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The Binding of CO_2 to Human Hemoglobin*

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SUMMARY

CO_2-dissociation curves of concentrated human deoxy- and carbonmonoxyhemoglobin at 37°, pH 7.6 to 7.0, p_{CO_2} = 10 to 160 mm Hg, have been obtained by a rapid mixing and ion exchange technique. The CO_2-dissociation curves for deoxyhemoglobin can only be fitted by assuming two classes of binding sites for carbon dioxide. The simplest way to account for the experimental data is to assume that the α-amino groups of the α and β chains react with carbon dioxide with affinities that differ by at least a factor of 3. No difference in reactivity with CO_2 was found among the four terminal α-amino groups of carbonmonoxyhemoglobin.

At physiological p_{CO_2}, pH, and temperature, carbon dioxide in blood or hemoglobin solutions exists mainly as: (*a*) dissolved CO_2, (*b*) bicarbonate, and (*c*) carbaminohemoglobin.

CO_2 combines, under physiological conditions, with the four α-NH_2 groups of valines at the end of the α and β chains of hemoglobin. The binding there is oxygen-linked, *i.e.* at constant p_{CO_2} and pH deoxyhemoglobin forms more $HbCO_2$[1] than HbO_2 or HbCO.

In the previous papers we have reported on the carbamate reactions of bovine hemoglobin by a new ion exchange/gel filtration method. The initial use of bovine hemoglobin, due to its excellent stability at alkaline pH, has always been preferred by all previous investigators in this field. We have now developed a new method suitable to the investigation of the CO_2 reactions of human hemoglobin. These studies not only have a considerable physiological interest, but represent an essential step for the study of the interaction between hemoglobin and other allosteric ligands. A thorough review of previous work in this field has recently appeared (1).

The method described in this paper requires 300 to 500 mg of hemoglobin for each determination. However, by using suitable microtechniques for CO_2 analysis, the total protein requirement can be reduced to ~100 mg. This represents a 20-fold improvement, at least, over previous techniques. Owing to the general validity of the method described, which can easily be adapted to the investigation of the CO_2 reactions of other proteins, a rather detailed description of the experimental procedure is given in the miniprint supplement.[2]

RESULTS

Coefficient of Solubility of CO_2—Values of Q', the apparent Henry's Law coefficient in hemoglobin solutions of varying concentrations, at 37°, are shown in Fig. 2. Q' gives the concentration of the sum (CO_2 + H_2CO_3) per liter of hemoglobin solution at 760 mm Hg CO_2, for the experiments at pH ~ 4.6. For the experiments at pH ~ 6.2, Q' expresses the true free CO_2 (H_2CO_3 is in "instantaneous" equilibrium with HCO_3^-, which is removed in the ion exchange process). The concentration of H_2CO_3 is, however, approximately 1/600 (15) of CO_2 and thus no practical difference between the two experimental approaches should be noticed. Fig. 2 shows that the data obtained by the experiments at pH 6.2 are more scattered than those yielded by the Van Slyke method. This was expected because of the greater number of independent measurements needed by the former method. However, the most scattered points are less than 3% off the least squares line drawn through the experimental data, and no systematic deviations between the two methods are observed.

Carbamates of Hb and HbCO—Fig. 3 shows the carbamate of deoxyhemoglobin as a function of pH at p_{CO_2} = 120 mm Hg. The slope of this curve at a given pH was used to correct data obtained at slightly different pH values (see "Methods," section *c*). These corrections were applied to both Hb and HbCO data obtained at different p_{CO_2} values. In fact, Perrella *et al.* (6) have shown that curves at CO_2 fractional saturation *versus* pH at different p_{CO_2} values run almost parallel to each other. CO_2 saturation curves at constant pH but with varying p_{CO_2} pressures are shown in Fig. 4 for deoxyhemoglobin, and in Fig. 5 for HbCO. Previous experiments (5) have shown that the CO_2 binding properties of HbCO and HbO_2 are identical, and data obtained

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[1] The abbreviations used are: $HbCO_2$, carbaminohemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; diamox (acetazolamide), 2-acetylamino-1,3,4-thiadizaole-5-sulfonamide; cardrase (ethoxzolamide), 6-ethoxy-benzo-thiadiazole-2-sulfonamide; Hb, deoxyhemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; $\alpha_2^c\beta_2$, human hemoglobin reacted with cyanate at the α chain α-amino groups; $\alpha_2\beta_2^c$, human hemoglobin reacted with cyanate at the β chain α-amino groups; TEA, triethylamine; K_e, K'_e, dissociation constant of hemoglobin carbamate; K_z, K'_z, ionization constant of the terminal α-amino groups of hemoglobin.

