Preparation, properties, and plasma retention of human hemoglobin derivatives: Comparison of uncrosslinked carboxymethylated hemoglobin with crosslinked tetrameric hemoglobin

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ABSTRACT   Human hemoglobin A has been crosslinked by diisothiocyanatobenzenesulfonate to give a limited number of products in a yield of ≈70%. The predominant product was crosslinked between subunits within a tetramer and had a M_r of 64,000; no higher M_r species were formed. This product had one crosslink per tetramer located between the NH_2 termini of its α chains, as established by HPLC analysis, amino acid analysis, Edman degradation, and mass spectrometry. This crosslinked derivative had a slightly increased oxygen affinity [P_{50} = 9 mmHg (1 mmHg = 133 Pa); P_{50} for unmodified hemoglobin = 11 mmHg], and the retention time of this derivative in the circulation of rats was 2.9 and 3.3 hr at two hemoglobin concentrations (7 g/dl and 14 g/dl, respectively). The half-life of an uncrosslinked carboxymethylated derivative, which has a low oxygen affinity (P_{50} = 28 mmHg), was 0.6 and 0.7 hr under the same conditions. Therefore, prolongation of the plasma-retention time of infused hemoglobin is dependent on the crosslinking of the tetramer but independent of the oxygen affinity of the derivative.

Studies on hemoglobin-based blood substitutes have used a variety of chemical modifications. Some reagents used for these chemical modifications are bifunctional crosslinking agents and others yield monosubstituted products. The latter derivatives must be subsequently crosslinked to prevent dissociation of the tetramer into dimers of M_r 32,000, which are rapidly filtered by the kidney and thereby removed from the circulation. Examples are the pyridoxal 5′-phosphate adducts with hemoglobin (1–5) and carboxymethylated hemoglobin (Cm-Hb) (6–9), which have low oxygen affinities but require subsequent treatment with a crosslinking agent to prevent subunit dissociation. One of the monofunctional aspirin-based derivatives, however, also has a low oxygen affinity but does not require crosslinking (10). Several of the bifunctional reagents, such as 2-nor-2-formyl-5-pyridoxal 5′-phosphate (2, 11), bispyridoxal tetraphosphate (12), bis(3,5-dibromosalicyl)fumarate (13), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) (14), yield a crosslinked hemoglobin derivative with a low oxygen affinity when the crosslinker is used with deoxyhemoglobin. In general, the reduced oxygen affinity of a hemoglobin derivative together with its inability to dissociate form the basis for its consideration as a blood substitute. However, the relationship between oxygen affinity and plasma retention had not been carefully analyzed to date.

Some crosslinked hemoglobin derivatives have a M_r of 64,000—i.e., crosslinking only within a given tetramer (12–15), and others have M_r values of 128,000 or greater—i.e., crosslinking between hemoglobin tetramers (16, 17). The

latter derivatives include the dextran–hemoglobin complexes (18), hemoglobin conjugates with polyethylene glycol (19), and glutaraldehyde-treated pyridoxylated hemoglobin, which are very large polymers of different molecular weights. The optimal molecular weight for a blood substitute has not yet been defined. Even though some investigators feel that the minimum molecular weight for a hemoglobin-based blood surrogate is 128,000 (20), good evidence suggests that a M_r of 64,000 is probably sufficient to sustain effective concentration of a blood substitute in the circulation. For example, serum albumin, which has a M_r of ≈60,000, is not cleared from the circulation. Furthermore, studies on the clearance time of crosslinked tetrameric hemoglobin derivatives also support this view (21–23).

In this communication we examine 2,5-diisothiocyanatobenzenesulfonate (DIBS) as a crosslinking agent with several objectives. (i) A bifunctional crosslinking reagent that generates a defined *tetrameric* crosslinked species was desirable. (ii) The plasma-retention time of a well-characterized crosslinked tetramer had to be measured to compare its clearance rate with that of the uncrosslinked Cm-Hb derivative and to ascertain the influence of the oxygen affinity of each derivative on its plasma-retention time.

METHODS AND MATERIALS

Whole blood from normal individuals was washed, lysed, and dialyzed as described (6–9). For studies with deoxyhemoglobin, the solution of oxyhemoglobin (≈200 μM) was bubbled with humidified N_2 for ≈45 min. Spectra were recorded on a Cary 2200 spectrophotometer. Inositol hexaphosphate (InsP_6) and glycylglycine were purchased from Sigma. DIBS was obtained from Trans World Chemical (Rockville, MD). Elemental analysis for $C_8H_4O_3N_2S_3$ monohydrate gave the following theoretical values: C, 33.1%; H, 1.4%; and N, 9.7%; experimental values were as follows: C, 32.9%; H, 2.8%; and N, 11.9% (average of two determinations). DIBS was dissolved in cold buffer just before use.

