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THE SOLUBILITY OF PROTEINS

BY S. P. L. SÖRENSEN

RECEIVED SEPTEMBER 9, 1924 PUBLISHED FEBRUARY 5, 1925

One of the most important questions today concerning protein studies is how far it is possible to apply to protein solutions the theories derived from the study of real solutions; or whether such colloid solutions as those of protein are so divergent in their behavior from ordinary real solutions that to parallel the behavior of colloid solutions and real solutions must be considered inadvisable, or even warned against, as Wo. Ostwald has often done.

I believe there is a growing tendency to think that Ostwald is not right in his view of the matter, but that the need simply is, by improved methods and particularly by the development of more exact analytical procedures to make it possible to deal with protein solutions in accordance with general physicochemical views.

The space allowed does not admit of a more particular discussion of the great work already done in this direction; I must be content to mention a few names in order to throw some light on the nature of the researches here under discussion.

The investigations of Harriette Chick and C. W. Martin[1] on the coagulation of protein have demonstrated, among other things, that the denaturation process is a monomolecular process, provided that the hydrogen-ion concentration be kept constant.

The elaborate investigations of Wolfgang Pauli and his collaborators on proteins, especially in acid or alkaline solutions, should also be mentioned. Pauli has succeeded in showing that proteins form salts of the same nature and character as simple ampholytes.

Further should be mentioned Jacques Loeb, recently and prematurely deceased, whose studies on the so-called "Donnan equilibrium" in connection with the physicochemical properties of protein solutions have thrown light on so many hitherto unknown matters.

Finally, among all these examples, to which many more might be added, I may perhaps venture to mention the investigations on hen's egg albumin conducted in the Carlsberg Laboratory, by which we succeeded in demonstrating that in many and essential cases the theories developed from the study of real solutions may be applied also to the solutions of egg albumin. This is true, for example, in such important cases as the state of equilibrium by salt precipitation, the application of Gibbs' phase rule, the question of the osmotic pressure of albumin solutions and in many more instances.

[1] Chick and Martin, *J. Physiol.*, **40**, 404 (1910); **43**, 1 (1911); **45**, 61, 261 (1912).

The present discussion, then, concerns the precipitation of some proteins, and the conditions of their solubility in water and salt solutions, these conditions being but imperfectly known, yet of no small importance.

I shall first briefly mention the equilibrium conditions of albumin when crystallized by means of ammonium sulfate and then somewhat more fully discuss the solubility of the globulins of horse serum, more particularly that of euglobulin.

The Precipitation of Albumins by Ammonium Sulfate

If Gibbs' phase rule, which applies to real solutions, be applicable also to protein solutions, a precipitation of the solution by means of a salt must result in the same state of equilibrium between the precipitate and the surrounding mother liquor, irrespective of whether the initial solution be concentrated or diluted, if only care be taken that, at the end of the process, the factors that influence the precipitation (temperature, salt concentration, hydrogen-ion concentration) are alike in all cases. Under such conditions much albumin should be precipitated from a strong albumin solution; and from a weak solution, on the other hand, only little; but the final albumin concentration in the solution must in every case be the same, provided that Gibbs' phase rule applies.

However, according to the generally accepted view, the protein solution does not behave in accordance with the phase rule, the common opinion being that, under otherwise equal circumstances, the protein is salted out the more completely, the higher the starting protein concentration. It is not necessary to enter on a discussion of the voluminous literature published on this subject. We may be content to elucidate the question by a single example. For this purpose I choose Harriette Chick and C. W. Martin's[2] otherwise most valuable work on the precipitation of egg albumin by ammonium sulfate, because the conditions of the experiments mentioned there are analogous to those under which our

TABLE I

PRECIPITATION OF PURE EGG-ALBUMIN WITH AMMONIUM SULFATE. INFLUENCE OF CONCENTRATION OF PROTEIN

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

Albumin G.	Water G.	Salt G.	G. of albumin in 100 g. of total mixture	G. of albumin to 31 g. of salt and 100 g. of H_2O	G. of albumin in 100 g. of filtrate	G. of albumin precipitated from 100 g. of total mixture	Protein pptd. %
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

[2] Chick and Martin, *Biochem. J.*, **7**, 380 (1913).

experiments were conducted. In a series of experiments, reproduced from their paper in Table I, Chick and Martin investigated the influence of the initial albumin concentration on the precipitation of egg albumin by ammonium sulfate. In all the experiments of the series, the ratio, *ammonium sulfate to water, was constant, namely, 31:100, but the amount of albumin* varied in the mixtures from 3.3 to 27.4 g. per 100 g. of water. As may be seen from the sixth vertical column in the table, Chick and Martin find that the albumin is precipitated more completely, the higher the initial concentration.

