Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7280–7284, October 1987
Medical Sciences

HbXL99α: A hemoglobin derivative that is cross-linked between the α subunits is useful as a blood substitute

(oxygen transport/cross-linking agent/methemoglobin/transfusion)

STEVEN R. SNYDER, EMILY V. WELTY, ROXANNE Y. WALDER, LAURA A. WILLIAMS, AND JOSEPH A. WALDER*

Department of Biochemistry, University of Iowa, Iowa City, IA 52242

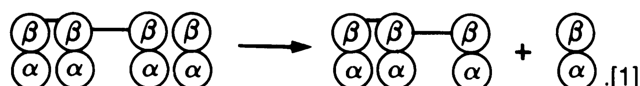
Communicated by Irving M. Klotz, June 11, 1987 (received for review March 11, 1987)

ABSTRACT Under deoxygenated conditions, bis(3,5-dibromosalicyl) fumarate reacts with hemoglobin selectively to cross-link the α subunits between Lys-$\alpha_1$99 and Lys-$\alpha_2$99. We have characterized further the properties of this recently described hemoglobin and have demonstrated its utility as a blood substitute. The oxygen transport characteristics of the cross-linked derivative are very similar to those of whole blood. Under physiological conditions, the partial pressure of oxygen at half-saturation of hemoglobin is increased to 29 mm Hg (1 mm Hg = 133.3 kPa), compared to 12 mm Hg for hemoglobin A, fully compensating for the absence of 2,3-bisphosphoglycerate outside of the erythrocyte. The Hill coefficient is 2.9. The dependence of the oxygen affinity of HbXL99α on CO_2 is also identical to that of hemoglobin A. The cross-link between the α subunits blocks dissociation of oxyhemoglobin into $\alpha\beta$ dimers and thereby prevents renal excretion of the modified hemoglobin. In the rat, the half-life of HbXL99α in plasma, at a 15% volume exchange, is increased to 3.3 hr, compared to 90 min for hemoglobin A. Cross-linking HbXL99α intermolecularly with bis(sulfosuccinimidyl) suberate to form predominantly a mixture of dimers and trimers further increased the half-life of the hemoglobin within the circulation by about 2-fold. The rate of autooxidation of the transfused hemoglobin was found to be markedly reduced because of the presence of an endogenous reducing system in plasma.

The difficulties in the storage of whole blood, the need for blood typing, and the risk of blood-borne infection make the development of a blood substitute an important goal. The current epidemic of AIDS has greatly heightened the interest in this area. Two potential acellular oxygen carriers have been studied most extensively: fluorocarbons and derivatives of hemoglobin. Because of limitations in oxygen-carrying capacity, perfluorochemicals can only be used with high partial pressures of inspired oxygen. They cannot be used under ambient conditions. Immunotoxicity associated with the uptake and slow rate of elimination of perfluorocarbons from the reticuloendothelial system may also limit their utility (1, 2).

There are two principal problems that must be overcome to utilize hemoglobin as a blood replacement. First, the derivative must be cross-linked to prevent dissociation of the tetramer. Oxyhemoglobin readily dissociates into $\alpha\beta$ dimers, which, having a molecular weight of only 32,000, are filtered by the kidneys (3). This leads to a rapid rate of clearance of hemoglobin from the circulation, and the massive hemoglobinuria that results poses the risk of renal injury. Secondly, the increase in the oxygen affinity of hemoglobin because of the absence of 2,3-bisphosphoglycerate (P_2-glycerate) limits the unloading of oxygen to the tissues. Under physiological conditions, the P_{50} (partial pressure of O_2 at half-saturation of hemoglobin) of isolated hemoglobin A is approximately 12 mm Hg (1 mm Hg = 133.3 kPa), compared to 26 mm Hg for whole blood (4). As a result, the amount of oxygen released by hemoglobin at the normal tissue partial pressures of oxygen (approximately 40 mm Hg) is decreased by nearly half.