Crosslinking of Hemoglobin with DIBS. Hemoglobin solutions (200 μM in the deoxygenated state, usually ≈3–5 μmol unless indicated otherwise) were treated with a 10-fold molar excess of the crosslinking agent DIBS. When present, InsP_6 was in 50-fold molar excess over hemoglobin. This solution was then incubated at 25°C in 0.1 M potassium phosphate, pH 7.2, for 15 min. By analogy with earlier experiments with sodium cyanate (24, 25), the reaction was terminated by adding glycylglycine in 30-fold molar excess; a further incu-

Abbreviations: DIBS, 2,5-diisothiocyanatobenzenesulfonate; InsP_6, inositol hexaphosphate; Cm-Hb, carboxymethylated hemoglobin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate.
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bation for 15 min was then performed. The solution was dialyzed at 4°C against the buffer used for the subsequent chromatographic step (described below). The amount of subunit crosslinking was determined by SDS/PAGE; the procedure of Laemmli (26) was used with a 1-mm-thick, 12% crosslinked gel. The gel was stained with Coomassie blue for 1 hr and then destained in 30% (vol/vol) methanol/5% (vol/vol) acetic acid. The amount of crosslinking was estimated by densitometry on a Gilford model 2520 instrument equipped with a Shimadzu Chromatopac recorder model C-R6A.

The number of DIBS moieties per hemoglobin tetramer was calculated from the ratio of absorbance at 295 nm to that at 540 nm. For unmodified hemoglobin, this ratio was found to be 1.42, and its millimolar extinction coefficient at 540 nm was 57; values exceeding this ratio were considered to be due to the DIBS on the protein. Dividing this value by 15, the extinction coefficient of DIBS ($mM^{-1} \cdot cm^{-1}$), and by the hemoglobin concentration, calculated from its absorbance at 540 nm, provided an estimate of mol of DIBS per mol of hemoglobin tetramer. Because absorbance at 295 nm is a shoulder on the main protein absorbance band, the calculation gives only a rough estimate; the accuracy is ±20%.

Chromatography and Gel Filtration of Crosslinked Hemoglobin. The crosslinked hemoglobin (total, 200–250 mg) was applied to a Whatman DE-52 column (2 × 30 cm) and eluted with a linear gradient of 50 mM Tris acetate from pH 8.3 to pH 6.3 (500 ml of each). For removal of the most adherent components, the column was further eluted with 500 ml of the pH 6.3 buffer. Recovery of hemoglobin from the column was 80–95%. For preparative purposes the crosslinked hemoglobin was first passed through a mixed bed resin, as described by Christensen *et al.* (27). The amount of methemoglobin was ≤5%.

Gel filtration was done either with or without 1 M $MgCl_2$ (12, 28, 29) to provide some information on the crosslinking pattern. Without $MgCl_2$, a large column of Sephadex G-100 (2 × 110 cm) in 50 mM Tris acetate, pH 7.3, was used to determine whether the M_r of the crosslinked product was 64,000 or 128,000 or greater. Gel filtration in 1 M $MgCl_2$ [Sephadex G-100 superfine (2.5 × 35 cm) equilibrated in 1.0 M $MgCl_2$/10 mM Tris·HCl, pH 7.0 (12, 28, 29)] permits dissociation of the tetramer along each α–β plane of hemoglobin, whether or not a crosslink occurs within the α–β pairs in the same tetramer. A crosslink between two α chains or between two β chains in the same tetramer would prevent dissociation by 1 M $MgCl_2$. This information, together with the results of SDS/PAGE electrophoresis, can be informative on the crosslinking pattern.

Determination of Oxygen-Dissociation Curves. Samples were first dialyzed against 50 mM bisTris, pH 7.5, and, when necessary, concentrated to ≈0.5–0.7 mM. The oxygen-equilibrium curves were measured at 37°C on a Hem-O-Scan instrument.

