not give data at 40 but at 38°; these values of K_2' are *assumed* to hold at 40°.

Corrections were then applied by means of the relation $x^2/(C - x) = K'/K_2'$ for each value of "C" listed in Tables I and II and for each value of $\mu^{1/2}$. The resulting values of $(C - x)$, the true $[CO_3^=]$, were then related to the ionic strength by plotting $1/m\pm$ against $\mu^{1/2}$. Extrapolation of these curves to zero value of $\mu^{1/2}$ yielded the following values.

TABLE III

EXTRAPOLATED VALUES OF $1/m\pm$ AT $\mu^{1/2} = 0$

Salt	$BaCO_3$		$SrCO_3$	
Temp., °C.	25	40	25	40
$[1/m\pm]_{\mu=0}$	42,880	58,100	95,800	151,500

These extrapolated values of $1/m\pm$ were used to calculate the values of the activity coefficients of the carbonates in their various solutions, according to the method of Lewis and Randall,[6] by means of the relation

$$f = [1/m\pm]_\mu/[1/m\pm]_{\mu=0}$$

The following tables present these calculated values, in which "Total m" is the ionic strength.

TABLE IV

ACTIVITY COEFFICIENTS OF BARIUM CARBONATE IN ALKALI CHLORIDE SOLUTIONS

Total m	Act. coeff. at 25° in LiCl	NaCl	KCl	Act. coeff. at 40° in LiCl	NaCl	KCl
0.001	0.919	0.933	0.944	0.873	0.864	0.873
.002	.877	.909	.930	.824	.811	.823
.005	.797	.863	.901	.732	.711	.730
.010	.716	.807	.865	.633	.611	.628
.020	.581	.716	.802	.513	.492	.508
.050	.338	.501	.606	.330	.321	.356
.100	.196	.326	.415	.211	.217	.260
.200	.110	.193	.263	.119	.138	.184
.500	.056	.091	.147	.050	.076	.118
1.000	.033	.054	.098	.024	.050	.084

TABLE V

ACTIVITY COEFFICIENTS OF STRONTIUM CARBONATE IN ALKALI CHLORIDE SOLUTIONS

Total m	Act. coeff. at 25° in LiCl	NaCl	KCl	Act. coeff. at 40° in LiCl	NaCl	KCl
0.001	0.881	0.931	0.946	0.840	0.892	0.876
.002	.815	.903	.933	.761	.856	.827
.005	.690	.847	.897	.596	.784	.725
.010	.566	.783	.856	.484	.700	.614
.020	.449	.678	.783	.363	.576	.486
.050	.321	.455	.568	.236	.372	.346
.100	.238	.278	.390	.161	.229	.251
.200	.175	.149	.251	.110	.117	.177
.500	.121	.067	.159	.069	.046	.111
1.000	.089	.041	.122	.043	.025	.078

Summary

1. The solubilities at 25 and 40° of barium and strontium carbonates have been determined in pure water and in solutions of lithium, sodium and potassium chlorides. The solubilities in solutions of the alkali chlorides are presented tabularly.

2. The activity coefficients of these carbonates in the alkali chloride solutions have been calculated and recorded.

3. The average heats of solution of these carbonates into their saturated solutions have been calculated for the temperature range 25–40°.

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The Magnetic Properties and Structure of Ferrihemoglobin (Methemoglobin) and Some of its Compounds

BY CHARLES D. CORYELL, FRED STITT AND LINUS PAULING

Our studies of the magnetic properties of hemoglobin derivatives containing ferrous iron,[1,2] including ferroheme, several hemochromogens, hemoglobin, and carbonmonoxyhemoglobin, led to the discovery that in two of these substances (ferroheme, hemoglobin) there are four unpaired electrons per heme, indicating that the bonds attaching the iron atoms to the rest of the molecule are essentially ionic in character, whereas the others contain no unpaired electrons, each iron atom being attached to six adjacent atoms by essentially covalent bonds. We have now investigated ferrihemoglobin[3] (acid methemoglobin), ferrihemoglobin hydroxide (alkaline methemoglobin), ferrihemoglobin fluoride, ferrihemoglobin cyanide, and ferrihemoglobin hydrosulfide, and have found a variety in magnetic properties greater than that for the ferrohemoglobin derivatives; the magnetic susceptibilities

(1) L. Pauling and C. D. Coryell, *Proc. Nat. Acad. Sci.*, **22**, 159 (1936).

(2) L. Pauling and C. D. Coryell, *ibid.*, **22**, 210 (1936).

(3) The nomenclature used in this paper is described in ref. 2.

of ferrihemoglobin and its fluoride appear to correspond to five unpaired electrons per heme, those of the cyanide and hydrosulfide to one, and that of the hydroxide to three. The structural significance of these results is discussed in the last section of this paper.

Technique of the Magnetic Measurements.—The Gouy method was used to determine the magnetic susceptibilities of solutions of hemoglobin derivatives, the difference in susceptibility of solution and water being measured with use of a glass tube divided by a glass partition into two compartments, one containing solution and the other water.[4] The tubes used were about 18 mm. in internal diameter and 30 cm. long, and were provided with ground glass caps for the ends and with suitable supports for suspension from the balance arm. Fields of about 7640 and 8810 gausses were used; forces measured at 8810 gausses were changed to 7640 gausses by multiplication by the experimentally determined factor 0.752, and all measurements made (usually four) averaged to give a mean value of Δw (in milligrams). Measurements were made in several tubes with slightly different diameters. Each tube was calibrated by the measurement of Δw for water against air, these values being close to 47. Experimentally determined corrections have been applied for dilution and for the diamagnetism of added reagents. All susceptibility measurements were made at temperatures between 22 and 26°.

The concentration of each hemoglobin solution used was determined by reducing to ferrohemoglobin with 0.3 or 0.6 g. of sodium hydrosulfite (for 30 ml. of solution), determining Δw, then saturating with carbon monoxide in the dark or in diffuse daylight and again determining Δw. The change in Δw corresponds to a change in molal susceptibility (per heme) of $12{,}430 \times 10^{-6}$ c. g. s. u. at 24°, the effective magnetic moment of ferrohemoglobin per heme being taken[2] as 5.46 Bohr magnetons. Representing Δw for ferrohemoglobin solution by Δw_{Hb}, that for carbonmonoxyhemoglobin solution by Δw_{COHb}, and that for the solution being studied by Δw (these Δw's corresponding to the same molal concentration), the molal susceptibility (per heme) at 24° for the solution being studied is given by the equation

$$\chi_{\mathrm{molal}} = \frac{\Delta w - \Delta w_{\mathrm{COHb}}}{\Delta w_{\mathrm{Hb}} - \Delta w_{\mathrm{COHb}}} \cdot 12{,}430 \cdot 10^{-6} \text{ c. g. s. u.} \quad (1)$$

and the effective magnetic moment per heme by the equation

$$\mu = \left(\frac{\Delta w - \Delta w_{\mathrm{COHb}}}{\Delta w_{\mathrm{Hb}} - \Delta w_{\mathrm{COHb}}}\right)^{1/2} \cdot 5.46 \text{ Bohr magnetons} \quad (2)$$

Measurements of *p*H values were made with a Beckman glass-electrode *p*H meter generously furnished by Professor A. O. Beckman of these Laboratories. As a standard 0.05 *M* potassium hydrogen phthalate solution, *p*H 3.97, was used. Corrections were applied in alkaline solutions for error due to sodium and potassium ions present as recommended by the manufacturer of the instrument.

