The reactivity of the amino groups of hemoglobin A toward reductive hydroxyethylation with glycolaldehyde in the presence of NaCNBH₃ has been investigated. The α-amino groups of the α- and β-chains of hemoglobin A are the most reactive amino groups of the protein for reductive hydroxyethylation; both sites are about equally reactive. Similarly, on reaction of hemoglobin A with glyceraldehyde in the presence of NaCNBH₃, the α-amino groups of both the α- and β-chains of the protein undergo selective reductive dihydroxypropylation. Modification of the α-amino groups of hemoglobin A either by reductive hydroxyethylation or reductive dihydroxypropylation apparently lowers the pKₐ of the α-amino groups and results in an early elution of the modified protein from CM52 chromatography compared with that of the unmodified protein as well as protein modified at the ε-amino groups. By hybridization of the modified chains with unmodified chains, new derivatives of hemoglobin with dihydroxypropyl or hydroxyethyl moieties on Val-1(α) or Val-1(β) of the protein have been prepared for the functional studies.

Modification of the α-amino group of Val-1(α) by reductive hydroxyethylation or by reductive dihydroxypropylation resulted in an increased oxygen affinity of hemoglobin A, whereas the same modification of the α-amino group of the β-chain resulted in a slight decrease in the oxygen affinity. The Hill coefficient of all four derivatives was 2.7, nearly the same as that of the native protein, showing normal heme-heme interactions in these derivatives. Both 2,3-diphosphoglycerate and chloride modulate the oxygen affinity of these hybrids; the influence of 2,3-diphosphoglycerate on decreasing the oxygen affinity of the hybrids with alkyl groups at Val-1(β) is relatively less than that of the native molecule. On the other hand, when the modification was on Val-1(α), the relative decrease in oxygen affinity of the hybrids upon binding of 2,3-diphosphoglycerate was of the same order as with the native protein. The chloride ion-mediated relative decrease in the oxygen affinity of the hybrids with alkyl groups at Val-1(β) was nearly the same as that seen with the Val-1(α) hybrids, and lower than that with the native molecule. The relative influence of hydroxyethylation or of dihydroxypropylation at a given site was nearly the same, suggesting that the size of the alkyl chain has little to do with the observed changes in the oxygen affinity. The influence of reductive alkylation of the α-amino groups with these two hydroxaldehydes probably reflects the change (decrease) in the pKₐ of the reductively hydroxyethylated or dihydroxypropylated α-amino group as compared with that of the unmodified α-amino groups.

Derivatization of the functional groups of proteins with group-specific reagents continues to be one of the powerful procedures in studies on the structure-function relationships of proteins. Of the many procedures available for the modification of the amino groups of proteins, reductive alkylation of the amino groups is becoming increasingly popular, especially since the reaction can be carried out in the neutral pH region, and the derivatized amino group still retains its original positive charge (1). Reductive alkylation involves the reaction of aliphatic carbonyl compounds (aldehydes or ketones) with proteins in the presence of reducing agents such as sodium borohydride (2) or sodium cyanoborohydride (3, 4).

We have been studying the reaction of the aliphatic aldehyde glyceraldehyde with Hb, especially in view of its anti-sickling potential (5–9). Glyceraldehyde forms stable adducts at some of the amino groups of proteins even in the absence of reducing agents (7, 8); this stability is a result of the potential of the Schiff base adducts of some of the amino groups of HbA to undergo Amadori rearrangement (ketoamine adduct). The formation of the ketoamine adduct appears to be specific; the major sites of reaction on Hb are Lys-16(α), Val-1(β), Lys-82(β), Lys-59(β), and Lys-120(β) (7). However, when HbA is incubated with glyceraldehyde in the presence of sodium cyanoborohydride, the selectivity of the reaction changes significantly (9). With limiting concentrations of glyceraldehyde, the α-amino groups showed a preferential reaction; the α-amino groups of the α- and β-chains react nearly to the same extent (9). The preferential reactivity of the α-amino groups, and the nearly same reactivity of the α-amino groups of the two chains are consistent with the fact that the two α-amino groups have low, and nearly the same, pKₐ values (10). With the objective of determining whether this preferential reactivity of the α-amino groups of Hb in the presence of sodium cyanoborohydride is unique to glyceraldehyde or common to other hydroxaldehydes, we have now investigated the reaction of Hb with glycolaldehyde (Fig. 1).

