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

The Amphoteric Properties of Certain Globulin Fractions of Normal Horse Serum[1]

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Globulins are classified as being insoluble in water but soluble in neutral salt solutions. They are also soluble when combined with acids and bases. Combined with acids they bear a positive charge and migrate to the negative pole in the electric field; combined with bases they bear a negative charge and migrate to the positive pole. The minimum solubility of the globulins has therefore been associated with their isoelectric state and "practice has consisted in reducing this range by the removal of salt, and then either in noting the maximum precipitation of the globulin, or the limiting hydrogen ion concentrations at which migration occurred in an electric field."[2]

In the presence of a second protein with a slightly different isoelectric point the zone of precipitation is, however, "usually widened, and the point of maximum precipitation shifted in the direction of the isoelectric point of the second protein. The magnitude of the shift, and therefore of the error, depends upon the difference in the isoelectric points of the two proteins, upon their relative concentrations, and upon their relative solubilities."[2]

The presence of more than one globulin in serum has generally been postulated. Thus in a solution of serum diluted tenfold with water and precipitated by the addition of acid a zone of maximum precipitation occurs between *p*H 5 and 6. Not all of the globulin is, however, so precipitated and the fraction that is so separated has generally been called the euglobulin fraction.[3,4]

Separation of Proteins by Salt Precipitation.—Most proteins are precipitated by sufficiently high concentrations of neutral salts, especially of sulfates. The euglobulin fraction of serum is largely precipitated by one-third saturated ammonium sulfate and the rest of the globulin, sometimes termed pseudoglobulin, by one-half saturated ammonium sulfate. It follows from the theory of salting out that the logarithm of the solubility is proportional to the ionic strength according to the relation[5] (p. 413)

$$\log S = \beta - K_s m$$

where S is the protein solubility, m the concentration of the salt per liter, β a constant characteristic of the protein, and K_s the salting out constant.[5,6] Separation by repeated precipitation with a neutral salt is, however, satisfactory only if the salting out constants of the respective proteins are sufficiently far apart.

Overlapping of salting out curves has been shown for serum in sodium sulfate by Howe[7] and in phosphate buffers by Butler.[8] Thus this method alone is inadequate for the separation of the serum globulins.

Separation of Proteins by Isoelectric Precipitation.—The isoelectric point of serum globulin was considered to be *p*H 5.5 by early investigators[9] and to coincide approximately with the point of maximum precipitation in the absence of salt. More recently Felton[10] obtained two fractions of serum globulin from anti-pneumococcus horse serum by precipitation at different *p*H values. One of these, which he called euglobulin, was precipitated at about *p*H 5 and was less soluble in dilute salt solution than the other which was precipitated near *p*H 6.8 and contained the antibodies. Working with both normal and immune horse serum, Reiner and Reiner[11] separated water insoluble serum globulin into two fractions, one with a zone of maximum precipitation at *p*H 5 and one above *p*H 6, and suggested that these fractions have different

(1) This study was supported in part by a grant from the Commonwealth Fund of New York. A preliminary paper on this work was presented to the Thirty-First Annual Meeting of the American Society of Biological Chemists [A. A. Green, *Proc. J. Biol. Chem.*, **119**, XXXIX (1937)].

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(2) E. J. Cohn, *J. Gen. Physiol.*, **4**, 697 (1922).

(3) H. Chick, *Biochem. J.*, **8**, 261 (1914).

(4) W. B. Hardy, *J. Physiol.*, **33**, 251 (1905); W. Kühne, "Lehrbuch der physiol. Chem.," Leipzig, 1868, pp. 168, 174; J. Mellanby, *J. Physiol.*, **33**, 338 (1905); A. Schmidt, *Arch. (Anat.) Physiol.*, 428 (1862).

(5) E. J. Cohn, *Physiol. Rev.*, **5**, 349 (1925).

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

(7) P. E. Howe, *ibid.*, **49**, 93 (1921).

(8) A. M. Butler and H. Montgomery, *ibid.*, **99**, 173 (1932); A. M. Butler, H. Blatt and H. Southgate, *ibid.*, **109**, 755 (1935).

(9) H. Chick, *Biochem. J.*, **7**, 318 (1913); L. Michaelis and P.5 Rona, *Biochem. Z.*, **28**, 193 (1910).

(10) L. E. Felton, *J. Infectious Diseases*, **42**, 248 (1928); **48**, 543 (1928).

(11) H. K. Reiner and L. Reiner, *J. Biol. Chem.*, **95**, 34 (1932).

nitrogen contents and different amphoteric properties.

Our studies upon placental extract[12] indicated the presence of at least two euglobulins other than the more readily salt precipitable tissue globulin which is a blood coagulant[13] and suggested the need of further study of the globulin from normal and immune horse serum.

