0273–2289/84/9000–0133$02.00

Preparation and In Vitro Characteristics of a Blood Substitute Based on Pyridoxylated Polyhemoglobin

P. E. KEIPERT AND T. M. S. CHANG*

Artificial Cells and Organs Research Centre, Faculty of Medicine, McGill University, 3655 Drummond St. Montreal, Quebec, Canada H3G1Y6

Received November, 1983; Accepted December, 1983

ABSTRACT

Stroma-free hemoglobin (SFHb) is prepared from outdated blood. Red cells are washed, lysed in hypotonic phosphate buffer, and stromal lipid is then removed by toluene extraction and high-speed centrifugation. Pyridoxal-phosphate (PP) added in a 4:1 molar ratio to deoxygenated SFHb, is covalently linked across the polyphosphate binding site of the Hb tetramer by reduction with $NaBH_4$ under N_2 for 18 h. Excess reagents are removed by dialysis. Subsequent crosslinking using 5.0% glutaraldehyde in the presence of lysine for 12–36 h yields soluble, macromolecular polyhemoglobin (PolyHb). Progress of the reaction is monitored by gel chromatography. In vitro work shows that different molecular weight (MW) PolyHb can be produced, ranging in weight from 130,000 to over 1,000,000. Intermediate MW PolyHb (<600,000) remains stable for months at 4°C, withstands prolonged incubation at 37°C, and has a viscosity similar to blood. SFHb has a low P_{50} = 15 torr, which has been raised to 26 torr by pyridoxylation. When PP–SFHb is crosslinked into PP–PolyHb, it can reversibly carry oxygen with a lower affinity (P_{50} = 16 torr) than nonpyridoxylated PolyHb (P_{50} = 11 torr). These favorable physicochemical properties of PP–PolyHb suggest that further progress toward a potential artificial blood substitute has been made.

Index Entries: Polyhemoglobin; pyridoxylated polyhemoglobin; artificial blood; blood substitutes; crosslinked hemoglobin; stroma-free

*Author to whom all correspondence and reprint requests should be addressed.

hemoglobin; polymerized hemoglobin; hemoglobin, crosslinked, stroma-free, pyridoxylated, or polymerized.

INTRODUCTION

The earliest endeavors to use hemoglobin solutions as a blood substitute were limited by such problems as renal damage and rapid removal from the circulation (*1*). In an attempt to overcome these problems, Chang prepared the first artificial red blood cells by microencapsulating hemoglobin solution (*2–5*), and also by crosslinking hemoglobin using agents such as sebacyl chloride (*2–5*) or glutaraldehyde (*6*). This crosslinking resulted in the formation of particulate polyhemoglobin artificial red cells with diameters of 1–6 μm. They survive only for a very short time in the circulation (*3,5*), and although they could carry oxygen, the affinity for oxygen was high (*3,5*). With the development of pyridoxylated hemoglobin with low oxygen affinity (*7*), there has recently been a return to the approach of hemoglobin crosslinking, originally proposed and developed by Chang (*2–6*). Using this method, polyhemoglobin is formed by intermolecular crosslinkage, and dimer formation is prevented by intramolecular crosslinks (*8*).

