XXXIX. THE PRECIPITATION OF EGG-ALBUMIN BY AMMONIUM SULPHATE. A CONTRIBUTION TO THE THEORY OF THE "SALTING-OUT" OF PROTEINS.

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(*Received June 11th, 1913.*)

Introduction.

The first systematic and quantitative researches upon the precipitation of proteins by salts in large amounts were made by Hofmeister and his pupils.

In the experiments of Kauder [1886] and Lewith [1888] with serum proteins, and Hofmeister [1888] with egg-protein, comparison was made of the precipitating power of a large series of electrolytes, as a result of which the latter were arranged in what is known as the Hofmeister series. Most of the experiments were made with sodium salts, among which sulphate, phosphate, acetate, citrate, tartrate, chromate, chloride, nitrate and chlorate formed a series arranged in descending power of precipitation; of the kations examined lithium was the most effective, and sodium, potassium, ammonium and magnesium came afterwards in order of decreasing efficiency.

Hofmeister [1889] came to the conclusion that the precipitation was caused by the electrolytes depriving the protein of the amount of water necessary to keep it in solution, and was confirmed in this view by the results of some experiments showing the influence of various salts in modifying the imbibition of water by gelatin [1891]; work in this direction was later extended by Pauli [1898] and Pauli and Rona [1902].

Spiro [1904] demonstrated that the precipitation from their solutions of caseinogen and gelatin by sodium sulphate was analogous to the "salting out" of alcohol recently studied in detail by de Bruyn [1900]. In neither case is the phenomenon one of simple precipitation, since, owing to the appropriation of water by the salt, separation into two phases occurs. Each phase contains all the constituents of the system and any alteration in one of

the three constituents leads to readjustment of the composition and relative volumes of the two phases. Spiro also pointed out that, since in the case of alcohol the effect of electrolytes is not attributable to the constituent ions, any influence of the latter in the salting out of proteins must be regarded as a subsidiary phenomenon.

Spiro's conception explains to some extent the divergent results obtained in the precipitation of proteins by the addition of neutral salts, when the whole conditions are not maintained constant.

The series of observations which we are about to record concern the "salting out" by ammonium sulphate of pure recrystallised egg-albumin. Our observations show that in this case also we have to deal with the separation of the original system (itself not homogeneous) into two distinct phases, and that the influence upon the volume of these phases of concentration of protein, salt and water in the system is, as Spiro found for caseinogen and gelatin, analogous to what occurs in alcohol, salt and water mixtures. In addition, however, we find that the charge carried by the protein particles is an important factor in the final equilibrium.

The results of the experiments will first be set forth and the proposed explanation discussed later.

Precipitation of pure egg-albumin by ammonium sulphate.

Material. The material employed was egg-albumin crystallised from egg-white in presence of ammonium sulphate according to the method of Hopkins and Pinkus [1898]. The albumin was recrystallised once or twice, separated from the mother liquor by pressing between filter paper, and finally dissolved in distilled water. A concentrated stock solution was thus obtained, the composition of which, as regards (1) protein, (2) ammonium sulphate, (3) water was accurately ascertained by analysis, and which, when diluted to a suitable degree, served for most of the following experiments.

Since the salt employed for "salting out" in these experiments was also ammonium sulphate, the small concentration of the latter always present in the original albumin solution presented no complication; an allowance was made for this amount in the calculations. In those cases where an electrolyte-free solution was required, the albumin solution was previously dialysed.

Egg-albumin prepared in this way we believe to be as homogeneous a protein as it is possible to obtain. Hopkins [1899–1900] came to the conclusion that egg-albumin, crystallised from faintly acid ammonium sulphate solutions by the above method, was a pure substance. The

rotatory power remained absolutely constant after repeated recrystallisations (p. 312) and the proportion of carbon, hydrogen, nitrogen and sulphur, as well as the ash, remained constant. His experiments were made with four different samples after three or four recrystallisations.

1. *Influence of concentration of salt upon the amount of protein precipitated.*

Mellanby [1907] made a quantitative study of the influence of concentration of ammonium sulphate on the precipitation of the proteins from horse serum, but, as far as we are aware, no experiments have as yet been made with a pure protein. In the present instance two sets of experiments were made, in both of which the concentration of protein was about 1 %. In the first the concentration of protein in the whole system was left constant = 1·11 % by weight, and the ratio salt to water was varied (Table I and Fig. 1). In the second set the ratio protein to water was kept constant and the precipitation studied by varying the amount of salt present; the concentration of protein in the whole system varied from 1·0 % to 0·93 % (by weight). (Table II and Fig. 2.)

TABLE I.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of salt.

Temperature, 20°.
Protein constant = 1·11 % by weight of total system.
Ratio salt/water varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total system	G. salt in 100 g. total system	G. albumin in 100 g. filtrate
1·00	69·00	20·00	1·11	22·22	1·089
1·00	68·00	21·00	,,	23·33	0·711
1·00	67·00	22·00	,,	24·44	0·302
1·00	66·00	23·00	,,	25·55	0·104
1·00	65·00	24·00	,,	26·66	0·0315
0·50	32·35	12·15	,,	27·00	trace
0·50	32·00	12·50	,,	27·77	trace

The method of experiment was as follows. Mixtures were prepared by weighing into stoppered bottles the required amount of water and protein, and the necessary amount of ammonium sulphate was then added in large crystals and gently shaken. This prevented over-saturation with ammonium sulphate in the neighbourhood of the crystals, the large size of which

prevented a too rapid solution. The bottles were placed in a thermostat at 20°, for from 1 to 2 hours, the contents filtered and the protein estimated in the filtrate by weighing the coagulum formed on heating.