Nonspecific cross-linking agents such as glutaraldehyde have been used to prolong the intravascular retention time of hemoglobin (5–8). However, the high level of modification that invariably occurs with such reagents may lead to problems of antigenicity and can increase the propensity of the hemoglobin toward autooxidation. Because a heterogeneous mixture of derivatives is formed, only products within a selected molecular weight range can be isolated, rather than a unique species. If the molecules within a complex are not also intramolecularly cross-linked, dimerization can still occur, as shown in the following example:



Moreover, the oxygen affinity following random cross-linking is frequently increased—the opposite effect of that which is desired for use of the hemoglobin as a blood substitute. Pyridoxal phosphate and other polyanionic affinity reagents that react with hemoglobin at the P_2-glycerate site have been used to lower the oxygen affinity of such derivatives (5–7, 9). The bifunctional analog 2-nor-2-formylpyridoxal 5′-phosphate, studied extensively by Ruth and Reinhold Benesch and their coworkers (10–12), provides a means of both cross-linking the tetramer between the β subunits and reducing the oxygen affinity. However, the reagent itself is difficult to synthesize, making it infeasible to prepare this derivative in the large amounts that would be needed for clinical use. Bis(3,5-dibromosalicyl) fumarate reacts with oxyhemoglobin to yield a derivative that is also cross-linked within the β-subunit cleft (13, 14). However, in this case the oxygen affinity is increased (14). Since the P_2-glycerate binding site is blocked, it is not possible to further modify the hemoglobin in this region to lower the oxygen affinity.

Recently, our laboratory has described (15) a new low-affinity hemoglobin derivative cross-linked between the α subunits, which is formed in the reaction of bis(3,5-dibromosalicyl) fumarate with deoxyhemoglobin A. The cross-linking bridge lies between Lys-$\alpha_1$99 and Lys-$\alpha_2$99. The specificity of the reaction and, hence, the yield are greatly

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: P_2-glycerate, 2,3-bisphosphoglycerate; HbXL99α, hemoglobin cross-linked between Lys-$\alpha_1$99 and Lys-$\alpha_2$99; P_{50}, the partial pressure of O_2 at half-saturation of hemoglobin.
*To whom reprint requests should be addressed.

increased by the addition of inositol hexaphosphate to block side reactions that occur within the P_2-glycerate site (15). This has made the large-scale preparation of the derivative possible. In studies reported here, we have characterized the properties of this new hemoglobin further to determine its utility as a blood replacement.

MATERIALS AND METHODS

Preparation of HbXL99α. Human adult hemoglobin A was isolated from outdated blood as described (14). The reaction of bis(3,5-dibromosalicyl) fumarate with deoxyhemoglobin A was carried out in 100 mM [bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane (Bistris; pH 7.2) at 37°C for 2 hr. The concentrations of hemoglobin and the cross-linking reagent were 1 mM and 1.5 mM, respectively. Inositol hexaphosphate (Sigma) was included in the reaction mixture at a concentration of 5 mM to block modifications of the β chains (15). After completion of the reaction, glycine was added as a solid to a final concentration of 1 M to consume any remaining cross-linking reagent. HbXL99α was purified by chromatography over DEAE-Sepharose (Pharmacia) as described earlier (15), except that the derivative was eluted isocratically with 0.03 M NaCl rather than with a salt gradient. The residual concentration of inositol hexaphosphate after DEAE-Sepharose chromatography, determined by phosphate analysis (16), was <0.3% of that in the original reaction mixture—i.e., <0.015 mol/mol of hemoglobin.

MetHbXL99α was prepared by reaction of the derivative with 2 equivalents of potassium ferricyanide per heme at 37°C for 30 min. The remaining ferricyanide and the ferrocyanide produced in the reaction were removed by chromatography over Sephadex G-25 resin in 0.2 M glycine buffer (pH 8), followed by chromatography over DEAE-Sepharose equilibrated with the same buffer. The fully oxidized derivative was eluted with 0.04 M NaCl and collected in a sterile container. A portion of the material was reduced under deoxygenated conditions with 40 mM sodium dithionite (G. Frederick Smith Chemical Co., Columbus, OH) in an Amicon ultrafiltration cell. The excess dithionite was subsequently removed by ultrafiltration under deoxygenated conditions. The reduced hemoglobin solution contained 6% methemoglobin (MetHb) as measured by the absorbances at 577 nm and 630 nm (17).

Oxygen-Binding Studies. Oxygen dissociation curves were measured with a Hemox-analyzer (TCS Medical Products, Southampton, PA). The conditions of the oxygen-binding studies are described in the legend to Table 1. The hemoglobin concentration was 100 μM tetramer. This high concentration was used to suppress autooxidation of the hemoglobin. In each experiment the final fraction of MetHb was <8%.

