TABLE II

Analysis	% H	% C	Total
	Chrysene, $C_{18}H_{12}$		
39	5.279	94.733	100.012
40	5.301	94.695	99.996
45	5.299	94.704	100.003
47	5.298	94.698	99.996
66	5.297	94.686	99.983
67	5.296	94.693	99.989
68	5.296	94.700	99.996
70	5.294	94.703	99.997
73	5.299	94.701	100.000
74	5.296	94.703	99.999
Average	5.296	94.701	99.997
Calculated	5.298	94.702	
	Triphenylbenzene, $C_{24}H_{18}$		
42	5.929	94.062	99.991
44	5.923	94.076	99.999
48	5.921	94.078	99.999
60	5.919	94.077	99.996
61	5.921	94.078	99.999
63	5.920	94.077	99.997
64	5.920	94.082	100.002
65	5.920	94.082	100.002
Average	5.922	94.077	99.999
Calculated	5.921	94.079	
	Anthracene, $C_{14}H_{10}$		
55	5.657	94.342	99.999
56	5.659	94.347	100.006
62	5.652	94.345	99.997
71	5.655	94.346	100.001
72	5.653	94.346	99.999
76	5.654	94.339	99.993
77	5.655	94.335	99.990
80	5.663	94.343	100.006
81	5.657	94.336	99.993
Average	5.656	94.342	99.998
Calculated	5.655	94.345	
	Pyrene, $C_{16}H_{10}$		
78	4.986	94.939	99.925
79	4.987	94.963	99.950
Calculated	4.983	95.017	

12.0005. This is 1/10,000 lower than the value reported by him a year earlier, 12.0018.[5] Bainbridge (private communication) finds 12.0012, corrected to the chemical scale. With the value 12.0012 for C^{12} and our atomic weight the calculated abundance ratio of C^{13} is 1/109. This value is lower than that computed from our earlier results. Jenkins and Ornstein[6] find 1/106; Vaughan, Williams and Tait[7] 1/92 and Aston[8] 1/138.

Summary

It seems unlikely that our final value would be altered materially by continuance of the experiments. Of the four hydrocarbons investigated, one, pyrene, proved incapable of being purified by any method we could devise. The other three yielded identical results for carbon, whether they had been initially separated from coal tar, or synthesized, from a simple coal tar product in two cases (chrysene and anthracene), from a natural product in the third (triphenylbenzene).

The average atomic weight of carbon obtained by combustion of chrysene, triphenylbenzene and anthracene, 12.010, may therefore be taken as a fair outcome of this method of attack.

CAMBRIDGE, MASS. RECEIVED DECEMBER 14, 1936

(5) Aston, *Nature*, **135**, 541 (1935).

(6) Jenkins and Ornstein, *Proc. Acad. Sci. Amsterdam*, **35**, 1212 (1932).

(7) Vaughan, Williams and Tait, *Phys. Rev.*, **46**, 327 (1934).

(8) Aston, *Proc. Roy. Soc.* (London), **A149**, 400 (1935).

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Studies in the Physical Chemistry of the Proteins. XIV. The Amphoteric Properties of Hemoglobin*

BY EDWIN J. COHN, ARDA A. GREEN AND MURIEL H. BLANCHARD

The behavior of proteins is determined largely by the number and distribution of their electrically charged groups. An accurate knowledge of the number of dissociable groups is therefore an essential requisite for the study of their distribution by such means as dielectric constant measurements and measurements of change in activity coefficient with change in ionic strength. In extending to proteins relationships developed by the study of amino acids and peptides, we shall first consider those that can be obtained as pure chemical individuals.

From certain points of view the hemoglobin of the horse may be considered the best known of the proteins. It is readily crystallizable by a number of procedures. It contains 0.335% iron[1] and

* For supplementary tables of data obtained in this work, order Document 1007 from Science Service, 2101 Constitution Ave., Washington, D. C., remitting 25¢ for microfilm form, or 50¢ for photocopies readable without optical aid.

(1) Zinoffsky, *Z. physiol. Chem.*, **10**, 16 (1886).

0.390% sulfur. The minimal molecular weight of hemoglobin estimated from its sulfur content is 8223, and that from its iron content is double this value, or 16,669. Svedberg and Nichols have studied "the influence of *p*H on the diffusion constant, molecular weight and specific sedimentation velocity of carbon monoxide hemoglobin. . . over a *p*H range of 5.4 to 10.2. The diffusion constant and the specific sedimentation velocity are normal. . . over the range of *p*H 6.0 to 7.56, and the molecular weight is normal, 68,000, at least from a *p*H of 6.0 to 9.05."[2] Osmotic pressure measurements upon aqueous hemoglobin solutions yield the same molecular weight,[3] though it has been suggested that this weight is halved in urea solutions[4] and perhaps under other conditions.

The solubility of horse hemoglobin also has been investigated under a wide variety of conditions, and has been demonstrated to be essentially independent of the amount of saturating body.[5,6,7] Hemoglobin is therefore not only monodisperse, but a chemical individual.

The isoelectric point of hemoglobin has been estimated to be near *p*H 6.8.[8] Its acid properties are increased to the same extent by combination with either oxygen or carbon monoxide, but this effect, important over the physiological range, does not extend to extremely acid or alkaline reactions.

The basic properties of globin depend upon the large amounts of arginine, histidine and lysine in the molecule. Isolation methods have yielded nearly the same values for histidine[9,10] as the nitrogen distribution method[11] (Table I).

The latter method, however, yielded slightly higher estimates for arginine and lysine, suggesting the difficulties of quantitative isolation of these most basic amino acids. The nitrogen distribution method often yields too high values, however. Horse hemoglobin may therefore be assumed to contain between 83 and 93 free basic groups derived from histidine, arginine and lysine.

