XLIX. SEPARATION OF PROTEINS. PART III. GLOBULINS[1].

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(*Received July 27th, 1913.*)

Up till the middle of last century it was generally held that the protein of blood serum and other kindred fluids was a single homogeneous substance —albumin. The word globulin, apparently first used by Berzelius, was applied by him to two substances, the protein part of haemoglobin (called by him haemato-globulin) and the protein of the lens, and came thus to be associated with cell-protein. That the serum protein could be split up, or that serum contained more than one protein was first suspected on account of some experiments of Liebig, Zimmerman and others, who found that great dilution of blood-serum produced a precipitate, especially after neutralisation with acetic acid. It was, however, Panum [1851] of Copenhagen who first discovered serum-globulin, and who developed the now classical method of preparation by first diluting the serum with water, and then adding acetic acid. Panum showed that the substance occurs constantly in human blood both in health and in disease, and called it serum casein from its resemblance to milk casein. Shortly after Zimmerman [1854] published the carbonic acid gas method of preparation. A few years later Alexander Schmidt [1862] in the course of his extensive observations on serum and kindred fluids showed in all cases that, side by side with the more soluble albumin, there was always the more insoluble globulin. In pursuance of his views on blood clotting he called the globulin in serum fibrino-plastic substance, though as often as not in his writings he calls it globulin.

Considerable activity followed on the publication of Schmidt's views: several ways of producing protein precipitates in serum were discovered and discussed; and there was some little confusion as to whether there was more than one substance, and as to nomenclature, till Heynsius [1869, 1876] gave

[1] Part I Haslam [1905], Part II Haslam, [1907].

good reasons for believing that, whether precipitated by simple dilution, carbonic acid gas, dilute acids, or saturation with sodium chloride there was only one substance—para-globulin as it was then called—a conclusion that, except in regard to sodium chloride, still holds good.

In 1878 Hammarsten [1878] published his great attempt at the purification and estimation of this substance. He introduced magnesium sulphate which produced a much larger precipitate than the reagents hitherto employed. In 1883 Burckhardt [1883], in repeating Hammarsten's experiment showed that, in addition to the globulin which was insoluble in water, a water-soluble substance was contained in the magnesium sulphate precipitate. Thus the probability of the existence of a third protein in serum was shown, though Hammarsten maintained that the magnesium sulphate precipitate was a single substance. Burckhardt's observation was, however, confirmed by Marcus [1899].

Meanwhile Hofmeister had conceived the idea of dividing serum and other liquids into fractions by means of taking precipitates at different degrees of concentration of one and the same salt solution, and thus isolating the different proteins. The first attempt was carried out by Kander [1886] when he precipitated globulin by half-saturation with ammonium sulphate, and albumin by complete saturation of the filtrate, the globulin roughly corresponding to Hammarsten's. Later on this system was elaborated by Pick [1902], Fuld and Spiro [1900], and others, both in the case of albumoses and serum. It was at first found that the globulin brought down at half-saturation could be split into two portions. The first was found to resemble the original water-insoluble substance of Panum, Schmidt, Heynsius and others, and was accordingly named eu-globulin; the second was more soluble and was called pseudo-globulin. In a further research Porges and Spiro [1903] thought there were three distinct fractions, both by ammonium, sodium, and magnesium sulphate; and Reiss [1904] also in Hofmeister's laboratory, decided on three—eu-globulin, pseudo-globulins I and II. Freund and Joachim [1902] on the other hand, taking the two fractions eu- and pseudo-globulin, showed that each contained a water-soluble portion, and took the view that there were four globulins. In regard to this system of fractionation I showed by direct experiment in Part I [1905] of this series that no single precipitation, both in the case of albumoses and serum proteins, whether by acid, salts of heavy metals, salting out or alcohol, ever produced a complete separation: that in the precipitate the substance of the filtrate could always be demonstrated, often up to 20 or 30 %; and similarly, mutatis mutandis, with the filtrate. And Wiener [1911], working on quantitative lines, showed that

reliable estimations of globulin could not be made by single precipitations. J. Mellanby [1907] from a determination of the percentage of protein at gradually increasing concentration of alcohol drew the conclusion that there were three different proteins in serum. Fractional methods, then, seemed to have left the subject in a more confused state than that in which they found it. I believe, however, and hope to show in these pages, that the method is inherently sound, and must for the present be regarded as one of the most important, and in many cases the only, method, we have of separating proteins.

Fractional precipitation.

The fundamental observation on which the method rests is that when a precipitant is added gradually to a protein fluid, and the resulting precipitate collected in successive portions, it is found that one portion differs from another. It is inferred from this that a separating process has been set up. And the proof of the correctness of this inference is found in the fact that if the process is continued sufficiently, substances that are undoubtedly distinct from each other can be obtained. Now in the case of the precipitant being a salt such as ammonium sulphate each increment of the salt in the protein fluid is followed by an increment of the precipitate. It is possible that at some concentrations the precipitate falls rather more thickly than at others: and indications may thus be afforded as to how a separation may be attempted. But it is entirely fallacious, as I have already pointed out, to suppose that one protein is wholly, or even nearly wholly precipitated before the next begins to come down. If there are two or more proteins their precipitation commences almost if not quite simultaneously, and they continue to come down together till the end of the precipitation, though at any one time different quantities of each might be coming down. Mellanby has demonstrated this point in the case of serum and ammonium and magnesium sulphate, having made quantitative experiments and plotted curves therefrom. He finds the precipitate falls very uniformly and that at no point is there a cessation of precipitation. His conclusion, however, that no splitting up of the serum can be brought about by these means, is erroneous. If no separation were brought about by the salting out, the protein in any one fraction would resemble that in every other. But this is obviously not the case. The chief cause of confusion hitherto has been of the opposite kind; that there has been no way of determining in the case of differing fractions, whether each fraction connotes a separate substance or not.

Let us suppose that there are two substance A and B; that A in a pure

state is precipitable more easily and is all precipitated at half[1] saturation; while B, in a pure state, does not commence to come down till the half-saturation point is reached, and only comes down completely on full saturation. Suppose now that three successive precipitates are taken at one-third, two-thirds, and full saturation. The portions would be composed as follows:

Fraction 1.	n_1 parts A,	m_3 parts B.
2.	n_2 parts A,	m_2 parts B.
3.	n_3 parts A,	m_1 parts B.

where $n_1 > n_2 > n_3$ and $m_1 > m_2 > m_3$.

