far from the first chlorine atom as possible. (c) Substitution of a second chlorine atom upon a carbon atom already holding a halogen substituent is difficult; a third chlorine atom cannot be introduced. (d) A bromine substituent is not displaced.

3. The chlorination of side-chains of aromatic hydrocarbons proceeds readily as shown by a study of the chlorination of toluene, *p*-chlorotoluene, ethylbenzene, isopropylbenzene, *t*-butylbenzene, *m*-xylene, and triphenylmethane. The study of the chlorination of toluene, with an excess of sulfuryl chloride, reveals that in this case also the substitution of chlorine upon a carbon atom ceases with the introduction of the second chlorine atom. Both ethylbenzene and isopropylbenzene chlorinate chiefly in the alpha position of the side-chain. Diphenylmethane and the nitrotoluenes cannot be chlorinated by this method.

4. This method of chlorination is of particular value for the chlorination of side-chains in molecules containing reactive nuclei. Thus, *m*-xylene is chlorinated in the side-chain without the simultaneous formation of any nuclear-substituted products; and *t*-butylbenzene reacts readily to form β-chloro-*t*-butylbenzene, a compound which cannot be prepared by the direct action of chlorine upon the hydrocarbon.

5. The characteristics of the reaction indicate that the slow decomposition of the peroxide at the reaction temperature initiates a chain reaction involving chlorine *atoms*.

CHICAGO, ILLINOIS RECEIVED MAY 24, 1939

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, NO. 711]

The Magnetic Properties of Myoglobin and Ferrimyoglobin, and their Bearing on the Problem of the Existence of Magnetic Interactions in Hemoglobin

BY DONALD S. TAYLOR

Myoglobin, or muscle hemoglobin, was first isolated in crystalline form by Theorell,[1a] who showed that it is very similar to hemoglobin but that it has certain important differences. The minimum molecular weight calculated from the iron analysis is 17,000,[1a] a value agreeing with that calculated from hemoglobin analyses. Theorell[1b] investigated the sedimentation velocity of crystallized myoglobin, and found two components, with molecular weights of 34,000 for the principal one and 68,000 for the secondary one. A redetermination by Polson[2] in the same laboratory gave the value 17,000, leading to the conclusion that there is only one iron atom, and therefore only one heme group per molecule. The oxygen saturation curve found for myoglobin[1e] is a rectangular hyperbola rather than a sigmoid curve as is found for hemoglobin. It has a much higher affinity for oxygen than hemoglobin has[1e] and thus acts as an oxygen reservoir for the muscle. Its oxygen equilibrium is much less affected by change in acidity than is that of hemoglobin.[1e] Myoglobin forms compounds analogous to those of hemoglobin, and is very easily oxidized to brown ferrimyoglobin[3] (metamyoglobin). The absorption spectra[1c] of myoglobin and its derivatives are very similar to those of the analogous hemoglobin substances, but the band positions are slightly shifted.

The magnetic studies of myoglobin are then of interest for the information they give about the structural chemistry of the myoglobin series, and also for the opportunity offered to make a comparison between this series in which the hemes are located on individual molecules (or at least are physico-chemically independent as judged by the oxygen equilibrium study[1e]) and the hemoglobin series in which there are four hemes per molecule with a large physico-chemical interaction effect (calculated to be about 6000 calories per mole according to the theoretical analysis of the oxygen equilibrium by Pauling[4]). In particular this comparison should shed light on the question as to

(1) H. Theorell, *Biochem. Z.*, (a) **252**, 1 (1932); (b) **268**, 47 (1934); (c) **268**, 55 (1934); (d) **268**, 64 (1934); (e) **268**, 73 (1934).

(2) T. Svedberg, *Nature*, **139**, 1051 (1937). Note added in proof: Dr. Theorell has informed us in a private communication that Polson worked with cow myoglobin, so that the reported differences from values obtained by Theorell on horse myoglobin may be real. However, the discussion or conclusions given here are not changed by this new information.