TABLE I

HORSE HEMOGLOBIN

		Amino acid in hemoglobin hydrolysate, %	Equivalent combining weight per g. hemoglobin moles × 10^5	Weight hemoglobin containing one mole amino acid, g. per moles	Moles amino acid per mole hemoglobin, no. of equivalents
Tyrosine		3.15	17.4	5749	12
Histidine	(10)	7.64	49.3	2030	33
	(9)	7.60	49.0	2041	33
	(11)[a]	7.74	49.9	2004	33
Arginine	(10)	3.32	19.1	5244	13
	(9)	3.60	20.7	4836	14
	(11)[a]	4.11	23.6	4236	16
Lysine	(10)	8.10	55.4	1804	37
	(9)	8.25	56.5	1771	38
	(11)[a]	9.55	65.4	1530	44
Trivalent bases	(10)	19.06	123.8		83
	(9)	19.45	126.2		85
	(11)[a]	21.40	138.9		93

[a] Values by Hunter and Borsook recalculated by Vickery and Leavenworth.[10]

Estimates of the dicarboxylic amino acids in hemoglobin are unfortunately incomplete, or unsatisfactory, but tyrosine has been analyzed by Folin and Marenzi[12] and found to be present to 3.15%. This analysis as well as those of Vickery and Leavenworth[10] were carried out on hemoglobin isolated and recrystallized in this Laboratory by the same methods as the preparations employed in the physical chemical studies.

Materials and Methods.—The method employed in the preparation of hemoglobin has been described repeatedly[6,13] and essentially consists in recrystallization at the isoelectric point. Experiments I to III were carried out with preparations made in 1930, and preparations IV to VIII in 1936.[14]

The repeatedly recrystallized and washed carboxyhemoglobin generally had a *p*H near 6.5. At this reaction, however, horse hemoglobin is relatively insoluble. It was dissolved by the addition of just sufficient sodium hydroxide of known concentration. The *p*H of these stock solutions was generally between 7.4 and 7.8. They were saturated with carbon monoxide and analyzed

(2) Svedberg and Nichols, THIS JOURNAL, **49**, 2934 (1927).

(3) Adair, *Proc. Roy. Soc.* (London), **A120**, 573 (1928).

(4) Burk and Greenberg [*J. Biol. Chem.*, **87**, 197 (1930)] give the molecular weight of hemoglobin in urea as 34,000, but Huang and Wu [*Chinese J. Physiol.*, **4**, 221 (1930)] give the molecular weight of hemoglobin in this solvent as 66,200. Steinhardt [*Nature*, **138**, 800 (1936)] has recently studied hemoglobin in urea solutions with the ultracentrifuge and reported a value close to 34,000.

(5) Cohn and Prentiss, *J. Gen. Physiol.*, **3**, 619 (1927).

(6) Green, *J. Biol. Chem.*, **93**, 495; **93**, 517 (1931); **95**, 47 (1932).

(7) Sörensen and Sörensen, *Compt. rend. trav. Lab. Carlsberg*, **19**, No. 11 (1933).

(8) Michaelis, "Nernst Festschrift," Verlag Wilh. Knapp, Halle an der Saale, Germany, 1912; *Biochem. Z.*, **47**, 250 (1912). Ferry, *J. Biol. Chem.*, **57**, 819 (1923).

(9) Abderhalden, Fleischmann and Irion, *Fermentforschung*, **10**, 446 (1929).

(10) Vickery and Leavenworth, *J. Biol. Chem.*, **79**, 377 (1928).

(11) Hunter and Borsook, *ibid.*, **57**, 507 (1923). The values of Hunter and Borsook by the nitrogen distribution method have been recalculated by Vickery and Leavenworth.

(12) Folin and Marenzi, *ibid.*, **83**, 89 (1929).

(13) Ferry and Green, *ibid.*, **81**, 175 (1929).

(14) These preparations were made by Ferry and Newman for studies on the solubility of hemoglobin in ethanol–water mixtures. Ferry, Cohn and Newman, *J. Biol. Chem.*, **114**, Proc. xxxiv (1936).

for the small remaining amounts of chloride and for total nitrogen. The nitrogen content of horse hemoglobin is taken as 16.86%.[10] The amounts of sodium chloride recorded in Tables II and III include, in the case of acid titrations, the neutralized alkali used to dissolve the hemoglobin.

TABLE II

ELECTROMOTIVE FORCE MEASUREMENTS ON SYSTEMS CONTAINING CARBOXYHEMOGLOBIN AND HYDROCHLORIC ACID

HCl concn. moles/liter	Log $1/a_{H^+}$, pH^+	Log $1/C_{H^+}$, $pH^+ - p\gamma_{H^+}$	HCl uncombined, moles/liter	HCl combined, moles/liter	HCl combined, moles × 10^5 per g. Hb
Experiment III: 48.8 g. Hb and 0.01 mole NaCl per liter					
0.0625	2.994	2.929	0.0012	0.0609	124.8
	2.965	2.900	.0013	.0608	124.6
.0701	2.610	2.542	.0028	.0672	137.7
	2.613	2.545	.0028	.0672	137.7
.0901	1.827	1.751	.0177	.0724	148.4
	1.823	1.747	.0179	.0722	144.0
Experiment III: 10.84 g. Hb and 0.0022 mole NaCl per liter					
0.0173	2.677	2.631	0.0023	0.0150	138.4
	2.656	2.610	.0025	.0149	137.5
.0213	2.300	2.252	.0056	.0157	144.8
	2.303	2.255	.0056	.0158	145.8
.0253	2.103	2.054	.0088	.0165	152.2
	2.080	2.031	.0093	.0160	147.6
.0273	1.996	1.946	.0113	.0160	137.6
	2.000	1.950	.0112	.0161	148.5
.0293	1.922	1.871	.0135	.0159	146.7
	1.934	1.883	.0131	.0163	150.4
Experiment IV: 20.95 g. Hb and 0.0042 mole NaCl per liter					
0.0368	2.239	2.186	0.0065	0.0303	144.6
	2.234	2.181	.0066	.0302	144.2
.0448	1.910	1.854	.0140	.0308	147.0
	1.915	1.859	.0138	.0310	148.0
.0568	1.644	1.584	.0261	.0307	146.5
	1.645	1.585	.0260	.0308	147.0
.0684	1.491	1.426	.0375	.0309	147.5
	1.496	1.431	.0371	.0313	149.4