Preparation of Globulin Fractions.—The essential characteristic of the method of separation of serum globulins here employed is isoelectric precipitation in a very low concentration of salt. The respective isoelectric points are close together and in order to effect a separation, precipitations must be performed repeatedly to obtain a product even relatively pure.

The most soluble "water insoluble" globulin from horse serum which is precipitated only in almost salt-free solution at an isoelectric point of *p*H 5 we have designated PI (1). PII has an isoelectric point of about *p*H 6.2 and is relatively soluble. PIII has an isoelectric point of about *p*H 5 and is so insoluble that it often precipitates with PII.

The preparations that have been studied were made by dialyzing horse serum[14] or the proteins precipitated from it in solutions one-third or one-half saturated with respect to ammonium sulfate against water in cellophane tubes until relatively salt free. The reaction was maintained close to neutrality and finally cautiously adjusted with dilute hydrochloric acid with stirring until a precipitate formed, usually at a *p*H of about 6.5, *i. e.*, blue-green to brom thymol blue when a drop or two of solution was placed in a cc. of distilled water. The solution was allowed to stand overnight in a refrigerator. The precipitate containing both PII and PIII was removed by centrifugation. To the solution more dilute hydrochloric acid was added until the *p*H was close to 5 (faint orange to chlor phenol red) and PI precipitated. Dilution with water or further dialysis brought down a further quantity of PI.

PII and PIII were dissolved in acid and separated by precipitation with alkali. When sufficient alkali was added to the acid solution to bring it to *p*H 5, PIII precipitated. The subsequent addition of more alkali to *p*H 6.2, precipitated PII.

(12) C. F. McKhann, A. A. Green, L. E. Eckles and J. A. V. Davies, *Ann. Internal Med.*, **9**, 388 (1935).

(13) R. C. Eley, A. A. Green and C. F. McKhann, *J. Ped.*, **8**, 135 (1936).

(14) Obtained from the Massachusetts Antitoxin Laboratory through the courtesy of its Director, Dr. Elliott S. Robinson.

The isoelectric points are thus sufficiently close together so that curves defining solubility as a function of *p*H overlap. A schematic representation is given in Fig. 1. Thus, complete separation can be effected only by approaching the more alkaline isoelectric point from the alkaline side and conversely by precipitating the proteins with the more acid isoelectric points by adding alkali to them in acid solution. At least three or four solutions in alkali or acid and isoelectric reprecipitations were carried out before the fractions were even approximately homogeneous.

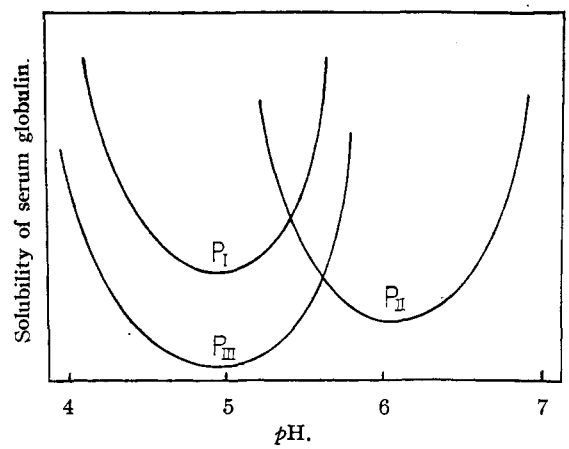

Fig. 1.

Higher yields may be obtained by reprecipitating proteins left in solution at various points in the procedure with one-half saturated ammonium sulfate and repeating the whole process of dialysis and separation.

Our criterion for adequate separation was to test the solution from which a given fraction had been precipitated, or with which it had been washed, for the presence of one of the other protein fractions by adjusting the *p*H to its isoelectric point.

Amphoteric Properties of Serum Globulins.—In order to determine the amphoteric properties of the various purified fractions, the isoelectric precipitates were dissolved in a minimum amount of alkali and diluted to a known volume. To aliquot portions of these solutions were added varying quantities of sodium hydroxide or hydrochloric acid and sufficient sodium chloride solution to make the final concentration of electrolyte 0.15 *N*.

E. m. f. measurements on these solutions were made and calculations of acid and base combined carried out precisely as in a recent paper on

carboxyhemoglobin.[15] E_0 for the 0.1 N calomel half cell was taken as 0.3353 at 25° and the pH of 0.1 N hydrochloric acid as 1.076. Apparent activity coefficients of the hydrogen or hydroxyl ions at total ionic strength 0.15 were determined under the same conditions used in the titration of the protein. No corrections were made for liquid junctions. The conditions under which the apparent activity coefficients were determined, and have been employed, differ, however, only by the small amount of protein present in the latter systems (Table I). In acid solutions, interpolated values of $p\gamma$ were employed. In alkaline solutions, the value of $p\gamma$ has been taken as 0.11 regardless as to whether the ions present were those of sodium chloride or of sodium hydroxide.

