STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

VII. THE SOLUBILITY OF FIBRINOGEN IN CONCENTRATED SALT SOLUTIONS.

By MARCEL FLORKIN.*

(*From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.*)

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In 1859, Denis (8) observed the appearance of a gelatinous precipitate which he called plasmine in blood saturated with respect to sodium chloride.[1] Plasmine was studied by Leon Fredericq (11) in 1877. He noted that upon heat coagulation, proteins separated at two different temperatures and, therefore, suggested that plasmine consisted of two proteins, one of them the postulated protein in the blood concerned with its coagulation and called fibrinogen by Virchow. In 1879 Hammarsten (14) precipitated one of these proteins from blood plasma by half saturating it with respect to sodium chloride. This method has ever since been the classical procedure in the preparation of fibrinogen.

Fibrinogen has generally been considered insoluble in water, but soluble in dilute salt solutions and, therefore, to belong to that class of substances, the globulins, which were first described by Denis (8) in the same monograph in which he described plasmine. The contention of De Waele (9) that fibrinogen is largely soluble in water involves both the characterization of this protein and the concept of solubility, and these we shall consider in terms of the measurements that are reported.

The early observations upon the temperature of heat coagula-

* Fellow of the Commission for Relief in Belgium; Educational Foundation.

[1] The blood was rendered incoagulable by the addition of one-sixth of its volume of saturated ammonium sulfate.

tion have since been supplemented, but in this protein, as in others, the coagulation temperature varies with the concentration and the nature of the solvent. A method of characterization which has generally proved satisfactory is the determination of the isoelectric point. Various investigators have, however, reported values for the isoelectric point of this globulin, varying from pH 4.4 to 8. In Table I are collected their observations, together with the methods that have been employed in the attempts to measure the isoelectric point of fibrinogen.

TABLE I.

Measurements of the Isoelectric Point of Fibrinogen.

Author.	Year.	Method employed	Isoelectric pH.
Resch (23).	1917	Cataphoresis in phosphate buffer.	5.0–8.0
Funck (12).	1921	Maximal flocculation in acetate buffer.	4.4–5.0
Stuber and Funck (28).	1921	Cataphoresis in acetate buffer.	5.0
Kugelmass (17).	1923	Maximal turbidity.	8.0
Wöhlish (30).	1924	" flocculation in acetate buffer.	4.9
Rabinovich (22).	1925	Maximal opalescence.	5.9–6.2
Quagliariello (21).	1926	" flocculation in acetate buffer.	4.9
De Waele (9).	1927	Cataphoresis and maximal precipitation by alcohol.	6.9
Wöhlish and Schloss (31).	1927	Maximal flocculation in NaCl.	4.46
Nordbö (19).	1927	Titration curve.	5.50

Globulins are rendered more soluble by low concentrations, but less soluble by high concentrations of neutral salts. The latter property has most often been taken advantage of in the preparation of globulins, and the observation of Hammarsten (14) that fibrinogen was precipitated by half saturation with sodium chloride indicates that this is the most readily precipitated of the blood proteins. Since the preparation of fibrinogen thus depends upon its solubility in concentrated salt solutions, and since the precipitation of proteins by neutral salts has recently achieved not only a

quantitative formulation but a theoretical significance, both for the characterization of proteins and for the interpretation of the effects of different salts upon them, it seemed desirable to begin our studies upon this protein by determining its solubility in concentrated salt solutions.

Several investigators have determined the concentration of a neutral salt at which the precipitation of the protein begins. Data of this kind have been compiled by Pickering (20) and by Davide (6). They give us information only concerning the protein concentration of the actual salt solution considered, for precipitation starts when the protein is in greater concentration than the amount corresponding to saturation. The so called salting out of proteins by neutral salts has been quantitatively investigated in recent years. Chick and Martin (1), and later Sørensen and Høyrup (26), studied the precipitation of egg albumin by ammonium sulfate and noted the influence of the concentration of the neutral salt, of the hydrogen ion, and the apparent influence of the amount of saturating body Sørensen and Høyrup (26) interpreted the apparent influence of the saturating body in terms of the water and the salt contained in the precipitate. They showed that, at any one temperature, the solubility of egg albumin was completely defined by the concentration of neutral salt and of hydrogen ions in the solution. Disturbances due to salt and water content of the precipitate, factors for which Sørensen and Høyrup corrected their results, become less significant if smaller amounts of saturating body are employed.

In 1925 Cohn (3) demonstrated that a linear relationship existed between the concentration of the added salt and the logarithm of the solubility. In sufficiently concentrated solutions solubility could, therefore, be described by the equation

$$\log S = \beta - Ks\mu \qquad (1)$$

He interpreted the constant β as representing "the solubility in water of the soluble protein compound in the absence of salt," ((3) p. 417) which is precipitated by the neutral salt. The proportionality constant he associated with the salting out constant employed by Debye and McAulay (7) in describing the effect of neutral salts upon the activity coefficients of non-electrolytes.

More recently Cohn and Prentiss (2) showed that the solvent action of neutral salts upon the globulin, hemoglobin, could be described by means of the first term of the Debye-Hückel equation for the activity coefficients of electrolytes

$$\log \frac{S}{S_0} = 0.5\, z_1 z_2 \frac{\sqrt{\mu}}{1 + \kappa b} - Ks\mu \qquad (2)$$

The last term in this equation may be associated with the last term in Equation 1 and the two equations considered identical under such circumstances that the first term is equal to zero, or assumes a constant value. Green and Cohn (13) have described the solubility of carboxyhemoglobin in solutions of sodium chloride and potassium chloride up to very concentrated solutions. Under these circumstances it became apparent that there was a salting out effect, and the value of the salting out constant, Ks, was calculated. Neither sodium chloride nor potassium chloride can, however, be regarded as precipitants of carboxyhemoglobin. Carboxyhemoglobin is, however, completely precipitated by the phosphates or sulfates of sodium, ammonium, or potassium.