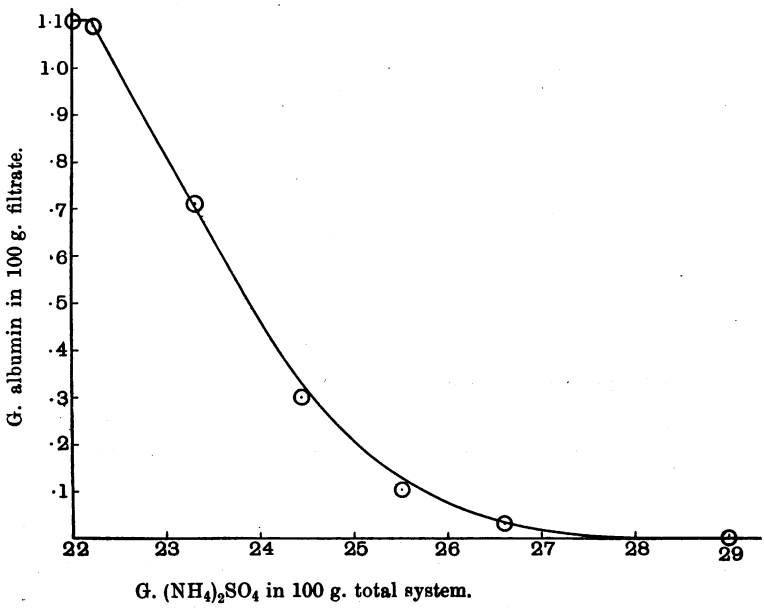

Fig. 1. Influence of concentration of the salt upon precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20°, see Table I; protein constant = 1·11 % (by weight) of total system, ratio salt to water varying.

TABLE II.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of salt.

Temperature, 20°.
Ratio protein/water constant = 1·3/100.

Albumin g.	Water g.	Salt g.	G. salt to 1·3 g. protein and 100 g. H_2O	G. albumin in 100 g. filtrate
1·30	100	29	29	0·998
1·3	100	29·7	29·7	no precipitation.
1·30	100	30	30	0·938
1·95	150	46·5	31	0·733
1·3	100	32	32	0·487
2·6	200	66	33	0·273
2·6	200	67	33·5	0·232
2·6	200	70·01	35·0	0·105
2·6	200	76	38·0	0·022

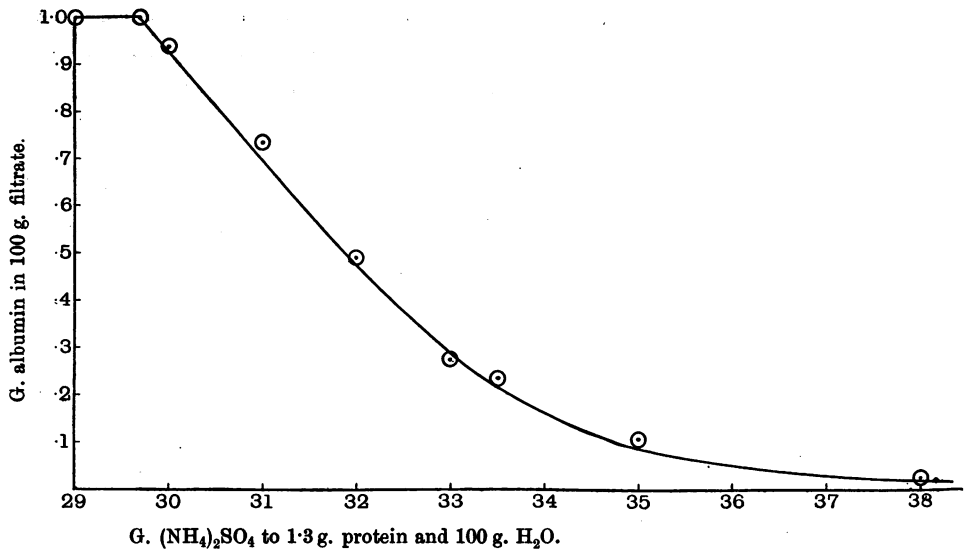

Fig. 2. Influence of concentration of the salt upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20°, see Table II; ratio protein to water constant=1·3 to 100, amount of salt present varying.

The curves in Figs. 1 and 2 are both of the same general form, in both cases the percentages of albumin in the filtrate are plotted as ordinates and the abscissae are respectively percentage by weight of ammonium sulphate in the whole system and grams of ammonium sulphate present, which explains the fact that the curve is steeper in the former instance. The point of commencing precipitation which is very sharply marked is at about the same concentration of salt in both cases, viz. 22·2 and 22·7 % ammonium sulphate respectively; the curve then descends steeply and approaches the base line asymptotically.