TABLE I

ACTIVITY COEFFICENTS OF HYDROCHLORIC ACID AND SODIUM HYDROXIDE IN THE PRESENCE OF SODIUM CHLORIDE

Total ionic strength, 0.15

Concn. acid or base	Concn. NaCl	pH HCl	$P\gamma$	pOH NaOH	$P\gamma$
0.01	0.14	2.046	0.046	2.114	0.114
.05	.1	1.356	.055	1.410	.109
.1	.05	1.070	.070	1.117	.117
.15	0	0.905	.081	0.947	.125

Concentration of protein in the system was determined by prolonged heat coagulation in a boiling water-bath at the pH of the isoelectric point in the presence of small quantities of sodium chloride. The coagulum was washed with boiling water on a weighed sintered glass filter and dried to constant weight at 110°. The titration curves reported are based on four preparations of PI, three of PII and two of PIII.

Acid Combining Capacities of Serum Globulins.—Concentration of acid or base is given in the first column and of protein in the second column of Tables II and III. The preparation number of each of the globulin fractions studied is designated by the superscript. It will be noted that the acid combining capacity of each fraction was the same at any pH for each of the preparations of both PI and PII. As in the previous study,[15] combination of acid was, moreover, essentially complete by pH 2. At that pH the acid bound was in excess of the free acid. Although satisfactory measurements were made up to pH 1.6 where the concentration of hydrogen ions was two and one-half times as great as the acid bound, there was but very small change in

(15) E. J. Cohn, A. A. Green and M. H. Blanchard, THIS JOURNAL, **59**, 509 (1937).

TABLE II

ELECTROMOTIVE FORCE MEASUREMENTS ON SYSTEMS CONTAINING HYDROGEN CHLORIDE AND HORSE SERUM GLOBULINS AT TOTAL IONIC STRENGTH OF 0.15

HCl concn., moles/liter	Protein concn., g./liter	Log $1/a_{H^+}$ pH	HCl uncombined moles/liter	HCl combined moles/liter	HCl combined moles $\times 10^5$ per g. prot.
Measurements on Serum Globulin PI					
0.02112	11.5[2]	2.048	0.01000	0.01112	95.9
		2.047	.01002	.01110	95.7
.03046	19.2[4]	1.966	.01217	.01829	95.3
		1.968	.01222	.01822	94.9
.03511	15.4[3]	1.745	.02028	.01483	96.3
		1.760	.01959	.01552	100.7
.03640	11.2[1]	1.637	.02606	.01034	91.8
		1.641	.02582	.01058	94.7
.03712	11.5[2]	1.645	.02553	.01159	100.7
		1.641	.02576	.01136	98.7
.03844	19.2[4]	1.760	.01963	.01881	98.0
		1.756	.01982	.01862	96.9
.04641	19.2[4]	1.612	.02773	.01868	97.3
		1.614	.02761	.01881	98.0
.04955	15.4[3]	1.513	.03491	.01464	95.0
		1.517	.03459	.01496	97.2
Measurements on Serum Globulin PII					
0.03251	20.2[2]	1.951	0.01259	0.01992	98.7
		1.960	.01233	.02018	100.0
.03094	16.9[3]	1.899	.01416	.01678	99.2
		1.900	.01413	.01681	98.6
.02960	11.6[1]	1.769	.01910	.01050	90.5
		1.771	.01901	.01059	91.3
.04049	20.2[2]	1.747	.02028	.02021	100.0
		1.758	.01972	.02077	102.6
.03892	16.9[3]	1.714	.02183	.01709	101.5
		1.706	.02223	.01669	98.8
.03760	11.6[1]	1.633	.02624	.01136	97.8
		1.627	.02661	.01099	94.7
.04846	20.2[2]	1.607	.02812	.02035	100.6
		1.608	.02805	.02042	101.0
.04690	16.9[3]	1.577	.03006	.01684	99.6
		1.565	.03090	.01600	94.7
.05760	11.6[1]	1.388	.04677	.01083	93.2
		1.389	.04667	.01093	94.2
Measurements on Serum Globulin PIII					
0.02160	13.44[1]	2.041	0.01016	0.01144	85.1
		2.040	.01019	.01141	85.0
.02160	12.30[2]	1.990	.01143	.01017	82.7
		1.997	.01125	.01035	84.2
.02960	13.44[1]	1.810	.01738	.01222	90.9
		1.794	.01803	.01157	86.2
.02960	12.30[2]	1.771	.01901	.01059	86.2
		1.782	.01858	.01102	89.7
.03760	13.44[1]	1.643	.02570	.01180	86.2
		1.634	.02594	.01166	87.8
.03760	12.30[2]	1.626	.02673	.01087	88.5
		1.627	.02667	.01093	88.9

combined acid over this range. The measurements reported in Table II yield average acid combining capacities of 97 and 98 $\times 10^{-5}$ moles per gram of protein for PI and PII, respectively, over this range.