Each fraction would thus show some difference from every other. Let us suppose that each fraction is redissolved and again precipitated at the same concentration. The three fractions would then differ from each other more markedly. The first would contain a greater proportion of A; the last would contain a greater proportion of B; while the middle one would remain about the same, much the greater part coming down between the precipitation limits. Generally speaking the separating power of the salt is not sufficient to cause any great change at any one precipitation. Thus far we might consider that we were dealing with three separate substances.

There are two principle methods by which to determine whether a fraction represents a substance or not.

1. Constancy of quantity under repetitions of the process. Fractions 1 and 3 would show this constancy after a time. Fraction 2 consisting of a mixture, would not, but would gradually disappear.

2. Subdivision of a fraction to find whether it is consistent or not. Fraction 2 on subdivision would show that it consisted of substances properly belonging to fractions 1 and 3. At half-saturation, in short, it can always be divided into a precipitate containing a higher proportion of A than B, and a filtrate containing more B than A.

Having decided on the number of substances into which the parent body can be split, there next arises the question as to how far each can be separated from the other. In the case of A, after a certain number of precipitations, the filtrates contain a constant quantity of organic nitrogen: that is to say, the separating process has entirely ceased. The separation, therefore, is presumably complete, but I will return to this point later. In regard to B, when we arrive at the point that half-saturation produces no precipitate, we cannot infer that the separation is complete. I have shown in previous

[1] As in previous papers I follow the usual convention of describing as "half" saturated a solution made by mixing equal volumes of saturated salt and protein solution. In reality such a solution is less than half saturated.

papers that, if a further small addition of salt be made, the resulting precipitate will consist largely of *A*; that is to say, a quantity of *A* remains dissolved in the *B* fraction. Means must be adopted, therefore, to continue the separation. Hitherto, the procedure has been to take a small fraction, dissolve it, and reprecipitate at the same concentration. The precipitate consisting mostly of *A*, is withdrawn, while the filtrate is returned to the main solution. This may be continued until no further trace of *A* can be found in the fractions. The separating process, then, comes to an end in this case also. But although the separating process has come to an end we cannot assume that the substances are completely separated, because in analogous separations in fractional distillation, crystallisation and precipitation it may happen that a certain amount of the substance being got rid of remains with the substance being purified, the two together, in this instance, acting as one substance towards the separating agent. Proof positive, therefore, can only be obtained by means of independent reactions. In the case of most proteins these, at present, are few in number: it is obvious that some separation must precede the discovery of typical reactions based on constitutional differences. In one of the separations to be described, we have such a reaction, and it goes far, I think, in demonstrating the validity of fractional precipitation in the case of serum proteins.

In regard to the question of the chemical individuality of the products of such separation, it can only be said that it is convenient to regard them as individuals until they are shown to be capable of further subdivision. Each can be tested with all the means at our command.

First Separation.

In the case of serum (ox-serum was used in all these experiments) my first procedure was to divide it by means of half-saturation with ammonium sulphate. The serum was diluted some four times with water, and an equal volume of the saturated salt solution was added. The resulting precipitate was collected, redissolved in water, and the process repeated until the separation, as shown by the sulphuric acid decomposition test[1], was complete. The separation was an easy one at this concentration; three or four precipitations got rid of all but a small quantity of albumin, and the

[1] 8–10 c.c. of the filtrate are mixed with an equal volume of concentrated sulphuric acid in a test-tube and warmed to boiling point. As the protein in the filtrate diminishes the tint gets lighter and when two successive filtrates give the same tint the amount of protein in the filtrates has become constant and the separation is at an end.

separation was completed in some six precipitations. The globulin fraction so obtained was almost entirely soluble in salt and water.

I then sought to divide it further by ammonium sulphate. Precipitation at one-third saturation (one volume of saturated solution being added to two volumes of the globulin solution) was carried out, and in this way the protein was divided into two fairly equal portions, the greater portion, perhaps, being that precipitated. The precipitate was re-dissolved and re-precipitated; and the process was repeated until the filtrate showed only small quantities of protein. During the progress of the precipitations, a larger and larger portion of the precipitate became insoluble in salt and water, and the experiment, on this account was not continued to a point of constancy in the usual way, since the insoluble matter might have held to itself soluble protein and so have vitiated the result. It served to indicate, however, that the globulin of half-saturation could be further divided into a water-insoluble part, precipitable at one-third saturation, and a more soluble portion not so precipitable. The latter was then treated by the process of fractional precipitation to remove any water-insoluble globulin that had remained dissolved in it. Powdered ammonium sulphate was added gradually and dissolved by stirring until a small precipitate, say some 15–20 % of the amount of protein present appeared. This was filtered off, redissolved in water, and saturated salt solution was added till the solution was at "one-third" saturation. This caused precipitation of a good proportion of the fraction. Examination showed it to consist largely of water-insoluble globulin. The filrate from the precipitation was returned to the main liquid, and the whole process was repeated some five times, the last fraction showing only quite a small amount of globulin. A further small amount of globulin was removed by dialysis.

The resulting substance was easily and completely soluble in water, and could be precipitated at half-saturation with ammonium sulphate. It was tested for albumin. A portion was dissolved in 50 c.c. water and precipitated by the addition of 50 c.c. saturated salt solution. This precipitate was redissolved and re-precipitated in the same way at the same volume[1].

90 c.c. of 1st filtrate gave by Kjeldahl 1·0 c.c. N/10 NH_3,
90 c.c. ,, 2nd ,, ,, ,, 0·95 c.c. ,, ,,

The small amount of protein in the filtrate, being constant in quantity, is presumably pseudo-globulin.

It was mostly precipitable by saturation with sodium chloride and nearly completely by magnesium sulphate. With ammonium sulphate most of it

[1] For details of this method see Haslam [1905].

(say five-sixths) was precipitated by the addition of only three-quarters of a volume of saturated solution (43 % saturation). Half-saturation precipitated all but traces. This, then, was the pseudo-globulin of Hofmeister, the existence of which was indicated by the experiments of Burckhardt and Marcus. Its solubilities are intermediate between those of the water-insoluble globulin and albumin.