To solve the original problem of rapid removal of the large particulate, crosslinked hemoglobin (*2–5*), stroma-free hemoglobin has been crosslinked with glutaraldehyde into much smaller, macromolecular, soluble polyhemoglobin (*9*). Preliminary results from animal studies showed that intravenously infused stroma-free hemoglobin is rapidly removed from the circulation of rats with a half-life of about 1.5 h, and only 25% of the stroma-free hemoglobin remains after 3 h. In the case of crosslinked polyhemoglobin, however, its level in the circulation after 3 h was still 75–80% of the initially injected amount (*9*). Subsequent, more extensive in vivo studies have been done, using a total of 54 rats (*10*). To compare the intravascular survival of injected polyhemoglobin to that of stroma-free hemoglobin, rats were chronically cannulated to allow intermittent vascular access (*11*), and then given a 10–15% isovolemic exchange transfusion with the different hemoglobin preparations. When unmodified stroma-free hemoglobin was infused to a plasma concentration of about 1.5 g/dL, it was rapidly cleared from the circulation with a half-life ($T_{\frac{1}{2}}$) of about 1 h. Once crosslinked into polyhemoglobin, however, the circulation survival time was significantly prolonged, having a $T_{\frac{1}{2}}$ of 3.5 h (*10*). This improved $T_{\frac{1}{2}}$ obtained from rats injected with the same dose, is evidence that the glutaraldehyde crosslinks prevent hemoglobin dissociation and therefore limit renal excretion. This is further supported by results obtained from the analysis of urine samples, during an 8-h period following infusion (*10*). At 4 h after infusion of stroma-free hemoglobin, as much as 30% of the initial plasma hemoglobin level can be recovered in the urine. However, no polyhemoglobin was detected in the urine 4 and 8 h after injection of polyhemoglobin (*10*). Thus, the

problem of rapid clearance of stroma-free hemoglobin from the circulation can be solved by the crosslinkage of hemoglobin into soluble, macromolecular polyhemoglobin.

The second problem is related to the high oxygen affinity of hemoglobin, because of the absence of 2,3-diphosphoglyceric acid (2,3-DPG) and other intracellular organic phosphate ligands. Pyridoxal-5'-phosphate has analogous effects to 2,3-DPG in lowering oxygen affinity, and furthermore, it can be covalently linked to the hemoglobin tetramer across the normal polyphosphate binding site (*7*). As a result, several researchers have used this technique to pyridoxylate stroma-free hemoglobin, so as to improve its oxygen releasing ability (*12–14*). To make use of this beneficial effect of pyridoxal-phosphate, stroma-free hemoglobin is now being treated with pyridoxal-phosphate prior to glutaraldehyde crosslinking into polyhemoglobin. The resulting preparations of pyridoxylated stroma-free hemoglobin, and pyridoxylated polyhemoglobin have both been tested in vivo to assess their potential to function as a blood substitute (*10*). Pyridoxylated stroma-free hemoglobin tested for intravascular retention in rats, using the same method just described, was found to have a plasma disappearance half-life ($T_{\frac{1}{2}}$) of approximately 2 h. Following crosslinking into pyridoxylated polyhemoglobin, however, the $T_{\frac{1}{2}}$ increased to over 7 h, which represents a sevenfold increase in the short survival time of about 1 h obtained for stroma-free hemoglobin (*10*).

The present report will describe in detail the methods of preparation for each of the different hemoglobin solutions that have been tested in vivo for intravascular retention (*9,10*). In addition, some in vitro characteristics of polyhemoglobin such as size, stability, and oxygen affinities will be described and compared.

METHODS

Preparation of Hemoglobin Solution

Stroma-free hemoglobin is prepared by a refinement of the method previously described (*9*). Out-dated whole blood obtained from a local blood bank is centrifuged at 6000 rpm (4000*g*) for 20 min at 4°C. The plasma and buffy coat containing the leukocytes and platelets is removed by aspiration and discarded. The sedimented erythrocytes that remain are washed four times by suspension in approximately 2–3 times their volume in ice-cold, sterile isotonic saline. Following each wash, cells are resedimented by centrifugation. These washed cells are then lysed by adding two volumes of hypotonic, 15 ideal milliosmolar phosphate buffer, pH 7.4 (*15*), to one volume of packed cells. Gentle swirling and repeated inversion of the vessel for 2–3 min ensures thorough mixing. After standing 20–30 min, the lysed cells are poured into a large separa-

