water. In calculating the quantity $-\Delta H_f^\circ$ we have used 68,313,[17] and 94,240,[18] respectively, as the heats of formation of liquid water and of gaseous carbon dioxide from graphite at 25.0°.

Summary

Experimental data are reported for the heats of combustion of hippuric acid and succinic acid, and from these are calculated values for the heats of formation. These data are compared with existing values.

Hippuric acid is shown to be a substance whose properties fulfil the requirements for a bomb calorimetric standard, and it is proposed that hippuric acid (crystallized from water) be used as a secondary standard, for investigations on nitrogen-containing compounds. The value proposed for the heat of combustion of hippuric acid crystallized from water is 23,546.3 NBS international joules, or 5628.6 calories, per gram mass, under the standard conditions for the bomb process at 25°. The "precision" and "accuracy" uncertainties are, respectively, ±0.009 and ±0.025%.

(17) Rossini, *Bur. Standards J. Research*, **6**, 34 (1931).

(18) Parks and Huffman, "The Free Energies of Some Organic Compounds," The Chemical Catalog Co., New York, N. Y., 1932.

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The Magnetic Susceptibility of the Iron in Ferrohemoglobin

BY DONALD S. TAYLOR AND CHARLES D. CORYELL

Introduction

The study of magnetic properties of hemoglobin derivatives and related compounds has been used as a method of elucidating their chemical structure.[1] It was found that the iron in oxyhemoglobin and carbonmonoxyhemoglobin is diamagnetic, and that the iron atom is bonded to six neighbors with covalent bonds. The iron atom in ferrohemoglobin[2] was found to have a magnetic moment about equal to that expected of a ferrous ion held in the structure by electrostatic bonds, although somewhat larger than usual for ionic ferrous complexes. It was suggested that the moment might be large because of interactions between the four heme groups of one molecule.

Interest in the absolute magnetic susceptibility of the iron of ferrohemoglobin is heightened by the fact that with the technique now in use hemoglobin concentrations can be determined as accurately by magnetic measurements as by the more tedious gasometric methods. Determinations have been made in this way by Coryell, Stitt, and Pauling[1c] in the study of magnetic properties and equilibria of ferrihemoglobin derivatives.

The possible presence in the blood of iron which does not combine with oxygen is a factor which should be considered in the determination of concentration and susceptibility of hemoglobin iron. Barkan[3] has shown that on acid denaturation a definite small fraction of the blood iron can be separated in the ionic form by ultrafiltration. He has called this fraction "leicht abspaltbares" iron and has postulated that it is formed from ferrous and ferric derivatives of hemoglobin with an altered heme structure which gives up iron more readily than ordinary hemoglobin does. By a comparison of the oxygen capacity and iron content of a number of blood samples (normal human blood, pathological human blood, and several animal bloods), Klumpp[4] has shown that there is about 5% (with a large spread of values) of the total iron in the blood which does not combine with oxygen. In the early magnetic investigations the possible effects of such iron were not taken into account, and they have been neglected in many other physico-chemical investigations.

It thus seemed desirable to redetermine the magnetic susceptibility of ferrohemoglobin with the superior technique now in use, taking into consideration the effect of the iron which does not combine with oxygen, and to ascertain how much variation there is from individual to individual and from species to species. It will be shown below that the value is constant within relatively small experimental error for individuals of one species,

(1) (a) L. Pauling and C. D. Coryell, *Proc. Nat. Acad. Sci.*, **22**, 159 (1936); (b) L. Pauling and C. D. Coryell, *ibid.*, **22**, 210 (1936); (c) C. D. Coryell, F. Stitt and L. Pauling, THIS JOURNAL, **59**, 633 (1937).

(2) The nomenclature used in this paper is that proposed by L. Pauling and C. D. Coryell.[1b]

(3) G. Barkan, (a) *Z. physiol. Chem.*, **171**, 179 (1927); (b) *ibid.*, **216**, 1 (1933); (c) G. Barkan and O. Schales, *ibid.*, **245**, 96 (1937). The last paper contains references to many other papers on the subject.

(4) T. G. Klumpp, *J. Clin. Investigation*, **14**, 351 (1935).

and varies only slightly among the four species studied.

