THE SEPARATION OF PROTEIDS. By H. C. HASLAM, *formerly John Lucas Walker Student.*

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Introduction. It is a fundamental principle of practical chemistry that in the separation of one substance from others such separation cannot be regarded as complete unless definite proof be afforded. This principle, however, has been much neglected in the separation of proteids[1].

Hammarsten[2] in his work on "Paraglobulin" was fully alive to the importance of this principle, and discusses the evidence for the purity of his preparation at considerable length: his experiments, however, were not sufficiently exhaustive for him to realise all the difficulties of the situation, and his method of estimation of globulin is not nearly as accurate as he supposes. He offers no evidence that *all* the globulin present is precipitated, or failing all, what proportion.

Kühne[3] in his well-known researches on digestion offers scarcely any evidence that the various albumoses which he discovers are either separated completely from each other or from other substances. Nor has this defect in Kühne's work been remedied in the subsequent investigations of Neumeister or Chittenden.

More recently one of the chief methods of separating proteids, more especially the proteids of serum and the albumoses, has been the fractional method. E. P. Pick[4] is perhaps the investigator chiefly identified with this method. But he does not produce anything like sufficient evidence that the various proteids he obtains are either separated from each other or from other substances. He never undertakes the demonstration of the purity of his preparations. Thus in his

[1] This does not apply to the method of crystallisation of proteids as used by Hopkins and others.

[2] Hammarsten, *Pflüger's Arch.* XVII. 1878.

[3] Kühne, *Zeitsch. für Biol.* XX. p. 11. 1884.

[4] Pick, *Zeitsch. f. physiol. Chem.* XXIV. p. 246. 1898. *Beit. zur chem. Physiol. u. Path.* I. p. 351. 1902.

research[1] on the primary albumoses, wherein he seeks to compare the constitutions of these bodies, although he prepares both bodies with the greatest care and takes numerous precautions against impurity, yet he does not actually demonstrate that his hetero-albumose is free from all traces of other albumoses, especially proto-albumose, nor that his proto-albumose is free from all but negligible quantities of other substances, especially hetero-albumose. And yet failing this demonstration his results cannot be accepted.

Nor can it be said that the generality of other investigators using this method recognise this necessity of demonstration of purity. Freund and Jóachim[2], for example, though careful to test their filtrates and thence to ensure that any particular fraction is largely at any rate free from substances of the succeeding fractions, yet make no attempt to test for substances of preceding fractions, which is, of course, equally necessary. As these authors point out, one of their globulins (the most insoluble sort) which clearly belongs to fraction II is precipitated continually in fraction III, even after the fractionation has been repeated four times in dilute solutions. I give this instance to show the great difficulty of the subject. Indeed it is by no means easy to obtain demonstration of the purity of these preparations. There are no simple reactions, for example, for determining traces of one albumose in presence of large excess of another. And as authors have not realised the necessity of complete demonstration, no general methods have been devised. Yet the greater the difficulty of separation the more conclusive must be the evidence that it is complete.

I propose the two following methods for testing purity, applicable to the processes of fractionation and salting out.

1. *Where it is sought to prove that the proteid precipitate is freed from the substances of the filtrate.*

The precipitate is dissolved in water and the whole made to given volume; the requisite amount of salt (or alcohol) is added; the mixture is allowed to stand 24 hours and is then filtered. If the filtrate can be shown to contain no proteid nor any other substance which is being got rid of the requisite proof is furnished. If not, the amount of organic nitrogen is estimated by Kjeldahl's method. The precipitate is then redissolved and again made to the same volume, again precipitated in the same way, allowed to stand 24 hours, and then filtered. The organic nitrogen in the filtrate is again estimated. If

[1] Pick, *Zeitsch. f. physiol. Chem.* XXVIII. p. 219. 1899.

[2] Freund und Jóachim, *Zeitsch. f. physiol. Chem.* XXXVI. p. 407. 1902.

the amount of nitrogen in the second filtrate is the same as that in the first, it is clear that it is due to small amounts of the precipitate soluble in that particular medium, and that the precipitate is therefore free from all nitrogenous matter of the filtrate, and therefore, as far as this particular separation is concerned, pure. If on the other hand the amount of nitrogen in the first filtrate is greater than that in the second it is clear that there is still nitrogenous impurity to be got rid of, and further precipitations are needed.

2. *Where the substance it is sought to purify is in the filtrate and the proteids that are being got rid of are of the precipitate, e.g.* serum albumin to be freed from and tested for globulin.

