constant of the uncatalyzed reaction, $k_2 = k_{23}k_{12}/k_{21} \approx 65\ M^{-1}\ sec^{-1}$, yields $k_{23} \approx 1 \times 10^6\ sec^{-1}$. Thus, there is (for the two systems under consideration) no appreciable dependence of the rate of (uncatalyzed) intramolecular proton transfer on the free-energy change of the reaction—analogous to intermolecular proton transfer reactions[25]—as long as the transfer occurs in the thermodynamically favored direction. As indicated by a molecular model of the zwitterion species, the relatively low value of k_{23} could possibly be due to a slight barrier to rotation around the C–N bond which might affect the efficiency of direct N–O proton transfer or of H_2O-mediated concerted proton transfer, respectively.

The analysis of the kinetic data obtained at low pH brought evidence for an additional catalytic rate term, which is to be interpreted either as general acid catalysis of the reaction with free piperazine by piperazine dication or as the reaction with piperazine monocation catalyzed by piperazine monocation. A distinction between the two possibilities cannot be made.

Acknowledgments. Thanks are due to Professor M. Eigen and Professor J. Kirsch for discussions. We would also like to thank Professor W. P. Jencks and Dr. J. M. Sayer for helpful comments. R. N. F. T. is grateful to the CIBA Fellowship Trust and the Max-Planck-Society for Postdoctoral Fellowship Awards.

Calorimetric Determination of Azide-Ion Binding by Ferrihemoglobin A in Water and in 5% *tert*-Butyl Alcohol[1,2]

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Contribution from the Laboratory for Biophysical Chemistry, Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received July 10, 1972*

Abstract: Ligand binding data for the ferrihemoglobins obtained by Beetlestone and coworkers demonstrate a linear relationship between standard enthalpy and entropy changes for variation of pH, salt concentration, and species. The slope is 285 ± 15°, the same range found in small solute processes in water. Since confirmation of these results at the highest possible resolution is required, the binding of a typical ligand, azide ion, to human ferrihemoglobin A has been carried out calorimetrically using highly purified protein preparations. The standard free energies and enthalpies of binding obtained in the absence of cosolvents conform within small errors to the previous van't Hoff results and thus confirm the existence of the pattern. The so-called "turn around" or characteristic pH is the same and the slope of the ΔS *vs.* ΔH plot is 280° within error. As a first check on the possibility that water participates directly in these reactions the experiments were repeated with 5% *tert*-butyl alcohol added as cosolvent. Available evidence indicates no effect of the alcohol on the protein through direct binding. The slope of the $\Delta H°$ *vs.* $\Delta S°$ line is the same but the intercept is 0.8 kcal/mol less negative showing that the alcohol cosolvent alters the binding process quantitatively but not qualitatively. The results appear to be attributable to alteration in water mediated through the protein.

The binding of azide to ferrihemoglobin has been studied by many workers[3–6] as a function of pH, species, and temperature and it has been concluded that the thermodynamic parameters resemble those of other ligands such as SCN^-, F^-, etc., *viz.* the standard free energy of binding is generally insensitive to pH variation but the standard enthalpies and entropies of binding are pH dependent and in such a way that $\Delta H°$ and $T\Delta S°$ tend to cancel each other (compensation).[7] Values of $\Delta H°$ as a function of pH show a maximum which correlates roughly with the isoelectric point of the species. In all previous studies $\Delta H°$ has been obtained by the van't Hoff method using spectral changes of the heme group. The high degree of linearity of the van't Hoff plots implied a negligible heat-capacity change. Confirmation of these interesting results is especially important because the same pattern of enthalpy–entropy compensation is being demonstrated in more and more protein systems. These demonstrations may be accidental and it is important to establish their validity in all cases at the highest possible level of precision. The calorimetric method is distinctly better than the van't Hoff method and particularly so when heat-capacity effects may be large. We have repeated the N_3^- binding studies of Beetlestone and coworkers with human ferrihemoglobin A at 25.9° using a flow microcalorimeter.