Cohn and Green (5) have studied the solubility of carboxyhemoglobin in solutions of these salts at different temperatures and acidities and have shown that, in sufficiently concentrated solutions, solubility is described, within the accuracy of their measurements, by Equation 1. Presumably, when the concentration of the solvent is sufficiently great, the first term on the right-hand side of Equation 2 either approaches a constant value or becomes negligible in comparison to the last term, and, under these circumstances, the simpler equation first employed in describing the properties of albumins suffices for the characterization of globulins. This investigation upon carboxyhemoglobin appears to confirm the deduction drawn from the study of the albumins that the salting out constant, Ks, is characteristic of a given protein and a given salt, and independent of pH. They also showed that it was independent of temperature. On the other hand, β is a variable describing the amphoteric behavior of the protein.

In the present investigation we have determined the values of Ks and β for fibrinogen in different salt solutions and at different acidities, and, as a result, have reduced to quantitative terms a

description both of those properties of the protein that depend upon its acid and basic dissociation, and of those upon which depends its precipitation by neutral salts.

Preparation of Fibrinogen.[2]

The method devised by Hammarsten (14) for the separation of fibrinogen depended upon its precipitation by the addition to plasma of an equal amount of a saturated solution of sodium chloride. Purification was achieved by repeatedly dissolving fibrinogen in dilute sodium chloride and precipitating it by half saturating the solution with respect to the salt. Heubner (16) showed that after precipitation at an alkaline reaction the resulting product was composed only to a small extent of fibrinogen, but to a much greater extent of a substance having very different properties. Only when purification was carried out at a neutral reaction did his product consist largely of fibrinogen. The following method of preparing fibrinogen, which depends upon the observations of Hammarsten and Heubner, was therefore attempted.

Method 1.—Citrated horse plasma, after remaining in a cold room for 1 day, was filtered, neutralized to litmus with dilute acetic acid, and rendered half saturated with respect to sodium chloride. The precipitation was carried out at room temperature, the neutral and calcium-free salt being slowly added. At least an hour was allowed to elapse after all the salt solution had been added before the precipitate was removed from the plasma by centrifugation. The precipitate was redissolved in an approximately normal sodium chloride solution (5 per cent) with the aid of gentle mechanical agitation. This process was thrice repeated, the solution remaining in the cold room only between the successive precipitations.

It was shown by Nolf (18) that preparations obtained by Hammarsten's method at room temperature were unsuitable for experiments upon the transformation of fibrinogen into fibrin. Presumably changes in the nature of the protein occurred under these circumstances which modified its reactions. Nolf, therefore, carried out his preparations in vessels cooled to 0°. These pre-

[2] We are indebted to the Massachusetts Antitoxin Laboratory for their courtesy in supplying us with the plasma from which fibrinogen was prepared.

cautions we observed in a second method of preparing fibrinogen, analogous to the first one, and differing only in this: that from the time the plasma was separated from the cells, the solutions were never taken from a cold room, maintained at 2° ± 0.2°. The following experiments indicate the necessity for this procedure, always subsequently employed.

Throughout this investigation the criteria of solubility were employed in determining whether a single protein had been isolated from the plasma. When the preparation had been carried out essentially as described in Method 1, solubility was studied in a phosphate buffer solution consisting of 0.49 mol of KH_2PO_4 and 0.53 of K_2HPO_4 and, therefore, having an ionic strength of 2.09 and a pH of 6.6 (4). The solubility of such a preparation did not remain constant, independent of the amount of saturating body. The results are recorded in Table II, in which it will be seen that, far from being constant, solubility increased progressively as the protein precipitate was successively triturated with fresh portions of the phosphate buffer.

When the precaution of carrying out the entire preparation at a low temperature was scrupulously observed, however, fibrinogen of constant solubility was prepared. The results of a single experiment are given in Table III, in which it will be observed that fibrinogen dissolved in the successive amounts of phosphate buffer with which it was equilibrated, to the same extent, that saturation was complete when 0.017 gm. of fibrinogen nitrogen per liter of the phosphate buffer had dissolved, that 2 hours appeared to be a sufficiently long time for saturation, and that more prolonged equilibration did not influence solubility. This protein must, therefore, be considered to have been separated from the other proteins of the plasma and to have been prepared in a state of purity with respect to other proteins and to the products of its own decomposition.

The difference in behavior of fibrinogen prepared by Methods 1 and 2 explains, we believe, statements such as those made by Wöhlish (30) that successive reprecipitations make fibrinogen more and more unstable. An examination of the methods used by the different authors quoted in Table I suggests that the fibrinogen solutions on which they made isoelectric determinations were prepared by methods which probably yielded a mixture of fibrinogen and its decomposition products.

If a solution, prepared by Method 2 and completely precipitable by half saturation with sodium chloride, is dialyzed in a collodion

TABLE II.

Experiment 5. Fibrinogen Preparation 14.

Phosphate ionic strength = 2.09, pH = 6.6, temperature = 25° ± 0.2°.