2. *Influence of concentration of protein.*

Kauder [1886] showed that serum albumin was more readily precipitated by ammonium sulphate if in more concentrated solution and determined the diminishing concentration of ammonium sulphate necessary to cause commencing precipitation in a series of solutions of increasing protein concentration. Hofmeister in 1888 published the results of similar experiments, using egg-white and potassium acetate and ammonium sulphate. Similar evidence has since been brought forward by other workers, e.g. Mellanby [1907], but in no case was a pure protein employed.

We have made an experiment with pure egg-albumin, estimating the

protein precipitated by a constant concentration of ammonium sulphate (ratio salt to water constant) when the amount of protein was varied. The results are given in Table III and graphically set forth in Fig. 3, where the proportion of the protein separated is plotted as ordinate against the concentration (percentage by weight of whole system) of protein in the original mixture as abscissa. Not only is more protein separated from the more concentrated

TABLE III.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of protein.

Ratio salt/water constant = 31/100.
Concentration of protein varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total mixture	G. albumin to 31 g. salt and 100 g. H_2O	G. albumin in 100 g. filtrate	G. albumin precipitated from 100 g. total mixture	Protein ppted. %
1·90	56·92	17·65	2·481	3·33	1·130	1·351	54·4
1·90	36·92	11·44	3·775	5·14	1·115	2·660	70·4
3·79	50·85	15·76	5·383	7·45	1·159	4·224	78·5
7·59	53·69	16·64	9·738	14·13	0·935	8·803	90·4
4·74	17·31	5·36	17·306	27·4	0·772	16·534	95·5

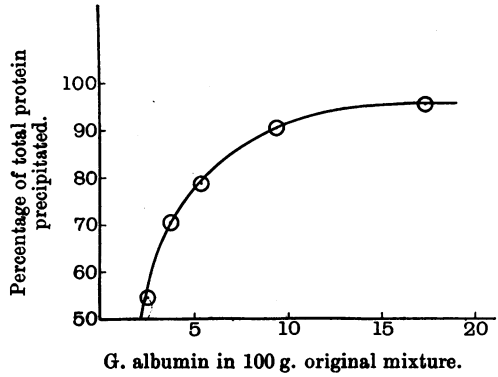

Fig. 3. Influence of concentration of protein upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20° C., see Table III; ratio salt to water constant = 31 to 100, amount of protein varying.

solution at a given concentration of salt, *but a greater proportion is precipitated* (see last column, Table III). The concentration of protein in the filtrate is not constant, but varies from 1·13 % to 0·77 % (col. 6, Table III), as the initial protein concentration is varied from 2·5 to 17·3 % (col. 4). This suggests that the precipitation is a phase separation, analogous to the case of "salting out" of alcohol with ammonium sulphate. As will be

seen later, when the results are given of determinations of the protein-salt-water content of filtrate and precipitate respectively, this was proved to be the case.

3. *Influence of hydrogen ion concentration.*

It is common experience, e.g. with serum, that the addition of a little acid enhances the amount of protein precipitated by the same concentration of ammonium sulphate, and that proteins not precipitated by saturation with sodium chloride are thrown down on acidification of the solution.

Mellanby [1907] called attention to the increased amount of precipitation of horse serum by neutral salts after addition of various acids, and gave some quantitative data, using sodium chloride. In the present investigation the influence of acidity was directly measured in a series of mixtures in which the concentration of protein and ammonium sulphate was maintained constant, and so chosen that precipitation had just begun in the control solution. The reaction of the solution was adjusted to various degrees of hydrogen ion concentration by the addition of small quantities of standard sulphuric acid.

Determinations of hydrogen ion concentration were made with the type of concentration cell described by Michaelis and Rona [1909], the contact fluid between the two cells being saturated potassium chloride solution. It is possible that the determinations of H^+ concentration are not very accurate owing to the high concentration of ammonium sulphate present; those in any one series are, however, perfectly comparable.

TABLE IV.

Precipitation of pure egg-albumin with ammonium sulphate; influence of hydrogen ion concentration.

Experiment I at 18°.
G. ammonium sulphate in 100 cc. original mixture = 30·4.
G. protein in 100 cc. original mixture = 0·575.

G. protein present in 100 cc. filtrate	Hydrogen ion concentration in filtrate, in terms of normality
0·572	$10^{-5.27}$ (54×10^{-7})
0·391	$10^{-5.00}$ ($101 \times$,,)
0·097	$10^{-4.64}$ ($231 \times$,,)
0·062	$10^{-4.53}$ ($296 \times$,,)
0·048	$10^{-4.31}$ ($492 \times$,,)
0·035	$10^{-4.11}$ ($780 \times$,,)

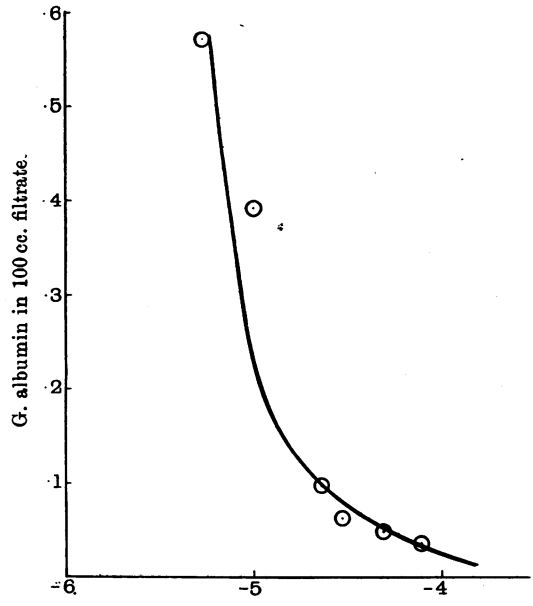

Fig. 4. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 18° (see Table IV).

