The Modification of Hemoglobin by Citrate*

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Citrate, when activated by a water-soluble carbodiimide, covalently modifies hemoglobin. At pH values near neutrality, complete modification of the N-terminal valine residues of α- and β-globin chains can be accomplished with a high degree of specificity. These groups react at a much more rapid rate than a slower reacting set of functional groups. Modification of hemoglobin with citrate alters the oxygen affinity of the protein. Although the p50 is not changed, the cooperative nature of oxygen binding is greatly decreased. Hemoglobin S modified with citrate is more soluble than unmodified hemoglobin S. The time taken for deoxygenated hemoglobin S to come out of solution in concentrated phosphate solutions is increased by citrate modification.

Interest in a cell-free blood substitute based on hemoglobin has led to the examination of chemical means of modifying hemoglobin structure to make it useful for oxygen transport and delivery to tissues. Modification is needed because outside the red blood cell the ionically bound allosteric effector 2,3-bisphosphoglycerate (BPG) dissociates and is lost during purification of hemoglobin resulting in an unacceptably high affinity of the purified protein for oxygen. In addition, concentrations of hemoglobin which can, because of osmotic considerations, be infused are sufficiently dilute that dissociation to dimers occurs to the extent that there is rapid renal clearance resulting in a high rate of removal of infused hemoglobin from circulation and possible renal damage. This leads to the need for chemical cross-linking of infused hemoglobin which is to be used as a blood substitute. Bifunctional anionic reagents able to react with amino groups which would be expected to bind with high affinity at the BPG binding site and both decrease the oxygen affinity and provide a covalent cross-link preventing dimer formation have received particular attention recently (Fantl et al., 1987; Kavanaugh et al., 1988; Benesch and Kwong, 1988; Vandegriff et al., 1991; Berbers et al., 1991). Another area where chemical modification of hemoglobin could be of clinical benefit is the modification studies. Hemoglobin concentrations were determined using a Corning Co-Oximeter and adjusted to required concentrations with MOPS buffer prior to treatment with citrate. Reactions were initiated by the addition of the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) obtained from Pierce Chemical Co. and carried out at 0 °C in an ice bath. [14C]Citrate (Du Pont-New England Nuclear) was used to determine the extent and site of chemical modification. To examine the effect of the modification on the intact protein, at time intervals samples were diluted 5-fold into ice-cold 0.5 M Tris-HCl buffer, pH 8.5, and dialyzed against 0.1 M Tris-HCl buffer, pH 8.5. Ion exchange chromatography was carried out on the dialyzed material using a Merck Superperformance 150 mm × 10-mm DEAE column at a flow rate of 1 ml/min. Radioactivity was determined in eluted fractions by scintillation counting of aliquots collected in a fraction collector at time intervals. Absorbance of the eluted material was monitored at 280 nm. For kinetic studies and for peptide mapping, reactions were stopped at time intervals by dilution of reaction mixtures into ice-cold trichloroacetic acid. Pellets of precipitated protein were obtained by centrifugation for 5 min in an Eppendorf centrifuge. The pellets were washed with acetone and redissolved in 70% formic acid. Radioactivity in the formic acid-soluble material was determined by scintillation counting of aliquots. Prior to peptide generation formic acid-soluble material was diluted into water and lyophilized. Peptides were generated in 0.1 M NH4HCO3, by digestion with trypsin for 6 h at 37 °C and subsequently separated by thin layer electrophoresis on Merck cellulose plates. Radioactive peptides located by radioautography were identified after further purification using reversed phase HPLC on Zorbax ODS (Du Pont) and FPLC on Superdex 75 (Pharmacia) columns. Amino acid compositions were determined after hydrolysis

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The abbreviations used are: BPG, 2,3-bisphosphoglycerate; MOPS, 4-morpholinepropanesulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; Hg, hemoglobin; OPA, o-phthalaldehyde.
and amino acid analysis on a Beckman System 6300 High Performance Analyzer.

Examination of the effect of modification of hemoglobin with citrate on the content of primary amino groups in hemoglobin was carried out using fluorescence measurement (Peterson, 1983) after reaction with o-phthalaldehyde (OPA). Globin was prepared from samples treated with citrate and from control samples in which either citrate or EDC had been omitted from the reaction mixtures. Weighed amounts of globin were dissolved in water. Aliquots were allowed to react with OPA and the fluorescence in 0.5 N NaOH was subsequently determined as described for OPA determination of intact proteins (Peterson, 1983). To determine total protein contents, aliquots were mixed with an equal volume of 12 N HCl and sealed in glass tubes. Hydrolysis was carried out for 16 h at 106°C. The acid was then removed using a SpeedVac concentrator (Savant Instruments). The dried hydrolyzed samples were dissolved in water and fluorescence after reaction with OPA was determined as before and compared with the fluorescence per μg of hydrolyzed bovine serum albumin.

