Quantitative Determination of Carbamino Adducts of $\alpha$ and $\beta$ Chains in Human Adult Hemoglobin in Presence and Absence of Carbon Monoxide and 2,3-Diphosphoglycerate*

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The principal component of normal adult human hemoglobin was equilibrated under various conditions with $^{13}$CO$_2$. Quantitative analysis of the carbamino resonance intensities over the pH range of 6.5 to 9.0 shows that the effects of conversion from the deoxy to the liganded state in reducing the carbamino adduct formation occur predominantly at Val-$\beta$. Analysis of the pH dependence of carbamino formation at constant total carbonates yields values of $pK_a$ and $pK_b$ for Val-$\alpha$ and Val-$\beta$ in the deoxy and liganded conditions. In contrast to the Val-$\alpha$ role as the allosteric site for CO, the Val-$\beta$ site is shown to be primarily an alkaline Bohr group. 2,3-Diphosphoglycerate is shown to reduce substantially the Val-$\beta$ carbamino resonance intensity in deoxyhemoglobin. Evidence for 2,3-diphosphoglycerate effects in carbon monooxide hemoglobin at both Val-$\alpha$ and Val-$\beta$ sites is presented. Enhanced carbamino formation in carbon monooxide hemoglobin at Val-$\beta$ is observed at pH values less than 7.8. Finally, chemical exchange analysis of the spectra shows the release rate of the deoxy Val-$\alpha$ carbamino adduct to be greater than that for deoxy Val-$\beta$. At pH 7.47, $k_{\text{obs, Val-$\alpha$}}$ is $\approx 1.0$ and $k_{\text{obs, Val-$\beta$}}$ is $\approx 11.0$ s$^{-1}$.

The identification in human deoxyhemoglobin of Val-$\beta$ as the dominant site of formation of carbamino adduct has been achieved by several methods (2–5). The present report extends the range of observation to yield quantitative estimates of CO$_2$ binding parameters for both subunits in liganded and unliganded states and in the presence and absence of the effector 2,3-diphosphoglycerate. Observations are also made concerning the rates of release of the carbamino CO$_2$ from the individual subunits.

The pH dependence of carbamino formation, provided the total carbonates vary little, is generally constrained to a bell-shaped form dictated by the equilibria

\[
R-NH_2 + \begin{array}{c} K_a \rightarrow \end{array} R-NH_3^+ + H^+ 
\]

\[
R-NH_3^+ + CO_2 \rightarrow \begin{array}{c} K_b \rightarrow \end{array} R-NHCOO^- + H^+ 
\]

\[
CO_2 + H_2O \rightarrow \begin{array}{c} K_c \rightarrow \end{array} HCO_3^- + H^+ 
\]

Here, $K_a$ is the dissociation constant of the amino group in question and $K_b$ is the formation constant of the carbamino adduct, expressed so as to include the step of dissociation of the relatively strong carbamic acid (6). At low pH, the concentration of the nonprotonated amino form will tend to be limiting, and at high pH, the concentration of dissolved CO$_2$ will tend to be limiting.

The assignments of carbamino resonances are again based principally on the observation of changes resulting from specific blockage of amino groups by modification with cyanate (5, 7–9). By these means it is possible to show a stabilization of the Val-$\alpha$ adduct in the liganded form at pH values near 7.8 and below. Evidence is also obtained showing that 2,3-diphosphoglycerate affects carbamino adducts in both the liganded and unliganded states.

**EXPERIMENTAL PROCEDURES**

**Normal Adult Hemoglobin**—Hemoglobin A, was prepared by DEAE-Sephadex chromatography following the procedure of Huisman and co-workers (10, 11) or that of Williams and Tsy (12). Other procedures such as removal of phosphates and paramagnetic ions, reduction of ferric forms when necessary, and equilibration with $^{13}$C-enriched CO$_2$ and bicarbonate were done as previously described (5, 13).

**Carbamylated Derivatives of Hemoglobin**—The method of Williams et al. (9) was applied for the separation of specifically carbamylated derivatives of hemoglobin A,$\alpha$ with modifications and analyses made as previously described (5). The presence of the carbamyl form of the NH$_2$-terminal residue in a given subunit is designated by a superscript, e.g. $\alpha^\gamma\beta$, to indicate the form blocked on the $\alpha$ subunit.

**Determination of 2,3-Diphosphoglycerate**—The concentration of 2,3-diphosphoglycerate as the sodium salt, derived by ion exchange chromatography from the pentacyclohexylaminium salt (Calbi ochim), was determined by an adaptation of the method of Lowry et al. (14).