[2] Supplementary data for The Binding of CO_2 to Human Hemoglobin are available as JBC Document Number 74M-851, in the form of 6 pages. Orders for supplementary material should specify the title, author(s) and reference to this paper, the JBC Document number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014 and must be accompanied by remittance to the order of the Journal in the amount of $1.00 for photocopy.

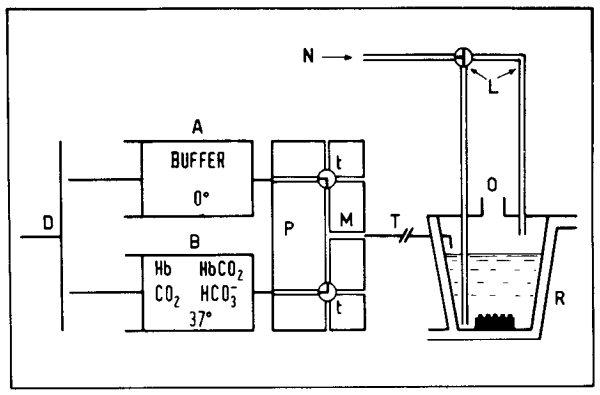

FIG. 1. The continuous flow apparatus for $HbCO_2$ determination. *A* and *B*, 2-ml syringes; *D*, pushing platform; P, Perspex mixing block; *R*, double-walled glass chamber; *L*, nitrogen inlet; *T*, plastic tubing; *M*, mixer; *t*, two-way taps.

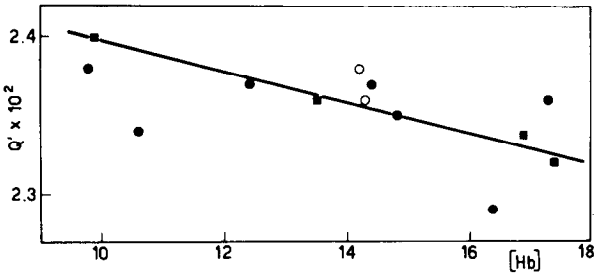

FIG. 2. Q', the apparent solubility coefficient of CO_2 in human hemoglobin solutions. ■, HbCO, as estimated by the method of Van Slyke (13) at pH ~ 4.5, $I \sim 0.14$ at p_{CO_2} = 80 to 160 mm Hg. ●, HbCO, and ○, Hb, as estimated by the ion exchange method of this paper.

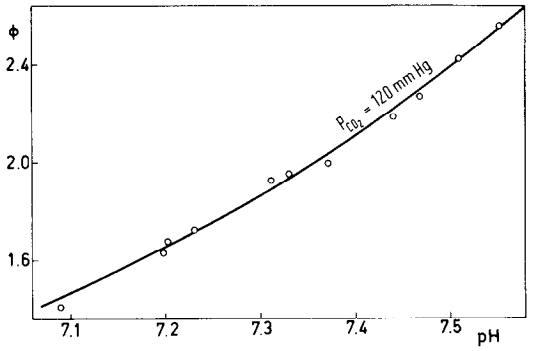

FIG. 3. Carbamates of human hemoglobin in moles of CO_2 per mole of hemoglobin tetramer (ϕ) *versus* pH at constant p_{CO_2}, $I \sim 0.2$, temperature = 37°.

with the CO form can be safely used for physiological applications.

DISCUSSION

Method for Determining Carbamates of Human Hemoglobin—The main methods for determining $HbCO_2$ have hitherto been based on (*a*) the classical $BaCO_3$ precipitation method (16, 17), and (*b*) the measurement of the change of p_{CO_2} in solution as carbamate is formed, originally by manometry (18), but more recently with the aid of p_{CO_2} electrodes (19–23). In the case of human hemoglobin, the CO_2 binding data presented in this paper can only be compared with the data of Ferguson (17), obtained with the barium method; and with those of Forster *et al.* (22) and Brenna *et al.* (23), both obtained with the p_{CO_2} electrode technique. Unfortunately, Ferguson did not measure the pH of his solutions, and a direct comparison with his data is not readily possible.

