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Studies in the Physical Chemistry of the Proteins. XIV. The Solvent Action of Sodium Chloride on Carboxyhemoglobin in 25 and 35% Ethanol at −5°

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Characterization of reactions in systems containing proteins and electrolytes demands knowledge of the activity coefficients both of the proteins and of the electrolytes. The logarithm of the activity coefficients of ions in dilute electrolyte solutions of low dielectric constant varies, according to Debye's theory, as the square root of the ionic strength and inversely as the three-halves power of the dielectric constant. As a first approximation "salting-out" effects can be neglected under these conditions.

In solutions of proteins, as of other dipolar ions, the conditions are more complicated, for not only is the dielectric constant high in such systems but there is specific interaction between protein and electrolyte, ascribable on the one hand to electrostatic forces, on the other to "salting-out" of the proteins by the salt. The higher the dielectric constant and the larger the protein, the greater this effect. The lower the dielectric constant, the more the activity coefficients of the protein vary with the ionic strength of the solution and the electric moments of the dipolar ions.

The activity coefficients of dipolar ions may be determined accurately by solubility measurements provided solubility is low. Under these conditions the solvent actions of neutral salts upon peptides have been shown to depend, as a first approximation, upon their dipole moments.[1] Recent measurements of the dielectric constants of aqueous solutions of egg albumin indicate that its electric moments are greater than those of peptides[2] and so is the solvent action of sodium chloride upon this protein under conditions such that it is but slightly soluble.[3] Together these observations suggest that the solvent action of neutral salts upon proteins depends upon the distribution of the electrical charges which they bear, and the resulting effective electric moments.

Although the solvent action of neutral salts upon horse hemoglobin has been investigated extensively,[4-6] the most satisfactory determinations of solubility for this protein have been at ionic strengths greater than 0.1. Under these conditions, hemoglobin is sufficiently soluble to influence the dielectric constant of the solution. Even in the absence of salt, it is so soluble that we cannot assume that the solution closely approaches the physical chemical conditions of the solvent. The dielectric constant of aqueous hemoglobin solutions is greater than the pure solvent by 0.33 per gram of protein per liter.[7]

In order to investigate the interaction of hemoglobin and electrolytes more adequately at lower concentrations both of hemoglobin and of electrolyte, we have studied its solubility at −5° in 25 and 35% ethanol under the same conditions employed in the case of egg albumin. At this temperature, the solubility of horse carboxyhemoglobin[8] in 35% ethanol 0.01 molal in sodium chloride is only 0.06 gram per liter.

Measurements were attempted at still lower electrolyte concentrations, but it has been our experience in these solvents as in water that their reproducibility decreased the lower the salt concentrations. Whether this depends on dissociation into molecules whose molecular weight is 33,350—or half that reported for more concentrated aqueous salt solutions—as has been demonstrated for horse hemoglobin in urea solutions[9-12] and under certain conditions in dilute aqueous solutions,[13] or whether, under these conditions, there is some other change in solution or saturating body remains to be investigated. The results reported are reasonably satisfactory, however, from ionic strengths of 0.01 to 0.2, and thus

(1) Cohn, McMeekin, Greenstein and Weare, THIS JOURNAL, **58**, 2365 (1936).

(2) Shutt, *Trans. Faraday Soc.*, **30**, 893 (1934); and unpublished data by Oncley.

(3) Ferry, Cohn and Newman, THIS JOURNAL, **58**, 2370 (1936).

(4) Cohn and Prentiss, *J. Gen. Physiol.*, **8**, 619 (1927).

(5) Green, *J. Biol. Chem.*, **93**, 495, 517 (1931).

(6) Green, *ibid.*, **95**, 47 (1932).

(7) Oncley, THIS JOURNAL, **60**, 1115 (1938).

(8) Human hemoglobin, which has been shown to be far more soluble in salt solutions [Green, Cohn and Blanchard, *J. Biol. Chem.*, **109**, 631 (1935)], is also far more soluble in these ethanol–water mixtures, and must, therefore, be considered to have quite different physicochemical properties.

(9) Burk and Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

(10) Steinhardt, *Nature*, **138**, 800 (1936).

(11) Wu and Yang, *Chinese J. Physiol.*, **6**, 51 (1932).

(12) The dielectric constant increments of horse hemoglobin in 4 molal urea are also smaller than in water.

