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Pyridoxylated Polymerized Hemoglobin Solution Processing

Interest of a Membrane Molecular Fractionation Step

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ABSTRACT

Glutaraldehyde hemoglobin polymerization gives too many high polymers, resulting in a too viscous solution. We describe here an alternate method leading to superior results, as compared to the classical one. This method includes a molecular fractionation step using a tangential flow ultrafiltration that secondarily lowers the unpolymerized tetramer's content of a mildly polymerized, pyridoxylated hemoglobin solution (Pyr–Poly Hb). This leads to an adequately polymerized product with a lesser high polymer content, implying a lower viscosity. We thus obtain a pyridoxylated, polymerized molecular fractionated solution presenting suitable features as a blood substitute: A 7.5 g% hemoglobin 2 g% albumin solution had a 16% unpolymerized tetramer's ratio, a 1.8 mPas viscosity, a P_{50} of 2.8 kPa, a Hill coefficient of 2.1, a binding coefficient of 1.3 mL/g, a colloid osmotic pressure of 2.4 kPa, and a methemoglobin concentration of 3% Male Sprague-Dawley rats undergoing an isovolumic blood exchange with this Pyr–Poly Hb solution, down to a 2% hematocrit, present a mean survival time of 20 h.

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Index Entry: Blood substitute; stroma-free hemoglobin; pyridoxylated polyhemoglobin; glutaraldehyde hemoglobin polymerization; molecular polyhemoglobin fractionation; polyhemoglobin tangential-flow ultrafiltration; polyhemoglobin viscosity; blood–exchanged rat.

INTRODUCTION

A stable product that is both a vascular filling liquid and a good oxygen carrier would solve many problems inherent in emergency transfusion. Recent works have shown that modified hemoglobin solutions give promising results. Best in vivo assays have been obtained with chemical modification of hemoglobin (Hb), such as pyridoxylation, which increases its P_{50}, and glutaraldehyde polymerization, which decreases its clearance.

Such a pyridoxylated, polymerized hemoglobin preparation (Pyr–Poly Hb) designed by Dudziak and Bonhard (*1*) and DeVenuto and Zegna (*2*) seems to present a too high intrinsic viscosity, related to a large proportion of high polymers. The membrane molecular fractionation described by Kothe et al. (*3*) would correct this weakness. We present here the characteristics and benefits of a Pyr–Poly Hb preparation obtained by this technique.

MATERIAL AND METHODS

Preparation

The hemoglobin used was obtained from outdated blood bank erythrocytes stored in CPD (citrate, phosphate, dextrose). The processing involved five steps: erythrocyte washing, stroma freeing, pyridoxylation, polymerization, and tangential ultrafiltration. All steps except pyridoxylation were carried out at a controlled temperature (+4°C).

Cells sustained four washings: A first one in 0.19*M* $NaHCO_3$; a second one in 0.09*M* $NaHCO_3$ and 0.07*M* NaCl; and two resuspensions in 0.15*M* NaCl.

After the last washing, 1.5 vol of ice-cold, distilled water were added to 1 vol of packed, washed erythrocytes, and a mild mixing was conducted for 90 min. The pH was then lowered to 5.6 with 1*M* HCl, and, 15 min later, all the stroma were flocculated and spun out by a 10-min centrifugation at 30,000*g*. The supernatant was neutralized to pH = 7.3 with 1*M* NaOH and clarified through successive filters of decreasing porosity down to 0.2 μm diameter.

The pyridoxylation method utilized was similar to that described by Benesh et al. (*4*). The Hb solution was buffered at pH = 7.3 by a Tris-HCl solution and deoxygenated by nitrogen bubbling in a Gambro G10 TP cell between 8 and 10°C. A droplet addition of pyridoxal-5′-phosphate (PP)

(Sigma), 85 m*M* in Tris 1*M*, was then set up so as to reach a molar ratio of 4:1 PP/Hb, as described by DeVenuto et al. (2). The pH was immediately adjusted to 6.8, and 30 min later nitrogen bubbling was stopped. Sodium borohydride ($NaBH_4$) (Aldrich) was added in a molar ratio of 20:1 ($NaBH_4$/Hb). The reaction was allowed to develop with a slight stirring for 90 min. Excess reactant was then dialyzed (Spiraflo SD 816) by continuous flow against isotonic saline solution.