Experimental Part

Fourteen hemoglobin preparations (defibrinated blood samples or hemoglobin solutions) were used in this investigation. They are designated by the letters A to P. For all preparations except Human Blood H fresh blood was defibrinated by stirring, oxygenated immediately to prevent formation of ferrihemoglobin, and packed in ice. Sheep hemoglobin preparations were mixtures from several individuals but all other preparations were from single individuals. Blood preparations were used without further treatment. For hemoglobin solutions D, E, F, and P, the blood was centrifuged and the corpuscles washed three times with 0.14 m. potassium chloride. The corpuscles were then hemolyzed for solutions D and E by treatment at 0° for twenty-four hours with toluene or toluene and water, and for solutions F and P by shaking with peroxide-free ether. The stromata emulsion was removed by centrifuging and in the case of ether-treated solutions the dissolved ether was removed by bubbling air through the solutions.

Human Blood H (C. D. C.) was prevented from coagulating by the addition of potassium fluoride, this having been shown in separate experiments to have no effect on the magnetic properties of hemoglobin.

All preparations were kept on ice until used. In all cases the entire interval of time between obtaining the blood and completing the measurements was less than five days, and in no case was the time interval long enough for measurable alteration to take place.

Determination of Concentration.—Standardization of the preparations was carried out in most cases both by the Van Slyke method of determination of oxygen content used previously[1b] and by a modified procedure intended to include heme iron which does not combine with oxygen. A comparison of the results of the two methods is of general interest in the analytical chemistry of hemoglobin.

Preparations were analyzed for oxygen content with the Van Slyke–Neill constant volume blood gas apparatus as in the earlier work. Great care was taken in the use of this apparatus to eliminate error, and good reproducibility was obtained. The gas pipet was calibrated, and the transfer pipet was calibrated for content and for retention of blood or solution on the walls. A correction was made for the change in the lower meniscus level on drainage. The data of Sendroy, Dillon, and Van Slyke[5] were used for the solubility of oxygen in blood at the temperature of saturation (room temperature). It was assumed that the solubility of oxygen is the same for hemoglobin solutions as for blood of the same general concentration. All determinations of concentration were carried out at least three times.

The following modified procedure was used to determine the concentration of all compounds capable of forming complexes with carbon monoxide. The preparations were reduced with sodium hydrosulfite ($Na_2S_2O_4$, 0.3 g. for 25 ml.), and their carbon monoxide capacities were determined by the method of Van Slyke and Hiller,[6] the same precautions being taken as in the oxygen capacity determinations.

(5) J. Sendroy, R. T. Dillon and D. D. Van Slyke, *J. Biol. Chem.*, **105**, 597 (1934).

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

The concentration of iron in preparations A and E was also checked by iron analysis. Twenty-five ml. of the preparation was heated with 25 ml. of concentrated sulfuric acid in a Kjeldahl flask until a clear solution was obtained (four to six hours). The iron was then precipitated with ammonium hydroxide and the precipitate was washed free from sulfate and redissolved in hydrochloric acid. The concentration of this solution was determined by an iodometric method[7] using carbon dioxide to prevent air oxidation. Titrations were made with 0.1 *N* sodium thiosulfate from a Koch micro-buret. Analyses of ferric chloride solutions of known concentration containing comparable amounts of iron showed that the method yields good results. Blanks showed that iron from impurities in reagents and glassware was negligible.

Determination of the Magnetic Constants.—The general technique used in the magnetic measurements by the Gouy method has been described earlier.[1c] Most of the measurements of this investigation were made at 25.0° with an air thermostat around the tube. It has been found that with controlled temperature and careful work a very high degree of precision can be maintained. The tube used in these measurements possessed the following characteristics: Δw in mg. at 7560 oersteds for water against water, +1.45; Δw in mg. at 7560 oersteds for air against water, +46.72.

Three magnetic constants were measured for four different samples of each preparation; the Δw of the oxygenated preparation, the Δw after reduction with weighed portions of sodium hydrosulfite, and the Δw after saturation of this preparation with pure carbon monoxide. Saturation was achieved by shaking for some time in a covered flask filled with carbon monoxide. Each Δw represents the mean of two measurements for different standard values of the current, the measurement for 9060 oersteds being multiplied by the experimental factor 0.6959 to make it comparable with that measured for 7560 oersteds. Corrections were made for diamagnetism of the added hydrosulfite.

Experimental Results

In Table I there are presented the complete data for the first six preparations, which show the order of magnitude of experimental inaccuracies. The columns headed f_{O_2} and f_{CO} give, respectively, the formality in iron as calculated from the oxygen capacities and from the carbon monoxide capacities after reduction with hydrosulfite. There has been added to the values Δw_{Hb} and Δw_{HbCO} a correction of +0.09 mg. for the addition of 0.3 g. of $Na_2S_2O_4$ in 25 ml. of preparation.