(13) Tiselius and Gross, *Kolloid-Z.*, **66**, 12 (1934).

extend considerably the range over which we have some knowledge of the interaction of hemoglobin and neutral salts.

Methods and Materials.—The solvents employed were made up to contain 25 or 35% ethanol by volume and varying amounts of C. P. sodium chloride at 20°. Thus, if the final volume of the solution was to be 1 liter, the sodium chloride was first added by weight, then 250 or 350 cc. of ethanol, and then water to volume. The water content of the solvents was thus slightly diminished in the solutions containing salt, but this variation can, for present purposes, be neglected.

Carboxyhemoglobin was prepared from horse erythrocytes by essentially the procedure generally used in this Laboratory.[5,14] The preparation was carried out in the cold room at 2°, or in a refrigerated centrifuge. In order to be certain that hemoglobin was always in the carboxy form, it was saturated with carbon monoxide whenever it was brought into solution. In this investigation, we generally brought about recrystallization from aqueous solution, by following the acid titration with the glass electrode, and stopping the titration at a *p*H of about 6.6, the point of minimum solubility.[6] This modification should provide a more uniform product; it also increased the yield.

To eliminate soluble impurities as completely as possible, we suspended the crystals in an equal volume of water, and let the suspension stand for six to eighteen hours before each centrifugation. After the final washing, a nearly saturated aqueous solution was prepared by suspending an excess of crystals in cold distilled water and leaving it overnight at 2°, then centrifuging out the undissolved crystals.

Ethanol at −5° in sufficient quantity to bring its concentration to 25 or 35 volume per cent. was added to this solution with vigorous stirring, and the alcoholic solution brought to −5°. After an hour or so crystals began to appear, but the yield was never satisfactory until sixteen to forty hours later. At the end of that time, beautiful rhomboid plates were visible under the microscope.

Crystallization from 25% ethanol was repeated once. The first crystals were suspended in a relatively large volume of 25% ethanol, centrifuged, dissolved in distilled water approximately equal in volume to that of the original solution. This solution was centrifuged to remove any insoluble material and ethanol again added to 25 volume per cent. Experiment showed that there was no decrease in solubility following this procedure. Carboxyhemoglobin was not recrystallized from 35% ethanol since preliminary experiments had shown that denaturation tended to occur during the inevitable rise in temperature during recrystallization.

In determining solubility the crystals were transferred to the special centrifuge bottles previously described[3] and washed three to five times with volumes of the solvent large in comparison with those of the crystals. Although the time for saturation of the solvents was varied within wide limits (one to fifteen days), it was apparent that, within the experimental error, equilibrium was attained within forty-eight hours. The short time necessary, as compared with egg albumin,[3] probably was due in part to the large surface presented to the solvent by the small carboxyhemoglobin crystals. In general, the time of rotation was from three to six days.

Since the small carboxyhemoglobin crystals settled imperfectly on standing and rapidly clogged the sintered glass filters, the equilibrium bottles were centrifuged before filtration. This was done from fifteen to thirty minutes in the brine-cooled centrifuge. In no case did the temperature rise more than 1.5° during centrifugation. More often the temperature fell slightly, but scrutiny of the results indicates that temperature variation at this point had a negligible effect upon the experimental results.

After centrifugation the supernatant solutions were filtered under nitrogen at fifteen pounds (1 atm.) pressure at −5°.[3] The equilibrium bottles were then refilled and the crystals again equilibrated with the fresh solvent (except at sodium chloride concentrations of 0.1 and 0.2 *N* where changes in the saturating body apparently occurred even at −5°).

Weighed aliquots of the saturated carboxyhemoglobin solutions were transferred quantitatively to Kjeldahl flasks, evaporated to a small volume to remove ethanol, digested, and the protein estimated by the Kjeldahl method, on the basis of the nitrogen factor of 16.86%.[15]

Solubility Measurements.—In Tables I and II appear the experimental values for the solubility of carboxyhemoglobin in ethanol–water mixtures containing sodium chloride, expressed as g. per 1000 g. of solution. The results, except for values in parentheses which appear to be abnormally high, have been averaged for each system investigated.