Interpretation of the pH dependence of $\Delta H°$ and $\Delta S°$ for the ferrihemoglobin ligand binding reactions has been based on changes in the hydration sheath provoked by tautomeric shifts of the imidazole proton.[6] As an initial test of the role of water in the hydration sheath and in the bulk water phase we have studied the reaction in the presence of *tert*-butyl alcohol and observed changes in $\Delta H°$ and $\Delta S°$ especially at the more acid pH values. The significant effects we observed even at low alcohol concentration provide some support for the idea that water plays a direct role in the reaction of ferrihemoglobins.

(1) This is paper no. 73 from this Laboratory. Please request reprint by this number.

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Experimental Section

Materials. Hemoglobin samples were prepared from outdated blood bank human blood using the method described by Beetlestone, *et al.*,[8] and were further purified by chromatography on a Sephadex G-100 column, using phosphate buffer pH 6.2 as eluent. Oxidation to ferrihemoglobin was achieved by addition of a two-fold excess of $K_3Fe(CN)_6$ to the sample. The excess $K_3Fe(CN)_6$ and the $K_4Fe(CN)_6$ were removed by passing the oxidized sample through a Dintzis column[9] equilibrated with buffer pH 6.2. This procedure provides a major additional step in purification. Phosphate buffer solutions were used in the region below pH 8.0 and borate buffers above pH 8.0. The ionic strength of these solutions was adjusted to 0.05 with NaCl. Concentration of phosphate and borate solutions was 0.01 *M*.

Purified NaN_3 obtained from the Fisher Scientific Co. was used without additional purification. The concentrations of stock azide solutions were determined by the back titration of residual ceric ammonium nitrate in acid solution, after excess ceric ammonium nitrate was added to an azide solution. The titrant was an acid solution of ferrous sulfate.[10,11]

Concentrations of methemoglobin samples were determined as the cyanomethemoglobin complex at 540 mμ using 10.9 as the millimolar extinction coefficient at this wavelength.[12,13] The determinations were made with a Cary Model 16 spectrophotometer just prior to calorimetric runs. The pH values of all solutions were determined prior to a run as well as in the effluent of each run. Usually the two values agreed within ±0.01 pH unit. Changes in pH reading caused by addition of *tert*-butyl alcohol at this small mole fraction can be neglected.[14]

Determination of Ionization Constant. The ionization constant for the ionization of the water at the sixth coordination position in ferrihemoglobin was determined in 5% *tert*-butyl alcohol by the method described by Beetlestone, *et al.*,[15,16] using a Cary Model 11 spectrophotometer with a constant temperature cell compartment held at 25.9 ± 0.05°. The method used for determination of the ionization constant of hydrazoic acid is that described by Burn and Chang.[17]

Calorimetric Measurements. Calorimetric determinations were made with an LKB 10700-1 microflow calorimeter. The signal from the calorimeter was amplified with a Fluke 845AB high impedance voltmeter and recorded on a Honeywell Electronik 194 recorder.

Electrical calibration of the calorimeter was checked from time to time by measuring the heat of dilution of sucrose. Approximately equal volumes of the reacting solutions were pumped into the calorimeter vessel using two LKB 10200 Perpex peristaltic pumps producing a flow rate of each solution of approximately 0.009 ml/sec. The steady heat flux produced under these conditions ranged from about 2 to 20 μcal/sec. Corrections for the heats of dilution of the Hb^+ and N_3^- solutions were made in the conventional way (*vide infra*).