The results of all of the experiments are summarized in Table II. Columns A and B contain the calculated quantities indicated. The bracketed figures in the last three columns are not experimental values as the carbon monoxide capacity was not determined for these preparations. They were obtained by assuming that the average of the difference between values in columns A and

(7) E. H. Swift, THIS JOURNAL, **51**, 2682 (1929).

TABLE I

GASOMETRIC AND MAGNETOMETRIC DATA FOR PREPARATIONS A–F

(Temp. = 25.0° for magnetic work)

Prepn.	f_{O_2}	f_{CO}	Δw_{HbO_2}	Δw_{Hb}	Δw_{HbCO}
A	0.00799	0.00832	−0.81	+5.29	−0.87
	.00808	.00832	− .79	5.30	− .81
	.00799	.00832	− .75	5.22	− .87
			− .83	5.34	− .93
Av.	.00799	.00832	− .80	+5.29	− .87
B	.00740	.00792	− .85	+4.91	− .88
	.00736	.00780	− .90	4.84	− .81
	.00743	.00782	− .85	4.95	− .99
	.00746		− .77	4.96	− .79
Av.	.00741	.00784	− .84	+4.92	− .87
C	.00969	.01017	− .80	+6.52	− .95
	.00950	.01015	− .83	6.43	−1.10
	.00959	.01010	− .86	6.48	−1.07
	.00965	.00997	− .91	6.45	−1.08
Av.	.00961	.01010	− .85	+6.47	−1.05
D	.00663	.00699	− .48	+4.46	−0.65
	.00658	.00700	− .58	4.62	− .60
	.00658	.00697	− .42	4.47	− .78
			− .40	4.67	− .74
Av.	.00659	.00699	− .47	+4.55	− .69
E	.00689	.00749	− .57	+4.75	− .73
	.00681	.00746	− .51	4.84	− .56
	.00683	.00747	− .38	4.95	− .66
			− .50	4.90	− .73
Av.	.00684	.00747	− .48	+4.86	− .67
F	.01048	.01109	− .65	+7.26	− .89
	.01037	.01103	− .68	7.32	− .96
	.01042	.01108	− .76	7.36	− .89
			− .79	7.16	− .97
Av.	.01042	.01107	− .72	+7.28	− .90

B obtained from other preparations represents the difference in these cases. These estimated values are probably rather close to the actual values, since the correction is small. Justification for this procedure is provided by the very good agreement between values calculated in this way for bovine solutions L to N and those obtained directly for bovine solutions A to F.

Iron analysis of preparations A and E gave the results (f_{Fe}) which are compared with iron formalities calculated from the carbon monoxide capacity of the reduced preparations in Table III.

Discussion

The Susceptibility of Ferrohemoglobin.—The susceptibilities (χ_m) given in Table II represent the paramagnetic contribution to the magnetic susceptibility of ferrohemoglobin per gram atom of iron at the temperature given. The values are obtained by multiplying the quantities in column B by the apparatus constant 1.655×10^{-5} (the values in A cannot be used for reasons discussed in the next section). In this way the difference between the Δw of the ferrohemoglobin and the Δw of the preparation with the iron transformed to a diamagnetic state (carbonmonoxyhemoglobin) is used for the calculation. The diamagnetism of water, dissolved salts, and proteins cancels out, since the entire change in magnetic properties is due to the change of state of the iron (except for negligible diamagnetism of added carbon monoxide).

The high degree of constancy of χ_m for the first six (bovine) preparations A to F attests the precision of the method. The constancy also proves that the magnetic susceptibility is within experimental error a constant for individuals within a species. The values obtained for these preparations give an average value of the suceptibility of cow ferrohemoglobin (χ_m at 25°) of $12{,}290 \times 10^{-6}$ c. g. s. u. with an average deviation of 60×10^{-6}