Compared with the results for egg albumin,[3] those now reported for hemoglobin show wide experimental variation. Change in temperature during centrifugation was demonstrated not to be

TABLE I

THE INFLUENCE OF SODIUM CHLORIDE ON THE SOLUBILITY OF HORSE CARBOXYHEMOGLOBIN IN 25% ETHANOL AT −5°

The raised figures give the number of the experiment

Concentration of NaCl, mole per liter				
0.0101	0.0202	0.0505	0.101	0.202
Solubility of hemoglobin, g. per 1000 g. of solution				
0.048^{17}	0.083^{21}	0.21^{15}	0.37^{16}	1.4^{18}
$.057^{17}$	$.083^{21}$	$.19^{15}$	$.38^{18}$	1.5^{19}
$.061^{17}$	$.088^{21}$	$.21^{15}$	$.56^{19}$	1.2^{23}
$.059^{17}$	$.110^{22}$	$.20^{18}$	$.49^{20}$	1.8^{24}
$.058^{17}$	$.102^{22}$	$.18^{18}$	$.54^{21}$	1.2^{26}
$.054^{17}$	$.107^{22}$	$.21^{18}$	$(.64)^{22}$	
$.066^{19}$	$.104^{23}$	$.19^{18}$	$.54^{25}$	
$.056^{22}$	$.101^{23}$	$.21^{18}$	$.36^{34}$	
$.060^{22}$	$.089^{24}$	$.20^{18}$	$.54^{34}$	
$.064^{22}$	$.096^{24}$	$.24^{19}$	$.50^{35}$	
$.038^{40}$		$.22^{19}$	$.49^{35}$	
$.044^{40}$			$.38^{36}$	
			$.41^{37}$	
			$.47^{40}$	
Av.				
0.055	0.096	0.21	0.46	1.4

(14) Ferry and Green, *J. Biol. Chem.*, **81**, 175 (1929).

(15) Vickery and Leavenworth, *ibid.*, **79**, 377 (1928).

TABLE II

THE INFLUENCE OF SODIUM CHLORIDE ON THE SOLUBILITY OF HORSE CARBOXYHEMOGLOBIN IN 35% ETHANOL AT −5°

The raised figures give the number of the experiment

Concentration of NaCl, mole per liter				
0.0101	0.0152	0.0203	0.0507	0.101
Solubility of hemoglobin, g. per 1000 g. of solution				
0.052^{29}	0.079^{33}	0.081^{29}	0.46^{32}	1.2^{32}
$.057^{29}$	$.093^{33}$	$.11^{29}$	$.48^{32}$	1.1^{32}
$.076^{30}$	$.076^{33}$	$(.16)^{30}$	$.41^{39}$	1.1^{39}
$.060^{33}$	$.077^{33}$	$.12^{33}$	$.42^{39}$	1.4^{39}
$.062^{33}$	$.083^{35}$	$.13^{33}$	$.32^{40}$	0.86^{40}
$.089^{36}$	$.079^{35}$	$.099^{34}$	$.32^{40}$	
$.085^{36}$		$.095^{36}$		
$.040^{37}$		$.093^{36}$		
$.035^{37}$				
$.064^{39}$				
$.076^{39}$				
Av.				
0.063	0.081	0.10	0.40	1.1

the cause of this variation. Qualitative spectroscopic examination did not reveal methemoglobin, though carboxyhemoglobin is denatured rapidly even at −5° when the concentration of ethanol exceeds 40%. It was for this reason that our experiments were not extended to lower dielectric constants. The per cent. variation was greater the lower the salt concentration. In part, this may be ascribed to analytical difficulties, but the possibility of change in molecular weight under these conditions is not precluded.

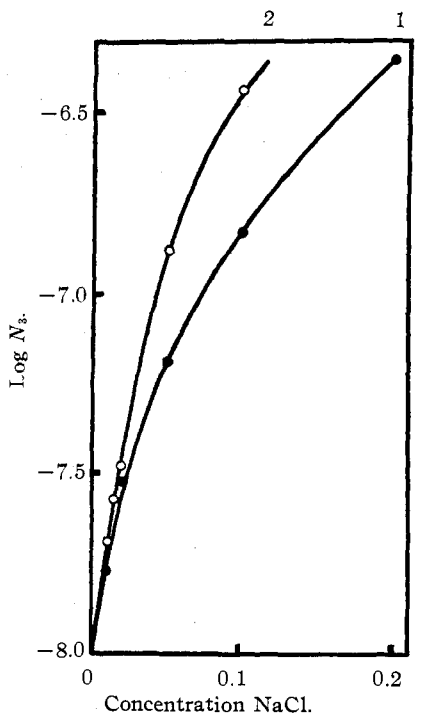

Fig. 1.—The solvent action of sodium chloride on carboxyhemoglobin in ethanol–water solutions at −5°: 1, 25% ethanol; 2, 35% ethanol.

