A binding method that detects only the strongest binding site for a ligand on a protein has been used to show that folates and folate analogs, conjugated with poly-γ-glutamates, are bound to hemoglobin. When the concentration of hemoglobin is much larger than that of the polyglutamate, as is the case in the red cell, the fraction bound is a direct function of the hemoglobin concentration and is independent of the total polyglutamate concentration. Binding to deoxyhemoglobin tetramers is competitive with 2,3-diphosphoglycerate. In oxyhemoglobin the folyl and methotrexate polyglutamates are bound preferentially by free αβ dimers, but removal of the pteridine moiety leads to tetramer binding even in oxyhemoglobin. Changes in the length of the polyglutamate side chain and alterations of the pteridine structure such as reduction and/or methylation have a much larger effect on the constant for binding to deoxyhemoglobin tetramers than on that for oxyhemoglobin dimers. The implications of these results for the storage of pteroylpolyglutamates in the erythrocyte and their release from the red cell under the influence of the degree of oxygenation and variations in the 2,3-diphosphoglycerate level are discussed.

Folic acid as well as analogs such as aminopterin or methotrexate, used in cancer chemotherapy, are conjugated in vivo with glutamic acid to form poly-γ-glutamates of varying chain length (Baugh et al., 1976; Galivan, 1979; Covey, 1980; Kisliuk, 1981; McGuire and Bertino, 1981; Jolivet et al., 1982). These highly charged compounds are, of course, bound electrostatically in a nonspecific fashion to many proteins (Zamierskowski and Wagner, 1974; Waxman et al., 1977; Holm et al., 1980; Wagner, 1982) including hemoglobin (Hansen et al., 1981). However, we have shown previously (Benesch et al., 1983) that a more specific binding of pteroylheptaglutamate (PteGlu7) to hemoglobin can be demonstrated under conditions where the concentration of the folate is of the same order as that in the red cell, i.e. about 1 μM, and hemoglobin is in large excess. In this way nonspecific binding is eliminated and only the strongest binding site is occupied. The binding constant is then equal to the hemoglobin concentration at which half the total folate is bound. In the case of deoxy-Hb it was found that 1 mol of PteGlu7 is bound per tetramer over a 10-fold range of hemoglobin concentration. This was not so with oxy-Hb where it was shown that free αβ dimers instead of tetramers are responsible for the binding of PteGlu7 (Benesch et al., 1983).

In this paper a variety of folyl- and antifolylpolyglutamates has been investigated to determine the effect of structural alterations on the binding energy. These include variations in the length of the polyglutamate side chain, as well as reduction, substitution, and removal of the pteridine portion of the molecule. It will also be shown that 2,3-diphosphoglycerate, the main intraerythrocytic regulator of oxygen transport, inhibits polyglutamate binding to deoxy-Hb competitively. The conclusions from these experiments agree well with the binding site for PteGlu7 on deoxy-Hb that has been identified crystallographically.

The methods used will illustrate a general strategy for treating the binding of a small molecule in very low concentration to a protein present in large excess. This is, of course, particularly relevant for those biological situations where proteins occur in high molar concentration, such as hemoglobin in the red cell (~5 mM) or serum albumin in plasma (~0.5 mM).

MATERIALS AND METHODS

Hemolysates of normal human blood were prepared as described previously (Benesch et al., 1972). The major hemoglobin component (HbA) was isolated by chromatography on DEAE-Sephrose-Fast Flow (Pharmacia) at 4 °C using a linear gradient of 0.05 M Tris buffer, pH 8.5-7.5. Hb Kansas (pH7-8.5) was isolated essentially as described by Bonaventura and Riggs (1968) except that the gradient was flattened by using 4 liters instead of 1 liter of each buffer. After 40 h a good separation between Hb Kansas and HbA was obtained. Hemolysates and purified hemoglobin were stored in liquid N2. Hemoglobin concentration was measured spectrophotometrically at 540 nm after conversion to methemoglobin cyanide (ε = 4.4 × 10^5 M^-1 cm^-1).