TABLE III

ELECTROMOTIVE FORCE MEASUREMENTS ON SYSTEMS CONTAINING SODIUM HYDROXIDE AND HORSE SERUM GLOBULINS AT TOTAL IONIC STRENGTH OF 0.15

NaOH concn., moles/liter	Protein concn., g./liter	Log $1/a_{H^+}$ pH 25°	NaOH uncombined moles/liter	NaOH combined moles/liter	NaOH combined moles $\times 10^5$ per g. prot.
		Measurements on Serum Globulin PI			
0.02240	7.5[1]	11.968	0.01528	0.00712	95.2
		11.958	.01489	.00751	100.5
.02688	11.5[2]	11.982	.01578	.01110	96.5
		11.972	.01545	.01143	99.4
.03324	19.2[4]	11.947	.01422	.01902	99.0
		11.938	.01400	.01924	100.2
.04119	19.2[4]	12.127	.02203	.01916	99.8
		12.104	.02163	.01956	102.0
.04360	11.2[1]	12.292	.03221	.01139	101.5
.04914	19.2[4]	12.254	.02972	.01944	101.2
		12.259	.02985	.01929	100.5
		Measurements on Serum Globulin PII			
0.03041	20.2[2]	11.944	0.01439	0.01602	79.3
		11.938	.01419	.01622	80.2
.02640	11.6[1]	12.033	.01774	.00866	74.4
		12.023	.01734	.00906	78.0
.03326	16.9[3]	12.059	.01884	.01442	85.2
		12.054	.01862	.01464	86.6
.03440	11.6[1]	12.192	.02559	.00881	75.9
		12.181	.02529	.00911	78.5
.04121	16.9[3]	12.200	.02612	.01509	89.2
		12.200	.02612	.01509	89.2
.04240	11.6[1]	12.316	.03404	.00834	71.8
		12.310	.03342	.00898	77.4
.05027	20.2[2]	12.324	.03451	.01576	78.0
		12.317	.03350	.01677	84.0
.04917	16.9[3]	12.345	.03639	.01278	75.6
		12.327	.03491	.01426	85.4
.05820	20.2[2]	12.414	.04246	.01574	77.9
.05689	16.9[3]	12.423	.04355	.01334	78.9
		12.410	.04236	.01453	85.9
.06616	20.2[2]	12.494	.05105	.01511	74.8
		Measurements on Serum Globulin PIII			
0.02640	13.44[1]	11.929	0.01390	0.01250	93.1
		11.919	.01355	.01285	95.6
.02644	12.30[2]	11.986	.01592	.01052	85.5
		12.006	.01663	.00981	79.6
.03046	13.44[1]	12.048	.01828	.01218	90.6
		12.030	.01758	.01288	95.6
.03440	13.44[1]	12.133	.02223	.01217	90.6
		12.111	.02113	.01327	98.7
.03446	12.30[2]	12.176	.02460	.00986	80.0
		12.171	.02432	.01014	82.6
.04248	13.44[1]	12.274	.03076	.01172	86.5
		12.257	.02958	.01292	96.0

The acid combining capacity of PIII appears to be appreciably smaller and may be taken as 87 $\times 10^{-5}$ mole per gram over this range, which is in good agreement with the value calculated[5] (p. 367) from Hitchcock's[16] early study of ox serum globulin.

Base Combining Capacities of Serum Globulins.—No increase in base combining capacity has been detected from pH 11.9 to 12.3 for either PI, PII or PIII although the concentration of hydroxyl ions doubled over this range. The average base combined by PI up to these alkalinities was 100 $\times 10^{-5}$ mole per gram of protein. Although it is possible that more base is bound at more alkaline reactions, the measurements that have thus far been made indicate that for PI acid and base combining capacity are essentially equal. Whereas the acid combining capacity of PI and of PII have been found to be identical (Table III); the base combined by PII appears to be somewhat smaller than by PI and to be close to 80 $\times 10^{-5}$ mole per gram of protein at the alkalinities studied. Certain of the measurements on one of the preparations reported show base combining capacities at this reaction somewhat greater than 80 but it is our impression that the lower values are more reliable.

The base combining capacity of PIII is smaller than that of PI but greater than that of PII at the same alkalinities, and is tentatively taken as 90 $\times 10^{-5}$ mole per gram. These results in round numbers may be summarized as shown in Table IV.