The average solubilities in Tables I and II are recalculated in Table III as mole per liter and mole fraction, N, on the basis of the observed densities of pure solvents and the apparent specific volume of 0.75 for hemoglobin.[16] Though the hemoglobin concentration never exceeded 0.2%, nor the ionic strength 0.2, in the systems for which measurements are reported, solubility varied more than twenty-fold. The logarithm of the mole fraction—log N—is plotted against salt concentration in Fig. 1. Solubility in 35% ethanol is higher than in 25% ethanol at all salt concentrations studied. This however might not be true at zero ionic strength since the solvent action of sodium chloride is also higher at the lower dielectric constant.

Further calculations involve consideration of the dielectric constant. The dielectric constants, D, for 25 and 35% ethanol are taken as 78.6 and 73.2, respectively.[17] Since our measurements are referred to a standard state in water, the value 90, obtained by extrapolation from Wyman's results, is employed as an hypothetical value for D_0, the dielectric constant of water, at that temperature (−5°). These values are used in calculating the quantity $D_0/D \times \Gamma/_2$ and $D/D_0 \times \log N$ in Table III.

TABLE III

THE INFLUENCE OF SODIUM CHLORIDE ON THE SOLUBILITY OF CARBOXYHEMOGLOBIN IN ETHANOL–WATER MIXTURES AT −5°

Concn. NaCl $\Gamma/2$	Soly. of hemoglobin Mole $\times 10^5$ C	Mole fraction $\times 10^7$ N	$\left(\frac{D_0}{D}\frac{\Gamma}{2}\right)$	$\left(\frac{D}{D_0}\log N\right)$	$\left(\frac{D}{D_0}\log\frac{N}{N'}\right)$
25% Ethanol, D = 78.6					
0.0101	0.081	0.17	0.0116	−6.78	0.25
.0202	.14	.30	.0231	−6.57	.46
.0505	.30	.64	.0578	−6.28	.75
.101	.67	1.43	.116	−5.98	1.05
.202	2.1	4.36	.230	−5.59	1.44
35% Ethanol, D = 73.2					
0.0101	0.091	0.21	0.0124	−6.24	0.26
.0152	.118	.27	.0187	−6.15	.37
.0202	.145	.33	.0248	−6.08	.44
.0507	.58	1.31	.0623	−5.60	.92
.1013	1.60	3.63	.124	−5.27	1.25

(16) Svedberg and Nichols, THIS JOURNAL, **49**, 2920 (1927). This value was obtained at 20°, but changes slightly with temperature. The use of this value leads to no appreciable error.

(17) Wyman, *ibid.*, **53**, 3292 (1931).

The dielectric constant of the solution cannot have been appreciably greater than that of the solvent for the most concentrated hemoglobin solutions for which measurements are now reported.

In earlier experiments upon hemoglobin the logarithm of the solubility was plotted against $\sqrt{\Gamma/2}$,[18] and the slope in dilute aqueous solution was approximately 2.0.[4,6] The measurements that have now been made in ethanol–water mixtures could also be approximately described in terms of the relation, for 25% ethanol

$$-D/D_0 \times \log N = 7.10 - 3.2\sqrt{(D_0/D)(\Gamma/2)} \quad (1)$$

and for 35% ethanol

$$-D/D_0 \times \log N = 6.76 - 4.5\sqrt{(D_0/D)(\Gamma/2)} \quad (2)$$

The first terms in the above equations would yield estimates of solubility in the absence of salt, provided this method of calculation were justified. The slope constants in the above equations would, however, vary with the dielectric constant, whereas our studies upon amino acids in ethanol–water mixtures suggest that at sufficiently low dielectric constant the limiting slope representing Coulomb forces should be nearly independent of the dielectric constant.[3,19–22]