Pteroyl tetra-, penta-, and heptaglutamate were prepared by the method of Krumdieck and Baugh (1980) and the concentration of the solutions was determined spectrophotometrically at 282 nm (ε = 2.7 × 10^4 M^-1 cm^-1). The corresponding ^14C-labeled compounds, i.e. PteGlu7 ^14C, PteGlu6 ^14C, and PteGlu5 ^14C, had a specific activity of about 1 μCi/μmol.

[^1]°C]-5-CH3H4PteGlu7 and [^1]°C]-5-CH3H4PteGlu6 were prepared from the corresponding unlabeled pteroylpolyglutamates as described by Matthews et al. (1982) using °C-formaldehyde (2 mCi/mmol from New England Nuclear).

[^2]p-Aminobenzoylheptaglutamate (p-AB-Glu7) and methotrexatehexaglutamate (MTX-Glu6) which also contains a total of 7 glutamate residues) were synthesized as described before (Nair and Baugh, 1973).

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The abbreviations used are: PteGlu7, pteroylolo-gamma-glutamate; DPG, 2,3-diphosphoglycerate; p-AB-Glu7, p-amino benzoylheptaglutamate; methotrexate, 4-NH2-10-CH2-pteroylglutamate; MTX-Glu6, 4-NH2-10-CH2-pteroylgluta-gammat, bistris, 2-[bis(2-hydroxylethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

Assay Methods—The very low concentrations of the polyglutamate conjugates which were essential for these measurements required...
sensitive assays, accurate to about 0.01 nmol.

All the 14C-labeled compounds were assayed in 5 ml of Aquasol-2 in a Packard Tri-Carb Liquid Scintillation Counter.

$p$-AB-Glu$_7$ was determined fluorimetrically after reaction with fluorescamine (Furness and Loewen, 1981). 0.2 ml of the sample was mixed with 0.1 ml of freshly prepared fluorescamine (10 mg/50 ml of acetone). After 5 min at room temperature, 1.5 ml of H$_2$O was added and the fluorescence was read at 500 nm using an excitation wavelength of 395 nm.

The assay of methotrexate or MTX-Glu was based on the stoichiometric inhibition of dihydrofolate reductase as described by Peterson et al. (1975). Various amounts of methotrexate were incubated at 37 °C for 5 min with 0.01 unit/ml of bovine liver dihydrofolate reductase (Sigma) and 100 μM NADPH (Sigma) in 0.6 M KCl and 0.6 M sodium acetate buffer, pH 6.0. The enzyme reaction was started by the addition of 3.3 mM dihydrofolate (Sigma)/2 ml. The decrease in the initial reaction rate was linear with methotrexate or MTX-Glu concentration from 5 to 25 pmol/ml.

DPG concentrations >10$^{-4}$ M were determined by phosphate analysis as described by Ames and Dubin (1960). For the low concentration range (down to 2 × 10$^{-8}$ M) the assay was based on the catalytic effect of DPG on the reaction: 2-phosphoglycerate → 3-phosphoglycerate, using a modification of the method of Towne et al. (1957). The reaction mixtures at 30 °C contained the sample, 40 units of enolase (Sigma), 1.6 μmol of 3-phosphoglyceric acid (Sigma) and 20 μmol of MgSO$_4$ in 5 ml of 0.07 M Tris buffer, pH 7.3. The reaction was started by the addition of 1.7 units of phosphoglycerate mutase (Sigma). The increase in absorbance at 240 nm was linear for the first 3 min and proportional to the DPG concentration from 2 to 8 × 10$^{-8}$ M.

**Binding Measurements**—All binding studies were carried out by ultrafiltration as previously described (Benesch et al., 1963) at room temperature with varying hemoglobin concentrations, 0.05 M bistris buffer, pH 7.3, was used throughout, except for the DPG binding experiments, which were conducted in 0.05 M Tris at the same pH, since Tris has no significant absorbance at 240 nm.

**Hemoglobin-Oxygen Equilibria**—The effect of PteGlu$_7$ on the oxygen equilibrium curve of hemoglobin was measured at 20 °C in 0.05 M bistris buffer, pH 7.3, 0.1 M CI$^-$, 0.1 mM EDTA, using previously described methods (Benesch et al., 1965, 1973). The concentration of the polyglutamate was kept constant at 2.5 × 10$^{-4}$ M and that of the hemoglobin was varied from 1.6 × 10$^{-4}$ M to 5 × 10$^{-3}$ M. For the lowest hemoglobin concentration the absorbances at 415, 420, and 430 nm were corrected for the absorbance of PteGlu$_7$.