Concentration of $(NH_4)_2SO_4$ in whole system, constant = 30·4 grams per 100 cc.
" " Protein " " " = 0·575 " "

The concentration of protein varied from 0·6 to 0·9 % and that of ammonium sulphate from 30·4 to 28·6 % (by volume) in the three different series of experiments set forth in Tables IV to VI. The mixtures were placed for two hours at 18° in order that equilibrium might be attained; they were then filtered and the concentration of protein and of hydrogen ions in the filtrate was determined.

The influence of hydrogen ion concentration was found to be very marked although the range through which it operates is not extensive. In one case (Exp. I, Table IV, protein concentration = 0·57 %, ammonium sulphate = 30·4 %) an increase in acidity from 54×10^{-7} normal (control) to 780×10^{-7} normal was enough to cause precipitation of nearly all the protein. In Exp. II, Table V (0·86 % protein and 30·03 % ammonium sulphate) and Exp. III, Table VI (0·9 % protein and 28·6 % ammonium sulphate) the effective range of hydrogen ion concentration was from 2·9 to 360×10^{-7} normal, and from 12 to 203×10^{-7} normal respectively.

TABLE V.

Precipitation of pure egg-albumin with ammonium sulphate; influence of hydrogen ion concentration.

Experiment II at 18°.
G. ammonium sulphate in 100 g. original mixture = 30·03.
G. protein in 100 g. original mixture = 0·856.

No. of cc. N/10 H_2SO_4 (or equivalent) added in total volume of 27 cc.	No. of cc. N/10 NH_4OH (or equivalent) added in total volume of 27 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentration (filtrate), in terms of normality
—	1·0	0·864	$10^{-6.54}$ ($2{\cdot}88 \times 10^{-7}$)
—	0·5	0·809	$10^{-6.01}$ ($9{\cdot}82 \times$,,)
—	0·3	0·795	$10^{-5.72}$ ($19{\cdot}0 \times$,,)
—	—	0·514	$10^{-5.40}$ ($39{\cdot}8 \times$,,)
0·5	—	0·056	$10^{-4.98}$ (104 × ,,)
1·0	—	0·01 (about)	$10^{-4.45}$ (358 × ,,)

The last trace of protein present, however, does not appear to be precipitated by alteration of reaction alone, a slight trace in Exp. III being still left in solution at a hydrogen ion concentration of about 1/100 normal or 73000×10^{-7} normal.

The range of reaction where hydrogen ion concentration has its great effect is presumably just on the acid side of the iso-electric point (see below p. 392). From Exp. III it is seen that for ammonium sulphate change in hydrogen ion concentration at and on the alkaline side of the neutral point is without much influence (viz. from $H^+ = 12 \times 10^{-7}$ normal to $0{\cdot}008 \times 10^{-7}$ normal), see Table VI.

TABLE VI.

Precipitation of pure egg-albumin with ammonium sulphate; influence of reaction, hydrogen ion concentration.

Experiment III at 18°.
G. $(NH_4)_2SO_4$ in 100 g. original mixture = 28·6.
G. protein ,, ,, ,, = 0·910.

Cc. N/10 H_2SO_4 (or equivalent) added in total volume of 25·4 cc.	Cc. N/10 $(NH_4)OH$ (or equivalent) added in total volume of 25·4 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentration (filtrate) in terms of normality
—	3·6	No precipitation	$10^{-9.11}$ ($0{\cdot}008 \times 10^{-7}$)
—	2·0	0·899	$10^{-7.23}$ (0·59 ,,)
—	1·0	0·908	$10^{-6.56}$ (2·7 ,,)
—	—	0·899	$10^{-6.08}$ (12·0 ,,)
0·25	—	0·533	$10^{-5.45}$ (35 ,,)
0·5	—	0·174	$10^{-5.01}$ (97·5 ,,)
1·0	—	0·063	$10^{-4.69}$ (203 ,,)
5·0	—	trace	$10^{-2.45}$ (35000 ,,)
10·0	—	slight trace	$10^{-2.14}$ (73000 ,,)