When $D/D_0 \times \log N$ for 35% ethanol is plotted against $D_0/D \times \Gamma/2$, the lowest three points fall on a straight line having a slope 14 and giving an extrapolated value for $D/D_0 \times \log N'$ of -6.42. This corresponds to a solubility of 0.038 g. per liter. The value 14 is, however, a minimal value for the slope, $K_{R'} = (D/D_0 \times \log N/N')/(D_0/D) \times (\Gamma/2)$. If we assume, tentatively, that the slope obtained at the lowest dielectric constant at which measurements were made also holds for 25% ethanol, we obtain an estimated value of -6.98 for $D/D_0 \times \log N'$, corresponding to a solubility of 0.031 g. per liter. These values would be even smaller if the limiting slope were considered higher, if the logarithm of the solubility were considered proportional to the square root of the ionic strength as in equations 1 and 2, or to an expression in which the denominator contains a term in the square root of the ionic strength, as in equation 21 of Kirkwood.[21]

Regardless of the method of extrapolation, there is no doubt that the solubility of carboxyhemoglobin is greatly diminished in relatively low ethanol concentration from its estimated value of 15 or 17 g. per liter in water at 25°. Over the range reported, further increases in ethanol have relatively little effect. This is reminiscent of the behavior of leucine over this range rather than that of glycine.[23]

Discussion

Coulomb Forces.—In the case of amino acids, such as glycine and its peptides, the concentration of ions and of uncharged molecules in equilibrium with dipolar ions at the isoelectric point may be considered negligible[24] and the change in activity coefficient with change in ionic strength may be related unequivocally to the electric moments of the dipolar ions.[1,21,22] In the case of proteins, analyses of titration curves in the neighborhood of their isoelectric points[25] demonstrate that appreciable concentrations of protein ions must be in equilibrium with the protein dipolar ions. The interaction of salt ions and protein ions, and the mean valence of the latter,[26] as well as change in molecular weight of saturating body, formation of new saturating bodies by combination with solvent,[27] and hydration of protein[28] may have to be taken into account as well as the electric moments of the protein.

Kirkwood's theory for the interactions of ions and dipolar ions permits consideration of the protein as a sphere of radius b and containing any number of charges located within the sphere. Although carboxyhemoglobin may have as many as seventy-five positive and seventy-five negative charges in the isoelectric state,[29] most of these must be arranged fairly symmetrically, for the influence on the dielectric constant may be explained by assuming carboxyhemoglobin to have but two positive charges at the one edge, and two negative charges at the other. Assuming a molecular weight of 66,700, and a specific volume of 0.75, hemoglobin has a molecular volume of 50,000, and a radius, b, of 27 Å. The dielectric constant increment per gram, 0.33, yields on this

(18) This practice has been followed by other investigators [Palmer, *J. Biol. Chem.*, **104**, 359 (1932); Joseph, *ibid.*, **116**, 353 (1936)] and there can be no doubt that the results may be satisfactorily represented in this way.

(19) Cohn, *Naturwissenschaften*, **20**, 663 (1932).

(20) Cohn, *Chem. Rev.*, **19**, 241 (1936).

(21) Kirkwood, *J. Chem. Phys.*, **2**, 351 (1934).

(22) Scatchard and Kirkwood, *Physik. Z.*, **33**, 297 (1932).

(23) Cohn, McMeekin, Edsall and Weare, THIS JOURNAL, **56**, 2270 (1934).

(24) Edsall and Blanchard, *ibid.*, **55**, 2337 (1933).

(25) Cohn, *J. Biol. Chem.*, **46**, Proc. iii (1921); *Phys. Rev.*, **5**, 349 (1925).

(26) Linderström-Lang [*Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924)], and more recent calculations by him and by J. T. Edsall subsequently to be reported.

(27) Sörensen, *ibid.*, **12**, 262 (1917).

(28) Adair and Adair, *Proc. Roy. Soc.* (London), **B120**, 422 (1936).

(29) Cohn, Green and Blanchard, THIS JOURNAL, **59**, 509 (1937).

basis an increment per mole of 22,000, and a dipole moment of 500×10^{-18} e. s. u.[7] If the charges are the same distance from the edge as in glycine, or 1.2 Å., the distance from the center, r, is 25.8 Å. and the maximum distance of separation 51.6 Å. Two such dipole pairs should yield a moment of 495×10^{-18} e. s. u.