Results

Insofar as can be judged from the spectra of ferrihemoglobin samples in aqueous solution and in 5% *tert*-butyl alcohol the addition of 5% *tert*-butyl alcohol does not change the state of the protein. Our determination of p*K* values which is generally believed to apply to the ionization constant for the water molecule attached to the sixth coordination position of the iron atom in 5% *tert*-butyl alcohol gave 8.07 ± 0.10 at 25.9° compared to a value of 8.10 obtained by Beetlestone, *et al.*,[15,16] for the same ionization at 24° in aqueous solutions. The agreement demonstrates that the alcohol even though a better nucleophile than water does not replace the water at the sixth coordination position of heme iron. The spectra were not the same in the two systems in the visible region but the isosbestic point at 525 mμ was preserved indicating only a quantitative difference. At 10% *tert*-butyl alcohol the quantitative differences in ferrihemoglobin behavior indicated more complications. Results reported here are for 5% *tert*-butyl alcohol only.

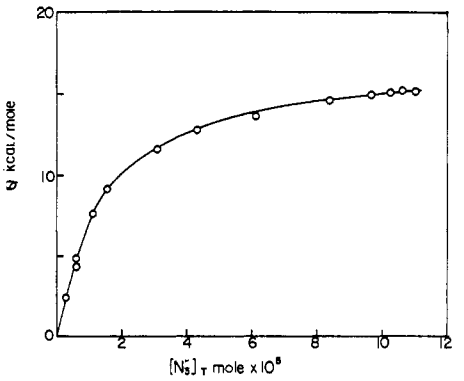

Figure 1. Plot of corrected heat of reaction *Q vs.* total azide concentration, for 5% *tert*-butyl alcohol system.

Heats of dilution for Hb^+ and N_3^- solutions did not differ significantly in the two systems. In the concentration range of our studies ([Hb] ≃ 5 × 10^{-5} g-mol of Fe/l.) the heats of 1:1 dilution of Hb^+ solutions were 0.5 kcal/mol or less; 1:1 dilution of N_3^- solutions ($[N_3^-]$ = up to a maximum of 10^{-3} *M*) produced heats of 0.4 μcal/sec or less; We have compared the heats of solvation and dilution in water and 5% *tert*-butyl alcohol by measuring the heats evolved when 10% *tert*-butyl alcohol was diluted to 5% *tert*-butyl alcohol with solutions containing about 10^{-3} *M* N_3^- and comparing this to dilutions of 10% *tert*-butyl alcohol with water. Heats of dilution of 10^{-3} N_3^- in pure water were also determined. From the aggregate of these determinations we could detect any changes in heat contributed by solvation of N_3^- in 5% *tert*-butyl alcohol. Within the calibration error of ±0.04 μcal/sec the heats of dilution and solvation of N_3^- were the same in water or 5% *tert*-butyl alcohol. It is therefore probable that at the low concentration of alcohols used there is no significant change in the heats of solvation of the species in the two systems.

Figure 1 shows the dependence of the corrected heat of reaction of the total N_3^- concentration in the final calorimetric solution. The experimental heat of reaction measures the amount of N_3^- complex formed. The reaction between N_3^- and ferrihemoglobin may in general be represented by

$$Hb^+OH_2 + N_3^- \overset{K}{\rightleftharpoons} HbN_3 + H_2O \qquad (1)$$

which yields

$$K = \frac{[HbN_3]}{[Hb^+OH_2][N_3^-]} \qquad (2)$$

As shown elsewhere[5,6] the following equilibrium constants are defined by

$$K_{obsd} = \frac{[HbN_3]}{([Hb^+OH_2] + [HbOH])[N_3^-]}$$

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Table I. Values for the Binding Constant K, $\Delta H°$, and n (the Stoichiometry)[a]

	$K \times 10^{-4}$, mol^{-1}	n	$\Delta H°$, kcal/mol	n
Hb^+ varied	3.61 ± 0.11	1.002 ± 0.05	14.97 ± 0.66	0.995 ± 0.10
N_3^- varied	3.59 ± 0.26		15.09 ± 0.53	

[a] Determined by varying concentrations of Hb^+ and N_3^-; pH 6.50; *tert*-butyl alcohol system. For the method of calculation see ref 19