Cross-Linking HbXL99α with Bis(sulfosuccinimidyl) Suberate. HbXL99α was cross-linked intermolecularly with bis-(sulfosuccinimidyl) suberate (Pierce) to form a mixture of higher molecular weight species. The reaction was carried out with 3.5 mM HbXL99α and 20 mM of the compound for 30 min at room temperature in 20 mM Bistris (pH 7.2). The reaction was quenched by the addition of solid glycine to a final concentration of 1 M. The products of the reaction were analyzed by HPLC using a TSK 3000 gel-filtration column. The buffer used was 50 mM sodium phosphate (pH 7.0). The retention time for the monomer (64 kDa) was 10.6 min.

Transfusion Experiments. Hemoglobin solutions were exchanged by ultrafiltration into 140 mM NaCl/5 mM KCl/2 mM sodium phosphate, pH 7.4 and concentrated to 7% (approximately isooncotic with plasma). The hemoglobin solutions were filtered through sterile 0.22-μm filters just before transfusion to remove microparticulates. Male Sprague–Dawley rats (350 g) were anesthetized with 1.2 g of urethane per kg of body weight. Catheters were placed within the femoral artery and the bladder. Approximately 15% of the blood volume, calculated on the basis of a total blood volume of 6% of the body weight (18), was removed through the femoral artery and immediately replaced with an equal volume of the hemoglobin solution. Blood samples were withdrawn at 5 min, 30 min, and at intervals of 1 hr thereafter for 5 hr. Plasma hemoglobin concentration was determined by using Drabkins reagent (Sigma). The % MetHb was determined by the absorbances at 577 nm and 630 nm after dilution of the sample at least 10-fold in 50 mM sodium phosphate buffer (pH 7.2). To exclude the possibility of intravascular hemolysis and the release of rat hemoglobins within the circulation, samples also were analyzed by isoelectric focusing (15). In transfusion experiments with derivatives of HbXL99α cross-linked intermolecularly, the distribution of cross-linked species in the plasma was determined by HPLC as described above. The amount of hemoglobin excreted in the urine was determined by the benzidine method (19).

Table 1. Oxygen-binding properties of HbXL99α

Hemoglobin	With 5% CO_2*: P_{50}, mm Hg	With 5% CO_2*: Hill coefficient	Without CO_2†: P_{50}, mm Hg	Without CO_2†: Hill coefficient
Hb A	12	3.0	10	2.9
HbXL99α	29	2.9	24	3.0

*Studies with 5% CO_2 were carried out in 10 mM sodium phosphate/105 mM NaCl/19.2 mM sodium bicarbonate, pH 7.4 at 37°C to simulate physiological conditions.

†Studies without CO_2 were carried out in 230 mM Bistris·HCl/105 mM NaCl/10 mM sodium phosphate, pH 7.4 at 37°C at the same ionic strength as the physiological buffer.

RESULTS

Oxygen-Binding Studies. Oxygen-binding curves for hemoglobin A and HbXL99α measured under physiological conditions are shown in Fig. 1. Despite the presence of the cross-linking bridge between the α chains, the hemoglobin remains fully cooperative—the Hill coefficient is 2.9 (see Table 1). The right shift in the oxygen equilibrium curve for HbXL99α corresponds to a decrease in oxygen affinity by a factor of approximately 2.5. The P_{50} of the cross-linked derivative, 29 mm Hg, is very similar to that of whole blood.

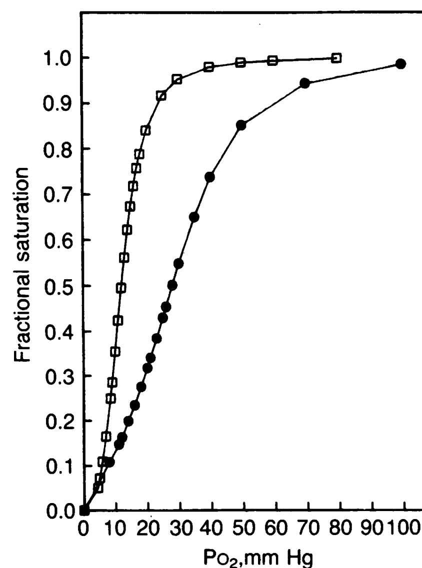


FIG. 1. Oxygen binding curves for hemoglobin A (□) and HbXL99α (●). Conditions are described in the legend of Table 1.