Kinetic examination of rates of incorporation of radioactivity used procedures appropriate to studies of the modification of proteins in which sets of functional groups with different reactivities could be present (Freedman and Radda, 1968; Anderson and Perham, 1970). Oxygen binding properties of hemoglobins were determined using a Hem-O-Scan (American Instrument Co.). Samples were dialyzed against Krebs-Ringer bicarbonate and oxygen binding was determined in the presence of 5.6% carbon dioxide. The pentacyclohexylammonium salt of BPG (Sigma) in the same buffer at a final concentration of 10 mM was used to determine effects on the oxygen binding properties of modified and control hemoglobin. Hemoglobin solubility was determined in solutions of 2.3 M potassium phosphate, pH 7.0, at 32°C in the presence of 10 mg/ml sodium dithionite. Concentrated protein solutions were diluted into the buffer at room temperature. Turpidity leading to an increase in light scattering was measured at 650 nm in a 1-cm light path water jacketed cell in a Hitachi 124 Spectrophotometer.

RESULTS

The Effect of Citrate on the Oxygen Binding Properties of Hemoglobin—Preliminary experiments indicated that the presence of citrate decreased the oxygen affinity of hemoglobin. When present at a concentration of 25 mM in a 1 mM hemoglobin solution, citrate increased the p50 value from 17 to 30 mm Hg as determined in Krebs-Ringer bicarbonate using the Hem-O-Scan. There was no effect on co-operativity of binding as the Hill coefficient was 2.8 in both cases. This is similar to previous reports of reduced oxygen affinity in the presence of aliphatic poly(carboxylic acids) (Shimizu and Bucci, 1974). However, when hemoglobin was treated with citrate in the presence of the carboxyl-activating carbodiimide, the effect on oxygen binding was much different. Fig. 1 shows the oxygen binding curves obtained when 1 mM hemoglobin was incubated with 32 mM citrate for 1 h at 0°C in 0.1 M MOPS buffer, pH 7.2, with and without 26 mM EDC and then dialyzed overnight against a large excess of Krebs-Ringer bicarbonate prior to the determination of oxygen binding. The curve for hemoglobin incubated in the absence of EDC indicates that citrate was removed during dialysis as the material had a p50 of 17 mm Hg. Hemoglobin treated with citrate in the presence of EDC clearly showed altered oxygen binding properties. Although the p50 value was similar to that of untreated hemoglobin, the binding curve was much less sigmoidal. The Hill coefficient decreases from 2.8 to 1.3.

The allosteric effector EDC had no effect on citrate-modified hemoglobin oxygen binding. An identical oxygen binding curve was obtained for the modified material described in Fig. 1 when 10 mM effector was present. This concentration of effector caused a right shift of the oxygen binding curve of the control hemoglobin to a p50 of 25 mm Hg. When hemoglobin was treated with EDC in the absence of citrate as described, no effect on the oxygen binding was subsequently observed. The effect on oxygen binding seems therefore to be a consequence of covalent attachment of citrate to hemoglobin due to activation of the carboxyl groups of the citrate by the carbodiimide and subsequent covalent bonding to functional groups on the protein.

