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Walder

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[54] **PRODUCTION OF ALPHA-ALPHA CROSS-LINKED HEMOGLOBINS IN HIGH YIELD**

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[*] Notice: The portion of the term of this patent subsequent to Jul. 15, 2003 has been disclaimed.

[21] Appl. No.: **528,331**

[22] Filed: **May 24, 1990**

Related U.S. Patent Documents

Reissue of:

[64] Patent No.: **4,600,531**
Issued: **Jul. 15, 1986**
Appl. No.: **713,348**
Filed: **Mar. 18, 1985**

U.S. Applications:

[63] Continuation of Ser. No. 101,502, Sep. 28, 1987, abandoned, which is a continuation-in-part of Ser. No. 625,360, Jun. 27, 1984, Pat. No. 4,598,064.

[51] **Int. Cl.**[5] **A61K 35/14**

[52] **U.S. Cl.** **530/385**; 530/408; 530/410

[58] **Field of Search** 530/385; 514/6

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Primary Examiner—Howard E. Schain
Attorney, Agent, or Firm—Zarley, McKee, Thomte, Voorhees & Sease

[57] **ABSTRACT**

A high yield method of production of a cross-linked hemoglobin derivative suitable for use as a blood substitute and plasma expander which is cross-linked between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ in high percentage by adding the cross-linker in the presence of an added polyanion which blocks competing reactions at other sites of the protein.

