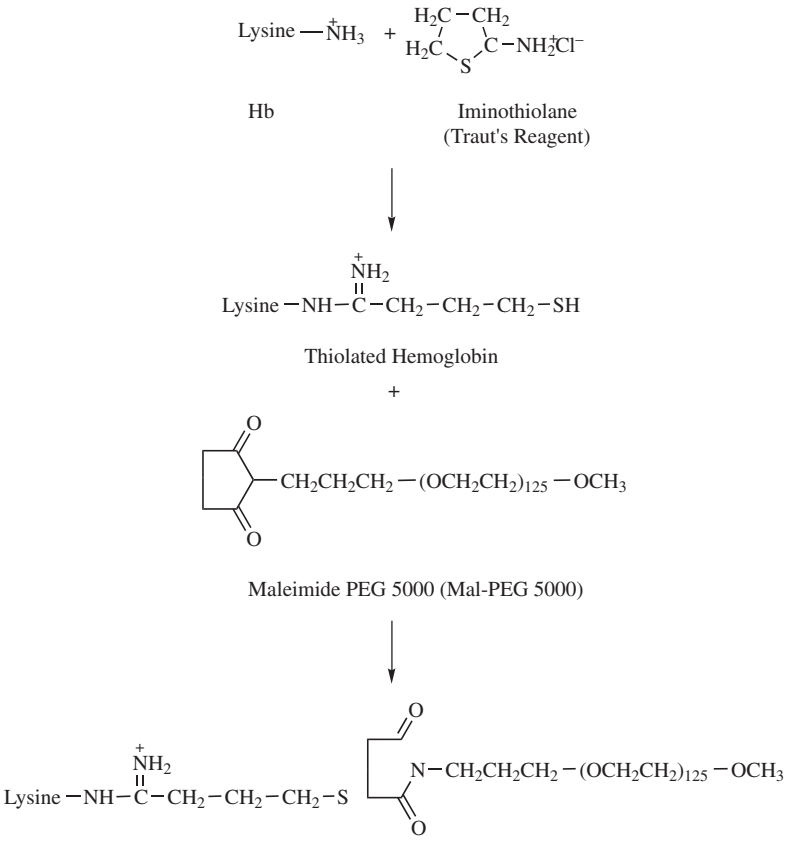

Fig. 8. Schematic of the maleimide-PEG modification of human hemoglobin (132).

reaction (132) (Fig. 8). The MP4 has been shown to be free of vasoconstriction in the hamster microcirculation and does not elevate systemic or pulmonary vascular resistance in pigs. It also does not cause myocardial lesions in rhesus monkeys. The chemistry, as shown in Figure 8, involves the surface modification of ε-amino groups of lysine followed with site-specific coupling of maleimide-activated PEG (133). The product is extremely homogeneous.

3.8. Recombinant Hemoglobin. Human hemoglobin has been expressed in a variety of recombinant systems, but in large scale in Escherichia coli (134,135). A novel molecule that was modified to genetically fuse the two α chains preventing subunit dissociation was called rHb1.1. The properties were almost identical to DCLHb and ααHb. This product was abandoned as a blood substitute candidate because of pronounced gastrointestinal side effects and hypertension. These effects were attributed to NO scavenging. Consequently, a series of mutants with altered NO affinity was developed (136). A product, rHb 2.2, shows reduced NO affinity, but has intact O_2 binding. It is free of hypertension in rats and did not produce microscopic necrotic lesions in monkey hearts (137). Some information about this product is available (138), but data have not yet been published to prove that reduced vasoactivity is a result of altered NO binding (139).

4. Hemoglobin Sources

4.1. Purification. Hemoglobin is provided by the red blood cell in highly purified form. However, the red cell contains many enzymes and other proteins, and red cell membranes contain components, such as phospholipids, that could be toxic. Furthermore, plasma proteins and other components could trigger immune reactions in recipients. The chemical modification reactions discussed here are not specific for hemoglobin they may modify other proteins as well. Indeed, multifunctional reagents could actually couple hemoglobin to nonhemoglohin proteins.