The varying amounts of standard acid or alkali were added to aliquot parts of the stock carboxyhemoglobin solutions and brought to constant volume shortly before they were transferred to hydrogen electrodes. Clark hydrogen electrode vessels, 0.1 N calomel electrodes and a saturated potassium chloride salt bridge were employed, no correction being made for the liquid junction potential. The electrodes were always standardized with 0.1 N hydrochloric acid, the pH of which was considered to be 1.075.[15] Four different electrodes were used, measurements generally being made in duplicate.

(15) Scatchard, THIS JOURNAL, 47, 696 (1925).

TABLE III

ELECTROMOTIVE FORCE MEASUREMENTS ON SYSTEMS CONTAINING CARBOXYHEMOGLOBIN AND SODIUM HYDROXIDE

NaOH Concn., moles/liter	Log $1/a_{H^+}$, $pH^+_{25°}$	Log $1/C_{OH^-}$, $p_{OH^-} - p\gamma_{OH^-}$	NaOH uncombined, moles/liter	NaOH combined, moles/liter	NaOH combined, moles × 10^5 per g. Hb
Experiment IV: 20.95 g. Hb and 0.0027 mole NaCl per liter					
0.0430	12.140	1.688	0.0205	0.0225	107.4
	12.142	1.686	.0206	.0224	106.9
.0629	12.398	1.420	.0380	.0249	118.9
	12.408	1.410	.0389	.0240	114.6
	12.390	1.428	.0373	.0256	122.2
.0828	12.586	1.224	.0597	.0231	110.3
	12.583	1.227	.0593	.0235	112.2
	12.571	1.239	.0577	.0251	119.8
	12.593	1.217	.0607	.0221	105.5
	12.593	1.217	.0607	.0221	105.5
.0987	12.693	1.111	.0774	.0213	101.7
	12.684	1.120	.0759	.0228	108.8
Experiment VI: 18.54 g. Hb and no NaCl added					
0.0676	12.494	1.323	0.0475	0.0201	103.3
.0836	12.603	1.207	.0621	.0215	116.0
	12.619	1.191	.0644	.0192	103.5
Experiment VI: 27.80 g. Hb and no NaCl added					
0.06522	12.334	1.483	0.0329	0.0323	116.3
.08516	12.535	1.274	.0532	.0320	114.9
.10510	12.651	1.151	.0706	.0345	124.0

Calculation of Results.—Acid and base combined by protein is calculated as the difference between the total and the free acid, C_{H^+}, or base C_{OH^-}, present. This calculation involves the activity coefficients of the hydrogen and hydroxyl ions, which were estimated by Lewis and Randall[16] from mean activity coefficients on the assumption that γ_{H^+} is equal to γ_{OH^-} in solutions more dilute than 0.01 N. They estimate $-\log \gamma_{H^+}$ in 0.1 N hydrochloric acid as 0.076. Scatchard's[15] subsequent revision yields slightly higher values for $-\log \gamma_{H^+}$ at low concentrations, but his result, 0.075, is in good agreement at 0.1 N. The standardization of our electrodes with 0.1 N hydrochloric acid has always given a value close to this for the hydrogen potential.[17]

"Although Lewis and Randall's . . . value 1.005×10^{-14} . . . is now the most probable value for the dissociation constant of water, its use in the calculation of our data leads to different values for p_{OH^-} in alkaline solution than those calculated from Lewis and Randall's coefficients for the activity of the hydroxyl ion. In all probability this de-

(16) Lewis and Randall, "Thermodynamics and the Free Energy of Chemical Substances," 1st ed., McGraw-Hill Book Company, Inc., New York, 1923.

(17) Cohn and Berggren, *J. Gen. Physiol.*, 7, 45 (1924).

pends not upon the value of K_w, but upon diffusion potentials, for which we have not corrected. If, however, we employ the Sørensen–Michaelis values for K_w at different temperatures, our p_{OH}-values conform approximately to those calculated from the activity of the hydroxyl ion"[17] (p. 53). Without making any assumption whatever as to the correct value for K_w we have adopted tentatively the latter.[18] The temperature variation of pH is also ascribed to K_w, and not to p_{OH}-(pK_w − pH$^+$).