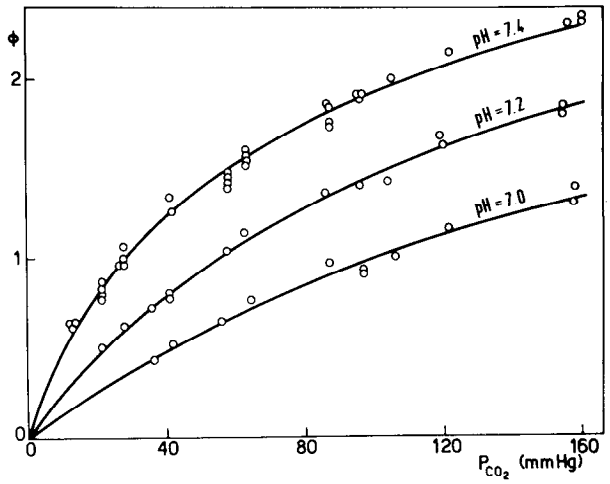

FIG. 4. ϕ *versus* p_{CO_2} for human deoxyhemoglobin at various pH values. The *solid lines* have been calculated from the constants reported in Table I.

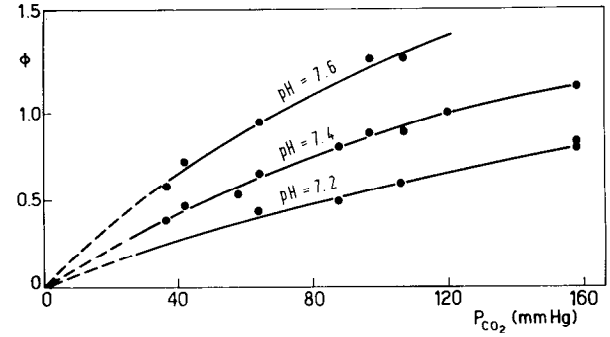

FIG. 5. ϕ *versus* p_{CO_2} for human carbonmonoxyhemoglobin at various pH values. The *solid lines* have been calculated from the constants reported in Table I.

However, in a limited number of the experiments reported in Figs. 4 and 5, the total CO_2 of the hemoglobin solutions used for $HbCO_2$ determination has also been measured. From such data it is thus possible to calculate the apparent pK (Equation 1), of carbonic acid under experimental conditions of protein concentration and ionic strength very similar to those of Ferguson. The equation used is a modified form of the Henderson-Hasselbach equation (24, 25), *i.e.*

$$pH = pK_1 + \log \frac{[\text{total } CO_2] - [HbCO_2 + CO_2]}{[CO_2]} \qquad (6)$$

Analysis of 12 experiments at pH 7.4 with p_{CO_2} values ranging from 21 to 160 mm Hg gave an average $pK_1 = 6.197 \pm 0.011$. Ferguson assumed in his calculation of pH an arbitrary value of $pK_1 = 6.1$. Revised values make it now possible to assign to each of Ferguson's $HbCO_2$ determinations a precise pH value. Ferguson's data can thus be compared with the corresponding values of $HbCO_2$ obtained by interpolation from the data of Figs. 4 and 5 at the same p_{CO_2} and pH. The comparison is shown in Fig. 6. No systematic deviations between the two methods of analysis are apparent. This is satisfactory, considering the different experimental approach and the size of the corrections used by Ferguson to calculate $HbCO_2$.

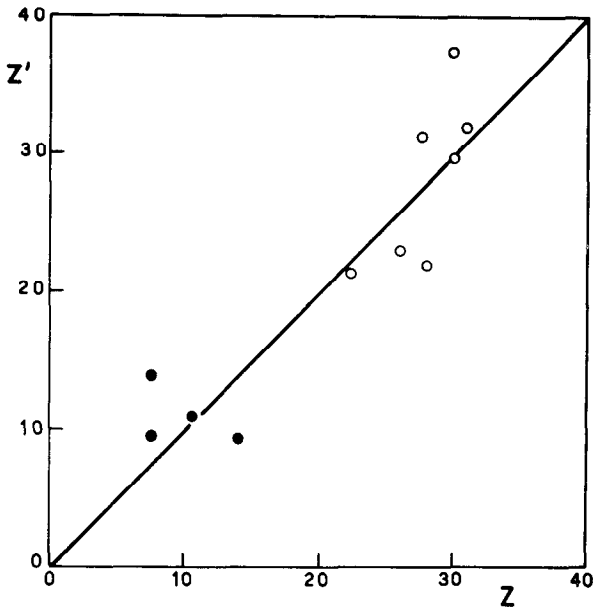

FIG. 6. The percentage saturation of human hemoglobin with CO_2 as obtained by Ferguson (17) is plotted on the ordinate *versus* that obtained at the same p_{CO_2} and pH by the method of this paper. Only the most complete experiments of Ferguson, *i.e.* Experiments. 24.viii.35, 23.x.35, and 8.xi.35, have been considered. ○, deoxyhemoglobin; ●, oxy- or carbonmonoxyhemoglobin.