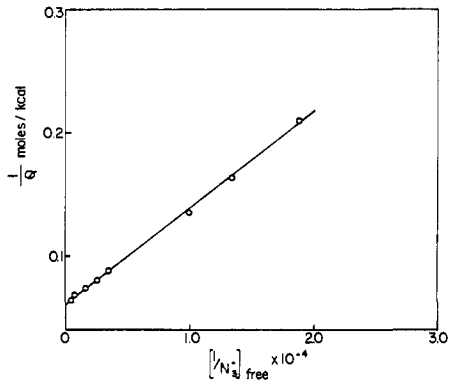

Figure 2. Double reciprocal plot of $1/Q$ (mol/kcal) *vs.* $1/[N_3^-]_{free}$ assuming $n = 1$; pH 7.0; for aqueous system.

$$K_a = \frac{[HbOH][H^+]}{[Hb^+OH_2]}$$

where $[Hb^+OH_2] + [HbOH]$ is the total concentration of ferrihemoglobin that has not reacted with N_3^-. $[HbN_3]$ is the concentration of ferrihemoglobin azide and K_a is the equilibrium constant for the ionization of the water at the sixth coordination position. It is readily shown that

$$K = K_{obsd}(1 + K_a/[H^+])$$

The final value of K used is corrected for the hydrazoic acid which is present at the more acid pH values. As Bjurulf and coworkers[18] have pointed out, the above equation can be expressed for a 1:1 complex in terms of the heat of reaction as eq 3

$$K_{obsd} = \frac{[E_t]Q/Q_{max}}{([E_t] - [E_t]Q/Q_{max})([I_t] - [E_t]Q/Q_{max})} \quad (3)$$

where in our study $[E_t]$ is the total concentration of Hb^+, $[I_t]$ is the total concentration of N_3^-, and Q_{max} is the heat of reaction per mole of Hb^+ for complete saturation of all four binding sites.

In a more general case where n represents the stoichiometry of the reaction following Bolen, *et al.*,[19] we can write eq 3 as eq 3a

$$K_{obsd} = \frac{[E_t]Q/Q_{max}}{([E_t] - [E_t]Q/Q_{max})([I_t] - n[E_t]Q/Q_{max})} \quad (3a)$$

which reduces to

$$\frac{1}{Q} = \frac{1}{Q_{max}} + \frac{1}{Q_{max}K[I_F]} \quad (4)$$

$[I_F]$ is the concentration of the free azide in the final calorimetric solution and is given by

$$[I_F] = [I_t] - nQ/Q_{max}[E_t] \quad (5)$$

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The best values of Q_{max} and K may be obtained from the data by iterative least-squares treatment according to eq 3a with proper weighting.

In Figure 2 we show a double reciprocal plot of $1/Q$ (moles/kilocalories) *vs.* $1/[N_3^-]_{free}$ assuming $n = 1$. There is an excellent linear relationship over a wide concentration range. For other values of n we obtain curvatures outside experimental error. We have also carried out the more rigorous test suggested by Bolen, *et al.*,[19] in their study of binding of 3′-cytidine monophosphate to ribonuclease A, in which $[N_3^-]$ is held constant and $[Hb^+]$ is varied. As can be seen from Figure 1 and the data in Table I the binding of N_3^- to Hb^+ involves one N_3^- per site for both the aqueous and 5% *tert*-butyl alcohol solutions which is in agreement with other results reported in literature[3,5,6] and shows that the stoichiometry of the reaction is unaffected by 5% *tert*-butyl alcohol. This also indicates the absence of heme–heme interaction.

For most determinations except at those pH values where the binding constant was also determined, $\Delta H°$ was obtained by using various saturating concentrations of N_3^-, varying from two- to tenfold excess, to obtain Q_{max} and hence $\Delta H°$ by extrapolation to infinite N_3^- concentrations. We have evaluated the thermodynamic quantities $\Delta G°$ and $\Delta S°$ from the relationships $\Delta G° = -RT \ln K$ and $\Delta G° = \Delta H° - T\Delta S°$. The standard states for N_3^- and Hb^+ are 1 M and the activity coefficient of N_3^- at 1 M was assumed to be unity which is satisfactory for all comparisons we make.