Geoghegan et al. (11) have used glycolaldehyde in the presence of sodium borohydride or sodium cyanoborohydride as a

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1 The abbreviations used are: Hb, hemoglobin; DHP, 2,3-dihydroxypropyl; HE, 2-hydroxyethyl; HMB, p-hydroxymercuribenzoate; DPG, 2,3-diphosphoglycerate; HPLC, high performance liquid chromatography; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethyl-propane-1,3-diol.
procedure for the hydroxyethylation of the amino groups of proteins. The hydroxyethyl groups on the amino groups are attacked by periodate to regenerate the free amino groups; reversible modifications of lysozyme and ovalbumin have been carried out (11). We have recently demonstrated that upon reaction of glycolaldehyde with proteins the initial Schiff base adduct undergoes Amadori rearrangement to generate a new aldehyde function which, in turn, reacts with another amino group to form covalent cross-links (12). In the presence of NaCNBH₃, this cross-linking reaction is inhibited, and hydroxyethylation of amino groups takes place (12). The present communication describes the reaction of glycolaldehyde or glyceraldehyde with Hb in the presence of sodium cyanoborohydride. The selectivity of the α-amino groups of HbA toward reductive hydroxyethylation (with glycolaldehyde) appears to be nearly the same as that with reductive dihydroxypropylation (with glyceraldehyde). Using these reductive hydroxyethylation and reductive dihydroxypropylation procedures, HbA with specific modifications at the α-amino group of α- and β-chains have been prepared and their functional properties have been studied.

MATERIALS AND METHODS

Erythrocytes from normal adults were washed with phosphate-buffered saline, pH 7.4, and then lysed with water, and diazoylated against 50 mM Tris-acetate, pH 8.5. HbA was isolated by chromatographing the lysate on DE52 (7, 8). Protein concentration was measured by its absorbance at 546 nm.

Hydroxyethylation of HbA—The erythrocyte lysate was diazoylated against phosphate-buffered saline, pH 7.4, and treated with 10 mM glycolaldehyde in the presence of 20 mM NaCNBH₃ at 37 °C for 30 min. The HbA concentration in this incubation mixture was 1 mM. After incubation, the excess reagents were removed by gel filtration on a column (2.2 × 35 cm) of Sephadex G-25 equilibrated and eluted with 50 mM Tris-acetate buffer, pH 8.5. The incorporation of the radioactivity of the tritium label (from the tritiated sodium cyanoborohydride) eluted at a position close to that of α-hydroxyethyllysine (11) and the remainder eluted near the void volume of the short column of the Moore-Stein analyzer. This acidic radioactive component eluted at a position close to that of threonine on the long column. In conjunction with the peptide map analysis (see below), this radioactive component appears to be α-hydroxyethylvaline derived from the NH₂ terminus of both the α- and β-chains of HbA.

On chromatography of glycolaldehyde-treated HbA on DE52 (Fig. 2), most of the protein eluted at the position of unmodified HbA and was radioactive. The material eluting at this position was isolated and referred to as HE-HbA. HE-HbA was subjected to structural analysis for determination of the site of modification by glycolaldehyde. The results of the separation of the HMB chains of HE-HbA are shown in Fig. 3. Four components designated A₁ to A₄ were present. From amino acid analysis and peptide mapping, A₁ and A₂ were found to be the modified β-chains, while A₃ and A₄ were found to be the derivatized α-chains. The position of A₄ corresponded to that of unmodified α-chain. The chromatographic behavior of A₃ and A₄ (both α-chains) seen in this study is very close to that observed in the separation of α-dihydroxypropyl α-chain from unmodified α-chains and α-chains that are dihydroxypropylated at their α-amino groups. This would suggest that compound A₃ could be the α-hydroxyethylated α-chain, which is confirmed by peptide mapping (see below).