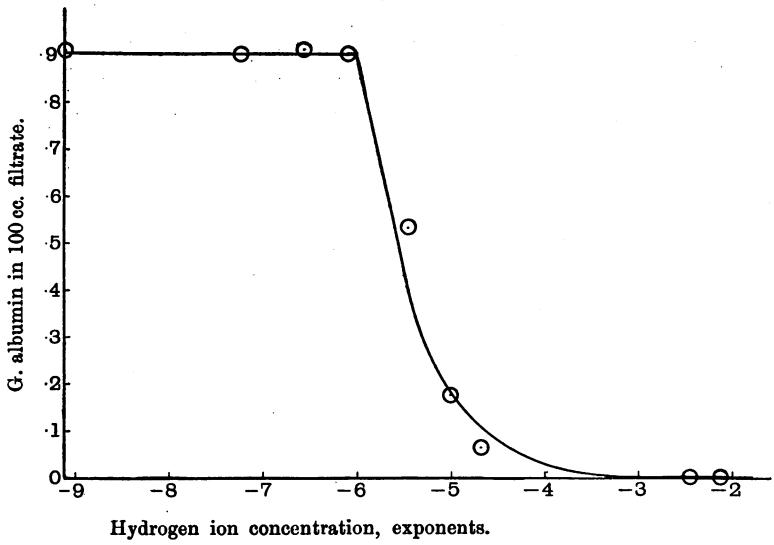

Fig. 5. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 18° (see Table VI).

Concentration of $(NH_4)_2SO_4$ in whole system, constant = 28·6 grams in 100 cc.
,, ,, protein ,, ,, ,, = 0·910 ,, ,,
H^+ concentration expressed as exponents.

4. *Influence of temperature.*

Lewith [1888] in case of ox-serum proteins, showed that rise in temperature assisted the precipitation by ammonium sulphate and Hofmeister [1888] made the same observation with egg-white and various salts. Spiro [1904] stated the same to be true of crystalline serum albumin and ammonium sulphate.

We have confirmed the above observations for serum proteins[1] and pure egg-albumin if the reaction be alkaline, but in faintly acid solution (10^{-5} normal) we have found the reverse to be true above 9°.

In Table VII and Fig. 6 are set forth the results of an experiment with 0·85 % protein and 28 % ammonium sulphate. A series of exactly similar solutions were placed for from 1 to 2 hours in a thermostat at the required temperature, after which they were rapidly filtered and the protein estimated in the filtrate. From 0° to 9° the temperature coefficient of precipitation was positive, above 9° it remained negative to 50°[2]. This can be readily

[1] Mellanby [1907, p. 294], on the other hand, states that the temperature coefficient of serum-protein precipitation with $(NH_4)_2SO_4$ is negative between the temperatures of 0° and 40° C. and so small as to be unimportant.

[2] No denaturation occurred.

demonstrated if ammonium sulphate be added to an egg-albumin solution at 9° to the point of opalescence, and just short of the formation of a definite precipitate. If, then, the mixture be divided into three portions, of which one is placed at 0° and a second at 20°, a definite precipitation will occur at both temperatures, whereas the portion maintained at 9° will remain merely opalescent.

TABLE VII.

Influence of temperature upon the precipitation of pure crystalline egg-albumin with ammonium sulphate (28 %).

Protein content = 0·85 %.
Hydrogen ion concentration about 10^{-5} normal.

Temperature	Concentration of protein in filtrate, %
50	0·828
36	0·703
20	0·364
9	0·268
0	0·318

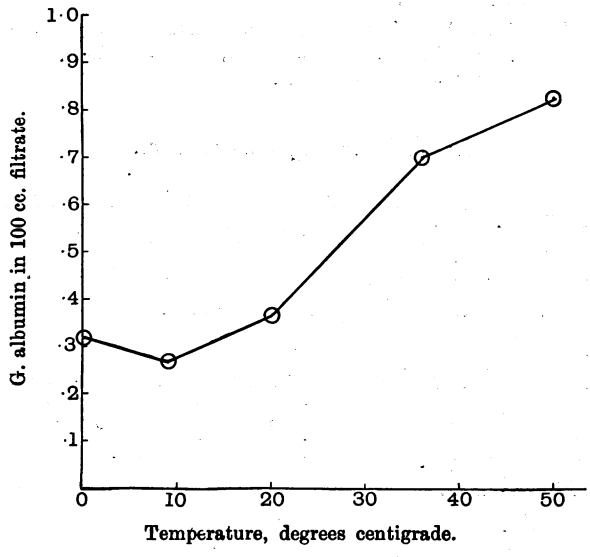

Fig. 6. Influence of temperature upon the precipitation of egg-albumin by $(NH_4)_2SO_4$, see Table VII.

Concentration of protein, salt, water and hydrogen ions in whole system constant.

Interpretation of results.

From the experiments detailed above, it is clear that the amount of protein precipitated from solution (volume of the protein-rich phase) is

dependent upon the amount of salt present, concentration of protein, concentration of hydrogen ions and temperature.

Spiro [1904] showed that, in the "salting out" of caseinogen and gelatin by sodium sulphate, the ratio of salt to water in the more protein-rich phase was less than in the watery phase—a similar relation to that found by de Bruyn [1900] in alcohol separation.