Rabiner's method (40) for the filtration purification of hemoglobin was thought to be a significant advance over older centrifugation methods (140,141). However, hemoglobin prepared in this way still caused unwanted reactions in human recipients (47). The crystallization method (142) showed fewer toxic effects in animals (142), but batch-to-batch reproducibility was uneven. Ultrapurification of hemoglobin using ion-exchange chromatographic technique is possible but is tedious and expensive (143).

4.2. Outdated Human Blood. If clinical efficacy and safety of hemoglobin solutions can be shown, the demand for product would soon outstrip the supply of outdated human blood. About 12 million units of blood (1 unit is ~480 mL) are used in the United States each year, and only about 1% outdate. The primary use of blood is in intraoperative and emergency settings. The quantity of blood available for use in production of blood substitutes depends on the willingness of donors who are qualified to donate, and the efficient matching of donor blood to the recipients.

4.3. Bovine Hemoglobin. One solution to the hemoglobin supply problem is to use nonhuman sources of blood as a starting material such as cows (bovine hemoglobin). The ultimate success of bovine, or any other hemoglobin, depends on demonstration of safety, not supply. One problem in using bovine hemoglobin is the fear of bovine spongiform encephalitis (BSE) virus. This virus, related to the Scrapie organism, has been detected in cows in Europe as well as other mammals in North America. The variant Creuzfield-Jacob Disease (vCJD) has been associated with BSE in Europe, and it is known that BSE can be transmitted by blood in animals. Although there are no known cases of human transmission by blood transfusion at this time, the FDA has placed restrictions on the importation of blood from Europe into the United States. At this time, there is no adequate test for BSE in donated blood that could be implemented on a large scale.

Under conditions found in the red cell, bovine hemoglobin has a lower oxygen affinity than human hemoglobin because of its greater sensitivity to anions (144–147). Thus instead of regulation by 2,3-DPG, as is the ease with human hemoglobin, bovine hemoglobin oxygen affinity is regulated by chloride ion.

Bovine hemoglobin has been cross-linked using the bifunctional reagent DBBF to obtain a product with a p50 in excess of 40 Torr under physiologic conditions (148). Although the reaction mixture was somewhat heterogeneous, no uncross-linked material was detected by sedimentation velocity analysis. The plasma retention in rats was prolonged 10-fold as compared to unmodified hemoglobin. It also has been found that the pyridoxylation reaction raises the

p50 from 28 to 38 Torr, and glutaraldehydc polymerization drops the p50 to 18 Torr (149). The polymerized material had essentially the same plasma retention time as human hemoglobin modified in the same way, and rats could also be supported at zero hematocrit.

4.4. Recombinant Hemoglobin. An alternative and novel source of hemoglobin, which is used for modification, is from microorganisms, the genome of which has been modified to contain globin genes for recombinant hemoglobin (rHb) production (see GENETIC ENGINEERING, MICROBES). Significant strides have been made in this approach, and it is possible to express both human α- and β-globin chains in E. coli (134,135).

Much of the development work with recombinant hemoglobin for commercial purposes has been done in commercial laboratories, so not all details of the process are available. However, it is likely that production on the scale needed for a viable red cell substitute product could be a problem. One unit of blood (500 mL) contains ∼15 g/dL of hemoglobin, so a total of 75 g of hemoglobin would be needed to produce a unit. If the yield is 0.1 g/L of culture medium, 750 L of cell culture would be needed. In the future, it might be possible to express hemoglobin genes in higher organisms; synthesis of functional human hemoglobin has already been reported in yeast, transgenic mice (150), and pigs (151), however, these approaches present additional purification, logistic, and economic problems. Purification of rHb could also be a significant challenge, since it would need to be separated from media components and other microorganism products. Endotoxin contamination could be a serious problem for bacteria.

5. Current Status of Artificial Blood

The magnitude of the undertaking to produce "Artificial Blood" cannot be overestimated. In the past 20 years, >$2 billion have been expended on attempts to do so. To date, only one product has been presented to the FDA for licensure approval. At the same time, several prominent attempts, fueled by significant investments from large pharmaceutical companies have failed.