Now, the error is due to the fact that Chick and Martin have taken it for granted that the proportion between ammonium sulfate and water does not change during the precipitation, and consequently they have made no determination of the amount of ammonium sulfate in the filtrates. It is, however, beyond doubt that a solution of egg albumin behaves during precipitation just as during crystallization, in which latter case we have demonstrated that 1 g. of egg albumin contains constantly 0.22 g. of water. If, however, it is clearly realized that the egg albumin by its precipitation removes water from the solution, it is evident that the higher the starting concentration of egg albumin is, the more water will be removed from the solution during the precipitation, and consequently the concentration of ammonium sulfate in the filtrate will be the higher. As an increase of the ammonium sulfate concentration causes a more complete precipitation of the egg albumin, Chick and Martin's experimental results may therefore, be accounted for.

Indeed, the experiments made by us in order to throw light on this question have proved that the amount of albumin remaining in the mother liquor by the crystallization of egg albumin is independent of the initial protein concentration, if only care be taken that at the end of the crystallization the ammonium sulfate concentration of the mother liquor is the same in all the experiments and that this is really so, must of course be demonstrated analytically.

In Fig. 1 is shown graphically the result of one of our series of experiments, the content of egg albumin in the mother liquor being used as ordinate and the crystallization time in days as abscissa. In all the experiments the ammonium sulfate concentration is the same, namely, 26.658 g. of ammonium sulfate per 100 g. of water; but the initial albumin concentration varies in the different experiments from 2.3 to 14.1 g. of albumin per 100 g. of water. There is a curve corresponding to each experiment, and it is plainly shown in the diagram that when the crystallization has proceeded for a few days, the amount of albumin in all the filtrates is practically the same. A more minute consideration of the experimental results will show that the small discrepancies between the results point in the opposite direction to what would be expected according to

the above-mentioned generally accepted theory; and besides, it is easy to give an explanation of these small discrepancies.

From what I have here stated, as well as from a series of other experiments which also include serum albumin, but which would carry us too far to enter on now, it is apparent that the behavior of albumins when precipitated with ammonium sulfate does not exclude the application of the phase rule in its usual form and with its usual consequences. What has been stated here about the precipitation of albumins by ammonium sulfate is probably also true of many other protein precipitations and whenever the opposite view has hitherto been commonly held, the reason must be sought in the defective experimental treatment of the subject.

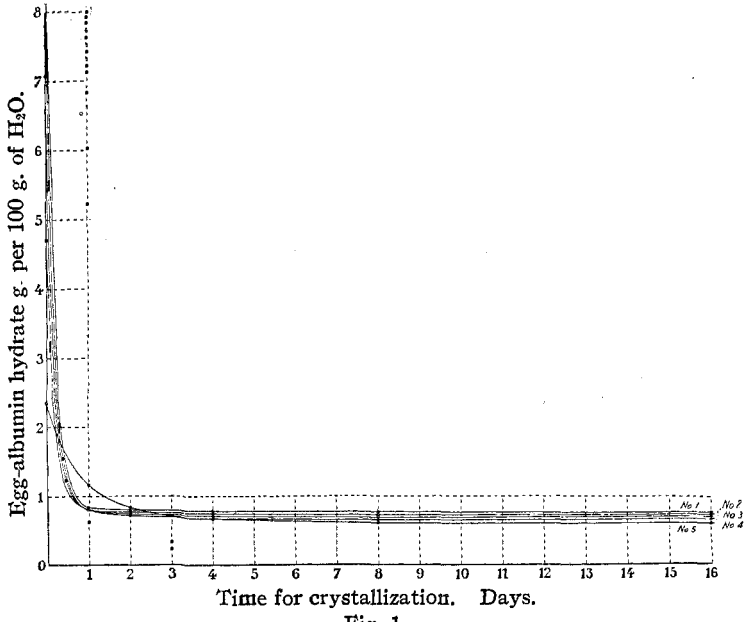
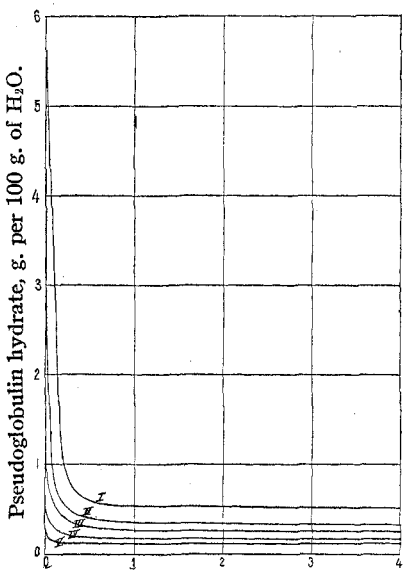