tory funnel, and 0.5 vol cold, reagent-grade toluene are added. The mixture is shaken vigorously for several minutes to completely emulsify the organic and aqueous phases, and then left to separate for 2–3 h at 4°C. Most of the organic phase containing the toluene-extracted stromal lipid and cellular debris is removed by aspiration, and the solution is then clarified by high-speed refrigerated centrifugation. A second toluene extraction is done, and the extraction mixture is then left to separate overnight in a cold room at 4°C. The lower layer of dark red, aqueous stroma-free hemoglobin is then separated and centrifuged at 25,000*g* for 1 h. Subsequently, the stroma-free hemoglobin is dialyzed for 3 h against physiological Ringer's solution using a standard hollow fiber dialyzer. This results in the removal of excess intracellular potassium, and balances electrolytes and pH.

Pyridoxylation of Hemoglobin

The stroma-free hemoglobin is intramolecularly crosslinked across the polyphosphate binding site using pyridoxal-5'-phosphate (Sigma Chemical Co.), by a slight modification of the method proposed by Benesch (*7*). First, stroma-free hemoglobin is deoxygenated under continuous nitrogen bubbling for 1–2 h at 4°C. Next, pyridoxal-phosphate in Tris-HCl is added in a 4:1 molar ratio to the deoxyhemoglobin, then reduced with excess $NaBH_4$ under N_2 for 18 h. Finally, excess reagents are removed by dialysis against an isotonic, buffered dialyzate solution† (pH 7.35), specially prepared to have electrolyte levels equivalent to normal rat plasma.

Crosslinking of Hemoglobin

Intermolecular crosslinking of stroma-free hemoglobin or pyridoxylated stroma-free hemoglobin is done in a cold room at 4°C, according to the method previously outlined (*9*). To regulate the speed of the reaction and to ensure formation of soluble polyhemoglobin, 10–20 equivalents of 1.3*M* lysine monohydrochloride is initially added. Aqueous 25% glutaraldehyde is diluted to a concentration of 0.5*M* in 0.1*M* phosphate buffer, then added to the hemoglobin solution in a 16:1 molar ratio. The resulting reaction mixture is left rotating on a mixer in the cold room. The progress of this reaction is monitored using agarose gel chromatography by periodically taking samples from the reaction vessel and running them on a Sepharose column. Once intermolecular crosslinking has produced the desired molecular weight range of either polyhemoglobin or pyridoxylated polyhemoglobin, the crosslinking is quenched by adding 16 mL of the 1.3*M* lysine-HCl solution. The solution containing the crosslinked polyhemoglobin is clarified by centrifuging at 25,000*g* for 1 h, and

†g/10 L: NaCl = 58.5; $NaHCO_3$ = 36.1; $NaH_2PO_4 \cdot H_2O$ = 4.4; KCl = 3.7; $CaCl_2 \cdot 2H_2O$ = 2.2; $MgCl_2 \cdot 5H_2O$ = 0.8.

then dialyzed for 3 h against buffered, isotonic dialyzate solution (pH 7.35), using a hollow fiber dialyzer. This removes unbound glutaraldehyde and excess lysine, and renders the final polyhemoglobin preparation physiological. Finally, the polyhemoglobin solution is filtered through a 0.45 μm Nalgene sterilization filter unit, and then stored under sterile conditions at 4°C to minimize methemoglobin formation and to retain critical functional characteristics (*16*).

In Vitro Characteristics

By controlling the length of time allowed for crosslinking, one can regulate the molecular weight range of the polyhemoglobin. Gel chromatography columns containing Sepharose 4B (60,000–20 million daltons) and Sepharose 6B (10,000–2 million daltons) are calibrated with appropriate high molecular weight globular protein standards (Pharmacia Fine Chemicals). Elution through these calibrated columns of the polyhemoglobin samples taken during the crosslinking reaction gives an approximate indication of the molecular weight range. In this manner, intermediate molecular weight and very high molecular weight polyhemoglobin can be prepared.