Preparation of Ferrihemoglobin Solutions.—In the preliminary work ferrihemoglobin solutions were prepared by oxidation of oxyhemoglobin solutions with potassium ferricyanide, the excess of this reagent then being converted to ferrocyanide (which has zero magnetic moment) by the addition of sodium sulfite, which does not reduce ferrihemoglobin. It was also found that on addition to oxyhemoglobin solutions of sodium hydrosulfite and then of potassium ferricyanide the hemoglobin is oxidized to ferrihemoglobin and the excess ferricyanide reduced to ferrocyanide by the sulfite formed earlier by oxidation of the hydrosulfite by oxyhemoglobin.

In order to avoid the possibility of magnetic effects of the added iron the following very satisfactory method of preparing ferrihemoglobin solutions was developed, involving the auto-oxidation of oxyhemoglobin solutions at *p*H 4.8 to 5.3. Corpuscles obtained by centrifuging bovine blood are washed three times with 0.14 *M* potassium chloride solution, laked with ether, and centrifuged, the ether then being removed by bubbling air through the solution. To this solution enough 6 *N* lactic acid solution is added (about 16 ml. per liter) to bring the *p*H to about 4.9. At this *p*H the auto-oxidation reaction is complete after the solution has remained for forty-eight hours at room temperature. The solution, now at *p*H about 5.15, is brought to *p*H 7.0 by the addition with vigorous stirring of 1 *N* potassium hydroxide solution and centrifuged to remove the small amount of denatured protein formed during acidification. For different preparations used in this work the concentration of ferrihemoglobin, determined magnetically as described above, ranged from 0.0113 to 0.0176 formal in heme-iron.

By following the concentration of oxyhemoglobin magnetically it was found that in twenty-four hours at *p*H 5.2 and temperature 22° the amount of oxyhemoglobin present had fallen to 1% of its initial value;[5] in order to ensure completion of the reaction it is recommended that the solution stand for another twenty-four hours.

Ferrihemoglobin and Ferrihemoglobin Hydroxide.—The pronounced change in spectrum of ferrihemoglobin solutions accompanying change in *p*H from the acid to the alkaline range corresponds to the addition of hydroxyl ion to ferrihemoglobin (acid methemoglobin) to form ferrihemoglobin hydroxide (alkaline methemoglobin). Spectrophotometric studies of this reaction made by Austin and Drabkin[6] have shown that the amounts of substances present at intermediate *p*H values correspond closely to the equilibrium[7]

$$\mathrm{HbOH} \rightleftharpoons \mathrm{Hb^+} + \mathrm{OH^-} \quad (3)$$

In Table I there are given magnetic data for ferrihemoglobin solutions of low ionic strength

(4) S. Freed and C. Kasper, *Phys. Rev.*, **36**, 1002 (1930).

(5) The reaction is approximately first-order in oxyhemoglobin and first-order in hydrogen ion; the rate-determining step may be

$$\mathrm{HbO_2} + \mathrm{H^+} \longrightarrow \mathrm{Hb^+} + \mathrm{HO_2}$$

with the production of hydrogen superoxide. The temperature coefficient of the rate is high, about 5 for 10°.

(6) J. H. Austin and D. L. Drabkin, *J. Biol. Chem.*, **112**, 67 (1935); see also F. Haurowitz, *Z. physiol. Chem.*, **138**, 68 (1924).

(7) We use the symbol $\mathrm{Hb^+}$ to represent the amount of ferrihemoglobin containing one heme, and HbOH, Hb, $\mathrm{HbO_2}$, etc., to represent corresponding amounts of ferrihemoglobin hydroxide, ferrohemoglobin, oxyhemoglobin, etc., respectively.

in the pH range 6.7 to 12.0. The data represent experiments made with three solutions, A, B and C. Two of these, A and B, were prepared by the lactic acid auto-oxidation method described above, and the third by a similar method, hydrochloric acid being used for acidification in place of lactic acid. The results for the three solutions show no differences greater than the experimental error. The hemoglobin concentration of each solution was determined by the ferrohemoglobin–carbonmonoxyhemoglobin magnetic method.

TABLE I

MAGNETIC SUSCEPTIBILITY OF FERRIHEMOGLOBIN SOLUTIONS OF LOW IONIC STRENGTH

Solution	pH	$\chi_{molal}\cdot10^{6}$ [a]	Solution	pH	$\chi_{molal}\cdot10^{6}$
B	6.73	13,975	A	8.32	10,790
B	6.73	13,800	A	8.32	10,840
B	6.73	14,010	A	8.34	10,930
B	6.73	13,980	A	8.49	9,950
A	6.86	13,900	A	8.57	9,835
A	6.86	13,760	A	8.60	9,890
A	6.86	13,620	A	8.61	9,620
A	6.86	13,725	A	8.63	9,660
A	6.86	13,630	C	8.97	9,080
A	6.86	14,000	A	9.02	8,960
A	6.86	13,770	A	9.06	9,250
A	6.86	13,820	A	9.54	8,775
A	6.86	13,900	A	9.61	8,340
C	6.88	13,730	A	9.82	8,730
C	6.88	13,630	C	10.01	8,420
C	6.88	13,450	A	10.02	8,020
C	6.88	13,630	A	10.30	8,040
C	6.88	13,730	B	10.78	8,765
A	7.05	13,785	A	10.81	8,380
A	7.34	13,220	B	10.82	8,310
C	7.69	12,190	B	10.90	8,470
A	7.87	12,080	A	10.92	8,355
A	7.87	12,200	A	11.73	8,150
A	8.12	11,320			

$\chi_{Hb^+}\cdot10^{6} = 14{,}060 \pm 50.$
$\chi_{HbOH}\cdot10^{6} = 8350 \pm 90.$
$pK_{HbOH} = 8.15 \pm 0.02.$
Ionic strength at pH 8.15 = 0.20.

[a] Paramagnetic contribution to susceptibility per mole of heme.