The activity coefficients of the hydrogen and hydroxyl ions depend not only on their concentrations, but on those of all other ions in solution and decrease as the valency of the latter increases. Thus the higher valencies of protein anions and cations might be expected to have a large effect on the activity coefficients of the hydrogen and hydroxyl ion. In an attempt to evaluate the kind of correction that should be employed in studies of amino acids and proteins, we studied some years since the activity coefficients of the hydroxyl ion in sodium hydroxide solutions containing other sodium compounds, including "four bivalent acids, sulfuric, oxalic, glutamic, and aspartic, the two latter amino acids. . . . The results with these acids. . . suggest that anions of higher valence have greater effects upon the activity of the hydroxyl ion. This effect might be expected to increase with the ionic strength, but the effect on the amino acids studied at this concentration was not so great as that of other divalent salts. . . . Because of their high valence, protein anions may, of course, further depress the activity of the hydroxyl ion. Uncertainty regarding the valence type to be ascribed to proteins makes it difficult, however, to apply a correction for this factor, and their very low concentration may make it unnecessary. Accordingly we have tentatively used the values of γ deduced from measurements of sodium hydroxide solutions in the calculation of our data"[17] (p. 57).

There is no doubt that activity coefficients, calculated on the basis of inorganic ions, are too low. There is also no doubt that those calculated on the basis of the valence of protein salts are far too high. Recent studies on the effect of the sodium salts of proteins, including hemoglobin, on the solubility of thallous chloride have been reported by Stone and Failey. Their results confirm the above conclusion, for the activity coefficients of the thallous chloride are far smaller than would be expected on the basis of ionic strengths calculated in terms of the molecular weights and the number of free groups of proteins. They calculated base combining capacity by assuming the mean activity coefficient of the thallous chloride, as determined by these solubility measurements, to yield the activity coefficient of the hydroxyl ion.[19]

In all of these methods, the activity coefficient of the hydroxyl ion varies with the sodium hydroxide concentration, and with the protein concentration. In order to minimize the influence of these factors we have carried out measurements in systems normal with respect to sodium chloride

TABLE IV

ELECTROMOTIVE FORCE MEASUREMENTS ON SYSTEMS CONTAINING CARBOXYHEMOGLOBIN, SODIUM HYDROXIDE AND MOLAL SODIUM CHLORIDE

NaOH concn., moles/liter	Log $1/a_{H^+}$, pH$^+_{25°}$	Log $1/C_{OH^-}$, p_{OH^-} − $p\gamma_{OH^-}$	NaOH uncombined, moles/liter	NaOH combined, moles/liter	NaOH combined, moles × 10^5 per g. Hb
Experiment V: 16.37 g. Hb per liter					
0.0400	12.015	1.650	0.0224	0.0176	107.5
.0560	12.229	1.436	.0366	.0194	118.5
.0800	12.459	1.206	.0622	.0178	108.7
.1200	12.676	0.989	.1026	.0174	106.3
Experiment VI: 27.80 g. Hb per liter					
0.08516	12.395	1.260	0.0550	0.03016	108.5
.10510	12.550	1.116	.0766	.02850	108.5
.12504	12.646	1.019	.0957	.02934	105.5
Experiment VII: 19.30 g. Hb per liter					
0.05982	12.258	1.407	0.0392	0.02062	106.8
	12.250	1.415	.0385	.02132	110.5
	12.263	1.402	.0396	.02022	104.8
	12.253	1.402	.0387	.02112	109.4
.09970	12.560	1.105	.0785	.02120	109.8
	12.564	1.101	.0793	.02040	105.7
.11964	12.664	1.001	.0998	.01984	102.8
	12.666	0.999	.1002	.01944	100.7
.15872	12.803	.862	.1374	.02132	110.5
	12.809	.856	.1395	.01942	100.6
Experiment VIII: 48.08 g. Hb per liter					
0.074410	12.028	1.637	0.0231	0.05131	106.7
	12.026	1.639	.0230	.05141	106.9
.08434	12.196	1.469	.0340	.05034	104.9
	12.190	1.475	.0335	.05084	105.7
.12411	12.535	1.130	.0741	.05001	104.0
	12.526	1.139	.0726	.05151	107.1
.24345	12.958	0.707	.1963	.04715	98.1
	12.958	.707	.1963	.04715	98.1
.28323	13.034	.631	.2339	.04933	102.6

(18) For interpolated values of −log K_w see W. Mansfield Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, 1928.

(19) Stone and Failey, *J. Phys. Chem.*, **37**, 935 (1933). Hydrolysis of the hemoglobin may, of course, have occurred during the long times of equilibration for the solubility measurements.

from acidities at which the hemoglobin chloride is precipitated by the neutral salt, to reactions at which the hemoglobin is completely bound by base.

Under these conditions the effect on the activity coefficient, due to the influence of the neutral salt on the hydroxyl ion, is sufficiently large to mask the changes due to variation with sodium hydroxide and presumably with protein concentration. "It has been shown that the Nernst formula for electromotive force, the solubility product and mass action law in the case of a complicated ionic equilibrium are applicable in their classical form to such concentrated salt solutions, the reason for this simplicity being the practical constancy of the activity coefficients in the practically constant medium. Utilization of these results would mean in many cases a great simplification in problems pertaining to electrolytic solutions"[20] (p. 431). Measurements upon 0.01 and 0.1 N sodium hydroxide in N sodium chloride, calculated by means of the Michaelis values of K_w, yield values of $-\log\gamma_{OH}$ which vary less than 0.01 and may be taken as 0.23 at 25° for all the systems containing salt that are reported.

Maximal Acid and Base Combining Capacity of Hemoglobin.—Several studies of the combination of hemoglobin with acid and base have been reported. One of these is the titration with the quinhydrone electrode by Lewis[21] of the carboxyhemoglobin of the ox with sulfuric acid in the presence of ammonium sulfate. His results yield an acid combining capacity of 145×10^{-5} equivalent per gram of hemoglobin. Pauli and Schwarzacher[22] report that hemoglobin combines 159×10^{-5} mole hydrochloric acid per gram and 124.5×10^{-5} mole lithium hydroxide per gram. The results of Stone and Failey[19] yield a base combining capacity of slightly over 100×10^{-5} mole per gram calculated on the basis of the activity coefficient of thallous chloride and the value 14 for pK_w. Recalculation by the methods we have employed yields essentially the same result.