TABLE IV

Serum globulins	PI	PII	PIII
Acid combining capacity	100	100	90
Base combining capacity	100	80	90
Excess basic groups	0	20	0
Possible dipole groups	100	80	90
Total combining groups	200	180	180

The acid and base combining capacities of these serum globulins may now be considered from various points of view. Weber[17] has determined acid and base combining capacities of the total serum globulin of horse and reports[17] (p. 34) values of 122 and 115 at pH 12.56 and 12.24, respectively, and values of 82 and 89 at pH 1.20 and 1.62, respectively, in the presence of 0.9% sodium chloride. The sum of the total acid and base combining capacities which they report thus

(16) D. I. Hitchcock, *J. Gen. Physiol.*, **5**, 383 (1923).
(17) H. H. Weber, *Biochem. Z.*, **218**, 1 (1930).

averages 204 × 10^{-5} mole per gram on the basis of a nitrogen factor of 6.3 (% N 15.85). Other measurements on serum proteins that have been reported are in less satisfactory agreement.[18] Reiner and Reiner[11] using an indicator method of estimating total combining capacity, give the value for their acid insoluble fraction as varying from 179 to 232 mole × 10^{-5} per gram. The range is therefore comparable to that now reported. Although the acid combining capacity of their neutral proteins was apparently in the same range as that of their acid proteins, base combining capacity and therefore total combining capacity was smaller. One of their acid proteins (no. 4) had an acid combining capacity close to that reported above for P_3 whereas that of all their other acid proteins was higher.

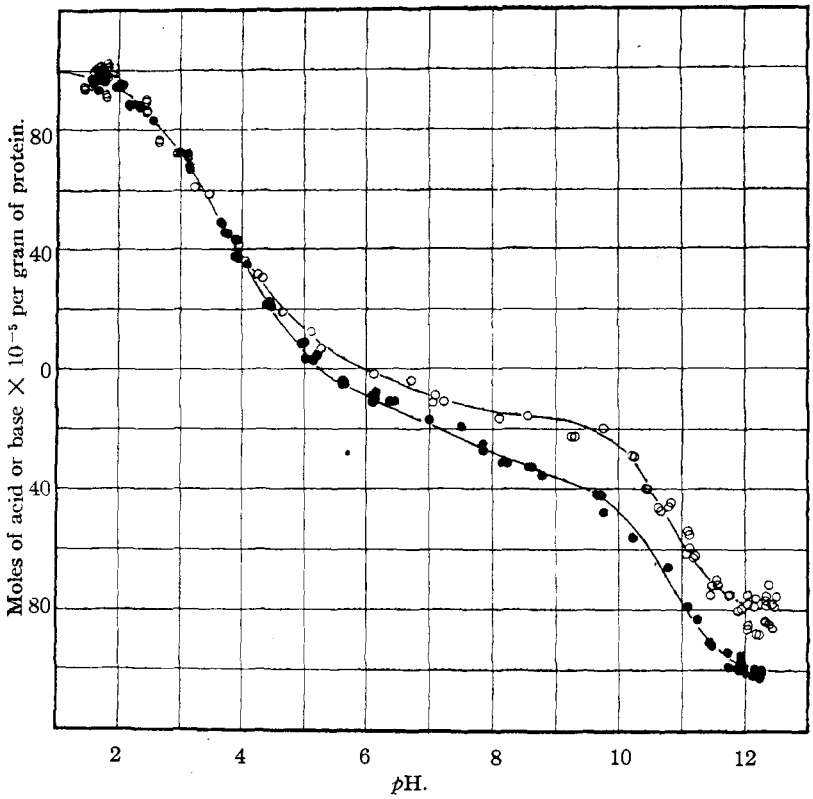

Fig. 2.—Titration curve of serum globulins PI, ●, and PII, ○, in the presence of sodium chloride, total ionic strength 0.15.

In previous studies it has been shown that acid combining capacity depends upon the histidine, arginine and lysine residues in the protein molecule.[5,15] Hartley[19] has studied the total globulin as well as variously prepared euglobulin and pseudoglobulin fractions of horse serum. Cohn[15] calculated certain of the results in Hartley's paper using the nitrogen factor 15.85 on which basis the results in Table V are computed.

TABLE V

Moles × 10^6 per gram protein

	Total globulin	Pseudo-globulin	Euglobulin Panum's method	Euglobulin $(NH_4)_2SO_4$ method
Histidine	18.1	21.8	19.6	20.5
Arginine	22.6	25.2	26.6	23.3
Lysine	61.0	55.4	57.0	56.2
Total	101.7	102.4	101.2	100.0

Preliminary results seem to indicate a somewhat lower nitrogen factor, in which case the values computed would be correspondingly lower. The methods of analysis for these bases is, however, not to be regarded as yielding strictly quantitative results. Their sum, in the Van Slyke method, is, however, more accurate than the estimate of their distributions. In any case, as in other proteins that have been studied adequately, the sum of the free groups of histidine, arginine and lysine would appear adequate to account for acid combining capacity.

