A Calorimetric Study of the Bohr Effect for the Reaction of Human Hemoglobin with Carbon Monoxide†

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ABSTRACT: The heat of reaction between carbon monoxide (CO) and human hemoglobin (Hb) in various buffer solutions has been measured using a gas–liquid microcalorimeter. The buffer solutions used cover the range pH 6–9. At each pH pairs of buffers were chosen with widely differing heats of ionization ΔH_i. The hydrogen ions released upon CO uptake are absorbed by buffers of differing ΔH_i with differing observable heats. From these heat measurements the number of protons released per mole of ligand bound was calculated. The values determined calorimetrically agree with titration measurements (Antonini *et al.* (1965), *J. Biol. Chem. 240*, 1096). The heat of reaction between CO and Hb, excluding heats of proton uptake by buffers, was found to vary from −17.7 to −23 kcal per mol of CO from pH 6.35 to 9.20. This variation agrees with the predictions of the model used by Antonini *et al.* ((1965), *J. Biol. Chem. 240*, 1096) whereby the origin of the Bohr effect is explained based on two ionizable groups in the hemoglobin molecule. A constant value (within experimental error) for the enthalpy of reaction of CO and Hb, *after correction for ionization processes in the protein*, is found to be −23.2 ± 1.5 kcal/mol of CO over the range pH 6–9 at 25°.

Owing to their physiological importance, the reactions between hemoglobin and gaseous ligands such as O_2 and CO have been the subjects of many thermodynamics studies over the past 40 years. These studies have been reviewed by Antonini and Brunori (1971). In spite of the considerable effort spent in determining the basic thermodynamic parameters of these reactions, there remains substantial uncertainty concerning the precise values of these quantities. This is due largely to the cooperative nature of the four-step reaction process. The high degree of cooperativity causes practical difficulty in determining individual equilibrium constants. There are even more serious problems in the determination of derivative properties, particularly the enthalpies of reaction obtained from the temperature variation of the equilibrium constants. Uncertainties in these individual reaction enthalpies are collected in turn in the uncertainty in the total heat of reaction. This unsatisfactory situation is inevitable where van't Hoff methods are used to obtain the total enthalpy for a hemoglobin ligand binding process, because the lack of complete cooperativity in the reaction makes the total enthalpy accessible only through the evaluation of heats for the separate steps.

The calorimetric determination of heats of reaction avoids the preceding problems. Roughton (1935, 1936) was first to employ a direct technique to obtain average heats of reaction between O_2 and Hb.[1] His values agreed within limits of experimental error with the average enthalpy values obtained from temperature variation of the saturation curve.

In an effort to obtain additional information on details of the enthalpy change of the hemoglobin–gas ligand reaction, we have used a gas–microcalorimetric technique (Rudolph *et al.*, 1972). In the present work we have studied CO in order to permit use of hemoglobin samples completely deoxygenated by sodium ascorbate and to avoid any formation of methemoglobin during the calorimetric run. In view of concern about possible deleterious effects to the protein of the ascorbate reduction, we also studied a few samples prepared by simple deoxygenation with N_2 without the use of ascorbate. Our attention has been directed primarily toward obtaining average heats of reaction in various buffers to study the Bohr effect (Bohr *et al.*, 1904) by direct calorimetry and to interpret our measurements in light of titration studies by Antonini *et al.* (1965) and Bailey *et al.* (1970).

Experimental Section

Adult human hemoglobin was used in all experiments. The hemoglobin was prepared at frequent intervals from small samples (10–20 ml) of freshly drawn blood. The blood was mixed immediately with 0.2 volume of 3.2% sodium citrate solution and centrifuged for 10 min at 10,000*g* at 4°. The plasma was then replaced with an equal volume of isotonic saline solution and the cells were recentrifuged. This was repeated four times or until a clear, colorless supernatant was obtained. The isotonic saline was then replaced with an equal volume of distilled water and allowed to sit for 2–4 hr at 4°. The hemolyzed cells were then centrifuged at 20,000*g* at 4° until a clear solution was obtained. The hemoglobin solution was then drawn off and the red cell ghosts were discarded. In order to remove small molecules, particularly DPG, the supernatant was dialyzed against two 2-l. changes of 2 M NaCl containing 0.02 M Tris-HCl at pH 8.5 (see Benesch *et al.*, 1969). This was followed by dialysis against distilled water and finally by dialysis against the buffer (prepared at 0.3 M concentration ionic strength by adding NaCl) to be used in the experiment. The procedure gave a solution of oxyhemoglobin having an α/β spectrophotometric peak height ratio of 1.065–1.070. Deoxygenation was accomplished either by the addition of sodium ascorbate to a concentration of 0.01 M or by swirling in a large flask under a stream of reagent grade nitrogen. Both methods of deoxygenation gave comparable results at pH 7.1. The hemoglobin solution was then stored in a