In several experiments we have titrated carboxyhemoglobin to saturation with hydrochloric acid (Table II). No appreciable increase in acid combining capacity was detected at reactions acid to pH 2.2. From pH 2.2 to 1.5 the free hydrochloric acid increased more than sixfold, with a change in combined hydrochloric acid only from 144.6 to 150.4×10^{-5} mole per gram. These results, as calculated, yield a maximal acid combining capacity of 148×10^{-5} mole per gram. The influence of protein in increasing the activity coefficients employed would result in a lower estimate of the combining capacity. The measurements in Table II are subject to correction at such a time as data are available for the activity coefficients of the hydrogen ion in the presence of hemoglobin hydrochloride.

The base combining capacity of hemoglobin has been calculated in Table III. The base bound by hemoglobin increased until the reaction was more alkaline than pH 12.2. At this reaction the concentration of free and combined base was approximately equal. At pH 12.68 the free base was three times that bound, and no appreciable increase in combining capacity was observed, provided the measurements were made soon after the hemoglobin was exposed to such extremely alkaline reactions. In either strongly acid or basic solutions, the hemoglobin changed from its characteristic red color to a deep brown. At either longer times or more alkaline reactions, greater apparent base combining capacities were noted and ascribed to hydrolysis. This was the case with many of the earlier experiments, which yielded satisfactorily constant acid combining capacities, but apparent base combining capacities which varied from experiment to experiment and were always greater than those reported.

The use either of higher activity coefficients, such as those employed by Stone and Failey, or of a larger value for pK_w, would result in lower estimates of base combining capacity than the value which results from the mode of calculation we have always employed (Table III). It was largely to test this point that measurements were undertaken in the presence of normal sodium chloride (Table IV).

Hemoglobin preparations V to VIII were studied in molal sodium chloride. Although the hemoglobin in these experiments varied from 16.37 to 48.08 g. per liter, the results with all but preparation V (which yielded a normal titration curve, but a maximal combining capacity of 148×10^{-5} mole sodium hydroxide per gram, and is not reported in detail since unconfirmed by any subsequent experiment) yield the same or slightly lower maximal combining capacity in the pres-

(20) Brönsted, *Trans. Faraday Soc.*, **23**, 416 (1927). See also Harned, "The Electrochemistry of Solutions," in Taylor's "Treatise on Physical Chemistry," D. Van Nostrand Company, New York, Vol. I, 1931, p. 805.

(21) Lewis, *Biochem. J.*, **21**, 46 (1927).

(22) Pauli and Schwarzacher in Pauli and Valkò, "Kolloidchemie der Eiweisskörper," Theodor Steinkopff, Leipzig, 1933.

ence of N sodium chloride as had the earlier experiments on systems to which no salt was added. The base combining capacity in the systems containing normal sodium chloride appears to pass through a minimum near pH 12.3. The apparent decrease in combined base at more alkaline reactions suggests that the activity coefficients employed may have been too large. The curve in Fig. 1 is constructed as though hemoglobin dissociated 261 $\times$ 10^{-4} mole of hydrogen ions between pH 1.5 and 13.

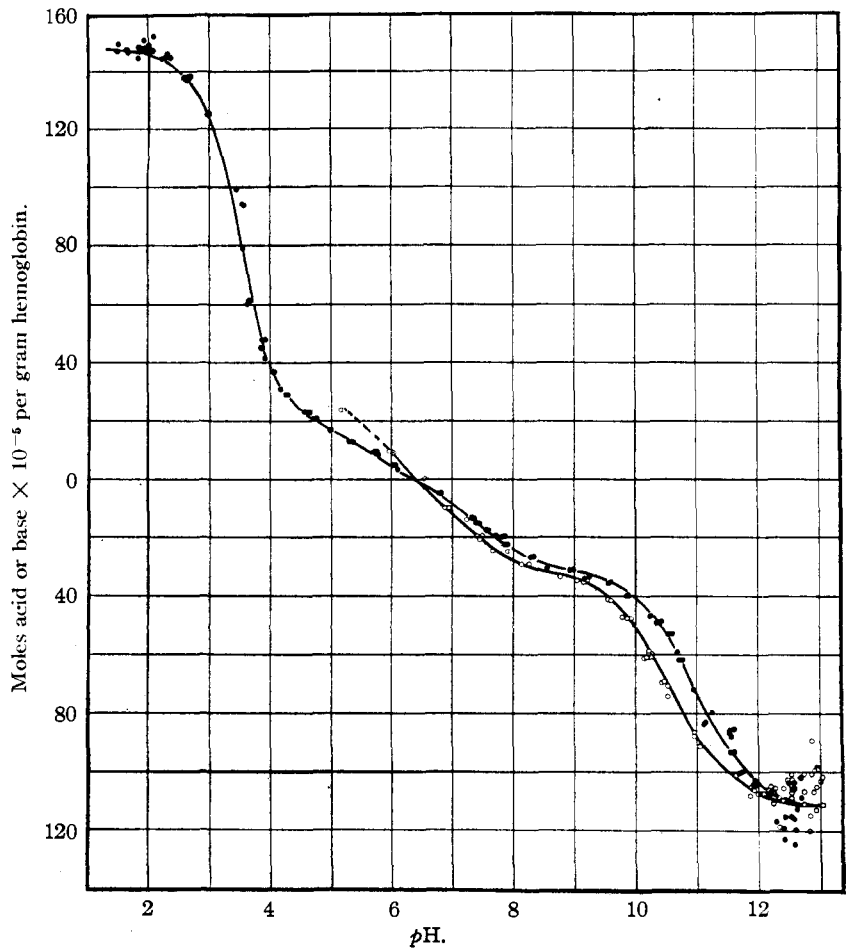

Fig. 1.—The titration curve of carboxyhemoglobin of the horse in the absence of added salt ● and in the presence of 1 M NaCl ⊙.