A portion of ferrihemoglobin solution was placed in one compartment of a tube and Δw determined. Successive small portions of potassium hydroxide solution (0.87 N) were then added with vigorous stirring, the values of pH and Δw being determined after the addition of each portion. The values of Δw were corrected for dilution and the diamagnetism of reagents, and converted into χ_{molal}, the paramagnetic contribution to the susceptibility per mole of heme, by means of equation 1. The data of Table I are shown by the open circles in Fig. 1.

The ionic strength of the hemoglobin solutions, due in part to the salts originally in solution in the erythrocytes and in part to added acid and base, changes somewhat with change in pH, the values at pH 7, 8 and 9 being about 0.17, 0.20 and 0.23, respectively. The contribution of hemoglobin to the ionic strength was ignored.

The data for these solutions represent a typical titration curve, the molal susceptibility changing rapidly over the range pH 7 to 9.5 from an asymptotic value of about $14{,}000\cdot10^{-6}$, representing ferrihemoglobin, Hb^+, to an asymptotic value of about $8300\cdot10^{-6}$, representing ferrihemoglobin hydroxide, HbOH. Using these estimated asymptotes to calculate concentrations of Hb^+ and HbOH, it is found that the experimental points when plotted on a graph of log ($[Hb^+]/[HbOH]$) against pH lie close to a straight line, with slope unity to within about five per cent., showing that the reaction is first-order in hydroxyl ion, as was reported by Austin and Drabkin. A theoretical susceptibility curve was fitted to the experimental points of Fig. 1 by the following procedure. The equilibrium constant K_{HbOH} for the reaction

$$HbOH \rightleftharpoons Hb^+ + OH^-$$

is related to the mole fraction x of total ferrihemoglobin in the form Hb^+ by the equation

$$\log x/(1 - x) = pK_{HbOH} - pH \qquad (4)$$

in which pK_{HbOH} is log (K_{HbOH}/K_W), with K_W the equilibrium constant for the ionization of water. The value of the molal susceptibility χ as a function of pH is then given by the expression

$$\chi = x\chi_{Hb} + (1 - x)\chi_{HbOH} \qquad (5)$$

in which x is determined by equation 4. With χ_{HbOH}, χ_{Hb^+} and pK_{HbOH} as variable parameters, this equation was fitted to the experimental points in such a way as to minimize the mean deviation from the curve. The final curve was found to have $\chi_{HbOH} = 8350 \pm 90\cdot10^{-6}$, $\chi_{Hb^+} = 14{,}060 \pm 50\cdot10^{-6}$, and $pK_{HbOH} = 8.15 \pm 0.02$, the indicated probable errors for the susceptibility values being half the mean deviations and that for pK_{HbOH} being an estimated value.

The data given in Table II and represented by the solid circles in Fig. 1 were obtained with a solution, D, to which potassium chloride had been added, the ionic strength being increased in this way to the value 1.3 (at pH 8.56). These data also are well represented by a curve corre-

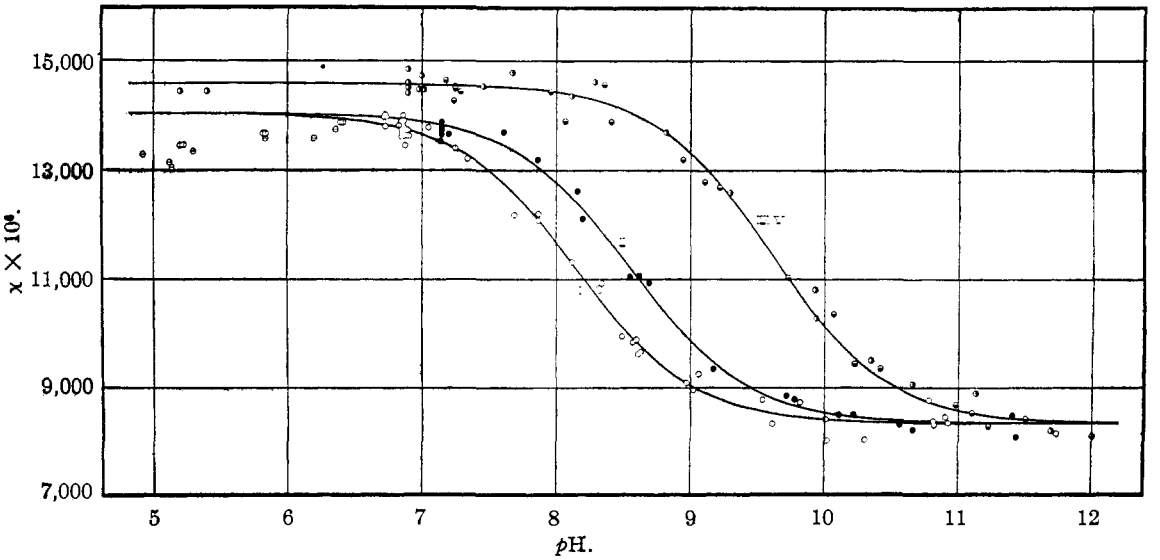

Fig. 1.—The dependence of the magnetic susceptibility of ferrihemoglobin solutions on pH: ○, solutions of low ionic strength, Table I; ●, solutions of high ionic strength, Table II; ⊖ ⊕, solutions in low pH range, Table III; ◑, solutions of low ionic strength with added fluoride, Table IV; ⊖, solutions of high ionic strength, with added fluoride, Table V.

sponding to equations 5 and 4, the values of the parameters for the curve giving the best fit being $\chi_{HbOH} = 8320 \pm 60 \cdot 10^{-6}$, $\chi_{Hb^+} = 14{,}000 \pm 70 \cdot 10^{-6}$, and $pK_{HbOH} = 8.56 \pm 0.02$.

TABLE II

MAGNETIC SUSCEPTIBILITY OF FERRIHEMOGLOBIN SOLUTIONS OF HIGH IONIC STRENGTH

Solution D

pH	$\chi_{molal} \cdot 10^6$	pH	$\chi_{molal} \cdot 10^6$
7.14	13,530	8.62	11,070
7.15	13,880	8.69	10,940
7.15	13,650	9.17	9,350
7.15	13,760	9.72	8,850
7.15	13,790	9.78	8,780
7.15	13,720	10.11	8,500
7.20	13,670	10.22	8,500
7.61	13,700	10.56	8,325
7.86	13,190	10.66	8,220
8.16	12,620	11.40	8,480
8.20	12,120	11.43	8,080
8.55	11,050		

$\chi_{Hb^+} \cdot 10^6 = 14{,}000 \pm 70$.
$\chi_{HbOH} \cdot 10^6 = 8320 \pm 60$.
$pK_B = 8.56 \pm 0.02$.
Ionic strength at pH 8.56 = 1.3.