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[1] Abbreviations used are: Hb, hemoglobin; Mb, myoglobin; DPG, 2,3-diphosphoglycerate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE I: Carbon Monoxide Plus Hemoglobin: Enthalpy of Reaction and Calorimetrically Determined Proton Release at 24°.

pH	ΔH_{CO} (kcal/mol of CO)	n (mol of H^+/ mol of CO)
6.35	−17.7 ± 0.3	0.3 ± 0.07
7.10	−17.3 ± 0.4	0.58 ± 0.05
7.85	−17.5 ± 1.0	0.4 ± 0.20
8.60	−22.8 ± 1.0	0.2 ± 0.10
9.20	−23.0 ± 1.0	0

graduated vessel having a spectrophotometric cell attached at the bottom. This arrangement allowed convenient checking of the visible spectrum of the preparation. After deoxygenation, the hemoglobin solution had a single shoulderless peak at 555 nm. The peak height was used to determine Hb concentration using ϵ_{555} = 12,300 M^{-1} cm^{-1} (Antonini and Brunori, 1971). Heme concentrations used in the experiments were in the range 1–4 mM (1.7–6.8% Hb).

The calorimeter is a highly sensitive gas–liquid reaction microcalorimeter that allows measurement of both heat evolution and gas uptake simultaneously. The calorimetric procedures and instrumentation used were the same as those used in a series of experiments on the myglobin–CO reaction and are described in detail in that paper (Rudolph *et al.*, 1972). The calorimetric sample cell used was an improved type constructed of gold-plated copper and requiring a correction for heat leakage of less than 2%.

Results

The heat evolved as a function of gas uptake for the reaction Hb(aq) + 4CO(g) → $Hb(CO)_4$(aq) was measured over the pH range 6.35–9.20. In this paper only average heats per mole of CO reacted will be reported; the determination of the individual heats for the four successive steps of the reaction is quite complex and will be discussed in detail in a subsequent publication.

It will be recalled that the binding of ligands to Hb changes the pH of certain ionizable groups on the protein resulting in a net loss of hydrogen ions in the alkaline pH range studied. This is known as the alkaline Bohr effect (Bohr *et al.*, 1904). The reaction can be written as

$$Hb(CO)_{i-1}(aq) + CO(g) \longrightarrow Hb(CO)_i(aq) + n_i H^- \quad (1)$$

where i = 1, 2, 3, 4. For the present we will assume that n_i is independent of i (*i.e.*, $n_i = n$ for i = 1–4). Because of this proton evolution, it was always necessary to have sufficient buffer present to absorb the protons and prevent large changes in pH. (Buffer concentrations were chosen to maintain $\Delta pH < 0.02$.) The observed average heat of reaction per mole of CO, ΔH_{obsd}, is given by

$$\Delta H_{obsd} = \Delta H_{CO} + n\Delta H_b \quad (2)$$

where ΔH_{CO} is the heat of the reaction per mole of CO averaged over the four reaction steps and ΔH_b is the heat of protonation of the buffer. Measuring the heat of reaction at the same pH, but in a buffer having a different heat of protonation ($\Delta H_b'$), gives

$$\Delta H_{obsd} = \Delta H_{CO} + n\Delta H_b' \quad (3)$$

TABLE II: Buffers Used with Heats of Ionization for Results Given in Table I.

pH	Buffers	$\Delta H_{protein}$ (kcal/mol)
6.35	Mes	−4.5[a]
	Sodium maleate	+0.8[b]
7.10	Tris-HCl	−11.3[b]
	Sodium maleate	+0.8[b]
7.85	Tris-HCl	−11.3[b]
	Hepes	−5.1[c]
8.20	Tris-HCl	−11.3[b]
	Sodium borate	−3.3[b]
9.20	Sodium borate	−3.3[b]

[a] Calculated from value of dpK_a/dT given by Good *et al.* (1966). [b] Sober *et al.* (1970). [c] Beres and Sturtevant (1971).