Fig. 1.

But this is not all. I think that one further step can and should be taken, namely, that such precipitations at different initial protein concentrations be employed as purity tests of the protein in question. There is at present hardly any better evidence as to whether one is dealing with a single protein, or with a mixture of or a compound of two or more proteins than just such precipitations at different starting concentrations of protein, and under such conditions that salt concentration and hydrogen-ion concentration are alike in all the experiments after the precipitation.

For the further elucidation of this I shall refer only to one instance. We have tested the degree of purity of a sample of pseudoglobulin prepared from horse serum and very carefully purified by repeated frac-

tionated precipitation, dialysis, solution in pure water and renewed fractionated precipitation. In Fig. 2 are graphically reproduced the results of five precipitation experiments, in all of which the ammonium sulfate concentration was 24.533 g. per 100 g. of water and the starting concentration of globulin varied from 5.6 g. of hydrated globulin (Curve I) to 0.28 g. (Curve V) per 100 g. of water. As in Fig. 1, the precipitation time expressed in days is used as abscissa and the globulin content of the mother liquor, as ordinate. It will be seen from the diagram that the globulin concentration of the mother liquor is by no means lower, the higher the starting concentration. On the contrary, the globulin concentration in the mother liquor is higher, the *higher* the starting concentration; and there is no doubt that this pseudoglobulin sample, in spite of the careful purification and fractionation, is not a homogeneous substance but, as demonstrated by other experiments on which I shall hardly have time to touch, probably consists of a combination of several globulin complexes. Possibly the small discrepancies observed in the experiments with egg albumin (Fig. 1) may be explained in the same way.



Time for precipitation days.
Fig. 2.

Solubility of Euglobulin in Water and Weak Salt Solutions

There are in serum two globulins: *euglobulin*, which contains phosphorus, is insoluble in water but soluble in weak salt solutions and is precipitated from its solutions by the addition of saturated ammonium sulfate solution to the extent of half the volume of the solution; and *pseudoglobulin*, which is free from phosphorus, is soluble in water and is precipitated completely from its solutions by the addition of an equal volume of saturated ammonium sulfate solution.

I shall not go into details regarding the methods we have adopted for the separation of euglobulin and pseudoglobulin, but merely mention that neither fractionation nor dialysis, nor a combination of the two methods, results in a complete separation of the two globulins. I have already mentioned an instance which proved that even very carefully purified pseudoglobulin is not a single substance and in the following

I shall mention a series of experiments which serve to show that it is not possible to prepare a pure euglobulin in this way either. In so doing I shall also have the opportunity of thoroughly elucidating the peculiar solubility conditions of euglobulin, which can be explained only from the viewpoint of a conception of the inter-relation of globulins which differs from the one hitherto accepted.

In all probability globulins occur in the serum itself as well as in the special globulin fractions obtained from it, not as mixtures of the two globulins, but as combinations of them. As to the nature of the combinations between the globulins, whether we have before us true compounds, as I am most inclined to think, or whether it is a question of solid solutions, or of a reciprocal adsorption between the different globulins, I shall venture no statement.

If a euglobulin complex molecule be designated E, and a pseudoglobulin complex or molecule, P, and if, for a provisional first guidance, we start out on the basis of the (most probably erroneous) supposition that the respective eu- or pseudoglobulin complexes are all alike, then such a combination may be written $EpPq$. The combination is readily dissolved in water and dilute salt solutions as long as the pseudoglobulin is in great excess, but gradually as the pseudoglobulin is split off by a simple dissociation process—as by diluting the solution with water—the remaining combination becomes richer and richer in euglobulin and therefore still more sparingly soluble and at last precipitates. Very characteristic of such a dissociation process is the circumstances that we are here in the presence of a real reciprocal alteration of equilibrium, so that the reaction (dilution with water, for example) does not occur *solely* between the combinations present in the solution, but the precipitate already once formed, takes part in the reaction until the new equilibrium is established between precipitate and mother liquor, corresponding to the decreased salt concentration brought about by the addition of water. Of course, the order of the process is reversed if the salt concentration be increased. I shall not go further into this, but merely mention that the behavior of salt precipitation in large salt concentrations may be explained from quite similar views.

