Tetrahedron, Vol. 37, pp. 1723 to 1726, 1981
Printed in Great Britain.

0040-4020/81/091723-04$02.00/0

SYNTHESIS OF POLYMER-BOUND HEMOGLOBIN SAMPLES

J. E. BALDWIN,* B. GILL and J. P. WHITTEN
Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, England

and

H. TAEGTMEYER
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, England

(*Received in UK 20 October 1980*)

Abstract—Dextran and hydroxyethyl starch have been chemically modified to give aldehyde-substituted polymers. These modified polymers were synthesised either by periodate oxidation of the starting polymer or by attachment of gluteraldehyde to amino-substituted side chains on the polymer. When such modified starches were allowed to react with hemoglobin soluble polymer-bound hemoglobins were formed. These were shown to be capable of binding oxygen but the observed oxygen-binding curves were shifted to the left relative to unbound hemoglobin. Heart perfusion experiments indicate that these polymer-bound hemoglobins are not suitable for use as blood substitutes.

The experimental search for a clinical blood substitute capable of delivering oxygen from the lungs to the body tissues has focused on two very different approaches. The first uses hemoglobin to transport oxygen, either in the form of stroma-free hemoglobin, or as a solution of cross-linked or polymer-bound hemoglobin. The use of hemoglobin solutions devoid of erythrocytes and therefore usable without prior blood typing has been considered for some time[1,2] and the method received considerable impetus from the discovery that such solutions could be made nontoxic on removal of erythrocyte stroma from the preparation.[3] More recently, attempts have been made to overcome the rapid excretion of hemoglobin through the kidneys and the massive hemoglobinuria which follows administration of stroma-free hemoglobin. This has usually involved increasing the effective molecular size of the hemoglobin, either by polymerisation or cross-linking[4-7] of the hemoglobin or through covalent coupling of the hemoglobin to a polymeric carrier.[8-10]

An entirely different approach to oxygen delivery is achieved through the use of perfluorochemicals. Here the mechanism is simply solution of oxygen. The ability of perfluorocompounds to sustain life was convincingly demonstrated in 1973 when Geyer described experiments in which the blood of rats was progressively but totally replaced by a fluorocarbon emulsion with subsequent survival of the animals.[11,12] More recently such emulsions have been used in clinical trails.[13]

We report a further method of preparing polymer-bound hemoglobins. These modified hemoglobins can be stored as freeze-dried powders and readily reconstituted when required. They have been shown to be capable of reversibly binding oxygen, the observed oxygen-binding curves being left-shifted relative to that for hemoglobin. Heart perfusion experiments, however, indicate that these modified hemoglobins release insufficient oxygen to be suitable for use as a blood substitute.

Materials and methods

Both dextran and hydroxyethyl starch were used in these experiments. The dextran (Sigma, Clinical grade) had an average molecular weight of 173,000. Three hydroxyethyl starch samples were used with average molecular weights 221,900 (Arnar–Stone Labs.), 400,000 (McGraw Labs.) and 479,900 (Arnar–Stone Labs.) respectively.

Aminoethylamino-substituted starches were prepared by a modification of the method of S–C Tam *et al.*[8] In a typical preparation, 1.5 g cyanogen bromide (Eastman Co.) was dissolved in 15 ml acetonitrile (Fisons SLR) and added to 500 ml 2% starch solution. The pH of the solution was maintained at 10.8 for 5–10 min by the addition of 1 M sodium hydroxide solution. The pH was then lowered to 2.0–2.5 with concentrated hydrochloric acid and 10 ml diaminoethane was added along with additional hydrochloric acid to prevent the pH from exceeding 9.5. The final pH was adjusted to 9.5 and the solution allowed to stand overnight at 4° before being dialysed against deionised water. A small sample for analysis was collected by freeze-drying, while the bulk of the dialysed solution was used for the following reactions. The ratio of cyanogen bromide/diaminoethane to starch has been varied, allowing a range of aminoethylamino-substituted hydroxyethyl starches and dextrans to be studied. Thus aminoethylamino-substituted starches with from 7 to 20% of the glucose residues in the starting polymer being substituted have been synthesised.