(3) The nomenclature adopted here is analogous to that proposed by Pauling and Coryell[5] for hemoglobin substances.

(4) L. Pauling, *Proc. Natl. Acad. Sci.*, **21**, 186 (1935).

whether magnetic interactions occur between the hemes in hemoglobin. The postulate of interaction was advanced by Pauling and Coryell[5] because the magnetic moment of ferrohemoglobin (calculated from the magnetic susceptibility assuming no interaction) is somewhat higher than expected for ferrous ion. It was thought that this might be caused by a slight tendency for the magnetic moments of the four hemes in a molecule to orient together when a magnetic field is applied. In myoglobin we have the hemes on separate molecules, so that no magnetic interaction can occur in dilute solution. The magnetic moment calculated for myoglobin from the susceptibility must then be the true magnetic moment of the heme group conjugated to globin, a value different from that found for hemoglobin if magnetic interaction does occur.

In the work that is to be described it will be shown that the magnetic properties of the iron in myoglobin (from horse heart muscle) in both the ferrous and ferric forms are to within the limits of experimental error equal to those of the corresponding hemoglobin substances.

Experimental Part

Preparation of Myoglobin Solutions.—Three myoglobin preparations were made, which will be designated as myoglobins I, II, and III. The first preparation was a pioneer preparation using the material from one heart. The last two yielded sufficient quantities for magnetic measurements. The method used in the preparations is essentially that described by Theorell[1a] with modifications as suggested by Morgan.[6] The procedure will be described here, as some variations were introduced.

Horse hearts were obtained from the animals less than two hours after death and placed in ice for the trip to the laboratory. They were then washed well with water and freed from fat as thoroughly as possible, and the meat was finely ground. An amount of water about equal to half the weight of the meat was added and the mixture allowed to stand about twenty hours in the cold room. A reddish extract was then obtained by squeezing out the liquid in a large press. It was found that additional myoglobin solution was obtained if the meat was again extracted in the same way.

The red liquid which was obtained rapidly darkened due to auto-oxidation to ferrimyoglobin when the reducing action of the tissues was no longer effective.

The *p*H was adjusted to 7.00 by addition of 1 *N* potassium hydroxide with rapid stirring. The solution was mixed at room temperature with one-fourth its volume of a saturated solution of basic lead acetate. This substance caused precipitation of the globulins in the form of a dense white glutinous mass which was easily removed by centrifuging. The *p*H was again adjusted to 7.00 by addition of 1 *N* potassium hydroxide, and 210 g. of potassium dihydrogen phosphate and 300 g. of potassium monohydrogen phosphate were added to each liter of solution, this being enough to give a solution approximately 3 molar in phosphate and buffered at about *p*H 6.6. The precipitate of lead phosphate and hemoglobin which then formed was filtered off. The myoglobin was obtained by repeatedly dialyzing this solution against saturated neutral ammonium sulfate solution. The first nearly colorless precipitate (albumins) was filtered off and discarded. The myoglobin finally precipitated as a brownish solid mixed with considerable albuminous material. Dialysis was carried out in cellophane casings in the cold room.

The crude myoglobin precipitate thus obtained was purified somewhat by several treatments involving redissolution in water and dialysis against saturated neutral ammonium sulfate until precipitation took place. No effort was made to get absolutely pure crystals since the only requirement for this work was the preparation of a hemoglobin free solution of sufficiently high myoglobin concentration.

All processes were carried out in the cold room or in the refrigerator except for the short periods for filtration and handling. Chemically pure reagents were used throughout except for some impure ammonium sulfate used in preparation II, it being thought that the inorganic iron present would not be carried through the purification process. Some seemed to have been carried through, however, and this rendered certain measurements on this solution valueless, as will be pointed out later.