**NMR Measurements**—NMR measurements were made as previously described (5) with the following changes. All spectra were accumulated in 16,384 and 8,192 memories giving digital resolution of 0.03 or 0.06 ppm. In several cases a high field instrument operating at 67.899 MHz was used (1, 15). For this instrument a recycle time of 5.5 s was used. Samples of the carbamyl hemoglobins were prepared with an additional internal reference compound, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate. Two $^{13}$C resonances in...
this compound with acid limits at 137.49 and 143.63 ppm, respectively, titrated with the expected pK value of 7.54 (16) and thus provided a direct measure of the pH of the sample.

Data Handling—The mole fraction of carbamino adduct of a given amino group is related to the concentrations of free CO₂ and protons by (15, 17):

$$Z = k_{e}C_{e}^{b} + (C_{1}^{d} + C_{2}^{d}) k_{e}^{(+)} + k_{e}^{(-)}$$

In our experiments the total carbonates rather than free CO₂ are measured. Thus Equation 4 was recast in terms of concentrations of total carbonates (TC) and total amine (TA) and is shown in Equation 5.

$$Z = \frac{b}{b} \left( b - 4 \frac{(TC)}{(TA)} + \frac{(TC)}{(TA)} \right)$$

where

$$b = \frac{K_{1}^{d} K_{2}^{d} K_{C}^{d} + (H^{+}) (K_{1}^{d} K_{C}^{d} + K_{1}^{d} + K_{2}^{d} + K_{2}^{d}) + (H^{+})^{2} (K_{1}^{d} + K_{2}^{d}) + (H^{+})^{3}}{K_{C}^{d}}$$

(5)

Here \(K_{1}^{d}\) and \(K_{2}^{d}\) are the ionization constants for CO₂ and bicarbonate ion (18, 19). \(K_{1}^{d}\) and \(K_{2}^{d}\) are evaluated at each experimental ionic strength by use of the Davies equation (20). Typical values of \(pK_{1}\) and \(pK_{2}\) obtained by this method are 6.20 and 9.82, respectively, for the conditions usually in the majority of the experiments.

Experimental values of \(Z\) are obtained from undecoupled NMR spectra by the following relation:

$$Z = \frac{I_{obs} - 329.1}{329.1 - \lambda_{CO_{2}}}$$

Here \(I_{obs}\) and \(I_{\lambda}\) are the integrated intensities, respectively, of the carbamino resonance and of the envelope of resonances due to all 328 natural abundance carbonyl carbon atoms per a/¿ dimer. \(\lambda_{CO_{2}}\) is the measured mole fraction of \(^{13}C\) in the enriched carbonates and the factor 1.1 represents the natural abundance of \(^{13}C\), both expressed in per cent.

Determination of \(K_{C}\) and \(K_{1}\) from measured values of \(Z\) is carried out by a least squares program (21) which fits Equation 5 for values of \(Z\) measured at constant total carbonates. The sensitivity of the

$$\frac{d\theta_{\alpha \beta}}{dt} + \Delta \omega_{\alpha \beta} M_{\alpha \beta} + k_{obs,\alpha \beta} M_{\alpha \beta} - k_{obs,\alpha \beta} M_{CO_{2}} = \frac{i (f_{\gamma} N H_{2}CO_{2})}{M_{\alpha \beta}}$$

$$\frac{d\theta_{\alpha \beta}}{dt} + \Delta \omega_{\alpha \beta} M_{\alpha \beta} + k_{obs,\alpha \beta} M_{\alpha \beta} - k_{obs,\alpha \beta} M_{CO_{2}} = \frac{i (f_{\gamma} N H_{2}CO_{2})}{M_{\alpha \beta}}$$

$$\frac{d\theta_{CO_{2}}}{dt} + \Delta \omega_{CO_{2}} M_{CO_{2}} + (k_{obs,CO_{2}} M_{CO_{2}} - k_{obs,CO_{2}} M_{\alpha \beta \gamma}) = \frac{i (f_{CO_{2}} N H_{2}CO_{2})}{M_{\alpha \beta \gamma}}$$

where

$$\Delta \omega_{j} = \omega_{j} - \omega - 1/T_{2 j},$$

and

$$M_{j} = M_{x j} + 1M_{y j}.$$

The effects of chemical exchange on NMR linewidths and resonance frequencies are well understood (22, 23). Here we examine