This gives us a calorimetric method for determining n, since

$$n = (\Delta H_{obsd} - \Delta H_{obsd}')/(\Delta H_b - \Delta H_b') \quad (4)$$

This value of n can be used to calculate ΔH_{CO}. The results of these experiments are summarized in Table I. Table II shows the buffer pairs used in these experiments and the values of ΔH_b used to compute n.

Discussion

The shape of the O_2 saturation curve for human hemoglobin was found by Allen *et al.* (1950) and by Roughton (1964) to be unaffected by pH except for possible deviations at very low or very high fractional saturations. This invariance with pH implies that each step of the reaction process is similarly affected by pH. In principle precise calorimetric data determined for different extents of reaction and in various buffers should also test this idea. We did observe linear displacement in heat values for different buffers at the same pH at different extents of reaction. However our heat measurements in this work could not be made with high accuracy in the regions of small and large extents of reaction due to the use of relatively small samples. It is in these limiting regions where one might find differences reflected if the initial and final steps of the reaction have different amounts of protons released. The calorimetric curves presently suggest that the release of hydrogen ions occurs similarly in each step of the reaction. Refined techniques will be needed to answer this question more certainly. The stereochemical theory of the Bohr effect proposed by Perutz (1970) predicts that a similar release of protons occurs with each reaction step.

A comparison of our calorimetric ΔH_{CO} values shown in Table I can be made with van't Hoff determined values for the average reactions

$$Hb(aq) + O_2(g) \longrightarrow HbO_2(aq) \qquad \Delta H_5 \quad (5)$$

and

$$HbO_2(aq) + CO(g) \longrightarrow HbCO(aq) + O_2(g) \qquad \Delta H_6 \quad (6)$$

At pH 7.3, ΔH_5 = −10 kcal/mol (Hill and Wolvekamp, 1936), at pH 7.0, ΔH_5 = −9.5 kcal/mol (Amiconi *et al.*, 1969) and at pH 9.5, ΔH_5 = −14.5 kcal/mol (Antonini *et al.*, 1965) for human hemoglobin at room temperature. The heat of the second reaction ΔH_6 was determined by Roughton (1954) as −5 kcal for sheep hemoglobin from the effect of temperature on the equilibrium constant. The combination of these values

TABLE III: Calculation of Intrinsic Enthalpies of Reaction ΔH_0 for Hemoglobin and CO at 25°.

pH	n_{NH} (mol of H^+/mol of O_2)[a]	n_{COOH} (mol of H^+/mol of O_2)[a]	ΔH_{ion} (= 9.0·n_{NH} − 1.5n_{COOH}) (kcal/mol)	ΔH_0 (kcal/mole)
6.35	+0.45	−0.37	4.6	−22.3 ± 0.6
7.10	+0.67	−0.12	6.2	−23.5 ± 0.7
7.85	+0.38	0.00	3.5	−21.0 ± 1.2
8.60	+0.15	0.00	1.3	−24.1 ± 1.2
9.20	+0.03	0.00	0.3	−23.3 ± 1.0

[a] Taken from Antonini *et al.* (1965).

gives an overall estimate for ΔH of −15 kcal/mol at pH 7 to −20 kcal/mol at pH 9.5 for

$$Hb(aq) + CO(g) \longrightarrow HbCO(aq) \quad (7)$$

Our direct calorimetric values show the same trend of increasing exothermicity with increasing pH by about 5–6 kcal from pH 7 to 9.5. The calorimetric values are slightly larger than the van't Hoff estimates, possibly due to oversimplification in using the van't Hoff equation for this multistep system.