The values found for χ_{HbOH} and χ_{Hb^+} in solutions at high and low ionic strength are in excellent agreement. For χ_{Hb^+} we accept the average value $14{,}040 \cdot 10^{-6}$, giving double weight to the asymptote of curve I. For χ_{HbOH} we obtain the mean value $8340 \cdot 10^{-6}$ by considering in addition to the two values $8350 \cdot 10^{-6}$ and $8320 \cdot 10^{-6}$ discussed above the values $8370 \cdot 10^{-6}$ and $8320 \cdot 10^{-6}$ obtained from solutions containing fluoride ion, discussed in the following section. The structural interpretation of the susceptibility values will be considered in the concluding section of the paper.

The equilibrium constant K_{HbOH} is dependent on the ionic strength of the solution, pK_{HbOH} changing from 8.15 to 8.56 with increase in ionic strength from 0.20 to 1.3. This dependence was observed by Austin and Drabkin, who found that pK_{HbOH} could be represented approximately by the equation

$$pK_{HbOH} = \text{constant} + \alpha\sqrt{\mu} \qquad (6)$$

in which μ is the ionic strength, the value for α being about 0.6. Our measurements support this,[8] the change for curves I and II corresponding to $\alpha = 0.59$.

The value $pK_{HbOH} = 8.12 \pm 0.01$ reported by Austin and Drabkin for canine hemoglobin at ionic strength 0.10 agrees reasonably well with our value 8.15 ± 0.02 at ionic strength 0.20 (corresponding to 8.07 at ionic strength 0.10) for bovine hemoglobin; complete agreement would, of course, not be expected for hemoglobins from different species.

It has been reported by Drabkin and Austin

(8) An experiment carried out involving the determination of change in Δw and pH for a portion of solution A, initially at pH 8.57, on the addition of successive portions of potassium chloride solution led to rough verification of the form of equation 6.

that ferrihemoglobin solutions become turbid at *p*H values less than 6. Two series of magnetic experiments were made in this *p*H range. In the first series a portion of solution A was made more and more acid by the addition, with rapid stirring, of successive small portions of 1 *N* hydrochloric acid solution, values of *p*H and Δw being determined after each acidification. These data are given in Table III and shown by the horizontally barred circles in Fig. 1. The formation of a small amount of coagulum in the acid solution was noticed; in order to eliminate possible error due to this coagulation, the following series of measurements was made. A portion of solution C was brought to *p*H 5.2 by the addition of hydrochloric acid, and centrifuged to remove the coagulum formed on acidification. The solution obtained in this way (solution E) was then made more and more alkaline by the addition of small portions of potassium hydroxide solution, values of *p*H and Δw being determined at each step. No coagulum was formed during this treatment. The ferrihemoglobin concentration, made uncertain by the loss of the coagulum resulting from the initial acidification, was determined by identifying the measured susceptibility at *p*H 7.25 (the most alkaline point) with that corresponding to the theoretical curve I. The data obtained in this way, given in Table III and represented in Fig. 1 by vertically barred circles, are in reasonably good agreement with those obtained by acidification. The measurements correspond to a decrease of about 5% in molal susceptibility in very acid solutions.

TABLE III

MAGNETIC SUSCEPTIBILITY OF FERRIHEMOGLOBIN SOLUTIONS OF LOW IONIC STRENGTH IN ACID SOLUTIONS

Solution	*p*H	$\chi_{molal}\cdot10^6$	Solution	*p*H	$\chi_{molal}\cdot10^6$
A	4.92	13,280	A	5.84	13,580
A	5.12	13,130	A	6.20	13,595
A	5.14	13,050	A	6.36	13,750
E	5.20	13,470	E	6.40	13,870
E	5.23	13,470	E	6.41	13,870
A	5.30	13,340	A	6.83	13,815
E	5.82	13,670	E	6.88	13,600
E	5.83	13,670	E	7.25	(13,410)

Ferrihemoglobin Fluoride.—The absorption spectrum of an acid ferrihemoglobin solution is changed in a pronounced manner by the addition of fluoride, indicating the formation of a compound. A crystalline compound was prepared and analyzed by Haurowitz,[9] who reported the substance to contain one atom of fluorine per atom of iron. Our magnetic studies have verified this, and have led to the evaluation of the equilibrium constants for the reactions

$$HbF + OH^- \rightleftharpoons HbOH + F^-, \text{ and} \quad (7)$$
$$HbF \rightleftharpoons Hb^+ + F^-$$

The data given in Tables IV (for solutions A, B, C) and V (for solution D) were obtained by adding 0.5 g. of sodium fluoride to a 32-ml. portion of ferrihemoglobin solution and adding small portions of 0.87 *N* potassium hydroxide solution, with vigorous stirring, *p*H and Δw being determined after the addition of each portion. The

TABLE IV

MAGNETIC SUSCEPTIBILITY OF FERRIHEMOGLOBIN SOLUTIONS OF LOW IONIC STRENGTH WITH ADDED FLUORIDE

Solution	*p*H	$\chi_{molal}\cdot10^6$	Solution	*p*H	$\chi_{molal}\cdot10^6$
A	5.2	14,430	A	8.12	14,355
C	5.4	14,430	A	8.29	14,620
A	6.90	14,620	A	8.82	13,700
A	6.90	14,510	A	9.30	12,590
A	6.90	14,410	A	9.93	10,820
A	6.90	14,845	A	9.94	10,280
B	7.0	14,730	A	10.34	9,500
B	7.0	14,520	A	10.66	9,060
C	7.0	14,480	A	11.13	8,900
C	7.0	14,480	A	11.69	8,210
A	7.46	14,530	A	12.0	8,115
A	7.68	14,790			

$\chi_{HbF}\cdot10^6$ = 14,630 ± 70 (uncorr.), 14,660 ± 70 (corr.).
$\chi_{HbOH}\cdot10^6$ = 8370 ± 140.
$pK_{OH,F}$ = 9.63 ± 0.01 (uncorr.), 9.65 ± 0.01 (corr.).
Ionic strength at *p*H 9.63 = 0.54.
$[F^-]$ = 0.34 at *p*H 9.63.