In the absence of CO_2, the P_{50} is decreased by nearly 20% for both HbXL99α and hemoglobin A. This last result indicates that the linkage between the binding of CO_2 and oxygen transport remains unaffected by the cross-linking bridge. CO_2 binds preferentially to deoxyhemoglobin at the amino termini of both the α and β chains (4, 20). These sites are far removed from Lys-α99 and the x-ray structure of the cross-linked derivative reveals no perturbations in these regions of the molecule (15). HbXL99α also binds P_2-glycerate normally (unpublished results), but unlike the interaction with CO_2, this is not physiologically relevant due to the lack of P_2-glycerate outside the erythrocyte.

Cross-Linking of the α Subunits Abrogates Renal Excretion of HbXL99α. In the rat, the half-life of unmodified hemoglobin A in the circulation is 90 min (Table 2). With a 15% volume exchange, between 20% and 25% of the hemoglobin infused was recovered in the urine over 5 hr. An even larger fraction may be filtered by the kidneys and in part retained within the renal parenchyma (3). In contrast, renal excretion of the cross-linked derivative appeared to be fully blocked. The small fraction of the hemoglobin that was recovered in the urine was probably due to trace contamination with unmodified hemoglobin. In this model, in which the animals were irreversibly anesthetized with urethane, the half-life of HbXL99α was increased to 3.3 hr. In a conscious rat model in which a 20% volume exchange was given, the observed half-life was 4.5 hr [T. N. Estep (Baxter Healthcare Corp., Round Lake, IL) personal communication]. A plasma half-life of 4.5 hr also has been found for a mixture of derivatives of bovine hemoglobin cross-linked intramolecularly with bis(3,5-dibromosalicyl) fumarate (21).

Effect of Intermolecular Cross-Linking on the Plasma Half-Life of HbXL99α. Although renal excretion of HbXL99α was prevented, the half-life was increased just 2- to 3-fold. It is likely that the tetramer remains susceptible to filtration through the somatic capillary beds. Substantially longer half-lives have been observed for high molecular weight polymers of hemoglobin prepared by using random cross-linking agents (5–8). To examine the possibility that the intravascular retention time of HbXL99α may be further prolonged by cross-linking the tetramer intermolecularly, bis(sulfosuccinimidyl) suberate was used to cross-link HbXL99α to produce a mixture of higher molecular weight species. The distribution of products was analyzed by HPLC using gel filtration to effect the separation (Fig. 2). Reaction conditions were chosen such that, among the cross-linked products formed, dimers and trimers of HbXL99α would predominate (Fig. 2*A*). Approximately half of the material remained as the monomer (i.e., 64 kDa). After transfusion, the fraction of higher molecular weight products increased, indicating that they are retained preferentially within the circulation (Fig. 2 *B–D*). The peaks due to the dimer and trimer were not sufficiently well-resolved to quantitate separately. The apparent half-life for the mixture of cross-linked species was increased to 6.4 hr, approximately 2-fold greater

Table 2. Plasma hemoglobin half-life and urinary excretion of HbXL99α

Hemoglobin	Plasma hemoglobin half-life	% hemoglobin excreted in urine after 5 hr	% MetHb in plasma	
			Initial	After 5 hr
Hb A	90 ± 7 min	22 ± 4	1.0	—*
HbXL99α	3.3 ± 0.2 hr	1.8 ± 1.3	5.9	4.9

Mean values and SEMs were determined from studies with four rats for hemoglobin A and with five rats for HbXL99α.

*The residual plasma concentration of hemoglobin A was too low at 5 hr to accurately determine the % MetHb. The fraction of MetHb at 2 hr was 2.0%.