In Tables II and III are given the $\Delta H°$ values at different pH values for the aqueous and 5% *tert*-butyl alcohol systems, respectively. In Table IV we present the thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ at selected pH values.

Table II. $\Delta H°$ Values for Azide Binding to Ferrihemoglobin[a]

Calorimetric		Spectrophotometric[b]	
pH	$-\Delta H°$, kcal/mol	pH	$-\Delta H°$, kcal/mol
5.65	14.1 ± 0.6	6.0	14.0
5.99	14.1 ± 0.23	6.4	15.6
6.41	14.8 ± 0.23	6.8	17.9
6.82	17.0 ± 0.8	7.0	18.6
7.0	17.7 ± 0.3	7.4	17.4
7.24	16.8 ± 0.2	7.8	15.5
7.60	16.8 ± 0.3	8.2	14.5
7.82	16.3 ± 0.3		
8.01	16.0 ± 1.0		
8.47	15.4 ± 0.4		
8.83	14.5 ± 0.3		
9.06	12.6 ± 0.3		
9.21	11.5 ± 0.3		

[a] As a function of pH in an aqueous system determined calorimetrically and spectrophotometrically.[5,6] [b] See ref 5 and 6.

Discussion

There is an excellent agreement between the results

Table III. ΔH° Values for Azide Binding to Ferrihemoglobin as a Function of pH in 5% *tert*-Butyl Alcohol

pH	$-\Delta H^\circ$, kcal/mol
5.80	13.8 ± 0.1
6.0	14.8 ± 0.7
6.41	14.7 ± 0.2
6.64	15.2 ± 0.1
6.80	15.1 ± 0.2
7.0	15.5 ± 0.4
7.25	16.6 ± 0.2
7.55	16.6 ± 0.6
8.02	15.2 ± 0.5
8.40	14.2 ± 0.8
8.56	14.3 ± 0.6
8.80	13.7 ± 0.6
9.01	12.4 ± 0.3

Table IV. Summary of Thermodynamic Parameters ΔG°, ΔH°, and ΔS° at Various pH Values

pH	$-\Delta G^\circ$, kcal/mol	$-\Delta H^\circ$, kcal/mol	$-\Delta S^\circ$, cal mol^{-1} deg^{-1}
	(a) Aqueous System		
6.0	7.21 ± 0.25	14.14 ± 0.23	23.19 ± 1.61
6.40	7.10 ± 0.37	14.63 ± 0.20	25.20 ± 1.91
7.0	6.89 ± 0.24	17.73 ± 0.29	36.27 ± 1.77
8.47	7.13 ± 0.28	15.41 ± 0.38	27.71 ± 2.21
	(b) 5% *tert*-Butyl Alcohol		
6.50	6.53 ± 0.24	15.07 ± 0.53	28.58 ± 2.58
7.00	6.40 ± 0.20	15.54 ± 0.38	30.58 ± 1.94
7.52	6.20 ± 0.31	16.60 ± 0.63	34.80 ± 3.15
7.94	6.31 ± 0.32	14.70 ± 0.40	28.07 ± 2.41
8.52	6.56 ± 0.36	14.30 ± 0.60	25.90 ± 3.21
9.00	6.50 ± 0.27	12.41 ± 0.30	19.78 ± 1.91

presented in this paper for the aqueous system and those of a previous study[6] using spectrophotometry. As can be seen from the data in Table II and from Figure 3, in which ΔH° is plotted against ΔS°, the "characteristic pH"—the turn around pH in the ΔH° *vs.* pH plot—the slope and linearity of the ΔH° *vs.* ΔS° confirms the existence of the "compensation" pattern reported earlier.[3,5,6] Our hemoglobin samples have been more extensively purified than that previously used so the slight differences are not surprising.