The most elaborate researches on the solubility of euglobulin have been carried out by I. Mellanby[3] and W. B. Hardy.[4] In these classical works it is stated that the solubility of euglobulin in neutral salt solutions increases with the salt concentration where fairly dilute salt concentrations are in question, but it is also stated that the amount of globulin dissolved by any given salt concentration is approximately proportional to the total amount of globulin employed in the solubility experiment.

[3] Mellanby, *J. Physiol.*, **33**, 338 (1905).

[4] Hardy, *ibid.*, **33**, 251 (1905).

This highly peculiar circumstance, which we have found corroborated by our experiments can be explained, according to our general chemical and physicochemical theories, only on the basis of the above-mentioned assumption that the euglobulin employed is not a single substance, but can be dissociated by salt solutions. I shall here give but a brief account of a few of our series of experiments.

To begin, I would mention that we have found also that the solubility of euglobulin increases with the increased salt concentration, at any rate

TABLE II

Total globulin used, mg. *N*.....	29.06	58.11	116.22
100 cc. of sat. soln. contains, mg. *N*.....	10.95	21.75	42.15

as long as fairly small concentrations are in question. Furthermore, a globulin obtained by dialysis and subsequent treatment with water, after several washings with water showed the solubility in 0.02 *N* sodium chloride solution given in Table II.

TABLE III

PURIFICATION OF THE GLOBULIN FRACTION B_{III}

Nature of the globulin fraction	Solubility of the globulin fraction in 0.02 *N* NaCl-solution: Total amount of globulin nitrogen per 100 cc. used for the experiment mg.	Amount of globulin nitrogen dissolved per 100 cc. of solution mg.	Mg. of P per g. of globulin nitrogen
	26.22	3.23	
B_{III}	52.44	5.39	4
	78.66	6.89	
B_{IV}	11.92	1.13	
(B_{III} dissolved in 125 cc. of H_2O + 15 cc. of *N* NaCl, precipitated with 1 liter of H_2O; decantation of the mother liquor, washing with 250 cc. of H_2O, decantation of the washing water and suspension of B_{IV} in 200 cc. of H_2O)	23.84	1.64	4
	35.76	2.05	
B_V	20.71	0.82	
(B_{IV} dissolved in 140 cc. of H_2O + 30 cc. of *N* NaCl, precipitated with 1 liter of H_2O; decantation of the mother liquor, washing with 200 cc. of H_2O, decantation of the washing water and suspension of B_V in 95 cc. of H_2O)	41.42	0.87	6
	62.13	1.28	
B_{VI}	17.08	0.62	
(B_V dissolved in 45 cc. of H_2O + 35 cc. of *N* NaCl, precipitated with 850 cc. of H_2O; decantation of the mother liquor, washing with 190 cc. of H_2O, decantation of the washing water and suspension of B_{VI} in 100 cc. of H_2O)	34.16	0.62	10
	51.24	0.72	

The solubility of this well-washed sample of euglobulin is thus very nearly proportional to the amount of globulin employed in the solubility experiment, just as stated by Mellanby and Hardy.

A really effective purification cannot, however, be obtained by washing with water, but only by dissolving the euglobulin in a weak salt solution and subsequent precipitation by dilution with water. To throw light on this question I shall mention a series of experiments, in which the purification of a globulin fraction, B_{III}, was followed in all its details. In the experiment, B_{III} which was taken as the primary substance had already been reprecipitated once and was consequently rather sparingly soluble. It was further reprecipitated three times by solution in sodium