The $HbCO_2$ binding data of deoxyhemoglobin at pH 7.4 reported in this paper can also be compared with data obtained under similar conditions of pH, p_{CO_2}, temperature, and ionic strength by Brenna *et al.* (23) with the p_{CO_2} electrode technique. At $p_{CO_2} = 40$, Brenna *et al.* reported a value of $\phi \sim 8\%$ higher than the corresponding value obtained with the ion exchange method of this paper. This discrepancy rises to approximately 10% at $p_{CO_2} = 120$ mm Hg. It should be noted, however, that in the experiments of Brenna *et al.*, the protein concentration was only 4 to 6 mM/Fe/liter. Furthermore, uncertainties as to the values of Q' do have a much higher effect in the calculation of $HbCO_2$ in the p_{CO_2} electrode technique, especially at high p_{CO_2} values. Such uncertainties, coupled with the well known difficulties of accurately measuring p_{CO_2} in streaming fluids, may well explain the difference noted between the two methods. The experiments of Forster *et al.* (22) are in general qualitative agreement with the results reported here. Very few data in the critical pH range 7.3 to 7.6, however, have been reported by the same authors, and a quantitative analysis of their data is not feasible. The method for determining $HbCO_2$ presented in this paper is also validated by the agreement found between the values of Q' obtained with the classical method of Van Slyke *et al.* (13) and the ion exchange procedure (Fig. 2).

Analysis of Binding Data—According to Kilmartin and Rossi-Bernardi (9), only the NH_2-terminal α-amino groups of horse hemoglobin react with CO_2 in the pH range 7.0 to 8.0. Van Kempen and Kreuzer (26) reached the same conclusions for bovine hemoglobin. For human hemoglobin, Kilmartin *et al.* (27) have recently found that CO_2 does not affect the oxygen affinity of hemoglobin carbamylated at the four α-NH_2 groups. Thus, we may reasonably assume that the data of Figs. 4 and 5 represent the equilibrium between the four terminal valines of human hemoglobin and CO_2. If all the four binding sites have equal affinity for CO_2, then, according to the analysis of Perrella *et al.* (6),

$$\phi = 4Z = \frac{4\lambda[CO_2]}{1 + \lambda[CO_2]} \tag{7}$$

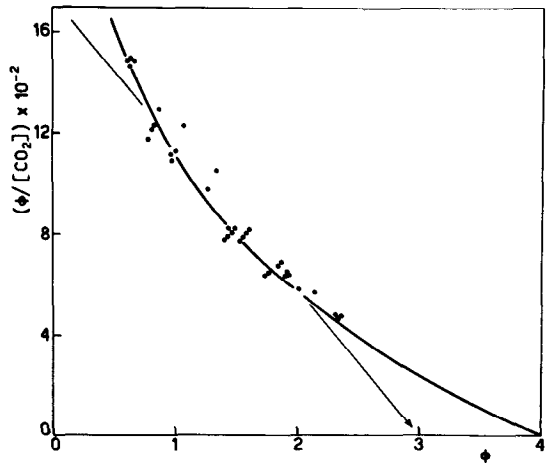

FIG. 7. Scatchard's plot of CO_2 binding data of human deoxyhemoglobin at pH 7.4. The *curved line* has been drawn by hand through the experimental points. The *straight line* has been obtained by least squares fitting assuming a linear relationship between $\phi/[CO_2]$ and ϕ.

where

$$\lambda = \frac{K_c K_z}{K_z[H^+] + [H^+]^2} \tag{8}$$

Fig. 7 shows a Scatchard plot of the data obtained for the most complete experiments of CO_2 binding, *i.e.* deoxyhemoglobin at pH 7.4. The "best" straight line through the points on this plot intercepts the abscissa at $\phi \sim 3$, suggesting some heterogeneity between the four reacting sites.