$$k_{obs,CO_{2}} = k_{obs,CO_{2}} + k_{obs,CO_{2}}$$

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The subscripts \(\alpha\), \(\beta\), and \(\gamma\) refer to Val-1α and Val-1β chain carbamates and free CO₂, respectively. Since the reaction is studied at equilibrium not all the fractional populations, \(f_{\alpha}\) and rate constants are independent. The equilibrium relations between the \(f_{\alpha}\) and rate constants are

$$k_{obs,CO_{2}} = k_{obs,CO_{2}} + k_{obs,CO_{2}}$$

Thus, for example, one can describe the system by the three fractional populations and two rates, \(k_{obs,CO_{2}}\) and \(k_{obs,CO_{2}}\). The desired steady state solution of these equations for the sum of the three magnetizations, in the \(x y\) plane was obtained using the matrix method described by Johnson and Moreland (23). A fortran program utilizing the above analysis has been written. In Fig. 2 the effects of increasing \(k_{obs,CO_{2}}\) on the linewidth and chemical shift of the carbamate resonances are shown. Both carbamate resonances are placed at 29.8 ppm in the absence of exchange (as in carbon monoxide hemoglobin), free CO₂ is at 68.4 ppm and the two values for \(k_{obs,CO_{2}}\) and \(k_{obs,CO_{2}}\) are the same. The population of free CO₂, \(f_{CO_{2}}\), is assumed to be 10 times greater than both \(f_{\alpha}\) and \(f_{\beta}\) as is typical

Data and sample calculations are presented as a miniprint supplement immediately following this paper. (Tables IV through VIII will be found on p. 2244.) For the convenience of those who prefer to obtain this supplementary material in the form of 7 pages of full-size photocopies, these same data are available as JBC Document No. 76M-1393. Orders for supplemental material should specify the title, authors, and reference to this paper and the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.05.
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Fig. 1. The dependence of $Z$ (mole fraction carbamino) on the parameters $K_c$ and $K_e$ is illustrated as a function of pH. The curves are based on typical values of $pK_c$ of 5.5 and $pK_e$ of 7.0, and show that $Z$ is much more sensitive to $K_c$ over the entire pH range of interest and $K_e$ exerts a negligible effect on observed $Z$ above pH $= 8.0$.

Fig. 2. Shows the predicted line width ($1/2$) and chemical shift dependence on release rate (parts per million from external CS$_2$) of two carbamino adducts exchanging with CO$_2$. The line width in the absence of exchange is assumed to be 5 Hz and the spectrometer frequency 25.2 MHz.

in these experiments for acidic values for pH. Since the difference in chemical shift for the carbamate resonances in deoxyhemoglobin is small (0.6 ppm) with respect to the difference in chemical shift between free CO$_2$ and carbamate (39 ppm), Fig. 2 is also relevant to the discussion of exchange in deoxyhemoglobin (5).

RESULTS

General Characteristics of Spectra—Fig. 3A shows representative $^{13}$C NMR spectra of deoxyhemoglobin $A_4$ equilibrated at various pH values with $^{13}$CO$_2$, A, deoxyhemoglobin; B, carbon monoxide hemoglobin. The pH values are listed at the right of each spectrum. The parts per million scale on the abscissa is referred to external CS$_2$. Total carbonates were 35 to 57 mM, and $^{13}$CO$_2$ (mole fraction $^{13}$CO$_2$, 0.82 to 0.92) was equilibrated at pressures of 4 to 374 torr. The hemoglobin concentration was usually 11.0 to 16.6 mM expressed as heme concentration in 0.05 M NaCl. The bicarbonate-carbonate resonance appears near 33 ppm, usually with spinning side bands in evidence. Measurements were obtained between 29-31°C.

Fig. 3B shows representative spectra of carbon monoxide hemoglobin equilibrated with $^{13}$CO$_2$ in the same manner and conditions specified in the legend. As the pH is lowered (Fig. 3B) the resonance at 29.8 ppm first broadens and then splits into two resolved resonances at exchange and is not shown in Fig. 3 since it occurs near 68.38 ppm.