Some of the major problems that have plagued the development of hemoglobin solutions appear to be solved. For example, renal failure, a consequence of renal filtration of dissociated hemoglobin molecules has been eliminated by cross-linking or polymerization chemistry and strict elimination of unreacted hemoglobin. Modern perfluorocarbon emulsions can be stored at refrigerator temperature and need not be reconstituted prior to use. Optimal sizing of the emulsion particles has decreased some of the side effects that have hampered development of earlier emulsions. However, some significant scientific problems remain to be solved or clarified in the field.

For hemoglobin-based products, there is still no agreement on the mechanism of vasoconstriction. This problem does not affect all products. Most studies in the published literature are based on αα-Hb or DCL-Hb, mainly because these products have been readily available to researchers. Understandably, this has been a sensitive subject with commercial developers, and fewer basic studies are available with polymerized products that are in advanced clinical trials.

Both theories of vasoconstriction, NO binding and O_2 autoregulation, are being studied. To date it is not certain if they are mutually exclusive or what their physiological and clinical consequences are.

The mechanism of the frequently reported gastrointestinal (GI) side effects of hemoglobin solutions is not clear. Studies have been done in animals (152) showing that some hemoglobin solutions interfere with esophageal motility and gastric emptying. However, how plasma hemoglobin can interfere with smooth muscle function is not established. Other possible concerns center around the effect of hemoglobin on cellular function. Some preparations stimulate monocytes and macrophages (153) and others degranulate basophils and reduce eosinophil mobility (154). Still other reports suggest that neutrophil function and platelet-endothelial adhesion can be affected. Work is still in the early stages on these phenomena, and it is not yet established whether or not they have clinical implications.

The relatively short intravascular persistence of any of the products remains a problem for certain clinical applications such as chronic anemia, but should not impact use in many elective surgical procedures or in trauma, where the patient's own marrow should replenish red cells in a few days. This issue is complicated by the fact that detailed metabolic pathways for many of the products are still not completely defined, so the impact of the rate of breakdown is not known.

On the positive side, clinical trials with all of the products studied so far have shown the ability to reduce the number of patients that receive allogeneic transfusions and the number of units transfused in elective surgical procedures. The U.S. FDA has stated publicly that such reduction or elimination of allogeneic blood could be a basis for approval of these products. Establishing reduced mortality as an end point is a more difficult task, and the FDA has stated that while it will not require such demonstration as a prerequisite to approval, it will require phase II clinical trials in trauma to establish safety in this application (155).

Finally, the cost of new products will be an important issue once regulatory approval has been won. Some commercial developers and financial analysts believe that a safe and effective product that can reduce the number of patients who receive transfusions or the number of units of allogeneic blood transfused, will command a price significantly higher than that of banked blood. However, as no products are as yet approved, this remains to be seen.

BIBLIOGRAPHY

"Blood, Artificial", in *ECT* 4th ed., Vol. 4, pp. 312–333, by Robert M. Winslow, University of California, San Diego; "Blood, Artificial" in *ECT* (online), posting date: December 4, 2000, by Robert M. Winslow, University of California, San Diego.