Addition of phosphate buffer to precipitate.	Time precipitate was equilibrated with phosphate buffer.	Solubility.
	hrs.	*gm. N per l.*
1st	1	0.035
	15	0.080
2nd	1	0.085
	2	0.090
	3	0.115
	6	0.262
3rd	6	0.575

December 3, 1928. To 2 liters of fibrinogen solution prepared by Method 1 were added 2 liters of a phosphate buffer consisting of 1 mol of KH_2PO_4 and 1 mol of K_2HPO_4. The precipitate was redissolved in 0.1 molal phosphate of similar composition, and reprecipitated by means of an equal volume of the 2 molal phosphate buffer.

The precipitate, collected by centrifugation, was placed in a 200 cc. flask. 50 cc. of a precisely 1.02 molal phosphate buffer (mol fraction K_2HPO_4 = 0.52), ionic strength 2.09, pH 6.6, were added. A few drops of toluene were also added to prevent bacterial action. The flask was put on a shaking machine in a water bath kept at 25° ± 0.2°. Samples were collected after different periods of shaking, filtered, and analyzed with respect to nitrogen. Fresh solution was added to the precipitate and the process repeated two more times. As shown by the figures in Table II, no equilibrium was attained, the solubility increasing with time. At the end of the experiment the remaining precipitate was entirely soluble in dilute phosphate. The protein dissolved during the third equilibration with the precipitate was, however, no longer completely precipitated upon the addition of an equal volume of a saturated sodium chloride solution. 4 volumes of saturated sodium chloride precipitated all of the coagulable protein present, but the filtrate from this precipitate still contained 0.020 mg. of nitrogen per cc.

membrane in the cold, under reduced pressure, and in the presence of toluene, against an alkaline solution, by the time the fibrinogen has become salt-free it has also become denatured. The addition

of an equal volume of saturated sodium chloride no longer yields a precipitate even after neutralization of any residual alkali. Since fibrinogen is characterized and prepared by virtue of its insolubility under these circumstances, the conclusion is inevitable that hydrolysis of the fibrinogen had proceeded during the dialysis, yielding a product of different properties than fibrinogen. In order to

TABLE III.

Fibrinogen Preparation 18.

Phosphate ionic strength = 2.09, pH = 6.6, temperature = 25° ± 0.2°.

	Addition of phosphate buffer to precipitate.	Time precipitate was equilibrated with phosphate buffer.	Solubility.	Fibrinogen.
		hrs.	*gm. N per l.*	*gm. per 1000 gm. H_2O*
Experiment 9.	1st	2	0.017	0.106
	2nd	2	0.017	0.106
	3rd	4	0.018	0.110
	4th	11	0.017	0.106
Experiment 10.	1st	2	0.017	0.106

December 20, 1928. To 1 liter of fibrinogen solution prepared by Method 2 was added 1 liter of phosphate buffer consisting of 1 mol of KH_2PO_4 and 1 mol of K_2HPO_4. The precipitate was redissolved in a 0.1 molal phosphate buffer of similar composition, reprecipitated, and redissolved, and again reprecipitated with the 2 molal phosphate buffer.

The precipitate, collected by centrifugation, was put in a 200 cc. bottle. 100 cc. of a precisely 1.02 molal buffer (mol fraction $K_2HPO_4 = 0.524$, $\mu = 2.09$, pH = 6.6) were added after washing with the same solution. The bottle was placed at 25° ± 0.2°. Samples were collected after a period of shaking, filtered, and analyzed with respect to nitrogen. Fresh solution was added to the precipitate and the process repeated three more times. At the end of the experiment, the precipitate was still completely soluble in dilute phosphate.

determine whether the hydrolysis of the fibrinogen during dialysis was dependent on maintaining an alkaline reaction we dialyzed, in two membranes, a fibrinogen solution prepared by Method 2 and completely precipitable by half saturation with sodium chloride. One membrane was dialyzed against the alkaline solution recommended by Heubner (16), an approximately 0.0007 molal sodium hydroxide solution, the other against distilled water at pH 6.

Even after 1 day the solution dialyzed against dilute alkali was only partially precipitable by half saturation with sodium chloride. That a modification of the fibrinogen had occurred, yielding a less readily precipitable product, was demonstrated by heat coagulation of the filtrate from a half saturated sodium chloride solution.

The fibrinogen dialyzed at a neutral reaction soon began to precipitate. This precipitate was relatively insoluble in water but completely soluble in 5 per cent sodium chloride solution, from which it could be completely precipitated by half saturation with sodium chloride. This product, therefore, still manifested the characteristics of fibrinogen[3] and led to the method of preparation that was finally adopted. This may be described as follows:

Method 3.—Citrated horse plasma is kept in the cold room for 1 day, filtered, and the reaction brought to about pH 6.0 by means of dilute hydrochloric acid. An equal volume of a saturated calcium-free solution of sodium chloride of pH 6.0 is slowly added through a glass tube extending below the surface of the plasma and close to a screw-shaped motor-driven glass stirrer which constantly brings new portions of plasma in contact with the salt solution and avoids the accumulation of a local excess of reagent. The precipitate is allowed to collect for 1 hour, and is then filtered. It is redissolved by stirring in 5 per cent sodium chloride at pH 6.0. The solution is filtered and toluene added. The process is thrice repeated. The solutions are never taken out of the cold room, and all the operations are performed there.

In order to reduce denaturation of fibrinogen as far as possible we have always maintained our solutions in the cold at a reaction near pH 6 and tested their complete precipitability in a half saturated solution of sodium chloride before and after each experiment.

The solutions prepared by this method are slightly opalescent, are completely precipitated on half saturation with sodium chloride, and when kept in the cold room with the addition of a few drops of

[3] Dialyzed fibrinogen deteriorates to some extent even under the best experimental conditions that we have thus far been able to devise. Fibrinogen precipitated by dialysis and kept suspended in distilled water saturated with toluene appears to change slowly and in two respects: on the one hand it loses its solubility in sodium chloride; on the other, that portion which remains soluble is no longer completely precipitable by half saturation with sodium chloride.

toluene, remain unchanged for weeks. They are coagulated by the addition of horse serum.