To test the structural stability of these two different molecular weight range polyhemoglobins at 4 and at 37°C, one sample of each is placed in a shaking incubator set at 100 rpm (A. O. Corp., Scientific Instruments Div.). Approximately every 3–4 h a sample is taken and run on the calibrated Sepharose 6B gel column, and the peak areas of the elution profile can then be compared to the original.

Viscosity measurements using a Cannon-Ubbelohde type viscometer equilibrated at 37°C, showed that intermediate molecular weight polyhemoglobin has a kinematic viscosity very similar to rat blood. For this reason, it was chosen in favor of the high molecular weight polyhemoglobin for any in vivo testing (*10*).

To compare the oxygen affinities of crosslinked polyhemoglobin versus stroma-free hemoglobin, oxygen dissociation curves were generated using an IL 282 Co-oximeter calibrated for hemoglobin, the technique of tonometry, and a Corning Model 175 pH/Blood Gas Analyzer. The P_{50} values derived from the dissociation curves have been corrected to standard conditions of pH and pCO_2 (pH = 7.4, pCO_2 = 40 mm Hg, temp. = 37°C).

RESULTS AND DISCUSSION

Following the preparation of the different stroma-free and crosslinked polyhemoglobin solutions, each has been dialyzed against a buffered dialyzate solution that has electrolyte levels, osmolality, and pH at normal rat plasma values. The data characterizing the final hemoglobin solutions are given in Table 1. These values apply to all four types of he-

TABLE 1
Analysis of Final Solutions of Stroma-Free and Crosslinked Hemoglobin

Assays	Range[a]	Mean ± SD
Hemoglobin, g/dL	8–12	10.2 ± 1.4
Osmolality, mOs/kg	280–310	295.5 ± 10.8
Na^+, mEq/L	134–150	142.0 ± 5.5
K^+, mEq/L	4.2–5.4	4.80 ± 0.4
Cl^-, mEq/L	108–114	111.5 ± 2.2
pH	7.3–7.4	7.35 ± 0.03

[a]From eight preparations.

moglobin preparations tested: stroma-free hemoglobin, polyhemoglobin, pyridoxylated stroma-free, and pyridoxylated polyhemoglobin.

The effect of the time allowed for crosslinking on the molecular weight of the resulting polyhemoglobin is shown in Fig. 1. Stroma-free hemoglobin (SFHb), having a molecular weight of only 68,000, elutes very close to the bed volume (V_t) on the Sepharose 6B column. Crosslinking for about 12–36 h yields intermediate molecular weight polyhemoglobin (molecular weight ranging from 130,000 to 600,000). If the reaction is allowed to proceed for several days, a very high molecular weight polyhemoglobin is obtained. This polyhemoglobin has a molecular weight greater than 1,000,000 daltons, and therefore elutes primarily at the void volume (V_o) of the column (Fig. 1).