For preparation II eleven hearts were obtained and the final step yielded 130 ml. of dark brown solution concentrated enough for magnetic measurements. In preparation III twelve hearts (45 pounds, 20 kg.) were used but only about 100 ml. of solution of slightly lower concentration was obtained. (From concentrations given later it can be calculated that in all about 15 g. of myoglobin was obtained.) The solutions were adjusted to *p*H 7.0 and kept in the refrigerator until used.

Standardization of Solutions.—Spectroscopic examination of solutions obtained in the manner described above indicated that all of the myoglobin was in the form of ferrimyoglobin. A Van Slyke–Neill[7] oxygen-capacity determination on solution III gave zero oxygen capacity, which also indicated the substance to be entirely in the ferric form, as Theorell[1c] has pointed out.

The concentration of the solutions was determined in the following manner. To 10 ml. of solution 0.15 g. of sodium hydrosulfite ($Na_2S_2O_4$) was added to reduce the ferrimyoglobin completely. The carbon monoxide capacity of the solution so obtained was then determined by the method of Van Slyke and Hiller[8] using the Van Slyke–Neill blood gas apparatus. Calculations from equilibrium data showed that the carbon monoxide affinity of myoglobin is not enough different from that of hemoglobin to affect the completeness of saturation when carried out in the manner recommended for hemoglobin. One-ml. solution samples were used, corrections being

(5) L. Pauling and C. D. Coryell, *Proc. Natl. Acad. Sci.*, **22**, 210 (1936).

(6) V. E. Morgan, *J. Biol. Chem.*, **112**, 557 (1936).

(7) D. D. Van Slyke and J. M. Neill, *J. Biol. Chem.*, **61**, 523 (1924).

(8) D. D. Van Slyke and A. Hiller, *ibid.*, **78**, 807 (1928).

made for retention on the walls of the transfer pipet and for change in the meniscus level on standing. The Van Slyke gas pipet had been calibrated previously. A volume of 0.25 ml. of acid ferricyanide reagent was used and correction for solubility of carbon monoxide in the sample and dissolved gases in the reagent was determined as is customary by analysis carried out on 1 ml. of water.

From the amount of carbon monoxide chemically bound as determined in this way the concentration of myoglobin was calculated in terms of moles of heme iron per liter. The gasometric determinations were carried out at least three times, and the agreement was thoroughly satisfactory.

Measurement of Magnetic Properties of Solutions.—The general technique used in the magnetic measurements by the differential Gouy method has been described elsewhere.[9] The standardized tube was surrounded by an air thermostat at 25°. The Δw values which are given below represent the mean of two determinations of the observed force in milligrams on the tube containing the sample when in a standard magnetic field. The two determinations were made in fields of different strengths and converted to a common basis by an empirically determined factor.

Each sample of myoglobin solution was treated in the following way. Twenty-five ml. of solution was placed in the tube and the value of Δw of the ferrimyoglobin ($\Delta w_{\mathrm{Mgb}^+}$) was measured. The solution was then reduced by the addition of 0.3 g. of sodium hydrosulfite and the Δw measured, which on the addition of an experimentally determined correction of +0.09 mg. for the diamagnetism of the added hydrosulfite gives the Δw for ferromyoglobin (Δw_{Mgb}). This solution was then removed from the tube, treated with 0.1 g. of sodium hydrosulfite and saturated with carbon monoxide by stirring in a dark stoppered flask with pure carbon monoxide. The solution so obtained was returned to the tube in the dark or diffuse daylight, and the Δw was measured, which on addition of a total correction of +0.12 mg. for diamagnetism of hydrosulfite gives Δw for carbonmonoxymyoglobin ($\Delta w_{\mathrm{MgbCO}}$). Enough of solution II was available to run four sets of measurements as described above and enough of solution III to run three sets of measurements. Averages of the values obtained were used in calculations.

