used instead of sodium acetate. When the hydrolysate was chilled in ice, potassium dialurate was obtained; yield 0.247 g. from 0.500 g. of 1'-phenylalloxantine, theoretical 0.252 g.

Anal. Calcd. for $C_4H_3O_4N_2K$: N, 15.38. Found: N, 15.48.

1-Phenylmurexide (1.000 g.), suspended in 100 cc. of oxygen-free water by the passage of a slow stream of hydrogen, was hydrolyzed with 16 cc. of approximately molar hydrochloric acid added a few drops at a time over a period of forty-five minutes. The solid material remaining when the color of the solution had been completely discharged was brought upon a filter and identified as 1-phenyluramil by its qualitative reactions and nitrogen content; yield 0.427 g., theoretical 0.605 g. From the filtrate from the 1-phenyluramil, concentrated at room temperature to 5 cc., alloxantine was obtained by reduction with stannous chloride; yield 0.205 g., theoretical 0.364 g.

Hydrolysis of 1'-phenylmurexide (1.000 g.) by the procedure used for the hydrolysis of 1-phenylmurexide yielded 0.383 g. of uramil; theoretical 0.397 g. Due to the instability of N-phenylalloxan[18] in acid solution, only traces of 1,1'-diphenylalloxantine could be obtained from the filtrate from the uramil by reduction with stannous chloride.

(18) 1,1'-Diphenylalloxantine was obtained in 73% yields by reduction of a freshly prepared acid solution of N-phenylalloxan with stannous chloride. When the acid solution was allowed to stand for forty-five minutes before reduction, the yield was only 1.5%.

Anal. Calcd. for $C_{20}H_{14}O_8N_4$: N, 12.79. Found: N, 12.87.

Summary

1. The isomers 1-phenylmurexide and 1'-phenylmurexide maintain their identity at least for a limited length of time under certain experimental conditions. 1-Phenylalloxantine and 1'-phenylalloxantine also maintain their isomeric identities.

2. These phenylmurexides constitute the third pair of isomeric N-substituted murexides for which maintenance of identity has been established. The importance of this fact in connection with the theories of color production and resonance in organic molecules has been discussed.

3. The following compounds not described in the literature have been prepared: 1-phenylmurexide, 1'-phenylmurexide, 1-phenylalloxantine, 1'-phenylalloxantine, 1,1'-diphenylalloxantine, 1-phenyluramil, N-phenylalloxan, and malonyl dimonophenylurea.

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

The Influence of Ionic Strength and *p*H on Electrophoretic Mobility

BY BERNARD D. DAVIS AND EDWIN J. COHN

Electrophoretic mobilities are generally determined in the presence of buffer solutions. It seemed of importance to investigate further the influence of the valence of the buffer and of the ionic strength of the solution. Tiselius has noted[1,2] the effect of ionic strength and of the valence of inorganic cations, and work in this Laboratory had indicated that the ionic strength of the buffer has a very profound influence on electrophoretic mobility. The influence of these variables also has been studied by Abramson[3] and by Smith,[4] who observed the movements of particles of quartz or collodion coated with egg albumin in an electric field. The latter studied especially the dependence of the isoelectric point upon the nature and concentration of neutral salts.

In the present investigation we have used the moving boundary method and have chosen horse carboxyhemoglobin as the protein to be investigated since it is a well characterized protein whose other properties have been investigated extensively.[5-9] Mobility has been determined from *p*H 5.65 to 7.2. Hemoglobin was therefore present as cation in most of these systems, whereas most of the other blood proteins would be present as anions. The ionic strengths vary from 0.02, the concentration often used by Tiselius, to ionic strengths characteristic of physiological systems. Two sets of buffers were employed, namely, phosphates in which monovalent and bivalent anions, and citrates in which bivalent and trivalent anions, are present. The measurements reported demonstrate the great influence

(1) A. Tiselius, Dissertation, Upsala, 1930.

(2) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

(3) H. A. Abramson, "Electrokinetic Phenomena and their Application to Biology and Medicine," Chemical Catalog Co., Inc., New York, 1934.

(4) E. R. B. Smith, *J. Biol. Chem.*, **108**, 187 (1935); *ibid.*, **113**, 473 (1936).

(5) R. M. Ferry and A. A. Green, *ibid.*, **81**, 175 (1929).