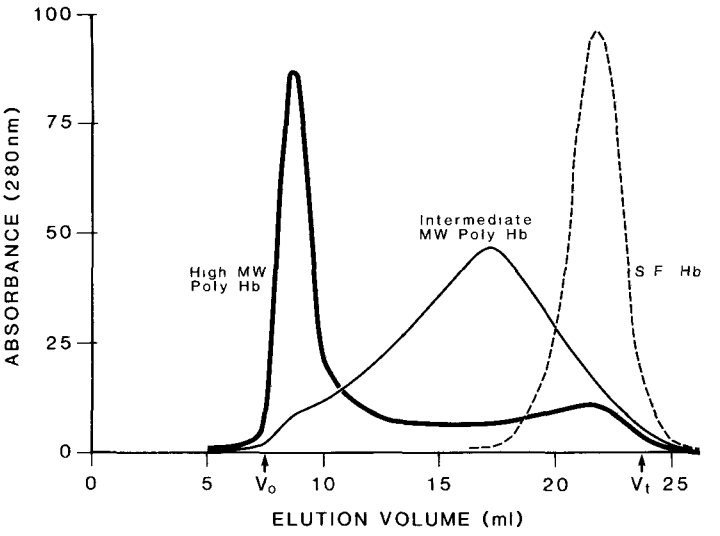

Fig. 1. Gel chromatography elution profiles of hemoglobin solutions run on Sepharose 6B column (10,000–2 × 10^6 daltons exclusion limit). Stroma-free hemoglobin (SFHb) crosslinked for 12–36 h yields intermediate molecular weight (MW) polyhemoglobin (PolyHb). High MW PolyHb is obtained after 3–5 d of crosslinking.

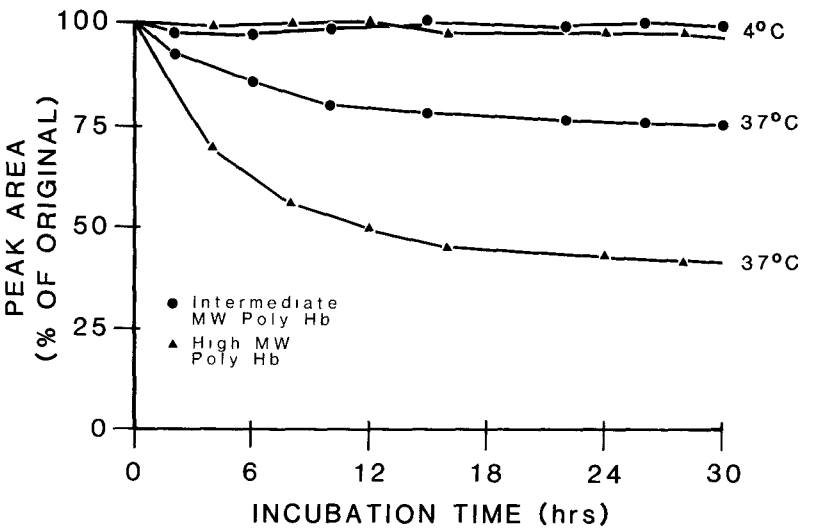

Fig. 2. Stability of polyhemoglobin samples kept in a shaking incubator at 100 rpm. Peak areas have been calculated from the elution profiles obtained by gel chromatography of polyhemoglobin samples on Sepharose 6B column.

Following incubation at 37°C with shaking at 100 rpm, it appears that the smaller, intermediate molecular weight polyhemoglobin is structurally more stable than the very high molecular weight polyhemoglobin. This is shown in Fig. 2, where the different peak areas from the elution profiles have been used as an approximation of the relative amounts of the different molecular weight ranges. At 4°C, on the other hand, no real difference in stability is seen between the two, and both have been found to remain stable for several months when stored at 4°C. Because of the excessive viscosity (2–3 times that of whole blood) of the very high molecular weight polyhemoglobin (>1,000,000 daltons), it was not used in vivo. Rather, the intermediate molecular weight range polyhemoglobin (<600,000 daltons) has been used, both for in vivo testing and for in vitro P_{50} determinations.

The effect that both pyridoxylation and crosslinking have on the oxygen affinity of hemoglobin, is represented by the partial pressure of oxygen necessary to produce 50% saturation of hemoglobin at pH 7.4 and 37°C (P_{50}). These P_{50} values are given in Table 2 for each of the different stroma-free and crosslinked hemoglobin preparations. Pyridoxylation lowered the high oxygen affinity of stroma-free hemoglobin (P_{50} = 14–16 torr), to a P_{50} of 25–26 torr for pyridoxylated stroma-free hemoglobin. This is essentially the P_{50} value of whole blood. Intermolecular crosslinking of stroma-free hemoglobin into soluble, macromolecular polyhemoglobin gives a P_{50} of about 11 torr. If stroma-free hemoglobin is pyridoxylated prior to glutaraldehyde crosslinking, then the resulting pyridoxylated polyhemoglobin is more capable of reversibly carrying oxygen with a lower affinity (P_{50} = 16 torr) than nonpyridoxylated polyhemoglobin (Table 2).