Effect of Citrate Modification on the Chromatographic Properties of Hemoglobin—When untreated purified hemoglobin that had been dialyzed against 0.1 M Tris-Cl buffer, pH 8.5, was applied to a Merck Superformance DEAE column equilibrated with 0.5 M Tris-Cl, pH 8.5, a single symmetrical absorbance peak was observed in the eluate after 7.5 min when the column was eluted at a flow rate of 1 ml/min with 0.5 M Tris, pH 8.5. Pretreatment of hemoglobin (0.5 mM) with EDC alone (36 mM) or citrate alone (32 mM) for 24 h at 0°C in 0.1 M MOPS, pH 7.2, followed by dialysis against 0.1 M Tris-Cl, pH 8.5, did not have any effect on the elution profile from DEAE under these chromatographic conditions. However, hemoglobin treated with both citrate and EDC for 24 h under these conditions and subsequently dialyzed against 0.1 M Tris-Cl, pH 8.5, was more strongly bound to the DEAE column. It could be eluted by making the pH 8.5, 0.5 M Tris-Cl buffer 0.5 M in NaCl. Fig. 2 describes elution conditions that were developed to separate unmodified and modified hemoglobins on the DEAE column. The DEAE elution profile of hemoglobin that had been modified by citrate radiolabeled with 14C in the presence of EDC for 10 min prior to dilution and dialysis is shown. The first absorbance peak corresponded in elution time to unmodified hemoglobin. It contained 21% of the detected absorbance. An absorbance peak at 9.6 min with a trailing shoulder accounted for 72.1% of the detected absorbance. Smaller peaks were detected at elution times of 24.5 and 26.8 min, accounting for 5.8 and 8.9% of the detected absorbance, respectively. The recovered radioactivity accounted for 97% of the radioactivity applied. Two radioactive peaks were detected corresponding to the absorbance peaks at 9.6 min (66% of the applied radioactivity) and at 26.8 min (30% of the applied radioactivity). These findings are consistent with a rapid limited incorporation of citrate into hemoglobin producing a form of the protein slightly more negatively charged than the untreated protein under the chromatography conditions and therefore having a moderately increased elution time. An accompanying lower rate, but more extensive modification, could account for the strongly bound

Fig. 1. Oxygen affinity of hemoglobin and modified hemoglobin. Hemoglobin (1 mM) in 0.1 M MOPS buffer, pH 7.2, was incubated for 1 h with 32 mM citrate in the absence (—) and presence (— — —) of 26 mM EDC. After dialysis against Krebs-Ringer bicarbonate buffer, oxygen binding of hemoglobin solutions as a function of oxygen concentration was determined at 37°C in gas mixtures containing 5.6% CO2 using a Hem-O-Scan Oxygen Dissociation Analyzer.
radioactive material eluted at 27.0 min. The small absorbance peak at 24.5 min that did contain radioactivity was not identified but could be free heme.

Additional support for a rapid initial limited incorporation of citrate into hemoglobin accompanied by a slower rate, more extensive, incorporation was obtained from DEAE chromatography of material that was modified by citrate in the presence of EDC for longer times. One hour of modification under the same conditions as for the 10-min modification produced an elution profile in which 91% of the detected absorbance and 96% of the recovered radioactivity was found in a peak eluting at 27.0 min. The remaining 9% of the absorbance and 4% of the radioactivity was found between elution times of 7 and 14 min. Radioactivity detected accounted for 98% of that applied. Chromatography of material which had been modified for 24 h under the same conditions produced a single major peak at 27.0 min, accounting for 98.5% of the detected absorbance applied and 99% of the applied radioactivity.

Determination of the Sites of Modification—The nature and extent of citrate modification of hemoglobin in the presence of EDC was further examined after digestion of modified protein with trypsin and subsequent separations of peptides. Fig. 3 shows a radioautograph of tryptic peptides generated from hemoglobin that had been incubated with radioactive citrate. It can be seen that two radioactive bands appear after a short period of reaction. Other bands indicative of slower reacting material appear after more extensive incubation, but the rapidly labeled bands were still present. Since the rapidly labeled bands were present after extensive times of modification, the subsequent formation of other labeled material does not appear to be due to further reaction of the initially labeled groups to form cross-links. Acrylamide gel electrophoresis in sodium dodecyl sulfate of material that had been extensively modified and shown to contain multiple bands when tryptic digests were electrophoresed and radioautographed gave no indication of subunit cross-linking.

Further purification of the rapidly labeled lower mobility radioactive band (m = 0.88 versus marker) on a Superdex 75 FPLC column followed by reverse phase HPLC on a Zorbax column gave material which had an amino acid composition of Asp (0.9), Ser (0.9), Ala (0.8), Val (1.0), Leu (1.0), Pro (1.0) when normalized for Lys content. No other amino acids were present in significant amounts. The composition is identical to that of a tryptic peptide arising from the N-terminal region of α-globin (Val-Leu-Ser-Pro-Ala-Asp-Lys), and it was therefore concluded that one of the residues rapidly labeled with citrate in the presence of EDC was the N-terminal valine of α-globin chains. From the higher mobility rapidly labeled radioactive band (m = 1.22 versus marker) similarly purified material was found to have a composition of Thr (0.9), Glu (1.7), Val (1.0), Leu (1.0), His (1.0), Pro (1.0), with no other amino acids present in significant amounts when normalized for Lys content. This composition is identical to that of a tryptic peptide arising from the N-terminal region of β-globin (Val-His-Leu-Thr-Pro-Glu-Glu-Lys). It was therefore concluded that the second residue that reacted rapidly with citrate in the presence of EDC was the N-terminal valine of β-globin chains. Other incorporation of radioactivity is presumably due to a slower reaction of amino acids.
groups from lysine residues with citrate in the presence of EDC.