### RESULTS

The binding of small molecules to proteins is usually carried out by titrating the protein with increasing amounts of the ligand and measuring the moles bound per mole of protein as a function of the free ligand concentration. Binding constants for a site or group of sites can then be obtained, provided the moles bound per mole of protein approach a plateau value (Klotz, 1982). We found earlier that with an excess of pteroylglutamates over hemoglobin no plateau is reached (Benesch et al., 1982). For this reason (as well as the sake of biological reality) we have reversed the usual concentration ratio of protein to ligand, using a large excess of hemoglobin. Under these conditions only the tightest binding site will be occupied and its binding constant is given by:

$$K = \frac{[\text{PteGlu$_7$}][\text{Hb}]}{[\text{Hb-PteGlu$_7$}]}$$

which reduces to

$$K = \frac{[\text{free PteGlu$_7$}]}{[\text{bound PteGlu$_7$}]} \times [\text{Hb}] \text{ total},$$

since [Hb-PteGlu$_7$] is negligible compared to the total hemoglobin concentration.

This formulation was first used successfully to demonstrate specific binding of PteGlu$_7$ to deoxy-Hb tetramers and oxy-Hb dimers (Benesch et al., 1983). The validity of Equation 2 is confirmed by two predictions: 1) The ratio of bound to free ligand should be a function only of the hemoglobin concentration and independent of the total ligand concentration when hemoglobin is in large excess. This is borne out by the data in Table I. 2) If a ligand binds with a 1:1 stoichiometry even when it is present in excess over the protein, as DPG does to deoxy-Hb (Benesch et al., 1968), then the two ways of defining and measuring the binding constant should be equivalent, i.e. the molar hemoglobin concentration at which half the total DPG is bound should be numerically equal to the free DPG concentration at which 0.5 mol is bound per mole of hemoglobin. The binding curve of DPG to deoxy-Hb was therefore measured (a) using 10$^{-4}$ M hemoglobin and varying the DPG concentrations from 3 × 10$^{-8}$ to 2 × 10$^{-4}$ M and (b) at a constant DPG concentration of 10$^{-4}$ M and hemoglobin concentrations from 7 × 10$^{-6}$ to 1.2 × 10$^{-4}$ M. Fig. 1 shows that the two curves are indeed identical.

### The Affinity of Different Polyglutamates for Hemoglobin

**Binding to Deoxyhemoglobin**—The effect of chain length (and therefore the number of negative charges) illustrates the contribution of electrostatic interactions to the binding energy (Table II, column 1). It is clear that the strength of binding to deoxy-Hb increases with the number of glutamates and that the effect of two additional ones is about the same, i.e. 2-fold, both for the pteroyl and the 5-methyltetrahydropteroyl compounds.

### Table I

**Effect of polyglutamate concentration on binding to hemoglobin**

| 5-CH$_3$H$_2$PteGlu$_7$ concentration | Total | Free | Bound | $K \times 10^6$ |
|--------------------------------------|-------|------|-------|---------------|
| Total                               |       |      |       |               |
| 0.40                                | 0.108 | 0.292| 73    | 5.6           |
| 1.10                                | 0.330 | 0.770| 70    | 6.5           |
| 2.20                                | 0.616 | 1.584| 72    | 5.9           |
| MTX-Glu$_7$ concentration            |       |      |       |               |
| Total                               |       |      |       |               |
| 0.40                                | 0.196 | 0.204| 51    | 15            |
| 1.10                                | 0.550 | 0.550| 50    | 15            |
| 2.20                                | 0.990 | 1.210| 55    | 12            |

![Fig. 1. Binding of DPG to deoxy-Hb. All measurements are in 0.05 M Tris buffer, pH 7.3, 0.1 M Cl$^-$ at 23 °C. Open symbols (left and bottom coordinates), Hb concentration constant at 1 × 10$^{-4}$ M with varying DPG concentrations; filled symbols (right and top coordinates), DPG concentration constant at 1 × 10$^{-3}$ M with varying Hb concentrations.](image-url)
were varied from 0.2 to 10 g/100 ml. Their values were constant for all the compounds between 15 and 85% binding.