There are, however, differences between the ΔH° values for the aqueous and 5% *tert*-butyl alcohol systems which are outside experimental error. Qualitatively the results are the same in the two systems. Plots of ΔH° *vs.* ΔS° are linear within limits of experimental error which is not surprising since similar compensation plots have been observed in other reactions involving water–alcohol systems.[20,21] Also the slopes are approximately equal conforming to the T_c value of 280 ± 5°K as is generally observed when lower concentrations of cosolvents are used.

Although the slopes are equal, the two lines are separated by about 0.8 ± 0.4 kcal/mol which is significant. As pointed out by Lumry and Rajender[7] and Lumry[22] for "compensation" between ΔH° and ΔS° in this kind of experiment we may write eq 6

$$\Delta H^\circ = \alpha + T_c \Delta S^\circ \qquad (6)$$

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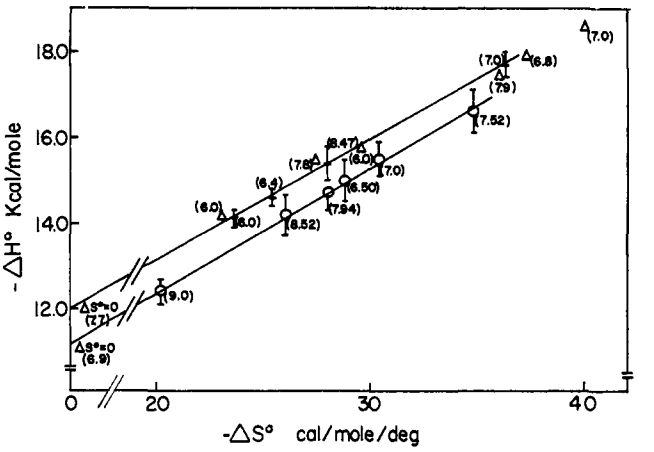

Figure 3. Plot of ΔH° *vs.* ΔS° for the aqueous and 5% *tert*-butyl alcohol systems compared to spectroscopic results obtained by Beetlestone and coworkers:[5,6] (○) 5% *tert*-butyl alcohol data; (+) aqueous data; (Δ) spectroscopic data.[5,6]

in which T_c is the compensation temperature and α is the intercept at $\Delta S^\circ = 0$. The intercept is the standard free energy of all the pH-independent chemical part processes of the total binding process. Our results show that the free energies of these chemical part processes in an aqueous system are different from those in 5% *tert*-butyl alcohol. The difference may arise from any combination of the following processes: (a) the solvation of the azide ion is different in the two systems; (b) the solvation of Hb^+ differs in the two systems; (c) the bond energies of the strong azide complex are different in the two systems. Our experimental results in this study tend to suggest that the first and second proposals are not significant in the two systems, since we could not detect any differences in heats of solvation in the two systems. Hence, our evidence tends to support alternative c.

As shown in the studies of ligand binding to Hb^+ [5,6] and pointed out elsewhere[7] there is a correlation between ΔG° for the different sixth position derivatives at any fixed pH and the measured magnetic susceptibilities of these derivatives as can be judged from the positions of the compensation lines on the ΔH° axis at $\Delta S^\circ = 0$ (see ref 7). The higher the magnetic susceptibility the lower the intercept.

It has been shown by Coryell and Stitt[23] and by a recent study here[24] that the addition of small monohydroxy alcohols increases the magnetic susceptibility of Hb^+ at pH values from at least 6 to 7. It is thus probable that the differences in the intercept are due to differences in the magnetic susceptibility of the N_3^- complex in the two solvent systems in which case it is not surprising that the values of α are different. It is interesting that our compensation pattern conforms to the predictions from previous studies[5–7] in the sense that the higher the susceptibility, the lower the intercept.