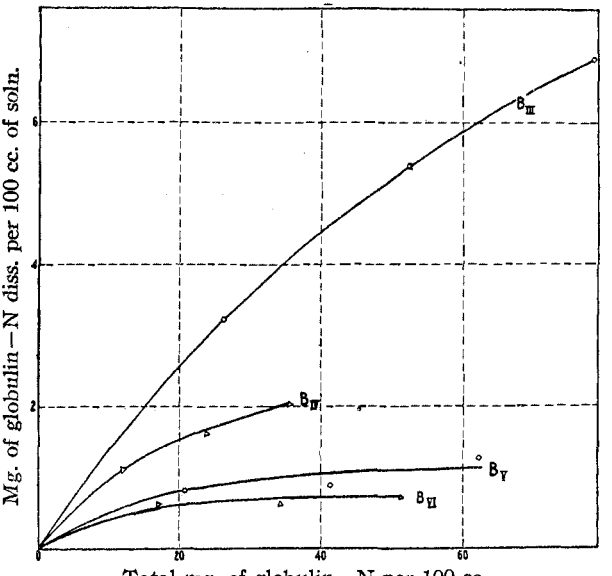


Fig. 3.

chloride solution and precipitation by water. B_{III} itself, as well as the products, B_{IV}, B_V, B_{VI}, obtained by the reprecipitations, was subjected to an analysis by which the solubility in 0.02 *N* sodium chloride solution was determined, three different but known globulin amounts of each fraction being used in the solubility deteminations.

The analytical results are grouped in Table III and graphically reproduced in Fig. 3, in which the total amount of globulin nitrogen is used as abscissa, while the globulin nitrogen in 100 cc. of saturated solution is used as ordinate.

From the table as well as from the figure it appears that the purification of the globulin not only causes a decrease of its solubility, but also makes

it approximately constant and independent of the total amount of globulin employed in the solubility experiment; graphically, this is shown by the curves in Fig. 3 more and more approaching a parallel with the abscissa axis.

If it were really a question of a single substance, this substance should have a constant solubility in water or in salt solution of a given concentration, irrespective of whether the surplus of globulin left undissolved is large or small. As just mentioned, however, euglobulin does not behave in this manner, neither after thorough washing with water nor after several reprecipitations.

Euglobulin can be treated so long and so often with water that the pseudoglobulin complexes, which can be dissociated in pure water are really split off and the remaining substance will consequently show—at least approximately—a constant solubility in water, independent of the amount of globulin employed. Thus Edwin J. Cohn,[5] in a series of carefully conducted and recently published experiments, has found that the solubility in pure water of well-washed euglobulin corresponds to about 1.2 mg. of globulin nitrogen per 100 cc. of saturated solution. We have been able to obtain exactly similar results, but if the purification of the globulin was continued, or was conducted not only by washing with water, but also by reprecipitation by means of neutral salt solutions, the reprecipitated globulin exhibited a considerably decreased solubility in water; furthermore, as shown in Table III, such an adequately reprecipitated fraction B_{VI} exhibits an approximately constant solubility in 0.02 N sodium chloride solution, which is lower than the solubility in pure water stated by Cohn.

However, even a substance such as B_{VI} cannot be regarded as a pure euglobulin; it exhibits indeed a fairly constant solubility in water and such weak sodium chloride solutions as 0.02 N, but in stronger salt solutions, where the dissociation of pseudoglobulin complexes can be carried still further, such a globulin preparation as B_{VI} shows a solubility dependent on the amount of globulin employed. On account of lack of material, we have unfortunately not been able to conduct such a solubility experiment with B_{VI}, but we have done so with another preparation called α_{a_4}.

The globulin sample α_{a_4} was purified by means of a series of reprecipitations, and was consequently one of the most sparingly soluble globulin fractions we have had to deal with. With this sample of globulin a series of solubility experiments was conducted in rather strong potassium chloride solutions, and with three different quantities of globulin in each concentration of potassium chloride. The experimental results are grouped in Table IV and graphically reproduced in Fig. 4, in which the potassium chloride concentration is used as abscissa, while the dissolved

[5] Cohn, *J. Gen. Physiol.*, **4**, 697 (1922).

euglobulin in terms of milligrams of nitrogen per 100 cc. of saturated solution is used as ordinate.