I next proceeded to ascertain whether this substance could be split up further by ammonium sulphate. Freund and Joachim found that the pseudo-globulin of Hofmeister was partly soluble, partly insoluble in water: but the insoluble portion was probably merely globulin left dissolved, as we have seen, in the pseudo-globulin. It was possible, however, that it could be split into two soluble bodies after Spiro and Reiss. A portion was divided into two roughly equal parts by ammonium sulphate in the following way. A dilute solution (about 0·25 %) was made. A small test portion was completely saturated with salt and placed in a cylindrical beaker. Saturated salt solution was then added to the main liquid until a small amount, double the volume of the test portion, placed in an exactly similar cylindrical beaker, showed on looking downwards the same degree of density of precipitate. I then assumed that about half the protein was precipitated. This, after being allowed to stand, was filtered off and the filtrate saturated to obtain the remainder. These two fractions closely resembled each other in their solubilities. Each was largely precipitable on the addition of three-quarters its volume of saturated salt solution, and the portions left over in solution appeared in no way different from those precipitated. Nor did further treatment on fractional lines show that the substance could be divided.

Since we had a water-soluble globulin commencing to fall at 33 % saturation, and nearly finishing at 43 %, that is to say when free or nearly free from other proteins, it might be supposed that a substance existed that commenced to fall at 43 % and was mostly precipitated at 50 % saturation. It might be supposed that if such a substance existed it might be nearly or entirely lost in the series of precipitations at 50 % in which albumin is got rid of. To test this point, I began afresh with some serum and endeavoured by suitable fractionations to find such a body; but entirely without success.

Experiment. 300 c.c. ox-serum were taken and diluted to 1000 c.c. and 500 c.c. saturated salt solution were added to get rid of some of the globulin. To the filtrate were added some 700 c.c. (over half-saturation) further of saturated salt solution, so that most of the searched for substance should be precipitated while as much albumin as possible should be left in the filtrate. The precipitate was dissolved again in 1000 c.c. water and the above

precipitations were repeated. The protein resulting was then dissolved in 900 c.c. water and 500 c.c. of saturated salt solution were added so that not only globulin but pseudo-globulin might be largely precipitated also. To the filtrate was added salt solution again to make rather more than half-saturation. I proceeded in this way to eliminate gradually portions of globulin and pseudo-globulin, on the one side, and albumin, on the other; always being careful to work well outside the precipitation limits of the body for which I was looking, so that as little as possible might be lost. No such substance, however, could be found. The process was continued until a quantity too small to work with remained, and up to the last, pseudo-globulin on the one hand and albumin on the other could be demonstrated. This experiment was repeated on another sample of ox-serum.

I concluded, therefore, that no substance exists in ox-serum precipitable between 43 and 50 % salt concentration, at any rate in quantities comparable to globulin and albumin.

Water-insoluble globulin.

As it was not possible to carry to completion the separation of globulin by means of ammonium sulphate, owing to its becoming increasingly insoluble in the salt solution, I tried precipitations with acetic acid. Some water-insoluble globulin that had been prepared from ox-serum by a few precipitations at one-third saturation and then diluting with water, was shaken well with water, thrown on the filter, and washed with water. It was then suspended in water, and a few drops of ammonia were added. The globulin completely dissolved. The volume of the solution was 400 c.c. About 0·65 g. of globulin was recovered at the end of the experiment, so that if we allow for some 20 % of other proteins and loss during the experiment, there would be some 0·8 g. protein. Thus the strength of the solution was about 0·2 %.

The globulin was then precipitated by the addition of dilute acetic acid. The precipitation began when the solution was about neutral, and was rapidly completed on the addition of further acid, the solution being finally faintly acid. The mixture was allowed to stand over-night. The precipitate was then filtered off, suspended in water, and redissolved by the addition of ammonia. Normal solutions of ammonia and acetic acid were used, the quantities required being added out of burettes. Before precipitation the volume of the solution was always made to 400 c.c. 16 c.c. alkali were used to dissolve: the precipitate was usually complete on the addition of some

13 to 14 c.c. of acid, but 16 were added. On one occasion a small excess was added, but no re-solution of the precipitate occurred. It was noted that the globulin took more time to dissolve as the experiment advanced.

In the second filtrate the presence of water-soluble protein was easily demonstrated. The fourth and fifth filtrate each showed a faint cloud on boiling, and a faint precipitate on saturation with salt. In each, however, by saturating the whole with salt, and collecting the small quantity of protein obtained, water-soluble protein could be demonstrated. The eighth and tenth filtrates were examined quantitatively for organic nitrogen by the method described in previous papers.

Eighth filtrate volume 345 c.c. gave 2·4 mg. N.
Tenth ,, ,, 343 c.c. ,, 2·4 ,,

Thus in precipitating the water-insoluble globulin by acetic acid a point can be arrived at in which the organic nitrogen in the filtrate is constant and the soluble pseudo-globulin presumably completely eliminated. Reckoning that globulin contains 16 % N, the amount in these filtrates would be 0·0073 g. or 0·004 %. It may be noted that the separation of globulin from pseudo-globulin is considerably more difficult than the separation of pseudo-globulin from albumin.

Physical Changes caused by Separation.

The substance thus obtained is distinctly less soluble than that prepared more rapidly by a smaller number of precipitations. Some of the essential peculiarities of globulin are, however, preserved. The addition of alkali causes it to swell up into a jelly-like consistence. It is, on the other hand, only slightly soluble in salt solutions. But the question of the alteration of the physical properties of proteins on precipitation is a difficult one.

Globulin prepared by a single precipitation with acetic acid from diluted serum is a comparatively soluble substance. It also has mixed with it considerable quantities of pseudo-globulin and albumin. The more it is precipitated, the more insoluble does it become, and it is very generally believed that this is due to a physical alteration. But it is not necessary to postulate physical alteration, at any rate, to the degree generally done. Globulin can be held in solution by the other proteins of serum. This can be shown by taking a portion of serum-protein, by precipitating with ammonium sulphate at half-saturation twice or thrice and dialysing until all the salt is removed. A certain amount of globulin will be precipitated; but no matter how long the clear solution from which the precipitate may be