(6) E. J. Cohn and A. M. Prentiss, Loeb Memorial Vol., *J. Gen. Physiol.*, **8**, 619 (1927).

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

(8) R. M. Ferry, E. J. Cohn and E. S. Newman, *ibid.*, **60**, 1480 (1938).

(9) M. Richards, *J. Biol. Chem.*, **122**, 727 (1938).

of the type of buffer and of the ionic strength of the solution both upon the observed electrophoretic mobility and upon the apparent isoelectric point of the protein.

Materials and Methods.—The carboxyhemoglobin of the horse employed in these experiments was twice recrystallized from aqueous solution by the method of Ferry and Green.[5,10] The crystals were then repeatedly triturated with distilled water, and centrifuged from the resulting saturated hemoglobin solutions to free them from salt. Solutions saturated with the hemoglobin after the crystals had been washed from three to five times in this manner, were employed in these studies.[11] The desired solutions of hemoglobin in buffer were dialyzed in cellophane bags against identical buffer solutions for from twelve to twenty-four hours at about 3°. This practice, employed by Tiselius,[1] minimizes the disturbances at the protein–buffer interface. The *p*H and the conductivity of the two solutions were found to be identical within the limits of experimental error.

The phosphate buffer solutions of known ionic strength and *p*H were those generally employed in this Laboratory.[12,13] Using the data of Hastings and Van Slyke,[14] which give the concentrations of primary, secondary and tertiary citrate at the successive stages in the titration of 0.1 *M* citric acid with sodium hydroxide, graphs were prepared from which could be read the concentration of citrate necessary to produce a solution of given *p*H and ionic strength. The *p*H values of all solutions were checked electrometrically after dialysis, and it is these values that are recorded in the tables.

The apparatus used consists of a U-tube connected through side-arms to large buffer chambers, at the bottom of which Ag–AgCl electrodes, coils of no. 18 wire 1.5 cm. in diameter and 4.5 cm. long, were immersed in reservoirs of 20% potassium chloride. The content of the U-tube below the tops of the stopcocks was about 15 cc.; the potassium chloride reservoir up to a level several mm. above the electrode, 30 cc. on each side; the rest of the buffer chamber and vertical column of the U-tube had a volume of 125 cc. on each side, ample to ensure a run of many hours without contamination by the transport of potassium chloride or electrode products. The diameter of the U-tube was 0.95 cm. The design of the electrode was largely adopted from Tiselius,[1,15] the U-tube was that previously used in investigations in this Laboratory.[16,17] The sharp boundary of the hemoglobin was read directly.

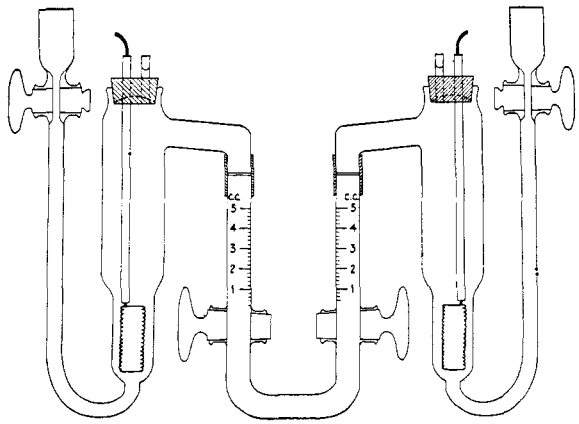

Fig. 1.

The top of each buffer chamber was closed with a two-holed rubber stopper, slightly hollowed on the under side to prevent the trapping of air bubbles. Through one hole of the stopper was passed the glass sealed electrode lead, and through the other a tube for the introduction and leveling of the buffer. The various solutions were evacuated and brought to the temperature of the bath before introduction, in order to remove dissolved air which would later form bubbles injurious to the boundary. When the protein, buffer and hydrochloric acid solutions had been introduced in the proper positions the assembled apparatus was immersed up to the side arms in a glass-walled thermostat at 25 ± 0.1°. The buffer solutions were carefully leveled in the tubes above the buffer chambers by addition of appropriate amounts of solution; the stopcocks were then opened and buffer solution slowly added to one chamber and removed from the other until one hemoglobin column was raised above the optical irregularities of the U-tube near the stopcock. A potential of 110 v. was applied to the electrodes and the movement of the protein in the field directly observed from time to time. The electrodes in successive experiments were alternated in polarity in order to keep them coated with silver chloride.