Aldehyde-substituted starches were prepared by reaction of the aminoethylamino-substituted starch solutions with glutaraldehyde. In a typical reaction approximately 500 ml dialysed solution of aminoethylamino-substituted hydroxyethyl starch or dextran was treated with 2 g sodium bicarbonate to give a solution 2% in starch and approximately 0.05 M in bicarbonate. 5 ml 50% glutaraldehyde solution (B.D.H.) was then added to the solution which was stirred at room temperature for 2 hr before being dialysed. Samples for analysis were collected by freeze-drying, the bulk of the solution being used directly for reaction with hemoglobin.

Hemoglobin (Warner Lambert) was supplied as a freeze-dried solid under carbon monoxide by Dr. R. P. Geyer. This was reconstituted under argon using deoxygenated deionised water at 4° to give a solution with approximately 2.5 g hemoglobin per ml. In a typical

reaction 500 ml dialysed solution of aldehyde-substituted starch prepared as described above was treated with sodium bicarbonate to give 500 ml solution approximately 2% in starch and 0.1 M in bicarbonate. 25 ml hemoglobin solution was then added and the reaction mixture stirred at room temperature for 4 hr, when gel filtration on Sephadex G 150 indicated that no unbound hemoglobin remained. Sodium borohydride (1.0 g) was then added to the solution which was stirred for a further 2 hr at room temperature. The sample was dialysed using an Amicon Ultrafiltration Unit with a 100,000 cut-off cartridge which enabled any trace of unbound hemoglobin to be removed. 10 g of glucose were then added to the solution which was freeze-dried and stored under carbon monoxide at 4° until required.

Polymer-bound hemoglobin samples were also synthesised from dialdehyde starches as follows. Using a modification of the method of Tam *et al.*,[8] 0.03 equivalents of starch were dissolved in 250 ml water and treated with 0.028 mol sodium periodate for 12 hr at 5° in the dark. The solution was dialysed until ion free and the % oxidation determined using a coulourimetric method.[14] The solution was then buffered to pH 8.0 by addition of sodium bicarbonate, cooled to 5° and treated with 5 g carbonylated hemoglobin. The reaction was allowed to proceed for 18 hr at room temperature after which gel filtration indicated complete binding of hemoglobin. The solution was dialysed against 1% aqueous ammonium carbonate then freeze-dried in the presence of glucose.

Oxygen-binding curves were obtained for polymer-bound hemoglobin samples prepared following either of these methods using an Amino Hem-o-scan unit.

Heart perfusion experiments were performed as follows. Rat hearts weighting approximately 1.2 g were perfused at near physiological work load in a perfusion apparatus for the working heart.[15] Krebs Henseleit bicarbonate saline (150 ml) was fortified with 1% polymer-bound hemoglobin and recirculated in the apparatus. The change in oxygen carrying capacity of the perfusion medium was determined. The oxygen content of the perfusion media used were determined by addition of 0.6% ferricyanide solution at 37° in a sealed Erlenmeyer flask using a precalibrated oxygen electrode.

RESULTS

When hemoglobin was covalently coupled to aldehyde-substituted dextran (av. mol. wt. 173,000) or hydroxyethyl starches (av. mol. wts. 221,900, 400,000 and 479,900) a polymer-bound hemoglobin was formed. The amount of bound hemoglobin obtained was found to vary with both the concentration of reactants and the reaction time. In general it was found that reactions between highly concentrated solutions of aldehyde starches and hemoglobin gelled within 1–2 hr, while very low concentrations of reactants meant that significant amounts of unbound hemoglobin remained after 24 hr. Although it was possible to remove unbound hemoglobin from the final product either by gel filtration or, more easily, on dialysis, the reaction conditions reported here reproducibly gave close to 100% binding of the hemoglobin used.

The polymer-bound hemoglobins were freeze-dried and stored in the solid form at 4°. Initial problems in reconstituting the dried samples, particularly those prepared from dialdehyde starches, were overcome by freeze-drying the samples in the presence of glucose.[16] Polymer-bound hemoglobin samples prepared using dialdehyde starch and dried with 4 g glucose per gram of hemoglobin could be reconstituted readily without apparent deterioration of the oxygen binding characteristics even after storage for several months. Samples prepared from aldehyde-substituted polymers were normally dried in the presence of 1 g of glucose per gram of hemoglobin added.