removed is allowed to dialyse, water-insoluble protein in large quantity can be demonstrated therein. Or, more simply, serum may be dialysed indefinitely and after filtering off the resulting precipitate, large quantities of globulin can be found in the filtrate. Again, during the course of fractional experiments, globulin is commonly found in solutions half-saturated and more than half-saturated with ammonium sulphate, there being excess of other protein present. Indeed, it is this property that makes the whole difficulty of the separation. It is to get rid of the globulin that remains dissolved in the pseudo-globulin that the many precipitations at high salt concentration have to be resorted to. It is this property that makes it impossible by any one precipitation by any agent to obtain more than a proportion of the globulin present. Pseudo-globulin and albumin are, in fact, the chief solvents of globulin in serum. But in this case it is clear that in any series of precipitations for its preparation and purification, where it loses at each precipitation a portion of the other protein, it loses a solvent. The more the other proteins are withdrawn from it, the more insoluble must the preparation become. Quite apart, therefore, from any changes that may go on in the globulin as a result of the physical action of the reagent used, we see that any preparation must become more insoluble as it becomes purer. But there is little doubt that some of the various reagents used may also cause insolubility. This is evident from the different behaviour of globulin when precipitated by acetic acid or ammonium sulphate. With ammonium sulphate, as we have seen, globulin gradually becomes so insoluble as to resemble coagulated protein. It may be noted that this change does not occur uniformly throughout the globulin, but a small portion of such insoluble matter appears, and continues to increase as the precipitations proceed. We may therefore argue that, apart from its separating power, ammonium sulphate has an action on globulin tending to render it insoluble. With acetic acid such action is extremely small; after many precipitations only quite a small proportion of this very insoluble matter appears.

But although it is quite easy to prepare solutions in which globulin is held in solution by the other proteins, it is apparently difficult to reproduce this condition when the various proteins have been separated from each other. At any rate, it cannot be produced by the simple addition of globulin to a solution of albumin or pseudo-globulin. I have no quantitative experiments on this point, but it is quite clear that only small quantities, if any, of completely separated globulin dissolve directly in watery solutions of separated albumin or pseudo-globulin. So far as my experiments have gone, it seems to be the case that, after a small degree of separation, say two or

three precipitations, the component parts can be restored to the *status quo*; but that the farther the separation is pushed, the more difficult does the restoration become. This would seem to show that each act of separation causes some secondary molecular change in the parts separated.

In this connection I may recall the remarkable fact to which Hardy draws attention: that while serum itself can be readily filtered through a porous pot, globulin, even in the early stages of preparation, cannot. Hardy also takes occasion to point out that neither alkali nor salt is capable of producing so high a grade of solution as that of globulin as it exists in serum, and that a further dissolving agent must be present. To say that that agent is the other serum protein is not very different from saying that there is some sort of combination among them; a conclusion to which Hardy is led on other grounds. In the first paper of this series I brought forward some evidence that this seems also to be the case with the albumoses.

Allowing, however, for the greatest amount of change in the process of separation, it can hardly be supposed that any radical change of constitution occurs: the changes are extra- rather than intra-molecular. So that from the point of view of chemical analysis, the matter is not of great importance.

Possibility of Substances between Globulin and Pseudo-Globulin.

In the series of precipitations at one-third saturation, it was found that, even after seven or eight precipitations at sufficient dilution, the product obtained was largely soluble in water. After dialysis a good proportion, perhaps 50 %, remained in solution. The clear solution certainly contained some pseudo-globulin, and might well have consisted entirely of this and globulin. We have seen that pseudo-globulin cannot be further split up, but there was a possibility that the bulk of the soluble matter consisted of a third substance, more soluble than globulin, but precipitable at one-third precipitation with the globulin. To ascertain whether such a substance existed, experiments were conducted on the following lines. Diluted serum is precipitated some eight or nine times at one-third saturation: this gets rid of all albumin, and a considerable quantity of pseudo-globulin. Globulin is then removed by fractional precipitation, care being taken to remove only insoluble matter, all doubtful portions being returned to the main solution, so that the quantity of substance sought for should not be diminished in this way.

This leaves us with a more soluble substance again, and from this, more pseudo-globulin is removed by precipitation at one-third saturation.

Thus alternately portions of globulin and pseudo-globulin are removed

from the protein under examination. This can be continued until the substance sought for is found, or there is no more substance to work with.

Two separate experiments were carried out, and on several occasions portions of protein were obtained which were precipitable at "one-third" saturation, and soluble in water—further examination, however, always showed them to consist of globulin and pseudo-globulin. I select the following for quotation.

500 c.c. ox-serum were diluted to 2,000 c.c., and a precipitate formed by the addition of 2,000 c.c. saturated salt solution. The precipitate was dissolved in 2,000 c.c. and a further precipitate formed by the addition this time of 1,000 c.c. saturated salt solution (one-third saturation). This latter precipitation was repeated eight times when the filtrates were found to contain only quite small quantities of protein. During the series of precipitations large quantities of insoluble matter appeared, and were removed from time to time. The final precipitate contained a fair proportion of protein that was soluble in water. From this I sought to remove any water-insoluble globulin that it might contain by means of the system of fractional precipitation before described. The protein was dissolved in 200 c.c. water (the first series of precipitations had considerably reduced it in bulk), and some 60 c.c. saturated solution were added. The small precipitate that fell was found to consist largely of water-insoluble globulin, but mixed with it was a certain amount of water-soluble substance, and this was returned to the main solution. To this a further quantity of salt was added and another fraction obtained, which was treated in the same way. Some six fractions were taken and in the last two only quite small quantities of water-insoluble matter were found. The experiment was accordingly brought to an end by nearly complete saturation of the liquid, and the resulting protein was examined. On being dissolved in 80 c.c. of water it was found that on addition of 40 c.c. saturated salt solution (one-third saturation), only some two-thirds of it were precipitated. The remainder came down on half-saturation. It was clear, therefore, that pseudo-globulin was present to a considerable extent. A fresh series of precipitations at one-third saturation was therefore undertaken, some eight in number. The resulting substance of which there was only a small quantity, was again found to contain both water-insoluble and water-soluble parts. Owing to the withdrawal of a large quantity of pseudo-globulin, the insoluble globulin was easily demonstrated again. The water-soluble part, after further precipitations of a similar character to those already described, was shown to contain both water-insoluble globulin and pseudo-globulin.