The determination of the mobility in units of cm.²/volt-sec. involved measurements of the rate of migration in cc./hour, of the conductivity of the buffer, and of the current flow. These quantities are related in the following formulas

$$IR = eq \quad (1)$$

$$M = ueq \times 3600 \quad (2)$$

$$u = M/3600\,IR \quad (3)$$

where M = rate of migration (cc./hour); u =

(10) A. A. Green, *J. Biol. Chem.*, **93**, 495 (1931).

(11) Many of these preparations were the same ones used by Ferry, Cohn and Newman[8] for their study.

(12) E. J. Cohn, THIS JOURNAL, **49**, 173 (1927).

(13) A. A. Green, *ibid.*, **55**, 2331 (1933).

(14) A. B. Hastings and D. D. Van Slyke, *J. Biol. Chem.*, **53**, 269 (1922).

(15) Since completion of most of these measurements Tiselius has perfected a new electrophoretic cell, and these measurements have been confirmed with this apparatus at 25°. The symbol ◐ in Fig. 2 represents measurements with the new Tiselius apparatus.[2]

(16) E. J. Cohn, J. Gross and O. C. Johnson, *J. Gen. Physiol.*, **2**, 145 (1919).

(17) L. Reiher, *Kolloid Z.*, **40**, 327 (1926).

mobility (cm.²/volt-sec.); I = current (amperes); R = specific resistance of buffer (ohms cm.); e = potential drop in U-tube (volts/cm.); and q = cross section of U-tube (sq. cm.). The cross section does not enter into the calculation, provided the U-tube is calibrated in cc. Current leakage through the joints and stopcocks was found to be negligible by measuring the current while the stopcocks were closed.

The advancing boundary, except when very near the isoelectric point, remained sharp indefinitely, showing less spreading than would be caused in the same interval of time by diffusion. On reversal of the current, the receding boundary became diffuse. Since the difference in conductivity and therefore the difference in potential drop at the boundary interface was very small, the influence of the hemoglobin concentration on its own mobility, whereby velocity increases with concentration, would appear the most probable explanation of the difference in velocity and diffuseness of the advancing and receding boundaries. The measurements reported are for the advancing boundary.

Influence of Hemoglobin Concentration.—The reproducibility of these electrophoretic measurements upon carboxyhemoglobin solutions may be illustrated by a series of experiments in which the hemoglobin concentration was varied systematically, the ionic strength of the buffer and the pH being maintained constant. The hour by hour readings in the seven experiments carried out at pH 5.93 at ionic strength 0.05 and hemoglobin concentrations from 1.67 to 13.3 g. per liter are recorded in Table I. They demonstrate the agreement between duplicate experiments, and the constancy of the rate of electrophoretic migration throughout the experiments. The current, which was always measured with a standardized Weston ammeter, generally increased slightly during the first period. The value when the current flow had become sensibly constant was employed in estimating the mobilities in absolute units recorded in Table I. The current flow, I, varied to be sure with the ionic strength, but the product of the current and the resistance, R—which measures the potential drop—did not vary systematically with pH or ionic strength.

The average mobility at each hemoglobin concentration is given at the bottom of Table I. There would appear to be a definite effect of the hemoglobin concentration upon mobility. This

TABLE I

INFLUENCE OF CARBOXYHEMOGLOBIN CONCENTRATION UPON ELECTROPHORETIC MOBILITY AT 25° IN PHOSPHATE BUFFERS OF IONIC STRENGTH 0.05 AND pH 5.93

Expt.....	92	96	86	98	88	91	99
Time in hours	Carboxyhemoglobin concentration 1.67 g./liter		3.3 g./liter		6.7 g./liter	13.3 g./liter	
	Migration in cc.						
1.0	0.42	0.46	0.42	0.46	0.44	0.48	0.52
2.0	.84	.90	.84	.92	.90	1.02	1.06
3.0	1.24	1.32	1.28	..	..	1.52	..
4.0	..	1.74	..	1.78	1.84	..	2.10
5.0	2.06	2.16	2.20	2.24	2.30	2.58	2.64
6.0	2.46	2.60	2.64	2.68	2.74	3.08	3.14
IR	2.43	2.48	2.38	2.41	2.43	2.39	2.37
	Mobility (Migration per hour × IR × 3600) × 10^5						
1.0	4.8	5.1	4.9	5.3	5.0	5.6	6.1
2.0	4.8	5.0	4.9	5.3	5.1	5.9	6.2
3.0	4.7	4.9	5.0	...	...	5.9	...
4.0	...	4.9	...	5.1	5.3	...	6.1
5.0	4.7	4.8	5.1	5.2	5.3	6.0	6.2
6.0	4.7	4.8	5.1	5.2	5.3	6.0	6.1
Av.	4.7	4.9	5.0	5.2	5.2	5.9	6.2
	4.8		5.1		5.2	6.1	