Polymer-bound hemoglobin samples prepared by either of these methods showed no gross spectral damage to the hemoglobin (Fig. 1) although in the deoxy form a 15% decreased absorbance at 430 nm compared to deoxyhemoglobin was observed.

The oxygen binding curves of both the dextran- and hydroxy-ethyl starch-bound hemoglobins in tris or phosphate buffers were shifted to the left relative to free hemoglobin (Fig. 2). The observed p_{50} values were between 3 and 4 mm Hg, indicating a 2.5-fold greater affinity for oxygen. The sigmoidicity of the observed curves was also considerably reduced relative to that of free hemoglobin—the polymer-bound hemoglobin shows no cooperativity—and no Bohr effect was observed. The oxygen binding curves obtained for both dextran-bound and hydroxyethyl starch-bound hemoglobins were very similar and neither were affected by addition of 2,3-diphosphoglycerate. Freshly prepared polymer-bound hemoglobin solutions showed curves with p_{50} 4.5 mm—only slightly higher than the values observed for samples which had been freeze-dried and stored under carbon monoxide for some weeks.

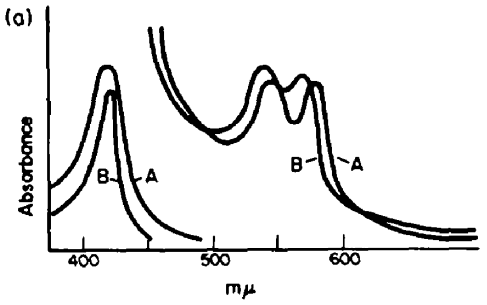

Fig. 1(a). Visible spectrum of aldehyde substituted-hydroxyethyl starch bound hemoglobin Spectrum A HbO_2-polymer. Spectrum B HbCO-polymer.

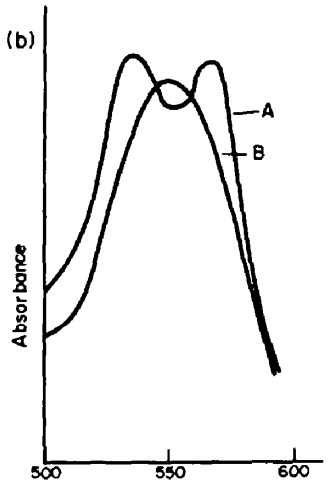

Fig. 1(b). Visible spectrum of dialdehyde-starch bound hemoglobin spectrum A HbO_2-polymer, spectrum B Hb-polymer.

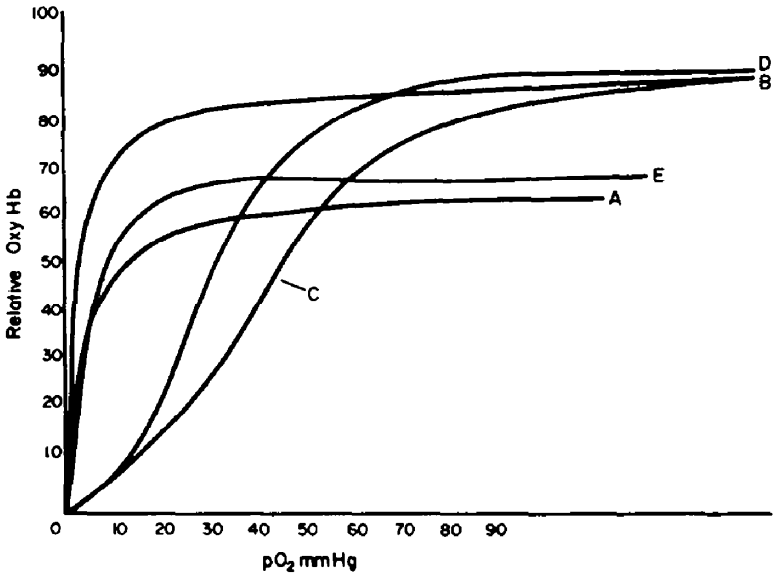

Fig. 2. Oxygen binding curves. (A) Aldehyde-substituted starch bound hemoglobin; (B) Aldehyde-substituted starch bound hemoglobin + 2,3-DPG; (C) Freeze-dried hemoglobin; (D) Fresh whole blood; (E) Dialdehyde-starch bound hemoglobin.