Determination of Plasma-Retention Time. Male Sprague–Dawley rats weighing ≈300 g were used for the plasma-clearance studies. A carotid-artery catheter was surgically implanted on the day before the study. Hemoglobin solutions were concentrated to 13 or 14 g/dl, dialyzed against saline, and subsequently diluted to 7 g/dl with saline where stated. Solutions were administered through the carotid catheter, and blood samples were taken at intervals. The plasma hemoglobin concentration was measured as the absorbance at 524 nm (26). Plasma-clearance rates were calculated from fits to exponential functions obtained by nonlinear least-squares methods. Rates were compared by using 95% confidence intervals about the rate as a measure of significant difference. Cm-Hb, a modified but uncrosslinked hemoglobin with a low oxygen affinity prepared as described (6–9), was

used as a clearance standard because this material is excreted as fast as unmodified hemoglobin.

Analytical Procedures. The elemental analyses of DIBS were done by Robert Buzolich (The Rockefeller University Microanalytical Services). UV spectra were obtained with a Cary 2200 instrument. HPLC separation of the globin chains, crosslinked or uncrosslinked, was performed with a Beckman–Altrex system on a C_{18} Vydac ODS column with a gradient from 45–65% of 0.1% trifluoroacetic acid/80% (vol/vol) acetonitrile with the balance 0.1% trifluoroacetic acid. Amino acids were analyzed either with a Beckman 6300 analyzer with an IBM-AT System Gold enhancement or on the original Moore and Stein amino acid analyzer (30). Protein sequencing was done by Sheenah Mische and Joseph Fernandez (Rockefeller University Protein Sequencing/Biopolymer Facility). MS was done as described by Beavis and Chait (31).

RESULTS

Effect of Incubation Time, pH, and Ratios of Reactants on the Crosslinking of Hemoglobin A with DIBS. To optimize selectivity of the crosslinking of hemoglobin by DIBS, a survey of the effect of pH, reaction time, and molar ratios of reactants was evaluated. A hemoglobin:DIBS ratio of 1:10 and a pH of 7.2 produced a substantial amount of crosslinking (Fig. 1); this is also the ratio used by Currell *et al.* (32) and Bellelli *et al.* (33) for treating hemoglobin with the monofunctional isothiocyanatobenzenesulfonate, a reaction that does not involve crosslinking. Because liganded hemoglobin treated with DIBS for either 15 or 60 min showed about the same amount of crosslinking, as determined by the SDS gel, incubations were limited to 15 min with DIBS solutions freshly prepared in cold buffer. Prolonged incubation (20 hr) in the absence of the terminator glycylglycine leads to progressively more crosslinking, but addition of the dipeptide terminator at any time stops further crosslinking. However, some of the crosslinked components are more stable than others either with or without glycylglycine. One of the crosslinked products, component B, is very stable and is the focus of the present study.

SDS/gel electrophoresis indicated that component A of Fig. 1 was unmodified hemoglobin and all of the other components (B–H) are crosslinked. Component B, which represents a substantial fraction of the total crosslinked
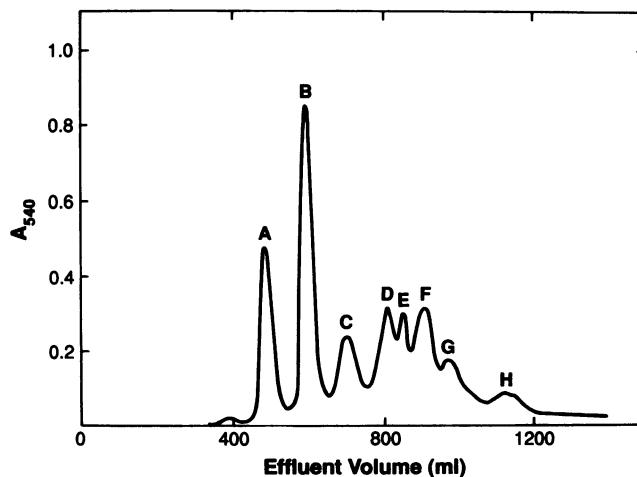

FIG. 1. Ion-exchange chromatography of hemoglobin crosslinked by DIBS. Deoxyhemoglobin A (200 μM) was treated with a 10-fold molar excess of DIBS and a 50-fold molar excess of $InsP_6$, as described in the text. After dialysis, each sample was applied to a DE-52 column. Letters correspond to the peak tubes of each component that were analyzed for the extent of crosslinking.

hemoglobin, has ≈50% crosslinked subunits as indicated by SDS/gel electrophoresis. Components C–E had a similar amount of crosslinking (36–51%) as component B. Components F–H, which are not completely resolved, had lesser amounts of crosslinking, which could result from some monofunctional attachment of DIBS to subunits without subsequent crosslinking to a second subunit. The slow chromatographic behavior of these species may be due to greater amounts of attached acidic DIBS moieties. The amount of DIBS on each hemoglobin component can be estimated as described above because DIBS itself has an absorption maximum around 295 nm. For component B, 1.0 ± 0.2 DIBS moiety per crosslinked tetramer was calculated, consistent with a single crosslink between two subunits within each tetramer to give the 50% crosslinking found by SDS/gel electrophoresis. Components C–H were estimated to have from 1 to 5 DIBS moieties per tetramer; amounts of modification increased for the components that eluted later in the chromatogram.