was not only true at pH 5.93, but in other experiments. Thus at an ionic strength of 0.02 and a pH of 7.11, alkaline to the isoelectric point, when the hemoglobin was moving as an anion, its mobility was -1.6×10^{-5} cm.²/volt-sec. in a solution of 0.3% with respect to hemoglobin (Expt. 81) but -2.3×10^{-5} cm.²/volt-sec. in a 1% hemoglobin solution (Expt. 80).

The observed increase in mobility with increase in hemoglobin concentration may well be associated with interaction of the protein dipolar ions with each other[18] or with buffer ions.[19] Tiselius,[20] working with serum proteins, has explained boundary irregularities at high protein concentration as due to the effect of the protein on the transport of the buffer ions, causing a shift of pH at the boundaries. In the range of hemoglobin concentration here described, all boundaries were sharp and all mobilities reasonably constant. In several experiments with hemoglobin concentrations below 0.1% at ionic strength 0.05, the boundary became convex and then diffuse. The hemoglobin concentration was maintained at 1% in studying the influence of ionic strength.

(18) E. J. Cohn, T. L. McMeekin, J. D. Ferry and M. H. Blanchard, *J. Phys. Chem.*, **43**, 1 (1939).

(19) Smith[4] has found that the mobility of egg albumin decreases with increasing protein concentration. She measured mobility of the protein adsorbed on quartz particles, and her observations may be related to this difference in technique, or to the far smaller effects of egg albumin on the dielectric constant of solvents.[21]

(20) A. Tiselius, *Biochem. J.*, **31**, 313, 1464 (1937).

(21) J. L. Oncley, unpublished work.

Influence of *p*H and Ionic Strength.—Observations on the influence of *p*H on the mobility of hemoglobin were early reported by Michaelis and Airila.[22] The influence of ionic strength on the mobility of carboxyhemoglobin is shown in Table II. The mobilities in phosphate buffers are graphically represented in Fig. 2, those in citrate buffers in Fig. 3. It will be noted that protein mobility is extremely sensitive not only to *p*H and ionic strength, but also to the valence and nature of the ions present; an effect noted also by others.[1,3,4,23] This sensitivity to the nature and strength of the ionic atmosphere (Fig. 2) would appear to be nearly as important as *p*H in

TABLE II

ELECTROPHORETIC MOBILITY OF 1% CARBOXYHEMOGLOBIN IN PHOSPHATE AND CITRATE BUFFERS AT 25°

Expt.	Concn. of salt mole/liter	Mole fraction K_2HPO_4	*p*H	Electrophoretic mobility $u \times 10^5$
		Phosphate, $\Gamma/2 = 0.02$		
52-III	0.0182	0.0424	5.65	10.3
55-III	.0180	.0550	5.81	9.3
60-IV	.0156	.140	6.22	6.5
53-IV	.0140	.230	6.49	4.4
54-IV	.0110	.395	6.82	1.8
80-V	.0093	.566	7.11	− 2.3
68-V	.0088	.616	7.18	− 3.0
57-IV	.0088	.616	7.21	− 3.1
		Phosphate, $\Gamma/2 = 0.05$		
61-IV	0.045	0.0535	5.68	6.7
63-IV	.0375	.165	6.21	4.0
62-IV	.032	.283	6.52	2.1
67-V	.0263	.444	6.82	− 0.2
69-V	.0235	.550	7.00	− 2.2
		Phosphate, $\Gamma/2 = 0.10$		
50-III	0.088	0.068	5.69	3.8
48-IV	.084	.088	5.79	3.5
49-III	.084	.088	5.79	3.4
89-VI	...	...	6.42	0.7
59-IV	.051	.477	6.83	− 1.1
		Phosphate, $\Gamma/2 = 0.15$		
75-V	0.129	0.080	5.72	2.5
77-V	.110	.176	6.12	1.1
73-V	.100	.249	6.30	0.3
74-V	.089	.341	(6.60)	− 1.0
76-V	.074	.500	6.82	− 2.2
		Citrate, $\Gamma/2 = 0.02$		
45-III	0.00481		5.92	4.9
47-IV	.00481		5.91	4.9
42-III	.00380		6.50	2.4
46-IV	.00380		6.55	2.3
71-V	.00358		7.09	− 1.3
		Citrate, $\Gamma/2 = 0.10$		
44-III	0.02403		5.68	2.0
64-V	.02035		6.10	0.7
41-III	.01821		6.56	− 0.5
65-V	.01821		6.54	− 0.6
		Citrate, $\Gamma/2 = 0.20$		
78-V	0.04384		5.77	0.2
79-V	.03718		6.31	− 1.3