TABLE 2
Oxygen Affinities of Stroma-Free and Crosslinked Hemoglobin Solutions Expressed as P_{50} Values

Hemoglobin solutions	Concentration, g/dL	In vitro P_{50}[a], torr
Stroma-free hemoglobin	9.0	14–16
Polyhemoglobin	10.0	10–12
Pyridoxylated stroma-free hemoglobin	8.5	25–26
Pyridoxylated polyhemoglobin	9.5	15–18

[a]At standard conditions: pH = 7.4; pCO_2 = 40 mm Hg; temp. = 37°C.

Thus, it appears that both of the major limitations restricting the practical use of stroma-free hemoglobin solutions have been improved upon by appropriate chemical modifications. The survival time in the circulation of only 1 h for unmodified stroma-free hemoglobin, has been increased to greater than 7 h by crosslinking with glutaraldehyde. In addition, the poor oxygen-releasing ability of stroma-free hemoglobin has been significantly improved by treatment with pyridoxal-5′-phosphate. This, in turn, has enabled the production of an intermediate molecular weight pyridoxylated polyhemoglobin with improved oxygen dissociation characteristics. These favorable physicochemical properties of this pyridoxylated polyhemoglobin has allowed us to successfully resuscitate lethal hemorrhagic shock in rats.

ACKNOWLEDGMENTS

We gratefully acknowledge Mr. Colin Lister from this research center for helpful technical advice, and would also like to thank Ms. Carmen Darway from the Blood Gas Laboratory, Royal Victoria Hospital, for the oxygen dissociation curves. This research has been supported by the Medical Research Council of Canada (MRC-SP-4 to TMSC).

REFERENCES

1. Hamilton, P. B., Hiller, A., VanSlyke, D. D. (1948), *J. Exptl. Med.* **85,** 477.
2. Chang, T. M. S. (1964), *Science* **146,** 524.
3. Chang, T. M. S. (1965), Semipermeable Aqueous Microcapsules. Ph.D. Thesis, McGill University, Montreal, Canada.
4. Chang, T. M. S., MacIntosh, F. C., and Mason, S. G. (1966), *Can. J. Physiol. Pharmacol.* **44,** 115.
5. Chang, T. M. S. (1972), *Artificial Cells,* Thomas, Springfield, IL, p. 93.
6. Chang, T. M. S. (1971), *Biochem. Biophys. Res. Comm.* **44**(6), 1531.
7. Benesch, R. E., Benesch, R., Renthal, R. D., and Maeda, N. (1972), *Biochem* **11**(19), 3576.
8. Chang, T. M. S. (1980), *Trans. Am. Soc. Artif. Intern. Organs* **26,** 354.

9. Keipert, P. E., Minkowitz, J., and Chang, T. M. S. (1982), *Int. J. Artif. Organs* **5**(6), 383.
10. Keipert, P. E., and Chang, T. M. S. (1983), *Trans. Am. Soc. Artif. Intern. Organs* **29,** 329.
11. Tabata, Y., and Chang, T. M. S. (1982), *Artif. Organs* **6**(2), 213.
12. Greenburg, A. G., Schooley, M., and Peskin, G. W. (1977), *J. Trauma* **17**(7), 501.
13. Sehgal, L. R., Rosen, A., Noud, G., Gould, S., Sehgal, H., DeWoskin, R., Rice, C., Kerstein, M., and Moss, G. S. (1979), *Eur. Surg. Res.* **11,** 43.
14. DeVenuto, F. (1982), *Crit. Care Med.* **10**(4), 238.
15. Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys.* **100,** 119.
16. Greenburg, A. G., Ginsburg, K., and Peskin, G. W. (1977), *Surg. Forum* **28,** 5.