For deoxyhemoglobin at higher values of pH, three carbamino resonances at 28.4, 29.2 and 29.8 ppm are observed. They are assigned, respectively, to carbamino adducts to e-amino groups, Val-la, and Val-1P. As the pH is lowered (Fig. 3B) the resonance at 29.8 ppm first broadens and then splits into two resolved resonances at

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pH values near neutrality. The identities of these resonances were established by examining the pH dependence of the carbamino adduct resonance positions of the two carbamyl carbon monoxide hemoglobin preparations, \( \alpha\beta_e \) and \( \alpha\beta_c \) (CO). These results are shown in Fig. 4. The Val-1\( \alpha \) carbamino adduct, represented by the form \( \alpha\beta_e \) (CO), is assigned to the smaller resonance (Fig. 3B) that moves upfield at low pH.

The data points included in Fig. 4 were obtained at the three different spectrometer frequencies of 15.1, 25.2, and 67.9 MHz, all yielding equivalent chemical shifts at a given pH. These results eliminate the possibility that the difference in chemical shift values for the Val-1\( \alpha \) and Val-1\( \beta \) adducts in carbon monoxide hemoglobin at low values of pH has its origin in any intermediate NMR rate process involving chemical exchange.

**Quantitation of Carbamino Adduct Formation**—The fraction, Z, of \( \alpha \)-amino group in each subunit in the carbamino form under given conditions was measured over the broad pH range from 6.5 to 9.0. The observed Z values are reported here as corrected to 55 mM total carbonates (5). Fig. 5 shows the values of Z as a function of pH for deoxyhemoglobin. In this case the chemical shift values distinguish the Val-1\( \beta \) adduct (29.8 ppm), the Val-1\( \alpha \) adduct (39.2 ppm), and the e amino adducts (28.4 ppm). At the high pH values, as previously reported (4, 5), the individual subunits exhibit nearly equal affinity for CO in the carbamino form. Fig. 5 shows that in the physiological pH range, however, Val-1\( \beta \) forms the predominant carbamino adduct.

In the case of carbon monoxide hemoglobin, in contrast, the individual carbamyl adduct resonances are clearly resolved only below pH 7.2, the Val-1\( \alpha \) adduct shifting upfield of the Val-1\( \beta \) adduct. Accordingly, the specifically carbamylated carbon monoxide hemoglobin derivatives, \( \alpha\beta_e \) (CO) and \( \alpha\beta_c \) (CO), were used to evaluate the contribution of each liganded chain over the entire pH range of interest. Fig. 6 shows the Z values as a function of pH for the unmodified carbon monoxide hemoglobin and for the two liganded, carbamylated derivatives. It may be seen that the sum of the Z values for the modified species is closely equivalent to the Z value at a given pH of the unmodified carbon monoxide hemoglobin. In the limited number of cases in which the Val-1\( \alpha \) and Val-1\( \beta \) adduct resonances in the unmodified carbon monoxide hemoglobin could be distinguished at pH 7.2 and below, the results corresponded well with those for the modified derivatives.

Fig. 6 also shows that the Z values for the Val-1\( \beta \) adduct are enhanced below pH 8, in a pH range in which carbamino formation would usually be expected to decrease as the proportion of nonprotonated \( \alpha \)-amino group decreases (Equations 1 and 2). This effect has the result that carbamino adducts are formed approximately equally well with the Val-1\( \alpha \) and Val-1\( \beta \) sites in the liganded hemoglobin near pH 7.4, in agreement with manometric measurements at this pH (4).

Table I summarizes the experimental conditions for the results in Figs. 5 and 6, and gives the values for pK, and pK, derived in each case from the two-parameter fit according to Equation 5. The curves are drawn according to Equation 5 using the values for pK, and pK, given in Table I. The insets in Figs. 5 and 6 show the per cent change in Z when pK, and pK, are caused to vary about their fit values. As expected from the computations illustrated in Fig. 1, \( \chi^2 \) is much more responsive to variations in pK, than in pK, for all data examined. Note that a unique solution exists for each parameter. The
contrast to the dominance of the Val-1P adduct in the absence of deoxyhemoglobin over a range of pH. This result is in clear distinction from the results in the absence of the organic phosphate. The predominance of the Val-1P adduct is much less pronounced than in the absence of 2,3-diphosphoglycerate (Fig. 3A). The mechanism can be seen in the sharp change in pK as observed in the presence of organic phosphate. The lower curve shows a two-parameter fit of these data according to Equation 5. The upper curve is taken from Fig. 6 and refers to Z values obtained in the absence of organic phosphate.