CITED PUBLICATIONS

1. W. Harvey, *Exercitatio Anatomica de Motu Cordiset Sanguinis in Animalibus*, Leach, London, 1653.

2. L. Diamond, A history of blood tranfsusion, in *Blood, Pure and Eloquent*, M. Wintrobe, ed., McGraw-Hill, New York, 1980, pp. 659–690.
3. M. Hollingsworth, *Ann. Med. Hist.* **X**, 213 (1928).
4. R. Winslow, *Hemoglobin-based Red Cell Substitutes*, Johns Hopkins University Press, Baltimore, M.D, 1992.
5. T. Thomas, *N.Y. State J. Med.* **27**, 449 (1878).
6. C. Jennings, *Brit. Med. J.* **1**, 1147 (1885).
7. J. Bovell, *Can. J.* **3**, 188 (1955).
8. J. Brinton, *Med. Rec.* **14**, 344 (1878).
9. C. Guthrie and F. Pike, *Am. J. Physiol.* **18**, 14 (1907).
10. W. Kühne, *Arch. Anat. Physiol. Med.* 769, 1859.
11. W. von Moraczewski, *Pflug. Arch. Physiol.* **77**, (1899).
12. S. Ringer, *J. Physiol. Cambridge* 380 (1880).
13. A. Hartmann and M. Senn, *Trans. Am. Pediat. Soc.* **44**, 56 (1932).
14. J. Miller and C. Poindexter, The effects observed following the intravenous and subcutaneous administration of fluid. An experimental study on dogs, *J. Lab. Clin. Med.* **18**, 287 (1932).
15. P. Lamson, B. Robbins, and M. Greig, *J. Pharm. Expe. Therapeutics* **83**, 225 (1945).
16. C. Ludwig, *S. B. Akad. Wiss. Wien* **48**, 731 (1863).
17. W. Bayliss, *Brit. J. Exper. Path.* **1**, 1 (1920).
18. Y. Henderson and H. Haggard, *J. Am. Med. Ass.* **78**, 697 (1922).
19. W. Amberson, *Biological Reviews* **12**, 48–86 (1937).
20. H. Bowditch, *Arbeit. Physiolog. Anstalt Leipzig* **6**, 139 (1871).
21. H. Kronecker and W. Stirling, *Beitrage zur Anatomie und Physiologie al Festgabe (av)*, Ludwig Gewidmet, Leipzig, 1875.
22. S. Ringer, *J. Physiol.* **6**, 361 (1885).
23. J. Moldovan, *Dtsch. Med. Wschr.* **2**, 2422 (1910).
24. O. Wangensteen, H. Hall, A. Kremen, and B. Stevens, *Proc. Soc. Exp. Biol. Med.* **43**, 616 (1940).
25. A. Kremen, H. Hall, H. Koschnitzke, B. Stevens, and O. Wangensteen. *Surgery* **11**, 333 (1942).
26. J. Dunphy and J. Gibson, *Surgery* **14**, 509 (1943).
27. E. Cohn, *Ann. Intern. Med.* **26**, 341 (1947).
28. J. Boldt, G. Haisch, S. Suttner, B. Kumle, and A. Schellhaass, *Brit. J. Anaesth.* **89**, 722 (2002).
29. T. Gan, E. Bennett-Guerrero, B Phillips-Bute, H. Wakeling, D. Moskowitz, Y. Olufolabi, S. Konstadt, C. Bradford, P. Glass, S. Machin, and M. Mythen, *Anesth. Analg.* **88**, 992 (1999).
30. L. Clark and F. Gollan, *Science* **152**, 1755 (1966).
31. H. Sloviter and T. Kamimoto, *Nature (London)* **216**, 458 (1967).
32. R. Geyer, R. Monroe, and K. Taylor, *Fead. Proc.* **27**, 384 (1968).
33. S. Gould, A. Rosen, L. Sehgal, H. Sehgal, L. Langdale, and L. Krause, *N. Engl. J. Med.* **314**, 1653 (1986).
34. P. Keipert, *Japan II(2)*, 47 (2003).
35. G. VonStark, *Deuts. Med. Wochenschr.* **24**, 805 (1898).
36. A. Sellards and G. Minot, *J. Med. Res.* **34**, 469 (1916).
37. H. Bunn and J. Jandl, *J. Exp. Med.* **129**, 925 (1969).
38. H. Bunn and J. Jandl, *Trans. Assoc. Am. Phys.* **81**, 147 (1968).
39. R. Benesch, R. Benesch, R. Renthal, and N. Maeda, *Biochemistry* **11**(19), 3576 (1972).
40. S. Rabiner, J. Helbert, H. Lopas, and L. Friedman, *J. Exp. Med.* **126**, 1127 (1967).
41. F. DeVenuto, A. Zegna, and K. Busse, *Surgery, Gynecol., and Obstet.* **148**, 69 (1979).