Gel Filtration of Crosslinked Hemoglobin. When isolated component B (Fig. 1) was analyzed by gel filtration either with or without 1 M $MgCl_2$ (12, 28, 29), practically all of the material eluted with a M_r of 64,000. Without 1 M $MgCl_2$, no more than 1% of the hemoglobin eluted with a M_r of 128,000 or greater. Therefore, DIBS leads primarily to intratetrameric crosslinking. The M_r of 64,000 with 1 M $MgCl_2$ suggests that the crosslink is between like subunits (either α–α or β–β) because such a crosslink, unlike an α–β crosslink, would prevent dissociation of the tetramer into a M_r 32,000 dimer that usually occurs with hemoglobin during gel filtration in 1 M $MgCl_2$.

The studies described above were done with 200 μM hemoglobin. When a more concentrated hemoglobin solution (350 μM) was crosslinked by DIBS, 95% of the crosslinked species was also of M_r 64,000 (data not shown). Lack of significant dependence of the crosslinking pattern on hemoglobin concentration is expected when crosslinking is within tetramers. Crosslinking between tetramers (i.e., intertetrameric) would show a dependence on hemoglobin concentration, as found with the crosslinking agent glycolaldehyde (9).

Characterization of Component B. This crosslinked tetramer is very stable at neutral pH—i.e., no hydrolysis of the crosslink was found under sterile conditions in neutral aqueous solution at room temperature for several months. The derivative can be separated into its constituent chains under acidic conditions by HPLC (Fig. 2). The protein in peak 1 is of M_r 16,000, whereas the protein in peak 2 is M_r 32,000 (Fig. 2 *Inset*). As shown in Table 1, amino acid analysis of the protein in peak 1 indicated that it was the β chain of hemoglobin; the protein comprising peak 2 had the amino acid composition of the α chain of hemoglobin.

Identity of the α–α crosslinked globin chains was confirmed by MS. The matrix-assisted laser desorption mass spectrum of the material labeled as peak 2 (Fig. 2) showed species $[M+H]^+$ and $[M+2H]^{2+}$ (Fig. 3). The species labeled $[Myo+H]^+$ and $[Myo+2H]^{2+}$ were derived from singly and doubly protonated horse muscle myoglobin added as a mass calibrant. The M_r of the α–α globin dimer was measured as 30,539, consistent with two α-globin chains (mass, 30,252) linked by a single DIBS moiety (mass, 272). The M_r of a β globin dimer with one DIBS crosslinker is 32,006.

For purposes of sequencing by Edman degradation, the DIBS-crosslinked α chains were first hydrolyzed from the α–α globin chains by treatment for 16 hr with 5% (vol/vol) acetic acid at 56°C. Subsequent SDS/gel electrophoresis indicated that most of the crosslink (80%) had been cleaved. When this sample was subjected to HPLC, under the conditions described in Fig. 2, one major peak eluted just before the remaining uncleaved α–α crosslinked globin (peak 2)
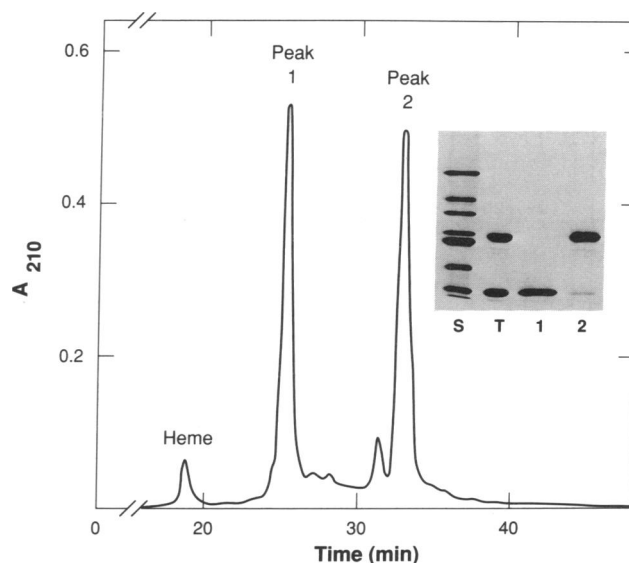
(data not shown). This material gave a discrete sequence in high yield beginning at the second amino acid residue of the α chain: $Leu^2-Ser^3-Pro^4-Ala^5-Asp^6-Lys^7-Thr^8-Asn^9-Val^{10}$ (α chain minus NH_2 terminus). Therefore, the original NH_2-terminal valine residue was removed during the cleavage in acetic acid together with the DIBS crosslink, presumably by a cyclization similar to the Edman reaction. In addition to the residual α–α chains (21%), two uncrosslinked chains were isolated in a pure state (45% yield). Each of these purified chains (50% yield) gave the same single sequence shown above; sequencing was done on 400 pmol of chain with an