The polymerization was started after an Hb concentration of up to 15 g% by negative pressure ultrafiltration. A slow addition of 2.5% glutaraldehyde (G) (Prolabo) was undertaken with rapid stirring until a molar ratio of 7:1 G/Hb was attained. The stirring was maintained for 10–12 h, and an excess of lysine was then added (3:2 lys/G). After a further 2 h of stirring, the solution was filtered through a 0.2-μm filter.

The molecular fractionation was operated with a Millipore Pellicon™ cell equipped with filtration membranes of 100-kdalton cut-off. The Hb solution recirculated through the cell, and its volume was kept constant with isotonic saline infusion. Clearance of unpolymerized tetramers was monitored by gel filtration.

In order to compare this glutaraldehyde polymerization followed by membrane molecular fractionation with the classical glutaraldehyde polymerization process without molecular fractionation, we assayed polymerization of pyridoxylated hemoglobin aliquots using various molar ratios of G/Hb ranging from 3 to 14.

For in vivo assays a 84% crosslinked Pyr–Poly Hb solution was adjusted to 7.5 g% concentration and supplemented with ions, glucose, and human serum albumin.

In Vitro Measurements

Hemoglobin concentration and saturation were assayed with the Radiometer OSM2 Hemoxymeter. Methemoglobin was measured as described by Kaplan (5). The Lexington Lex-O_2-Con was used for coulometric titration of the O_2-binding coefficient at +23°C, with a PO_2 of 20.6 kPa.

The oxygen dissociation curve was plotted using an Oxymeter DCA 1 from Radiometer at 37°C, pH = 7.4, and PCO_2 = 4.7 kPa, as described by Duvelleroy et al. (6) and Teisseire et al. (7). Viscosity measurements were made with a Low Shear Contraves viscometer at 37°C and a 87/s shear rate.

Other determinations included a Thomas BMT 921, a Roebling osmometer, and a Superose 12™ column (10 × 300 mm) for gel filtration in Pharmacia FPLC.

In Vivo Assays

Male Sprague-Dawley rats (400 ± 30 g) underwent an isovolumic blood exchange stopped at 2% hematocrit. Animals were bled at a 1.6

mL/min rate and simultaneously infused with a Pyr–Poly Hb solution or with Plasmagel™ for control experiments.

After completion, catheters were withdrawn and animals set in a physiologically and thermostatically controlled environment. They received a 5-mL intraperitoneal isotonic (glucose/NaCl 1:1) injection every 2 h for 10 h in order to prevent hypovolemia.

RESULTS

Elution diagram of Pyr–Poly Hb solutions shows three main fractions (Fig. 1): High polymers were first eluted, then an intermediate pool, which we call oligomers, and, finally, a last fraction, which represented the unpolymerized tetramers. Throughout the ultrafiltration, unpolymerized tetramers firmly decreased in the retentate, whereas high poly-