Measurement of Solubility.

In order that solubility measurements may have a theoretical significance they must be so carried out that solubility is independent of the amount of saturating body and of the time of equilibration. The results recorded in Table III demonstrated that it was possible to prepare fibrinogen sufficiently pure to satisfy these conditions at least within the limits of error obtained in these measurements. The temperature has been maintained constant at 25° ± 0.2° in all of these experiments, and the pH of each solution was always measured.

Certain modifications of the procedures that have generally been employed in measuring solubility in this laboratory were imposed by the properties of fibrinogen that have already been described. For the most part it was found desirable to reach equilibrium by precipitation of a fibrinogen solution rather than by dissolving precipitated fibrinogen. That equilibrium was reached from either side is indicated by the experiments recorded in Table III. In these experiments fibrinogen was precipitated by the phosphate buffer, was centrifuged, and the supernatant liquid decanted. The precipitate was then partially dissolved by being triturated with the more dilute phosphate buffer solution indicated, and solubility was measured. That this procedure gives the same result as that which has generally been employed may be seen by comparing this result with those in Table VI. The measurements recorded in Table III are also graphically represented in Fig. 1 where they fall upon the straight line which described solubility in phosphate buffers of this pH.

Sørensen and Høyrup (27) have demonstrated that equilibrium is reached more rapidly in the case of egg albumin when crystals are dissolved than when crystallization is brought about by increase in the concentration of neutral salt, and this phenomenon appears to be of general occurrence. Experiments were undertaken to determine the length of time required for precipitation to be completed, within the accuracy of our measurements, of a fibrinogen solution that had been added to a concentrated salt solution.

The procedure that has generally been followed has been to

reprecipitate the fibrinogen sufficiently often with the salt in the solution in which its solubility was to be determined, so that no other ions were present. The reaction of fibrinogen in this neutral salt solution was adjusted to the desired pH by means of acid or alkali. Aliquot parts were then placed in bottles in a water bath at 25° and permitted to come to this temperature. A water-driven screw-shaped glass stirrer dipped into each bottle and after temperature equilibrium was reached the solutions were stirred and the desired amounts of saturated salt solution slowly added from a pipette, the tip of which reached below the surface of the liquid and just above the stirrer which constantly forced fresh portions of the solution past. After the salt solutions were added, stirring was continued for the length of time that had been found necessary for equilibrium. The solutions were then filtered on No. 42 Whatman filter papers. The temperature of the filtration was maintained as close as possible to that of the bath by the use of water-jacketed filter funnels. Solubility was always estimated by analyzing the filtrate with respect to nitrogen by the Kjeldahl method. The concentration of neutral salt in the filtrates was analyzed by methods appropriate for the specific ions. Hammarsten (15) estimated that fibrinogen contained 16.66 per cent of nitrogen. The nitrogen analyses made on the various saturated fibrinogen solutions have, therefore, been multiplied by 6 to yield fibrinogen concentrations.

Solubility in Sodium Chloride Solutions.

Since the preparation of fibrinogen has, in the past, depended largely upon its precipitation by sodium chloride, the solubility of this protein in solutions of this neutral salt was quantitatively investigated. The fibrinogen for these studies was prepared as a solution in 5 per cent sodium chloride. The solubility experiments were carried out in the manner that has been described, the concentration of sodium chloride in the saturated solutions being estimated by chloride analysis carried out by Wilson's modification (29) of Van Slyke's method. The pH was measured electrometrically. The results obtained at pH 5.8 are recorded in Table IV and are defined by the equation

$$\log S = 1.61 - 0.91\mu$$

in which 0.91 is the value for Ks, and 1.61 for β.

Solubility in Potassium Phosphate Solutions.

From certain points of view phosphate buffer solutions must be considered preferable for the extraction and the precipitation of proteins to most other salts. This is especially true where small changes in reaction are likely to denature a protein, or, from an analytical point of view, where solubility is changing rapidly with change in pH. The determination of the activity coefficients and,

TABLE IV.

Solubility of Fibrinogen in Concentrated Solutions of Sodium Chloride.

Temperature = 25° ± 0.2°, pH = 5.8. Defined by the equation $\log S = \beta - Ks\mu$.

	Concentration of total protein.	Period of stirring.	Sodium chloride concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility.	$\frac{\log S_1 - \log S_2}{\mu_1 - \mu_2}$.	Log S + 0.91μ.
	gm. per l.	*hrs.*	*M per l. solution*	*M per 1000 gm. H_2O*	μ	*s = gm. per l. solution*	*S = gm. per 1000 gm. H_2O*	*log S*	*Ks*	β
Experiment 20, Preparation 29.	1.360	1	2.35	2.46	2.46	0.230	0.241	$\bar{1}$.38	0.91	1.61
	1.310	1	2.43	2.55	2.55	0.192	0.206	$\bar{1}$.31	0.93	1.61
	1.270	1	2.49	2.62	2.62	0.168	0.176	$\bar{1}$.24	0.89	1.62
	1.230	1	2.61	2.75	2.75	0.132	0.139	$\bar{1}$.14	0.90	1.64
	1.220	1	2.64	2.78	2.78	0.120	0.126	$\bar{1}$.10	0.86	1.62
Experiment 25, Preparation 36.	0.980	1	2.04	2.12	2.12	0.540	0.562	$\bar{1}$.74	1.02	1.66
	0.980	3	2.04	2.12	2.12	0.480	0.500	$\bar{1}$.69	0.87	1.61
	0.945	3	2.18	2.27	2.27	0.378	0.394	$\bar{1}$.59	0.97	1.65
	0.810	3	2.28	2.38	2.38	0.264	0.276	$\bar{1}$.44	0.92	1.60
	0.820	3	2.55	2.68	2.68	0.132	0.139	$\bar{1}$.14	0.91	1.57
Average									0.91	1.61

therefore, of the apparent dissociation constant of mixtures of KH_2PO_4 and K_2HPO_4 has facilitated the preparation of phosphate buffers of known pH and ionic strength, and it is believed that for purposes of the characterization of proteins, solubility in such solutions will prove most valuable.