Table 1. Identification of DIBS-crosslinked chains of component B by amino acid analysis

Amino acid	Theoretical		Experimental	
	β Chain	α Chain	Peak 1	Peak 2
Lysine	11	11	10.8	10.9
Histidine	9	10	9.3	9.9
Arginine	3	3	2.9	2.8
Aspartic acid	13	12	13.0	12.2
Threonine	7	9	6.8	8.3
Serine	*5*	**11**	*5.3*	**10.1**
Glutamic acid	*11*	**5**	*11.8*	**5.6**
Proline	7	7	8.2	8.3
Glycine	*13*	**7**	*12.8*	**7.2**
Alanine	*15*	**21**	*15.1*	**20.4**
Valine	*18*	**13**	*16.9*	**12.5**
Isoleucine	0	0	0	0
Leucine	18	18	18	18
Phenylalanine	8	7	8.1	7.2

Amino acids shown in boldface (α chain) or in italics (β chain) are those for which significant differences exist between α and β chains. Values for cysteine, methionine, tyrosine, and tryptophan are not included because these amino acids are either partially or completely destroyed during acid hydrolysis; the special conditions for hydrolysis or analysis to protect these amino acids were not needed for identification of the chain. Because recovery of the listed amino acids is nearly complete, the DIBS crosslink is probably completely hydrolyzed upon acid hydrolysis in 6 M HCl with regeneration of the amino acid side chain to which the DIBS was attached.
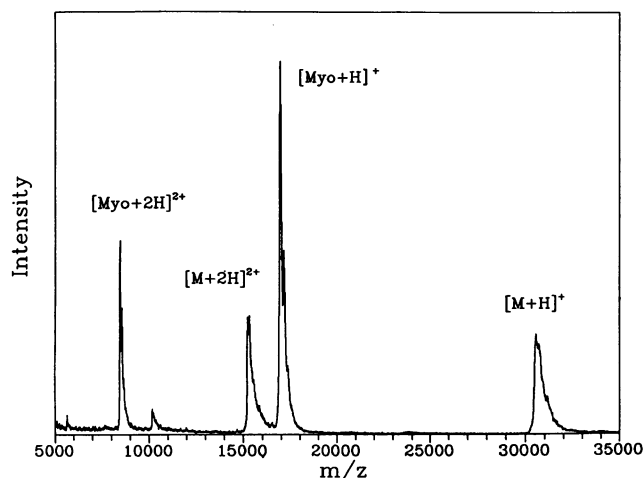
FIG. 3. Analysis of an α–α crosslinked globin by MS. Protein (1 pmol) from peak 2 of Fig. 2 was subjected to laser desorption mass analysis. Horse myoglobin $[\text{Myo}+2\text{H}]^{2+}$ was added as calibrant. M, molecule being studied—in this case, α–α crosslinked chains of human globin.

FIG. 4. Plasma-retention times of uncrosslinked and crosslinked hemoglobin in rats.

average repetitive yield of 88%. One component probably represents the intact α chain from which DIBS together with the original NH_2-terminal valine had been cleaved by cyclization in 5% acetic acid. The second, minor component probably represents the same deblocked α chain but with an additional cleavage at the labile Asp^{94}-Pro^{95} bond; the COOH-terminal fragment of this chain was not further characterized.

Oxygen-Equilibrium Properties and Plasma-Retention Times of Cm-Hb and of Tetrameric Crosslinked Hemoglobin. Component B was found to have a somewhat increased affinity $[P_{50} = 9$ mmHg; unmodified hemoglobin $= 11$ mmHg (1 mmHg $= 133$ Pa)] and full cooperativity ($n = 2.5$). As described (6–9), Cm-Hb has a significantly lowered oxygen affinity of 28 mmHg.