(22) L. Michaelis and Y. Airila, *Biochem. Z.*, **118**, 144 (1921).

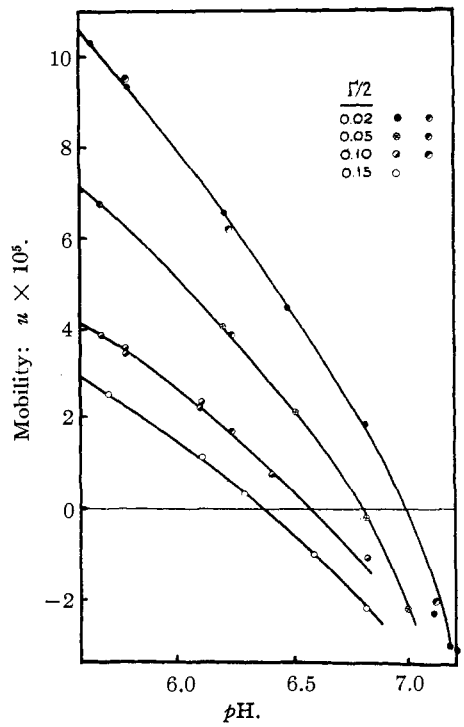

Fig. 2.—Phosphate buffers.

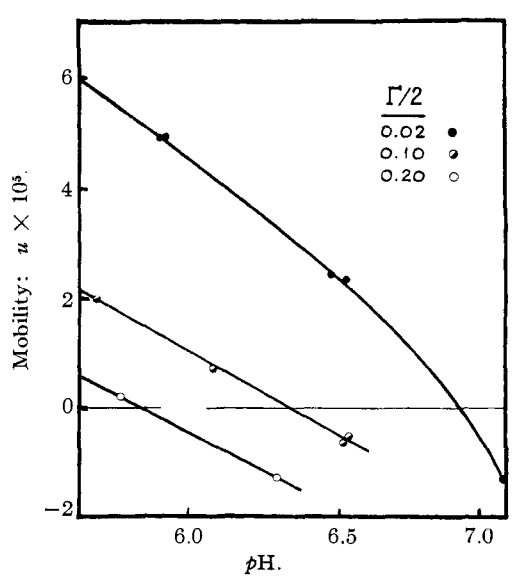

Fig. 3.—Citrate buffers.

(23) L. S. Moyer and H. A. Abramson, *J. Biol. Chem.*, **123**, 391 (1938).

determining the optimal conditions for the electrophoretic separation of proteins.

The effect of ionic strength[24] and ionic species on the apparent isoelectric point is shown in Fig. 4, the conditions at which there is no mobility being graphically estimated from Figs. 2 and 3. As might be expected on theoretical grounds, mobility in citrate and phosphate buffers appears to approach the same value at low ionic strengths.

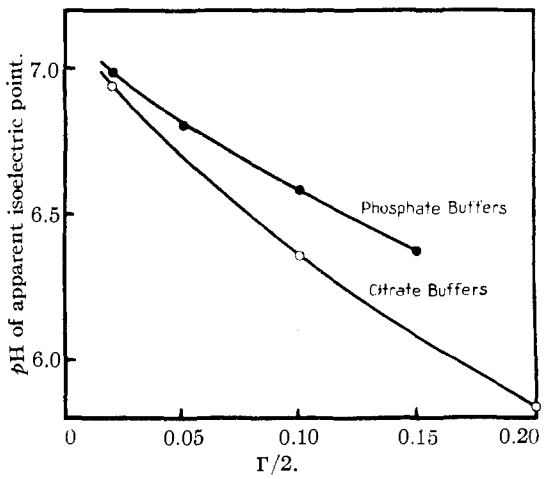

Fig. 4.