It was thus seen that no body precipitable at one-third saturation and at the same time water-soluble, could be obtained that remained constant in quantity, and could not be resolved into globulin and pseudo-globulin. Such fractions, therefore, must be looked upon as mixtures. Or if we look upon the serum proteins as one molecular structure in the serum, such fractions must be regarded as portions of that molecular structure which have so far resisted the disintegrating action of the salt; but which, by further action can be resolved into globulin and pseudo-globulin. And it may be further remarked that by precipitation at suitable concentrations fractions of material or portions of the original molecular aggregate may be obtained having any required solubility, and exhibiting a certain appearance of constancy. This is much more the case with mixtures of globulin and pseudo-globulin than with those containing albumin. The two former are much more closely connected at any rate in regard to their behaviour to salt, than are pseudo-globulin and albumin. As we have already seen, albumin can be removed comparatively readily.

I thus arrived at the general conclusion that there are two, and only two different proteins that are precipitable at half-saturation with ammonium sulphate; the historic water-insoluble globulin and the protein soluble in water corresponding to Hofmeister's pseudo-globulin. In addition to the precipitation differences between these two bodies, there are also certain differences in appearance. The soluble substance comes down in finer particles and has not the flocculence of the globulin precipitate. It is perhaps rather whiter and does not become discoloured so readily as globulin.

The Separation and Purification of Pseudo-Globulin.

We are now in a position to discuss the separation and purification of pseudo-globulin in greater detail, with a view of determining the limits of fractional methods, and the most convenient way of applying those methods to this particular case.

To obtain crude pseudo-globulin for further experiment, diluted ox-serum was taken; globulin and albumin got rid of at one-third and half-saturation respectively. After some two to three repetitions, a sample of pseudo-globulin can be obtained, giving no precipitate at one-third saturation, and leaving only traces of protein in the filtrate at half saturation. I will now describe the method of fractional precipitation I have hitherto employed in greater detail.

The crude protein, in this case pseudo-globulin, is dissolved in water and saturated salt solution is added until a precipitate begins to fall. This

is usually just after one-third saturation. In the early stages of the experiment a good precipitate is obtained at "one-third" saturation of the fraction. After the first few removals of globulin, however, this is no longer the case. The concentration of protein in the solution at this point may be 2–0·5 %. The readiest way of determining this is by a Kjeldahl determination of the total organic nitrogen, calculating the protein as containing 16 % N. The precipitate formed may be some 20 to 25 % of the whole quantity of the protein present. This is determined by comparing the opacity of the precipitate formed with that in a small quantity of the solution in which the protein has been completely precipitated by the addition of solid salt as has been described before. In using this method it must be remembered that the precipitate continues to fall gradually for some hours after the addition of a quantity of salt, so that only a rough determination can be made at the time of adding salt. The rate of fall of the precipitate appears to depend, to some extent, on the concentration of the protein : the diluter the solution the slower the fall. After the formation of the precipitate the solution is allowed to stand 24 hours. If the precipitate has sunk to the bottom, most of the liquid can be decanted. The remainder is filtered and the precipitate is redissolved. Some 35–30 % of this is then precipitated at about the same concentration as in the previous experiment.

This precipitate being some 9–6 % of the total protein present, is removed, and the filtrate is added to the main solution. The amounts withdrawn are gradually lessened as the experiment is repeated and may be tested for the protein it is desired to eliminate, in this case dialysis being used to detect globulin.

The process can be repeated indefinitely. When, however, the separation proceeds slowly, that is, when at each precipitation the proportions of the substances to be separated do not differ much in the precipitate and filtrate, it is expensive both of material and time. If the fractions are large the material is soon used up, and if small the process becomes very slow.

The amount of the fraction should correspond to some extent with the amount of protein present which it is desired to eliminate. As will be seen from the experiment quoted, some six or eight repetitions of this process will reduce the insoluble globulin in pseudo-globulin from 8–10 % to 2–3 %. The volume of the solution is increased at the end of each pair of precipitations. When it becomes inconveniently large it can be reduced by salting out the protein completely from a portion, dissolving the protein so obtained in a minimum of water, and returning it to the main solution.

In employing this method in the separation of the albumoses in some

cases I had no means of determining the progress of the separation, beyond that of noting the increasing solubility of the protein being purified and obtaining from successive fractions diminishing quantities of the more insoluble protein. Where hetero-albumose was being salted out there was the more independent test of precipitation by dialysis. This is the case in the present instance. This test, however, is only a solubility one, and cannot be relied on in the same way as some independent reaction. In the present case we have this latter: since, while globulin contains phosphorus, pseudo-globulin does not contain any. Globulin only contains about 0·1 % P, so that in spite of the possibility of detecting very minute quantities of phosphorus the test is limited in its usefulness. It is sufficient, however, to show the general course of the separation, and to enable us to place a value on the solubility tests.

Hardy [1905] first pointed out that globulin contained phosphorus and that other fractions of serum-protein containing less globulin contained less phosphorus. The relationship of phosphorus to globulin is not quite simple as will be pointed out more fully later on. It is sufficient for present purposes to note that salting out operations and dialysis apparently leave the phosphorus content of globulin unchanged; while samples of pseudo-globulin can be obtained which, by the most careful tests, give no phosphorus.

For detecting and roughly estimating minute quantities of phosphorus the ammonium phosphomolybdate method of Neumann was used; the decomposition of the protein being effected by Bayliss and Plimmer's modification to avoid using more sulphuric acid than is necessary. Ammonium phosphomolybdate contains only some 1·6 % P, and as very small quantities can be precipitated and detected, it forms, under proper conditions, a very delicate test. Further, owing to the way in which minute quantities of the precipitate fall, it is possible, simply by inspection, to estimate the amounts with rough accuracy.

Taking known quantities of a solution of sodium phosphate I have found that 0·005 mg. P can certainly be detected, sometimes 0·0025 mg. P. And if the conditions as to total volume of solution, quantities of reagents present, and heat used to effect precipitation, are maintained equally, the differences between such quantities as 0·005, 0·0075, 0·01, 0·015, and 0·02 mg. P, can be readily appreciated; and, by comparison, a rough estimate can be formed of the trace of phosphorus under consideration.

Good results were obtained with solutions differing slightly from Neumann's:

30 c.c. Water.
30 c.c. 50 % ammonium nitrate solution.
30 c.c. 3 % ammonium molybdate solution.
Heat to 85° C.