This is by no means so simple as would at first sight appear. In the first place there has to be taken into account the solubility of globulin in the half-saturated solution. (Let us suppose ammonium sulphate is being used.) But this solubility, whatever it may be, is very largely increased by the presence of serum albumin and the other constituents of serum; so that in the filtrate containing the albumin there is a notable quantity of globulin. This is also the case with albumoses and peptones, and has been noted by Kühne and other investigators. It may be simply demonstrated as follows: the globulins are precipitated from serum by addition of an equal volume of saturated ammonium sulphate; to the clear filtrate saturated ammonium sulphate solution is added until a small precipitate appears; this is filtered off and redissolved in water; if now to this solution an equal volume of saturated salt solution is added a precipitate will appear. Thus the presence of globulin in the original filtrate is demonstrated.

Now as I shall show later on this globulin can be removed by a system of fractional precipitation. Let us suppose this process to have been applied to our albumin solution, and that a small fraction obtained in the way above described gives no precipitate on the addition to its solution of an equal volume of the saturated salt solution. This does not necessarily prove that there is no globulin in the fraction. A further quantity of the salt solution is added till a small precipitate appears, that is, a sub-fraction, or fraction of a fraction is taken; and this sub-fraction may show evidence of globulin, that is to say, a precipitate may be formed by the addition to its solution of an equal volume of saturated salt solution. This process of taking fractions and sub-fractions can be continued as long as there is enough substance.

It is obvious that if pushed far enough it is an extremely delicate

test. It is based on the principle that on the addition of the precipitant the more easily precipitable bodies must always tend to come down before the more soluble.

By means of the first test, then, we can tell whether any given proteid preparation contains as impurity some more soluble substance; by means of the second test whether it contains any of the substances less soluble than itself. A preparation of proto-albumose, to take an example, can thus be tested for the presence of secondary and hetero-albumose.

I will now proceed to apply these methods to the study of the proteids of the digestive mixture.

Section I. Separation of albumoses from peptones and other more soluble bodies.

Since the salt was first used in Kühne's laboratory saturation with ammonium sulphate has been generally considered the best means of separating albumoses from peptones. In fact, albumoses are defined as those biuret-giving substances which can be separated out of a digestive mixture by means of complete saturation with ammonium sulphate. It is now known that other salts possess very similar properties; but ammonium sulphate having come to be regarded as a standard in this respect I determined on its use. Since, however, the nitrogen of small amounts of organic matter had to be determined in the presence of large excess of this salt, a sufficiently exact method of doing this had first to be devised.

1. *To estimate the organic nitrogen in the presence of a not large excess of ammonium sulphate.* This is the case of a precipitate of albumose mixed with the salt. There are already several ways of doing this. There are the indirect methods: the total nitrogen is estimated and then the amount of ammonia or sulphate; the organic nitrogen being found by deduction. These are open to the grave objection that as the amount of salt becomes larger the error also increases, and the nitrogen of the salt may be 5—6 times as great as that in the albumose. There is also the direct method of Stutzer and employed by Bömer, in which the ammonia is got rid of by warming with barium carbonate and the barium sulphate got rid of by filtration. This, however, gives an error, since the barium sulphate always retains a certain amount of organic matter which cannot be separated from it. I found by experiment, however, that if magnesia be used instead of barium

carbonate this loss is avoided and at the same time the process is simplified and shortened. The ammonia in fact may be got rid of without introducing any error at all. The procedure is as follows. The liquid to be estimated is placed in a small dish on a water-bath. Magnesia is added gradually to small excess, which is easily seen. Evaporation is continued until no more ammonia is given off as tested by Kossel's method. It is best to continue evaporating a quarter of an hour after this, since it is not easy to get rid of the last traces. The whole is then transferred to a Kjeldahl flask and the total nitrogen estimated in the usual way, the magnesium sulphate formed not interfering.

To test accuracy of this method I have performed the following experiment.

Some albumose was dissolved in water and made up to a definite volume. Two quantities, each of 5 c.c., were taken and a Kjeldahl performed in each:

I. 20·05 c.c. $\frac{N}{10}NH_3$. II. 19·95 c.c. $\frac{N}{10}NH_3$.

Average 20·00.

Two quantities, each of 5 c.c., were then mixed with some ammonium sulphate and the total nitrogen estimated as described.

III. 20·00 c.c. $\frac{N}{10}NH_3$. IV. 20·1 c.c. $\frac{N}{10}NH_3$.

Average 20·05.