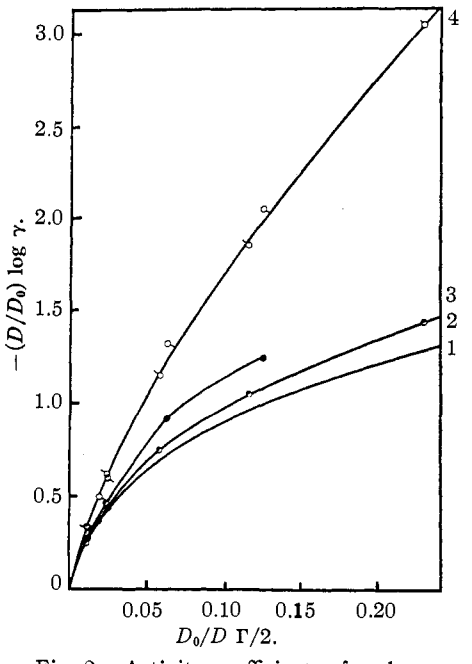

Fig. 2.—Activity coefficients of carboxyhemoglobin in sodium chloride at −5°: 1, calculated from Kirkwood's equation on the assumption of a molecular weight of 66,700 and a dipole moment of 500×10^{-18} e. s. u.; 2, ◐, observed in 25% ethanol; and 3, ●, in 35% ethanol; 4, measurements in 25% ethanol; ◔, and in 35% ethanol; ◔, corrected by a "salting-out" constant of 7.9.

Kirkwood's theory involves but one other quantity, the mean radius of protein and salt ions, a, taken as 28.2 Å. Evaluating activity coefficients from his equation 21, and employing all terms from the first to the seventh order, we have for the ionic strengths at which convergence is reasonably satisfactory

$(D_0/D)\ \Gamma/2$	0.0120	0.0480	0.108	0.192
$-(D/D_0)\ \log \gamma_\epsilon$	.274	.630	.935	1.19
$\left(\frac{-(D/D_0)\ \log \gamma_\epsilon}{(D_0/D)\ \Gamma/2}\right)$	22.8	13.1	9.4	6.2

in which γ_ϵ is that part of the activity coefficient due to Coulomb forces.[30]

(30) We are indebted to J. G. Kirkwood for extending this theory to proteins and to J. D. Ferry for calculating the higher terms for the model estimated for hemoglobin.

The very rapid decrease in the slope from the limit for this configuration, 50.2, should be noted. Indeed the logarithms of the activity coefficients calculated on this basis are very nearly linear in the square root of the ionic strength (see refs. 3 and 18).

If we divide the observed solubilities at the lowest five ionic strengths investigated by these calculated activity coefficients, we obtain values of (D/D_0) log N', respectively, of −7.03 and −6.52 for 25 and 35% ethanol, or intermediate between those obtained by linear or square root extrapolation. Values of (D/D_0) log N/N' estimated on this basis are given in the last column of Table III and graphically represented in Fig. 2. The curve calculated from the Kirkwood equation is also given (Curve 1). Though of the same order, it is definitely lower than that for the measurements in 25% ethanol, and far lower than those in 35% ethanol.

The "Salting-out" Effect.—The curves for hemoglobin in 25 and 35% ethanol (Fig. 2) diverge at the higher values of the ionic strength. Since the concentration of hemoglobin is so low and so nearly the same in 25 and 35% ethanol, this difference cannot depend upon the influence of the solute on the dielectric constant of the solution. It is probable, however, that "salting-out" cannot be ignored in these systems. How large a "salting-out" constant would suffice to cause these points to fall on the same curve, may be estimated by plotting (D/D_0) log $N/N' + K_s$ $(\Gamma/2)$ against (D_0/D) $\Gamma/2$ in the manner that has proven satisfactory for studies upon cystine.[31,32] Smaller values of K_s than 5 do not suffice. The "salting-out" constant calculated by means of Kirkwood's equation (Eq. 4, Ref. 30) on the basis of a molecular weight of 66,700 for hemoglobin is 7.90. (If the molecular weight of hemoglobin were one-half of this, the value of K_s should be 4.85 according to this equation.)

That part of the change in free energy due to Coulomb forces can be estimated from activity coefficients only if correction is made for the "salting-out" effect.[31] In the ethanol–water mixtures that have now been studied this correction is smaller than in aqueous solutions. Moreover, linear in the concentration, its effect will be small in comparison with Coulomb forces at low ionic

(31) McMeekin, Cohn and Blanchard, THIS JOURNAL, **59**, 2717 (1937).