Calculations.—The molal susceptibilities (χ_m) of the paramagnetic iron atoms in any compound x were calculated by means of the following equation, which holds for determinations made in the manner described.

$$\chi_m \times 10^6 = \frac{(\Delta w_x - \Delta w_{\mathrm{MgbCO}})}{m} 18.60 \qquad (1)$$

Here m is the molar concn. of heme iron and 18.60 is the apparatus constant determined by standardization with air and water.

In using Equation (1) we are assuming that the iron in carbonmonoxymyoglobin is in a diamagnetic state, and that therefore $\Delta w_{\mathrm{MgbCO}}$ may be used to correct for the diamagnetism of the solutions. This assumption is partially justified by the very close chemical similarity between carbonmonoxyhemoglobin and carbonmonoxymyoglobin. The magnetic results obtained by making this assumption agree so well with results for hemoglobin that we do not hesitate to accept them. If carbonmonoxymyoglobin were paramagnetic its magnetic moment would be due to unpaired electrons, of which there would necessarily be at least two. These two unpaired electrons would give a magnetic moment of at least 2.83 Bohr magnetons, an amount which would certainly cause a large difference in the calculated results for the free ferrous and ferric forms from results for the corresponding hemoglobin compounds arrived at in the same manner.

Magnetic moments μ in Bohr magnetons were calculated assuming Curie's law in the form

$$\mu = 2.84 \sqrt{\chi_m T} \qquad (2)$$

Experimental Results

All important experimental and calculated values are collected in Table I. It is to be noticed that the value of $\Delta w_{\mathrm{Mgb}^+} - \Delta w_{\mathrm{MgbCO}}$ for solution II is noticeably low. We ascribe this to the presence of inorganic iron in some form which undergoes increase in susceptibility on reduction. However, the value for solution III compares favorably with hemoglobin values, and since in this case care was taken to avoid contamination with inorganic iron we use this value to calculate the susceptibility of ferrimyoglobin. For ferromyoglobin measurements inorganic iron can have no effect, since the change in going from ferromyoglobin to carbonmonoxymyoglobin, the addition of carbon monoxide, would not change the susceptibility of the inorganic iron, but only that of the heme iron. In taking the difference we calculate the paramagnetic susceptibility of the heme iron independently of the other constituents of the solution. The close check between results for the two solutions indicates the precision that was obtained for even these rather dilute solutions.

The mean value of the molal susceptibility (χ_m) of ferromyoglobin is accordingly found to be 12,400 $\times$ 10^{-6}, corresponding to a magnetic moment of 5.46 Bohr magnetons; the value of the molal susceptibility of ferrimyoglobin (solution III) is found to be 14,200 $\times$ 10^{-6}, corresponding to a magnetic moment of 5.85 Bohr magnetons.

Experiments made on a very dilute solution of

(9) C. D. Coryell, F. Stitt and L. Pauling, THIS JOURNAL, **59**, 633 (1937).

TABLE I

EXPERIMENTAL DATA AND CALCULATED RESULTS

	Concn., m	Δw_{Mgb^+}	Δw_{MgbCO}	Δw_{Mgb}	Ferromyoglobin	Ferrimyoglobin
			Solution II			
	0.00437	+0.68	−2.28	+0.93		
	.00437	+ .85	−2.38	+ .94		
	.00438	+ .73	−2.25	+ .84		
		+ .77	−2.29	+ .88		
Av.	.00437	+ .76	−2.30 ± 0.04	+ .90 ± 0.4		
			$\Delta w_{Mgb^+} - \Delta w_{MgbCO}$ = +3.06 mg.			
			$\Delta w_{Mgb} - \Delta w_{MgbCO}$ = +3.20 mg.		$\chi_m = 12{,}300 \times 10^{-6}$	
			Solution III			
	0.00352	+0.92	−2.21	+0.48		
	.00350	+ .68	−2.23	+ .38		
	.00354	+ .59	−2.31	+ .29		
Av.	.00352	+ .73 ± 0.13	−2.25 ± 0.04	+ .38 ± 0.06		
			$\Delta w_{Mgb^+} - \Delta w_{MgbCO}$ = +2.98 mg.			$\chi_m = 14{,}200 \times 10^{-6}$
			$\Delta w_{Mgb} - \Delta w_{MgbCO}$ = +2.63 mg.		$\chi_m = 12{,}500 \times 10^{-6}$	
				Mean χ_m	$12{,}400 \times 10^{-6}$	$14{,}200 \times 10^{-6}$
				μ	5.46	5.85