42. F. DeVenuto, H. Friedman, R. Neville, and C. Peck, *Surgery, Gynecol., Obstet.* **149**, 417 (1979).
43. F. DeVenuto, K. Busse, and A. Zegna, *Surg. Gynecol. Obstet.* **153**(3), 332 (1981).
44. F. DeVenuto, *Crit. Care Med.* **10**, 238 (1982).
45. K. Bonhard, *Experimentelle Erfahrungen mit Ha(umlaut)moglobin—Infusion-slo(umlaut)sungen.* S. Rappaport and F. Jung. Akademie-Verlag, Berlin (1975), pp. 221–225.
46. L. Djordjevich, J. Mayoral, and A. Ivankovich, *Anesthesiology* **63**(3A), A109 (1985).
47. J. Savitsky, J. Doczi, J. Black, and J. Arnold, *Clin. Pharmacol. Therap.* **23**, 73 (1978).
48. J. Payne, *Biochem. J.* **135**, 867 (1973).
49. U.S. Pat. 554-051, M. Laver, P. Boysen, and K. Morris, 1975.
50. L. Sehgal, A. Rosen, S. Gould, H. Sehgal, L. Dalton, J. Mayoral, and G. Moss, *Fed. Proc.* **39**, 718 (1979).
51. F. DeVenuto, and A. Zegna, *Transfusion* **21**, 599 (1981).
52. S. A. Gould, E. E. Moore, D. B. Hoyt, J. M. Burch, J. B. Haenel, J. Garcia, R. DeWoskin, and G. S. Moss, *J. Am. Coll. Surg.* **187**, 113 (1998).
53. T. Chang, *Biomat., Art Cells., Art. Org.* **16**(1–3), 1 (1988).
54. C. Hunt, R. Burnette, R. MacGregor, A. Strubbe, D. Lau, and N. Taylor, *Science* **230**, 1165 (1985).
55. R. Rabinovici, W. T. Phillips, G. Z. Feuerstein, and A. S. Rudolph, Encapsulation of hemoglobin in liposomes, In: *Red Blood Substitutes. Basic Principles and Clinical Application*, A. S. Rudolph, R. Rabinovici, and G. Z. Feuerstein, eds., Marcel Dekker, New York, 1998, pp. 263–286.
56. E. Tsuchida, D. Eng, and H. Nishide, *Biomat., Art Cells, Art Org.* **16**(1–3), 313 (1988).
57. J. Manning, *Preparation of hemoglobin carbamylated at specific NH2-terminal residues* E. Antonini, L. Rossi-Bernardi, and E. Chiancone, eds., Academic Press, New York, 1981, pp. 159–167.
58. W. Fantl, L. Manning, H. Ueno, A. DiDonato, and M. Maning, *Biochemistry* **26**, 5755 (1987).
59. J. Manning, W. Fantl, L. Manning, and H. Ueno, *Biomat., Art Cells, Art Org.* **17**(5), 639 (1989).
60. W. Fantl, A. Donato, J. Manning, P. Rogers, and A. Arnone, *J. Biol. Chem.* **26**, 12700 (1987).
61. R. San George and H. Hoberman, *J. Biol. Chem.* **261**(15), 6811 (1986).
62. R. Benesch and R. Benesch, *Preparation and properties of hemoglobin midified with derivatives of Pyridoxal*, E. Antonini, L. Rossi-Bernardi, and E. Chiancone, eds., Academic Press, New York, 1981, pp. 147–158.
63. A. Arnone, R. Benesch, and R. Benesch, *JMB* **115**, 627 (1977).
64. R. Benesch, R. Benesch, and S. Kwong, *JBC* **257**, 1320 (1982).
65. A. Bellelli and M. Brunori, *Ann. Ital. Med. Int.* **1**, 275 (1986).
66. L. Sehgal, A. Rosen, G. Noud, H. Sehgal, S. Gould, R. DeWoskin, C. L. Rice, and G. S. Moss, *J. Surg. Res.* **30**, 14 (1981).
67. F. DeVenuto and A. Zegna, *J. Surg. Res.* **34**, 205 (1983).
68. D. Hoyt, A. Greenburg, G. Peskin, S. Forbes, and H. Reese, *Surg. Forum* **31**, 15 (1980).
69. F. Jesch, W. Peters, J. Hobbhahn, M. Schoenberg, and K. Mesmer, *Crit. Care. Med.* **10**, 270 (1982).
70. L. Sehgal, S. Gould, A. Rosen, H. Sehgal, and G. Moss, *Surgery* **95**(4), 433 (1984).
71. S. Gould, L. Sehgal, A. Rosen, H. Sehgal, and G. Moss, *Ann. Emer. Med.* **15**, 1416 (1986).