TABLE II

CORRELATION OF MAGNETOMETRIC AND GASOMETRIC DATA FOR ALL PREPARATIONS

Prepn.	f_{O_2}	f_{CO}	$\frac{f_{CO} - f_{O_2} \times 100}{f_{CO}}$	T	A $\frac{\Delta w_{Hb} - \Delta w_{HbO_2}}{f_{O_2}}$	B $\frac{\Delta w_{Hb} - \Delta w_{HbCO}}{f_{CO}}$	$10^6\chi_m$	Means ($10^6\chi_m$) (corr. to 25°)
Bovine Bl. A	0.00799	0.00832	3.9	25.0	762	740	12,260	
Bovine Bl. B	.00741	.00784	5.5	25.0	777	738	12,220	
Bovine Bl. C	.00961	.01010	4.9	25.0	761	744	12,320	12,290
Bov. Hb Sol. D	.00659	.00699	5.5	25.0	762	750	12,400	
Bov. Hb Sol. E	.00684	.00747	8.4	25.0	780	740	12,250	
Bov. Hb Sol. F	.01042	.01107	5.9	25.0	767	739	12,240	
Horse Bl. G	.00835	.00874	4.5	25.0	773	740	12,260	12,260
Human Bl. H	.00851	.00902	5.7	25.0	754	719	11,910	11,910
Sheep Bl. J	.00773			27	766	(738)	(12,210)	(12,390)
Sheep Bl. K	.00769			27	778	(750)	(12,420)	
Bovine Bl. L	.00907			25	767	(739)	(12,240)	
Bovine Bl. M	.01002			26	766	(738)	(12,240)	(12,350)
Bovine Bl. N	.01004			26	780	(751)	(12,460)	
Bovine Hb. P	.01682			27	735	(707)	(11,700)	

TABLE III

COMPARISON OF IRON ANALYSES WITH CARBON MONOXIDE CAPACITIES AFTER REDUCTION

Blood A		Solution E	
f_{Fe}	(0.00805)	f_{Fe}	0.00736
	.00831		.00737
	.00825		.00743
	(.00809)		.00736
	.00817		.00737
Mean	0.00824	Mean	0.00738
Mean f_{CO} (Tab. I)	0.00832	Mean f_{CO}	0.00747

c. g. s. u. This corresponds to a moment of 5.435 ± 0.015 Bohr magnetons calculated by assuming that Curie's law holds and that the iron atoms act independently. The bracketed values for preparations L to N, obtained with the small empirical correction, agree well with this one. The value for solution P is unaccountably low and will be disregarded. The result obtained by Pauling and Coryell[1b] was 5.46 Bohr magnetons with a much higher uncertainty.

The earlier work left some doubt as to whether breaking the corpuscles affects the magnetic properties. The agreement of blood and hemoglobin solutions shown here definitely proves that it does not. Additional experiments confirming this directly were also carried out by measuring the Δw before and after hemolysis with saponin.

Examination of the data for the other species studied shows that the susceptibilities for sheep and horse hemoglobin are within experimental error equal to that for cow hemoglobin. The value for human hemoglobin, however, is lower by 3%. According to the hypothesis that the moment observed is increased by interactions between the hemes, some slight variation between species might be expected, as different oxygen dissociation curves indicate differences in the amount of interaction with respect to oxygen equilibrium. Also the differences in globins from species to species might lead us to expect some small effect.

The fact that the difference between the values obtained in this research and the value obtained in the previous one is less than the experimental error of the previous research makes modification of the theoretical interpretations unnecessary.

Discussion of the Iron Not Combining with Oxygen.—It will now be shown that the conclusions given above are essentially independent of the presence of iron in the original preparation which does not combine with oxygen. The results presented in Table III show that the determination of the carbon monoxide capacity of a hemoglobin solution after reduction is a measure of the total iron in the solution.[8] In fact, about one per cent. less iron was found by direct analysis than by gasometric analysis, but the occurrence of low values in the iron determinations for blood A (which are not included in the mean) indicates that results tend to be low unless the analysis is made very carefully. The frequently reported agreement between iron determination and oxygen determination of hemoglobin concentration probably rests on the tendency for the former to give low results.

The oxygen or the carbon monoxide capacity of a hemoglobin preparation, corrected for the physical solubility of the gas, measures the amount of ferrous iron present in the form of hemoglobin or closely related compounds, since ferriheme compounds do not combine with these gases under any known experimental conditions. Since it was found in our work that the carbon monoxide capacity after reduction of the whole blood or hemoglobin solution is always greater than the oxygen capacity by amounts of the order of magnitude of 5%, it is concluded that there was originally present an appreciable fraction of iron in the ferric form. This iron may have been in the form of normal ferrihemoglobin, or of a slightly altered derivative with similar properties, or of ferriheme itself, or of a compound differing quite widely in structure but capable of combining with carbon monoxide after reduction. Any of these ferric compounds would be expected to be paramagnetic and the presence of one or more of them would tend to make values of Δw_{HbO_2} high, since the ferric atom in any form of chemical binding has an odd number of electrons, and is found to have molal susceptibilities ranging from 2000 to 14,600 $\times 10^{-6}$ c. g. s. u.

On saturation of the reduced solutions with carbon monoxide to measure Δw_{HbCO}, essentially all of the iron combines with carbon monoxide. Carbonmonoxyhemoglobin and carbonmonoxyferroheme[9] are both diamagnetic and it is likely that similar compounds are also diamagnetic. For these reasons we have used reduced solutions saturated with carbon monoxide as the diamagnetic reference throughout this investigation.