14 Claims, 2 Drawing Sheets

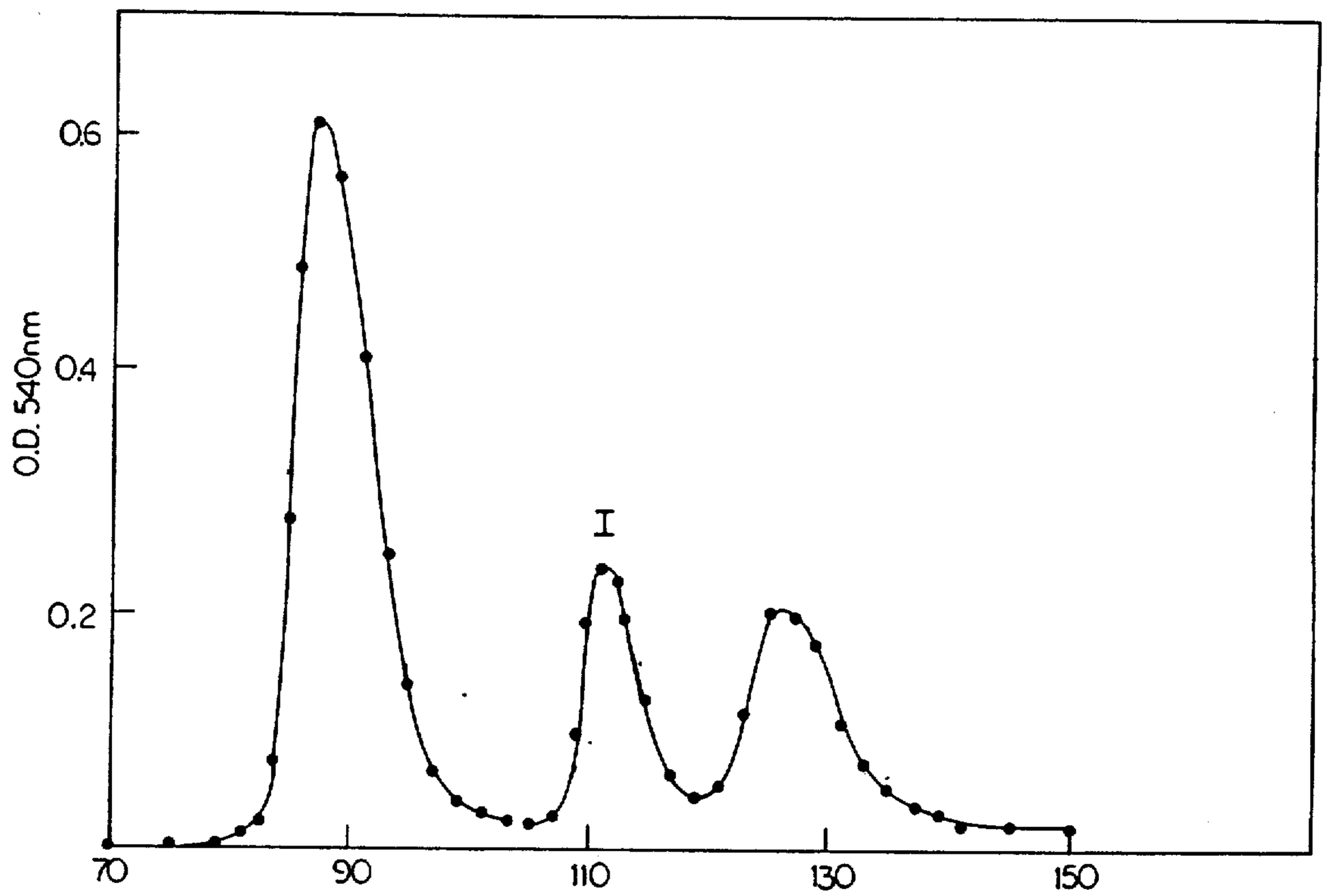

0.6
0.4
0.2
O.D. 540nm
I
70
90
110
130
150
FRACTION

Fig. 1

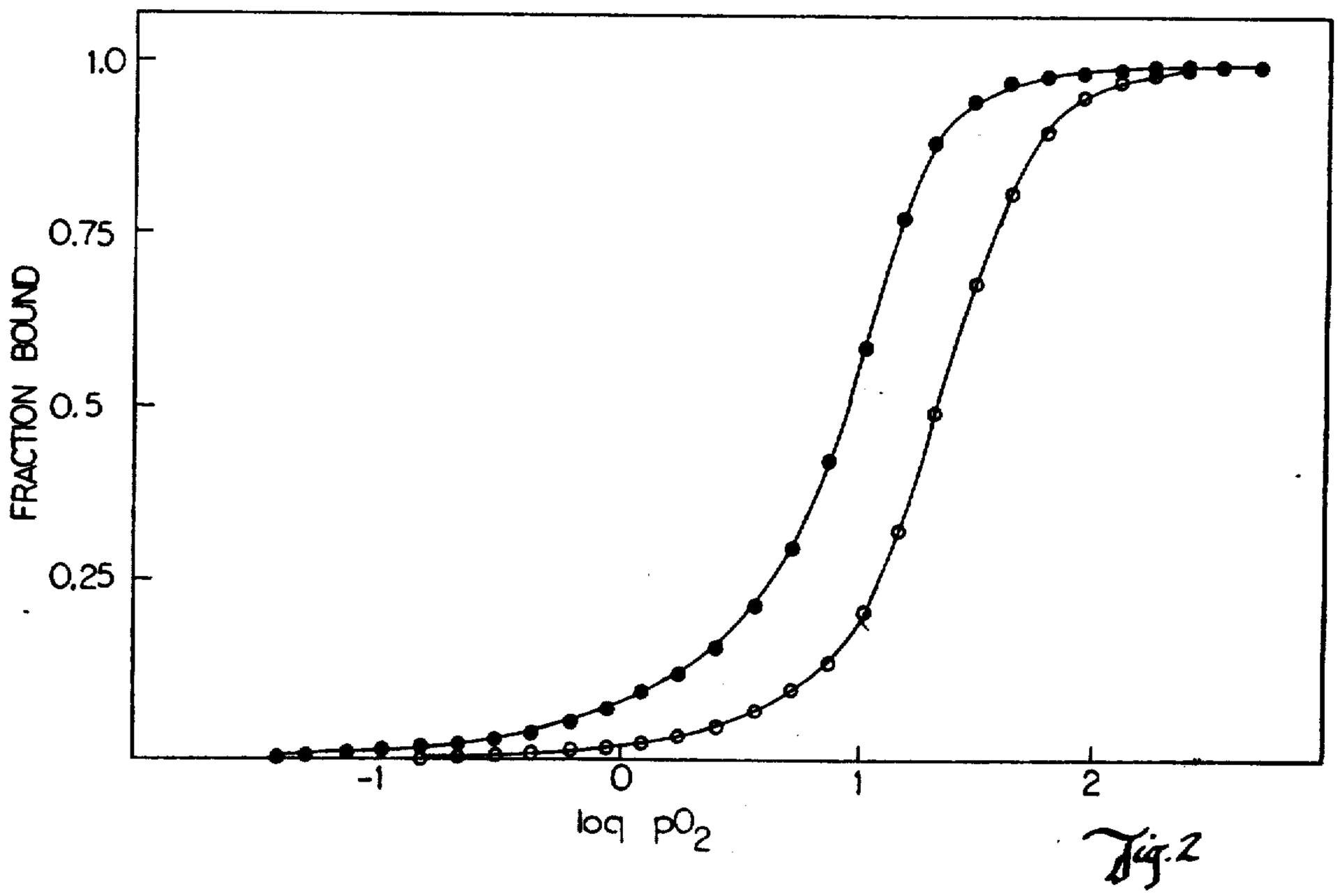

1.0
0.75
0.5
0.25
FRACTION BOUND
-1
0
1
2
log pO_2

Fig. 2

Fig. 5

PRODUCTION OF ALPHA-ALPHA CROSS-LINKED HEMOGLOBINS IN HIGH YIELD

Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.

This is a continuation of copending application Ser. No. 07/101,502 filed on Sep. 28, 1987 now abandoned and a reissue of Ser. No. 713,348, filed Mar. 18, 1985, now U.S. Pat. No. 4,600,531 which is a continuation-in-part of Ser. No. 625,369 filed Jun. 27, 1984, now U.S. Pat. No. 4,598,064.

[CROSS REFERENCE TO A RELATED APPLICATION

This application is a continuation-in-part of co-pending commonly assigned application Walder, Ser. No. 625,360, filed June 27, 1984.]

BACKGROUND OF THE INVENTION

This invention relates to production of a modified hemoglobin composition, used as a blood substitute and a blood plasma expander. There is a critical need within the medical industry for blood substitutes and blood volume expanders. This need occurs not only because of the shortage of donor blood in blood banks, but also because of many problems that commonly exist with donor bloodbank practices. For example, there is an increasingly significant risk of disease transmission such as acquired immunodeficiency syndrome, commonly referred to as "AIDS", and even much more commonly, a real hepatitis risk. The shelf life of whole blood is also relatively short, not usually lasting longer than 30 days. There is also the problem of the need for blood typing, etc. with donated whole blood samples.

Accordingly, there is a very real and continuing need which has existed for some time, for blood substitutes or blood plasma expanders which can be conveniently prepared from a base hemoglobin source, such as outdated blood. This invention has as its primary objective, the fulfillment of this continuing need.

Currently there are two available possible routes for blood substitutes and blood plasma expanders which are being investigated. The first is fluorocarbons and the second is modified hemoglobins. The modified polyhemoglobins are represented by U.S. Pat. No. 4,001,401. Fluorocarbons are also receiving much active investigation at the present. However, it is believed unlikely that fluorocarbons will every successfully take over the market for blood substitutes or blood plasma expanders because these are known to at times block the natural immune system. In addition, the use of fluorocarbons is limited to situations in which high partial pressures of oxygen can be administered. They do not have a sufficiently high oxygen binding capacity for use under normal environmental conditions. Thus, while currently available materials do represent a contribution and some advancement in medical sciences directed towards the concept of a blood substitute and blood plasma expander, there is currently nothing of significant commercial affect available on the market.

There is also the problem of not only developing an effective oxygen carrying blood substitute which will effectively release the oxygen for body use, but also developing a composition which will not be renally

eliminated. A natural mammalian hemoglobin is a tetramer, which in plasma will in the oxy form have a tendency to split into two alpha-beta dimers, each having a molecular weight of approximately 32,000. These dimers are small enough to be filtered by the kidneys and be excreted, with the result being a potential for renal injury and a substantially decreased intravascular retention time.

It therefore becomes readily apparent that there is a continuing need for a therapeutic product useful as a blood substitute and blood plasma expander, which will effectively bind oxygen, but not bind it so tightly that it will not be released for body use; and, for development of a product which will not split into alpha-beta dimers, capable of rapid elimination by the renal route as well as loss from the circulation through capillary beds in other tissues. The composition of my prior application accomplishes the primary objective of fulfilling this expressed need.

Accordingly, another primary object of my prior invention was to prepare an effective blood substitute and blood plasma expander from modified hemoglobin.

Another objective of the prior invention was to prepare a blood substitute and blood plasma expander based on a derivative of hemoglobin cross-linked specifically between the alpha chains, at Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$.

The composition of my prior invention was initially prepared as only a minor reaction product at yields of approximately 10% to 15% of theoretical. Such yields are too low to be of commercial significance, and must therefore be increased significantly if the composition of my prior invention is to be available at practical cost for use as a blood substitute and blood plasma expander.

A primary objective of the present invention is the production of the composition of my prior invention in high yields. In particular, at yields which are commercially significant, that is within the range of at least 50% to 60% of theoretical such that the alpha-alpha cross-linked hemoglobin composition can be prepared in yields that make it practical as a blood substitute and blood plasma expander.

A further objective of the present invention is to prepare the blood substitute and plasma expanders of my prior invention in the presence of an added polyanion such as 2,3-diphosphoglycerate, inositol hexaphosphate or inositol hexasulfate, it having been discovered that when the cross-linking reaction with deoxygenated hemoglobin described in my prior invention occurs in the presence of one of these polyanions, the yield of product is significantly increased from 10% to 15% up to as high as 60–70%. The formation of unwanted side products is correspondingly reduced. These results greatly simplify the purification of the cross-linked hemoglobin and make its production feasible on a commercial scale.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an elution profile monitored by the optical density at 540 nm for the purification of the alpha-alpha cross-linked derivative by chromatography on a DEAE (diethylaminoethyl) cellulose column.

FIG. 2 is an oxygen equilibrium curve showing the fraction of oxygen bound as a function of the log of the partial pressure of oxygen, for both normal adult hemoglobin (closed circles) and the alpha-alpha cross-linked derivative (Lys 99 $Alpha_1$-Lys 99 $Alpha_2$) (open circles).

FIG. 3 is the difference electron density contour map between the alpha-alpha cross-linked derivative and native deoxyhemoglobin superimposed upon the atomic model of hemoglobin in the region of the cross-link.