The physico-chemical data reported indicate that the acid combining capacities of PI and PII are identical and equal to the residues of basic amino acids revealed by Hartley's analysis whereas the acid combining capacity of PIII is somewhat smaller. The difference between PI and PII would thus appear to depend not on the number of basic groups in these molecules but on the number of free acid groups. PII has a smaller number of acid groups than PI and, consistent with this, a more nearly neutral isoelectric point. Whereas PI and PII appear to have closely the same number of free basic groups, PII and PIII appear to have a smaller number of free acid groups. Whereas PII has an excess of basic groups over acid groups and a nearly neutral isoelectric point near *p*H 6, for both PI and PIII the number of free acid groups is approximately

(18) W. Pauli and E. Valkó, "Kolloidchemie der Eiweisskörper," Verlag Theodor Steinkopff, Dresden, Germany, 1933, p. 43.

(19) P. Hartley, *Biochem. J.*, **8**, 541 (1914).

equal to the basic groups and the apparent isoelectric points of these two proteins at the ionic strengths studied are according to our measurements approximately 5.0 for PIII and 5.2 for PI.

Further consequences of these results are that (1) the total combining capacities, and (2) the possible number of dipole pairs contributing to the electric moments of these proteins cannot be greater than 80×10^{-5} mole per gram protein for PII and 90×10^{-5} mole per gram for PIII, whereas it would be possible for all the groups in PI, or 100×10^{-5} mole per gram, to contribute to the multipolar moment of these proteins. It is perhaps for this reason that PI is more soluble than PII or PIII.

Dissociation Constants of Serum Globulins.—The titration curves of the serum globulins that have been studied are reported in Figs. 2 and 3. It should be noted that not only the maximal acid combining capacity but the titration curve over the most acid range is identical for both PI and PII (Fig. 2). The segment of the titration curves between *p*H 9.5 and 12 also appears to be the same. The difference between these two curves is thus between *p*H 4 and 9.5. Assuming that the base combining capacity gives the total number of acid groups (though measurements at still more alkaline reactions might reveal other acid groups) the titration curves in Fig. 2 are constructed on the assumption that twenty less acid groups are dissociating in the neighborhood of *p*H 4 in the case of PII than of PI. At all other reactions comparable numbers of groups are considered to be present in PI and PII though the ranges in which they are dissociating have been shifted somewhat in order that the calculated curve may more closely describe the experimental measurements.

The titration curve for serum globulin PIII, Fig. 3, has been constructed on the assumption that the number of acid groups dissociating in the neighborhood of *p*H 2.9 is identical to that of PI and PII and that the number of groups dissociating near *p*H 4 is intermediate between PI and PII. The other significant difference occurs at the most alkaline reactions studied where ten fewer basic groups are assumed to be dissociating.

Thus the smaller the base combining capacity, the smaller the number of groups dissociating near *p*H 4; conversely, the smaller the acid combining capacity, the smaller the number of groups assumed to be dissociating in the neighborhood of *p*H 10.5. This is the range in which free amino groups situated far from other groups may be expected to dissociate and is characteristic of the ε-amino group of lysine. The hydroxy group of tyrosine present, to the extent of 37×10^{-5} mole per gram in cow serum globulin,[20] might also be expected to dissociate in this range. The histidine groups might be expected to dissociate at more nearly neutral and arginine groups at more alkaline reactions. The number of groups and the negative logarithms of the apparent dissociation constants at ionic strength 0.15 that have been tentatively assumed are reported in the accompanying table.