72. G. Moss, S. Gould, L. Sehgal, H. Sehgal, and A. Rosen, *Biomat., Art Cells, Art Org.* **16**(1–3), 57 (1988).
73. E. Bucci, A. Razynska, B. Urbaitis, and C. Fronticelli, *J. Biol. Chem.* **264**, 6191 (1989).
74. E. Bucci, C. Fronticelli, A. Razynska, and B. Urbaitis, *Biomat., Art Cells, Art Org.* **17**(5), 637 (1989).
75. R. Benesch, R. Benesch, S. Yung, and R. Edalji, *Biochem. Biophys. Res. Commun.* **63**, 1123 (1975).
76. H. Sloviter, B. Mukherji, R. Benesch, R. Benesch, and S. Kwong, *Fed. Proc.* **42**, 652 (1981).
77. P. Keipert, M. Verosky, and L. Triner, *Biomat., Art Cells, Art Org.* **16**, 643 (1988).
78. L. Triner, R. Benesch, R. Benesch, S. Kwong, and M. Verosky, *Physiologist* **26**, A69, 1983.
79. J. VanderPlas, V-V. A. Rossen, W. Bleeker, and J. Bakker, *J. Lab. Cun. Med.* **108**, 253 (1986).
80. P. Keipert and L. Triner, *Catabolism and excretion of crosslinked hemoglobin*, G. Brewer, ed., Alan R. Liss, New York, 1989, pp. 383–405.
81. G. Ayer and A. Gauld, *Arch. Pathol.* **33**, 513 (1942).
82. W. Bleeker, J. VanderPlas, R. Feitsma, J. Agterberg, G. Rigter, A. de Vries-van Rossen, E. Pauwels, and J. Bakker, *J. Lab. Clin. Med.* **113**, 151 (1989).
83. W. Bleeker, J. VanderPlas, R. Feitsma, J. Agterberg, G. Rigter, E. Pauwels, and J. Bakker, *Nucl. Med. Biol.* **16**(4), 365 (1989).
84. R. Benesch, L. Triner, R. Benesch, S. Kwong, and M. Verosky, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2941 (1984).
85. J. Bakker, W. Bleeker, and J. VanderPlas, *Prog. Clin. Biol. Res.* **211**, 49 (1986).
86. J. VanderPlas, J. Damm, and J. Bakker, *Transfusion* **27**, 425 (1987).
87. R. Benesch and S. Kwong, *Biochem. Biophys. Res. Commun.* **156**, 9 (1988).
88. P. Keipert, A. Aderiran, S. Kwong, and R. Benesch, *Transfusion* **29**, 768 (1989).
89. R. Winslow, *Transfusion* **29**(9), 753 (1989).
90. R. Jones, C. Head, T. Fujita, A. Grant, and R. Kluger, *Biomat., Art Cells, Art Org.* **17**(5), 643 (1989).
91. R. Zaugg, L. King, and I. Klotz, *BBRC* **64**(4), 1192 (1975).
92. J. Walder, R. Zaugg, R. Walder, J. Steele, and I. Klotz, *Biochemistry* **18**, 4265 (1979).
93. J. Walder, R. Walder, and A. Arnone, *J. Mol. Biol.* **141**, 195 (1980).
94. L. Wood, D. Haney, J. Patel, S. Clare, G.-Y. Shi, L. King, and I. Klotz, *JBC* **256**, 7046 (1981).
95. J. Walder, R. Chatterjee, and A. Arnone, *Fed. Proc.* **41**, 651(abstr 2228) (1982).
96. S. Snyder, E. Welty, R. Walder, L. Williams, and J. Walder, *PNAS* **84**, 7280 (1987).
97. R. Tye, F. Medina, R. Bolin, G. Knopp, G. Irion, and S. McLaughlin, *Modification of hemoglobin—Tetrameric stabilization*, R. Bolin, R. Geyer, and G. Nemo, eds., Alan R. Liss, New York, 1983, pp. 41–49.
98. V. Macdonald and R. Winslow, *J. Appl. Physiol.* **72**, 476 (1992).
99. K. Vandegriff, F. Medina, M. Marini, and R. Winslow, *J. Biol. Chem.* **264**, 17824 (1989).
100. T. Estep, M. Bechtel, T. Miller, and A. Bagdasarian, *Artif. Cells Blood Substit Immobil Biotechnol.* **16**(1–3), 129 (1988).
101. T. Estep, M. Bechtel, S. Bush, T. Miller, S. Szeto, and L. Webb, *The purification of hemoglobin solutions by heating*, G. Brewer, ed., Alan R. Liss, New York, 1989, pp. 325–338.
102. R. M. Winslow, *Vox sang* **79**, 1 (2000).
103. L. Sehgal, A. Rosen, S. Gould, H. Sehgal, L. Dalton, J. Mayoval, and G. Moss, *Fed. Proc.* **39**, 718 (1980).