SUMMARY OF THE INVENTION

A method of production of a new hemoglobin composition, which is intramolecularly cross-linked between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ in commercially significant yields. In the process of production the unmodified hemoglobin is deoxygenated and cross-linked with cross-linking occurring in the presence of an added polyanion which binds electrostatically to deoxyhemoglobin at the 2,3-diphosphoglycerate binding site, located between the beta chains, blocking side reactions of the cross-linker within this site and neighboring regions of the protein, thus significantly enhancing the probability of reaction at the desired Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ site, access to which is not blocked by the polyanion.

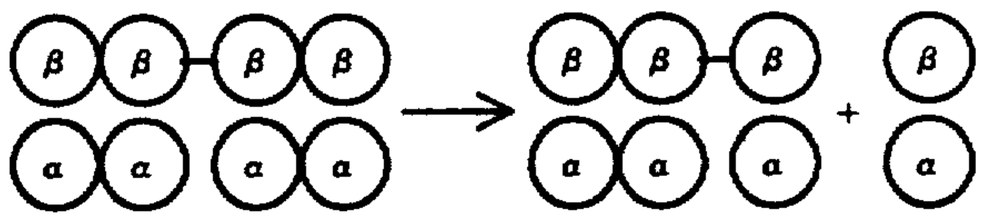
DETAILED DESCRIPTION OF THE INVENTION

Normal hemoglobin useful in animals is a tetramer, commonly referred to by the symbol Hb_4. The tetramer has a molecular weight of about 64,000 and is comprised of four polypeptide chains, two identical alpha chains and two identical beta chains noncovalently linked together. The tetramer Hb_4, under oxygenated conditions, readily dissociates into two alpha-alpha dimers. Dissociation of the tetramer into two alpha-alpha and beta-beta dimers, or alpha and beta monomers does not occur to any significant extent under physiological conditions. With regard to modifying hemoglobin such that it has a beta-beta intramolecular cross-link, see a previously published article, Walder, et al. Journal of Molecular Biology (1980), 141, 195–216. The referenced article deals with selectively cross-linking oxyhemoglobin at the beta chains of the tetramer, between Lys 82 $Beta_1$ and Lys 82 $Beta_2$ with bifunctional acylating agents and the potential use of this modification in the treatment of sickle cell disease. The cross-link is advantageous in that it markedly increases the solubility of sickle cell hemoglobin (hemoglobin S) in the deoxygenated form while having relatively little effect on the intrinsic oxygen binding properties of hemoglobin. In another article, a second derivative cross-linked between the beta chains with the same reagents as reported by Walder, was prepared and tested as a blood substitute, Tye et al. (1983) Advances in Blood Substitute Research (Bolin, R. B.; Geyer; R. P. and Nemo, G. J. eds), pp. 41–49, Alan R. Loss, New York.

Surprisingly, in accordance with the invention of my prior application, Ser. No. 625,360, it was discovered that deoxyhemoglobin can be cross-linked selectively at a novel site between the alpha chains of the tetramer. The site of cross-linking was established by x-ray crystallographic studies to be from Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$. The result was a molecule which will not dimerize, and a molecule whose oxygen binding properties was improved, that is, the oxygen affinity is decreased, if compared to that of unmodified natural hemoglobin.

It was important to my prior invention and to an appreciation of its contribution, to recognize that in the derivative of hemoglobin described the cross-linking occurs intramolecularly and at a specific site on the hemoglobin molecule, from Lys 99 of one alpha chain to Lys 99 of the second alpha chain. This is distinctly different from random cross-linking. It is a specific intra molecular cross-link, as opposed to both inter and intra molecular cross-linking occurring in a random fashion as in Bonsen, U.S. Pat. No. 4,001,401. Previous studies, Bunn, et al., Journal of Experimental Medicine, (1969), 129, 909–924, had shown that hemoglobin is filtered from the circulation by the kidney as alpha-beta dimers, and that derivatives of hemoglobin that are cross-linked so as to inhibit the dissociation of the tetramer have a decreased filtration rate, and prolonged intravascular retention time, and hence may be useful as a blood substitute. For this purpose, Bonsen et al. have described the use of a number of known nonspecific cross-linking agents which react with hemoglobin nonselectively, potentially at as many as 40–50 different sites on the hemoglobin molecule. In contrast, the cross-linked hemoglobin described in my prior application was a specific derivative cross-linked at a unique site on the hemoglobin molecule, Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$. The advantages of this composition as a blood substitute are there described but also set forth below.

Where the cross-linking reagent reacts with hemoglobin in a nonselective fashion, the mixture of reaction products would include, in addition to derivatives which are intramolecularly cross-linked, substantial amounts of hemoglobin, both modified and unmodified, that is not cross-linked, as well as higher molecular weight aggregates due to intermolecular cross-linking between hemoglobin tetramers. In Bonsen, et al., this entire mixture, without further fractionation constitutes the cross-linked hemoglobin composition of the product. For a therapeutically useful product, it would probably be essential to at least remove the non-cross-linked hemoglobin which can dissociate into alpha-beta dimers capable of filtration by the kidney, and hence poses the risk of renal injury. Even the isolation of higher molecular weight aggregates of polymerized hemoglobin due to intermolecular cross-linking may not be sufficient for this purpose since in this case, it is still possible for an alpha-beta dimer to split out and dissociate from the complex as in the following schematic:



Such is not possible with the alpha-alpha cross-linked hemoglobin composition of my earlier referenced invention in which cross-linking occurs intramolecularly. The alpha-alpha cross-linked hemoglobin may, of course, be used as the substitute for further intermolecular cross-linking should it prove that higher molecular weight aggregates of polymerized hemoglobin are cylinically useful.

Even with attempts to isolate a particular molecular weight fraction from the reaction mixture, where the cross-linking reagent reacts nonselectively with hemoglobin, the final product would still in general contain a mixture of hemoglobin derivatives that are modified at a number of different sites on the molecule. This random modification increases the risk of antigenic reaction to the foreign protein. With a single specific site of modification as in the alpha-alpha cross-linked derivative of the present invention, this risk is decreased. This is particularly true in this case since the site of cross-linking Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$, is at a relatively

inaccessible region of the molecule near the center of the hemoglobin tetramer.

Finally, the heterogeneity of the reaction products resulting from nonspecific cross-linking makes it difficult to isolate a final product of reproducible composition and to purify that product from other red cell proteins.

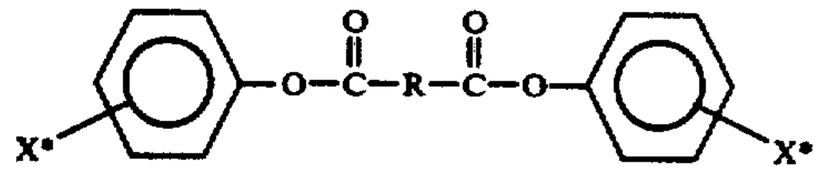
In a preferred embodiment of my prior invention, for certain applications such as ischemia, as in myocardial infarction, an even further modification of the alpha-alpha cross-linked derivative occurs with a second reagent which introduces a negatively charged group at the 2,3-diphosphoglycerate binding site between the beta chains, with the result being a further enhanced capability for oxygen release. In the derivatives cross-linked between the beta chains described above, the site of cross-linking is located within the 2,3-diphosphoglycerate binding site potentially precluding or inhibiting further modifications within this region.