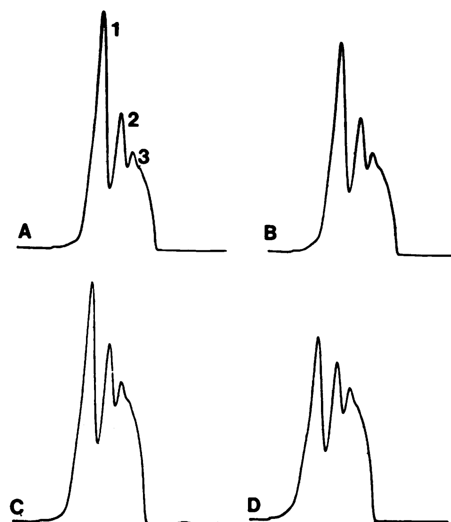


FIG. 2. HPLC analysis of derivatives of HbXL99α cross-linked intermolecularly with bis(sulfosuccinimidyl) suberate. The reaction conditions were as described. The cross-linked hemoglobin species were separated on a TSK 3000 gel-filtration column. (*A*) The original reaction mixture, Peaks 1, 2, and 3 correspond to the HbXL99α monomer, dimer, and trimer, respectively. (*B–D*) Plasma hemoglobin samples withdrawn at 1, 3, and 5 hr, respectively, after transfusion of the mixture of cross-linked hemoglobins in the rat.

than the original derivative. The ratio of trimers to dimers also appears to have increased, suggesting that there may be a progressive increase in the intravascular retention time with increasing size of the complex.

Intravascular Reduction of MetHbXL99α. In the transfusion experiments with HbXL99α and with hemoglobin A, we were surprised to observe that there was little increase, and in some cases actually a small decrease, in the percentage of MetHb (see Table 2). In contrast, when HbXL99α or hemoglobin A was incubated *in vitro* under physiological conditions, there was an increase of approximately 7% in the fraction of MetHb over 5 hr. These results imply either that there is a reducing system present in plasma or that MetHb is cleared preferentially from the circulation. To distinguish between these two possibilities, we carried out transfusion experiments in the rat in which HbXL99α was first fully oxidized to the MetHb derivative. HbXL99α that was fully oxidized and then reduced with sodium dithionite served as a control. The cycle of oxidation and reduction had no effect on the lifetime of the hemoglobin within the circulation (data not shown). When metHbXL99α was transfused, the hemoglobin was progressively reduced—i.e., converted to Fe^{2+}-containing hemoglobin (see Fig. 3). After 5 hr, only 27% of the hemoglobin remaining in the circulation was MetHb (73% was Fe^{2+}-containing hemoglobin). To rule out the possibility that this result was due to intravascular hemolysis and the release of reduced rat hemoglobin into the circulation, plasma hemoglobin samples were analyzed by isoelectric focusing (Fig. 4). Because dissociation of the tetramer was blocked by the cross-link, each of the five stoichiometric oxidation states (0 to +4) could be resolved electrophoretically (see Fig. 4, lane 3). This same ladder of bands was observed after partial reduction of the hemoglobin within the circulation (Fig. 4, lane 2). None of the species of rat hemoglobin (Fig. 4, lanes 5 and 6) were detectable in the plasma. The half-life of the oxidized hemoglobin within the circulation was not significantly different from that of the derivative in the reduced form—i.e., between 3 and 4 hr (see the upper curve in Fig. 3); nor was metHbXL99α excreted in the urine.

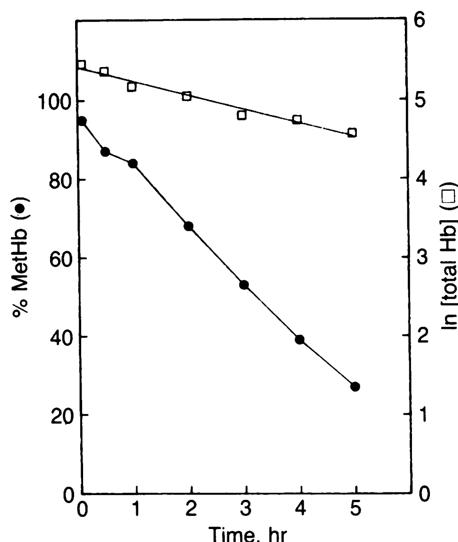


FIG. 3. Reduction and rate of clearance of MetHbXL99α from the circulation. Male Sprague–Dawley rats were transfused with HbXL99α that had been fully oxidized. A 15% blood-volume exchange was carried out. Blood samples were withdrawn at the various times indicated for determination of the residual plasma hemoglobin concentration and the % MetHb. The % MetHb plotted on the ordinate is the fraction of the hemoglobin remaining in the circulation that is in the oxidized form. Ln (total Hb) is the natural logarithm of the micromolar hemoglobin concentration.