TABLE V

MAGNETIC SUSCEPTIBILITY OF FERRIHEMOGLOBIN SOLUTIONS OF HIGH IONIC STRENGTH WITH ADDED FLUORIDE

Solution D

*p*H	$\chi_{molal}\cdot10^6$	*p*H	$\chi_{molal}\cdot10^6$
7.18	14,640	9.11	12,780
7.24[a]	14,270	9.22	12,680
7.25	14,490	9.73	11,030
7.25	14,520	10.07	10,360
7.29	14,430	10.23	9,440
7.96	14,430	10.42	9,350
8.07	13,900	10.98	8,690
8.36[b]	14,570	11.10	8,530
8.41	13,880	11.22	8,290
8.94[a]	13,190	11.50	8,425

$\chi_{HbF}\cdot10^6$ = 14,500 ± 80 (uncorr.), 14,550 ± 80 (corr.).
$\chi_{HbOH}\cdot10^6$ = 8320 ± 40.
$pK_{OH,F}$ = 9.60 ± 0.01 (uncorr.), 9.64 ± 0.01 (corr.).
Ionic strength at *p*H 9.60 = 1.6.
$[F^-]$ = 0.34 at *p*H 9.60.

[a] By adding 1 *N* HCl to solution of *p*H 10.98. [b] By adding 1 *N* HCl to solution of *p*H 9.22.

(9) F. Haurowitz, *Z. physiol. Chem.*, **138**, 68 (1924).

points for solutions A, B and C, with low ionic strength (0.54 at pH 9.6), and those for solution D, with high ionic strength (1.6 at pH 9.6), lie close to the same curve, showing that there is no appreciable salt effect for the reaction. This provides support for the postulate that the reaction consists essentially in the conversion of ferrihemoglobin fluoride into ferrihemoglobin hydroxide (eq. 7), for which no appreciable salt effect would be expected. Further evidence for equation 7 will be mentioned later.

The molal susceptibility values of Tables IV and V can be approximated closely by curves of the type used above for the ferrihemoglobin–ferrihemoglobin hydroxide equilibrium. For reaction 7 the equilibrium constant $K_{OH,F}$ has the value

$$K_{OH,F} = [HbOH][F^-]/[HbF][OH^-] \quad (9)$$

and the molal susceptibility is given by the equation

$$\chi = y\chi_{HbF} + (1 - y)\chi_{HbOH} \quad (10)$$

in which y is the mole fraction of total ferrihemoglobin in the form of ferrihemoglobin fluoride, given by the equation

$$\log y/(1 - y) = pK_{OH,F} - p\mathrm{H} \quad (11)$$

in which $pK_{OH,F}$ is $-\log (K_{OH,F}K_W/[F^-])$. The asymptotes of the curves which fit the data most closely are $14{,}630\cdot10^{-6}$ and $8{,}370\cdot10^{-6}$ for solutions A, B and C, and $14{,}500\cdot10^{-6}$ and $8320\cdot10^{-6}$ for solution D. The values $8370\cdot10^{-6}$ and $8320\cdot10^{-6}$, representing χ_{HbOH}, are in excellent agreement with the values $8350\cdot10^{-6}$ and $8320\cdot10^{-6}$ found for solutions without added fluoride.

The values for the other asymptote represent molal susceptibility not of ferrihemoglobin fluoride itself but of ferrihemoglobin fluoride containing a small fraction of ferrihemoglobin (Hb^+). From the equilibrium constants given below the values of the concentration ratio $[Hb^+]/[HbF]$ are found to be 0.047/0.953 for solutions A, B and C, and 0.094/0.906 for solution D. Using these ratios and the known value of χ_{Hb^+} to correct for the ferrihemoglobin present, the values $\chi_{HbF} = 14{,}660 \pm 70\cdot10^{-6}$ and $14{,}550 \pm 80\cdot10^{-6}$ are obtained; we accept for χ_{HbF} the mean of these closely agreeing values, $14{,}610\cdot10^{-6}$.

The values 9.63 and 9.60 for $pK_{OH,F}$ given by the curves lead on similar consideration of the $[Hb^+]/[HbF]$ ratio to the corrected values 9.65 and 9.64, which agree to within their estimated probable error of 0.01. Introducing the values of $[F^-]$ (0.34 at pH 9.6) and K_W ($10^{-14.01}$), we obtain for the equilibrium constant $K_{OH,F}$ at 24° the value $0.78\cdot10^4$.

The equilibrium constant K_{HbOH} for reaction 3 is given by the equation

$$\log K_{HbOH} = -6.12 + 0.59\sqrt{\mu} \quad (12)$$

Combining with this the value of $K_{OH,F}$, we obtain for K_{HbF}, the equilibrium constant for the reaction

$$HbF \rightleftharpoons Hb^+ + F^- \quad (13)$$

the expression

$$\log K_{HbF} = -2.23 + 0.59\sqrt{\mu} \quad (14)$$

Three sets of measurements were made to test these equilibrium constants by direct titration with potassium fluoride solution. In the first run a portion of solution A was brought to pH 8.60, and values of pH and Δw were measured after the addition of successive small portions of known volume of 2.00 f potassium fluoride solution. The average of nine values of K_{HbF} corresponding to the nine added portions of fluoride solution is 0.0145, with a mean deviation of 0.0012; this value is in good agreement with the value 0.013 given by equation 14 for μ = 0.34. When the formality of fluoride in the solution had reached 0.28, the pH was observed to have changed to the value 8.89, an increase of 0.29; this agrees well with the calculated change in pH expected because of the replacement of hydroxyl in ferrihemoglobin hydroxide by fluorine, 0.30. A less reliable set of six measurements made on solution D at pH 8.6 gave the average value 0.049 for K_{HbF}. This is somewhat larger than the value 0.029 given by equation 14 for μ = 1.4, perhaps because of experimental error, the total change in Δw values during the run being less than 1 mg. A set of five measurements made on solution A at pH 6.2 gave for K_{HbF} the average value 0.008, somewhat lower than the value 0.010 expected for μ = 0.22; the difference may be due to error in the measurement of Δw, which changed by only 0.40 mg. during the run.

A determination of the value of K_{HbF} by a spectrophotometric method has been reported by Lipmann.[10] The value found, about 0.015, for a solution of swine hemoglobin of uncertain ionic strength agrees with that calculated from equation 14 with μ given the reasonable value 0.5. Lipmann also reported a decrease of the constant in very acid solutions.