In order to analyze further the measurements of electrophoretic mobility that are reported, it seemed desirable first to consider them from the points of view that have been developed for ions of ordinary valence. Two generalizations may first be made: that the greater the valence, the greater the net charge and therefore the greater the mobility. On the other hand, the greater the valence the more the mobility will diminish from that which would obtain in infinitely dilute solution.

The mobility of an ion is given, following Onsager's theory, by an equation of the form

$$u = u_0 - (\alpha u_0 + \beta)\sqrt{\Gamma/2} \qquad (4)$$

in which u is the mobility at ionic strength $\Gamma/2$, u_3 the mobility at infinite dilution and α and β constants depending, respectively, on the relaxation and on the electrophoretic migration of the ionic atmosphere. The former effect is a complicated function of the valence and mobilities at infinite dilution of all ions present.

This equation, valid only in very dilute solution, has been extended by Shedlovsky to the case of more concentrated solutions[25–27] as follows

$$u = u_0 - (\alpha u_0 + \beta)\sqrt{\Gamma/2} + B(\Gamma/2) - \alpha B(\Gamma/2)^{3/2} \qquad (5)$$

The third term is small, in part because of the exponent of the ionic strength, in part because α is small in comparison with β (0.22 and 28.9, respectively, for a univalent ion at 25°). The empirical constant B for simple ions often has been found to be nearly equal to $(\alpha u_0 + \beta)$. Neglecting the third term in the above equation, we may write

$$u = u_0 - A'\sqrt{\Gamma/2} + B'(\Gamma/2) \qquad (6)$$

Values of u_0, A' and B' estimated from the observed mobilities by this equation show no significant variation with ionic strength, and are given in Table III. As in the case of simple ions the values of A' are close to those of B' and it is therefore possible to use for interpolation the still simpler equation for the influence of ionic strength

$$u = u_0 - A'(\sqrt{\Gamma/2} - \Gamma/2) \qquad (7)$$

TABLE III

CONSTANTS DEFINING THE MOBILITY OF CARBOXYHEMOGLOBIN IN PHOSPHATE AND CITRATE BUFFERS VARYING IN *p*H AND IONIC STRENGTH AT 25° IN TERMS OF EQUATIONS (6) AND (7)

	Three constant equation			Two constant equation	
*p*H	u_0	A'	B'	u_0	$A' = B'$
		Phosphate Buffers			
5.65	18.5	66.7	66.7	18.5	66.8
5.8	16.6	59.2	56.5	17.1	62.7
6.0	14.5	53.0	49.7	15.0	56.8
6.2	12.6	48.4	45.9	12.9	51.5
6.4	10.2	41.6	38.9	10.6	45.1
6.6	7.9	35.3	31.9	8.4	39.4
6.8	5.6	20.9	29.3	5.9	33.1
		Citrate Buffers			
5.8	9.57	34.7	30.4	10.37	41.2
6.0	8.54	32.4	27.5	9.37	39.4
6.2	7.38	29.3	23.7	8.34	37.3
6.4	6.14	26.1	20.0	7.18	34.8

For the studies in phosphate and citrate buffers, deviations in linearity of u_0 in *p*H over this *p*H range are scarcely greater than the experimental error. The straight lines drawn in Fig. 5 for phosphate and citrate, respectively, are given by the equations

$$u_0 - 4.0 = 11.0\ (7.00 - p\text{H}) \qquad (8)$$

$$u_0 - 4.0 = 5.5\ (7.00 - p\text{H}) \qquad (9)$$

It would, however, not be safe to estimate isoelectric points by extrapolation of these equations.

(24) The possibility that the change in mobility with ionic strength might partly be due to differences in heat production was tested in one experiment with high conductivity (acetate) by the introduction in series of a resistance which halved the potential drop, but which did not sensibly influence the mobility.

(25) T. Shedlovsky, THIS JOURNAL, **54**, 1405 (1932).

(26) D. A. MacInnes and T. Shedlovsky, *ibid.*, **54**, 1429 (1932).