TABLE V

Protein	Acid combining capacity, moles × 10^5	Base combining capacity, moles × 10^5	Total combining capacity, moles × 10^5
Ox-oxyhemoglobin (21)	145		
Horse—carboxy-hemoglobin (19)	159	105 124.5	283.5
(22)	148	113	261

These results are compared with those previously reported in Table V. The final column gives the total acid and base combining capacity of hemoglobin.

Titration Curve of Hemoglobin and its Apparent Dissociation Constants.—Although the acid combining capacity of a protein depends on the histidine, arginine and lysine in the molecule, these basic amino acids dissociate at alkaline reactions. The titration curve of hemoglobin,[23] given in Fig. 1, is readily divisible into at least three well-defined sigmoid curves, one extending from saturation with acid roughly to pH 5, the second from pH 5 to 9 and the third from pH 9 to saturation with base. Although analyzable into a larger number of component parts, we have preferred describing this curve—and that in the presence of N sodium chloride—in terms of the smallest possible number of dissociation constants. The curve in the absence of salt in Fig. 1 is constructed on the basis of the following groups.

Taking the molecular weight of hemoglobin as 67,000, each free reactive group combines with 1.5 $\times$ 10^{-5} mole of acid or base per gram. On this basis one may assume 12 tyrosine, from 13 to 16 arginine and from 37 to 44 lysine

TABLE VI

pK_1'	pK_2'	pK_3'	pK_4'	pK_5'	pK_6'	pK_7'
3.7	4.0	4.8	5.7	7.5	10.8	11.6
Number of groups of each constant dissociating						
83	4	13	20	40	14	
Carboxyl groups		Histidine		Lysine and Arginine or Tyrosine		

(23) The large number of measurements at neutral reactions, on which the titration curve is constructed, are not reported in detail for lack of space, but have been filed as a Science Service Document.

molecules per hemoglobin molecule on the basis of the analyses in Table I.

The solid curve in Fig. 1 is constructed on the assumption that 60×10^{-5} mole dissociate with a pK of 10.8 and 21×10^{-4} mole with a pK of 11.6. In the free condition the pK of the ϵ-amino group of lysine is 10.53 and of the guanidine nucleus 12.48.[24]

The hydroxyl group of tyrosine dissociates at pK 10.28[25] and analytical data suggest 12 molecules of tyrosine in the hemoglobin molecule. The titration curve does not reveal a sufficient number of dissociable groups in the range alkaline to pH 9 to account for arginine, lysine and tyrosine,[26] but enough to account for lysine and either tyrosine or arginine. Groups dissociating beyond pH 13 would not, it is true, be described by our measurements. The experimental errors at more alkaline reactions are, however, formidable.

The unusual feature of the titration curve of hemoglobin is the extremely well-defined sigmoid curve in the neutral range. This part of the curve has previously been investigated by Hastings,[27] and Sörensen,[7] and is now being reinvestigated by German and Wyman[28] by means of the glass electrode, over the region in which it is shifted to more acid reaction as a result of combination with oxygen or carbon monoxide.

Hemoglobin combines with almost exactly 50×10^{-5} mole per gram between pH 5 and 9. That is to say, over this range 33 groups dissociate on the hemoglobin molecule, or exactly the number of histidine molecules revealed by analysis. The difference in dissociation of reduced and carboxyhemoglobin does not involve as many as 33 groups. The free group of histidine has a pK of 6.04.[24] In histidyl-histidine the comparable groups have dissociation constants of pK 5.6 and 6.8.[29] The titration curve in Fig. 1 is constructed by assuming 12 groups dissociating with pK 5.6 and 21 groups with pK 7.4. The latter groups, or a portion of them, may be considered as combining less acid in the oxygenated than in the reduced state, and as arranged with some sort of symmetry with respect to the four hematin groups of the hemoglobin molecule.[30]

The basic amino acids revealed by analysis suffice to account for the groups dissociating from pH 5 to saturation with base. Over this range 87 groups per molecule dissociate. From this reaction to saturation with acid (Table II) roughly the same number of groups would appear to dissociate.

Since phenolic hydroxyl groups do not generally dissociate in a range more acid than pH 5, all of the rest of the groups have been assumed to be carboxyl groups. The curve, acid to pH 5, is a sigmoid curve with a point of inflection near pH 3.6. Amino acid and protein titration curves are generally analyzed by assuming the independence and additivity of the groups dissociating.[31] The most acid segment of the hemoglobin curve appears to be too steep to be accounted for in this manner, suggesting that the acid groups are not independent of each other. Larger activity coefficients, and therefore a smaller acid combining capacity and estimate of dissociable carboxyl groups, might yield a simpler form of sigmoid curve. The curve tentatively has been described in terms of an equation of the type previously suggested, having the form[32]

$$\frac{A^{-n}}{(H_nA)} = \frac{K_1}{(H^+)\gamma_1} + \frac{2K_1K_2}{(H^+)^2\gamma_2} + \frac{3K_1K_2K_3}{(H^+)^3\gamma_3} + \ldots \frac{nK_1K_2K_3 \ldots K_n}{(H^+)^n\gamma_n}$$

The first two right-hand terms suffice, pK_1 being taken as 3.7, and pK_2 as 4.0. The total curve fits somewhat better if a small number of groups—possibly representing four of the groups of hematin—is assumed to dissociate in the neighborhood of pH 4.9.