(32) Cohn, McMeekin and Blanchard, Sörensen Jubilee Volume, *Compt. rend. trav. lab. Carlsberg*, **22**, 142 (1938).

strengths and dielectric constants, and large at high ionic strengths and dielectric constants. The maximum effect, for the influence of the protein in diminishing polarization of the solvent by the salt, given by taking K_s from Kirkwood's equation equal to 7.90, is represented graphically in Fig. 2 by curve 4. This curve represents maximal values of the activity coefficients due to Coulomb forces. At low ionic strengths they are presumably not much greater than the total effects for hemoglobin in the ethanol–water mixtures investigated, or than the calculation for that part of the interaction due to the double-dipole configuration. Provided K_s is as big as 7.90, additional interactions due to the higher moments of the other more symmetrically arranged charged groups will have to be taken into account. Like the "salting-out" effect, these are, according to Kirkwood's theory, more nearly linear the higher the order of the moment. For the time being the effects of these moments on the activity coefficient, as well as effects due to the protein ions in equilibrium with protein dipolar ions, may be considered roughly equal, but somewhat greater and opposite in sign, to the "salting-out" effect for hemoglobin in sodium chloride at $-5°$.

Activity Coefficients of Hemoglobin in Aqueous Sodium Chloride.—Without further discussion of equilibrium between ions and dipolar ions, or of the nature of the configuration leading to the higher electric moments, curve 4 can be considered to give the activity coefficients due to Coulomb forces at $-5°$, and to be expressible by Kirkwood's equation (21). Then, since the temperature enters into each term in equation (21) in the same way, it is possible to evaluate the temperature dependence of these activity coefficients without knowledge of the individual terms or the actual charge configuration involved. From the form of the equation it follows that $-(D_0/D) \log \gamma$ at any absolute temperature T is determined by taking points from the curve at $-5°$ and multiplying the abscissas by $T/268.1$ and the ordinates by $268.1/T$. When this is performed for $T = 298.1°$, at the same time changing from $D_0 = 90$ to $D_0 = 78.54$, the curve for 25° is obtained. The difference between values of $-(D/D_0) \log \gamma_e$ at -5 and $+25°$ is not great, since increase in T is compensated by decrease in the value D_0 (Table IV). Now it is only necessary to subtract from this curve (given in the second column) the "salting-out" effect, given by $K_s(\Gamma/2)$, where $K_s = 8.14$ at 25°, and the remainder should predict the experimental activity coefficients in water at 25°.

TABLE IV

ACTIVITY COEFFICIENTS CALCULATED FOR 25°

$(D_0/D)\ \Gamma/2$	$-(D/D_0) \log \gamma_e$	$K_s(\Gamma/2)$	$-(D/D_0) \log \gamma$
0.0097	0.330	0.079	0.251
.0194	.536	.158	.378
.0485	1.091	.395	.696
.097	1.776	.790	.986
.146	2.32	1.19	1.13
.199	2.84	1.58	1.26

The curve of $-(D_0/D) \log \gamma$ against $(D/D_0)\ \Gamma/2$ thus derived for hemoglobin at 25° is quite similar to the experimental curve at $-5°$. Its limiting slope is 53.7, or slightly greater than at $-5°$, but it flattens out more markedly with increase in ionic strength. In dilute hemoglobin solution D may be considered equal to D_0. If the dielectric constant of more concentrated hemoglobin solutions be calculated with the value of 22,000 for δ,[7] the last column of Table IV agrees satisfactorily, as a first approximation, with the solubility measurements of Green[6] (see also Ref. 20, p. 261).

Activity Coefficients of Salts in Hemoglobin Solutions.—From equations (24) and (25) of Kirkwood's paper[21] it follows that the logarithm of the activity coefficient of a dilute salt in the presence of a dipolar ion should be linear in the concentration of the dipolar ion, and for a uniunivalent salt the slope of this line should be one-half the slope at infinite dilution of the corresponding curve for the activity coefficient of the dipolar ion as influenced by the salt, assuming tentatively that the saturating body is the uncombined dipolar ion. On the basis of the double dipole configuration for hemoglobin previously described, the slope of the curve of $-\log \gamma_e$ of salt plotted against dipolar ion concentration at 25° should at infinite dilution be 27. Allowing for "salting-out" should not greatly increase this value. However, the value would be smaller at the finite concentrations of salt studied to an extent calculable from the data of the above table by means of the Duhem equation.