The simplest way to account for the experimental data, which would also be in agreement with the known difference in chemical bonding of the two pairs of the α-amino groups of the α and β chains (28, 29), is to attribute a different value of λ (Equation 8) to the two pairs of NH_2-terminal amino groups. Equations 7 and 8 can accordingly be transformed into

$$\phi = 4Z = \frac{2\lambda_1[CO_2]}{1 + \lambda_1[CO_2]} + \frac{2\lambda_2[CO_2]}{1 + \lambda_2[CO_2]} \tag{9}$$

where

$$\lambda_1 = \frac{K'_c K'_z}{K'_z[H^+] + [H^+]^2} \text{ and } \lambda_2 = \frac{K''_c K''_z}{K''_z[H^+] + [H^+]^2} \tag{10, 11}$$

It should be clear that the λs of Equations 7 and 9 can also be considered as apparent CO_2 binding constants, independent from the mechanism of CO_2 combination to α-NH_2 groups expressed by Equations 4 and 5. If the mechanism indicated by the two equations is correct, then the dependency of λ or λ_1 and λ_2 on pH can be described by Equation 8 or Equations 10 and 11. Values for λ_1 and λ_2 on the two classes of binding sites hypothesis have been obtained by a standard least squares procedure (30).

Table I summarizes the values of λ so obtained together with their standard errors. In the case of HbCO, due to the low carbamate concentration and to the fewer number of experimental points, the model expressed by Equation 9 cannot be safely tested. In such a case, an empirical constant λ has been calculated according to Equation 7 to fit the data. The *solid lines* of Figs. 4 and 5 have been calculated assuming the constants reported in Table I. The results of the analysis of the CO_2 binding data of human hemoglobin are, so far, in good agreement with the results previously reported by Perrella *et al.* (6) on bovine hemoglobin at pH 7.4, at 25°.

TABLE I

Computed values of λ_1 *and* λ_2 *(Equation 9) and associated standard errors at various pH values for Hb and HbCO*

Hb

pH	λ_1 (M^{-1})	λ_2 (M^{-1})
7.4	774 ± 4.1%	113 ± 6.2%
7.2	348 ± 8.3%	87 ± 12.6%
7.0	186 ± 19.4%	50 ± 38%

HbCO

pH	λ
7.6	λ = 156 (equation 7)
7.4	λ = 92 (equation 7)
7.2	λ = 55 (equation 7)

To calculate values of K'_c, K''_c and K'_z, K''_z, Equations 10 and 11 can be transformed into the corresponding linear forms, of which Equation 12 is an example.

$$\frac{1}{\lambda_i[H^+]} = \frac{1}{K'_c} + \frac{[H^+]}{K'_c K'_z} \qquad (12)$$

A plot of $1/\lambda_i[H^+]$ *versus* $[H^+]$ should give a straight line having $1/K'_cK'_z$ as angular coefficient and giving $1/K'_c$ as intercept on the ordinate. When the values of Table I are treated according to Equation 12, however, no meaningful values of K'_c, K''_c and K'_z, K''_z can be obtained. The results of this analysis show that a considerable decrease in the standard errors of the parameters is required if a proper test of the effect of pH and of oxygen or carbon monoxide binding of human hemoglobin is to be made.

Effect of Carbon Monoxide on $HbCO_2$ Binding—The effect of carbon monoxide on $HbCO_2$ binding can be deduced from the CO_2 binding data shown in Fig. 4 for deoxyhemoglobin and in Fig. 5 for carbonmonoxyhemoglobin. According to Reactions 4 and 5, carbamates should only be a function of pH and p_{CO_2}. A decrease in CO_2 binding at constant p_{CO_2} and pH can only mean that K_c or K_z (or both) are affected by the conformational changes that are known to occur in the hemoglobin molecule upon heme-ligands binding (25).

Values of F, the difference in carbamate bound by deoxy over HbCO at constant pH, can be obtained from the data of Figs. 4 and 5 and compared with those obtained from the difference in total CO_2 of human oxy- and deoxyhemoglobin solutions at p_{CO_2} = 40 mm Hg, pH 7.4 (31). The agreement, F = 0.2 *versus* 0.21, is very satisfactory. F, as obtained from the data of Figs. 4 and 5, is not linearly related with p_{CO_2}, as previously supposed (31).