The method of obtaining the starting material for the preparation of the cross-linked derivative, that is, stroma-free hemoglobin, represents state of the art knowledge. It is known how hemoglobin is separated from cells, including isolating it in substantially free form from cellular debris and stroma. For example, hemoglobin to be modified in accordance with this invention can be isolated from discarded blood samples, whose shelf life has exceeded ordinarily regarded safe limits. For details of a suitable isolation technique, see for example, U.S. Pat. No. 4,001,401, at column 4, line 49 through column 5, line 13, which is incorporated herein by reference. In addition, see Rabiner, et al., Journal of Experimental Medicine (1967), 126, 1127–1142; and Feola, et al., Surgery Gynecology and Obstetrics (1983), 157, 399–408, which are also incorporated herein by reference. As in Feola's work, the source of hemoglobin may be other species, for example, bovine or porcine hemoglobins. Bacterial strains engineered to produce hemoglobins by recombinant DNA techniques may also be used as the source of hemoglobin.

The isolated hemoglobin Hb_4 is now ready for modification and treatment and cross-linking in accordance with the present invention. It is essential for the desired alpha-alpha cross-linking reaction to occur, that the hemoglobin for reaction be deoxygenated. If the hemoglobin is oxygenated, cross-linking will occur between the beta chains, in accordance with the references cited above. Deoxygenation is accomplished by extensively purging the stroma-free hemoglobin prior to cross-linking with inert gases such as nitrogen or argon. Deoxygenation is essential to render the reactive site at Lys 99 of the alpha chains accessible to the cross-linking regent. In oxyhemoglobin the conformation is such that this region of the molecule is totally inaccessible.

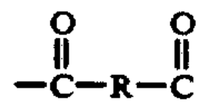
It has been mentioned that the reaction can be controlled to assure deoxygenation by purging with argon or nitrogen or other inert gases. Alternatively, or in combination, deoxygenation of hemoglobin may be achieved by reaction with sodium dithionite or other conventional reducing agents, such as ferrous citrate. Deoxygenation by purging under a blanket of appropriate inert gas should occur for from about one hour to about three hours, at normal atmospheric pressure at a temperature of from about 0° C. to about 40° C. Purging for a time within this framework assures that deoxygenation will have occurred, making accessible the site of cross-linking between the alpha chains, Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$. After purging, the composition is now ready for reaction with the cross-linking reagent.

The cross-linking agent employed must react with a substantial degree of specificity at Lys 99 of the alpha chains in deoxyhemoglobin. The reaction with the cross-linking agent to form a stable covalent adduct occurs at the ϵ-amino group of the side chain of the lysine residue. There are 42 other lysine residues and the amino-terminal amino groups of the four polypeptide chains of hemoglobin at which competing reactions may occur. The general formula of suitable cross-linkers which may be employed is:

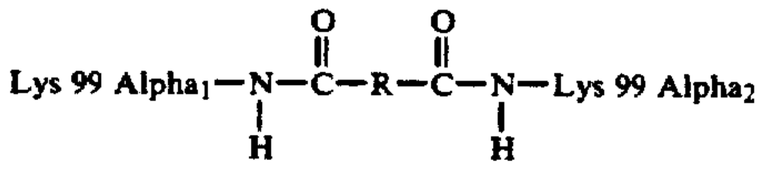


•X can be any organic moiety attached to the ring.

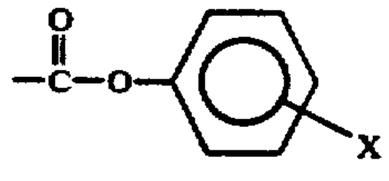
This preferred formula represents phenyl esters which are effective cross-linkers. An important fact is that the cross-link bridge



is formed. This is the cross-link bridge which links the amino groups of the two lysine residues, i.e.,

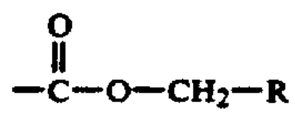


Substitution of groups on the cross-link bridge may affect the properties of the modified derivative, as well as the reactivity of the compound. It is known that R may vary in length, and could, for example, be C_2H_4, C_3H_6, or an unsaturated chain. Generally R can be any organic moiety, whether substituted or unsubstituted of chain lengths varying from 2 to about 8. R may also be substituted with a specific functional group such as carboxyl group in which case the additional carboxyl group would become attached to the hemoglobin molecule on the cross-link bridge. The functional group that reacts with the amino group of the lysine residue is:

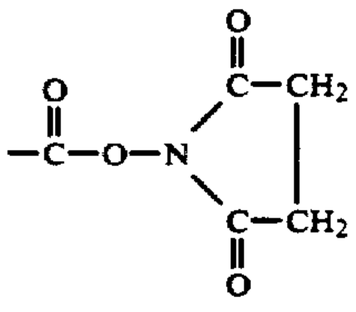


a phenyl ester.

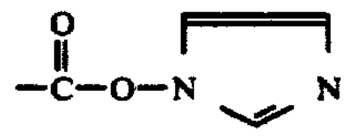
Other possibilities for reaction at amino groups include:



an aliphatic ester;

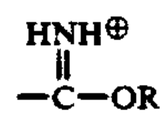


a hydroxysuccinimide ester;



an acyl imidazole.

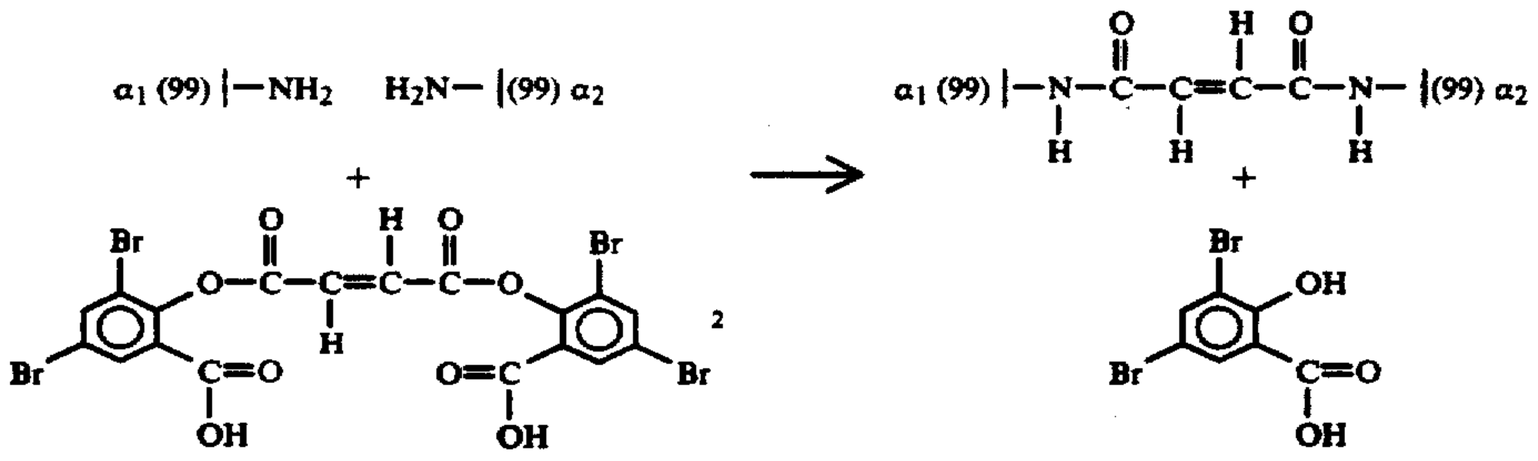
All of these can be thought of as acylating agents. In addition, the reactive group may also be an imidoester:



or a related amidinating reagent, or a sulfonyl halide. It is possible that certain dialdehydes may also be used to cross-link the two Lys 99 Alpha residues by formation of a Shiff's base and reduction with sodium borohydride or sodium cyanoborohydride carried out to convert the Shiff's base to an amine linkage as in the following equation:

Alkyl halides, or sulfonate esters or other alkylating agents may also be used to cross-link the amino groups of the lysine residues.