### Table I

| Chemical shift | pK<sub>A</sub> | pK<sub>2</sub> | pK<sub>3</sub> | λ<sub>2</sub> M<sup>-1</sup> | λ<sub>3</sub> M<sup>-1</sup> | λ<sub>4</sub> M<sup>-1</sup> |
|----------------|---------------|---------------|---------------|----------------|----------------|----------------|
| Deoxyhemoglobin |               |               |               |                 |                 |                 |
| Val-1β         | 29.8          | 6.91 ± 0.32   | 6.84 ± 0.12   | 4.64 ± 0.08     | 244             | 455             | 761             |
| Val-1α         | 29.2          | 7.83 ± 0.19   | 7.79 ± 0.10   | 4.89 ± 0.10     | 48              | 110             | 251             |
| Carbon monoxide hemoglobin |       |               |               |                 |                 |                 |
| Val-1β<sup>a</sup> | 29.8    | 7.05 ± 0.05   | 5.8<sup>a</sup> | 35              | 60              | 86              |
| Val-1α<sup>a</sup> | 28.8    | 7.16 ± 0.36   | 6.95 ± 0.13   | 5.5 ± 0.08      | 26              | 50              | 92              |

<sup>a</sup> Referenced to external CS<sub>4</sub>.
<sup>b</sup> For comparison values of pK<sub>A</sub> determined by Garner et al. (7) are included.
<sup>c</sup> Carbamylated α chain NH<sub>2</sub>-terminal hemoglobin derivative.
<sup>d</sup> Carbonmonoxy β chain data does not fit two-parameter pK, pK<sub>A</sub> function.
<sup>e</sup> Carbamylated β chain NH<sub>2</sub>-terminal hemoglobin derivative.

### Discussion

**Roles of α-Amino Groups of α and β Subunits**—The results in Table I confirm that the α-amino groups of the α and β subunits are adapted to clearly different roles with respect to the heterotropic effectors (5), in addition to any discrimination with respect to the homotropic effector, O<sub>2</sub> (27-29). One role is that taken by Val-1α in responding to the change in heme ligand state by undergoing a change in hydrogen ion binding, thereby contributing to the so-called alkaline Bohr effect (17, 30). The mechanism can be seen in the sharp change in pK for the Val-1α, a change that is not observed with Val-1β.

The other role is that taken by Val-1β which undergoes a
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HbA,CO + DPG

FIG. 7. Shown here are 25.2 MHz 13C NMR spectra of human adult hemoglobin A, in the presence of 2,3-diphosphoglycerate (DPG) equilibrated at various pH values with 13CO2. A, deoxyhemoglobin and 5 mM 2,3-diphosphoglycerate; B, carbon monoxide hemoglobin and 8 mM 2,3-diphosphoglycerate. The pH values are listed at the right of each spectrum. The parts per million scale on the abscissa is referenced to external CS3. Total carbonates were 48 to 60 mM and WO2 (mole fraction WO2 0.82 to 0.92) was equilibrated at pressures of 4 to 374 torr. The average hemoglobin concentration were 11.97 mM heme and 11.32 mM heme for A and B, respectively. Conditions are more fully described in Table II.

change in binding of CO2 in the carbamino form, thereby making the major contribution to the so-called Haldane effect (17, 31). The mechanism in this case depends again on the differences in pK values between the subunits and, in addition, on the reduced stability of the carbamino derivatives in both subunits in the ligand state as expressed by the pK values in Table I. Since Val-1β undergoes the change in pK, but not in pKc, it experiences a clear decrease in carbamino derivative that is mirrored in the values for l in Table I. On the other hand, the same trend of pK values for Val-1α is nearly compensated by the differences in pKc, between the liganded and unliganded forms. In effect, Val-1α in the deoxyhemoglobin is primarily protonated at physiological pH and is much less free to form the carbamino derivative than is Val-1β which is primarily unprotonated. Since the two pKc values in deoxyhemoglobin lie about 0.5 unit on either side of the physiological pH of 7.4, Val-1β forms and discharges much more carbamate than does Val-1α.

The pK values of 4.64 and 4.89 listed in Table I are comparable in magnitude to those observed with many simple amines (13). The large increases in these values in the l

FIG. 8. The effect of 2.3-diphosphoglycerate (DPG) on the pH-dependent carbamino formation for deoxy Val-1β and Val-1α. No insets of best fit pK and pKc are included as the equilibria assumed in the fitted line do not apply rigorously in the presence of organic phosphate.

FIG. 9. The effect of 8 mM 2,3-diphosphoglycerate (DPG) on the unmodified carbon monoxide hemoglobin resonance at 29.8 ppm (referenced to external CS3). The computer-calculated curve (from Fig. 6) for the unmodified carbon monoxide hemoglobin in the absence of organic phosphate is included for comparison as the upper solid curve.