The binding curves of four different heptaglutamates are shown in the form of Hill plots in Fig. 2. It is noteworthy that they are all parallel with a slope of one, as required by Equation 2. Surprisingly, the introduction of a methyl group at N₇ and especially at N₁₀ weakens the binding more than the removal of the entire pteridine moiety. The 4-amino group of methotrexate is unprotonated at neutral pH (Poe, 1977) and is therefore unlikely to contribute significantly to the weaker binding of this compound.

The effect of DPG on polyglutamate binding by deoxy-Hb was investigated with molar ratios of DPG to hemoglobin close to unity as is the case in the red cell. As before, the concentration of polyglutamates was 1 × 10⁻⁷ M, so that both DPG and hemoglobin were in great excess. From the data in Table III it is clear that the fraction of polyglutamate bound is decreased progressively by increasing the DPG concentration at each hemoglobin concentration. Furthermore, if DPG and the polyglutamates compete for the same site on the protein, then only those hemoglobin molecules which are not complexed with DPG should be "active" in binding polyglutamates. The calculated values in Table III were therefore derived by replacing the total hemoglobin concentration in Equation 2 by the free hemoglobin concentration. This was calculated from the known DPG binding constant (Benesch et al., 1968) (Fig. 1) at each total hemoglobin and total DPG concentration. In view of the agreement between the values calculated in this way and the experimentally determined fraction of polyglutamate bound (Table III), it can be concluded that these polyglutamates and DPG do indeed compete for the same binding site on deoxy-Hb.

Binding to Oxyhemoglobin—We have shown previously (Benesch et al., 1963) that the binding of PteGlu₇ to oxy-Hb cannot be described by Equation 2. However, when the concentration of aβ dimers [D] is substituted for the total hemoglobin concentration, i.e.

\[ K_D = \frac{\text{free PteGlu}_7}{\text{bound PteGlu}_7} \times [D] \]  

constant values of \( K_D \) are obtained over the whole range of total hemoglobin concentrations. This demonstrates that the strongest binding site for PteGlu₇ on oxy-Hb is located on the aβ dimer and not on the intact tetramer. An early example of a similar case was described by Metzger et al. (1967), who showed that glycogen cannot bind to the tetramer form of phosphorylase a and that the dimer-glycogen complex is therefore the active form of this enzyme.

Since the tetramer/dimer dissociation constant of oxy-Hb is only about 10⁻⁷ M (Edelstein et al., 1970; Chu and Ackers, 1980), the concentration of dimers in oxy-Hb is much less than that of tetramers in deoxy-Hb at the protein concentrations used for this work. Therefore, the fraction of polyglutamate bound is always less for oxy-Hb than for deoxy-Hb (Table II). It is also evident that the effect of the polyglutamate chain length on the binding constant is noticeably smaller in the case of the oxy-Hb dimers (Table II).

The selective affinity for oxy-Hb dimers was observed with all the polyglutamates included in this study with one notable exception. This is shown in Fig. 3 where the Hill plots based on Equation 3 are linear with a slope of one except for the p-aminobenzoylheptaglutamate. This compound evidently binds to tetramers even in oxy-Hb, since the binding curve fits Equation 2 rather than 3 (Fig. 3).

Just as is the case for the glutamate chain length, methylation of the pteridine ring and its state of oxidation has much less effect on the affinity for oxy-Hb dimers than for deoxy-Hb tetramers. The numerical values of the binding constants of the four heptaglutamates to oxy- and deoxy-Hb are listed in Table IV.

The binding of polyhepta-glutamate to oxy-Hb dimers is borne out by two other lines of evidence.

(a) In Hb Kansas the mutation (\( ^{8(928)Asp-738} \)) gives rise to a
FIG. 3. Binding of different heptaglutamates to oxy-Hb. The conditions and symbols are the same as in Fig. 2. $[D]$ is the concentration of $\alpha_2\beta_2$ dimers calculated from the tetramer/dimer dissociation constant $K = [D]^4/[T] = 1.5 \times 10^{-8}$ M (Edelstein et al., 1970). The squares with the dashed line are the $p$-AB-Glu$_i$ data plotted against the total Hb concentration instead of the dimer concentration.