Ferrihemoglobin Cyanide.—Crystalline ferrihemoglobin cyanide was first obtained by Zey-

(10) F. Lipmann, *Biochem. Z.*, **206**, 171 (1929).

nek,[11] who by analysis showed the substance to contain one cyanide per heme. We have verified this by titration of a ferrihemoglobin solution with cyanide. The data represented in Fig. 2 were obtained with a solution containing equal volumes

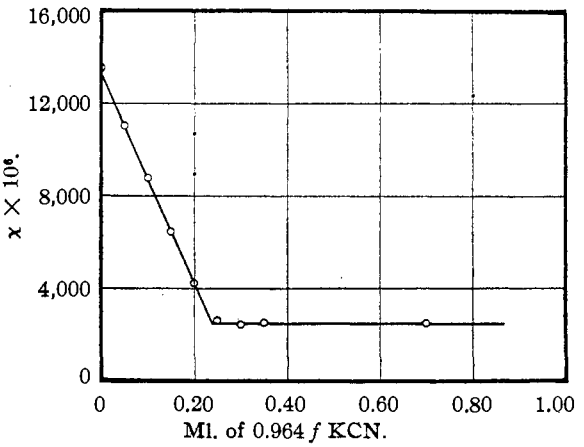

Fig. 2.—The magnetic titration of ferrihemoglobin with potassium cyanide at *p*H 6.75.

of ferrihemoglobin solution B and a phosphate buffer, the *p*H of the solution being 6.75. To 31.25 ml. of this solution there was added from a 1-ml. glass syringe graduated in hundredths successive measured volumes (0.050 ml.) of 0.964 *f* potassium cyanide solution. When the molal susceptibility is plotted against the amount of cyanide added the first five points fall close to a straight line and the last four to a horizontal straight line. The intersection of these lines occurs at 0.235 ml., which agrees to within the experimental error with the value 0.236 ml. calculated for one cyanide per heme. The value found for χ_{HbCN} in this experiment is $2520 \cdot 10^{-6}$. A similar experiment performed at *p*H 10.8 gave similar results.

In order to determine the molal susceptibility of ferrihemoglobin cyanide accurately sets of duplicate measurements were made with unbuffered solution B at *p*H 6.7 and with solution B brought to *p*H 10.9 by the addition of potassium hydroxide solution, an excess of potassium cyanide solution being added in each case. The values found for $\chi_{HbCN} \cdot 10^6$ are 2590 and 2620 at *p*H 6.7 and 2630 and 2590 at *p*H 10.9. There is accordingly no dependence of χ_{HbCN} on *p*H. The average of the measured values is $2610 \cdot 10^{-6}$. Values approximating this were also obtained in several preliminary measurements and in measurements made incidental to other experiments.

In order to obtain an approximate value for the equilibrium constant K_{HbCN} for the reaction

$$HbCN \rightleftarrows Hb^+ + CN^- \qquad (15)$$

two cyanide titration experiments were made with ferrihemoglobin solution B brought to *p*H 4.77 by the addition of an equal volume of an acetate buffer solution 2 *M* in acetic acid and 2 *M* in sodium acetate. A portion of the solution was placed in one compartment of a tube, which was then closed with a thin rubber stopper. Portions of 0.964 *f* potassium cyanide solution were then added by means of a syringe, the needle being inserted through the rubber stopper, and Δw values were measured. The corresponding values of χ_{molal} for the two runs are shown in Fig. 3. When about one-half of the stoichiometric amount of cyanide has been added it is almost entirely in the form of HbCN. With larger amounts of cyanide the formation of HbCN is incomplete, the amount of cyanide present as HCN becoming appreciable. The ratio $[CN^-]/[HCN]$ is about 10^{-4}, so that the equilibrium measured is essentially

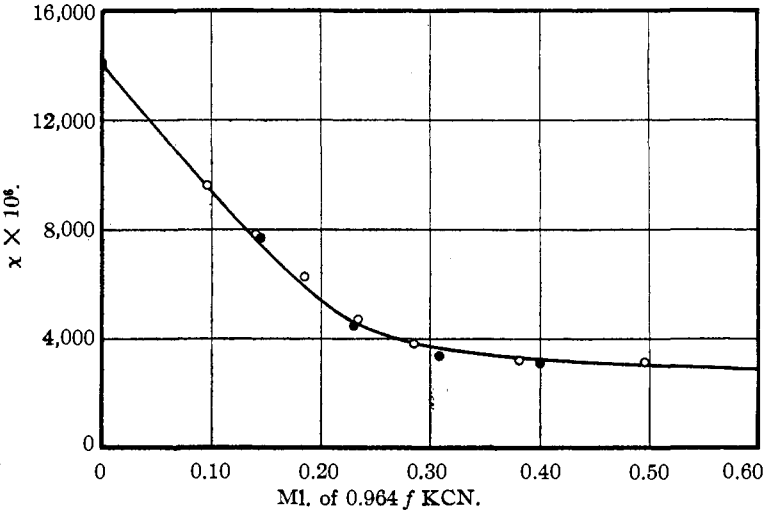

Fig. 3.—The magnetic titration of ferrihemoglobin with potassium cyanide at *p*H 4.77.

$$Hb^+ + HCN \rightleftarrows HbCN + H^+ \qquad (16)$$

The experimental points lie close to the theoretical equilibrium expression, represented by the curve in Fig. 3; the value found for the corresponding

(11) R. v. Zeynek, *Z. physiol. Chem.*, **33**, 426 (1901).

equilibrium constant (presumably essentially independent of ionic strength) is 18. This value multiplied by the ionization constant of HCN, $2.0 \cdot 10^{-9}$, gives for K_{HbCN} the value $3.6 \cdot 10^{-8}$, at zero ionic strength.[12] The dependence on ionic strength is probably nearly the same as for K_{HbOH} and K_{HbF}; we hence write for K_{HbCN} the equation

$$\log K_{HbCN} = -7.44 + 0.59\sqrt{\mu} \qquad (17)$$

Ferrihemoglobin Hydrosulfide.—It was discovered by Keilin[13] that ferrihemoglobin forms a compound with hydrogen sulfide,[14] containing one sulfur atom per heme. By analogy with other compounds of ferrihemoglobin we consider it likely that this compound is ferrihemoglobin hydrosulfide, HbSH.

Solutions of ferrihemoglobin hydrosulfide made by addition of sodium hydrosulfide to ferrihemoglobin solution buffered to *p*H values in the range 5 to 7 were found to decompose rapidly, apparently undergoing auto-reduction to ferrohemoglobin, as shown by its spectrum and molal susceptibility and by the spectrum (that of oxyhemoglobin) observed after air is admitted to the solution. In order to evaluate χ_{HbSH} the following experiments were performed. To a portion of ferrihemoglobin solution B an equal volume of acetate or phosphate buffer was added. About 30 ml. of the solution was then placed in a compartment of a tube, which was closed with a thin rubber stopper, and 0.5 ml. (or, in one experiment, 0.1 ml.) of $4f$ sodium hydrosulfide solution was added through the stopper with a syringe. The tube was then placed in position, and readings of Δw were taken at intervals of about two minutes, the first being made about six minutes after the addition of the hydrosulfide. The measured values of Δw correspond to a rapid increase of the molal susceptibility with time, χ being at first linear in t and then approaching an asymptote. The data are represented by the theoretical expression for a reaction first order with respect to HbSH, with the asymptotic value of χ_{molal} equal to that for ferrohemoglobin. To evaluate χ_{HbSH} the susceptibility values over the nearly linear portion of the curve were extrapolated to zero time; for four solutions, with *p*H 5.1, 5.7, 5.7, and 7.0, respectively, the values 2240, 2110, 2260 and $1930 \cdot 10^{-6}$, average $2140 \cdot 10^{-6}$, were obtained. A value for the dissociation constant of the substance is not provided by our data.[15]