Table II

Carbamino formation data for human hemoglobin A, in presence of 2,3-diphosphoglycerate

|                | Val-1β 5 mM | Val-1α 5 mM | Carbon monoxide hemoglobin |
|----------------|------------|------------|----------------------------|
|                | DPG        | DPG        |                            |
| Hemoglobin     |            |            |                            |
| DPG            |            |            |                            |
| NeO concentration was 50 mM for deoxyhemoglobin and 100 mM for carbon monoxide hemoglobin samples. Hemoglobin concentration averaged 12.0 mM for deoxyhemoglobin and 11.3 mM for carbon monoxide hemoglobin samples. In both cases average total carbonates were 55 mM. Ionic strength varied from 213 to 252 mM for deoxy and 294 to 402 mM for carbon monoxide hemoglobin samples due to progressive ionization of hydrated CO2.

 Chemical shift $\chi_{13}$ M$^{-1}$ $\chi_{14}$ M$^{-1}$ $\chi_{15}$ M$^{-1}$

Deoxyhemoglobin

Val-1β 5 mM

DPG

29.8 80 167 333

Val-1α 5 mM

DPG

29.2 72 154 313

Carbon monoxide hemoglobin

Hemoglobin 8 mM DPG

29.8 21 41 76

* Referenced to CS3.

* 2,3-diphosphoglycerate.

* These values represent calculated association constant for Val-1α and Val-1β assuming equal contributions from both sites to the combined resonance in unmodified liganded hemoglobin.
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formation at this site in the liganded hemoglobin. This consequence for the α subunit would run counter to the allosteric effector role of CO with respect to the β subunit. Secondly, the maintenance of a low level of carbamino formation with Val-1α under both liganded and unliganded conditions allows this site to serve its function as essentially a Bohr group without significant interference.

Dependence of Carbamino Formation on pH, pCO₂, and Bicarbonate—Fig. 10 shows plots for the α-amino group of both α and β subunits in the liganded and unliganded states. The plots show values of Z versus pH corresponding to various values of the concentration of bicarbonate and pCO₂. For reference, each panel is marked with the symbols θ and φ to indicate the conditions corresponding to arterial and venous blood, respectively. The plots in Fig. 10 are useful for observing the response of Z values for the individual chains to acidosis and alkalosis of respiratory or metabolic origin (32, 33).

Relationships between Bohr and Haldane Effects—Fig. 11 shows results of computations describing the changes in bound hydrogen ions and carbamino derivatives at the α-amino groups between the two ligand states under given conditions of pH and pCO₂. Fig. 11A shows plots of ΔZ versus pH for the carbamino formation with the α-amino groups of both subunits at various values of pCO₂. The expression for the change in Z, ΔZ, is given by Equation 13:

\[ ΔZ = \ln \left( \frac{k_{pCO₂}}{1 + k_{pCO₂}} \right) \]

Fig. 11. A, the change in mole fraction of carbamate, Z, in going from carbon monoxide to deoxyhemoglobin is shown for Val-1β (- - -) and Val-1α (------) at the indicated pCO₂ values. B, the Bohr effect, ΔΗ⁺, due to Val-1α is shown for the indicated pCO₂ values.
In these equations (Equations 13 and 14, below) the subscripts 1 and 2 refer to the initial and final ligand states and $k$ is the Henry's law proportionality constant.

The change in hydrogen ions bound, representing the contribution to the alkaline Bohr effect, is given in Fig. 11B for Val-$\alpha$ only since the corresponding plot for Val $\beta$ would not be meaningful (Table I). The expression for the change in hydrogen ions bound by Val-$\alpha$, $\Delta H^+$, is

$$\Delta H^+ = (H^+) \frac{K_{21} - K_{22} + K_{22}Z_1 - K_{21}Z_2 + (H^+)(Z_1 - Z_2)}{(K_{21} + (H^+)) (K_{22} + (H^+))}$$

(14)

where $Z_j = \lambda_j k PCO_2 / (1 + \lambda_j k PCO_2)$

Fig. 11A shows how much more marked the change in carbamino formation is for Val-$\beta$ and also how marked is the effect of $pCO_2$ at physiological pH. The results in Fig. 11B show the increasing effect of $pCO_2$ on limiting the proton uptake by the $\alpha$-amino group of Val-$\alpha$, an effect that is relatively modest at pH 7.4.