TABLE IV

Binding constants of heptaglutamates to hemoglobin

|          | Deoxyhemoglobin $K_T$ | Oxyhemoglobin $K_T$ |
|----------|-----------------------|---------------------|
| PteGlu$_7$ | $2.5 \times 10^{-5}$   | $2.5 \times 10^{-5}$ |
| 5-CH$_3$H$_2$PteGlu$_7$ | $6.5 \times 10^{-3}$   | $2.8 \times 10^{-9}$  |
| MTX-Glu$_6$ | $14 \times 10^{-5}$   | $1.8 \times 10^{-3}$  |
| $p$-AB-Glu$_7$ | $4.3 \times 10^{-5}$   | $5.8 \times 10^{-5}$  |

FIG. 4. Binding of PteGlu$_7$ by Hb Kansas. Open symbols, CO Hb Kansas; filled symbols, CO HbA. The PteGlu$_7$ concentration was kept constant at $1 \times 10^{-5}$ M in 0.05 M bistris buffer, pH 7.3, 0.1 M Cl$^-$. Inset, the dimer concentration for Hb Kansas was calculated from the dissociation constant $K_{Hb\text{Kansas}} = [D]^4/[T] = 5.4 \times 10^{-8}$ M (Atha et al., 1979; Pettigrew et al., 1982).

low oxygen affinity and a 50–100-fold increase in the dissociation of the liganded form into $\alpha_2\beta_2$ dimers (Atha and Riggs, 1976). Fig. 4 shows that COHb Kansas does indeed bind more PteGlu$_7$ than HbA over the whole range of hemoglobin concentrations. The binding curve of the mutant hemoglobin fits Equation 3 very well (Fig. 4, inset) using the published value for the tetramer-dimer dissociation constant $(K_{d2} = 5.4 \times 10^{-8}$ M). The resulting binding constant of Hb Kansas dimers for PteGlu$_7$ is $6.6 \times 10^{-5}$ M compared to $2.5 \times 10^{-5}$ M for HbA. Evidently the substitution which loosens the contact between the $\alpha_2\beta_2$ dimers also reduces the affinity of PteGlu$_7$ for these dimers.

(b) Another consequence of folylpolyglutamate binding to oxy-Hb dimers is that the allosteric shift in the oxygen affinity should vary with the hemoglobin concentration. In the case of PteGlu$_7$ the affinities for deoxy-Hb tetramers and oxy-Hb dimers happen to be equal (Table IV). Therefore $P_{50}$ should be unaffected by this polyglutamate at a hemoglobin concentration at which the molar concentrations of oxy-Hb dimers and deoxy-Hb tetramers are equal, i.e. $7.5 \times 10^{-7}$ M or 0.005 g/100 ml hemoglobin. Above this concentration the $P_{50}$—in the presence of a constant concentration of PteGlu$_7$—should increase progressively with increasing hemoglobin concentration since the proportion of oxy-Hb dimers decreases with the total hemoglobin concentration. The results in Fig. 5 confirm the expectation that, in contrast to other compounds like DPG (Benesch et al., 1969), the allosteric effect of PteGlu$_7$ increases with increasing hemoglobin concentration.

DISCUSSION

The method developed here is unusual because binding is measured with a large excess of protein over ligand. The binding constant is then defined as the protein concentration for half-saturation of the small molecule. This has several notable advantages: 1) It permits the recognition of the tightest binding site even in the case of weakly bound ligands. 2) In a dissociating protein system the affinity of the ligand for either the monomer or the polymer can be measured easily without disturbing the equilibrium. 3) The simplicity of the equation under these conditions avoids the ambiguities associated with measurements of multiple binding sites in the presence of excess ligand (Klotz, 1982). Therefore the association constants for the tightest binding site are easily determined.