In order to determine the exact location of the hydroxyethyl groups in A₁ and A₄, the globin chains from these components were subjected to tryptic digestion after aminooethylation and the tryptic digests were separated on an HPLC column (Fig. 1).
FIG. 2. Chromatography of HbA reductively alkylated with glycolaldehyde (using $^3$H-labeled NaCNBH$_3$) on DE-cellulose. Carbonmonoxy-HbA (1 mM) was treated with 10 mM glycolaldehyde in the presence of 20 mM sodium cyanoborohydride ($^3$H-labeled). The reaction mixture was passed through a column (2.2 × 45 cm) of Sephadex G-25 equilibrated and eluted with 50 mM Tris-Ac, pH 8.5, to separate the protein from excess reagent. The fractions containing protein were pooled, concentrated, and applied to a column (2.2 × 40 cm) of DE52. The protein was eluted with a linear gradient of 500 ml each of 50 mM Tris-Ac buffer, pH 8.3, and 50 mM Tris-Ac buffer, pH 7.3. The fractions in the region marked + were pooled and concentrated. This sample is referred to as HE-HbA. The position of HbA is indicated by 1, as described under "Materials and Methods." The tryptic peptide map of component A$_1$ was identical with that of $\beta$-chain of unmodified HbA (14). Almost all of the radioactivity eluted at the position of unmodified $\beta$-T$_1$ (Fig. 4A). The fractions containing radioactivity were pooled and hydrolyzed. The amino acid composition of this peptide $\alpha$ was identical with that of $\beta$-T$_1$ (Table I), except for a loss of about 0.8 mol of valine. This peptide appears to be a mixture of two peptides; about 20% is unmodified, the remaining 80% being modified at its NH$_2$-terminal valine residue. Thus, the major site of modification in the $\beta$-chain is the NH$_2$-terminal residue.

When the tryptic peptides of component A$_2$ were analyzed (Fig. 5B), the maps were found to be identical with that of the $\alpha$-chain (14). All the radioactivity of this component was located in two peaks. Nearly 75% of the radioactivity eluted at a position corresponding to that of $\alpha$-T$_1$ (b), and the remainder at the position corresponding to that of $\alpha$-T$_{1+2}$ (c). The radioactive peptides $b$ and $c$ were pooled, lyophilized, and hydrolyzed. Amino acid analysis of peptide $b$ corresponded well with that of $\alpha$-T$_1$ except for the loss of a single residue of valine, identifying this peptide as $\alpha$-T$_1$ modified at its NH$_2$ terminus by glycolaldehyde. The amino acid composition of peptide $c$ was the same as that of $\alpha$-T$_{1+2}$ (Table I) except for a decrease of one residue of valine; the lysine content was not changed. This peptide arises from incomplete tryptic cleavage at Lys-7 of the $\alpha$-chain and is known to be relatively more resistant to trypsin in the unmodified $\alpha$-chain. Thus, it is concluded that component A$_2$ is the $\alpha$-chain that has been hydroxyethylated at its $\alpha$-amino group.
The peptides designated a, b, and c are those isolated by HPLC of the tryptic peptides of the chains (Fig. 4). The labeled peptides were hydrolyzed and subjected to amino acid analysis as described in the text. Values are expressed as residues per peptide, using lysine as one residue for peptides a and b, and as two residues for peptide c.

### Table 1

| Amino acid | Peptide a | Found | β-T₁ | Found | α-T₁ | Found | α-T₁×1 |
|------------|-----------|-------|------|-------|------|-------|--------|
| Asp        | 0         | 0.0   | 1.0  | 1.0   | 1.9  | 2.0   |
| Thr        | 0.8       | 1.0   | 0.9  | 1.9   | 1.0  | 1.0   |
| Ser        | 0.0       | 0.0   | 0.0  | 0.0   | 0.0  | 0.0   |
| Glu        | 1.9       | 2.0   | 1.2  | 1.0   | 1.0  | 0.0   |
| Pro        | 1.0       | 1.0   | 0.9  | 1.0   | 1.9  | 2.0   |
| Gly        | 0.2       | 0.0   | 0.1  | 0.2   | 0.0  | 0.0   |
| Ala        | 0.2       | 0.0   | 1.1  | 1.0   | 1.0  | 2.0   |
| Val        | 0.2       | 1.0   | 0.0  | 1.0   | 1.0  | 1.0   |
| Leu        | 1.1       | 1.0   | 0.9  | 1.0   | 1.0  | 1.0   |
| Tyr        | 0.0       | 0.0   | 0.0  | 0.0   | 0.0  | 0.0   |
| Phe        | 0.1       | 0.0   | 0.0  | 0.0   | 0.0  | 0.0   |
| His        | 1.0       | 1.0   | 0.0  | 0.0   | 0.0  | 0.0   |
| Lys        | 1.0       | 1.0   | 1.0  | 2.0   | 2.0  | 2.0   |
| Arg        | 0.0       | 0.0   | 1.0  | 0.0   | 0.0  | 0.0   |

HbA

Chromatography of the hybrid HbA generated on mixing reductively alkylated HbA with 20 mM NaCNBH₃. The hybrid HbA with modifications at two of its four α-amino groups was purified by chromatography on CM52. Chromatography of the hybrid HbA generated on mixing reductively alkylated α- or β-chains with unmodified β- or α-chains, respectively, is shown in Fig. 6. The major component in these two hybrids prepared eluted ahead of the position of unmodified HbA, and represents the species with two of its four α-amino groups reductively hydroxyethylated.

Fig. 5. Schematic representation of the procedure used for the preparation of the hybrid hemoglobins containing hydroxyethylation or dihydroxypropylation at the α-amino groups of α- or β-chains.

The component A₂ accounts for nearly 70% of the α-chain, i.e., about 70% of the α-chains are modified at the α-amino group. From the amount of free valine present in peptide a (Table I), it is calculated that 70% of the β-chains in A₂ are hydroxyethylated at their α-amino groups. About 10% of the β-chain elutes at A₀, and the amino acid analysis of this component has shown that all of the radioactivity associated with this peak is present as hydroxyethyllysine. Thus, the amount of the β-chains of HE-HbA hydroxyethylated at their α-amino group accounts for about 67%, a value close to that of the α-amino group of the α-chain (≈70%). Thus, the extent of modification of the α-amino group of α- and β-chains of HbA by glycolaldehyde in the presence of sodium cyanoborohydride is nearly the same as that seen with the aldriose, glyceraldehyde, in the presence of sodium cyanoborohydride to form ketoamine adducts (9).

Preparation of Hemoglobin Hybrids Reductively Alkylated with Glycolaldehyde and Glyceraldehyde and Specific α-Amino Groups—Previous studies have shown that on CM52 columns HbA reductively dihydroxypropylated at the α-amino groups elutes ahead of unmodified HbA and HbA reductively dihydroxypropylated only at the ε-amino groups of lysine (9). The observation in the present study (Fig. 3) that the α-chains hydroxyethylated at the α-amino groups also elute ahead of unmodified α-chain or the α-chain modified at the ε-amino groups suggests that the HbA tetramer hydroxyethylated at the α-amino groups could also be separated from unmodified HbA. Thus, this would provide a route for the preparation of hybrid HbA molecules with specific modifications at the α-amino groups. Furthermore, the reductive alkylation of amino groups does not eliminate the original positive charge of the group and fact affords the opportunity to introduce hydrophilic alkyl groups at the α-amino groups without perturbing the net positive charge, to study their influence on the functional properties of HbA.

The preparation of hybrid hemoglobins specifically modified at α-amino groups of either α- or β-chains either by hydroxyethylation or by dihydroxypropylation was carried out following the protocol shown in Fig. 5. Appropriately modified HMB α- and β-chains were hybridized with unmodified β- or α-chains, respectively, in the presence of β-mercaptoethanol. The hybrid HbA with modifications at two of its four α-amino groups was purified by chromatography on CM52. Chromatography of the hybrid HbA generated on mixing reductively hydroxyethylated α- or β-chains with unmodified β- or α-chains, respectively, is shown in Fig. 6. The major component in these two hybrids prepared eluted ahead of the position of unmodified HbA, and represents the species with two of its four α-amino groups reductively hydroxyethylated.

Chromatography of the hybrid HbA prepared by mixing the reductively dihydroxypropylated α- and β-chains of HbA with unmodified β- and α-chains, respectively, is shown in Fig. 7. Again the major (radioactive) protein component of these two preparations eluted ahead of the position of unmodified HbA, and the position corresponded well with that obtained with hybrids generated from the α-hydroxyethylated chains. Thus, the early elution of the hybrids appears to be only a function of the substitution at the α-amino groups of the tetramer and independent of whether the substitution of the α-amino groups is by dihydroxypropylation or by hydroxyethylation, as well as whether the substitution is at the α-amino group of either chain. The early elution of the HbA derivatives reductively alkylated at their α-amino groups could be a reflection of a decrease in the pKₐ of the α-amino groups as a result of reductive alkylation.

Oxygen Equilibrium Curves of Hybrid Hemoglobins—From the hybridization studies described above, four species of hybrid hemoglobins designated α₂β₂(α-HE), α₂β₂(β-DHP), α₂β₂(α-HE/β-DHP), and α₂β₂(β-DHP) were isolated and concentrated. Oxygen equilibrium measurements on these four hybrids have been determined at 37 °C and pH 7.4 (Table II). Hill coefficients of all four hybrids were about 2.7, close to that of the native molecule (Fig. 8), suggesting that modification of the α-amino groups either by hydroxyethylation or by dihydroxypropilation has little influence on the heme–heme interaction. However, modification of the α-amino group of the ε-chain either by hydroxyethylation or by dihydroxypropilation resulted in a significant increase in the oxygen affinity of the hybrids. On the other hand, modification of the α-amino group of the
HbA hydroxyethylated or dihydroxypropylated at Val-1(α) showed a considerable increase in its oxygen affinity. The $P_{50}$ values of these hybrids are lower than that of native HbA; however, with the binding of DPG, the relative increase in the $P_{50}$ of these hybrids and native HbA is nearly the same, i.e. about 2.3. From these results, it can be concluded that modification of Val-1(β) by reductive alkylation decreases the influence of DPG on the oxygen affinity of the molecule, whereas modification of the α-amino group of the α-chain has

**FIG. 6.** Purification of the hybrid hemoglobins with hydroxethylolation at the α-amino group of α- or β-chains by chromatography on CM-cellulose. The hybrids were dialyzed against 10 mM phosphate buffer, pH 5.85 (1 mM in EDTA), and loaded on CM52 columns (2.2 × 35 mm) equilibrated with the same buffer. The protein was eluted with a linear gradient of 250 ml each of 10 mM potassium phosphate buffer, pH 5.85, and 15 mM potassium phosphate buffer, pH 7.6 (1 mM in EDTA). A, hybrids prepared by mixing α-chains hydroxethylolated at α-amino groups (component A5 of Fig. 3) with unmodified β-chain. The major radioactive peak eluting between 270 and 290 ml was pooled and designated α$_2$β$_2$(α-HE). A small amount of radioactive component was eluted around 250 ml, and has not been characterized further. B, hybrids prepared by mixing hydroxethylolated β-chains (component A5) with unmodified α-chain. The major radioactive component eluting between 270 and 290 ml was pooled and designated α$_2$β$_2$(α-HE). The minor component eluting at the position of unmodified HbA (~306 ml) was pooled and designated α$_2$β$_2$. A small amount of radioactive component was eluted around 250 ml, and has not been characterized further. B, hybrids prepared by mixing hydroxethylolated β-chains (component A5) with unmodified α-chain. The major radioactive component eluting between 270 and 290 ml was pooled and designated α$_2$β$_2$(α-HE). The minor component eluting at the position of unmodified HbA was radioactive, and is apparently generated from the β-chains that are hydroxethylolated at α-amino groups, and unmodified β-chains present in component A5 (Fig. 1).

β-chain by hydroxethylolation, as well as by dihydroxypropylation decreased the oxygen affinity of HbA slightly.

**Influence of Diphosphoglycerate on the Oxygen Equilibrium Properties of Hybrid Hemoglobins**—2,3-Diphosphoglycerate binds tightly to deoxyhemoglobin and leads to a decrease in the oxygen affinity. By x-ray crystallography, it has been shown that residues Val-1, His-2, Lys-82, and His-143 of the β-chain are in the binding site of 2,3-DPG (17). In addition, there are indications from the x-ray data that Val-1(β) and Lys-82(β) may provide the binding site for inorganic phosphate. Therefore, it was of interest to determine whether hydroxethylolation or dihydroxypropylation of the amino groups of Val-1(β) decreased or abolished the influence of DPG binding on the oxygen equilibrium properties of the molecule; as seen in Table II, hydroxethylolation or dihydroxypropylation significantly reduced the overall influence of DPG on the oxygen affinity of the molecule. The relative increase in $P_{50}$ of these hybrids is about 1.6, as compared with the value of 2.5 for the native molecule. On the other hand,

**TABLE II**

| Sample | No addition | With DPG (1 mM) | +DPG/ −DPG | With chloride (500 mM) | +Cl/ −Cl |
|--------|-------------|----------------|------------|-----------------------|--------|
| α$_2$β$_2$ | 8 | 23 | 2.9 | 19 | 2.4 |
| α$_2$β$_2$(α-HE) | 5 | 12 | 2.4 | 8 | 1.6 |
| α$_2$β$_2$(α-DHP) | 11 | 18 | 1.6 | 20 | 1.8 |
| α$_2$β$_2$(β-DHP) | 5 | 13 | 2.6 | 9 | 1.8 |
| α$_2$β$_2$(α-HE) | 10 | 15 | 1.5 | 16 | 1.6 |
with glycolaldehyde extensive covalent cross-linking of the protein takes place (12). The present study shows that when seen with glyoxylic acid (22).

The present study shows that when seen with glyoxylic acid (22).

sodium cyanoborohydride only hydroxyethylation of the pro-

the functional properties of derivatized HbA, such as oxygen binding, and the influence of DPG and chloride on the oxygen affinity.

clearly demonstrated that the α-amino groups of both the α- and β-chains are modified to nearly the same extent by the NH₂-terminal valine. Analysis of the tryptic peptides with our earlier studies with glyceraldehyde (9) and probably reflect the similar pKₐ of the two α-amino groups of HbA (10). Similar reactivity of the α-amino groups of HbA is also seen with glyoxylic acid (22).

The separation of the α-chains hydroxyethylated at the α-amino group from the unmodified α-chain, and from α-chain hydroxyethylated at the ε-amino groups observed in the present study, is very similar to that found after dihydroxypropylation of the α-chains and β-chains (9). This result provided a procedure to prepare hybrid HbA specifically modified at the α-amino groups of HbA.

Modification of the α-amino group of the α-chain by dihydroxypropylation or by hydroxyethylation resulted in an increase in the oxygen affinity of the molecules, similar to that seen upon carbamylation of this residue with cyanate (21). On the other hand, the influence of modification of the α-amino group of the β-chain by dihydroxypropylation or by hydroxyethylation is to decrease the oxygen affinity, also similar to the slight decrease in oxygen affinity observed with carbamylation of Val-1(β) (21).

The modification of Val-1(β) by reductive alkylation appears to perturb the modulation of the O₂ affinity by DPG-binding to HbA. Binding of DPG to HbA lowers the oxygen affinity (23). The relative changes in oxygen affinity of αβ, α₁⁺ε-CH₂⁺β, and α₂⁺ε-NO₂β on DPG binding are nearly the same. On the other hand, the hybrids with modifications at Val-1(β) had a much lower influence of DPG on their O₂ affinity as compared with the native molecule. The titration of Val-1(β) hybrids with DPG as measured by the changes in P₅₀ showed a sharp break at 1:1 of DPG to HbA, clearly demonstrating that the binding of these hybrids with DPG is complete (data not shown). Thus, it is clear that the DPG bound to the Val-1(β) hybrid has a decreased effect on the oxygen affinity of the molecule as compared with the native molecule. It may be pointed out that HbA₀, which contains covalently bound glucose at its β-amino termini, also shows a reduced influence of organic phosphate (inositol hexaphosphate) on its oxygen affinity (24, 25).