The same is true in the case of egg-albumin. An albumin solution was precipitated by ammonium sulphate and a complete analysis made of the original solution, the precipitate and the filtrate. In order to free the precipitate from adherent mother-liquor, it was not simply dried between filter paper, as done by Spiro, but placed between filter paper, surrounded by kieselguhr and submitted to a pressure of about 3 tons to the square inch. This pressure was, however, more than enough to squeeze out adherent mother-liquor, and actually removed some of the imbibed water of the protein-rich phase, for the concentration of salt in the liquid expressed, in the one case where it was collected and analysed, was only about half of that in the watery phase. The analyses given in Table VIII, where the results of these experiments are set out in detail, do not therefore express the composition of the two phases which were in equilibrium. The experiments prove, however, that the protein had appropriated some of the water for, notwithstanding this squeezing out of weak salt solution, the ratio salt to water in the compressed cake was considerably less than in the watery phase. See Table VIII.

This means that egg-albumin, like caseinogen and gelatin, dissolves or imbibes water just as alcohol does, and a mixture of protein, salt, water can be made to separate into two phases, either by the further addition of salt or of protein in the same way as a mixture of alcohol, salt, water separates on the addition of either salt or alcohol. In either case it amounts to increasing the concentration of salt in the watery phase.

To explain the influence of hydrogen ion concentration in the precipitation of egg-albumin, we must suppose that the electrical condition of the colloidal particles is a factor which modifies the ease with which they aggregate under the influence of the salt present. Hofmeister [1889] was influenced by a similar idea when he made parallel experiments with colloidal ferric hydroxide and egg-white, and Posternak [1901, 1 and 2], when investigating the precipitation of vegetable globulins, found that the efficiency of various electrolytes was influenced by the reaction of the suspension and presumably by the charge originally carried by the protein particles.

TABLE VIII.

Precipitation of pure egg-albumin by ammonium sulphate; composition of filtrate, pressed precipitate and press-liquor.

Exp.	Composition % by weight of	In original mixture	In pressed precipitate	In filtrate	In press liquor
I	Egg-albumin ...	9·56	64·65	0·84	—
	$(NH_4)_2SO_4$	23·35	29·83	26·67	—
	H_2O	67·08	6·39	72·49	—
	Salt present in 100 parts salt and water	25·82	17·6	26·9	—
II	Egg-albumin ...	12·48	63·69	0·59	—
	$(NH_4)_2SO_4$	22·60	29·91	27·04	—
	H_2O	64·92	8·47	72·37	—
	Salt present in 100 parts salt and water	25·82	22·1	27·2	—
III	Egg-albumin ...	9·47	73·57	1·58	0
	$(NH_4)_2SO_4$	24·09	22·04	27·66	15·2
	H_2O	66·42	6·39	70·76	84·8
	Salt present in 100 parts salt and water	26·62	22·47	28·1	15·2

The particles of proteins in acid or alkaline solutions carry respectively a positive or negative electric charge. Only at the iso-electric point, which is slightly to the acid side of the true neutral point in all cases hitherto investigated, does the charge disappear.

Salts like ammonium sulphate and sodium sulphate will cause separation into two phases, one protein-rich and the other protein-poor, whatever the charge upon the protein aggregates of the colloidal solution, if enough of the salt be added. Such phase separation is very materially assisted if the particles are positively charged, i.e. in solutions more acid than the iso-electric point. In the case of sodium chloride, indeed, acidification is necessary with many proteins.

From the analogy of the "salting out" of alcohols, phenols, etc. one must suppose that, by the gradual withdrawal of water from the protein aggregate by the salt, a critical dispersion point is reached when the surface tension at the interfaces causes the particles to run together. Supposing the particles are not iso-electric with the continuous phase, either from the original solution being acid or alkaline, or from the preferential adsorption of one of the ions of the electrolyte, the possession of charge will lower surface tension, so that, with charged particles, a higher concentration of salt will be required to arrive at the critical dispersion point. With negatively charged particles, in an alkaline solution, the charge cannot be neutralised by the more potent

ion SO_4'' which, if adsorbed, would still further increase the negative charge. We find that once the solution is more alkaline than the neutral point the amount of alkali added makes no difference[1] to the amount of salt required for precipitation, see Exp. III, Table VI and curve Fig. 5. The moment the reaction is made more acid than the iso-electric point and the protein particles carry a positive charge, this will at once be neutralised by the adsorption of SO_4'' ions. As these are in such high concentration they are apparently able to counteract in this way the maximum positive charge imposed upon the particles by the addition of acid (see Exp. III, Table VI, $H^+ = 0{\cdot}007$ normal).

We venture to put forward the above interpretation from the analogous action of SO_4'' upon protein particles under conditions which permit of the demonstration of the existence of charge. In acid solution, protein particles, carrying a positive charge, have been shown to be sensitive to the anion of any electrolyte they may encounter. (Hardy [1900] for heated egg-white; Chick and Martin [1912] for denaturated serum proteins and egg-white; Chick [1913] for euglobulin and caseinogen.) Arguing from analogy we may suppose that in the case of egg-albumin the SO_4'' ion of ammonium sulphate will also be more readily adsorbed and any charge on the protein neutralised if the solution be originally on the acid side of the iso-electric point, i.e. if the protein particles carry a positive charge.