As far as the location of this site on deoxy-Hb is concerned,
the demonstration that DPG competes with the polyglutamates suggests that these compounds are bound at the same site, i.e., at the entrance to the central cavity between the $\beta$ chains (Arnone, 1972). Crystallographic evidence obtained by Arthur Arnone$^2$ has shown that the pteroyl group of PteGl$_7$ is buried deep inside the central cavity of deoxy-Hb where it interacts with phenylalanine $\alpha$36. The glutamate residues can be seen at the entrance to the central cavity near the basic residues of the DPG binding site, although, of course, they give rise to a much weaker positive density in the difference map. This evidence accounts very well for the binding of all the pteroylpolyglutamates to the tetramer in deoxy-Hb but not in oxy-Hb where the entrance to the central cavity is considerably smaller (Perutz, 1965). It also explains why the $p$-aminobenzoylheptaglutamate, which lacks the bulky pteridine residue, is the only one of these compounds which is able to penetrate the oxy-Hb tetramer (Fig. 3).

The oxy-Hb dimer binding site for pteroylpolyglutamates is obviously inaccessible in the undissociated tetramer. It is therefore likely that it involves the same region by which these dimers are linked within the tetramer. This is reminiscent of the interaction between oxy-Hb dimers and haptoglobin (Benesch et al., 1976; Wejman et al., 1984). Other ligands have been shown to have an affinity for oxy-Hb dimers, for example Zn$^{3+}$ (Gray, 1980; Gray and Dean, 1982) and inositol hexaphosphate (Hensley et al., 1975; White, 1976) and even for deoxy-Hb dimers (Weidner and Olson, 1975).

Erythrocytes contain relatively high concentrations of folylpoly- $\gamma$-glutamates but their function there is not known. The major forms are 5-CH$_3$H$_4$ folates with 4-7 glutamate residues (Shin et al., 1974; Perry and Chanarin, 1977). The chemotherapeutic folate analog, methotrexate, is similarly conjugated with glutamic acid, and accumulates in the red cell in high concentrations (daCosta and Iqbal, 1981; Kamen et al., 1981, 1984).

In view of the results reported here, hemoglobin binding probably plays an important role in storing folates and antifolates in the red cell. By analogy with results on other cells (Rosenblatt et al., 1978; Balinska et al., 1981; Galivan and Balinska, 1983; Poser and Sirotzak, 1983), free pteroylpolyglutamates are likely to pass through red cell membranes, albeit more slowly than the monoglutamate forms. The rate of efflux would, of course, only be proportional to the concentration of polyglutamates not bound to hemoglobin, and it is therefore very relevant that Cooper and Peyman (1982) found a significant efflux of intact pteroylpentaglutamate from red cells with a low hemoglobin concentration. Since we have also shown that the proportion bound is directly influenced by DPG concentration (Table III) and oxygen pressure (Table IV), these two variables would participate in regulating the rate of efflux from the erythrocyte.

Using the dissociation constants in Table IV, the fraction of free 5-CH$_3$H$_4$PteGlu$_7$ or MTX-Glu$_6$ can be calculated at red cell hemoglobin concentrations and various oxygen pressures and DPG levels. Thus, at a red cell hemoglobin concentration of about 5 mmol and a normal venous $p$O$_2$ of 40 mm where hemoglobin is about 75% oxygenated and with normal DPG levels of about 0.8 mol of DPG/mol of hemoglobin, only 27% of the total 5-CH$_3$H$_4$PteGlu$_7$ and 17% of the MTX-Glu$_6$ would not be bound to hemoglobin. Under anoxic conditions, however, where the molar DPG/Hb ratio rises well above 1, much greater amounts of the polyglutamates will be released.

In conclusion, it seems probable that the binding of folates and antifolates may be just one example of a hitherto neglected role of hemoglobin. Other biologically important molecules such as vitamins, coenzymes, and peptides, as well as some drugs (Perutz and Poyart, 1983), which occur in the red cell in minute molar concentrations compared to that of hemoglobin might be similarly bound. Thus, hemoglobin could act as a storage reservoir from which these compounds are released under the influence of such regulatory factors as oxygen and DPG. In the erythrocyte the enormous hemoglobin concentration (about 30 g/100 ml) would lead to significant binding even of substances which have a relatively low affinity for the protein.

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