104. J. Carson, H. Noveck, J. Berlin, and S. Gould, *Transfusion* **42**, 812 (2002).
105. A. Rosen, L. Sehgal, S. Gould, H. Sehgal, and G. Moss, *Crit. Care Med.* **14**(2), 147 (1986).
106. M. Marini, G. Moore, R. Fishman, R. Jesse, F. Medina, S. Snell, and A. Zegna, *Biopolymers* **29**, 871 (1990).
107. M. Marini, G. Moore, R. Fishman, R. Jesse, F. Medina, S. Snell, and A. Zegna, *Biopolymers* **28**, 2071 (1989).
108. A. Agostoni, R. Stabilini, M. Perrella, P. Pietta, M. Pace, V. Russo, and L. Sabbioneda, *Trans. Am. Soc. Artif. Intern. Org.* **33**, 372 (1987).
109. D. Guillochon, M. Vijayalakshmi, A. Sow-Thiam, and D. Thomas, *Biochemistry* **64**(1), 29 (1986).
110. E. Gendler, S. Gendler, and M. Nimni, *J. Biomed. Mater. Res.* **18**, 727 (1984).
111. J. McPherson, S. Sawamura, and R. Armstrong, *J. Biomed. Mater. Res.* **20**, 93 (1986).
112. D. Speer, M. Chvapil, C. Eslekson, and J. Ulreich, *J. Biomed. Mater. Res.* **14**, 753 (1980).
113. P. Scannon, *Crit. Care Med.* **10**, 261 (1982).
114. A. Greenburg and P. Maffuid, *Modification of hemoglobin—ring opened dials*, R. Bolin, R. Geyer, and G. Nemo, eds., Alan R. Liss, New York, 1983, pp. 9–17.
115. M. McGarrity, K. Nightingale, and J. Hsia, *J. Chromatogr, Biomed App.* **415**, 136 (1987).
116. W. Mok, D. Chen, and A. Mazur, *Fed. Proc.* **34**, 1458 (1975).
117. B. Matheson, A. Razynaka, H. Kwansa, and E. Bucci, *J. Appl. Physiol.* **93**, 1479 (2002).
118. S. Tam, J. Blumenstein, and J. Wong, *PNAS* **73**(6), 2128 (1976).
119. J. Chang and J. Wong, *Can. J. Biochem.* **55**, 398 (1977).
120. J. Blumenstein, S. Tam, J. Chang, and J. Wong, *Experimental transfusion of dextran-hemoglobin*, G. Jamieson and T. Greenwalt, eds., Alan R. Liss, New York, 1978, pp. 205–212.
121. R. Humphries, P. Killingback, J. Mann, J. Sempik, and J. Wilson, *Brit. J. Pharm.* **74**, 266P (1981).
122. P. G. Cunnington, S. N. Jenkins, S. C. Tam, and J. T. Wong, *Biochem. J.* **193**, 261 (1981).
123. J. Wong, *Artif Cells Blood Substit Immobil Biotechnol* **16**(1–3), 237 (1988).
124. X. Wu, L. Porter, and J. Wong, *Biomat., Art Cells, Art Org.* **17**(5), 645 (1989).
125. K. Iwasaki, K. Ajisaka, and Y. Iwashita, *BBRC* **113**, 513 (1983).
126. K. Schmidt, *Klin. Wochenschr.* **57**, 1169 (1979).
127. K. Ajisaka and Y. Iwashita, *BBRC* **97**, 1076 (1980).
128. M. Leonard and E. Dellacherie, *Biochim. Biophys. Acta* **791**(2), 219 (1984).
129. M. Matsushita, A. Yabuki, P. Malchesky, H. Harasaki, and Y. Nose, *Biomat., Art Cells, Art Org.* **16**(1–3), 247 (1988).
130. M. Matsushita, A. Yabuki, J. Chen, T. Takahashi, H. Harasaki, and P. Malchesky, Y. Iwashita, and Y. Nose, *ASAIO Trans* **34**(3), 280 (1988).
131. M. R. McCarthy, K. D. Vandegriff, and R. M. Winslow, *Biophys. Chem.* **92**, 103 (2001).
132. K. D. Vandegriff, A. Malavalli, J. Wooldridge, J. Lohman, and R. M. Winslow, *Transfusion* **43**, 509 (2003).
133. A. S. Acharya, B. N. Manjula, and P. Smith, *Hemoglobin crosslinkers* Albert Einstein College of Medicine of Yeshiva University, New York (5,585,484), 1996, pp. 1–16.
134. S. Hoffman, D. Looker, J. Roehrich, P. Cozart, S. Durfee, J. Tedesco, and G. Stetler, *Proc. Natl. Acad. Sci., U.S.A.* **87**, 8521 (1990).
135. D. Looker, D. Abbott-Brown, P. Cozart, S. Durfee, S. Hoffman, and A. Mathews, *Nature (London)* **356**, 258 (1992).