We do not yet understand by what mechanism the addition of small amounts of alcohol changes the magnetic susceptibility of the complex, but there is abundant evidence in literature to show that alcohols in general have a subtle effect on the reactions of ferrihemoglobin[23–25] even though there is no indication that small

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(25) J. Cassat and J. Steinhardt, *Biochemistry*, **10**, 3738 (1971).

alcohols are bound by Hb^+ in the concentration range of our study. Steinhardt, *et al.*,[25] have noted that in the presence of trace amounts of alcohol the rate of acid denaturation is increased but their evidence also suggests no direct binding between alcohols and ferrihemoglobin.

Kotani, *et al.*,[26,27] have shown that they could generate a compensation line by consideration of the high spin–low spin equilibria as a function of ligand using both ferrimyoglobin and ferrihemoglobin. Studies here[24] also suggest that the pH dependence of ΔH° and thus of the compensation behavior is closely associated with the change in spin state. In each case the implication is that there is a part process of the total ligand-binding process which generates the compensation behavior and which can be modified to alter α but not T_c (eq 6) by the formation of different derivatives.

The aggregate of these studies suggests that the binding of ligands is significantly influenced by the nature of the environment outside the heme and very likely as much in the peripheral water as in the protein itself. In previous studies[5,6] the thermodynamic results have been explained by changes in the hydration sheath which accompanies changes in pH. It is likely that modifications in the hydration sheath tend to change the low spin–high spin equilibrium. The involvement of the low spin–high spin equilibrium in the binding of ligands to Hb^+ is suggested by our results. It is interesting to note that independent results from infrared spectroscopy of N_3^- binding to ferrimyoglobin and ferrihemoglobin[28] have been interpreted as showing that in the high spin complex the iron–N_3^- bond is "ionic" and in the low spin complex it is "covalent."

Possibly the ability of *tert*-butyl alcohol to change the magnetic susceptibility is a result of its influence on the structure of the hydration sheath. Studies to test this hypothesis are in progress.

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Calcium Binding to Carbohydrates. Crystal Structure of a Hydrated Calcium Bromide Complex of Lactose

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Abstract: The crystal structure of a hydrated calcium bromide complex of lactose (4-*O*-β-D-galactopyranosyl-D-glucopyranose) was determined by use of three-dimensional X-ray diffractometer data. Crystals of $C_{12}H_{22}O_{11} \cdot 7H_2O \cdot CaBr_2$ are orthorhombic, space group $P2_12_12_1$, with $a = 21.952$ (3), $b = 13.705$ (3), and $c = 8.792$ (4) Å. The structure was solved by the heavy-atom method and was refined by least squares to $R = 0.043$. The absolute configuration was confirmed by anomalous dispersion effects. The crystal structure contains a mixture of the α and β anomers of lactose, with an α/β ratio of about 88/12. An outstanding feature of the crystal packing is the interaction of lactose molecules with calcium ions. The calcium ion binds two lactose molecules and four water molecules. One lactose molecule is coordinated to the calcium ion through O(3) and O(4) of its galactose moiety, and the second is coordinated through O(2′) and O(3′) of its glucose moiety. Similar interactions probably account for the chelation of calcium ions by lactose in aqueous solution, and may be involved in the mechanism by which lactose, as well as other carbohydrates and polyols, increase intestinal absorption of calcium.

Interactions of calcium ions with carbohydrates have been implicated in such biological processes as calcium transport,[1,2] calcification,[3–7] cell–cell adhesion,[8,9] and binding of glycoproteins to cell surfaces.[10] It has been demonstrated that calcium ions complex with both uncharged[11] and anionic[6,12] carbohydrates in aqueous solution, but little is known about either the factors involved in calcium–carbohydrate interactions, or the stereochemistry of the resultant complexes. We are currently examining the crystal structures of calcium–carbohydrate complexes[13] to obtain information concerning the structural factors that govern calcium–carbohydrate interactions in biological systems. This paper describes the crystal structure of a hydrated calcium bromide complex of lactose (4-*O*-β-D-galactopyranosyl-D-glucopyranose).

It is established that lactose, a component of milk, can increase the rate at which calcium is absorbed from

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