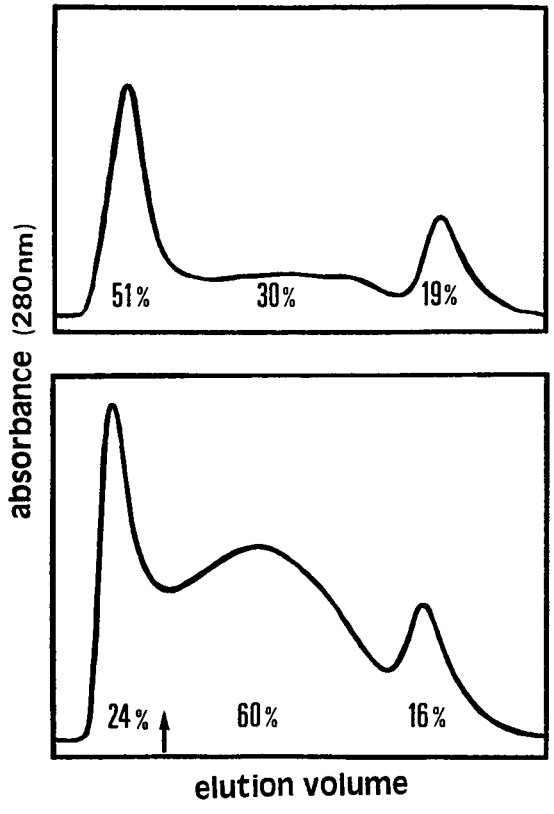

Fig. 1. Gel filtration elution profiles of Pyr-Poly Hb solutions run on Superose 12™ column. Three main fractions can be differentiated: high polymers in void volume, oligomers, unpolymerized tetramers. (↑) shows the thyroglobulin (660 kdalton) elution volume. (upper) classical glutaraldehyde polymerization process. (lower) glutaraldehyde polymerization + molecular fractionation process.

mers increased, and the oligomers ratio was unchanged and predominated (Table 1).

In addition to unpolymerized tetramers, the ultrafiltrate contained a few percent of oligomers and high polymers (Fig. 2).

When G/Hb ratios were increased in the classical processing without ultrafiltration, we found a drastic increase of high polymers. The ratio of 12 (G/Hb) led to a gelatinous consistency, whereas the percentage of unpolymerized tetramers remained above 19% (Table 2).

TABLE 1
Evolution of the Molecular Pattern in Pyr–Poly Hb During the Process Including a Molecular Fractionation[a]

Time, h TO	Unpolymerized tetramers, %	Oligomers, %	High polymers, %
	45	47	8
1	36	54	10
2	30	59	11
3	26	61	13
4	25	60	15
5	21	59	20
6	16	60	24

[a]Percentages were calculated from gel filtration chromatography elution profiles. (TO: initial state, a membrane regeneration was undertaken after 4 h).

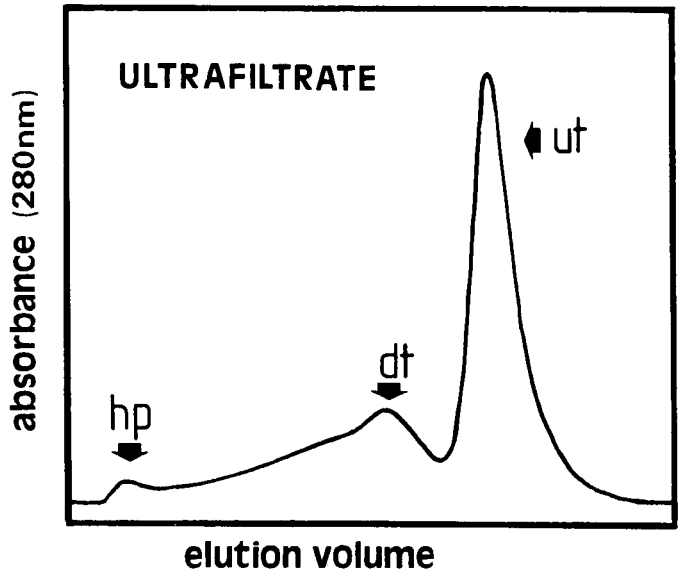

Fig. 2. Gel filtration chromatography elution profile of ultrafiltrate issued from Pyr–Poly Hb preparation, showing unpolymerized tetramer (ut), 130-kdalton oligomer (dt) and high polymers (hp).

TABLE 2
Molecular Pattern of Pyr–Poly Hb as a Function of Molar Ratio of Glutaraldehyde/Hb[a]

Molar ratio, G/Hb	Unpolymerized tetramers, %	Oligomers, %	High polymers, %
3	79	21	0
5	61	37	2
7	42	47	11
9	31	41	28
10	25	32	43
12	19	30	51
>12		gelatinous consistency	

[a]Percentages were calculated from gel filtration chromatography elution profiles.