The reproducibility in the determination of Z (the percentage saturation of hemoglobin with CO_2), by the method described in this paper, is 1 to 2%. It seems unlikely that this figure can significantly be improved. Thus, the present experimental approach seems unsuitable for the calculation of statistically significant values of K_c, K'_c and K_z, K'_z and for the elucidation of the mechanism of the change of CO_2 affinity with pH and ligand binding. This is mainly due to the rather small difference between λ_1 and λ_2, and to the narrow range of p_{CO_2} and pH investigated. A glance at the CO_2 binding curves of Figs. 4 and 5 shows that the study of the CO_2 equilibrium has been limited to less than 50% saturation. Unfortunately, technical reasons do not allow extension of the p_{CO_2} range much beyond 160 mm Hg. Furthermore, at a pH lower than 7.0, the amount of $HbCO_2$ becomes too low to be determined with reasonable accuracy. At a pH higher than 7.6, on the other hand, the reaction of CO_2 with the ϵ-amino groups of the molecule might become significant, making the analysis of the CO_2 binding data even more complicated.

The results reported, however, show for the first time the behavior of human hemoglobin in respect to the binding of the physiologically important non-heme-ligand CO_2. The data should be useful for a proper analysis of the role of carbamate compounds in regulating oxygen affinity, and of their importance in the transport of CO_2 by circulating blood.

Arnone (32) has recently found by x-ray diffraction methods, an unexpected positive difference peak behind the β-heme pocket. The dimensions of the peak are consistent with the binding of CO_2 or HCO_3^-, but the hydrophobic nature of this binding site would favor the neutral CO_2 molecule. Arnone's studies suggest another form of CO_2 is "bound" to hemoglobin. It is relevant to ask whether this finding would complicate the scheme of CO_2 reactions with human hemoglobin presented in this paper. As Arnone has clearly pointed out, however, the CO_2 bound near the β-heme pocket would be at least 5 to 10 times less for the p_{CO_2} range investigated in this paper. In addition, any form of loosely bound CO_2 other than carbamate would be accounted for in our solubility coefficient for CO_2 in hemoglobin solution. Finally, it should be pointed out that at a pH below 6.8, Rossi-Bernardi and Roughton (25) have found that solutions of oxy- and deoxyhemoglobin at the same pH and p_{CO_2} contain the same amount of CO_2. This would rule out a possible "oxygen linkage" of the CO_2 present in the β-heme pocket.

Little doubt is left in regard to the inhomogeneity of the CO_2 reactions of the four α-NH_2 terminal groups of the molecule. This finding explains the failure of previous attempts at analyzing CO_2 binding data in terms of only two constants, K_c and K_z (25). The data reported in this paper are in agreement with the results of Kilmartin *et al.* (27), who showed that the effect of CO_2 on the oxygen equilibrium of $\alpha_2\beta_2^c$ was different from that in the case of $\alpha_2^c\beta_2$. The present data cannot be used alone to identify which of the two pairs of α-amino groups of human hemoglobin has the highest affinity for CO_2. The method described, however, can now be applied to the study of the CO_2 binding properties of $\alpha_2\beta_2^c$ and $\alpha_2^c\beta_2$, making this identification possible.

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Supplemental Material
to
The Binding of CO_2 to Human Hemoglobin
by
Michele Perrella, Dario Bresciani and Luigi Rossi-Bernardi

EXPERIMENTAL PROCEDURE

Materials

Hemoglobin— DPG-free hemoglobin solutions were prepared using a combination of the Adair and Adair (2) and Berman *et al.* (3) methods. Enzymatic assay of DPG (4) showed that the residual organic phosphate concentration was less than 1% of the concentration of hemoglobin tetramer. The carbonic anhydrase inhibitors "diamox" (1 mg/ml) and "cardrase" (0.2 mg/ml) were added to a small sample (*ca* 20 ml) of either Hb or HbCO, kept under N_2 or CO in 100 ml bottles at room temperature. This sample was used for one day's experiments.

Gases and Reagents— Cylinders of CO_2 + N_2 of known CO_2 content were obtained from SIAD (Bergamo), and analysed using a Lloyd apparatus. Dextran gels were obtained from Pharmacia (Upsala). Other chemicals were analytical grade.

Methods

The method of Rossi-Bernardi *et al.* (5) and Perrella *et al.* (6) has been modified to make the procedure both suitable for the study of human hemoglobin and less time and material consuming.