The statements detailed above have not actually been substantiated in case of pure egg-albumin, nor has the iso-electric point been determined. This has, however, been done in case of serum-albumin [Michaelis and Mostynski, 1910; Michaelis and Rona, 1910], and these two proteins otherwise display close similarity as regards the conditions of their solution or precipitation. We have not been able to put our interpretation to the direct test because it is impracticable to determine the charge carried by the particles of egg-albumin in the presence of excess of ammonium sulphate, nor under these conditions were we able to study the influence of addition of ions of varying valency. To test our hypothesis we therefore had

[1] In the case of Na_2SO_4, which as regards the influence of acid behaves in an analogous manner to $(NH_4)_2SO_4$, excess of alkali (NaOH) favours precipitation, but, compared with the acid, a high concentration is required. In accordance with the explanation of the influence of reaction set forth above we presume that, while in acid solution the positively charged protein particles attract the SO_4 ion, in alkaline solution the Na ion of the sodium salt is preferentially absorbed. The electric charge on the particles is neutralised in both cases, but more readily in the first, owing to the greater potency of the SO_4 ion. With $(NH_4)_2SO_4$ no effect in solutions made alkaline with ammonia can be demonstrated, owing, presumably, to the low ionisation of $(NH_4)OH$, especially in the presence of excess of $(NH)_2SO_4$.

recourse to the expedient of withdrawing the water from the protein-water-combination by the addition of alcohol to the point where a surface tension is just manifest at the interfaces. In other words, the alcohol was added until the solution became opalescent but short of commencing precipitation, which could then be brought about by addition of a small amount of various electrolytes. Two sets of experiments were made; in one set the original protein solution was acid, and in the second set alkaline, and it was found, as was expected, that an electrolyte was efficient in causing precipitation of the protein in order of increasing valency of its anion in the first case and of its kation in the second. All solutions contained the same concentration of alcohol.

The results of a series of experiments with serum proteins are given in Table IX. In solution A (acid) salts containing divalent (ammonium sulphate) and trivalent (sodium citrate) anions caused precipitation in respective concentrations of 0·00055 and 0·00036 molar, whereas, in case of a monovalent anion (magnesium nitrate) about ten times the concentration was required. The valency of the kation was not without effect, but worked in the opposite direction; a small concentration of lanthanum nitrate (0·0007 molar) cleared up the original opalescence of the solution. For the same reason magnesium sulphate proved to be a much less efficient precipitant than ammonium sulphate.

An exactly converse set of results was obtained when the protein solution was originally made alkaline (B. Table IX). In this case lanthanum nitrate was the most powerful precipitant of all the salts tried and the sulphate of magnesium was much more effective than that of ammonium. At the same time a small concentration of sodium citrate (0·0007 molar) caused the solution to become clear.

With alkaline solutions higher concentration of lanthanum nitrate (0·0036 molar) prevented the formation of a precipitate, no doubt owing to the acquisition of a positive charge in excess of that needed to neutralise the negative one originally possessed. In acid solution an analogous result followed addition of sodium citrate to a concentration of 0·0007 molar.

Some experiments made with pure egg-albumin are set out in Table X. The original solution was acidified and after addition of sufficient alcohol to cause turbidity, determination was made of the concentration of a series of sodium salts necessary to cause a precipitate to form. The influence of increasing valency of the anion is very marked, sodium citrate and sulphate being respectively about 800 and 25 times as powerful in this respect as sodium chloride.

TABLE IX.

Precipitation of serum proteins by various electrolytes in presence of alcohol (almost to precipitation) at 0°.

Protein content = about 0·1 %.

A. In acid solution.

Salt	Conc. (molar)	Conc. (normal)	Degree of precipitation	Conc. (molar) required for complete precipitation
$Na_3\overline{Cit}$	0·00007	0·00022	+	0·00036
	0·00036	0·0011	+ +	
	0·00071	0·0022	+	
	0·0036	0·011	−	
$(NH_4)_2SO_4$	0·00011	0·00022	+ −	0·00055
	0·00055	0·0011	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
$MgSO_4$	0·00055	0·0011	+	0·0011
	0·0011	0·0022	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
$Mg(NO_3)_2$	0·0011	0·0022	+ −	0·0055
	0·0055	0·011	+ +	
$La(NO_3)_3$	0·00071	0·0022	−	
	0·0036	0·011	−	

B. In alkaline solution.

$La(NO_3)_3$	0·000071	0·00022	+ −	0·00036
	0·00036	0·0011	+ +	
	0·00071	0·0022	+ +	
	0·0036	0·011	+	
$Mg(NO_3)_2$	0·00011	0·00022	+ −	0·00055
	0·00055	0·0011	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
$MgSO_4$	0·0011	0·0022	+ +	0·0011
	0·0055	0·011	+ +	
$(NH_4)_2SO_4$	0·0011	0·0022	+ − *	No precipitation
	0·0055	0·011	+ − *	
$Na_3\overline{Cit}$	0·00071	0·0022	−	,, ,,
	0·0036	0·011	−	

* Clearer than control solution containing alcohol only.

− = clear solution, clearer than the control, containing alcohol only.

− + = opalescent solution.

\+ = partial precipitation.

+ + = complete precipitation, filtrate protein-free or containing trace only.