Influence of Sodium Chloride upon the Titration Curve.—Apparent dissociation constants are not independent of hemoglobin or of salt concentration. In the systems reported salt concentration was maintained below 0.01 N, and hemoglobin concentration varied from 10.8 to 88 g. per liter. Over this range, no large effect of protein concentration was observed, but the largest effect of hemoglobin upon its dissociation occurs at very low concentrations, at which it is not practicable to work in strongly acid or alkaline solutions. The influence of sodium chloride concentration on pH in systems differing from each other only in re-

(24) Schmidt, Kirk and Appleman, *J. Biol. Chem.*, **88**, 285 (1930).

(25) Simms, *J. Gen. Physiol.*, **11**, 629 (1928).

(26) Lewis[21] titrated the carboxyhemoglobin of the ox in the presence of formaldehyde with the glass electrode. His results suggest a base combining capacity of 63 moles per mole hemoglobin. This is half again as great as that to be expected from the lysine present.

(27) Hastings, Sendroy, Murray and Heidelberger, *J. Biol. Chem.*, **61**, 317 (1924).

(28) German and Wyman, *ibid.*, **117**, 533 (1937).

(29) Greenstein, *ibid.*, **93**, 479 (1931).

(30) Pauling, *Proc. Nat. Acad. Sci.*, **21**, 186 (1935).

(31) Von Muralt, THIS JOURNAL, **52**, 3518 (1930); Simms, *ibid.*, **48**, 1239 (1926); Weber, *Biochem. Z.*, **189**, 381 (1927).

(32) Cohn, *Physiol. Rev.*, **5**, 349 (1925).

spect to concentration of electrolyte is given in Fig. 2. The smallest effect of salt on the *p*H of protein solutions is near the isoelectric point, as pointed out by Sörensen, and is opposite in direction on either side of the isoelectric point.[5,33,34]

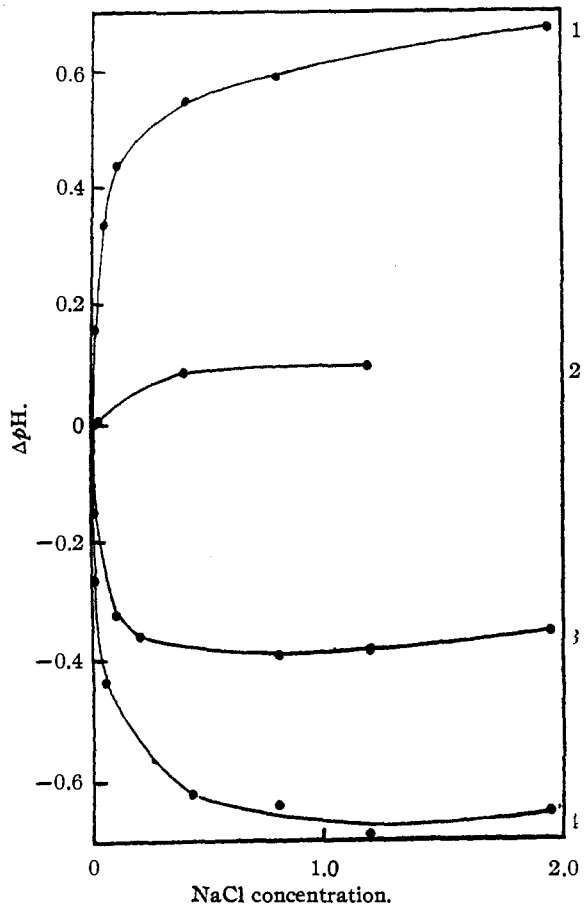

Fig. 2. *Influence of NaCl on the pH of Hemoglobin Solutions.*—Change in *p*H with increasing salt concentration, Δ*p*H, measured at 37° with glass electrode standardized with 0.1 *M* phosphate buffers. The *p*H of the hemoglobin solutions to which no salt was added were: (1) 4.90; (2) 6.60; (3) 7.73; (4) 8.7.

The titration curve in the presence of 1 *M* sodium chloride (Fig. 1) is constructed using the values of 5.2, 6.1, 7.1, 10.4 and 11.2 for pK_3', pK_4', pK_5', pK_6' and pK_7', respectively. pK_3' and pK_4' are thus 0.4 greater and pK_5', pK_6' and pK_7' are 0.4 less than the values in Table IV. Although apparent dissociation constants vary with temperature, salt and protein concentration, total base combining capacity we calculate to be independent of the presence or absence of salt.

(33) Sörensen, Höyrup, Hempel and Palitzsch, *Compt. rend. trav. Lab. Carlsberg*, **12**, 68 (1917).

(34) Sörensen, Linderstrøm-Lang and Lund, *J. Gen. Physiol.*, **8**, 543 (1927).

Relation between Acid and Basic Dissociation and the Isoelectric Point.—The method of calculation that has been employed may be expected to yield a maximal estimate of dissociable groups.[35] The number of these derived from the histidine may be considered well known. It follows that basic groups dissociate on both sides of the isoelectric point, which bears no simple relation to the reaction, near *p*H 5, on the one side of which acid and on the other side basic groups are presumably dissociating. That certain of the free groups of basic amino acids dissociate in the protein at acid reactions previously has been noted.[36]

The dipolar ion structure of amino acids and proteins demanded reinterpretation of the reactions at which the various groups dissociate. Such analyses of titration curves have been reported,[36–38] but it has often been assumed that the apparent dissociation constants of protein groups bear simple relation to the true dissociation constants of the groups of the free amino acids, a conclusion which by no means follows. The apparent dissociation constants of proteins, as of amino acids and peptides, vary with the number and distribution of the dissociable groups on the molecule.[31–32] The basic strength of an amino group is diminished by carboxyl groups and this is true whether the latter are dissociated or in the non-dissociated condition. Conversely, carboxyl dissociation is increased by juxtaposition of amino and carboxyl groups, and most other substituents. The result is that the closer amino and carboxyl groups are to each other, the more acid the molecule. On the basis of analytical data, casein has half again as many free carboxyl as basic groups, and gelatin twice as many basic as carboxyl groups. In egg albumin the numbers of free acid and basic groups are nearly equal. None the less, all of these proteins have very nearly the same isoelectric point.