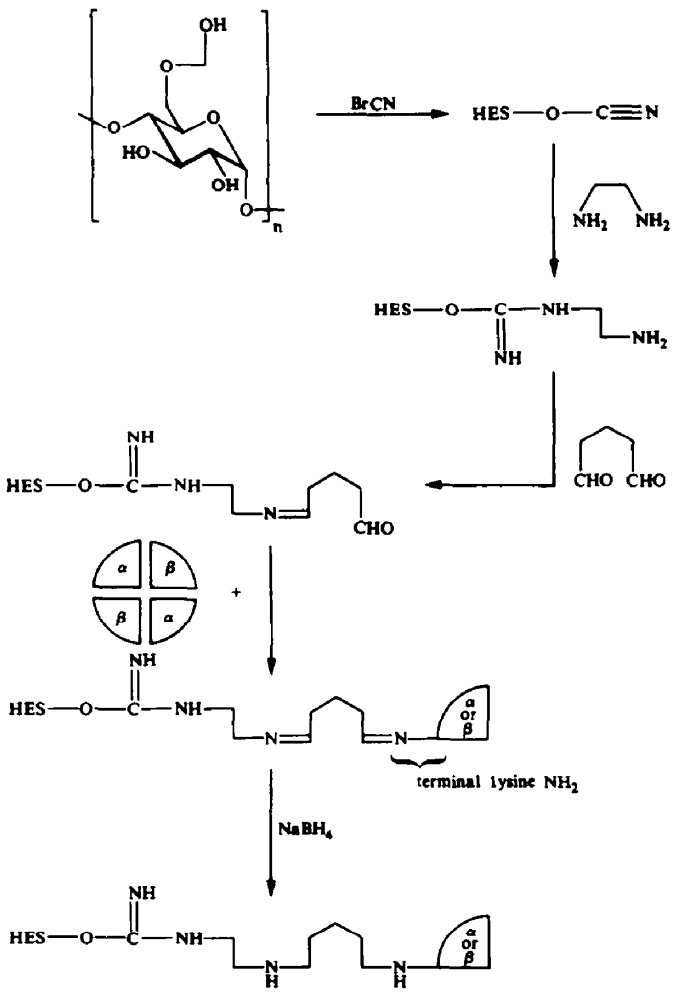

Fig. 3. Modification of HES.

Rat heart perfusion experiments showed that when the Krebs Henseleit bicarbonate saline used as perfusion medium was fortified with 1% polymer-bound hemoglobin the oxygen carrying capacity of the perfusion medium was increased by 35%. The "arterial" oxygen content of this saline/hemoglobin was 0.888 μmol/ml, the measured "venous" oxygen content was 0.272 μmol/ml. Myocardial oxygen consumption of the perfused rat heart at high work load was 14.8 μmol/min or 4.63 mmol/hr per g dry weight. The performance of the hearts was stable for 2.5 hr and at the end of the perfusion the visible spectrum of the hemoglobin containing solution indicated the formation of only a small amount of methemoglobin. Treatment of the polymer-bound hemoglobin solution with 0.6% ferricyanide after equilibration with oxygen in a sealed Erlenmeyer flask at 37° gave an increase in oxygen content of 11% compared to that obtained for the saline solution.

DISCUSSION

This paper describes a simple modification of soluble starches to give in good yield a material capable of binding hemoglobin. The chemical steps involved in the synthesis of the aldehyde-substituted starches (on the basis of the known reactions of the reagents) are outlined in Fig. 3 for hydroxyethyl starch samples. The reaction with hemoglobin is not site specific, there are a number of amino groups on both the alpha and beta chains of hemoglobin which could be capable of undergoing such a reaction. The reactivity of the amino-groups of carbonmonoxy hemoglobin S have been determined by reaction with ^{14}C labelled glyceraldehyde followed by peptide mapping;[17] we are not aware of any comparable study on hemoglobin A.