Fibrinogen preparations employed in these measurements were first purified of other proteins by precipitation with sodium chloride by the method that has already been described (Method 3).

Finally the fibrinogen was purified of sodium chloride by being twice reprecipitated by a 2 molal phosphate buffer (mol fraction $K_2HPO_4 = 0.52$).

TABLE V.

Solubility of Fibrinogen in Concentrated Phosphate Solutions. Fibrinogen Preparation 26.

Mol fraction $K_2HPO_4 = 0.52$, temperature = 25° ± 0.2°, pH = 6.6. Concentration of total protein in the system = 2.80 gm. per liter; concentration of phosphate = 0.82 mol per liter.

Time of equilibration.	Solubility.
hrs.	*gm. fibrinogen per l.*
1	2.586
2	2.580

TABLE VI.

Solubility of Fibrinogen in Concentrated Solutions of $KH_2PO_4 + K_2HPO_4$.

Mol fraction $K_2HPO_4 = 0.52$, pH = 6.6, temperature = 25° ± 0.2°, period of stirring = 1 hour. Defined by the equation $\log S = \beta - Ks\mu$.

	Concentration of total protein.	Phosphate concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility.	$\frac{\log S_1 - \log S_2}{\mu_1 - \mu_2}$.	Log S + 1.95μ.
	gm. per l.	*M per l. solution*	*M per 1000 gm. H_2O*	μ	*s = gm. per l. solution*	*S = gm. per 1000 gm. H_2O*	*log S*	*Ks*	β
Experiment 15, Preparation 26.	3.040	0.712	0.736	1.49	1.650	1.706	0.24	1.92	3.14
	2.800	0.820	0.852	1.73	0.522	0.542	$\bar{1}$.73	1.93	3.16
	2.690	0.867	0.902	1.84	0.288	0.299	$\bar{1}$.47	1.97	3.05
	2.560	0.919	0.958	1.95	0.186	0.194	$\bar{1}$.28	2.05	3.08
Experiment 16, Preparation 26.	3.170	0.664	0.685	1.39	2.580	2.664	0.42	1.94	3.13
	3.040	0.719	0.744	1.51	1.680	1.739	0.23	2.02	3.17
	2.920	0.775	0.802	1.64	0.780	0.809	$\bar{1}$.90	2.12	3.09
	2.840	0.809	0.841	1.71	0.540	0.561	$\bar{1}$.74	1.78	3.07
Experiment 19, Preparation 28.	0.752	1.021	1.071	2.18	0.072	0.075	$\bar{2}$.87	1.81	3.12
	0.752	0.973	1.017	2.09	0.093	0.097	$\bar{2}$.98	2.00	3.05
	0.752	0.925	0.976	1.98	0.162	0.169	$\bar{1}$.22	1.98	3.08
	0.752	0.877	0.914	1.86	0.300	0.312	$\bar{1}$.49	1.93	3.11
	0.752	0.829	0.861	1.76	0.450	0.467	$\bar{1}$.66	2.02	3.09
Average.....								1.95	3.09

Solubility measurements were carried out by means of the same procedure as was employed in the case of sodium chloride. A