TABLE IV

SOLUBILITY OF THE GLOBULIN FRACTION α_{a_4} IN SOLUTIONS OF POTASSIUM CHLORIDE

Equilibrium by precipitation; 1 day's standing; 18°

Concentration of potassium chloride N	Total globulin nitrogen per 100 cc.		
	Mg.	Mg.	Mg.
..	4.53	9.31	18.61
	Globulin nitrogen dissolved per 100 cc. of soln.		
0.05	0.62	0.98	1.70
.10	2.63	4.22	6.33
.20	4.12	7.57	12.25
.50	4.17	9.27	18.07
1.00	4.12	9.27	18.27
2.00	3.91	8.18	16.00
2.80	..	5.20	7.36

From the table as well as from the figure it is apparent that the solubility of α_{a_4} in these comparatively strong potassium chloride solutions

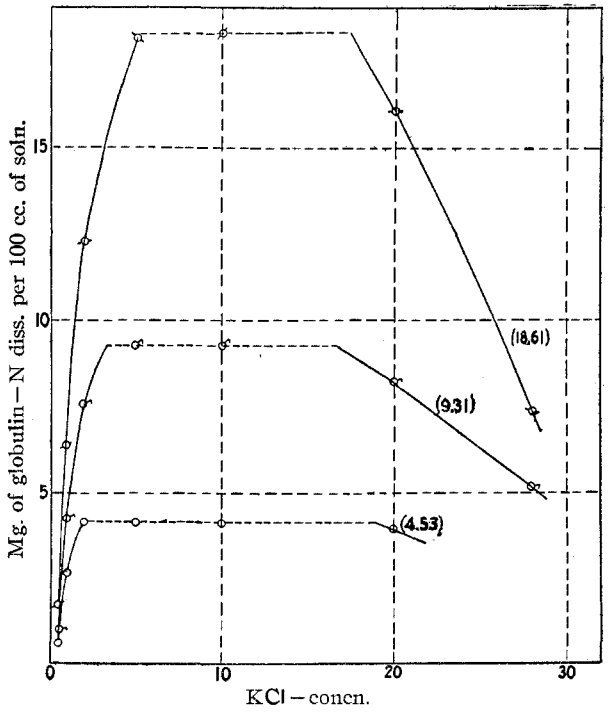


Fig. 4.

is largely dependent on the quantity of globulin employed in the solubility experiment, so that we here find exactly similar conditions to those

indicated by solubility determinations of less thoroughly purified euglobulin samples in water or dilute salt solutions.

It is further seen that in these experiments such salt concentrations have been used that the solubility of the globulin again decreases, no doubt owing to the precipitation of combinations between the globulin and the salt or one of its components. The middle parts of the curves are dotted, because in these cases we have hardly had wholly saturated solutions; at any rate, in these experiments the residue was extremely slight and the presence of small quantities of denaturized globulin must always be reckoned with.

We have in these experiments a striking instance of the fact that even a highly purified euglobulin can be further split up by dissolving it in concd. potassium chloride solution if the subsequent dilution with water is so slight that the precipitation of the globulin is only partial. When, on the other hand, the addition of water is abundant, all the globulin is precipitated anew. By further addition of water, the precipitate first deposited will combine with the globulin complexes present in the solution until a state of equilibrium corresponding to the decreased salt concentration has been established between the precipitate and the globulin in the solution. That such an interaction occurs between the precipitate first deposited and the globulin present in the solution, and that it is not a question of a continuous precipitation of the same compound, can be demonstrated by a fractionated precipitation with water. This fractionation must naturally be so conducted that the first deposit, which must contain relatively the least amount of pseudoglobulin and consequently be most sparingly soluble, is filtered off before a further addition of water takes place.

Such a fractionation was conducted with α_{a_4}, which was dissolved in 0.5 N potassium chloride solution; from this solution α_{a_4}I was precipitated by the addition of a little water, from the filtrate of this precipitate by addition of more water, α_{a_4}II, and finally from the filtrate of this precipitate, α_{a_4}III.

Of the solubility of these globulin fractions in 0.1 N potassium chloride solution as compared with the solubility of the original substance α_{a_4}, Fig. 5 gives particulars.

It appears from the figure, in which the total amount of globulin nitrogen is used as abscissa, while the dissolved amount of globulin nitrogen per 100 cc. of saturated solution is taken as ordinate, that the three fractions had widely different solubility and that—as was to be expected—especially Fraction I, but also very markedly Fraction II, showed less solubility; Fraction III, on the other hand, showed greater solubility than the starting material, α_{a_4}.

The peculiar solubility conditions, characteristic for euglobulin, which

are mentioned here, can scarcely be explained in any other way than that I have adduced, namely, on the basis of the supposition that ordinary euglobulin must be regarded as a combination of euglobulin and pseudoglobulin, *EpPq*.