Effect of Citrate Modification of Hemoglobin on Subsequent Reaction o-Phthalaldehyde—Chromatography on DEAE and peptide mapping indicated that a slow rate modification of a number of functional groups in addition to the N-terminal α amino groups occurred when hemoglobin was allowed to react with citrate in the presence of EDC for long periods of time. The extent of this modification and an indication of the nature of the reacting groups were examined in greater detail using a reagent that reacts with primary amino groups with the subsequent production of fluorescence species which can be quantified. The fluorescence produced when globin prepared from hemoglobin that had been modified with citrate for 24 h under the conditions described in Fig. 3 was reacted with OPA was compared to the fluorescence produced when identical amounts of globins from hemoglobin treated with citrate in the absence of EDC or EDC in the absence of citrate were reacted with OPA. Globin from the modified hemoglobin produced 49.3 ± 2.0 fluorescence units/µg protein. Globin from hemoglobins in which only EDC or citrate were present during the reaction produced 68.0 ± 2.6 fluorescence units/µg protein. These findings are compatible with the reaction of 27.5% of the primary amino groups of hemoglobin with citrate in 24 h under the reaction conditions described in Fig. 3.

Kinetics of Citrate Incorporation into Hemoglobin—Preliminary experiments were carried out to determine conditions that would allow the kinetic examination of the incorporation of radioactivity from citrate into hemoglobin. The most common application of carbodiimides in protein modification studies is to activate the protein carboxyls that subsequently react with added nucleophilic reagents or with nucleophilic functional groups within the protein. In the present case, carbodiimide activation of citrate carboxyls occurs under conditions in which hemoglobin carboxyl groups would also be expected to be activated. It was therefore decided to use limiting amounts of the carbodiimide with a view to selecting for activated carboxyl groups most reactive with nucleophilic functional groups in hemoglobin. Initial experiments were carried out at pH 7.2. At constant carbodiimide concentrations, citrate and hemoglobin concentrations were varied until conditions were found under which the amount of radioactivity incorporated after extensive incubation depended directly on the amount of hemoglobin present. At an EDC concentration of 26 mM and a citrate concentration of 32 mM, citrate incorporation into hemoglobin after a 24-h incubation at 0 °C increased in a linear fashion with hemoglobin concentrations from 0.25 to 1.0 mM. The time course of the incorporation of citrate into hemoglobin was then determined under these conditions and analyzed assuming first order kinetics. Linear semi-logarithmic plots were taken as evidence that the analytical method was appropriate. Fig. 4 shows the plot of data obtained in an experiment in which trichloroacetic acid-insoluble radioactivity was determined at intervals with the radioactivity incorporated at 20 h taken as the maximum incorporation. The best fit line obtained by linear regression has an R value of 0.99. The first order rate constant given by the slope is 0.021 min⁻¹. However, it can be seen that the line does not pass through the origin. This could be explained by the presence of some reactive groups, which react completely very quickly compared to other groups. From the specific radioactivity of the citrate used, the amount of citrate incorporated at the much faster rate can be calculated. From this and the amount of hemoglobin present, it was calculated that 3.85 mol of citrate/mol of hemoglobin or 0.96 mol of citrate/mol of globin chain was rapidly incorporated. Together with the other data on the sites and extent of modification, this suggests that N termini of both α- and β-globins react with citrate very rapidly compared to a set of other groups, probably amino groups contributed by lysine residues, all of which appear to react at similar rates to each other.