136. D. H. Doherty, M. P. Doyle, S. R. Curry, R. J. Vali, T. J. Fattor, J. S. Olson, and D. D. Lemon, *Nature Biotechnol.* **16**, 672 (1998).
137. K. E. Burhop and T. E. Estep, *Art Cells, Blood Subs., Immob. Biotech.* **29**, 101 (2001).
138. K. Burhop, M. Doyle, M. Schick, and M. Matthews, *The Development and Preclinical Testing of a Novel Second Generation Recombinant Hemoglobin Solution*. The 9th International Symposium on Blood Substitutes Program II(1), Vol. 50, 2003.
139. T. Resta, B. Walker, M. Eichinger, and M. Doyle, *J. Appl. Physiol.* **93**, 1327 (2002).
140. G. Brown and H. Dale, *J. Physiol.* **86**, 42P (1936).
141. P. Hamilton, A. Hiller, and D. Van Slyke, *J. Exp. Med.* **86**, 477 (1947).
142. F. DeVenuto, T. Zuck, A. Zegna, and W. Moores, *J. Lab. Clin. Med.* **89**, 509 (1977).
143. S. Christensen, F. Medina, R. Winslow, S. Snell, A. Zegna, and M. Marini, *J. Biochem. Biophys. Methods* **17**, 143 (1988).
144. H. Bunn, *Science* **172**, 1049 (1971).
145. M. Feola, H. Gonzalez, P. Canizaro, D. Bingham, and P. Periman, *Surgery, Gynecolosy, Obstetrics* **157**, 399 (1983).
146. C. Fronticelli, E. Bucci, and C. Orth, *J. Biol. Chem.* **259**, 10841 (1984).
147. C. Fronticelli, T. Sato, C. Orth, and E. Bucci, *Biochim. Biophys. Acta* **874**, 76 (1986).
148. H. Friedman, F. DeVenuto, B. Schwartz, and T. Nemeth, *Surgery* **159**, 429 (1984).
149. F. DeVenuto, *Biomat., Art Cells, Art Org.* **16**(1–3), 77 (1988).
150. R. Behringer, T. Ryan, M. Reilly, T. Asakura, R. Palmiter, R. Brinster, and T. Townes, *Science* **245**, 971 (1989).
151. M. Swanson, M. Martin, K. O'Donnell, K. Hoover, W. Lago, and V. Huntress, *Biotechnology* **10**, 557 (1992).
152. C. D. Conover, L. Lejeune, K. Shum, and R. G. Shorr, *Life Sci.* **59**, 1861 (1996).
153. S. McFaul, P. Bowman, and V. Villa, *Blood* **84**, 3175 (1994).
154. A. L. Baldwin, *Am. J. Physiol.* **277** (2 Pt 2), H650 (1999).
155. T. Silverman, *Regulation of Blood Substitutes*, The 9th International Symposium on Blood Substitutes Program II(1), Tokyo, Vol. 34, 2003.

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