(27) T. Shedlovsky and A. S. Brown, *ibid.*, **56**, 1066 (1934).

Indeed their use would imply a higher isoelectric point for hemoglobin in the presence of very small concentrations of citrate than of phosphate ions. Rather linearity would appear to depend on the almost linear combining capacity of carboxyhemoglobin for acid and base over this pH range, one of the reasons for the choice of these particular systems for this study. For the evidence would appear to be conclusive[28-30] that the changes in net charge of the carboxyhemoglobin molecule over this pH range is ascribable to the imidazole ring of histidine. There are 32 or 33 histidine residues in horse hemoglobin on the basis of a molecular weight of 66,800, of which 12 or more dissociate at reactions acid to the isoelectric point. We thus estimate a large number of imidazole groups dissociating over this pH range. The values of A' in Table IV vary only from 20 to approximately treble this value. Moreover, in the systems containing phosphate buffer A' as well as u_0 is linear in pH (Fig. 5) and is given over the range investigated by the relation

$$A' - 27.6 = 29.0\ (7.00 - p\mathrm{H}) \qquad (10)$$

whereas in the systems containing citrate buffer the relation appears more complicated.

Although the above equations are useful for interpolation, their significance is doubtful. If the valence be assumed to reach approximately 12 at the most acid pH investigated, on the basis of the titration curve of hemoglobin,[30] far higher values of u_0 and A' should be expected.

Moyer and Abramson[23] have employed an equation for the electrophoretic mobility of proteins of the form

$$u = u_0[1/(1 + \kappa r)] \qquad (11)$$

Taking the radius of hemoglobin as 27 Å., u_0 may be estimated by this equation as

$$u_0 = u(1 + 9\sqrt{\Gamma/2}) \qquad (12)$$

The values of u_0 calculated by means of our data and equation (12) vary with ionic strength. Thus for the four phosphate ionic strengths investigated at pH 5.65, this equation yields 23.37, 20.78, 15.22 and 12.15; whereas equation (7) yields 18.38, 18.51, 18.45 and 18.54. At other reactions also values of u_0 calculated by equation (7) are essentially independent of ionic strength, whereas those calculated by means of equation (12) show wide variations.

(28) B. German and J. Wyman, Jr., *J. Biol. Chem.*, **117**, 533 (1937).
(29) J. Wyman, Jr., *ibid.*, **127**, 1 (1939).
(30) E. J. Cohn, A. A. Green and M. H. Blanchard, THIS JOURNAL **59**, 509 (1937).

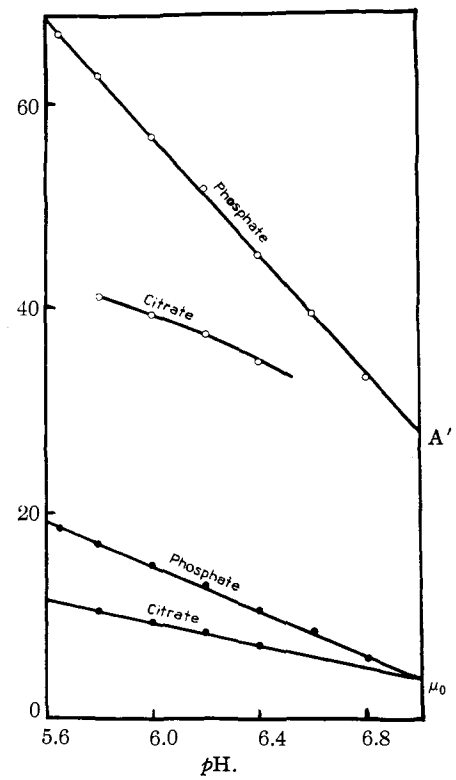

Fig. 5.—Analysis of results in terms of equation (7).

In the electrophoretic term of the Onsager equation, as generally employed, there is omitted a factor of $1/(1 + \kappa r)$, which would take into account as a first approximation the finite size of the ion[31] (p. 325). For dilute solutions of small ions κr is small in comparison with unity, but for proteins this is not the case. Including the above factor but omitting the relaxation term in α equation (4) becomes

$$u = u_0 - \beta\sqrt{\Gamma/2}/(1 + (r/3)\sqrt{\Gamma/2}) \qquad (13)$$

To use equation (13) for extrapolation to infinite dilution, we have again taken r for carboxyhemoglobin as 27 Å. Values of u_0 and β estimated by means of this equation for the phosphate and citrate systems investigated are recorded in Table IV. These values of u_0 are satisfactorily constant and independent of ionic strength at each pH. They are moreover appreciably greater than the estimates of u_0 derived from either equation (7) or (12).