Further examination of the reactivity of functional groups in hemoglobin with citrate was carried out by determining the radioactivity in specific tryptic peptides generated from hemoglobin aliquots taken at intervals throughout incubation. After separation of peptides by thin layer electrophoresis, the radioactivity in regions from which peptides could be isolated corresponding in composition to the N-terminal tryptic peptides of α- and β-globins was determined. There was no difference detectable in the amount of radioactivity in the two regions over the time intervals and the incorporation of radioactivity was complete after a 20-min incubation. Fig. 5 shows the plot obtained using the radioactivity incorporated after 20 min as the maximum. The R value for the line obtained by linear regression is 0.99 and comes very close to passing through the origin. The first order rate constant given by the slope of the line is 0.133 min⁻¹. The rate of reaction was increased by increasing the hydroxide ion concentration. Under conditions identical to those used to generate the data of Figs. 4 and 5 except that 0.1 M MOPS at pH 6.6 was used instead of 0.1 M MOPS, pH 7.2, and analysis of data as described, the rate constants calculated from slopes were 0.067 min⁻¹ for the slow group and 0.317 min⁻¹ for the fast group of N-terminal residues. When the reaction was carried out as before but in 0.1 M MOPS, pH 7.8, the rate of incorporation of radioactivity decreased. Radioautographs of tryptic digests did not indicate a more rapid incorporation into the N-terminal peptides. The kinetic analysis indicated that a first order single rate constant of 0.005 min⁻¹ applied to all the reactive groups.

Modification of Hemoglobin S—Incorporation of citrate radioactivity into hemoglobin S was the same as incorporation into normal hemoglobin when carried out under identical conditions.
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FIG. 5. Rate of incorporation of radioactivity into the rapidly reacting functional groups of hemoglobin. Incubation conditions with [14C]citrate were the same as described in Fig. 4, and the data were plotted in the same way. Aliquots were taken in triplicate at time intervals. Tryptic peptides were generated from trichloroacetic acid-insoluble material obtained from each aliquot and subsequently separated and detected as described in Fig. 3. Radioactivity in the rapidly labeled bands was quantitated by scintillation counting at time intervals. Trypsic peptides were generated from trichloroacetic acid-insoluble material obtained from each aliquot and subsequently separated and detected as described in Fig. 3. Radioactivity in the rapidly labeled bands was quantitated by scintillation counting after elution from scraped areas. The radioactivity in the areas from which the N-terminal peptides can be purified reached a maximum in aliquots taken after 20 min incubation. This amount of incorporated radioactivity was used as Rc.

FIG. 6. Time course of the development of turbidity in hemoglobin S solutions. Hemoglobin S at concentrations of 0.42 mg/ml (- - ) or 0.82 mg/ml (---) or mixed with citrate-modified hemoglobin S (0.42 mg/ml + 0.42 mg/ml) (- - -) was incubated in 2.3 M potassium phosphate buffer, pH 7.0, at 32 °C. The increase in absorbance due to light scattering was monitored for a 1-cm light path at 650 nm.

conditions. The effect of modification with citrate on the solubility of deoxygenated hemoglobin S is shown in Fig. 6. Dilute solutions of hemoglobin S aggregated rapidly when incubated in the deoxy form at 32 °C in concentrated phosphate. Increasing the protein concentration decreased the time required for the onset of aggregation. However, if citrate-modified hemoglobin S was present, the aggregation of unmodified hemoglobin S was impeded. Solutions of only citrate-modified hemoglobin S showed no evidence of aggregation at concentrations of up to 1 mg/ml for at least 1 h at 32 °C in concentrated phosphate.

DISCUSSION

The use of carbodiimide activation in protein modification studies has mainly involved applications in which an exogenous nucleophilic amine is added in large excess to proteins in which carboxyl residues are activated resulting in the covalent attachment of the amine to the protein (Carraway and Koshland, 1972). At high concentrations, EDC has been used to react with protein functional groups in the absence of added exogenous nucleophile (Verburg et al., 1992). High concentrations of EDC may also result in protein cross-links (Mejillano and Himes, 1991). In this study a different approach has been used. The carboxyl groups of an exogenous reagent are activated with carbodiimide and allowed to react with nucleophilic protein functional groups at close to neutral pH. It has been shown that citrate can be used to modify hemoglobin with a high degree of selectivity with respect to the functional groups of hemoglobin with which it reacts. Citrate is highly negatively charged at the pH values at which selective modification has been shown to occur and can decrease the oxygen affinity of hemoglobin presumably by binding to the positively charged region between β-subunits. However, carbodiimide-activated citrate at less than physiological pH reacts preferentially with the α amino groups of the N-terminal residues of α- and β-globins. There are a number of possible explanations for this. Activation of carboxyl by carbodiimides leads to a positively charged adduct (Carraway and Koshland, 1972). Ionic interactions leading to citrate binding to the protein may not be the most important factor. The α amino groups may be more nucleophilic or more accessible to the reagents under the conditions used. Examination of citrate reaction with other proteins under conditions similar to those that have been used for hemoglobin may be useful in determining the major factors involved in the reaction. In the present study, the increased rate of reaction observed with increasing hydrogen ion concentration is to be expected since formation of the carbodiimide-carboxyl adduct requires protonation (Carraway and Koshland, 1972) and the carbodiimide would be expected to be relatively stable over the pH range used (Gilles et al., 1990).