The most preferred cross-linking agent is bis(3,5-dibromosalicyl)fumarate. It will effectively cross-link the two Lys 99 Alpha residues in accordance with the following schematic:



In accordance with the improved process of the present invention wherein the hemoglobin intramolecularly cross-linked between Lys 99 Alpha$_1$ and Lys 99 Alpha$_2$ is prepared in high yields, it has been surprisingly discovered that if the reaction between deoxygenated hemoglobin and the cross-linker occurs in the presence of an added polyanion, preferably in substantial excess of equimolar amounts, that the yield of the desired intramolecularly cross-linked hemoglobin, cross-linked at the preferred Lys 99 Alpha$_1$ to Lys 99 Alpha$_2$ site is substantially increased. In particular, when practicing the improved process of this invention, yields can be increased from typical yields according to my prior invention of from 10% to 15% of theoretical up to yields within the range of at least 50% to 60%.

Polyanions of a wide variety of different structures bind to deoxyhemoglobin by electrostatic interactions with the cluster of positively charged groups of the beta chains located at the entrance to the central cavity of the hemoglobin tetramer. This is the natural binding site on hemoglobin for 2,3-diphosphoglycerate. Apparently the major source of side reactions which occur with the cross-linking agent is due to reactions with the amino groups at this site and neighboring portions of the protein which can be blocked by the binding of polyanions such as inositol hexaphosphate within the region. There may also be secondary binding sites of lower affinity on deoxyhemoglobin for polyanions such as inositol hexaphosphate which further contribute to the suppression of side reactions with the cross-linker. Surprisingly, in connection with the present invention, the binding of a polyanion at the 2,3-diphosphoglycerate binding site does not block access of the cross-linker to the two Lys 99 Alpha residues which are located within the central cavity of the hemoglobin tetramer near the very middle of the molecule. Presumably, the cross-linking agent normally enters the central cavity in deoxyhemoglobin either near the interface between the alpha and beta chains or between the alpha chains. Importantly, in follow-up purification steps, the polyanion is removed leaving the purified cross-linked hemoglobin.

Suitable polyanions capable of electrostatic binding to deoxyhemoglobin while leaving Lys 99 Alpha$_1$ and Lys 99 Alpha$_2$ accessible for cross-linking include 2,3-diphosphoglycerate, inositol hexaphosphate and inositol hexosulfate, preferably inositol hexaphosphate. These block potential sites at which the side reactions may occur as earlier described.

The reaction with the cross-linkers is substantially as described in my previous application and as described hereinafter. Generally the concentration of the polyanion should be within the range of from equimolar amounts with hemoglobin up to as much as twenty fold excess, preferably from about 5 times to about 10 times the molar amount of hemoglobin. With regard to the cross-linking agent, it is preferred that the amount of cross-linking agent be from about one to about three fold molar excess with the amount of hemoglobin, preferably from about 1.3 times to about 2 times the molar amount of hemoglobin.

The reaction with the cross-linking agent and the deoxygenated hemoglobin can occur at a temperature of from about 0° C. to about 40° C., preferably from about 35° C. to about 40° C. The pH of the reaction can vary from about 5.5 to about 10, preferably from about 6 to about 8, with the reaction occurring in an aqueous

solution of salts, typically 0.2 molar Bis-Tris buffer in a salt solution up to a concentration of about one molar. The ratio of cross-linking agent to hemoglobin can be from about 1:1 to about 3:1, preferably 1.3:1 to about 2:1. While one may use a greater excess of cross-linking agent to assure completion of cross-linking, the narrow ranges expressed here are preferred. The time for the reaction again will vary, but can be up to two hours for a sufficient degree of cross-linking to have occurred.

The cross-linked derivative can be separated from the unreacted hemoglobin and impurities modified at other sites by ion exchange chromatography, gel filtration, and other chromatographic techniques. Chromatographic procedures using high pressure liquid chromatography may also be used. In certain cases, it may be possible to sufficiently purify the cross-linked derivative by nonchromatographic methods such as ultrafiltration. The purification also removes the polyanion from the modified hemoglobin.

The hemoglobin is now cross-linked at the Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$ position, and may be used as is, as an effective blood substitute, it having been found to have a lowered oxygen affinity and suitable oxygen release, and having also been found resistant to dimerization and hence rapid removal from the circulation by renal elimination.

For parental use, the purified derivative can be dialyzed or exchanged by ultrafiltration into a physiological saline solution at a pH of 7.4 and concentrated to approximately 7% (7 g hemoglobin per 100 milliliter). The material, of course, must be substantially endotoxin free and packaged under sterile conditions. It may also be possible to store the hemoglobin as a lyophilized powder which would be reconstituted when needed by the addition of saline.

The cross-linked modified hemoglobin, cross-linked as heretofore discussed intramolecularly between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ can be used as is, for a blood substitute and blood plasma expander, as a pharmaceutical composition with an acceptable carrier, and with other plasma substitutes and plasma expanders. The pharmaceutical carriers can be crystaloids, including physiological saline, a mixture consisting of saline and glucose, Ringer's solution, lactated Ringer's solution, Locke-Ringer's solution, Krebs-Ringer's solution, Hartmann's balanced saline, and heparinized sodium-citrate-citric acid-dextrose solution.

The cross-linked hemoglobin can be mixed with water soluble physiologically acceptable polymeric plasma substitutes such as poly(ethylene oxide), polyvinylpyrrolidone, polyvinyl alcohol, and ethylene oxidepolypropylene glycol condensates. Additionally, it can be mixed with colloidal-like plasma substitutes and plasma expanders such as linear polysaccharides, including dextrans, albumin, other plasma proteins, pectins, balanced fluid gelatin and hydroxyethyl starch. Generally, the pharmaceutical compositions will contain about 1% to about 10% by weight of the modified hemoglobin admixed with one of the above carriers, or a mixture thereof. Conventional methods for administering the therapeutic agents are known medical state of the art, see for example, Tares and King (1980) in Remington's Pharmaceutical Sciences, (Osol, A. ed.) pp. 1488–1497, Mack Publishing Company, Easton, Pa., which is incorporated herein by reference.