ferrimyoglobin recovered from carbonmonoxymyoglobin III indicated that the formation of ferrimyoglobin hydroxide is accompanied by a decrease of magnetic susceptibility to approximately 8000 × 10^{-6}, very much like that attending the formation of ferrihemoglobin hydroxide.[9] These experiments were not of sufficient accuracy to allow quantitative conclusions to be made. A similar experiment also indicated that ferrimyoglobin fluoride has a susceptibility somewhat greater than that of ferrimyoglobin itself.

Discussion

The value 12,400 × 10^{-6} for the molal magnetic susceptibility found for ferromyoglobin is to within experimental error equal to that of 12,290 × 10^{-6} reported by Taylor and Coryell[10] for ferrohemoglobin. The susceptibility found for ferrimyoglobin is also to within the experimental error equal to that of ferrihemoglobin reported by Coryell, Stitt and Pauling.[9]

The values of the magnetic moments obtained for ferromyoglobin and ferrimyoglobin may be interpreted in the same manner as for ferrohemoglobin[5] and ferrihemoglobin,[9] leading to the conclusion that the iron atoms are held to the porphyrin by essentially ionic bonds.

Since myoglobin has one (or possibly two chemically independent) hemes per molecule as contrasted with the four of hemoglobin, the existence of interaction between the hemes of hemoglobin might lead to some difference in the magnetic moments observed for the two substances. That no difference greater than the experimental error is observed for either the ferrous or ferric forms of these substances is a strong indication that there is little or no magnetic interaction between the hemes in hemoglobin. We believe, therefore, that 5.44 Bohr magnetons is the magnetic moment of ferroheme conjugated with globin, and that 5.80 Bohr magnetons is the magnetic moment of ferriheme conjugated with globin. Magnetic titrations carried out by Coryell, Pauling, and Dodson[11] on systems involving the transformation of ferrohemoglobin to oxyhemoglobin and to nitric oxide hemoglobin also failed to give evidence for magnetic interactions between the hemes in ferrohemoglobin. These measurements would not be expected to be so sensitive as those described in this study.

That the observed moment of ferromyoglobin and ferrohemoglobin is considerably higher than that calculated for spin alone (4.92 for ferrous ions) may possibly be explained by assuming that the difference is due to orbital contribution to the moment. This contribution seems to be greater than that usually found for ferrous ions (0.2–0.3 Bohr magnetons). However, it should be remembered that the ferrous ion in the heme compounds is surrounded by the nitrogen atoms of the rigid planar heme group, and the closeness of approach of the nitrogen atoms to the iron atom is so restricted by the porphyrin structure that the separations may be greater than they would be

(10) D. S. Taylor and C. D. Coryell, THIS JOURNAL, **60**, 1177 (1938).

(11) C. D. Coryell, L. Pauling and R. W. Dodson, *J. Phys. Chem.*, in press (1939).

if the groups were free to move radially; and hence the orbital moment might not be quenched so much as it is in simpler compounds. A large amount of orbital contribution to the moment is also observed in the case of ferrihemoglobin cyanide and hydrosulfide,[9] which, however, have only one unpaired electron per iron atom.

Summary

The magnetic moments of the iron atoms in ferromyoglobin and ferrimyoglobin have been determined to be 5.46 and 5.85 Bohr magnetons, respectively. The magnetic moments indicate that the iron atoms are held by essentially ionic bonds in ferromyoglobin and ferrimyoglobin. The agreement of the magnetic moments with those calculated for the analogous hemoglobin compounds leads to the conclusion that magnetic interactions do not occur between the four hemes of hemoglobin.