The rate constant $k = -\mathrm{d}(\ln[\mathrm{HbSH}])/\mathrm{d}t$ has the approximate value $5 \cdot 10^{-3}$ (with t measured in minutes), the observed values of $k \cdot 10^{3}$ being 5.0 at *p*H 5.08, 12.0 at *p*H 5.73, 5.2 at *p*H 7.02, and 3.0 at *p*H 5.73. (The third experiment was made with phosphate buffer, the others with acetate buffers; the fourth was made with 0.1 ml., the others with 0.5 ml. of $4f$ sodium hydrosulfide solution added.) It is seen that no more than about 2-fold variation was observed over the *p*H range 5.1 to 7.0, and that the rate seems to increase with increase in the concentration of hydrosulfuric acid.

The Interpretation of the Molal Susceptibility Values

The values of the paramagnetic part of the molal susceptibility (per heme) at 24° of the substances studied in this investigation are collected in Table VI. If it be assumed that these values result from the independent orientation of the magnetic moments of the hemes and that Curie's law is applicable, they correspond to the values of the magnetic moment μ, in Bohr magnetons, shown in the last column of the table, calculated with the equation

$$\mu = 2.84\sqrt{\chi_{molal} T}$$

in which T is the absolute temperature.

TABLE VI

VALUES OF THE PARAMAGNETIC MOLAL SUSCEPTIBILITY AND EFFECTIVE MAGNETIC MOMENT (PER HEME) OF FERRIHEMOGLOBIN AND SOME OF ITS COMPOUNDS

	$\chi_{molal} \cdot 10^{6}$ [a]	μ [b]
Ferrihemoglobin, Hb^+	14,040	5.80
Ferrihemoglobin hydroxide, HbOH	8,340	4.47
Ferrihemoglobin fluoride, HbF	14,610	5.92
Ferrihemoglobin cyanide, HbCN	2,610	2.50
Ferrihemoglobin hydrosulfide, HbSH	2,140	2.26

[a] At 24°. [b] In Bohr magnetons.

(12) An effort to check this result was made by determining magnetically the ratio ferrihemoglobin–ferrihemoglobin cyanide in a solution in equilibrium with solid silver cyanide and solid silver chloride. Three measurements, made with concentrations 0.20, 0.43 and 1.00 f of chloride ion, gave for the ratio $[Hb^+]/[HbCN]$ the values 0.20, 0.093 and 0.040, respectively. Taking $1.7 \cdot 10^{-10}$ as the solubility product of AgCl and $7 \cdot 10^{-16}$ as that of AgCN [as given by M. Randall and J. O. Halford, THIS JOURNAL, **52**, 178 (1930)], these measurements lead to $K_{HbCN} = 1.6 \cdot 10^{-6}$, in rather poor agreement with the value $1 \cdot 10^{-7}$ given by equation 17. It is possible that the disagreement is due to error in the solubility product of AgCN, inasmuch as an older value $4.5 \cdot 10^{-17}$ [Bodländer and Eberlein, *Z. anorg. Chem.*, **39**, 197 (1904)] leads to $K_{HbCN} = 1 \cdot 10^{-5}$.

(13) D. Keilin, *Proc. Roy. Soc.* (London), **B113**, 393 (1933).

(14) Keilin emphasized the fact that this compound is different from the green substance formed from ferrohemoglobin in the presence of hydrogen sulfide and oxygen.

(15) Keilin performed experiments to determine this dissociation constant. No mention of the auto-reduction reaction which we observed is made in his paper.

The possibility should be considered that the moments of the four hemes in a molecule of molecular weight 68,000 are not oriented independently, but instead are combined to a resultant constant moment for the molecule, with magnitude twice that given for μ in the table. This possibility was discussed in connection with ferrohemoglobin,[2] and reasons were advanced for rejecting it in favor of the alternative simple interpretation in terms of hemes which interact with one another only weakly; it was suggested that the observed effective moment per heme for ferrohemoglobin, 5.46, is somewhat larger than the expected value for four unpaired electrons (spin moment alone, 4.90; expected orbital contribution, about 0.1 to 0.4) because of a partial stabilization of parallel orientations of the heme moments through heme–heme interaction. Some of the arguments advanced in support of this interpretation for ferrohemoglobin are applicable to ferrihemoglobin and its compounds also, and we have, moreover, been able to interpret the susceptibility values in a reasonably satisfactory manner on this basis and not on the basis of constant moments for the four-heme molecules; hence we believe that in these substances too the heme moments are entirely or almost entirely independent of one another.[16]

The effective moment per heme observed for ferrihemoglobin fluoride is 5.92, which is identical with the theoretical spin moment for five unpaired electrons, 5.917. Moreover, for five unpaired *d* electrons the total moment is equal to the spin moment, the orbital contribution vanishing because of the occupancy of each orbit in the subgroup by one electron; there is actually very close agreement between the theoretical spin moment and the experimental values for iron-group ions and complexes with five unpaired electrons, representative observed moments being 5.94 for Mn^{++} and 5.86–5.98 for Fe^{+++} in aqueous solution, 5.88 for the fluoferriate complex $[FeF_6]^{=}$, and 5.91 for the complex $[FeF_5 \cdot H_2O]^{=}$. The observed moment for ferrihemoglobin fluoride shows that in this substance, as in the fluoferriate complex, the bonds from iron to the surrounding atoms (fluorine, the four porphyrin nitrogens, one globin nitrogen atom) are essentially ionic in character.

The effective moment per heme for ferrihemoglobin itself, 5.80, shows that in this complex too the bonds from the iron atom to the surrounding atoms are essentially ionic. It is possible that the coördination number of iron is here only five; it seems to us probable, however, that the sixth octahedral position is occupied by a water molecule,[17] the complex being $[HbOH_2]^+$ rather than Hb^+. The transition to ferrihemoglobin hydroxide would then involve loss of a proton (with change in bond type—*v. infra*) rather than addition of an hydroxyl ion.

The difference[18] between the observed moment 5.80 and the theoretical value 5.92 we attribute to heme–heme interaction operating to stabilize configurations in which the heme moments are opposed. Why the interaction should decrease the effective moment of ferrihemoglobin, increase that of ferrohemoglobin, and leave that of ferrihemoglobin fluoride unchanged we do not know.