Not only is u_0 more than half again as great as estimated by means of equation (13) but β is nearly five times as great as A' of equation (7). Whereas A' is approximately of the magnitude

(31) D. A. MacInnes, "The Principles of Electrochemistry," Reinhold Publishing Corporation, New York, 1939.

TABLE IV

CONSTANTS DEFINING THE MOBILITY OF CARBOXYHEMOGLOBIN IN PHOSPHATE AND CITRATE BUFFERS VARYING IN pH AND IONIC STRENGTH AT 25° IN TERMS OF EQUATION (13)

pH	u_0	β	β/u_0
	Phosphate Buffers		
5.65	30.78	325.4	10.57
5.8	28.84	308.6	10.71
6.0	25.71	280.3	10.91
6.2	22.62	253.4	11.21
6.4	19.17	222.7	11.62
6.6	15.83	194.5	12.29
6.8	12.11	163.1	13.47
	Citrate Buffers		
5.8	17.70	197.5	11.16
6.0	16.63	191.9	11.54
6.2	15.36	183.7	11.96
6.4	13.85	172.8	12.48

to be expected of a univalent or bivalent ion, the values of β from equation (13) approach those that might be expected for decavalent ions, and this, as we have seen, is approximately the mean valence of the hemoglobin at the greatest acidities studied.

Since the apparent isoelectric point is different for each solvent (Fig. 4), it seemed desirable to consider mobility at fixed pH intervals from that pH at which there was no mobility in a buffer of the same ionic strength. Applying the same equations to the measurements under these conditions yields the constants in Table V. The values of all the constants are smaller when computed in this way. Moreover, the ratios (A'/u_0) and (β/u_0) would appear to be more nearly independent of the nature of the buffer, as might be expected if both were largely dependent upon the net charge of the protein.

TABLE V

CONSTANTS DEFINING THE MOBILITY OF CARBOXYHEMOGLOBIN IN PHOSPHATE AND CITRATE BUFFERS AT CONSTANT pH INTERVALS FROM APPARENT ISOELECTRIC POINTS

	Equation (7)			Equation (13)		
δ pH	u_0	A'	A'/u_0	u_0	β	β/u_0
	Phosphate Buffers					
0.1	1.74	5.54	3.20	2.08	27.2	13.32
.2	3.30	10.49	3.20	5.28	51.8	9.82
.4	5.89	18.3	3.11	9.33	90.1	9.66
.6	8.43	26.1	3.10	13.37	128.7	9.63
.8	10.86	33.7	3.11	17.22	166.2	9.65
	Citrate Buffers					
0.1	1.13	3.57	3.16	1.39	12.22	8.78
.2	1.94	5.89	3.07	2.43	21.02	8.67

The quantity u_0 estimated from measurements in systems containing buffer may well have no significance for salt-free solutions. Indeed values of u_0 independent of the nature of the buffer are not quite realized on the basis of these measurements and of any of the equations considered. Whether this failure depends upon the form of these equations, upon the approximate method of estimating equal net charges for comparison or upon ion association between protein and buffer must await further investigation. The results do not preclude the latter interpretation, especially for buffers with polyvalent anions, and such association, if it occurs, must be taken into account as well as the electrostatic forces between buffer ions, protein ions and protein dipolar ions.

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Summary

1. The electrophoretic mobility of carboxyhemoglobin has been studied at 25° in phosphate and citrate buffers, varying in pH from 5.65 to 7.2, and in ionic strength from 0.02 to 0.20.

2. Over this range the influence of ionic strength upon mobility is of the same order as that of pH. Corrections for the influence of electrostatic forces both in diminishing mobility and in changing the isoelectric point are considered.

3. Whereas the computations attempted on the basis of these results do not preclude ion association, they yield constants for the effect of the buffers at constant pH, and at constant pH intervals from the isoelectric condition.

4. Carboxyhemoglobin has a net charge due to the dissociation of twelve or more imidazole groups at the most acid reactions investigated.

5. Not only mobility but diminution in mobility due to electrostatic forces is a function of the net charge, and therefore increases with distance from the isoelectric condition.

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