Relation between Basic Groups and Acid Combining Capacity, and between Acid Groups and Base Combining Capacity.—In earlier investigations the relation between the number of basic groups and acid combining capacity and between the number of acid groups and base combining

(35) If larger activity coefficients were assumed, not only the base combining but the acid combining capacity would be smaller. The possibility of measuring these activity coefficients by the simultaneous use of cells without liquid junction remains to be accomplished. See Harned and Åkerlöf, *Physik. Z.*, **27**, 411 (1926), and Joseph, *J. Biol. Chem.*, **111**, 479, 489 (1935).

(36) Simms, *J. Gen. Physiol.*, **14**, 87 (1930).

(37) Kerwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

(38) Russell and Cameron, THIS JOURNAL, **58**, 774 (1936).

capacity has been stressed.[17,32] Reinterpretation of the regions in which the free groups of proteins dissociate in no way alters this relation. Indeed new analyses of the amino acids[39] in proteins have on the whole confirmed the estimates of the number of free groups derived from physical chemical measurements.[40]

Our measurements yield an acid combining capacity of 148×10^{-5} and a base combining capacity of 113×10^{-5} mole per gram of carboxyhemoglobin. The total combining capacity of 261×10^{-5} mole per gram thus represents 174 ± 2 dissociable groups on the hemoglobin molecule, of which 75 would be acid and 99 basic groups. On this basis not more than 75 dipole pairs can be considered dissociated at the isoelectric point.

There remains a discrepancy between these results and those derived from analytical data, for whereas no more than 85 basic amino acids have been isolated from hydrolysates, or 93 estimated by the nitrogen distribution method (Table I), the acid combining capacity suggests the presence of 99 trivalent basic amino acid groups in the hemoglobin molecule.[41] Some of these might conceivably titrate only after partial splitting of the molecule in acid solution, where heme is liberated from globin and the latter yields molecules of smaller molecular size.

(39) Among them investigations of the dicarboxylic amino acids of edestin [Jones and Moeller, *J. Biol. Chem.*, **74**, Proc. liv (1927)] and of egg albumin [Calvery, *J. Biol. Chem.*, **94**, 613 (1932)] and of the basic amino acids of the latter protein [Calvery, *ibid.*, and Vickery and Shore, *Biochem. J.*, **26**, 1101 (1932)].

(40) The acid combining capacity of zein, not detected in aqueous systems in which the protein is insoluble [Cohn, Berggren, Hendry, *J. Gen. Physiol.*, **7**, 81 (1924)] has been investigated in the dry state with gaseous hydrogen chloride [Czarnetzky and Schmidt, *J. Biol. Chem.*, **105**, 301 (1934)], and in ethanol–water mixtures [Cohn, Edsall and Blanchard, *ibid.*, **105**, 319 (1934)], and reveals approximately the number of basic groups previously estimated to be present from the results of analyses. See also Neuberger, *Biochem. J.*, **28**, 1982 (1934).

(41) Note added to proof: This discrepancy suggested that part of the acid combining capacity might be ascribable to the heme. Accordingly a study of the acid combining capacity of globin was undertaken and the first preparation investigated combined 138×10^{-5} mole per gram, representing 92 free basic groups, or almost exactly those expected from the analyses of basic amino groups in globin and hemoglobin. Other differences between the titration curves of globin and hemoglobin will be reported subsequently.

Summary

1. Electromotive measurements have been made with the hydrogen electrode on systems containing carboxyhemoglobin and sodium hydroxide and hydrochloric acid.

2. The methods of calculating the combining capacity of a protein from such measurements is discussed, and the acid combining capacity estimated to be 148×10^{-5}; the base combining capacity 113×10^{-5} mole per gram.

3. On the basis of a molecular weight of 66,700 hemoglobin thus has 174 dissociable groups. Of these not more than 75 dipole pairs can exist at the isoelectric point.

4. Since various analyses reveal between 83 and 93 trivalent basic amino acids per mole of hemoglobin, approximately half the dissociable groups in hemoglobin are derived from the imino group of histidine, the guanidine nucleus of arginine and the ϵ-amino group of lysine.

5. The free groups of arginine and lysine dissociate at very alkaline reaction, but those of histidine near neutrality. There are 33 histidine molecules in hemoglobin. The free groups of approximately 13 of these appear to dissociate at reactions acid to the isoelectric point. Not more than the remaining 20 dissociate at physiological reactions, in the regions affected in dissociation by oxygenation and reduction of the hemoglobin.

6. The titration curve reported is described in terms of the following pK' values: 3.7, 4.0, 4.8, 5.7, 7.5, 10.8, 11.6. Of these the first three are considered to represent acid groups, the next two histidine groups, and the most alkaline lysine and tyrosine or arginine groups.

7. Although the titration curve is very sensitive to ionic strength, the maximal base combining capacity is demonstrated to be the same in solutions to which no salt was added and in N sodium chloride.

BOSTON, MASS. RECEIVED NOVEMBER 13, 1936