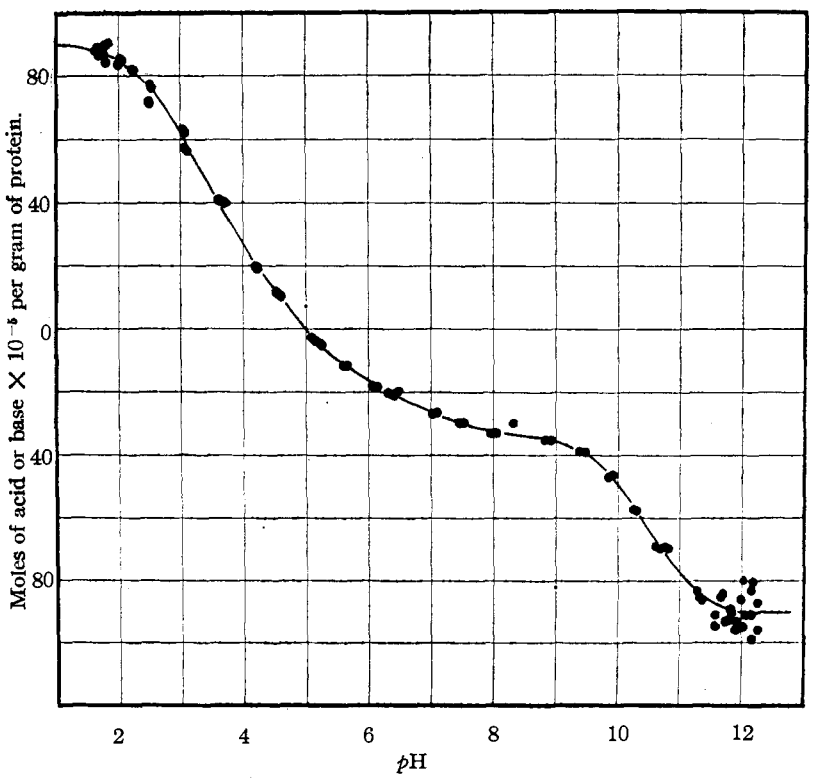

Fig. 3.—Titration curve of serum globulin PIII in the presence of sodium chloride, total ionic strength 0.15.

(20) O. Folin and J. M. Looney, *J. Biol. Chem.*, **51**, 421 (1922).

TABLE VI

PI		PII		PIII	
pK'	Moles $\times 10^5$	pK'	Moles $\times 10^5$	pK'	Moles $\times 10^5$
2.9	50	2.9	50	2.9	50
4.2	50	4.0	30	4.1	40
6.0	20	5.1	20	5.5	20
8.0	15	7.0	15	7.2	15
10.7	65	10.7	65	10.5	55

The method of construction of titration curves from these constants is that previously employed.[15] The underlying assumption on the basis of which this analysis has been made is that the number of acid groups revealed by base combining capacity are dissociating at acid reactions. Whereas it is possible that the shifts in the various groups that have been assumed are due to the presence of larger numbers of charged groups on the molecule of PI than on PII, the analysis of the constants is somewhat arbitrary and is reported as a convenient method of describing and analyzing the accumulated data.

Discussion.—The presence of more than one serum globulin has been suspected on various grounds, some chemical and some immunological.[21] Most recent studies suggest that both the euglobulin and pseudoglobulin fractions of serum have the same molecular weight estimated to be 175,000 on the basis of osmotic pressure[22] and 150,000 on the basis of ultracentrifugal[23] measurements. The ultracentrifuge also reveals the presence of a small fraction of higher molecular weight. With the exception of this small fraction, one must therefore look for some difference other than the size of these molecules to account for their specific chemical and immunological properties; the latter to be discussed in another place.

The observation that serum globulins could be separated from each other by precipitation at their isoelectric points led to the present analysis of their amphoteric properties. The isoelectric point of the nearly neutral serum globulin, PII,[1,11,12] is related to the excess of basic over acid groups in the case of this protein. Its separation by isoelectric precipitation rendered possible the isolation first of one globulin, PI,[1] and then of a second, PIII, both with isoelectric points close to pH 5, but differing from each other by the total number of groups and therefore presumably in the electric moments of the isoelectric molecules.

A beautiful study of the amphoteric properties of the serum globulins over the range from pH 5 to 8 has been completed recently by Tiselius[24] using an improved method for the study of electrophoretic mobility. The results of this investigation indicate the presence of two globulin fractions (α and β in the notation of Tiselius) with isoelectric points at 5.06 and 5.12, respectively, and one fraction ($\gamma + \delta$) with an isoelectric point at pH 6. The isoelectric points of these proteins, though determined in the presence of different electrolytes than used in this study, suggest that certain of them are the globulins that have been separated by isoelectric precipitation.

The evidence from these two quite distinct and supplemental experimental techniques, electrophoretic mobility and electromotive force titration curves, would appear to offer an explanation of the differences between the serum globulins in terms of differences in the number and the apparent dissociation constants of the free groups of these closely related proteins.

Summary

1. Three distinct serum globulins have been separated from each other by the method of isoelectric precipitation.

2. Two of the serum globulins, PI and PII, have the same acid combining capacity of approximately 100×10^{-5} mole per gram whereas the third, PIII, has a somewhat lower acid combining capacity.

3. The base combining capacity of PI—up to pH 12.3—is equal to its acid combining capacity. The acid combining capacity and the base combining capacity of PIII—up to pH 12.3—are also identical and close to 90×10^{-5} mole per gram. Both PI and PIII have isoelectric points near pH 5. The protein with the larger number of dissociating groups, PI, is much the more soluble.