(8) The experiments of Van Slyke and Hiller[6] show that prior to reduction the carbon monoxide and oxygen capacities of blood are identical.

(9) Unpublished researches by Richard W. Dodson in these Laboratories.

The measurement of Δw_{Hb} gives an average susceptibility for the various forms of ferro compound. If the original ferri compound in the solutions had been normal ferrihemoglobin, there would be no error. If it had been ferriheme, the presence of as much as 6% of it on reduction would make the observed value low by 0.015 Bohr magneton, a figure which is the same order of magnitude as the estimated error. It is probable that the actual ferri compound after reduction is more closely related to ferrohemoglobin than to ferroheme, and that the true moment of the pure bovine ferrohemoglobin is accordingly very close to the observed value of 5.435. The close agreement between susceptibilities observed for preparations differing widely in the amount of excess iron (preparations A and E, for instance) provides support for this argument.

The average amount of iron in the original preparations of bovine blood (A to F) not combining with oxygen is 5.7% of the total iron (Table II). This value is in satisfactory agreement with that determined by Klumpp[4] by determinations of oxygen capacity and iron content. From the differences between Δw_{HbO_2} and Δw_{HbCO} and the concentration of the ferric iron measured by the differences between f_{CO} and f_{O_2} (Table I), values of the average paramagnetic susceptibility of the iron in the ferric state may be obtained; these are however of only qualitative significance because of the relatively large experimental error to be expected. The average value of $10^6\chi_{molal}$ calculated for solutions A to F is 5000 with an average deviation of 2000. It is possible that complete saturation was not attained in preparing the carbonmonoxyhemoglobin solutions for the magnetic measurements. Assuming that such an error had been made and using only the most negative one or two of the Δw_{HbCO} values for each blood, the molal susceptibility is found to be 7000 with essentially the same average deviation. It should be pointed out that relatively small constant errors in any of the analytical methods would influence this determination greatly, and that the two types of gas analysis are somewhat different in method. Nevertheless, these values are considerably lower than the value of $10^6\chi_m$ for ferrihemoglobin,[1c] 14,000. If the porphyrin of the iron has been altered, as suggested by Barkan and Schales,[3c] it would be difficult to predict how much of a change would occur in the susceptibility of the ferric atom. A low value is to be correlated with an increase in the covalent character of the iron bonds.[10]

It is to be noted that the presence of ferric compounds in normal blood and oxyhemoglobin solutions prepared from it should be taken into account in the determination of various physicochemical constants. The erroneous assumption that the oxygen capacity gives a measure of the total hemoglobin concentration of preparations is widespread, and the effect of an appreciable concentration of a substance of undefined character is difficult to estimate. Barkan has shown that repeated recrystallization does not effect a change in the fraction of iron cleaved on acid denaturation.

Summary

Values of the paramagnetic susceptibilities per formula weight of the iron in hemoglobins of the cow, horse, sheep, and human have been found to be 12,290, 12,260, 12,390, and 11,910 $\times 10^{-6}$ c. g. s. u., respectively, at 25°. In an extensive study of cow hemoglobin this quantity is found to be constant from individual to individual and to be not affected by hemolysis. The magnetic moment calculated for cow hemoglobin (assuming independent iron atoms) is 5.435 ± 0.015 Bohr magnetons; for horse, sheep, and human hemoglobin the moments are 5.43, 5.46, and 5.35, respectively.

The presence in normal blood and in oxyhemoglobin solutions of hemoglobin-like compounds which do not combine with oxygen has been noted. There also has been presented evidence that the iron in these compounds is in the ferric state, and a rough value for its atomic paramagnetic susceptibility has been determined. The hemoglobin concentration determined from the carbon monoxide capacity after reduction agrees with that determined from the iron content. The importance of this fact in hemoglobin chemistry has been pointed out.

With the accurate determination of the paramagnetic susceptibility of the iron of hemoglobin, it is now possible to determine hemoglobin concentrations magnetometrically with high precision.

PASADENA, CALIF. RECEIVED FEBURARY 25, 1938

(10) In this connection compare values[1c] of $10^6\chi_m$ of 8300 and 2600 for ferrihemoglobin hydroxide and ferrihemoglobin cyanide, respectively. The observed value for the ferric iron of approximately 6000 is not to be ascribed to normal ferrihemoglobin hydroxide because the preparations had a *p*H of approximately 7.4, at which acidity only about one-sixth of the ferrihemoglobin would be combined with hydroxide.