A comparison of heats of reaction of CO and O_2 with myoglobin, or single isolated chains of hemoglobin, at pH 7–8 can also be made. Values of ΔH at pH 7 and 25° for the reaction

$$Mb(aq) + CO(g) \longrightarrow MbCO(aq) \quad (8)$$

have been measured calorimetrically as −18.1 kcal/mol (horse) and −20.7 kcal/mol (Rudolph *et al.*, 1972; Keyes *et al.*, 1971). van't Hoff determinations for the reaction of isolated α or β of Hb

$$Hb(chains)(aq) + O_2(g) \longrightarrow HbO_2(chains)(aq) \quad (9)$$

give ΔH = −13.5 kcal/mol at pH 7 (De Renzo *et al.*, 1967). This value modified by inclusion of the −5 kcal heat for O_2 replacement by CO would provide an estimate of ΔH = −19 kcal/mol for the Hb (chain) reaction with CO(g). Thus the single chain reaction with CO is perhaps only slightly more exothermic than the Hb reaction with CO at neutral pH values. For alkaline pH values of 9.5 the normal hemoglobin reaction is considerably more exothermic by approximately 3–4 kcal/mol. Enthalpy differences between single-chain species and normal hemoglobin are not surprising in view of presence of the Bohr effect in normal hemoglobin.

The pH dependence of the overall heats of reaction ΔH_{CO} between hemoglobin and CO(g) is shown from the results of Table I at 25°. These results can be interpreted in the light of the release amount of H^+ upon reaction with CO. Antonini *et al.* (1965) carried out an extensive set of pH and titration measurements on deoxy- and oxyhemoglobin from which they concluded that the release of hydrogen ions upon oxygenation at different pH values depends upon two ionization processes, one probably associated with a carboxyl group and the other with an imidazole group. By suitable selection of ionization constants for these two groups in the oxy and deoxy forms of hemoglobin their data for the proton release could be accurately fitted over the range of pH values from 5 to 9. Their data taken at the different temperatures could be fitted to a single curve by using an enthalpy of ionization for the carboxyl group of −1.5 kcal and for the imidazole group of +9.0 kcal. They concluded that the inherent heat of oxygenation of hemoglobin when corrected for the heat effects accompanying the process is a constant. They state: "The most critical test of the interpretation just given would lie in direct determination of the heat of oxygenation at the two ends of the region occupied by the Bohr Effect. Unfortunately, hemoglobin is too unstable at low pH to allow of this . . ." Our direct measurements in the higher pH range do give the means for testing the constancy of the inherent heat of the carbon monoxide reaction with hemoglobin. In order to do this we have read from their Figure 4 the moles of protons released per mole of oxygen reaction at the appropriate pH values from the imidazole groups n_{NH} and from the carboxyl groups n_{COOH} at 25°. We then calculate the heat effect, ΔH_{ion}, of these ionization effects using +9.0 kcal/mol of H^+ and −1.5 kcal/mol of H^+, respectively. Values of n_{NH}, n_{COOH}, and ΔH_{ion} are shown in Table III. The intrinsic heat of reaction ΔH_0 is calculated from the observed calorimetric values ΔH_{CO} and the values of ΔH_{ion} by the equation

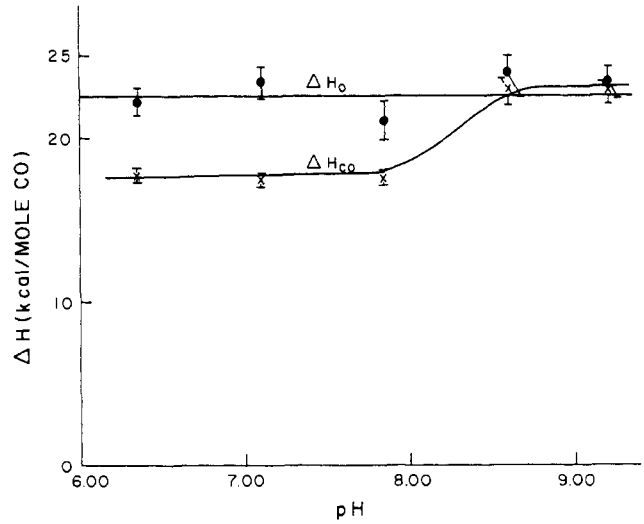

FIGURE 1: Heat of reaction of CO with hemoglobin at various pH values. ΔH_{CO} is the heat reaction corrected for buffer reaction with released Bohr protons. ΔH_0 is the heat of reaction corrected for heats of ionization of Bohr protons as assumed by Antonini *et al.* (1965).