As heretofore mentioned an additional important advantage of the Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$ cross-linked hemoglobin is that the 2,3-diphosphoglycerate binding site is still accessible for further modification with other reagents. It has also been found, and is therefore preferred in certain circumstances such as ischemia, with respect to the intramolecular cross-linked hemoglobin of the present invention, that the attachment of a negatively charged group within this region will act as a permanently bound anion and decrease the oxygen affinity of the hemoglobin. This is, of course, desirable since it means that the hemoglobin will more easily release its oxygen for use by the tissues.

In principle, a number of different derivatives could be prepared with a range of oxygen affinities, depending on the attached group, for different applications. Hemoglobin derivatives having a very low oxygen affinity may be particularly useful in the treatment of ischemia (heart attacks and strokes), for example. Even for simple blood replacement, it may be beneficial to have an oxygen affinity lower than that produced by the alpha chain cross-link alone.

Pyridoxal phosphate and other aldehyde derivatives have been used previously to introduce negatively charged groups within the 2,3-diphosphoglycerate binding site, Benesch, et al., Biochemistry, (1972), 11, 3576–3582. These compounds react with deoxyhemoglobin and require reductive alkylation for permanent covalent attachment. The compounds described here for this purpose react with oxyhemoglobin, and hence do not require deoxygenation of the sample, nor is any further reaction, such as reductive alkylation, required to give the final derivative.

The prototype of these compounds and the one most preferred is mono(3,5-dibromosalicyl)fumarate. This compound reacts selectively with oxyhemoglobin at lysine 82 beta and introduces a negatively charged carboxylate group within the 2,3-diphosphoglycerate (DPG) binding site.

The following examples are offered to further illustrate, but not limit, the process, product and medical techniques of the invention.

EXAMPLE 1

The Reaction of Bis(3,5-Dibromosalicyl)Fumarate with Deoxyhemoglobin and Isolation of the Derivative Cross-Linked Between the Alpha Chains

A solution of hemoglobin is prepared at a concentration of 2.0 mM in 0.2M Bis-Tris buffer at pH 7.2. The hemoglobin is initially in the oxy form. Oxygen is removed by purging with argon or nitrogen. A solution of bis(3,5-dibromosalicyl)fumarate at a concentration of 2.0 mM is prepared in the same buffer as hemoglobin and deoxygenated with a nitrogen purge. To the hemoglobin solution is added an equal volume of the solution of bis(3,5-dibromosalicyl)fumarate and the reaction allowed to proceed for two hours at 37° C. The final concentrations of hemoglobin and the compound are each 1.0 mM. Under these conditions, the yield of the derivative cross-linked between the alpha chains at Lys 99 $Alpha_1$ to Lys $Alpha_2$ is 10–15%. At the end of the reaction, glycine is added to a final concentration of 1.0M to consume any remaining amount of the cross-linking reagent and prevent further reaction with hemoglobin which may occur during the isolation of the cross-linked derivative.

After the reaction the hemoglobin solution is dialyzed against 0.2M glycine buffer at pH 7.8 or exchanged with the glycine buffer by ultrafiltration. In this step the hemoglobin becomes oxygenated. The

cross-linked derivative is then separated from unreacted hemoglobin and impurities due to modifications at other sites by chromatography on DEAE cellulose. The column is initially equilibrated with 0.2M glycine buffer at pH 7.8. After application of the hemoglobin, the sample is eluted from the column with a 0.03 to 0.06M NaCl gradient in the same buffer. The elution profile is shown in FIG. 1. The first peak at fraction 90 is unmodified hemoglobin. The peak at fraction 110 (I) is the desired alpha-alpha cross-linked derivative. The third peak at fraction 128 is a mixture of derivatives modified at other sites and includes derivatives cross-linked between the beta chains. Minor remaining non-cross-linked impurities (<5%), coeluting with the alpha-alpha cross-linked derivative can be removed by gel filtration in the presence of 1M $MgCl_2$. An improved purification in the first step particularly suited for large scale commercial applications may be obtained using cross-linked DEAE Sepharose as the chromatographic media.

The yield of the isolated alpha-alpha cross-linked derivative in the example shown in FIG. 1 was approximately 15%. Since most of the remaining material is unmodified hemoglobin, we had hoped to improve the yield of the alpha-alpha cross-linked derivative simply by increasing the concentration of the cross-linking agent or the reaction time or by driving the reaction to completion at higher temperatures. All of these variations in reaction conditions, alone or in combination, failed to increase the yield of the desired product. The yield of the alpha-alpha cross-linked derivative was in general actually decreased due to a greater extent of side reactions occurring at other sites of the protein. The yield of the derivative cross-linked specifically between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ was found to be increased only by the method described in the examples below in which the reaction is carried out in the presence of a polyanion, such as inositol hexaphosphate, blocking competing reactions of the cross-linking reagent at other sites of the protein.

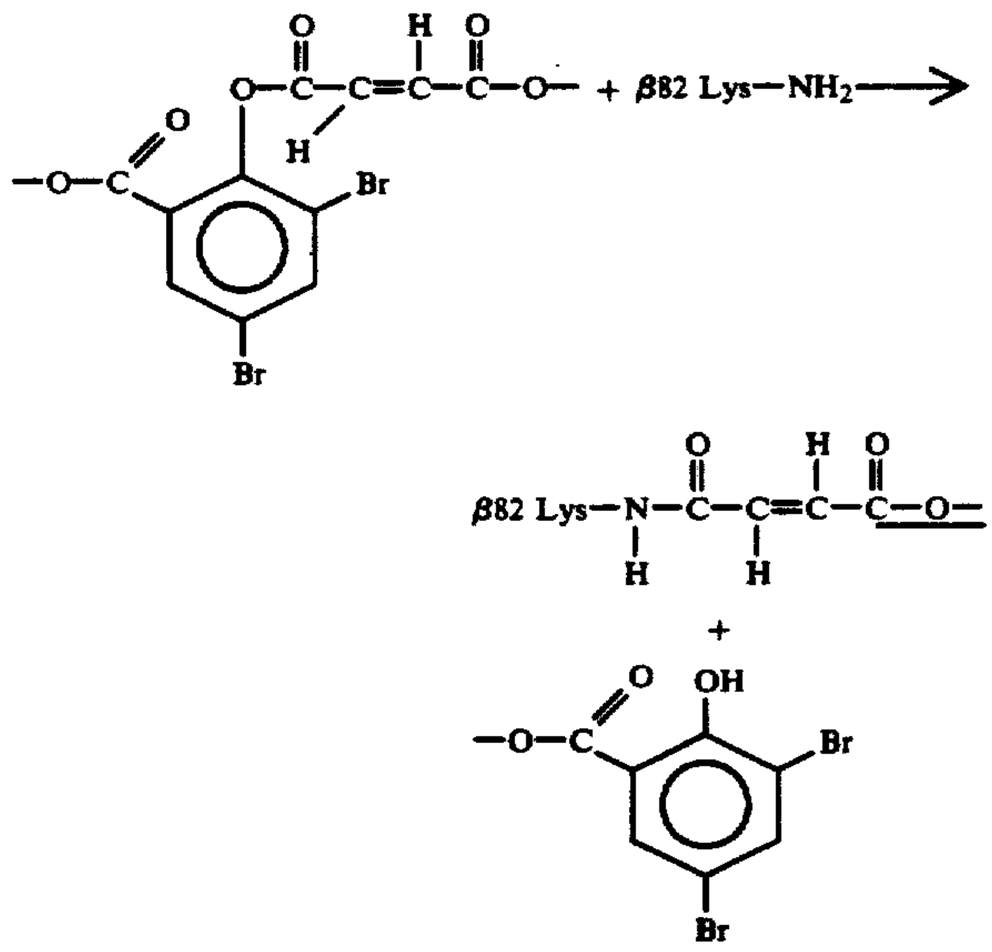
FIG. 2 illustrates the oxygen equilibrium curve of the alpha-alpha cross-linked derivative prepared as described above. The oxygen equilibrium curve is plotted as the fraction of oxygen bound as a function of the log of the partial pressure of oxygen. In the graph the closed circles represent normal adult human hemoglobin and the open circles, the cross-linked derivative (Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$). The conditions were 0.05M Bis-Tris buffer pH 7.0 with 0.1M NaCl at 25° C. The concentration of hemoglobin in both cases was 0.2 mM. Under these conditions the P_{50} (the partial pressure of oxygen at which half-saturation of the hemoglobin occurs) for native hemoglobin was 6.3 mm Hg and for the cross-linked derivative 15.1 mm Hg. The right shift in the oxygen binding curve for the alpha-alpha cross-linked derivative (i.e., a greater P_{50}) indicates a decrease in the oxygen affinity. Correspondingly, the release of oxygen from the cross-linked derivative is greater than from unmodified hemoglobin at higher partial pressures of oxygen. The cooperatively of the cross-linked derivative is not substantially decreased. The Hill coefficient determined in the experiments shown for the cross-linked derivative was 2.2.