(a) The Equilibration of Hb and HbCO with CO_2— Samples of Hb or HbCO (2 to 3 ml) were equilibrated with a gas phase containing CO_2 in a glass tonometer thermostatted at 37°. The tonometer was designed to obtain 99% deoxygenation of concentrated (up to 250 g/liter) normal hemoglobin in *ca* 30 min. Complete CO_2 equilibrium with a gas phase containing CO_2 can be obtained in *ca* 15 min.

(b) Separation of $HbCO_2$ from HCO_3^-–CO_3^{--} and Determination of $HbCO_2$— The two-layer column previously used (6) to separate $HbCO_2$ from other forms of combined CO_2 in solution, has been replaced by a small vessel where the ion-exchange process occurs. This procedure considerably shortens the time required for each $HbCO_2$ determination and allows a considerable (*ca* 10 times) saving of hemoglobin.

The reader is reminded that the reactions of CO_2 in an aqueous solution containing amino groups of amines or proteins ($R-NH_2$, where R may be hemoglobin) conform to the following scheme:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^- \qquad K_1 \qquad [1]$$

$$CO_2 + OH^- \rightleftarrows HCO_3^- \qquad K_{OH^-} \qquad [2]$$

$$HCO_3^- \rightleftarrows CO_3^{--} + H^+ \qquad K_2 \qquad [3]$$

$$CO_2 + R-NH_2 \rightleftarrows R-NHCOO^- + H^+ \qquad K_c \qquad [4]$$

$$R-NH_3^+ \rightleftarrows R-NH_2 + H^+ \qquad K_a \qquad [5]$$

K_c is the fundamental carbamate constant of the particular amino group and K_a its ionisation constant. (The undissociated carbamic acid species $R-NHCOOH$ can be neglected at pH > 6.8, [7]). In hemoglobin only two kinds of groups bind CO_2 carbaminowise, the ε-amino groups of lysine ($pK_a \sim 10$, [8]) and the four terminal α-amino groups ($pK_a \sim 7.5$, [9]). Eqs. [1-5] were first proposed by Faurholt (10).

Fig. 1 shows a schematic representation of the apparatus used. The hemoglobin solution, equilibrated with a known P_{CO_2}, is transferred anaerobically from the tonometer to syringe B and mixed with a triethylamine–HCl buffer in a simplified, hand-driven, continuous flow apparatus. A and B are 2 ml tuberculin syringes provided with thermostating jackets for temperature control. The mixture from M (at pH *ca* 11) is led into a 1 mm bore, 25 cm long plastic tubing, to ensure complete mixing of reagents within a few milliseconds, and from there delivered into the double-walled chamber R, thermostatted at 0 to 1°. The mixture is then left for 5 sec, during which time the free dissolved CO_2 reacts mainly with the newly deprotonated ε-amino groups of lysine residues of hemoglobin and disappears from solution. The rather low pH (~ 11), which is obtained a few milliseconds after mixing, ensures that CO_2 is taken up almost exclusively through reaction [4] and not through reaction [2]. The carbonic anhydrase catalysed reaction [1] is inhibited by the addition of carbonic anhydrase inhibitors. Next, 0.3 to 0.5 ml of ice cold 1 N KOH and 2 ml of a suspension of Bio-Rad AG 1 x 8 resin in the OH^- form (3 volumes of H_2O per volume of packed resin, pH 9) are added in rapid succession, thus raising the pH to 12 to 12.5. The purpose of this step is (*i*) to remove any HCO_3^- or CO_3^{--} by ion-exchange with OH^-, leaving $HbCO_2$ as the only form of CO_2 in solution; and (*ii*) to stabilise $HbCO_2$, which, at this pH, only slowly dissociates to carbonate. The hemoglobin solution is sampled from R and its carbamate content determined by gasometric analysis.

The overall elapsed time from mixing to sample collection was usually 150 ± 10 sec. The amount of resin and the stirring time needed to exhaustively remove carbonate ions from the resulting mixture in R were determined by replacing the hemoglobin solution with a carbonate–bicarbonate buffer. By determining the CO_2 content of this solution at different times and resin concentrations, it was found that a 30 to 40 sec reaction time and 2 ml of resin suspension diluted as described above were sufficient to remove all the HCO_3^- and CO_3^{--} ions.