I should like to discuss the solubility conditions of pseudoglobulin as fully as I have discussed those of euglobulin, but time will not permit. I must be content to point out a single circumstance, which is closely connected with that which I have just stated about euglobulin.

Ordinary pseudoglobulin solution, in my opinion is also composed of a complex such as *EpPq*, but with a very slight proportion of euglobulin to that of pseudoglobulin, as the quite minimal content of phosphorus goes to show. When such a solution is precipitated by ammonium sul-

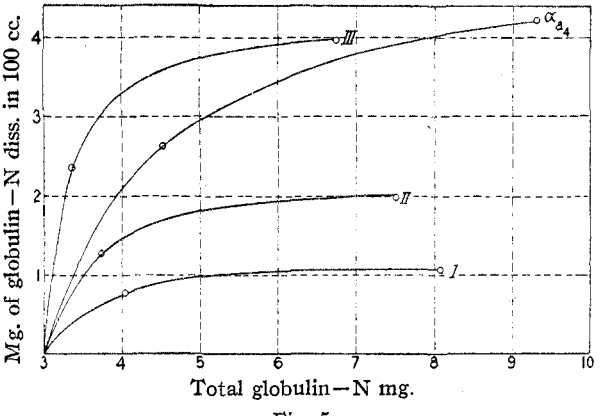


Fig. 5.

fate solution, the precipitate first deposited, which is richest in euglobulin, will not remain passive by the further precipitation but will, when the state of equilibrium is altered by renewed addition of salt, combine with the pseudoglobulin of the solution. Therefore the solution, being precipitated to the same ammonium sulfate concentration, will be poorer in globulin if the precipitate first deposited is allowed to be present during the continued precipitation than if it is filtered off before the precipitation is proceeded with.

In Fig. 6 the bottom curve shows such a precipitation of a pseudoglobulin solution to different ammonium sulfate concentrations which are used as abscissas, while the ordinates are the quantities of globulin left in the solution. The one above the bottom curve shows the same conditions for the same globulin solution, but such that the solution has first been precipitated to an ammonium sulfate concentration of 20 g. per 100 g. of water, whereupon the precipitate has been filtered off, and the precipitation of the filtrate continued *without the presence of the first precipitate*. It is evident

from the curves that, under otherwise equal conditions, the precipitate is greater and the amount of globulin in the filtrate is therefore less, when the first precipitate is allowed to remain.

I perfectly realize that some of the phenomena which I have adduced here come under what is generally termed "mechanical carrying down" or "adsorption;" others come under the so-called "protective colloid" cate-

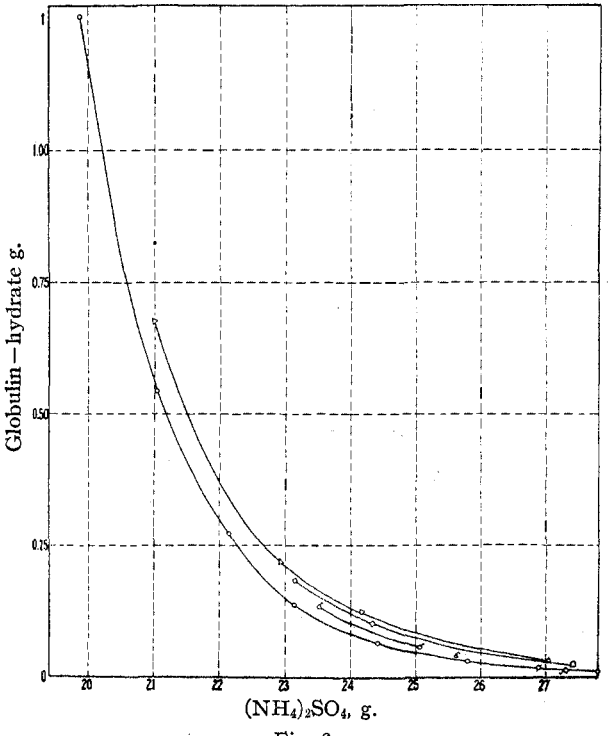


Fig. 6.

gory. The reason why I have left these categories out of consideration is that I have desired to make an attempt at approaching the question somewhat more closely than it is possible by the application of these very capacious, but not very elucidating classification terms.

Only by the application of purely chemical and physicochemical theories shall we succeed, I think, in bringing this very important problem of serum globulins, which occupies so many minds but is still unsolved, one step further toward solution.

COPENHAGEN, DENMARK