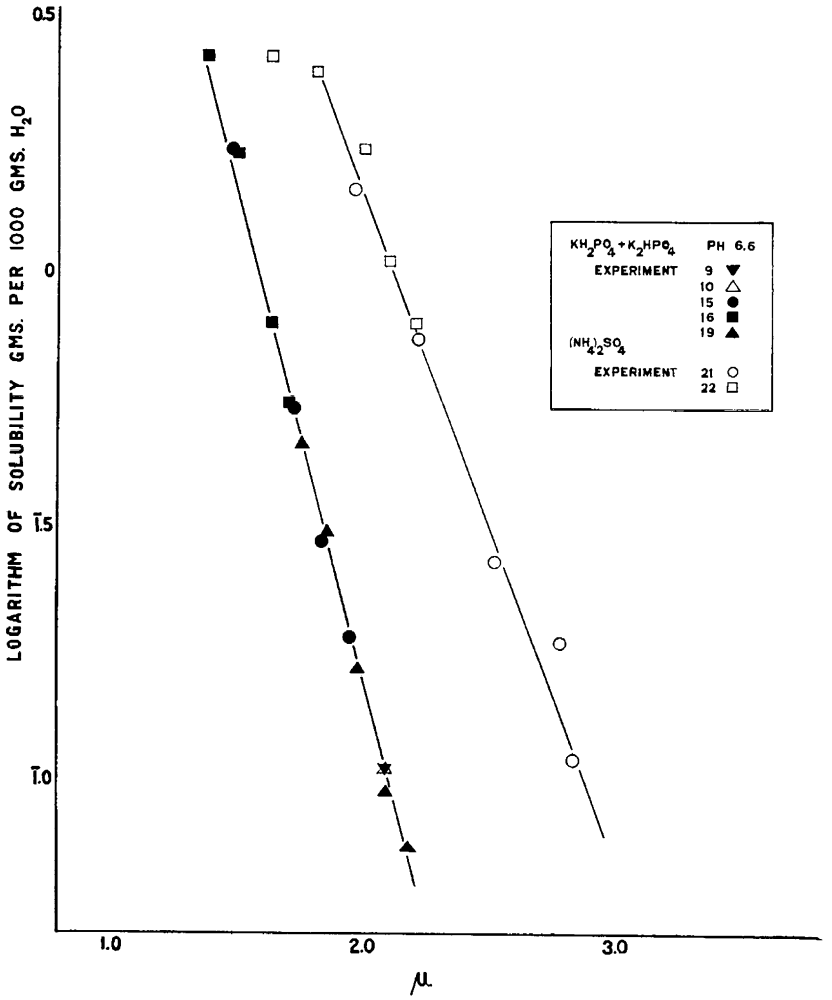

FIG. 1. Solubility of fibrinogen in concentrated phosphate and sulfate solutions at varying ionic strength.

preliminary experiment, recorded in Table V, suggested that equilibrium was reached within an hour. The results of a large

number of measurements all made at pH 6.6 are recorded in Table VI. The amount of potassium phosphate in the saturated fibrinogen solutions was estimated by analyzing the filtrates for phosphate by the method of Fiske and Subbarow (10).

The results in Tables III, V, and VI are graphically represented in Fig. 1. They leave little doubt that the logarithm of solubility is inversely proportional to the molecular concentration, or the ionic strength. This proportionality is represented by the equation

$$\log S = 3.09 - 1.95\mu$$

in which $\beta = 3.09$ and $Ks = 1.95$.

TABLE VII.

Solubility of Fibrinogen in Concentrated Ammonium Sulfate Solutions. Fibrinogen Preparation 32.

Temperature = 25° ± 0.2°. Concentration of total protein in the system = 2.91 gm. per liter; concentration of $(NH_4)_2SO_4$ = 0.562 mol per liter.

Time of equilibration.	Solubility.
hrs.	*gm. fibrinogen per l.*
1	2.586
2	2.580

Solubility in Ammonium Sulfate Solutions.

Ammonium sulfate has been the classical salt employed in the precipitation of proteins and their separation from each other. The fibrinogen preparations were first precipitated in sodium chloride in the conventional manner, and then twice precipitated and redissolved in ammonium sulfate solutions.

The presence of ammonia in the filtrate very much complicates the measurement of solubility in such solutions, however, and it is in part for this reason that phosphate buffers are superior for purposes of characterization.

The procedure employed in this investigation was based on that adopted by Sørensen and Høyrup (25) in their study on egg albumin. The fibrinogen in the filtrates was coagulated on a water bath, then cooled, and filtered. The filters on which the precipitates

TABLE VIII.

Solubility of Fibrinogen in Concentrated Solutions of Ammonium Sulfate.

Temperature = 25° ± 0.2°, pH = 6.0, period of stirring = 1 hour. Defined by the equation $\log S = \beta - K s\mu$.

	Ammonium sulfate concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility	Log S + 1.33μ.
	M per l. solution	*M per 1000 gm. H_2O*	μ	*s = gm. per l. solution*	*S = gm. per 1000 gm. H_2O*	*log S*	β
Experiment 11, Preparation 20.	0.640	0.665	1.99	0.780	0.811	$\bar{1}$.90	2.54
	0.690	0.719	2.15	0.360	0.375	$\bar{1}$.57	2.42
	0.770	0.807	2.42	0.258	0.272	$\bar{1}$.43	2.64
	0.850	0.896	2.68	0.090	0.094	$\bar{2}$.97	2.53
	0.950	1.008	3.02	0.042	0.044	$\bar{2}$.64	2.45
Average							2.51

TABLE IX.

Solubility of Fibrinogen in Concentrated Solutions of Ammonium Sulfate.

Temperature = 25° ± 0.2°, pH = 6.6, period of stirring = 1 hour. Defined by the equation $\log S = \beta - K s\mu$.

	Concentration of total protein.	Ammonium sulfate concentration.		Ionic strength.	Solubility of fibrinogen.		Log of solubility.	$\frac{\log S_1 - \log S_2}{\mu_1 - \mu_2}$	Log S + 1.33μ.
	gm. per l.	*M per l. solution*	*M per 1000 gm. H_2O*	μ	*s = gm. per l. solution*	*S = gm. per 1000 gm. H_2O*	*log S*	Ks	β
Experiment 21, Preparation 31.	2.65	0.633	0.657	1.97	1.416	1.471	0.16	1.30	2.78
	2.65	0.710	0.741	2.22	0.726	0.758	$\bar{1}$.87	1.42	2.82
	2.65	0.803	0.843	2.52	0.264	0.277	$\bar{1}$.43	1.29	2.78
	2.65	0.850	0.896	2.68	0.180	0.189	$\bar{1}$.27	1.29	2.83
	2.65	0.893	0.944	2.83	0.105	0.111	$\bar{1}$.04	1.41	2.80
Experiment 22, Preparation 32.	2.91	0.562	0.581	1.74	2.586	2.675	0.42	1.01	2.73
	2.91	0.588	0.609	1.82	2.418	2.507	0.39	1.21	2.81
	2.91	0.645	0.670	2.01	1.680	1.747	0.24	1.58	2.91
	2.91	0.678	0.706	2.11	1.032	1.075	0.02	1.36	2.82
	2.91	0.706	0.737	2.21	0.762	0.795	$\bar{1}$.90	1.50	2.83
Average								1.33	2.81

were deposited, were washed with hot water until free of sulfate. The precipitate was then considered to be free from ammonia nitrogen and was analyzed for fibrinogen nitrogen by the Kjeldahl method. The filtrates were collected in volumetric flasks, brought to known volumes with boiled distilled water, and the ammonium sulfate concentrations estimated by the determination of ammonia.