Two-dimensional gel electrophoresis of the cross-linked derivative and amino acid analysis of the isolated cross-linked polypeptide chains first established that the site of the cross-link was between the alpha chains. X-ray crystallographic studies of the modified hemoglobin were carried out to determine the exact site of cross-linking. FIG. 3 shows the different electron density contour map between the cross-linked derivative and native deoxyhemoglobin superimposed upon the atomic model of the native structure. Contours due to negative difference electron density have been omitted for clarity. The band of positive difference electron density containing the two intense peaks near the center of the diagram is due to the cross-link bridge, which can be seen to connect lysine 99 (99K) of one alpha chain to lysine 99 of the second alpha chain. The other low level positive contours are due to small changes in the structure as a result of the cross-link. No other sites of modification were observed in the difference map.

EXAMPLE 2

New Protein Modifying Agents which Attach Negatively Charged Groups within the 2,3-Diphosphoglycerate Binding Site

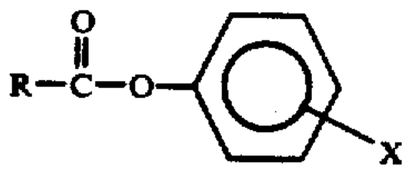
This example first describes the reaction of mono(3,5-dibromosalicyl)fumarate with native hemoglobin. The reaction of interest occurs with oxyhemoglobin, in which case the compound reacts selectively at Lys 82 beta as shown in the following equation and introduces a negatively charged carboxylate group (underlined) within the DPG binding site:



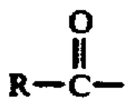
The reaction conditions are the same as described in Example I, except that hemoglobin is maintained in the oxy form. Normal ambient partial pressures of oxygen in room air are sufficient for this purpose. With a 1.5 molar excess of the reagent over hemoglobin the yield of the product was approximately 20%. The derivative was purified by chromatography on DEAE cellulose as described in the previous example. Two-dimensional gel electrophoresis of the modified hemoglobin showed that both of the beta chains were modified. X-ray crystallographic studies showed that the site of modification was at Lys 82 of the beta chains. This was confirmed by tryptic peptide mapping. The oxygen affinity of the modified hemoglobin is decreased by approximately 1.6 fold. At a pH of 7.0 in 50 mM Bis-Tris buffer, the P_{50} was found to be increased from 7.9 mmHg for native hemoglobin to 12.9 mmHg for the modified derivative.

The reaction of mono(3,5-dibromosalicyl) fumarate with the alpha-alpha cross-linked derivative described

in Example I under oxygenated conditions, occurs similarly to native hemoglobin. Correspondingly, oxygen binding studies as those in FIG. 2 show that the oxygen affinity of the alpha-alpha cross-linked derivative is further decreased by the addition of the negatively charged carboxylate group within the DPG binding site. In principle, a number of different derivatives could be prepared, having a range of oxygen affinities, by modification of the alpha-alpha cross-linked derivative with analogs of mono(3,5-dibromosalicyl)fumarate. The resulting oxygen affinity will depend on the negatively charged group which is added within the DPG binding site. In the following general structure:



the group



becomes covalently attached to the protein. The number and type of negatively charged substituents within this group may be varied. In addition to the carboxyl group these would include phosphonate, phosphate, sulfonate, and sulfate groups. In general, the greater the number of negative charges on the attached group, the lower would be the oxygen affinity of the modified hemoglobin.

In summary, the results of the studies performed demonstrate that the alpha-alpha intramolecularly cross-linked hemoglobin, described herein has the properties of an effective blood substitute, plasma expander and in general can be used for this purpose where conventional donor blood samples are now used.

In the series of examples below, reactions were carried out under substantially the same conditions as in Examples 1 and 2, but in the presence of inositol hexaphosphate. Conditions were determined which would provide the optimal yield of the alpha-alpha cross-linked derivative.

Following each of the reactions with bis(3,5-dibromosalicyl)fumarate, the modified hemoglobins were analyzed on pH 6–8 isoelectric focusing gels. Approximately 200 ug of Hb is loaded on each gel. Electrophoresis is first carried out for 1.5 hrs. at 500 V, sodium dithionite is then added at the cathodic end of the gel and the electrophoresis then continued at 600 V for 0.5 hrs. In the first 1.5 hrs, the various hemoglobin derivatives focus in the oxy form on the basis of their isoelectric pH. Generally the derivatives have a lower isoelectric point than native hemoglobin due to the loss of the positive charge of the modified amino group. Twenty ul of a solution of 74 mM sodium dithionite is added to the gel to convert the hemoglobins to the deoxy form. Dithionite is an anion and therefore migrates down the gel toward the anode to reach the Hb. The net gain of positive charge upon deoxygenation (the Bohr effect, +2 for native Hb) results in an upward shift (toward the cathode) of the Hb bands. For human, bovine, and procine Hb, the Bohr effect is in each case greater for unmodified hemoglobin than the cross-linked derivative. This makes the separation between unmodified Hb and the alpha-alpha cross-linked derivative much greater under deoxygenated conditions than with hemoglobin in the oxy form.

In the examples below three types of hemoglobin were studied: human, bovine, and porcine. In general, the cross-linking agent was first dissolved in the reaction buffer and then added immediately to the hemoglobin sample to minimize the spontaneous hydrolysis of the ester. Identical results were obtained using a concentrated stock solution of the compound prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in the reaction was then 1.5%. For reactions with deoxyhemoglobin, it ws not necessary to deoxygenate the DMSO solution.

EXAMPLE 3

Reaction of Deoxyhemoglobin with Bis(3,5-dibromosalicyl)Fumarate in 0.1M Bis-Tris Buffer, with Varying Concentrations of Inositol Hexaphosphate (IHP)

These reactions were all carried out in 0.1M Bis-Tris buffer pH 7.2 for a period of 2 hrs. at 37° C. with the concentrations of hemoglobin and bis(3,5-dibromosalicyl)fumarate equal to 1.0 mM and 1.5 mM, respectively. All solutions were deoxygenated by purging with N_2. Zero, 1.5, 5, 10, and 20 mM concentrations of IHP were tested.