The relationship between the Bohr and Haldane effects is most simply seen from the computations shown in Fig. 12. For each pH the bars show changes that accompany the transition from the liganded to the unliganded state of the hemoglobin with $pCO_2$ equal to 40 torr. The left hand member of each pair shows the uptake by the two identified alkaline Bohr groups, His-$\alpha\beta$ and Val-$\alpha$. The $pK_a$ change experienced by His-$\alpha\beta$ has been found to be virtually identical with that of Val-$\alpha$ (34). It is assumed that His-$\alpha\beta$ is not directly affected by CO$_2$, whereas allowance is made for this effect on Val-$\alpha$ as shown in Fig. 11B. The right hand member for each pH represents the net release of protons due to the Haldane effect from the two groups Val-$\alpha$ and Val-$\beta$. Here the total release consists of the contribution given by Equation 2, and also the effect of the carbamino formation in removing from the equilibrium of Equation 1 the conjugate base form of the amine.

The results in Fig. 12 show that the process of carbamino formation in the absence of organic phosphates can provide a substantial fraction of the protons taken up by these two Bohr groups that are responsible between them at pH 7.4 for approximately 80% of the alkaline Bohr effect (17, 34, 35).

**Carbamino Formation with $\beta$ Subunit in Carbon Monoxide Hemoglobin**—The anomalous stability of the carbamino derivative of Val-$\beta$ in carbon monoxide hemoglobin in the pH range below 8 cannot be described by the equilibria given by Equations 1 to 5. Reasonable stabilizing mechanisms could involve either electrostatic interactions with nearby groups or local conformational rearrangements leading to a pH-dependent $pK_a$. The stabilization process involving the imidazole group of His-$\beta$ for each pair shows the uptake by the two identified alkaline Bohr groups, His-$\alpha\beta$ and Val-$\alpha$. The $pK_a$ change in going from carbon monoxide to deoxyhemoglobin.

**Effect of 2,3-Diphosphoglycerate**—Comparison of Table II with Table I and of Fig. 8 with Fig. 5 shows that 2,3-diphosphoglycerate reduces the formation of carbamino adduct to Val-$\beta$. As expected, the effect is most obvious at the lower pH values, in the range where the organic phosphate binds most strongly to the deoxyhemoglobin (38, 39). A more detailed interpretation will require direct measurement of the binding of the 2,3-diphosphoglycerate to the hemoglobin. The present experiments involve higher ionic strength in the presence of the organic phosphate; furthermore, bicarbonate ion may act somewhat like chloride ion in reducing the binding of the polymer (39, 40).

The results in Fig. 9 show that total carbamino formation at Val-$\beta$ and Val-$\alpha$ in carbon monoxide hemoglobin is suppressed by 2,3-diphosphoglycerate. Here again direct binding measurements are planned for the future on specifically carboxylated hemoglobin preparations. Since the carbamino formation in Fig. 9 shows a suggestion of dropping off relatively sharply below pH 7.5, it is tempting to conclude that the adduct to Val-$\beta$ is preferentially suppressed (cf. Fig. 6).

The results in Fig. 7B showing that the upfield chemical shift of the Val-$\alpha$ carbamino adduct resonance at lower pH values (Fig. 4) is suppressed in the presence of 2,3-diphosphoglycerate constitute evidence both for a good degree of binding of the organic phosphate to the protein and for an effect on the
environment of Val-1α. From the kinetic analysis given below and the treatment presented under "Data Handling" it is clear that this chemical shift has its origin not in an exchange process but in a conformational or electrostatic change controlled by proton binding that appears to be suppressed in the presence of 2,3-diphosphoglycerate. In this regard it is interesting that the presence of the organic phosphate has no detectable effect on the chemical shift of resonances attributable to carbamino adducts of Val-1β; if concurrent binding of the phosphate and carbamino adducts occurs in the cleft containing the NH-terminus regions of the β subunits (4), it occurs without perturbing the rather sensitive carbamino resonance (13).

Analysis of Rates of Release of CO₂—The case of three-site exchange involving CO₂ in the free form and in the adducts to Val-1α and Val-1β was presented in the data-handling section and in terms of the populations of the sites and of the two first order release rate constants as the independent variables (Equations 7 to 11). The analysis deals with the observed variables of line width, resonance frequency, and resonance intensity. The line widths of carbamino resonances in Figs. 3, A and B, are typically less than 12 Hz (25.2 Hz = 1 ppm in these spectra) with the Val-1α adduct showing the greater broadening at low pH. In terms of Fig. 2 these observations mean that the first order rates of release of CO₂ from the hemoglobin are typically less than 25 s⁻¹. This result is in agreement with the studies of Caplow on release of CO₂ from model carbamate compounds (24).