Solubility has been studied in ammonium sulfate solution at pH 6 and 6.6. 1 hour appeared to be sufficient for the establishment of equilibrium in this, as in the other salts investigated (Table VII). The results at pH 6 are represented in Table VIII and those at pH 6.6 in Table IX. In Fig. 1 is contrasted the solubility of fibrinogen at pH 6.6 in concentrated phosphate and sulfate solutions. There can be no doubt that the data are adequately represented by a straight line for the sulfates, as well as for the phosphates, although the technical difficulties in making the measurements are far greater in the case of ammonium sulfate. The equations defining solubility in ammonium sulfate are

$$\text{For pH } 6.0,\ \log S = 2.51 - 1.33\,\mu$$
$$\text{“ “ } 6.6,\ \text{“ “} = 2.81 - 1.33\,\text{“}$$

Solubility of a Protein in a Salt Solution at Different Acidities.

Sørensen and Høyrup (26) studied the solubility of egg albumin at different acidities, and the recalculation of their results by Cohn (3) indicated that for the same salt solution Ks is independent of pH. Our results with fibrinogen are consistent with this generalization. Moreover, the studies upon egg albumin and carboxyhemoglobin indicate that solubility is minimal near the isoelectric point of a protein. That is to say, β has a minimal value in the case of egg albumin near pH 4.5 and for carboxyhemoglobin near 6.6. Their respective isoelectric points are 4.7 and 6.78. Since the solubility of fibrinogen is lower in ammonium sulfate at pH 6 than at pH 6.6, these data suggest that the isoelectric point of this protein occurs at a more acid reaction. These observations thus throw a certain light on the isoelectric measurements in Table I and favor the acceptance of an acid value for this constant.

Solubility of Different Proteins in the Same Salt Solutions.

The solubility of egg albumin, of serum albumin, of pseudoglobulin, of carboxyhemoglobin, and of fibrinogen have now been

studied in concentrated solutions of ammonium sulfate. The value of Ks varies in the proteins under investigation, as shown in Table X. The order is presumably a measure of the extent to which these proteins alter the dielectric properties of solutions. It does not necessarily yield the order in which these proteins will be precipitated from the same solution. A second set of influences is simultaneously operative. These influences are designated by β and ascribed to the amphoteric nature and state of the protein (3). A change in pH, which will not within wide limits affect the value of Ks changes the dissociation of a protein as acid and as base and,

TABLE X.

Values of β and Ks in the Equation Log $S = \beta - Ks\mu$, Defining Solubility of Different Proteins in Concentrated Solutions of Ammonium Sulfate.

Protein.	Author.	pH	Ks*	β
Fibrinogen.		6.6	1.33	2.81
		6.0	1.33	2.51
Pseudoglobulin.	Sørensen (25).		1.00	5.44
Egg albumin.	Chick and Martin (1).		0.96	7.85
" "	Sørensen and Høyrup (27).	4.7	0.91	6.22
Serum albumin.				
Fraction A.	Sørensen (28).	4.8	0.67	3.79
" B.	"	4.8	0.61	4.22
" γ.	"	4.8	0.33	2.97
Carboxyhemoglobin.	Green and Cohn.†	6.6	0.55	2.71

* All of the results have been recalculated on the basis of the ionic strength per 1000 gm. of water.

† Personal communication.

as a result, β may vary in such a way as to alter the order of precipitation of a protein in a series arranged according to values of Ks. A diagram representing the precipitation of proteins at their respective isoelectric points would appear very different from a diagram representing their precipitation at the same pH.

With the exception of egg albumin all of the proteins in Table X are blood proteins. Of them all fibrinogen has the highest value of Ks. The values of β of fibrinogen at pH 6.0 and 6.6 are 2.51 and 2.81, and β presumably becomes smaller at those acid reactions which are in the neighborhood of its isoelectric point. But,

whereas fibrinogen, egg albumin, and serum albumin have acid isoelectric points, the isoelectric point of pseudoglobulin is at pH 5.44 and of hemoglobin at 6.78. The minimal solubility of egg albumin is near pH 4.5 and of hemoglobin near pH 6.6. Acidification from 6.6 to 5.4 increases the solubility of hemoglobin, but decreases that of the other proteins considered. Further acidification, let us say to pH 5.0, increases the solubility of serum globulin as well as hemoglobin, whereas the solubility of albumin and presumably of fibrinogen diminishes. At pH 6.6 hemoglobin precipitates over approximately the same range as serum globulin. At 5.4 pseudoglobulin precipitates at lower, hemoglobin at higher concentrations. In how far the separations conventionally performed at a neutral reaction would be complicated at the isoelectric point of serum albumin must await further study. The

TABLE XI.

Values of β and Ks in the Equation Log $S = \beta - Ks\mu$, Defining Solubility of Fibrinogen in Concentrated Solutions of Different Salts.

Salt.	pH	*Ks*	β
NaCl	5.8	0.91	1.61
$(NH_4)_2SO_4$	6.0	1.33	2.51
	6.6	1.33	2.81
$KH_2PO_4 + K_2HPO_4$	6.6	1.95	3.09

behavior of fibrinogen, however, may be clearly postulated. It possesses the highest value of *Ks*, and values of β at pH 6.6 of the same order as the β of carboxyhemoglobin, minimal at this reaction. Preliminary investigations indicate, however, that β for fibrinogen becomes progressively lower, at least to beyond pH 5.0. The readily precipitable condition of this blood protein is thus manifest and its physiological significance demands consideration.