Figure 3 shows the viscosity of the preparation as a function of the unpolymerized tetramer percentage when four different concentrations of Pyr–Poly Hb were assayed; we compared results obtained with the classical polymerization and the method with an ultrafiltration step. In all cases ultrafiltered solutions led to lower viscosities, particularly when the unpolymerized tetramer percentage went lower than 25%. Table 3 shows the viscosity of 81–82% crosslinked, molecular fractionated Pyr–Poly Hb solutions. The 13.5 g% solution has the same viscosity as the whole blood.

If η is the viscosity of the Pyr–Poly Hb and η_0 the viscosity of a 0.15*M* NaCl solution, specific viscosity, η_{sp}, has the following ratio:

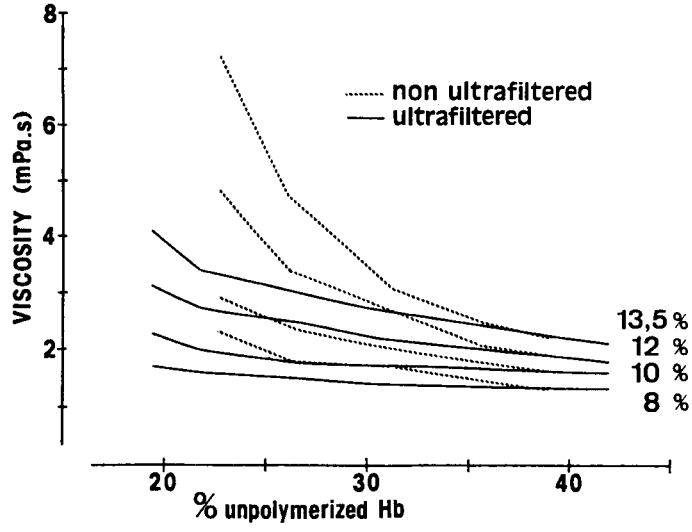

Fig. 3. Viscosity as a function of unpolymerized tetramers at various Hb concentrations: 13.5 g%, 12 g%, 10 g%, 8 g%. (——) glutaraldehyde polymerization + molecular fractionation process. (----) classical glutaraldehyde polymerization process.

TABLE 3
Viscosity Values of 81–82% Crosslinked Molecular Fractionated Pyr–Poly Hb Solutions at Different Hb Concentrations (mean ± SD), ($n=4$)

Hb, g%	Viscosity, mPas
8	1.78 ± 0.09
10	2.42 ± 0.22
12	3.39 ± 0.35
13.5	4.30 ± 0.45

$$\frac{\eta - \eta_0}{\eta_0}$$

In Fig. 4, the specific viscosity per unit of Hb concentration is plotted against the Hb concentration for different unpolymerized tetramer's percentages. The curves obtained show an upward concavity. The zero concentration extrapolation gives the intrinsic viscosity ([η]): a value of 4 mg/mL for the stroma free Hb was found, and [η] ranged from 10 to 13 mg/mL for the Pyr–Poly Hb preparation.

These results account for moderately elongated and flexible structures. In such a case [η] can be related to the mean molecular weight (mw) with the following equation:

$$[\eta] = K.mw^{\alpha}$$

where K and α are constant parameters.

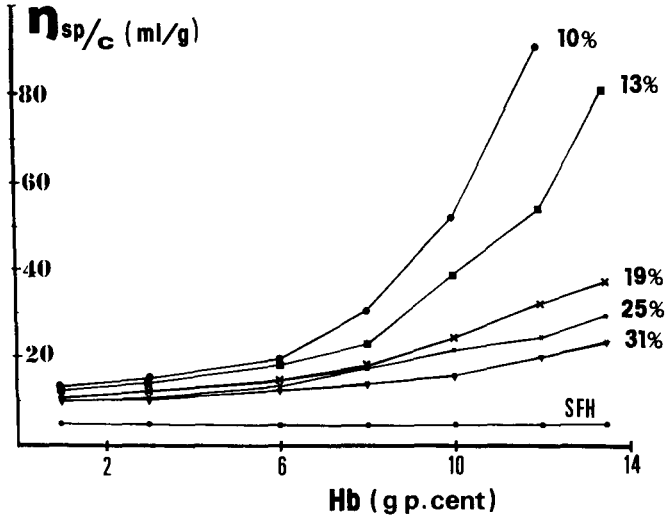

Fig. 4. Molecular fractionated Pyr-Poly Hb. Specific viscosity/Hb concentration ratio as a function of Hb concentration when the remaining unpolymerized tetramers is observed at 10%, 13%, 19%, 25%, 31%. Stroma free Hb (SFH) is plotted as reference.