TABLE X.

Precipitation of pure egg-albumin, in acid solution, by sodium salts, in presence of alcohol almost to precipitation; influence of anions.

Protein content = 0·7 %.
Temperature, 0°.

Salt	Concentration required to cause precipitation in presence of alcohol (Molar)	Concentration necessary to commence precipitation in absence of alcohol (Molar)
Citrate (neutral)	0·00013	1·37
Phosphate	0·00016	2·50
Tartrate	0·003	1·60*
Sulphate	0·004	1·69
Acetate	0·09	—
Chloride	0·10	4·3*
Chlorate	0·35	—
Chromate	0·4*	1·71*

* Did not precipitate.

In the same Table are given the results of a series of experiments in which precipitation took place in absence of alcohol and it will be seen that no such influence of valency can be detected here. The relation of the various electrolytes, with the exception of sodium citrate, is that expressed by the Hofmeister series [1888] and doubtless conditioned by their relative water-drawing capacity. When the protein is on the point of being precipitated after the necessary water-withdrawal has taken place, the influence of the anion or kation of the electrolyte present can complete precipitation by neutralising a charge. This occurs for example when protein almost precipitated by ammonium suphate is made slightly acid, or when to protein almost thrown out by alcohol a trace of an appropriate electrolyte is added. In the former case, as long as the solution remains alkaline, the effect of presence of ammonium sulphate with its divalent anion will rather be to increase the negative charge on the protein.

An additional piece of evidence in support of the view that the charge carried by the protein particles is an important factor in determining the concentration of salt requisite to occasion separation into two distinct phases, was obtained from studying the "salting out" of egg-albumin with calcium chloride. In this case the positive ion of the electrolyte is prepotent and we should expect it to act more efficiently if the solution containing the protein is made more alkaline than the iso-electric

point. This proved to be the case[1]. It was found that in a solution containing 0·8 % protein and 37·7 % $CaCl_2$ an opalescence was developed on standing for about 2 hours at 18° when the hydrogen ion concentration was about 23×10^{-7} normal. In presence of a small concentration of $Ca(OH)_2$, under otherwise similar conditions, the hydrogen ion concentration fell to about $0{\cdot}05 \times 10^{-7}$ normal. In this case almost all the protein was precipitated and only a trace remained in the filtrate.

Precipitation by calcium chloride differs from that by ammonium sulphate in the fact that the process is irreversible, and the precipitate is formed slowly and becomes insoluble. We were not able to make satisfactory experiments with other electrolytes of the same character, e.g. the nitrates and chlorides of magnesium and barium, because with egg-albumin precipitation is only very partial even in alkaline solution, but in these cases also, we were able to satisfy ourselves that alkalinity of the solution assisted the separation of a precipitate (protein-rich phase).

Summary.

1. The precipitation of egg-albumin by ammonium sulphate is, as Spiro demonstrated to be the case with sodium sulphate, and caseinogen and gelatin, due to the separation of the system into a protein-rich phase and a watery phase, and to a certain extent is analogous with the salting-out of alcohol.

2. The first effect of concentrated salt is to withdraw water from the protein aggregates. A surface tension is in consequence developed at the interfaces, which causes the protein particles to aggregate, thus dividing the system into two distinct phases (precipitate and filtrate).

3. All three constituents of the system, viz. protein, water and salt, are present in each phase; the proportion, however, is different. The precipitate (protein-rich phase) contains relatively little water and salt, and the filtrate (watery phase) relatively little protein. Under appropriate conditions practically all the protein may be precipitated, only a trace remaining in the filtrate.

The two phases are in equilibrium, and any alteration in the amount of any one of their three constituents is followed by a change both in their composition and volume. Thus increase in concentration of salt or of protein

[1] Precipitation by calcium chloride is also assisted in cases where the solution is markedly acid, a comparatively large concentration of acid (HCl) being needed to demonstrate the phenomenon. The explanation is exactly analogous to that offered for the case of sodium sulphate, see footnote, p. 393, but in the reverse sense, much more acid being required to produce this effect than is the case with alkali.

is followed by a corresponding increase in the protein-rich phase (precipitate). Owing to rigidity of the latter readjustment is, however, slowly accomplished.

4. The relative volume of the two phases (amount of precipitate) is altered by varying the temperature.

5. The exact proportion of salt, protein and water at which phase separation (precipitation) occurs and the relative volume of the two phases (amount of the precipitate) is very sensitive to hydrogen ion concentration in the neighbourhood of the iso-electric point. In the case of $(NH_4)_2SO_4$, when the hydrogen ion concentration varied from 10^{-6} to 10^{-5} normal, the amount of the precipitate increased from a negligible amount to a maximum.

6. With $CaCl_2$, in which the kation is prepotent, a similar effect was observed, but in the opposite direction.

7. An interpretation of the dominating influence of hydrogen ion concentration over this range is put forward and some experiments in support of it adduced.

The principles discussed in this paper must be borne in mind whenever salting-out is made use of in the fractionation of protein solutions and the purification of isolated fractions by re-solution and reprecipitation. Conclusions as to the homogeneity of proteins isolated by salt precipitation must also be reconsidered in the light of these results.

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