It is of interest to compare such calculations with measurements upon the activity coefficients of salts in aqueous hemoglobin solutions. Stadie and Hawes[33] have studied the effect of reduced and carboxyhemoglobin upon the activity coef-

(33) Stadie and Hawes, *J. Biol. Chem.*, **77**, 265 (1928).

ficients of sodium bicarbonate. They found over the wide range of protein concentrations studied, and at reactions from *p*H 7 to 8, that $-\log \gamma/C_{Hb}$ had the value 14 for carboxyhemoglobin and 20 for reduced hemoglobin, where the concentration C_{Hb} was based on a molecular weight of 16,700. Assuming a molecular weight of 66,700 the corresponding values are 56 and 80. Comparison with our results is however difficult, since the ratio of ionic to dipolar ionic hemoglobin changes markedly over the *p*H range studied by them.

Solubility studies on insoluble salts in the presence of hemoglobin may be more directly compared. Pauli and Stenzinger[34] observed a 5% increase in the solubility of calcium sulfate in 0.5% oxyhemoglobin. This leads to a very high value of the slope $(\log S/S_0)/C_{Hb}$. Stone and Failey's[35] more recent study upon the solubility of thallous chloride in the presence of concentrations of electrodialyzed reduced hemoglobin ranging from 1 to 3% yields far lower values for the slope, increasing with increasing concentration of from 14 to 26. At the ionic strength of saturated thallous chloride, slightly greater than 0.016, the slope calculated from the final column of Table IV is approximately eleven.

(34) Pauli and Stenzinger, *Biochem. Z.*, **205**, 71 (1929).
(35) Stone and Failey, *J. Phys. Chem.*, **37**, 935 (1933).

Summary

1. The solubility of horse carboxyhemoglobin has been studied at $-5°$ in 25 and 35% ethanol containing sodium chloride at ionic strengths from 0.01 to 0.2.

2. Under these conditions the solubilities of carboxyhemoglobin are 0.081 and 0.085×10^{-5} mole per liter at an ionic strength of 0.01 in 25 and 35% ethanol, or far lower than in aqueous solution Solubility ratios may therefore be considered to yield activity coefficients.

3. The experimentally determined activity coefficients have been compared with the expectation on the basis of Kirkwood's theory, assuming hemoglobin to be a double dipole with a moment of 500×10^{-18} e. s. u. Effects due to electric moments of higher order and to "salting-out" have also been considered.

4. Activity coefficients for hemoglobin in aqueous sodium chloride at 25° have been estimated and compared with the measurements of Green in such systems, and the activity coefficients of the sodium chloride estimated and compared with the measurements of Stone and Failey on thallous chloride.

5. The possibility of change in the nature of the saturating body under these various conditions is not excluded.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

The Interaction of Chlorine with Different Types of Organic Sulfur Compounds

BY IRWIN B. DOUGLASS[1] AND TREAT B. JOHNSON

As a result of the recent researches conducted in this Laboratory on the production of sulfonyl chlorides by the chlorination of isothiourea salts[2] in aqueous solution, we have been led to a study of the action of aqueous chlorine at low temperatures on various other types of sulfur compounds. In this paper the authors describe results of the chlorination of certain representations of the following types: mercaptans, disulfides, thiol esters, alkyl thiosulfate salts, a thiosulfonate ester, alkyl ethylxanthates, potassium xanthates, and an acyldithiocarbamate.

(1) Sterling Professorship of Chemistry Research Assistant 1937–1938.
(2) Johnson and Sprague, THIS JOURNAL, (a) **58**, 1348 (1936); (b) **59**, 1837 (1937); (c) **59**, 2439 (1937).

Schiller and Otto[3a] reported that diphenyl disulfide interacted with chlorine in the presence of water to give phenylsulfonyl chloride. They also treated phenylthiol benzoate[3b] with aqueous chlorine and obtained benzoic acid, phenylsulfonyl chloride, and phenylsulfonic acid. They postulated that the first action of the halogen is to produce benzoyl chloride and diphenyl disulfide, but they did not report the isolation of benzoyl chloride.

Zincke and others in a series of papers dealing with the chemistry of aryl sulfur chlorides[4]

(3) (a) Schiller and Otto, *Ber.*, **9**, 1638 (1876); (b) *ibid.*, 1635 (1876).
(4) Zincke and Frohneberg, *ibid.*, **42**, 2728 (1909), and later papers by Zincke and co-workers.