The polymer-bound hemoglobins reported in this and a number of previously reported studies[8,10,18] all possess characteristics that would contribute to a potentially useful blood substitute; they can bind oxygen reversibly, they are cleared from circulation much more slowly than free hemoglobin and they can be prepared readily in high yield. In all reported cases, however, the shape and position of the oxygen dissociation curve of hemoglobin were considerably altered and the p_{50} very much decreased. In the case of the polymer-bound hemoglobins reported here, the heart perfusion results show clearly that this low p_{50} value severely restricts the usefulness of these materials which do not release enough oxygen to make a substantial contribution to the oxygenation of the perfused rat heart. It is interesting to note that the dextran-bound hemoglobins reported by Tam *et al.*[19] and which show very similar p_{50} values to those reported here have been shown to be capable of supporting life.

It has generally been assumed that hemoglobin on binding to a water soluble polymer, essentially retains its tetramer character. It is, however, possible that the hemoglobin dissociates either prior to binding or, more probably, after binding of the first monomer unit. Such polymer-bound hemoglobin monomers would be expected to show greater oxygen affinity than hemoglobin nor would cooperative effects be possible. The oxygen dissociation curves reported here are consistent with the expected properties of polymer-bound monomeric hemoglobin. In addition, dialysis of the bound hemoglobin samples against triethylamine hydrochloride does not result in any dissociation of hemoglobin.

If hemoglobin is in fact bound in monomer form, the observed oxygen dissociation curve is then a property of the monomeric nature of the hemoglobin itself and will not therefore be significantly affected by changing either the binding site or the nature of the hemoglobin-polymer bridge. To obtain a right-shifted oxygen dissociation curve for such a polymer-bound hemoglobin would therefore be impossible without first linking the hemoglobin units in some way.

Acknowledgements—We wish to thank Dr. J. Darley and the John Radcliffe Hospital for access to their Hem-o-scan, and Dr. R. P. Geyer for samples of hemoglobin and hydroxyethyl starch.

REFERENCES

[1]W. R. Amberson, *Biol. Rev.* **12**, 48–86 (1973).
[2]P. B. Hamilton, L. E. Farr, A. Hiller and D. D. Van Slyke, *J. Exp. Med.* **86**, 455–463 (1947).
[3]S. F. Rabiner, J. R. Helbert, H. Lopas and L. H. Friedman, *Ibid.* **126**, 1127–1142 (1967).
[4]W. Mok, D. E. Chen and A. Mazur, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 1458–1460 (1975).
[5]H. Foerster, I. Hoos, M. Scheider and H. Hauk, *Infusionsther. Klin. Ernaehr-Forsch. Prax.* **4**(2), 122–126 (1977).
[6]P. Bonsen, M. B. Laver and K. C. Morris, *Chem. Abstr.* **86**, 195201 (1977); **85**, 198152 (1976).
[7]K. Bonhard and U. Boyson, *Ibid.* **85**, 130508 (1976).
[8]S.-C. Tam, J. Blumenstein and J. T.-F. Wong, *Proc. Natl. Acad. Sci. U.S.A.* **73**(6), 2128–2131 (1976).
[9]J. E. Chang and J. T.-F. Wong, *Can. J. Biochem.* **55**, 398–403 (1977).
[10]K. Schmidt, *Klin. Wochenschr.* **57**(21), 1169–1175 (1979).
[11]R. P. Geyer, *N. Engl. J. Med.* **289**, 1077 (1973).
[12]R. P. Geyer, R. C. Monroe and K. Taylor, *Fed. Proc.* **32**, 927 (1973).
[13]A. E. Nourse, *Good Housekeeping* **190**(2), 56. New York (1980).
[14]C. S. Wise and C. L. Mehltretter, *Anal. Chem.* **30**, 174 (1958).
[15]H. Taegtmeyer, R. Hems and H. A. Krebs, *Biochem. J.* **186**, 701 (1980).
[16]F. DeVenuto, A. I. Zegna and K. R. Busse, *Surg. Gynec. Obst.* **148**, 69–75 (1979).
[17]A. S. Acharya and J. M. Manning, *J. Biol. Chem.* **255**, 1406 (1980).
[18]P. Labrude, F. Bonneaux, E. Dellacherie, J. Neel and C. Vigneron, *Ann. Pharm. Fr.* **37**, 291–299 (1979).
[19]S.-C. Tam, J. Blumenstein and J. T.-F. Wong, *Can. J. Biochem.* **56**, 981–984 (1978).