Experiments were also made to determine how far Neumann's method could be used to estimate small quantities of phosphorus. Using a decinormal in place of a seminormal standard solution, I found that the method did not lose in accuracy until the quantities were below 0·2 mg. P, when the estimations became too high. Below 0·1 mg. P (which corresponds to 0·9 c.c. decinormal solution) the values were quite unreliable:

(1)	0·52 mg. P	gave	4·8 c.c.	N/10	solution	...0·539 mg. P
(2)	0·26 ,,	,,	2·5 ,,	,,	,,	...0·275 ,,
(3)	0·26 ,,	,,	2·5 ,,	,,	,,	...0·275 ,,
(4)	0·208 ,,	,,	2·1 ,,	,,	,,	...0·231 ,,
(5)	0·208 ,,	,,	2·0 ,,	,,	,,	...0·22 ,,
(6)	0·104 ,,	,,	1·1 ,,	,,	,,	...0·121 ,,
(7)	0·104 ,,	,,	1·0 ,,	,,	,,	...0·11 ,,
(8)	0·104 ,,	,,	1·15 ,,	,,	,,	...0·126 ,,
(9)	0·078 ,,	,,	0·9 ,,	,,	,,	...0·099 ,,
(10)	0·078 ,,	,,	0·8 ,,	,,	,,	...0·088 ,,
(11)	0·052 ,,	,,	0·65 ,,	,,	,,	...0·0675 ,,
(12)	0·052 ,,	,,	0·8 ,,	,,	,,	...0·088 ,,

For the experiments about to be described some three different samples of ox-serum (each of about one to two litres) were mixed. In order to determine the amount of phosphorus in the globulin of this mixture of serums, some globulin was separated and purified, first by salt precipitation, and then by acetic acid in the way previously described.

200 c.c. of 7th filtrate gave 1·2 c.c. N/10 NH_3.
200 ,, 8th ,, ,, 1·1 ,, ,,

The globulin was washed on the filter paper until no more acid was shown in the filtrates and then washed with alcohol and ether. It was dried at 110° to constant weight.

1·0965 g. globulin gave 1·2055 mg. P = 0·11 % (by Neumann).
0·732 ,, ,, 0·785 ,, = 0·107 %.
Mean = 0·1085 %.

Therefore, 1 mg. P indicates 0·921 g. globulin.

I. Crude pseudo-globulin, giving no precipitate at one-third saturation and containing traces only of albumin. To determine the amount of globulin contained in it phosphorus was estimated by Neumann's method.

2·172 g. gave 0·19 mg. P corresponding to 8·4 % globulin.

The fractional process described above was then carried out six times in succession; large fractions were taken, some 28 % in the first instance and

35–40 % in the second. There was a marked increase in the solubility of each successive fraction, until, in the last fraction removed, I was unable, even after four sub-fractionations, to find any protein insoluble at one-third saturation. On the other hand, the fraction gave a distinct, though small, precipitate on dialysis. The experiment was terminated by salting out all the protein. To get rid of the small amount of albumin it was redissolved in water and thrice precipitated at half-saturation.

200 c.c. of 2nd filtrate gave 2·4 c.c. N/10 NH_3.
200 ,, 3rd ,, ,, 2·3 ,, ,,

Thus albumin was eliminated.

On dialysis of a portion a good cloud appeared. The whole amount of protein was precipitated by the addition of alcohol in the cold (below 15° as advised by Mellanby) and washed with alcohol and ether. A portion was dried at 110° to constant weight and the phosphorus estimated:

(1) 0·4115 g. gave 0·0125 mg. P by inspection...2·8 % globulin.
(2) 0·442 ,, 0·015 ,, ,, ...3·1 % ,,

If we take a mean and allow 20 % error, the amount of globulin in the sample is from 2·3–3·5 %.

The great contrast between the solubility in salt and the precipitability on dialysis was surprising and will be referred to later. This inconsistency was no doubt the basis of the observation by Freund and Joachim, who found that the fraction which gave no precipitate of globulin at one-third saturation gave a precipitate on dialysis so large that it was considered another substance.

II. Crude pseudo-globulin as in I. This experiment was on a much larger scale, and the process was repeated 36 times. The fractions were smaller than in I, being at first 20 %, but dropping later to 12–15 %. The dilution varied from 1–0·5 % in the earlier stages to 0·2–0·1 % in the later.

The salt solubility of succeeding fractions was noted, and a general agreement with the results in I was found.

After the ninth and twentieth fractions, the whole protein was salted out and dried. On each occasion it was found on re-solution that the solubility had decreased, and further that fractions taken immediately after re-solution were less soluble. The tenth fraction had the same solubility as the fifth, and the twenty-first as the fifteenth.

All the sub-fractions were tested by dialysis. It was sought by this means to follow the progress of the separation. The quantity of protein precipitated showed in general a decline. The decline was not a regular one, however. The first nine tests showed a steady decrease of substance. The tenth (after

drying) showed an increase, and from this to the seventeenth a slower and more irregular decline was noted. The twenty-fifth fraction showed no precipitate on dialysis, but the sub-fraction gave a cloud. The thirtieth fraction and sub-fraction remained clear. The thirty-first sub-fraction, however, gave a good cloud and the fraction a faint cloud. The thirty-fourth fraction and sub-fraction were again clear and also the thirty-fifth.

In general it was noted that when the fractions taken were small, the separation seemed to make very little progress. Observations were made as to the composition of the precipitates on dialysis, since in some cases they appeared out of proportion to the amounts of globulin that could be present. It was found that a considerable amount of pseudo-globulin was precipitated with the globulin, as the experiment became more advanced. The precipitate caused by the dialysis of serum or of the mixture of proteins obtained by one or two precipitations by "salting out" consists mostly of globulin, a certain proportion of pseudo-globulin and a small amount of albumin. The earlier fractions on dialysis gave such precipitates. In the later fractions, after the twelfth, it was found that on dialysis a large quantity of pseudo-globulin was precipitated with the globulin—in some cases an excess of pseudo-globulin. In one case where some 6–7 % of the protein present was estimated to be globulin, the precipitate was some 15–20 % of the protein present. Allowing, therefore, for 2 % globulin remaining in solution, the precipitate must have contained some 70–80 % pseudo-globulin, to 20–30 % globulin. Without attaching too much importance to these estimates, it is clear that considerably more pseudo-globulin than globulin was in this precipitate.