In the absence of IHP the yield of the alpha-alpha cross-linked derivative was only 5%. Approximately 10–15% of the hemoglobin was unmodified. Side reactions at other sites on the protein accounted for the remaining material which appeared as a diffuse series of bands overlapping the position on the isoelectric focusing gel of the Lys 82 to Lys 82 beta-beta cross-link formed preferrential in the reaction with oxyhemoglobin. At 1.5 mM IHP the yield of the alpha-alpha cross-linked derivative determined by isoelectric focusing was increased to 40%. At 5 mM IHP the yield of the alpha-alpha cross-linked derivative was 60–65% with less than 6% impurities due to side reactions at other sites of the protein. There were no further improvements in yield at the higher concentrations of IHP.

EXAMPLE 4

Reaction of Oxyhemoglobin with Bis(3,5-dibromosalicyl)Fumarate in 0.1M Bis-Tris Buffer pH 7.2, with Varying Concentrations of IHP

The reactions in Example 3 were repeated with oxyhemoglobin. In no instance was there a detectable yield of the alpha-alpha cross-linked derivative. Even in the presence of a large excess of a polyanion such as IHP, hemoglobin must be in the deoxy conformation for access of the reagent to the site of cross-linking between the alpha chains, Lys 99 $Alpha_1$ to Lys 99 $Alpha_2$.

EXAMPLE 5

Reaction of Deoxyhemoglobin with Varying Concentrations of Bis(3,5-dibromosalicyl)Fumarate in the Presence of 5 mM IHP

The reaction conditions were the same as in Example 3. The concentrations of bis(3,5-dibromosalicyl)fumarate studied were 1.0, 1.3, 1.5, 2.0, 2.5, and 3.0 mM. At a concentration of 1.0 mM of the compound the yield of the alpha-alpha cross-linked derivative was 40%. At 1.3 and 1.5 mM the yield was between 55% and 65%. At concentrations of 2.0 mM and greater the yield of the desired product progressively decreased due to side

reactions at other sites of the protein. The impurities probably include derivatives that are cross-linked between the two Lys 99 Alpha residues but are also modified at one or more other sites of the protein.

EXAMPLE 6

The Reaction of Bis(3,5-dibromosalicyl)Fumarate With Bovine and Porcine Hemoglobin Under Deoxygenated Conditions in the Presence of IHP

Reaction conditions were the same as in Example 3. The concentration of bis(3,5-dibromosalicyl)fumarate was 1.5 mM. In both cases the increase in yield of the alpha-alpha cross-linked derivative in the presence of IHP paralleled that observed with human hemoglobin. The maximal yield with bovine hemoglobin was 35% to 40%, and with porcine hemoglobin was 60% to 70%, the latter being essentially the same as with human hemoglobin.

Other modifications may be made without necessarily departing from the scope and spirit of the invention, but it can be seen that the use of a polyanion, specifically preferred inositol hexaphosphate (IHP), greatly increases the yield of the alpha-alpha cross-linked derivative from the range of 10% to 15% up to as much as 60% to 70%.

While the above examples have been presented using purified hemoglobin as the starting material, the cross-linking process can also be conducted with crude hemolysates. This can be accomplished by simply lysing the red blood cells, adjusting the hemoglobin concentration to between 1 and 2 mM, adding inositol hexaphosphate, deoxygenating the solution with N_2, and then adding the cross-linking reagent. The cross-linking will occur even in the presence of stromal materials. The desired product cross-linked as heretofore described between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ can then be isolated. This process is more convenient for commercial practice than purifying the hemoglobin both before and after cross-linking.

It therefore can be seen that the invention accomplishes at least all of the objectives heretofore stated.

What is claimed is:

1. A method of producing in commercially significant yields, a modified hemoglobin which is capable of functioning as a blood substitute and blood plasma expander, comprising:

isolating unmodified hemoglobin or preparing a crude red cell lysate;

deoxygenating said hemoglobin;

selectively cross-linking said deoxygenated hemoglobin intramolecularly between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ in the presence of [an] *from about a 1.5 molar up to about a 20 molar excess* added polyanion selected from the group consisting of 2,3-diphosphoglycerate, inositol hexaphosphate, inositol hexasulfate; and

purifying said alpha-alpha cross-linked hemoglobin.

2. The method of claim **1** wherein said polyanion is 2,3-diphosphoglycerate.

3. The method of claim **1** wherein said polyanion is inositol hexasulfate.

4. The method of claim **1** wherein said polyanion is inositol hexaphosphate.

5. The method of claim **1** wherein the reaction is conducted at a temperature of from about 0° C. to about 40° C.

6. The method of claim **5** wherein the reaction is conducted at a temperature of from about 35° C. to about 40° C.

7. The method of claim **6** wherein the reaction is conducted at a temperature of about 37° C.

8. The method of claim **1** wherein the cross-linking between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ is with a cross-linking agent which is amino group-specific.

9. The method of claim **8** wherein said cross-linking agent is a phenyl ester cross-linking agent.

10. The method of claim **9** wherein said cross-linking agent is bis(3,5-dibromosalicyl)fumarate.

11. The method of claim **8** wherein the amount of cross-linking agent employed is up to a three fold molar excess of the amount of hemoglobin.

12. The method of claim **11** wherein the amount of cross-linking agent is from about 1.3 times to about 2.0 times the molar amount of said hemoglobin.

[**13**. The method of claim **11** wherein the amount of the polyanion is from an amount equimolar to the amount of hemoglobin up to a twenty fold excess of the equimolar amount.]

14. The method of claim [**13**] *1* wherein the amount of the polyanion is from about 5 to about 10 times the molar amount of said hemoglobin.

15. A method of producing in commercially significant yields, a modified hemoglobin which is capable of functioning as a blood substitute and blood plasma expander, comprising:

isolating unmodified hemoglobin or preparing a crude red cell lysate;

deoxygenating said hemoglobin;

selectively cross-linking said deoxygenated hemoglobin intramolecularly between Lys 99 $Alpha_1$ and Lys 99 $Alpha_2$ in the presence of from about a 1.5 molar up to about a 20 molar excess of added polyanion which binds electrostatically at the 2,3-diphosphoglycerate binding site located between the beta chains of said deoxyhemoglobin; and

purifying said alpha-alpha cross-linked hemoglobin.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : RE 34,271

DATED : June 1, 1993

INVENTOR(S) : Joe Walder

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, line 22, please add the following grant reference clause above the heading, "BACKGROUND OF THE INVENTION":

--Work for this invention was funded in part by a grant from the United States Army, grant number HL26745. The Government may have certain rights in this invention.--

Signed and Sealed this

Twenty-second Day of November, 1994

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : Re. 34,271

DATED : June 1, 1993

INVENTOR(S) : Joseph A. Walder

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, line 22, in the grant reference, added by the Certificate of Correction, delete the reference to "United States Army" and insert therefor --National Institute of Health--.

Signed and Sealed this

Twenty-fourth Day of June, 1997

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks