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# Effect of Heme and Non-Heme Ligands on Subunit Dissociation of Normal and Carboxypeptidase-digested Hemoglobin

## GEL FILTRATION AND FLASH PHOTOLYSIS STUDIES

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## SUMMARY

**The dissociation of normal and carboxypeptidase-digested human hemoglobin has been studied by gel filtration under several experimental conditions. These include (*a*) different derivatives, notably deoxy-, oxy-, and CO-hemoglobin, (*b*) changes in solvent composition and in pH, and (*c*) addition of inositol hexaphosphate.**

**In normal hemoglobin, in agreement with previous results, the deoxygenated derivative is much less dissociated than the oxy or CO ones. This difference is observed also in some of the digested hemoglobins, but tends to vanish in those proteins in which, as a result of extensive digestion, the conformational change accompanying ligand binding is abolished.**

**The dissociation of normal and digested hemoglobins is affected by solvent composition, is at a minimum at pH near 8, and is decreased by the addition of inositol hexaphosphate.**

**Parallel flash photolysis experiments, performed under conditions identical with those used in the gel filtration studies, indicate that the appearance of quickly reacting material parallels dissociation into dimers in normal hemoglobin.**

**Both in normal and digested hemoglobins conditions which decrease dissociation decrease the fraction of rapidly reacting material.**

**In the digested hemoglobins the fraction of rapidly reacting material may be much higher than can be accounted for by the amount of dimers, indicating in these cases that the tetramers may be rapidly reacting.**

**The data point once again to the critical role of the COOH-terminal residues in maintaining the subunit structure of hemoglobin and the interaction effects associated with it.**

Tetrameric human hemoglobin undergoes reversible dissociation into subunits under conditions where the oxygen-binding capacity is maintained. The dissociation is affected by ligands, pH, solvent composition, and protein concentration (1–3).

The work reported here brings together concentration-dependent features of data from flash photolysis and gel filtration studies with the attempt to correlate side by side the results from the two types of measurements.

Both normal and carboxypeptidase-digested hemoglobins were used in this study in order to gain further insight into the subunit dissociation. Many of the experiments reported here were done under hitherto unexplored conditions; others under previously investigated conditions, with the aim of clarifying still confused aspects of the problem.

The results on the dissociation of normal hemoglobin confirm previous reports obtained by other techniques (2, 4–7). Those on the dissociation of the carboxypeptidase-digested hemoglobins yield quantitative information on the role of the COOH-terminal residues of both the $\alpha$ and $\beta$ chains in the stabilization of the tetramer.

### MATERIALS AND METHODS

Human hemoglobin was prepared by the ammonium sulphate method and freed from organic ions by passage through a mixed bed ion exchange column (8). The digestions with carboxypeptidases A and B were performed as previously described (6, 9). The checks for the homogeneity of the digestion products were performed as described by Bonaventura *et al.* (6, 7). The carboxypeptidase-digested hemoglobins used in this study were (*a*) HbCPA,[1] (*b*) HbCPB, (*c*) (HbCPA)CPB, and (*d*) (HbCPB)CPA. Inositol hexaphosphate solutions were prepared by dissolving the sodium salt of phytic acid (Sigma) in water and neutralizing the solution with concentrated phosphoric acid. Bis-tris buffers were prepared by pH adjustment of 0.1 M *N*,*N*-bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (General Biochemicals) with 1 N HCl.

Gel filtration experiments were performed with the apparatus described by Chiancone *et al.* (10). For most of the experiments a column (50 × 2.0 cm) of Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, California) was used; in a few cases a smaller column

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[1] The abbreviations used are: HbCPA, des $\beta$ 146 His, $\beta$ 145 Tyr; HbCPB, des $\alpha$ 141 Arg; (HbCPA)CPB, des $\alpha$ 141 Arg, $\beta$ 146 His, $\beta$ 145 Tyr, $\beta$ 144 Lys; (HbCPB)CPA, des $\alpha$ 141 Arg, $\alpha$ 140 Tyr, $\beta$ 146 His, $\beta$ 145 Tyr; Hb, hemoglobin; IHP, inositol hexaphosphate; bis-tris, *N*,*N*-bis(2-hydroxyethyl)imino-tris(hydroxymethyl)-methane; $L_{2,4}$, dimer-tetramer association constant expressed in deciliters per g.

(50 × 0.8 cm) was employed. The flow rates were maintained constant at about 20 and 6 ml per hour, respectively, by a peristaltic pump (LKB, 4912 A). All experiments were carried out at 7–10°. In gel filtration experiments with CO derivatives, all the buffers were saturated with CO (resulting in about $10^{-3}$ M CO solutions). Buffer reservoirs were then layered with about 4 cm of paraffin oil. Deoxygenated conditions were maintained by a similar layering after about 0.5% dithionite was added to degassed buffers. The columns were calibrated with sperm whale myoglobin (Seravac Laboratories, Ltd., Maidenhead, England). The limiting value of the elution volume at high protein concentration (>10 mg per ml) was taken as the elution volume of the hemoglobin tetramer ($V_4$). The elution volume of the hemoglobin dimer ($V_2$) was then calculated assuming a linear relationship between elution volume and the logarithm of molecular weight.

Flash photolysis experiments were performed with a modified version of an apparatus previously described (11).

## RESULTS

### *Gel Filtration Studies*

*Effect of Solvent Composition on Different Hemoglobin Derivatives*—The effect of solvent composition was studied at moderate ionic strength (0.05 to 0.1 M) and at a constant pH of 7.0. The buffers chosen were those most commonly used for functional studies: NaCl, inorganic phosphate, and bis-tris. In addition, because of its marked effect on the function of hemoglobin, the role of IHP was analyzed. In Fig. 1 the results obtained with the oxygenated derivative are reported. The extent of dissociation is clearly dependent on the nature of the supporting buffer.

Previous studies have shown that the predominant equilibrium underlying the increase in elution volume at low protein concentrations involves $\alpha_2\beta_2$ tetramers and $\alpha\beta$ dimers (10–13). The curves in Fig. 1 were therefore calculated on the basis of a dimer-tetramer equilibrium. In agreement with the results obtained by Kellett (4) the deoxygenated derivative does not show any increase in elution volume down to 0.005 mg per ml (Fig. 1). Thus, in contrast to the behavior of oxyhemoglobin, the dissociation of deoxy-HbA is not detectable under our experimental conditions. An intermediate case is represented by the CO derivative, where the extent of dissociation in a given buffer is generally less marked than for oxyhemoglobin (see Fig. 5). Experiments with the CO form were performed both in the presence and in the absence of dithionite without noticeable difference.

Estimates of the dimer-tetramer association constants for the oxy, CO, and deoxy derivatives are summarized in Table I. They confirm earlier findings of a marked difference in dissociation between ligand-free and ligand-bound forms (2, 4), but they focus attention on another point, namely the effect of non-heme ligands. This is especially evident in the case of the oxygenated derivative. The most dramatic change in this respect is the association of $HbO_2$ induced by IHP at a concentration of 30 μM. Additional experiments showed that a 10-fold increase in IHP concentration (*i.e.* to 300 μM) did not produce any further significant changes. The effect observed implies finite binding of the organic phosphate to oxyhemoglobin and is consistent with the general idea of stabilization of the tetramer by organic polyphosphates, in view of their stoichiometry of binding (5).

Another series of experiments was carried out in phosphate buffer at pH 7.8. In the case of the oxygenated derivative, the molecule is considerably more associated at pH 7.8 than at pH 7.0 (Table II).

Gel filtration experiments were also performed at higher pH values with CO and deoxyhemoglobin in phosphate (at pH 8.8) and in borate (at pH 9.1) buffers. For both derivatives differences between the leading and trailing boundaries were observed, which became more evident as the protein concentration was

TABLE I

*Effect of solvent composition on dimer-tetramer association constants ($L_{2,4}$) of hemoglobin at pH 7.0*

| Solvent | $L_{2,4}$ | | |
|---|---|---|---|
| | $HbO_2$ | HbCO | Hb |
| | *dl/g* | | |
| NaCl (0.09 M) plus phosphate (0.01 M as $Na^+$) | 300 | 2,000 | $>10^5$ |
| Phosphate (0.1 M) | 1,200 | 6,000 | $>10^5$ |
| Bis-tris (0.05 M) | 1,200 | 3,000 | $>10^5$ |
| Bis-tris (0.05 M) with IHP (30 μM) | 40,000 | | $>10^5$ |

TABLE II

*Effect of pH on dimer-tetramer association constants ($L_{2,4}$) of hemoglobin derivatives in 0.1 M phosphate buffer*

| pH | $L_{2,4}$ | | |
|---|---|---|---|
| | $HbO_2$ | HbCO | Hb |
| | *dl/g* | | |
| 7.0 | 1200 | 6000 | $>10^5$ |
| 7.8 | 4000 | | $>10^5$ |

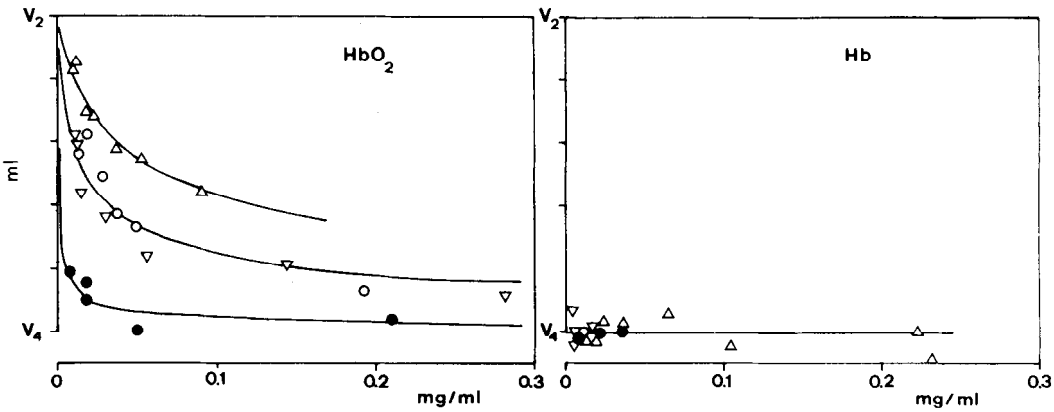


FIG. 1. Relationship between elution volume and protein concentration for oxygenated and deoxygenated human hemoglobin in different solvents. Solvent: △, 0.1 M $Na^+$ (0.09 M $Cl^-$, 0.01 M phosphate); ▽, inorganic phosphate (0.1 M); ○, bis-tris (0.05 M); and ●, bis-tris (0.05 M) with IHP (30 × $10^{-6}$ M). $V_2$ and $V_4$ are the elution volumes for the hemoglobin dimer and tetramer, respectively. ——, theoretical curves which correspond to the dimer-tetramer association constants given in Table I.

lowered. This effect was attributed to adsorption of the protein on the gel, leading to loss of material on the column. Thus, any quantitative analysis was prevented at these higher pH values.

*Effect of Digestion by Carboxypeptidases A and B*—These experiments were designed to elucidate the role of the COOH-terminal residues of the $\alpha$ and $\beta$ chains in the stabilization of the tetramer. For this reason they were all differential experiments, involving layering of the modified protein over HbA at the same total protein concentration. In such experiments the presence of a bump or a dip in optical density at the junction of the two solutions is indicative of a difference in their elution volumes. Furthermore, the area of the dip or bump may be easily correlated with the difference in the association constants of the two solutions (12, 13).

In the experiments with the oxygenated derivatives in 0.05 M bis-tris at pH 7.0, the presence of the dip after digestion with carboxypeptidase A indicates that removal of the COOH-terminal histidine and tyrosine of the $\beta$ chains results in an increase in dissociation. On the other hand, the presence of the bump after digestion with carboxypeptidase B indicates that removal of the COOH-terminal arginine of the $\alpha$ chains enhances association into tetramers. Subsequent digestions of either HbCPA or HbCPB have qualitatively a similar effect: carboxypeptidase A digestion of HbCPB increases its dissociation, while carboxypeptidase B digestion of HbCPA decreases the dissociation of the parent molecule.

Similar differential experiments were performed with the deoxygenated derivatives. As observed with the oxygenated forms, carboxypeptidase A digestion enhances dissociation into dimers; both HbCPA and CPA-digested HbCPB show detectable dissociation at concentrations above 0.01 mg per ml. Digestion of hemoglobin with carboxypeptidase B has no measurable effect since the deoxy form is fully associated in the accessible concentration range in both Hb and HbCPB. Digestion of HbCPA with carboxypeptidase B decreases its dissociation.

To summarize these results, we may say that the effects of carboxypeptidase digestion are qualitatively the same for both liganded and unliganded hemoglobins, and in general removal of His-Tyr from the $\beta$ chains enhances dissociation, while removal of Arg from the $\alpha$ chains decreases the extent of dimer formation.

A quantitative presentation of the differential experiments on HbCPA and HbCPB is given in Fig. 2 where the effect of $30 \times 10^{-6}$ M IHP is also shown. The curves correspond to the association constants summarized in Table III. Both the removal of oxygen and the presence of IHP significantly shift the subunit equilibrium toward tetramer formation.

Additional digestion of either molecule alters the association constant, but what is more important, it completely abolishes the ligand-linked and IHP-linked changes in extent of dissociation (Fig. 3). The curves of Fig. 3 correspond to the association constants given in Table III.

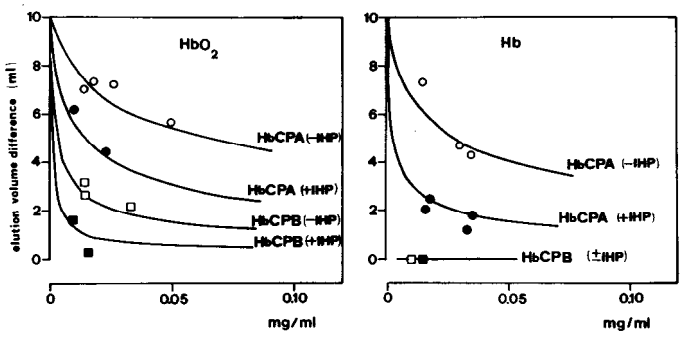


FIG. 2. Difference gel filtration of human hemoglobin *versus* HbCPA and HbCPB in the presence and absence of IHP. Oxygenated derivatives (*left*) and deoxygenated derivatives (*right*) in 0.05 M bis-tris at pH 7.0. The elution volume difference is given in absolute values. HbCPA (○, ●); HbCPB (□, ■). *Filled symbols* refer to experiments done in the presence of 30 $\mu$M IHP. ——, theoretical curves, which correspond to the dimer-tetramer association constants given in Table III.

## *Flash Photolysis Studies*

*Protein Concentration on Dependence of Reaction with CO after Flash Photolysis at pH 7.0 and 20°*—The subunit dissociation of normal hemoglobin results in a protein concentration dependence of some aspects of ligand binding. Certain rapid mixing and flash photolysis experiments have features that are dependent on protein concentration (14, 15). After complete photodissociation of HbCO the process of recombination is biphasic even at neutral pH. The phases correspond to the so-called "quickly" and "slowly" reacting forms (see Ref. 8 for review).

The protein concentration dependence of the fraction of quickly reacting material in 0.1 M phosphate buffer, pH 7 and 20°, is shown in Fig. 4. Within experimental error there is no noticeable effect of: (*a*) preparation of hemoglobin; (*b*) order of addition of the various reagents (Hb, dithionite, and CO); (*c*) CO concentra-

TABLE III

*Dimer-tetramer association constant ($L_{2,4}$) of oxy and deoxy forms of normal and carboxypeptidase-digested hemoglobins in 0.05 M bis-tris at pH 7.0*

| Protein | $L_{2,4}$ | | | |
|---|---|---|---|---|
| | Oxygenated | | Unliganded | |
| | − IHP | + 30 $\mu$M IHP | − IHP | + 30 $\mu$M IHP |
| | *dl/g* | | | |
| HbA | 1,500 | 40,000 | $>10^5$ | $>10^5$ |
| HbCPA | 300 | 1,500 | 700 | 7,000 |
| HbCPB | 7,000 | 40,000 | $>10^5$ | $>10^5$ |
| (HbCPB)CPA | 150 | 150 | 150 | 150 |
| (HbCPA)CPB | 1,500 | 1,500 | 1,500 | 1,500 |

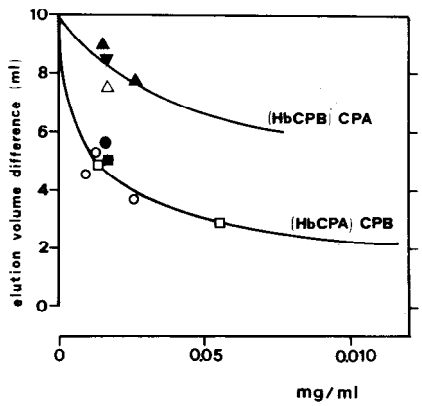


FIG. 3. Difference gel filtration of human hemoglobin *versus* (HbCPA)CPB and (HbCPB)CPA and effect of IHP. (HbCPB)-CPA (△, ▲, ▼); (HbCPA)CPB (○, ●, □, ■). Oxygenated derivatives (△, □) in the absence and (▲, ■) in the presence of 30 $\mu$M IHP. Deoxygenated derivatives (○) in the absence and (●, ▼) in the presence of 30 $\mu$M IHP. All experiments were done in 0.05 M bis-tris at pH 7.0. ——, theoretical curves which correspond to the dimer-tetramer association constants given in Table III.

tion (from 5 to 50 $\mu$M); (*d*) light path of the flash photolysis cell (from 0.1 to 5 cm); or (*e*) flash intensity (100 to 300 J), provided that photodissociation is complete. The percentage of the fast phase does, however, depend on solvent composition. The curves of Fig. 5 best fit the gel filtration data on HbCO (△) and correspond to the dimer-tetramer equilibrium constants given in Table I. A comparison of the percentage of quickly reacting material (○) with the percentage of dimers in the various solvents shows a reasonably good correlation in agreement with previous results (16). The data suggest that the percentage of quickly reacting material may be slightly higher than the percentage of dimers. We note, however, that the small temperature dependence of the dissociation process would account for a slightly higher percentage of dimers at 20° (as in flash experiments) than at 7° (as in the gel filtration studies).

*Effect of pH*—In phosphate buffer, the pH profile for the percentage of the fast phase (at constant protein concentration and wavelength) shows a minimum centered around pH 7.8. Addition of ATP has no effect, in agreement with the competition existing between organic and inorganic phosphates (Fig. 6).

In acetate buffers at pH values below 6, the amount of quickly reacting material is much higher than in phosphate buffer at the same pH and protein concentration. The percentage of quickly reacting material decreases on addition of phosphates or ATP to the solution in acetate buffer. For example, at a hemoglobin concentration of $1.5 \times 10^{-5}$ M and pH 5.4 the amount of quickly reacting material decreases from 55 to 30% when the solution is made $10^{-4}$ M in ATP. This finding is qualitatively in agreement with the notion that acetic acid enhances dissociation of liganded hemoglobin into subunits (17), while ATP stabilizes the tetramer.

*Effect of Temperature*—The temperature dependence of the quickly reacting form was measured over the range 7–35°. The results yield an apparent $\Delta H$ for a tetramer-dimer dissociation process of ~3 Cal per mole. The small temperature dependence of the tetramer-dimer equilibrium estimated by this kinetic parameter is in agreement with the results of molecular weight measurements (10).

*Effect of IHP*—For the carboxypeptidase-digested proteins

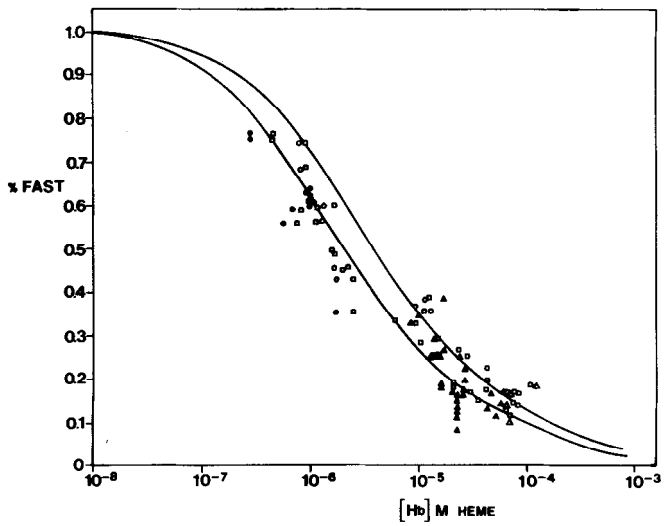


FIG. 4. Percentage of quickly reacting material on complete photodissociation as a function of hemoglobin concentration. Phosphate buffer (0.1 M) at pH 7.0. Light path of flash photolysis cell: △, 0.1 cm; ▲, 0.2 cm; □, 1 cm; ○, 2 cm; ●, 5 cm. The points with 1-cm light path were obtained using several hemoglobin preparations. The curves correspond to dimer-tetramer quilibrium constants of $1 \times 10^6$ M$^{-1}$ and $2 \times 10^6$ M$^{-1}$, respectively.

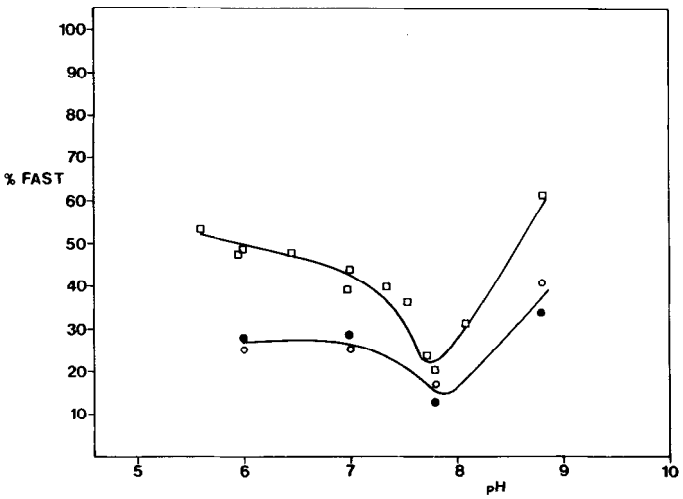


FIG. 6. Percentage of quickly reacting material as a function of pH in 0.1 M phosphate buffer. Hemoglobin concentration: □, $2.7 \times 10^{-6}$ M; ○, ●, $1.5 \times 10^{-5}$ M. ●, obtained after addition of $10^{-4}$ M ATP.

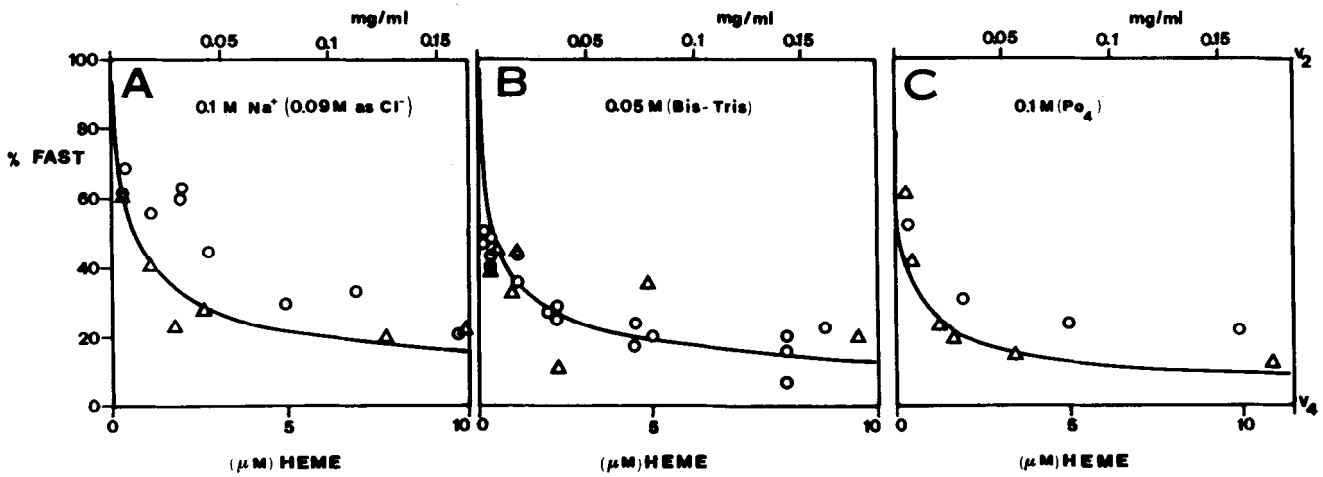


FIG. 5. Comparison of gel filtration (△) and flash photolysis (○) data of human hemoglobin in various solvents at pH 7. *A*, 0.1 M $Na^+$ (0.09 M as $Cl^-$ + phosphate); *B*, 0.05 M bis-tris; *C*, 0.1 M inorganic phosphate. Gel filtration plotted as percentage dimers *versus* protein concentration. Flash data plotted as percentage of the quickly reacting form *versus* protein concentration. ——, theoretical curves which correspond to the dimer tetramer association constants given in Table I.

used in this study the CO combination after flash photolysis is all fast in the absence of IHP, even at concentrations up to 5 μM in heme where the proteins in solution are largely tetrameric (see Figs. 2 and 3). This point is of particular importance since it illustrates a case where the percentage of the fast phase has no correlation with the percentage of dimers. IHP decreases the percentage of the fast phase for HbCPA and HbCPB, but does not affect the CO combination of both (HbCPA)CPB and (HbCPB)CPA. These molecules have a high rate of recombination with CO, independent of IHP and protein concentration (7). As previously mentioned, gel filtration studies indicate that IHP does not affect the dimer-tetramer equilibrium of these molecules. Accordingly, their state of aggregation does not affect their ligand-binding rates.

In view of the marked effect of IHP on the fraction of quickly reacting material in HbCPB, a series of experiments was performed to study the dependence of the phenomenon on IHP concentration. The results show that the fraction of quickly reacting forms at a given IHP concentration depended on the CO concentration. This result implies a decay of material from "quickly" to "slowly" reacting just after photodissociation. Due to the great number of species present in the system, an identification of the relevant process is at present difficult.

## DISCUSSION

The results presented here are pertinent to many long debated questions concerning structure-function relationships in hemoglobin, especially those related to the linkage between ligand binding and subunit interaction.

*Effect of Heme and Non-Heme Ligands on Dissociation of Normal Human Hemoglobin*—Previous results pertaining to subunit dissociation have been discussed in recent reviews, and need not be reconsidered in detail here (3, 8). Experiments with deoxyhemoglobin have shown in the past the largest scatter; the most recent ones, obtained by ultracentrifugation methods, however, indicate that deoxyhemoglobin is very little, if at all, dissociated under conditions which produce extensive dissociation of the ligand-bound derivatives (4). Liganded forms have always shown similar dissociation behavior, although differences between oxy- and CO-hemoglobin have been observed and reported (2).

The gel filtration data presented here show that deoxyhemoglobin does not dissociate appreciably at neutral pH and moderate ionic strength, even at very low concentration. Thus the very large difference in dissociation between oxy- and deoxyhemoglobin demonstrated by ultracentrifuge experiments (4) is confirmed in the present work.

In addition, a significant difference in dissociation between $HbO_2$ and HbCO is also confirmed (2). It is striking that the nature of the buffer affects dissociation of $HbO_2$ and HbCO to a different extent, the difference being larger in sodium chloride and smaller in inorganic phosphate.

*Dissociation of Digested Hemoglobins*—The difference in the tendency to dissociate into subunits between deoxygenated and liganded hemoglobin derivatives reflects the conformational changes associated with ligand binding, especially the so-called quaternary changes, involving the interaction between the various subunits. Thus it would be expected that in cases where the conformational (quaternary) change is abolished, the difference in dissociation between deoxy- and ligand-bound hemoglobin would vanish.

The difference between normal and digested hemoglobins is greater for the deoxy derivative than for the ligand-bound one; this implies that the conformation of the deoxy form has been most severely altered. These results are in agreement with previous conclusions based on information of a different nature, where removal of the COOH-terminal residues was shown to affect primarily the conformation of the protein in the deoxy form (18). In (HbCPA)CPB and (HbCPB)CPA these effects are most evident and even after deoxygenation these molecules remain "frozen" in a state resembling that of the ligand-bound derivative of normal hemoglobin. Also the dissociation of the ligand-bound form is sensitive to changes in the COOH-terminal region (Fig. 2 and Table III). This is of particular interest in the case of HbCPA, since the residues which have been removed (β 146 His and β 145 Tyr) were shown by x-ray crystallography to have considerable freedom of movement in liganded hemoglobin (18, 19).

*Correlation of Dissociation of HbCO with Flash Photolysis Behavior*—Flash photolysis of HbCO at low protein concentration and neutral pH yields ligand-free photoproducts, some of which show a high rate of recombination with CO. The fraction of rapidly reacting material increases with dilution and has therefore been ascribed to subunit dissociation products of HbCO. Attempts to correlate the flash photolysis data with previously available molecular weight data lead to conflicting conclusions. The quickly reacting form was initially identified as hemoglobin chains (15); then it was realized that the amount of monomers, or free chains, present under the condition of the flash experiments was too small to account for the rapidly reacting material. Finally on the basis of better molecular weight data it was concluded that hemoglobin dimers ($\alpha\beta$ dimers) were primarily responsible for the rapid phases of CO combination after photodissociation (16).

The results presented here show fairly good correlation between the fraction of dimers and the fraction of the quickly reacting form of normal human hemoglobin. The gel filtration data indicate that the amount of monomers is very small even at the lowest hemoglobin concentrations.

In contrast, in the carboxypeptidase-digested hemoglobins there is clear evidence that tetramers may also contribute to the fast phase at neutral pH and after complete photodissociation.

*Correlation of Dissociation Data with X-Ray Data*—The results on subunit interactions presented here point out once again the important role that the COOH-terminal residues play in the structural and functional properties of hemoglobin. The conformational state of the deoxy form is altered on removal of COOH-terminal residues in agreement with the x-ray studies which show that these residues are important in stabilizing the normal deoxy structure (18, 19).

The large effect of IHP on the dissociation behavior and on the reactivity of normal and digested hemoglobins can also be interpreted, in structural terms, by the stabilizing effect of the polyanion on the conformation corresponding to normal deoxyhemoglobin. Similar effects of IHP, recently reported in the case of Hb Bethesda (β 145 Tyr → His), provide additional evidence that the COOH-terminal residues and polyphosphates help stabilize the deoxy structure (20).

According to Perutz (18, 19), the stabilization of normal deoxyhemoglobin occurs through the formation of six salt bridges. When the COOH-terminal histidine and tyrosine residues of the β chains are removed by carboxypeptidase A digestion it is no longer possible to form some of the salt bridges that normally stabilize the deoxy conformation. After digestion with carboxypeptidase B, the salt bridges normally formed in deoxyhemoglobin by the COOH-terminal arginine of the α chains are replaced by salt bridges formed by the penultimate tyrosyl residues and the

$\alpha$ chains move closer together (19). This perhaps explains the greater stability of oxy-HbCPB relative to oxy-HbA. In the sequentially digested hemoglobins (HbCPA)CPB is a more stable tetramer than (HbCPB)CPA, presumably due to the influence of the penultimate tyrosine which is absent in the latter one. Both doubly digested proteins, however, have lost ligand- and IHP-linked changes in extent of dissociation and ligand binding. Since the functional properties of both HbCPA and HbCPB are ligand- and IHP-linked, the results imply that the additional loss of residues following the second digestion ($\beta$ 144 Lys and $\alpha$ 140 Tyr) are of considerable importance in the stereochemistry of the hemoglobin molecule.

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