$$\Delta H_0 = \Delta H_{CO} - \Delta H_{ion}$$

The values of ΔH_0 are found to be essentially constant over the range of pH from 6.35 to 9.20. Thus the interpretation that there is an intrinsic heat of reaction independent of pH along with a pH-dependent heat effect associated with two ionization processes is strongly supported by these results. The average ΔH_0 value of −23.2 ± 1.5 kcal/mol of CO is about −3 kcal/mol of CO more exothermic than the measured values for the myoglobin CO reaction with horse and whale species. The structural features of hemoglobin and the changes which occur (Perutz, 1970) upon reaction with ligands very likely are the basis for the observed differences in the enthalpies of reaction between myoglobin and hemoglobin.[2]

The use of different buffer systems with different heats of protonation as shown in Table II provided a means for an independent determination of the total moles of protons n released per mole of reacted CO. Our values for n agree within

[2] Some more detailed measurements in our laboratory on the heat of reaction between hemoglobin and CO as a function of extent of reaction indicate that the initial addition of CO produces a different heat effect than the terminal addition of CO. For a detailed discussion of this phenomenon in the Hb–O_2 system, see Noll *et al.* (1974).

experimental error with the results of Antonini *et al.* (1965) who studied the Bohr effect on solutions made in 0.25 M NaCl. The more recent work by Bailey *et al.* (1970), who took particular care to remove DPG, also is in reasonably close agreement with the results of Antonini *et al.* (1970). The general consistency of the n determinations from our heat measurements in different buffers with these other workers also suggests that the buffers used in our work do not produce any structural changes in hemoglobin which affect the CO reaction. The buffers chosen, in particular those introduced by Good *et al.* (1966), would not be expected to have strong tendencies to associate with proteins. Indeed Tris buffer has been shown to have no effect on the oxygen saturation curve of hemoglobin (Benesch *et al.*, 1969).

We feel the most important conclusion of the present work as shown in Figure 1 is the verification of a constant component of the enthalpy of reaction with CO that is independent of the release and rearrangement of hydrogen ions in the region of the alkaline Bohr effect. This result provides corroboration of those of Antonini *et al.* (1965).

References

Allen, D. W., Guthe, K. F., and Wyman, J. (1950), *J. Biol. Chem. 187*, 393.

Amiconi, G., Antoni, E., Brunori, M., and Wyman, J. (1969), unpublished data.

Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobin and Their Reactions with Ligands, New York, N. Y., American Elsevier Publishing.

Antonini, E., Wyman, J., Brunori, M., Frontincelli, C., Bucci, E. and Rossi-Fanelli, A. (1965), *J. Biol. Chem. 240*, 1096.

Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronticelli, C., and Rossi-Fanelli, A. (1963), *J. Biol. Chem. 238*, 2950.

Bailey, J. F., Beetlestone, J. G., and Irvine, D. H. (1970), *J. Chem. Soc. A 5*, 756.

Beres, L., and Sturtevant, J. M. (1971), *Biochemistry 10*, 2120.

Benesch, R. E., Benesch, R., and Yu, C. I. (1969), *Biochemistry 8*, 2567.

Bohr, C., Hasselbach, K., and Drought, A. (1904), *Skand. Arch. Physicol. 16*, 402.

De Renzo, E., Ioppolo, C., Amiconi, G., Antonini, E., and Wyman, J. (1967), *J. Biol. Chem. 242*, 4850.

Good, N. E., Winget, G. D., Winter, W., Connally, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5*, 467.

Hill, R., and Wolvekamp, H. P. (1936), *Proc. Roy. Soc., Ser. B, 120*, 484.

Keyes, M. H., Falley, M., and Lumry, R. (1971), *J. Amer. Chem. Soc. 93*, 2035.

Noll, L., Barisas, B. G., and Gill, S. J. (1974), *Biochem. Biophys. Res. Commun. 56*, 555.

Perutz, M. F. (1970), *Nature (London) 228*, 726.

Roughton, F. J. W. (1935), *Biochem. J. 29*, 2605.

Roughton, F. J. W. (1936), *Biochem. J. 30*, 2117.

Roughton, F. J. W. (1954), *Physiology 126*, 259.

Roughton, F. J. W. (1964), Oxygen in the Animal Organism, London, Pergamon Press, p 5.

Rudolph, S. A., Boyle, S. O., Dresden, C. F., and Gill, S. J. (1972), *Biochemistry 11*, 1098.

Sober, H. A., Harte, R. A., and Sober, E. K. (1970), Handbook of Biochemistry, Cleveland, Ohio, The Chemical Rubber Co., Chapter J-58.

Wyman, J. (1939), *J. Biol. Chem. 127*, 581.