Conditions have been developed under which a limited modification of hemoglobin with citrate can be obtained. At pH values near neutrality with defined concentrations of reactants, limited incorporation of citrate into two sets of functional groups based on rates of reaction can be obtained. The conclusion that the functional groups reacting rapidly with citrate in the presence of EDC are the N-terminal amino groups of the α- and β-globins is based on direct evidence. When [14C]citrate was used, there was rapid radiolabel incorporation into material produced by tryptic digestion, which, when purified and hydrolyzed, corresponded in amino acid composition to the amino acid compositions of the N-terminal peptides that would be expected from tryptic digestion of α- and β-globin. Rates were determined directly on this material after electrophoretic separation of peptides.

It seems likely that the more slowly reacting groups obtained under the reaction conditions used represent a set of lysine residue amino groups. The chromatographic properties on DEAE of hemoglobin after extensive times of modification suggest highly negatively charged molecules as would be produced by replacement of lysine amino group positive charges by negative charges from carboxyl groups of coupled polyalcohol citrate. Peptide maps and OPA treatment of material produced under the reaction conditions specified indicate that
only a limited number of groups reacted. The tryptic peptide maps contained fewer bands than would be expected if all 11 lysine amino groups of α-globin and all 11 lysine amino groups of β-globin reacted equivalently. After 24 h of modification at pH 7.2 with citrate under the define concentration conditions, 72.5% of the OPA-reactive material remained.

When modified to a limited extent with citrate as described, hemoglobin co-operativity with respect to oxygen binding is lost. Although the p50 of citrate-modified hemoglobin is similar to that of unmodified hemoglobin, the oxygen affinity at oxygen tensions relevant to physiological situations is lower. Similar effects on oxygen binding have been reported after carboxymethylolation of N-terminal residues of hemoglobin (Fantl et al., 1987). Limited citrate modification abolishes the response of hemoglobin to BPG. This can be accounted for by the proximity of the α amino group of β-globin to the binding site for this regulator in the cavity between β chains (Perutz et al., 1980). It would be of interest to determine the effects of citrate modification on carbon dioxide binding (Van-degriff et al., 1991).

Citrate contains three carboxyl groups. It is therefore possible that after reaction with the N-terminal amino group further reaction leading to intra- or intersubunit cross-linking could occur involving an additional carboxyl group of the citrate. However, there was no evidence of cross-linking of either type when reactions were carried out as described. If the modified hemoglobin were to be developed as a potential oxygen-delivering blood substitute an additional step in which subunits would be covalently linked would be necessary. The free carboxyl groups incorporated from citrate could possibly be exploited for this purpose.

The preliminary data on the properties of hemoglobin S modified with citrate suggest that the modification could have value in preventing the aggregation to form a gel that causes sickling when it occurs inside a red blood cell. The method used to determine hemoglobin S solubility is similar to a procedure in which deoxygenation is carried out in an anaerobic cell flushed by nitrogen and the temperature is raised from 0 to 30 °C and the time to onset of increasing turbidity is measured at 700 nm (Adachi and Asakura, 1979). Aggregation of dilute solutions of hemoglobin in concentrated phosphate solutions under these conditions has been proposed to be similar to the mechanism leading to the gelation of concentrated solutions of hemoglobin S (Adachi et al., 1987). Accordingly, retardation of aggregation of dilute solutions suggests conditions that might lead to the retardation of gelation of concentrated solutions in red blood cells. The altered conditions used in the present study to detect aggregation in which room temperature (22 °C) solutions were warmed to 32 °C and nitrogen flushing was eliminated give similar results in that the onset of turbidity occurs more rapidly with increasing temperature and increasing hemoglobin S concentration. Since aggregation of deoxy hemoglobin S was clearly retarded in mixtures of hemoglobin S and hemoglobin S modified with citrate and the modified protein by itself showed no indication of aggregation under conditions in which hemoglobin S showed substantial aggregation, citrate modification may prove to be a useful method to prevent gelation.

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