DISCUSSION

HbXL99α possesses the two essential properties required for the use of hemoglobin as a blood substitute. The decrease in oxygen affinity fully compensates for the absence of P_2-glycerate outside of the erythrocyte, giving rise to oxygen-transport properties nearly identical to those of whole blood; and, the cross-link between the α subunits prevents dissociation of the tetramer and, thereby, blocks renal excretion of the modified hemoglobin. Correspondingly, the plasma half-life of the derivative is prolonged 2- to 3-fold. A hemoglobin derivative having a half-life between 3 and 5 hr may be useful in many acute clinical situations. In applications where the derivative is used solely for the purpose of oxygen transport, as in the prevention of cardiac ischemia during balloon angioplasty (22, 23), a relatively short half-life may actually be advantageous. A longer intravascular retention time may be desirable in the treatment of massive hemorrhage or during surgery when a large fraction of the blood volume is replaced. As demonstrated in the present studies, this can be attained by further cross-linking the tetramer intermolecularly to produce higher molecular weight species. Polymerization of hemoglobin also makes it possible to use higher hemoglobin concentrations, comparable to that of whole blood, without exceeding the normal oncotic pressure of plasma (6). HbXL99α is a particularly attractive substrate for this modification. The intramolecular cross-link between the α subunits would prevent dissociation of all of the tetramers within the complex (see Eq. 1). Since the P_2-glycerate binding site remains accessible in this derivative, it should be possible to design specific affinity reagents targeted toward this region to yield cross-linked species of precisely defined size. Although the optimal size of the complex remains to be established, it appears that derivatives comprised of three or more hemoglobin molecules are preferentially retained in the circulation relative to the dimer (Fig. 2).

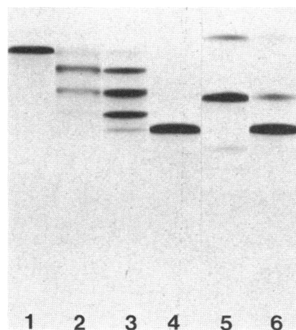


FIG. 4. Isoelectric focusing gels of oxidized derivatives of HbXL99α. Lanes: 1, metHbXL99α; 2, a 1-hr plasma hemoglobin sample from the transfusion experiment with metHbXL99α described in Fig. 3; 3, 50% metHbXL99α obtained by autooxidation of the hemoglobin at 37°C for 48 hr (the five bands seen correspond to the progressive oxidation states of the tetramer); 4, reduced HbXL99α; 5, rat hemoglobins; 6, a comixture of rat hemoglobins and HbXL99α.

Autooxidation of hemoglobin is an important concern in the use of hemoglobin derivatives as blood substitutes. The formation of MetHb compromises oxygen transport not only because the ferric hemes are unable to bind oxygen but also because the remaining ferrous hemes within the same tetramer have an increased affinity for oxygen (24). In addition, active oxygen species (i.e., $O_2^{\cdot-}$, H_2O_2, and OH·) generated concomitantly with oxidation may give rise to toxicity (25, 26). Reducing agents and antioxidant systems present in plasma may largely mitigate these problems. The low rate of autooxidation of hemoglobin A and HbXL99α when transfused in the rat first suggested to us that MetHb free within the circulation may be reduced (Table 2). Transfusion experiments with fully oxidized HbXL99α established that this does indeed occur (see Fig. 3). Although the reduction of MetHbXL99α may have resulted from intravascular hemolysis and release of the MetHb reductase system from within the erythrocyte, the absence of detectable levels of rat hemoglobin within the plasma argues strongly against this possibility. There are several endogenous reducing agents present in plasma, such as ascorbate and glutathione, capable of reducing MetHb (24, 27). *In vitro*, these compounds are often found to increase the rate of MetHb formation because of oxidizing species generated by the partial reduction of O_2 (28). The presence of oxygen radical scavengers in plasma (i.e., superoxide dismutase and uric acid) may shift the balance toward reduction. Antioxidant activities of transferrin and ceruloplasmin, which sequester Fe and Cu, respectively, may also play an important role (27). Such protective agents may prove essential for the use of hemoglobin as a blood substitute. MetHb is also reduced when incubated *in vitro* with human blood (unpublished results), providing an assay which should enable us to define the components of the reducing system. It may be necessary to augment this system by the addition of exogenous reducing agents or antioxidants to retard the formation of MetHb at higher levels of blood volume exchange (29).