The binding of the inorganic anion chloride to hemoglobin results in a decrease in the O₂ affinity of the protein. The relative decrease in the oxygen affinity of all four hybrids in the presence of 500 mM Cl⁻ was lower than that of the unmodified HbA (Table II). Thus, modification of the α-amino group of either chain decreased the influence of chloride. This is consistent with the presence of chloride-binding sites at the NH₂ termini of the α- as well as the β-chains. It may also be seen that the P₅₀ value of αβ₁⁺ε-CH₂⁺ in the presence of 500 mM chloride is close to the P₅₀ value of unmodified Hb (Table II). It is conceivable that reductive alkylation of the α-amino group of the β-chain results in a perturbation of the interplay of the positive charges between the β-chains in the central cavity. This could result in the stabilization of the T-state quaternary conformation of deoxyhemoglobin relative to the R-state conformation of the oxyhemoglobin. The stabilization of the deoxy conformation of hemoglobin would lead to a lower oxygen affinity. Such a mechanism has also been suggested to explain the influence of chloride binding at Lys-82(β), resulting in an increase in the P₅₀ of HbA (25).

Modification of the α-amino groups of the α-chain by reductive hydroxyethylation as well as by dihydroxypropylation decreases the relative influence of chloride on the oxygen affinity of these hybrids. This result is consistent with the observation that the α-amino group of the α-chain is one of the chloride ion-binding sites of the molecule (20, 26, 27).

An interesting aspect of the present studies is the close similarity of the effects on the oxygen affinity of HbA reductively alkylated and carbamylated at the α-amino groups (21). Reductive alkylation at the amino groups has been assumed not to change the net charge on the protein (1, 2), whereas carbamylation of the amino groups results in complete removal of the positive charge on the amino group. The func-
tional properties of the hybrids prepared in the present study can be interpreted to suggest the removal of the positive charge at the α-amino groups upon reductive alkylation. Consistent with this analysis is the observation that HbA modified at its α-amino groups elutes ahead of the unmodified protein on CM52, i.e., these derivatives are acidic. However, the derivatives co-elute with unmodified HbA on DE52. The interpretation of these results would be that reductive alkylation of α-amino groups of HbA lowered the pKα of the α-amino group. This phenomenon appears to be a function of reductive alkylation rather than that of the size of the alkyl groups introduced. The nearly similar influence of hydroxyethylation and dihydroxypropylation on the functional properties of HbA suggests that these changes may be related to a removal of the positive charge of the amino group at the physiological conditions due to the decrease in the pKα of the amino group. Dixon (28) has estimated a pKα of 6.6 for the glucosyl adduct of valyl histidine as compared with about 7.5 for the peptide α-amino group. For an ε-amino group, a comparable reduction in pKα would still leave it largely in the cationic state under physiological conditions. In view of the observation that the chromatographic position of αβ[HE] and αεβ[HE] of (or the dihydroxypropylated derivative) is nearly the same as that of HbA, on CM52 columns, it is conceivable that the pKα of the α-amino groups reductively alkylated with glyceraldehyde or glycolaldehyde is also decreased by about two pH units. If indeed this is the case, at pH 7.4 most of the derivatized Val-1(α) of αεβ[HE]βε and also of αεεβ[HE] should be present as the unprotonated form. The anion-mediated salt bridge between the protonated α-amino group of Val-1(α) and the terminal carboxyl of Arg-141(α) that is normally present in HbA should be absent in these derivatives. This salt bridge has been theorized to contribute to about 25% of the Bohr effect (32). A decrease in the pKα of Val-1(α) to about 6 should completely eliminate the Bohr effect contributed by Val-1(α)-Arg-141(α) interaction under physiological conditions. It may be added here that modification of the carboxyl group of Arg-141(α) by forming a peptide bond with glycine ethyl ester also apparently interferes with the Val-1(α)-Arg-141(α) interaction, and reduces the alkaline Bohr effect of HbA by nearly 30% (33). Precise definition of the pKα of Val-1(α) and Val-1(β) in reductively hydroxyethylated and dihydroxypropylated α and β-chains, and the titration of the Bohr protons at various pH values, will be needed for a more detailed analysis of the influence of a decrease in the pKα of the α-amino groups on the functional properties of HbA. These reductively alkylated derivatives of HbA, which could be prepared very easily, should also prove very useful for a study of CO2 binding to Hb (34), and their influence on O2 affinity under a variety of experimental conditions.

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