TABLE 4
Characteristics of an In Vivo Tested
Pyr–Poly Hb Solution

Hemoglobin, g%	7.5
Human albumin, g%	2
Glucose, mmol/L	5.6
Sodium, mmol/L	138
Potassium, mmol/L	4.5
Calcium, mmol/L	1.3
Lactate, mmol/L	4
Standard bicarbonate, mmol/L	14
Unpolymerized tetramers, %	16
Viscosity, mPas	1.8
Osmolality, mOsm/kg	320
Oncotic pressure, kPa	2.4
P50, kPa	2.8
Hill's coefficient	2.1
Oxygen binding coefficient, mL/g	1.3
Methemoglobin, %	3

Thus, the interest in reducing the mean mw of crosslinked Hb becomes more evident.

In Fig. 5, the oncotic pressure values are plotted against the percentage of the unpolymerized tetramer at different concentrations of Pyr–Poly Hb. For an optimally polymerized solution (i.e., containing 20% or less unpolymerized tetramer) and whatever the surveyed concen-

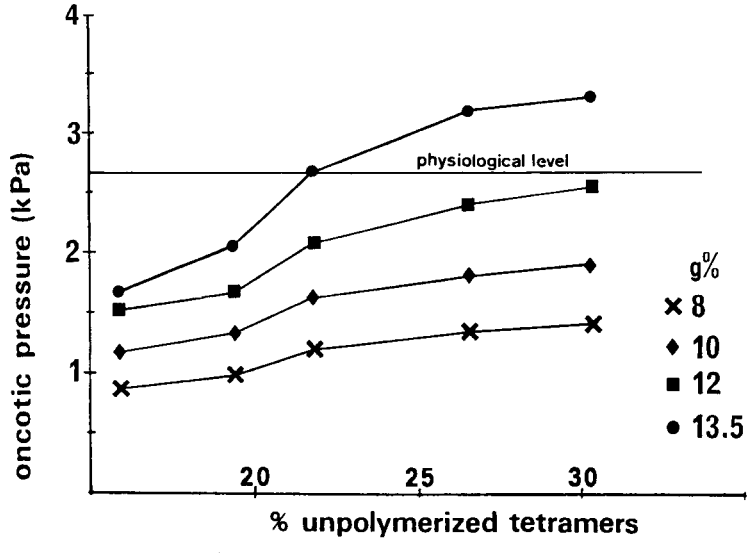

Fig. 5. Molecular fractionated Pyr-Poly Hb. Oncotic pressure as a function of the remaining percentage of unpolymerized tetramers at various Hb concentrations: 13.5 g%, 12 g%, 10 g%, 8 g%.

tration of Pyr–Poly Hb is, the solution would require an oncotic pressure adjustment to reach the physiological levels.

The oxygen dissociation curves of Pyr–Poly Hb and of whole blood are plotted in Fig. 6. Curves were computerized using Hill's equation for values of oxygen saturation between 30 and 70%.

The Pyr–Poly Hb solution showed a P_{50} of 2.8 kPa (SD = 0.07, n = 5) and a Hill's coefficient of 2.1, as compared to the standard values of 3.4 kPa and 2.6. The O_2-binding coefficient was 1.3 mL/g, and the methemoglobin ratio was below 3%.

Control rats, blood exchanged with Plasmagel™ down to an hematocrit of 2%, survived 5 min. Animals transfused with the Pyr-Poly Hb preparation showing the characteristics displayed in Table 4 presented a mean survival time of 20 h (±5) and were able to move and feed themselves.

DISCUSSION

The Hb polymerization by glutaraldehyde leads to a very heterogeneous preparation that often has an excessive viscosity.