We thank James Luper for assistance in the preparation and characterization of the derivatives of HbXL99α that were further cross-linked intermolecularly with bis(sulfosuccinimidyl) suberate. This work was supported in part by a grant from Baxter Healthcare Corporation. J.A.W. is the recipient of an Established Investigatorship Award from the American Heart Association.

1. Bucala, R., Kawakami, M. & Cerami, A. (1983) *Science* **220**, 965–967.
2. Geyer, R. P. (1983) in *Advances in Blood Substitute Research*, eds. Bolin, R. B., Geyer, R. P. & Nemo, G. J. (Liss, New York), pp. 157–168.
3. Bunn, F. H., Esham, W. T. & Bull, R. W. (1969) *J. Exp. Med.* **129**, 909–924.
4. Kilmartin, J. V. & Rossi-Bernardi, L. (1973) *Physiol. Rev.* **53**, 836–890.

5. DeVenuto, F. & Zegna, A. (1983) *J. Surg. Res.* **34,** 205–212.
6. Sehgal, L. R., Gould, S. A., Rosen, A. L., Sehgal, H. L. & Moss, G. S. (1984) *Surgery* **95,** 433–438.
7. Keipert, P. E. & Chang, T. M. S. (1985) *Biomater. Med. Dev. Artif. Organs* **13,** 1–15.
8. Feola, M., Gonzalez, H., Canizaro, P. C., Bingham, D. & Periman, P. (1983) *Surg. Gynecol. Obstet.* **157,** 399–408.
9. Benesch, R. E., Yung, S., Suzuki, T., Bauer, C. & Benesch, R. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2595–2599.
10. Benesch, R., Benesch, R. E., Yung, S. & Edalji, R. (1975) *Biochem. Biophys. Res. Commun.* **63,** 1123–1129.
11. Arnone, A., Benesch, R. E. & Benesch, R. (1977) *J. Mol. Biol.* **115,** 627–642.
12. Benesch, R., Triner, L., Benesch, R. E., Kwong, S. & Verosky, M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2941–2943.
13. Walder, J. A., Zaugg, R. H., Walder, R. Y., Steele, J. M. & Klotz, I. M. (1979) *Biochemistry* **18,** 4265–4270.
14. Walder, J. A., Walder, R. Y. & Arnone, A. (1980) *J. Mol. Biol.* **141,** 195–216.
15. Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. P., Rogers, P. H., Arnone, A. & Walder, J. A. (1986) *J. Biol. Chem.* **261,** 9929–9937.
16. Chaluardjian, A. & Rudnicki, E. (1970) *Anal. Biochem.* **36,** 225–226.
17. Assendelft, O. W. & Zilstra, W. G. (1975) *Anal. Biochem.* **69,** 43–48.
18. Wang, L. (1959) *Am. J. Physiol.* **196,** 188–192.
19. Crosby, W. H. & Furth, F. W. (1956) *Blood* **11,** 380–383.
20. Arnone, A., Rogers, P. H. & Briley, P. D. (1980) in *Biophysics and Physiology of Carbon Dioxide*, eds. Bauer, C., Gros, G. & Bartels, H. (Springer, Berlin), pp. 67–74.
21. Fronticelli, C., Toshihide, S., Orth, C. & Bucci, E. (1986) *Biochim. Biophys. Acta* **874,** 76–81.
22. Anderson, V. H., Leimgruber, P. P., Roubin, G. S., Nelson, D. L. & Gruentzig, A. R. (1985) *Am. Heart J.* **110,** 720–726.
23. Cleman, M., Jaffee, C. C. & Wohlgelernter, D. (1986) *Circulation* **74,** 555–562.
24. Bunn, H. F. & Forget, B. G. (1986) *Hemoglobin: Molecular, Genetic and Clinical Aspects* (Saunders, Philadelphia), pp. 637–640.
25. Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247,** 6960–6962.
26. Sadrzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E. & Eaton, J. W. (1984) *J. Biol. Chem.* **259,** 14354–14356.
27. Halliwell, B. & Gutteridge, J. M. C. (1985) *Free Radicals in Biology and Medicine* (Oxford Univ. Press, Oxford, U.K.), pp. 73, 184–186.
28. Kawanishi, S. & Caughey, W. S. (1985) *J. Biol. Chem.* **261,** 4622–4631.
29. Sehgal, L. R., Sehgal, H. L., Rosen, A. L., Gould, S. A., Rice, C. L. & Moss, G. S. (1981) *J. Surg. Res.* **31,** 13–17.