Precipitates of this kind remain insoluble, or nearly insoluble in water. They may be largely dissolved by the addition of salt, or better, a trace of alkali. In the latter case, if the solution be neutralised, no reprecipitation occurs, and further, if the solution be then dialysed there may be only quite a small proportion of the protein precipitated, or even none at all. This latter result could be attributed to traces of acid or alkali being left over after imperfect neutralisation. This was shown to be the case in many fractions containing small quantities of globulin. A portion dissolved in water and dialysed gave a small precipitate. Another portion which had been dissolved in dilute ammonia and then neutralised gave no precipitate on dialysis: nor did subsequent additions of traces of acid or alkali, nor prolonged dialysis, have any effect whatever in producing a precipitate.

The size and formation of the precipitate on dialysis appeared also to be connected with the rate at which the salt was withdrawn from the solution. In the majority of the experiments the precipitate reached its maximum in

24 hours; although there was then further salt in the solution, further dialysis produced no precipitate, even though continued many days. If, however, the solution was diluted four or five times with distilled water, a further precipitation was sometimes obtained.

It was also frequently noted that if pseudo-globulin containing a small proportion of globulin were dried, the precipitate on subsequent dialysis was increased. Both globulin and pseudo-globulin are rendered more insoluble by drying: by repeated drying both may be rendered entirely insoluble. So that the physical condition of pseudo-globulin would seem to be an important factor in inducing its precipitation with globulin on dialysis, and may have been one of the causes of the increase in the amount carried down with globulin already referred to.

It was also noted that, as the experiment advanced, a tendency to mechanical coagulation developed. This was chiefly noted in filtering, after a fraction had been precipitated. The protein in the solution then was just on the point of being precipitated. Unless the filtration was conducted slowly, small flakes and strings of protein appeared, which tended to adhere together at the end of the filter.

Towards the end of the experiment, also, the fractions were more affected by drying than in the early stages. Thus there is distinct evidence that the prolonged contact with salt induces slow physical changes in pseudo-globulin.

Only one intermediate observation of the amount of phosphorus was made. After nine fractions a portion was freed from salt, and precipitated by alcohol and dried.

0·347 g. gave 0·01 mg. P by inspection.....2·6 % globulin.

This shows a rather slower rate of progress than in I.

Of the final substance 0·561 g. was tested for phosphorus with a negative result.

The physical changes noted during the experiment were:

1. A gradual increase of solubility in salt solutions which, after a time, appears to cease. In no case did the increase go so far that pseudo-globulin became soluble in half-saturated, or nearly half-saturated solutions (that is at ordinary dilutions, 5–0·2 %).

2. The substance gradually became more liable to pass into the insoluble or coagulated state.

In regard to the dialysis test it was found:

1. That the amount of pseudo-globulin carried down with globulin on dialysis increases as the experiment proceeds; the pseudo-globulin, after a time, being in excess of the globulin.

2. That if the substance be dried before dialysis a considerably larger precipitate results.

3. That the amount and constitution of the precipitate is also dependent on various chemical conditions.

In regard to the phosphorus estimations, assuming that 0·005 mg. P is the smallest amount that can be detected, the final result would mean that the pseudo-globulin might contain a quantity less than 0·00087 % P, but not more. This, together with the rather better result in III, where nearly a gram gave a negative result, is practically conclusive that pseudo-globulin does not contain phosphorus. We can, therefore, attribute the small and diminishing amounts of phosphorus found in the experiments to the presence of globulin, and so far as we can estimate the phosphorus we can estimate the globulin.

Experiments I and II, therefore, show:

1. That by the method described all but some 2–3 % of the globulin can be eliminated in some six or eight fractionations.

2. That the larger the fractions taken, the more rapid the separation. So that the method becomes too lengthy if used economically as regards material.

3. That both solubility tests as guides to the progress of the separation are fallacious.

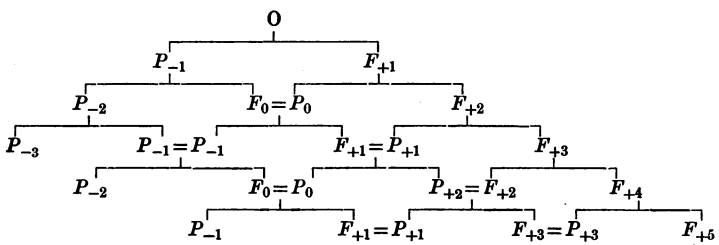
As to the progress of the separation in II after the ninth precipitation, it is clear from the negative result in the final test for phosphorus, that most of the remaining 2·6 % of globulin is eliminated. It would seem probable that the elimination is a very gradual one.

In the albumose separation I relied on solubility tests, especially the salt solubility one. In no case, however, did the indications point to such a rapid conclusion of the separation as in I: nor were such large divergences as those between I and II met with. Although, therefore, there may have been some small alterations in solubilities which would tend to falsify results, it is hardly possible that it could have been so extensive as in the present case. In all respects the albumoses showed themselves more stable under treatment than the serum protein.

Finally, I tried a different method with a view of obtaining greater rapidity. In place of taking only a small proportion of protein at each precipitation, which is only efficient when a comparatively large proportion of globulin is contained therein, I tried the following plan which is based on the principle of dividing the protein into two equal parts at each precipitation.

III. Some 10 g. of crude pseudo-globulin which had been fractioned

four times on the method described above, and contained about 4 % of globulin, were dissolved in 2,000 c.c. water. By the addition of solid salt about half the amount of protein as estimated in the way above described of comparing opacities was precipitated. The precipitate P_{-1} was dissolved in 2,000 c.c., and again by solid salt equally divided into P_{-2} and F_0. The filtrate F_{+1} was further divided equally as far as possible into P_0 and F_{+2} by the further addition of salt. At this stage, there were four divisions each containing about a quarter of the original protein. The process can be best explained by means of a diagram.



F_0 and P_0 were mixed being considered nearly of the same grade in purity; the precipitate being dissolved in a minimum of water and added to the filtrate. The mixture was then precipitated by the addition of solid salt as before and divided into P_{-1} and F_{+1}.

At each precipitation the globulin is divided into two portions, most going into the precipitate; so that the amount of globulin in the filtrates on the right F_{+1}, F_{+2} and F_{+3} is constantly diminishing. The process which involved a considerable number of precipitations was continued until a sufficiency of material of grades 3, 4, and 5 was obtained. It was found that an exactly equal division of the protein in any given experiment could not be obtained without an undue amount of time being spent. The method used was that already described of comparing opacities of precipitates. Owing, however, to the gradual fall of the precipitate which is the more accentuated the more dilute the solution, the precipitate cannot properly be estimated until twenty-four hours have elapsed. To save time, therefore, I endeavoured to allow for the extra fall on standing, by precipitating only some 35 or 40 % as estimated shortly after adding the salt. This led to several inexactitudes and probably accounts, in part, for the slight inequality of the results.