DeVenuto et al. (*2,8*) started their preparation with hemoglobin purified by crystallization, and obtained from a 1-h polymerization (G/Hb = 7) a solution containing 20% of unpolymerized tetramer and 17% of high polymer. This solution has the same viscosity as whole blood, but the concentration was not mentioned.

Sehgal et al. (*9*) polymerized hemoglobin in the presence of lysine. The reaction was stopped between 12 and 18 h after slow infusion of glutaraldehyde, when the G/Hb ratio reached 14–17. This preparation

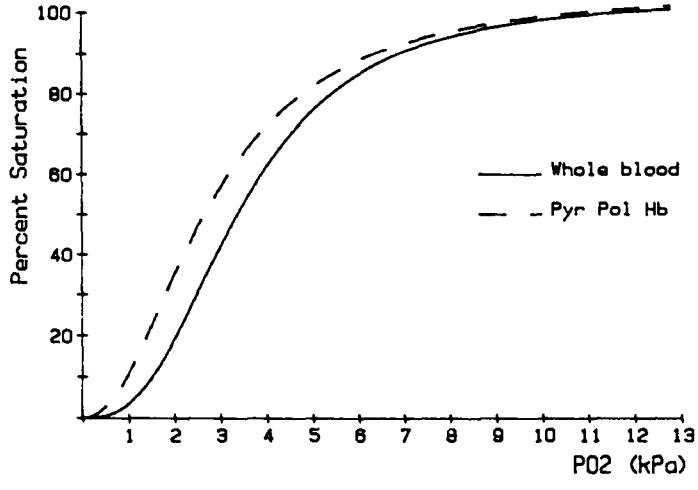

Fig. 6. Oxygen dissociation curves of Pyr–Poly Hb and whole blood at pH = 7.40, PC02 = 4.7 kPa, t = 37°C.

contained 20% of unpolymerized tetramer and 60% of high polymer, showing a viscosity of 4.5 cp for a 14-g% concentration.

Keipert and Chang (*10*) suggested that addition of lysine could control the rate of reaction, this resulted in more soluble complexes, whereas the final G/Hb ratio reached 16. When polymerizing between 12 and 36 h, they obtained an "intermediate mw polyhemoglobin"; for a concentration of 8–12 g%, its viscosity was similar to whole blood. A 4–5-d polymerization led to a mixture of "high mw" presenting the same gel chromatography elution profile as that obtained by Sehgal et al. (*9*). Its viscosity was up to three times the total blood viscosity for a concentration of 8–12 g%.

When we used the classical polymerization process with a G/Hb ratio of 12, we obtained a similar (high mw) elution profile. The viscosity was identical to that stated by Keipert and Chang (*10*), but much higher than that stated by Seghal et al. (*9*).

Kothe et al. (*3*) initially proposed a membrane molecular fractionation to lower the unpolymerized tetramer. Their preparation had a relative viscosity of three for a concentration of 8.5 g% and a percentage of 15% residual unpolymerized tetramer.

This variability observed from different author's reports is related to numerous factors, such as the quality of the starting hemoglobin or the various processings or the quality of glutaraldehyde employed; in fact, glutaraldehyde is reported to be a complex mixture of self-reacting molecules (*11*). Nevertheless, the mw heterogeneity of the polymers obtained stands as the common feature presented by all authors.

A Pyr–Poly Hb solution designed to be used as a resuscitation fluid should ideally contain the 130 kdalton oligomer. Such a solution at 14 g% would be isooncotic to plasma and retains the same O_2 content of the whole blood. Moreover, this solution should have a long intravascular retention, a maximum rate of cooperativity, a lower potential immunogenicity, and a minimal viscosity. The latter requirement seems to be essential, since we observed a shortening of the survival time when animals received solutions presenting a viscosity greater than 3.5 mPa.s. Thus, we agree with Kothe et al. (*3*) in emphasizing the importance of the viscosity parameter.

This work demonstrates the beneficial effects of a molecular fractionation step, after a moderate glutaraldehyde polymerization, thus leading to an important oligomer content that minimizes the viscosity of the Pyr–Poly Hb solution.

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