PASADENA, CALIF. RECEIVED MAY 24, 1939

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

Activity Coefficients of Barium and Strontium Carbonates in Aqueous Solutions of Alkali Chlorides

BY ERNEST HOGGE AND HERRICK L. JOHNSTON

Townley, Whitney and Felsing[1] have computed activity coefficients of strontium and barium carbonates in alkali chloride solutions from solubility measurements in carbon dioxide-free, aqueous solutions at 25 and at 40°. We have been unable to check the calculations recorded in their tables and, while details of their computations are not given, it appears that they incorrectly computed the mean ion molality and have misinterpreted (K_2')'s taken from tables of MacInnes and Belcher[2] as stoichiometric second dissociation constants of carbonic acid in salt solutions whereas these K's are in reality the products of the stoichiometric constants by the square of the mean activity coefficients of hydrochloric acid in the same solutions.[3] Correction for the activity coefficients of hydrochloric acid is important in the higher concentrations of the salt solutions employed by Felsing and co-workers.

We have accordingly recalculated the activity coefficients and obtained results that differ from those of T., W. and F. by as much as 100% in the higher salt concentrations. We employed the same equation used by T., W. and F. to correct for hydrolysis of carbonate ion, namely

$$\frac{x^2}{C - x} = \frac{k_w}{k_m'} \qquad (1)$$

with C set equal to *total* carbonate concentration in solution (equal as well to divalent metal ion concentration); x, to the concentration of bicarbonate ion; $(C - x)$ to that of carbonate ion; k_w to the stoichiometric ionization constant of water in the salt solutions and k_m' to the correct stoichiometric second ionization constant of carbonic acid in the solutions. The latter is computed from MacInnes and Belcher's tabulations of pK_2' [2] by the relationship

$$\log k_m' = -pK_2' - 2 \log \gamma_{HCl} \qquad (2)$$

In solving equation (1) for x, necessary in the eventual computation of the mean molality of barium (or strontium) and carbonate ions and the evaluation of the activity coefficients of barium (or strontium) carbonate, we employed the following sources of data.

Values of C are taken from Tables I and II of Townley, Whitney and Felsing. These are smoothed values of their solubility measurements in potassium, sodium and lithium chloride solutions, respectively.

The values of k_w are taken from the data of Harned and co-workers[4] who have determined the

(1) Townley, Whitney and Felsing, THIS JOURNAL, **59**, 631 (1937).

(2) (a) MacInnes and Belcher, *ibid.*, **55**, 2630 (1933); (b) **57**, 1683 (1935).

(3) The above misinterpretation is easily made for MacInnes and Belcher refer to their (K_2')'s as "apparent second ionization constants" and use them, without correction, as the stoichiometric constants in constructing their Tables V and VI. For their own purpose this use was justified for they were employed in computing a small correction term in solutions of relatively low ionic strength and neglect of the factor γ^2_{HCl} was without significance. The correct interpretation was further clouded by a misprint in equation (12) of MacInnes and Belcher's first paper (ref. 2a) which is, however, given in correct form as equation (3) of their second paper (ref. 2b). The latter equation shows clearly the dependence on γ^2_{HCl} in relating (K_2') to the stoichiometric constant

$$\left(K_2 \frac{\gamma_{HCO_3^-}}{\gamma_{H^+}\ \gamma_{CO_3^=}}\right)$$

(4) (a) Harned and Hamer, THIS JOURNAL, **55**, 2194 (1933); (b) Harned and Copson, *ibid.*, **55**, 2206 (1933); (c) Harned and Mannweiler, *ibid.*, **57**, 1874 (1935); (d) Harned and Cook, *ibid.*, **59**, 2304 (1937).