The phosphorus estimations were all done at the same time, and compared with each other as well as with known amounts:

Parent substance	0·335 g. gave	0·015 mg. P...4·1 % globulin.	(By inspection)
Third grade	0·511 ,,	0·0075 ,, ...1·3 % ,,	,,
Fourth grade	0·867 ,,	0·0075 ,, ...0·79 % ,,	,,
Fifth grade	0·883 ,,	negative, *i.e.* contains less than 0·5 % globulin.	

The figures, allowing for the roughness of method of estimation, are sufficient to show that this method is more suited to eliminate small quantities than the other. It also has the advantage of economising time and material, as the precipitations, though numerous, can be carried on simultaneously, after the first few; and all material in various grades can be labelled and kept ready for use at any time.

These estimations of phosphorus, restricted as they have been owing to the small amount present, are, I think, sufficient to establish the main point that the separation of these two proteins can be completed, or nearly completed, by means of fractional precipitations.

In regard to the practical point of the preparation of pseudo-globulin containing, say, not more than 1 % globulin, I do not think any very extensive procedure will be necessary. I found that in precipitating pseudo-globulin containing some 2–3 % of globulin with alcohol in the cold, that a small fraction could be obtained which was comparatively rich in globulin. I have not concluded my experiments on this point, but it is not unlikely that after a few fractions with salt have got rid of all but some 3 % or so of the globulin, that a large part of the latter can then be eliminated by a few fractions with alcohol.

The Preservation of Globulin and Pseudo-Globulin.

I have already pointed out that both globulin and pseudo-globulin on being dried, at any rate with salt, become more insoluble. To keep them without drying I have found that, by the addition of a little ether to their solutions in dilute saline, they will keep for considerable periods in stoppered bottles. The solutions should be well shaken with the ether.

I have samples which I have kept over a year which are quite clear and show no tendency to precipitate.

Relation of Phosphorus to Globulin.

In the preceding section I have assumed that globulin contained phosphorus as an integral part of itself. This was because throughout such reactions as there described the globulin always retains phosphorus. It is difficult to believe that if the phosphorus could be split off by salt, ammonia,

or acetic acid, that any except a trace would have remained with the globulin after the prolonged operations entailed by its purification. However, to obtain further assurance I estimated the phosphorus in a sample of crude globulin prepared from serum by four precipitations of salting out at one-third saturation, followed by two by acetic acid, after solution in ammonia.

1·4725 g. globulin gave 1·462 mg. P...0·099 %.

This is distinctly less than in the case of the more purified globulin although it had suffered less than half the number of precipitations.

I have already quoted (page 507) the phosphorus determinations in one sample of purified globulin. Another sample from ox-serum was analysed:

0·5495 g. globulin; 0·575 mg. P...0·105 %,

a very similar result.

Treatment of dried globulin with alcohol or ether gives a yellow, rather fatty extract, which contains phosphorus. This extract is only separated from globulin with some difficulty. I have tried various means; treatment of the finely powdered globulin in a Soxhlet apparatus with ether; prolonged shaking with alcohol and ether; and boiling with alcohol, after Plimmer. The latter was the most effective; an extract could generally be obtained by its means from samples which had been previously treated in other ways. I found, however, that though most of the extract could be obtained after some eight to ten hours boiling, that further small quantities could be got by more prolonged boiling. In no case could I get anything like a phosphorus-free globulin. I quote two experiments in which the globulin, well powdered, was boiled with successive portions of alcohol for periods of seven hours, until no more extract was obtained.

(1) 0·5265 g. globulin gave ext. cont. 0·3096 mg. P; 0·056 %. Boiled 21 hrs.
(2) 0·68625 g. ,, ,, ,, 0·3207 ,, ; 0·048 %. ,, 35 ,,

Thus about half the original phosphorus is got rid of in the extract. In previous experiments in which prolonged shaking (200 to 300 hrs.) was adopted, not more than 30 or 35 % of the phosphorus was eliminated. I also shook solutions of crude globulin and diluted serum directly with ether. In the former case the results were negative, though in one or two experiments minute traces of an extract similar to that looked for were obtained. With serum, no trace of the extract looked for was found, but an orange pigment was extracted without much difficulty. From pure or nearly pure pseudo-globulin no extract could be obtained by any of these means, though

small quantities were found in samples containing 3 or 4% of globulin, as would be expected. The amount of extract obtained from globulin measured by weight was some 8–10% of the globulin treated.

In view of the difficulty of completely extracting solids by liquids and especially the very slow action of the extracting liquids in this instance, it is tempting to suppose that the whole of the phosphorus in globulin belongs to some lecithin-like body or bodies which would appear closely connected with the globulin, but not part of the molecule. These would then amount to no less than 15–20% by weight of the globulin. On the other hand the present facts do not warrant us in accounting for more than half the phosphorus in this way.

Summary.

1. There are two proteins of ox-serum insoluble in half-saturated solutions of ammonium sulphate, saturated magnesium sulphate, or sodium chloride; the historic water-insoluble globulin, and the water-soluble body pseudo-globulin.

2. These two bodies cannot be split up further by means of fractionation with salt and water.

3. Intermediate fractions are shown to be mixtures of these two bodies.

4. Globulin contains, or is closely associated with phosphorus, rather more than 0·1 mg. P% being found. About half this belongs to a fatty, lecithin-like body which amounts to some 8–10% of the globulin freed from pseudo-globulin. Apparently no part of this body is detached from globulin through prolonged treatment with acids, alkalies, or salts.

5. Pseudo-globulin does not contain phosphorus.

6. Repeated precipitations of globulin at constant volume finally give filtrates in which the amount of protein is constant. Globulin, presumably, can thus be freed from pseudo-globulin.

7. Pseudo-globulin can be freed in a similar way from albumin, the separation being considerably easier.

8. Pseudo-globulin can also be freed, or nearly freed, from globulin by suitable methods of fractional precipitation.

9. The presence of phosphorus has given us an independent test for following the progress of the latter separation. The result has been to establish the validity of the general method of fractional precipitation in this case, to give important indications as to the most suitable method to use, and to estimate the value of the solubility tests.

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