4. The acid combining capacity, and the number of free basic groups, is greater in the case

(21) S. P. L. Sørensen, THIS JOURNAL, **47**, 457 (1925); *Compt. rend. trav. Lab. Carlsberg*, **15**, 11 (1925); T. Svedberg and Sjögren, THIS JOURNAL, **52**, 2855 (1930); A. McFarlane, *Biochem. J.*, **29**, 407, 660, 1175, 1209 (1935); T. Harris and H. Eagle, *J. Gen. Physiol.*, **19**, 383 (1935); F. E. Kendall, *J. Clin. Investigation*, **16**, 921 (1937).

(22) G. S. Adair and M. E. Robinson, *Biochem. J.*, **24**, 1864 (1930); N. F. Burk, *J. Biol. Chem.*, **121**, 373 (1937).

(23) P. Mutzenbecher, *Biochem. Z.*, **266**, 250 (1933); P. Mutzenbecher and T. Svedberg, *Naturwissenschaften*, **21**, 331 (1933); T. Svedberg, *Chemical Reviews*, **20**, 81 (1937).

(24) A. Tiselius, *Biochem. J.*, **31**, 313, 1464 (1937); *Trans. Faraday Soc.*, **33**, 524 (1937).

of PII than is its base combining capacity. Possessed of an excess of basic over acid groups, PII has a more nearly neutral isoelectric point than the other serum globulins studied.

5. The titration curves of these proteins from *p*H 1.6 to 12.3 are reported and analyzed in terms of apparent dissociation constants.

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Studies of the Dielectric Properties of Protein Solutions. I. Carboxyhemoglobin[1,2]

BY J. L. ONCLEY

Many methods have been employed in determining the nature and structure of colloidal materials. As a first approximation the colloidal particle generally has been considered a sphere of given radius and density. Further advances have involved consideration of models of lower degrees of geometrical symmetry. At the present time there are several methods which may be employed for determining accurately the size, or size distribution, of colloidal particles or of molecules of high molecular weight, and which give consistent results for many systems. There are also methods for the study of the shapes of molecules. The next step in our attempt to understand substances of colloidal dimensions is the determination of their electrical symmetry. Such investigations upon substances of low molecular weight have revealed much concerning their structure.[3,4] The importance of the configuration of electrical charges in the interpretation of a variety of physical chemical properties has been considered among others by Scatchard, Kirkwood and Cohn.[5,6]

In the present communication the theory of dielectrics in polar solution is developed briefly, the bridge method that has been adopted is described, and an empirical method is given for correcting dielectric values at low frequency for polarization effects. Studies upon carboxyhemoglobin are reported which supplement other studies of this well-known crystalline protein from the laboratory, and demonstrate the methods of characterizing proteins by their dielectric properties. Comparable studies upon other proteins will be reported subsequently.

I. Theory

The electrical symmetry of molecules may be studied by measuring the dielectric constants of their solutions. The dielectric constant, ϵ, of any polar liquid or solution can be interpreted as being almost entirely a measure of the number of molecules oriented by an external electrical field of unit strength. These molecules are oriented by a torque depending on the field strength and the dipole moment μ, a constant for each molecular species. Orientation is hindered by the frictional forces in the solution depending on the frequency ν, and a constant τ, designated as the "relaxation time."[7] Thus we find that the number of molecules oriented at unit field strength will decrease in the frequency region where the hindering frictional forces and the orienting torque become of the same order of magnitude. At lower frequencies the orienting torque is sufficient to overcome completely the resisting forces, and we have a high dielectric constant, ϵ_0.[8] At very high frequencies the resisting forces completely overcome the orienting torque and we again have a constant but low value of ϵ.

If we now consider a binary mixture of two polar molecules (dipoles), we find a more complicated behavior. Figure 1 represents the typical

(1) A preliminary report of this investigation was presented to the Fourteenth Colloid Symposium, held at Minneapolis, Minn., June 10–12, 1937.

(2) This investigation has been supported in part by grants from the Committee of the Permanent Charity Fund, Inc., and from the Farnsworth Fund, Harvard Medical School.

(3) Debye, "Polar Molecules," Chemical Catalog Co., New York, N. Y., 1929.

(4) Smyth, "Dielectric Constant and Molecular Structure," Chemical Catalog Co., New York, N. Y., 1931.

(5) Cohn, "Annual Review of Biochemistry," Vol. IV, 1935, p. 122.

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

(7) Other forces might be important in certain cases, but for large molecules in low viscosity solvents this effect has been shown to be the most important. The "relaxation time" is defined as the time required for $1/e$ of the molecules to become randomly distributed if they were completely oriented by a field, and then released at $t = 0$.

(8) Some workers use the term "specific inductive capacity" so that the term variable dielectric "constant" may be avoided. The phrase "specific inductive capacity" has an unfortunate connotation derived from induced dipoles, whereas the molecules with which we are concerned are permanent dipoles.