To estimate the exchange rates over a range of conditions a portion of the digitized spectrum (4096 data points) obtained for deoxyhemoglobin was fit to the case of the three-site exchange process. The actual spectra and the corresponding best fit simulations are shown paired in Fig. 14. The values used for the independent population and release rate parameters are listed in Table III, and are in accord with the approximate estimate of order of release rates given above. The release rate for the Val-1α adduct in deoxyhemoglobin is somewhat greater than that for the Val-1β adduct.

Forster et al. (42) have also estimated the rates of association and dissociation of CO₂ by deoxyhemoglobin. The expressions used for the association, \(v_a\), and dissociation, \(v_d\), velocities were

\[
v_a = k_d(R-NH_2)(CO_2) \quad (16)
\]

and

\[
v_d = k_d(R-NHCO_2) - k_d(R-NHCO_2^+)(H^+)/K_a \quad (17)
\]

where \(K_a\) is the acid dissociation constant of the carbamate. Since the rates studied here are generally low, broadening effects due to magnetic field inhomogeneity and spectrometer drift are nearly of the same degree as the exchange broadening itself.

The form of Equation 2 takes into account an estimate of \(pK_a\) of approximately 5.0 (6). This value is reasonable in view of the invariance of chemical shift of carbamino resonances in deoxyhemoglobin examined down to pH 6.6. The pH dependence of ¹³C chemical shifts in carboxyl or carboxamide groups normally covers a range of the order of 8 to 10 ppm (13). With a digital resolution of 0.03 ppm applying in the present studies, it follows that the \(pK_a\) almost certainly falls at least 1.3 units below the pH of observation, 6.6.
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Table III
Carbamino dissociation rate constants in deoxyhemoglobin

The dissociation constants for the carbamino adducts of deoxyhemoglobin were calculated on the basis of a least squares fit to the observed spectrum based on three site exchange with CO₂ and the two NH₂-terminal carbamino adducts. The apparent T₁ used for the fitting procedure was 0.064 s. The values reported below must be regarded as only semiquantitative, due to the uncertainties involved in the fitting procedures. The column designated populations gives the relative populations of the resonances at 29.2, 29.8, and 68.3 ppm (CO₂) as estimated by the fitting procedures.

| pH   | Estimate variance of fit | kₐ₋₁ (s⁻¹) | Populations |
|------|--------------------------|-------------|-------------|
|      |                          | Val-1α      | Val-1β      |             |
| 8.47 | 0.027                    | 5           | 6           | 0.39/0.51/10|
| 7.47 | 0.021                    | 11          | 1           | 0.27/0.46/27|
| 7.35 | 0.003                    | 18          | 1           | 0.23/0.40/37|
| 7.16 | 0.018                    | 27          | 1           | 0.16/0.31/53|
| 7.06 | 0.003                    | 21          | 3           | 0.18/0.39/51|
| 6.81 | 0.004                    | 55          | 2           | 0.11/0.20/59|

In the terminology used here

\[ v_{\text{a}} = k_{\text{a}}(R-NHC(O)⁻) \]  (18)

Eliminating kₐ from Equations 16 to 18, and introducing Equation 2 to express Kₐ, yields

\[ k_{\text{a}} = \frac{k_{\text{b}}(H⁺)}{Kₐ} \]  (19)

Forster et al. (42) measured kₐ equal to 11,000 s⁻¹ for deoxyhemoglobin. Taking this value at pH 7.47 and assuming that pKₐ is 4.6, corresponding to the value (Table I) for the Val-1β adduct that would be dominant under the experimental conditions (42), kₐ₋₁ is found to be 17 s⁻¹. This value is somewhat higher than those listed in Table III for the Val-1β site, but within the errors inherent in the methods.

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Table VII

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table VIII

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table IX

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table X

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table XI

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table XII

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table XIII

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table XIV

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table XV

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |

Table XVI

| Component | Concentration (mM) | molar extinction coefficient (mL/mM/cm) | molar absorptivity (mL/mM/cm²) |
|-----------|-------------------|----------------------------------------|-------------------------------|
| O2       | 1.0               | 10.0                                   | 100.0                         |
| H2O      | 1.0               | 1.0                                     | 1.0                           |
| H2       | 1.0               | 1.0                                     | 1.0                           |
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