$HbCO_2$ in syringe B can be calculated by multiplying the gasometric value by a factor which takes into account the dilution undergone by the hemoglobin in the various mixing steps from B to R. The dilution factor is obtained from the ratio between hemoglobin concentration in B and that in R. $HbCO_2$ so determined, however, consists of two quantities: (*i*) carbamate of hemoglobin, and (*ii*) CO_2 which, originally present in B as dissolved CO_2, was taken up by hemoglobin in the first mixing step with TEA–HCl buffer. The dissolved CO_2 can be calculated from Q', the solubility coefficient of CO_2 in the hemoglobin solutions, and the P_{CO_2}, and subtracted from $HbCO_2$ to give the carbamate in the hemoglobin solution contained in B. By knowing the hemoglobin concentration it is thus possible to obtain ϕ, the total CO_2 bound per hemoglobin tetramer, and Z, the fractional saturation of CO_2 per subunit. The Hb concentration was obtained by first diluting the protein in 0.1 N KOH and then reading its OD at 390 nm.

(c) pH Determination and Control— The pH of the equilibrated solution of hemoglobin was measured as previously described (11), and adjusted to the required value by the addition of 1 or 2 M $KHCO_3$. Perrella *et al.* (6) have shown that it is desirable, for further theoretical analysis, to obtain CO_2 binding data at constant pH. Since pH adjustments were accurate to within 0.01 pH units, binding data were corrected to a constant pH by the use of factors derived from data at constant P_{CO_2} and varying pH (see text and Fig. 3). Corrections for such deviations never exceeded 1% saturation of the protein with CO_2.

(d) CO_2 Analysis— Total CO_2 determinations were carried out according to Peters and Van Slyke (12) in a standard Van Slyke apparatus. The presence of the volatile triethylamine buffer made it necessary to avoid the use of strong alkali for CO_2 absorption and a glycine–NaOH buffer, pH 9, was used instead.

(e) Determination of the Solubility Coefficient of CO_2 in Hemoglobin Solutions— Two different methods were used: (*i*) the original Van Slyke *et al.* method (13), and (*ii*) the carbamate method described in this paper.

(i) HbCO solutions at pH 4.6 were prepared by rapidly mixing hemoglobin with appropriate amounts of 1 N HCl in a continuous flow apparatus of the Hartridge–Roughton type (14). The solution was then spun at 15,000 x *g* x 20 min to remove insoluble material, and equilibrated as soon as possible with a gas phase of known P_{CO_2}. The use of this procedure was essential to avoid extensive precipitation of the protein which inevitably occurs if the acidification is carried out by dropwise addition of the acid. After equilibration the total CO_2 of the acidified sample is determined. At pH ~ 4.6, *ca* 4% of this total CO_2 is present in solution as HCO_3^-. This, in turn, can be estimated from the Henderson–Hasselbach equation, assuming pK_1' values (eq. [1]) taken from Edsall and Wyman (15) by interpolation of data for dissolved CO_2 in NaCl solutions. The ionic strength of the acidified hemoglobin solutions at pH ~ 4.6 was 0.15 to 0.20, and, therefore, a value of $pK_1' = 6.032$ was assumed to correct for the HCO_3^- present in solution. If the apparent pK of carbonic acid in concentrated hemoglobin solutions, as calculated in this paper (see later), is used, a value of Q' *ca* 1% higher is obtained.

(ii) The second method for CO_2 solubility measurements consisted of the determination of the carbamate formed by rapidly mixing a hemoglobin solution, equilibrated at pH *ca* 6.2 with a gas phase of suitable P_{CO_2}, with a TEA–HCl buffer as described in *(b)*. At P_{CO_2} = 160 mm Hg and pH = 6.2, deoxyhemoglobin forms approximately 2 to 4% $HbCO_2$ per Hb tetramer. Therefore, only data obtained at pH = 6.2 using HbCO and data obtained with Hb at low P_{CO_2} have been considered for Q' determination by this method. It should be added that it was not possible to use a value of pH lower than 6.2, as at acid pH both acetazolamide and ethoxzolamide do not fully inhibit carbonic anhydrase and some free CO_2 (up to 20 to 30%) escapes detection since reaction [1] becomes competitive with reaction [4].

An extensive series of blank experiments was carried out to validate the method used for $HbCO_2$ determination. Such experiments conclusively proved that (*1*) the dissolved CO_2 is completely taken up by the amino groups of hemoglobin upon mixing with the TEA–HCl buffer, (*2*) the $HbCO_2$ originally present in solution does not appreciably dissociate during the time interval between mixing with the buffer and sample collection for CO_2 analysis, (*3*) the hemoglobin concentration of the sample from R is correctly estimated, and (*4*) no denatured hemoglobin is bound to the resin.