For the plasma-retention studies, 2.5 g of component B (Fig. 1) was isolated by a scale-up of the procedure described above. This material behaved as one component upon rechromatography on DE-52. The isoelectric focusing pattern of component B was similar to that of purified hemoglobin A_0. Each sample was dialyzed against sterile isotonic saline and then sterilized by filtration through a 0.2-μm filter into a sterile tube. The endotoxin content, as measured by the Limulus test, was 0.2 ng/mg of hemoglobin. The same amount of Cm-Hb (2.5 g) was isolated as described (6, 9). Each hemoglobin derivative was injected into the circulation of rats as described above. The plasma concentration half-life of Cm-Hb was 35 and 41 min (0.6 and 0.7 hr) at the two concentrations tested (Fig. 4). The DIBS-crosslinked hemoglobin had a plasma concentration half-life of 2.9 and 3.3 hr. After equilibrium, pseudo-first-order kinetics for the clearance were seen. Differences in plasma-concentration half-lives between the modified hemoglobin species were highly significant. The small increase in plasma concentration half-life seen in each species with increased dose was not significant at the doses studied.

DISCUSSION

Kavanaugh *et al.* (14) described the crosslinking of hemoglobin A with a compound similar to the one used in the present study. However, they showed that DIDS crosslinked the NH_2-terminal residues of the β chains, unlike the smaller reagent used in this study that crosslinks the major component B between the NH_2-terminal residues of the α chains. Component B of DIBS-crosslinked hemoglobin resembles
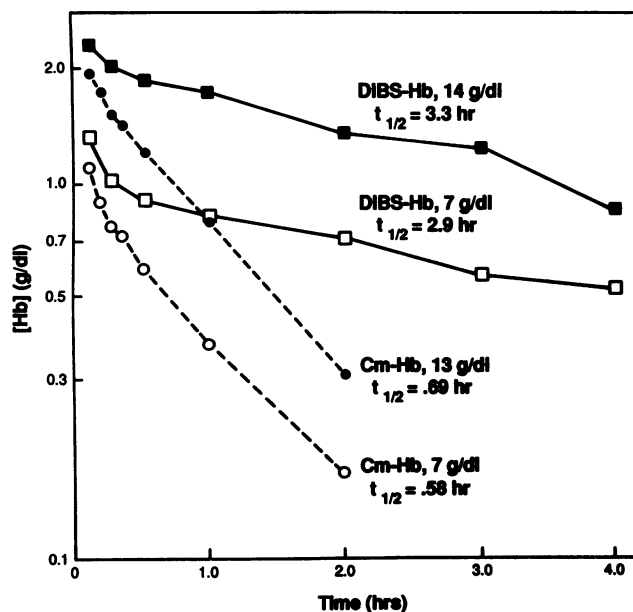
the hemoglobin derivative described by MacLeod and Hill (29), which is also crosslinked between the two α chains. However, there is no cooperativity for this latter derivative ($n = 1.0$), whereas the DIBS-crosslinked hemoglobin has full cooperativity ($n = 2.5$). The difference in oxygen affinity between DIDS- and DIBS-crosslinked hemoglobins is reminiscent of the effects of sodium cyanate on the oxygen affinity of hemoglobin (24). Thus, carbamoyl or DIBS groups on the α chains lead to increased oxygen affinity, whereas carbamoyl or DIDS groups on the β chains lead to decreased oxygen affinity.

Whereas the plasma-concentration half-life of extracellular hemoglobin is a function of both administered dose and animal species used in testing, the values reported here were estimated in rats with solutions in which volume and concentration are comparable with previous reports (34). The DIBS-crosslinked hemoglobin has a prolonged retention in plasma, comparable to that of other crosslinked hemoglobins that are under consideration as blood substitutes. DIBS-crosslinked hemoglobin would be expected to have a longer half-life when administered in greater dose and to larger animals. The results of this study clearly show that uncrosslinked carboxymethylated hemoglobin, which has a low oxygen affinity, has about the same retention time as unmodified hemoglobin A_0 (35). Hemoglobin component B crosslinked by DIBS does not have a lowered oxygen affinity, but its retention time was the same as that of hemoglobin crosslinked with an aspirin derivative; the latter has a M_r of 64,000 and lowered oxygen affinity (36). Therefore, the retention time of a hemoglobin derivative appears to be a function of its ability to dissociate into dimers and not a function of its oxygen affinity.

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