The observed decrease in susceptibility (by about 5%) of ferrihemoglobin solutions in the low *p*H range may be due to change in the heme–heme interactions. Further experimental data are needed before a reliable explanation of this phenomenon can be given.[19]

The susceptibilities of ferrihemoglobin cyanide and ferrihemoglobin hydrosulfide correspond to the effective heme moments 2.50 and 2.26, respectively. These are close to the value expected for one unpaired electron (1.732 plus an orbital contribution of 0.3 to 0.5), showing that in these molecules two *d* orbitals of each iron atom are involved in covalent bond formation. Without doubt the structures are similar to that of the ferricyanide ion[20] (with $\mu = 2.33$), the iron atom being attached by essentially covalent bonds to six surrounding atoms arranged octahedrally, consisting of the four porphyrin nitrogens, one globin nitrogen, and the carbon of cyanide or sulfur of hydrosulfide. The observed moments

(16) The possibility that the moments of the hemes in pasri are combined to constant resultants also seems unlikely to us.

(17) It is of interest that one fluorine in $[FeF_6]^{=}$ is easily replaced by a water molecule, forming $FeF_5 \cdot H_2O^{=}$.

(18) The reality of the difference is shown by the directly observed increase in susceptibility of ferrihemoglobin solution on the addition of fluoride.

(19) During the cyanide titrations it was observed that in solutions heavily buffered (with acetate) at *p*H 4.8 the molal susceptibility of ferrihemoglobin has the value $14{,}020 \cdot 10^{-6}$, which agrees well with the asymptotic value given by the curves rather than with the values observed for unbuffered solutions.

(20) We have made a determination of the molal susceptibility of potassium ferricyanide in solution by the differential method, the diamagnetic correction being made by using the susceptibility of the ferrocyanide solution obtained by reduction with sodium sulfite. Five measurements made at concentrations of 0.608 and 0.304 *f* gave for the paramagnetic molal susceptibility at 22.0° the value $2280 \pm 10 \cdot 10^{-6}$, corresponding to $\mu = 2.33 \pm 0.01$ Bohr magnetons.

are somewhat higher than expected, indicating some heme–heme interaction; it is possible, on the other hand, that the orbital contribution is greater than usual.

None, or one, or two of the 3*d* orbitals of trivalent iron atom in a complex may be involved in covalent bond formation, the number of unpaired electrons being five, three, or one, respectively, and the moment 5.92, about 4.2, or about 2.0 Bohr magnetons. Many complexes of the first type and many of the third type are known, whereas iron complexes of the intermediate type are very rare.[21] Ferrihemoglobin hydroxide apparently is of this type; the observed effective moment 4.47 is only slightly larger than that expected for three unpaired electrons (spin moment 3.88, orbital contribution about 0.4).

The nature of the bonds in this complex is somewhat uncertain, since, although the value of the magnetic moment is that which is associated with square coördination (as in nickel protoporphyrin[1]), there is little doubt that the configuration about the iron atom is octahedral. Four dsp^2 covalent bonds directed to the corners of a square would utilize one *d* orbital; in ferrihemoglobin hydroxide it is probable that these four covalent bonds resonate among the six adjacent atoms, each of which is then attached to the iron atom by a bond with roughly two-thirds covalent character (or perhaps somewhat less).

(21) Measurements of χ made by L. Cambi and A. Cagnasso, *Rend. Ist. Lombardo Sci.*, **67**, 741 (1934), for complexes of $Fe(CNS)_3$ and $Co(CN)_3$ with *o*-phenanthroline and 2,2'-bipyridyl indicate structures of this type.

Summary

Magnetic measurements at approximately 24° of solutions of ferrihemoglobin and some of its compounds have been made, leading to values of the paramagnetic part of the molal susceptibility which correspond to the following values of the effective magnetic moment per heme, in Bohr magnetons: ferrihemoglobin, 5.80; ferrihemoglobin hydroxide, 4.47; ferrihemoglobin fluoride, 5.92; ferrihemoglobin cyanide, 2.50; ferrihemoglobin hydrosulfide, 2.26. For ferrihemoglobin and its fluoride these correspond to five unpaired electrons per heme, indicating essentially ionic bonds; for the cyanide and hydrosulfide to one, indicating essentially covalent bonds; and for the hydroxide to three, indicating bonds of an intermediate type.

Values determined by magnetic titrations are reported for the dissociation constants of ferrihemoglobin hydroxide, fluoride and cyanide.

PASADENA, CALIF. RECEIVED FEBRUARY 8, 1937

[CONTRIBUTION FROM THE AVERY LABORATORY OF CHEMISTRY, UNIVERSITY OF NEBRASKA]

Arsonated Derivatives of Mixed Ketones

BY ROBERTA ELEANOR OMER AND CLIFF S. HAMILTON

In a study of arsonated aliphatic–aromatic ketones[1-4] a number of attempts were made to condense *p*-hydroxyphenylarsonic acid with nitriles in various solvents with and without catalysts. There was no evidence of condensation in any case, results which were not entirely unexpected in view of the fact that the literature does not report condensations with nitrophenols and hydroxybenzenesulfonic acids.

Although direct arsonation of resorcinol[5] gives good yields of resorcinol arsonic acid, 2,4-dihydroxyacetophenone[6] and the corresponding propiophenone derivative did not react with arsenic acid to form more than traces of the arsonic acids. Protection of the phenolic hydroxyl groups by acetylation[7] or methylation[8] before heating with arsenic acid failed to give any arsonic acid although a few instances of direct arsonation of compounds containing no free hydroxyl groups are recorded.[5] Methylation of the products before their separation from the reaction mixture gave 10% yields.

The introduction of arsenic into hydroxyaromatic–aliphatic ketones through the diazo reaction[9] was exceedingly difficult. Attempts to condense *p*-nitrophenol and nitroresorcinol[10] with

(1) Lewis and Cheetham, THIS JOURNAL, **43**, 2117 (1921).
(2) Lewis and Cheetham, *ibid.*, **45**, 510 (1923).
(3) Deutsche Gold- und Silber-Scheideanstalt vorm. Roessler, Austrian Patent 100,211 (1922).
(4) Margulies, British Patent 220,668 (1923).
(5) Bauer, *Ber.*, **48**, 509 (1915).
(6) Hoesch, *ibid.*, **48**, 1122 (1915).
(7) Brüll and Friedlaender, *ibid.*, **30**, 297 (1897).
(8) Perkin, Robinson and Turner, *J. Chem. Soc.*, **93**, 1085 (1908).
(9) Bart, *Ann.*, **429**, 55 (1922).
(10) Kauffmann and Kügel, *Ber.*, **44**, 753 (1911).