Solubility of Fibrinogen in Concentrated Solutions of Different Salts.

Ammonium sulfate is a precipitant for most proteins. The solubility of proteins in phosphate buffer mixtures of the same pH is very similar, but the lower solubility of the phosphates themselves has appeared to limit their usefulness. The value of *Ks* and of β

for carboxyhemoglobin in ammonium sulfate and in potassium phosphate is identical within the error of measurement. The same constants vary but slightly for fibrinogen at pH 6.6., as shown in Table XI.

In Table XI the values of Ks for fibrinogen in solutions of sodium chloride, ammonium sulfate, and potassium phosphate are contrasted, showing that the relative order of these salts is that ordinarily given for the Hofmeister series. Most proteins are not precipitated by the neutral chlorides, but the insolubility of fibrinogen in the presence of relatively concentrated solutions of these salts renders it ideal for the further investigation of interionic forces in solutions of neutral salts and proteins.

I wish to express my appreciation of the kind advice and assistance given me by Dr. Edwin J. Cohn throughout this investigation.

SUMMARY.

Fibrinogen is readily transformed even at room temperature and at neutral reactions to substances which are no longer precipitated by half saturation with sodium chloride. Conditions of acidity and of temperature have been determined, however, under which fibrinogen may be sufficiently purified of other proteins and its own denaturation products to yield a chemical individual of constant and reproducible solubility.

The solubility of fibrinogen in concentrated salt solutions is defined by the equation $\log S = \beta - Ks\mu$.

The values of β and of the salting out constant, Ks, have been determined at 25° for sodium chloride at pH 5.8, ammonium sulfate at pH 6.0 and 6.6, and for potassium phosphate at pH 6.6.

The results that have been obtained with ammonium sulfate at pH 6.0 and 6.6 are consistent with the postulate that Ks is a constant for a given protein and salt, independent, over wide ranges, of temperature and pH (5).

The amphoteric properties of the protein are reflected by the values of β. The variation of β from 2.81 at pH 6.6 to 2.51 at pH 6.0 suggests that fibrinogen has an acid isoelectric point.

A comparative study of the solubility of the different blood proteins in concentrated solutions of the same salt, $(NH_4)_2SO_4$, shows that, of all of them, fibrinogen has the highest value of Ks. This

study is thus a further contribution towards the description of the "salting out" of the blood proteins by neutral salts, in terms of physicochemical constants.

BIBLIOGRAPHY.

1. Chick, H., and Martin, C. J., *Biochem. J.*, **7**, 380 (1913).
2. Cohn, E. J., and Prentiss, A. M., *J. Gen. Physiol.*, **8**, 619 (1927).
3. Cohn, E. J., *Physiol. Rev.*, **5**, 359 (1925).
4. Cohn, E. J., *J. Am. Chem. Soc.*, **49**, 173 (1927).
5. Cohn, E. J., and Green, A. A., *J. Biol. Chem.*, **78**, p. xxxii (1928).
6. Davide, H., *Acta med. Scand.*, suppl. 1 (1925).
7. Debye, P., and McAulay, J., *Physik. Z.*, **26**, 22 (1925).
8. Denis, P. S., Mémoires sur le sang, Paris (1859).
9. De Waele, H., *Ann. physiol. et de physicochim. biol.*, **3**, 375 (1927).
10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
11. Fredericq, L., *Bull. Acad. roy. méd. Belgique*, **44**, 56 (1877).
12. Funck, A., *Biochem. Z.*, **124**, 148 (1921).
13. Green, A. A., and Cohn, E. J., *Am. J. Physiol.*, **90**, 366 (1929).
14. Hammarsten, O., *Arch. ges. Physiol.*, **19**, 563 (1879).
15. Hammarsten, O., *Arch. ges. Physiol.*, **22**, 431 (1880).
16. Heubner, W., *Arch. exp. Path. u. Pharmakol.*, **59**, 229 (1903).
17. Kugelmass, I. N., *Arch. internat. physiol.*, **21**, 139 (1923).
18. Nolf, P., *Arch. internat. physiol.*, **7**, 280 (1909).
19. Nordbö, R., *Biochem. Z.*, **190**, 150 (1927).
20. Pickering, J. W., The blood plasma in health and disease, London (1928).
21. Quagliariello, G., *Arch. sc. biol.*, **8**, 55 (1926).
22. Rabinovich, R., *Rev. soc. Argentina biol.*, **1**, 499 (1925).
23. Resch, A., *Biochem. Z.*, **78**, 297 (1917).
24. Sørensen, S. P. L., *J. Am. Chem. Soc.*, **47**, 55 (1926).
25. Sørensen, S. P. L., and Høyrup, M., *Compt. rend. trav. Lab., Carlsberg*, **12**, 164 (1915–17).
26. Sørensen, S. P. L., and Høyrup, M., *Compt. rend. trav. Lab., Carlsberg*, **12**, 213 (1915–17).
27. Sørensen, S. P. L., Proteins, New York (1925).
28. Stuber, B., and Funck, A., *Biochem. Z.*, **126**, 142 (1922).
29. Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, **79**, 221 (1928).
30. Wöhlish, E., *Z. ges. exp. Med.*, **40**, 137 (1924).
31. Wöhlish, E., and Schloss, J., *Z. Biol.*, **85**, 542 (1927).