Thus it is seen that this process scarcely adds to the error of the Kjeldahl.

2. *To estimate small quantities of organic nitrogen in presence of large excess of ammonium sulphate.*

This is the case where in the filtrate of saturated salt solution small quantities of organic nitrogen have to be estimated. There are no methods in use dealing with this case. I have devised the following method, which gives an error not exceeding 5 %.

The volume of the filtrate having been estimated, it is placed in a dish on a water-bath. Potassium bicarbonate is then added, one gram for every cubic centimetre of liquid (this gives the amount required to produce a small excess). The ammonia is then evaporated off; the evaporation being continued *half-an-hour after* testing shows no more ammonia to be present. The results of this method are surprisingly accurate. I quote some preliminary experiments.

I. An amount of albumose solution shown by Kjeldahl to contain 5·25 mg. nitrogen was mixed with 20 c.c. saturated ammonium sulphate solution. 20 grains of potassium

bicarbonate were added and the ammonia evaporated off as described. Kjeldahl showed 5·04 mg. nitrogen. Error 4 %.

II. Albumose solution contained 10·8 mg. N. Estimation after addition of 20 c.c. saturated ammonium sulphate solution gave 10·15 mg. N. Error 3·5 %.

10 c.c. of another albumose solution gave 5·81 mg. N.

III. 10 c.c. + 7·5 c.c. saturated salt solution gave 5·60 mg. N. Error 3·6 %.

IV. 10 c.c. + 10 c.c. „ „ „ 5·88 mg. N. Error 1·4 %.

V. 10 c.c. + 12 c.c. „ „ „ 5·53 mg. N. Error 4·8 %.

Having thus got sufficiently accurate methods for the estimation of the organic nitrogen in presence of ammonium sulphate, I proceeded to the separation of albumoses from peptones and other constituents of the digestive mixture. As a convenient material I used Witte's peptone, from which a larger part of the primary albumoses had been separated.

In a solution of about 10 % strength the albumoses were precipitated by complete saturation with the powdered salt. This process was repeated four times. The salt in the precipitate was then got rid of by dialysis, the albumose solution evaporated to small bulk and thrown into large excess of alcohol. It was washed with alcohol and ether and dried. A small quantity was then taken and dissolved in 100 c.c. of water in a measuring flask. Total organic nitrogen was then estimated in three portions, each of 5 c.c.

(1) 22·26 mg. N. (2) 22·05. (3) 22·19. Average 22·16 mg. N.

40 c.c. containing 177·13 mg. N or about 1·1 g. of albumose were then taken and evaporated to 11 c.c. so that the strength of the solution was 10 % (I selected this strength of solution because most authors on albumoses recommend it). The solution was completely saturated with the powdered salt and allowed to stand 24 hours, after which excess of salt was noted proving complete saturation. The precipitate was filtered off and washed with a very small quantity of saturated solution and the organic N was estimated in the filtrate and washings. Before a constant amount of nitrogen was found in the filtrate this process had to be repeated several times. Each experiment was always repeated in precisely the same way, the precipitate being always redissolved in the amount of water to make a 10 % solution.

Great care must be used to avoid all loss of substance during these operations. In filtering, the sticky albumose is retained as far as possible in the beaker, only a small amount passing on to the filter-paper. After the filtration the paper is removed from the filter and placed in a small dish in which it is soaked in water which dissolves the precipitate. This solution is then poured into the beaker containing the bulk of the precipitate and the paper is again further treated with a small quantity of water. After this process has been repeated three or four times all matter is removed from the paper and all the albumose without any loss whatever is in the beaker. The albumose in the beaker will gradually dissolve and the process may be hastened by warming to 37° C. When it is dissolved the volume of the solution is made to the requisite amount, for which purpose it is best that the beaker itself should be graduated.

In salting out great care must be taken that the solution be fully saturated: on the other hand only a very small excess of salt must be present since if there is a large excess it will not be found possible to redissolve the precipitate in the same volume as before. I give the results in tabulated form.

TABLE I. *Nitrogen in original mixture* 177·13.

Filtrate	Vol. in c.c.	N in filtrate in mg. N	Calculated impurity in filtrate	Calculated impurity retained in precipitate	Calculated percentage impurity retained in precipitate
1	14	4·55	3·29 mg. N	5·92 mg. N	64
2	11·5	3·15	1·89	4·03	68
3	12·5	3·08	1·82	2·21	54
4	11·5	2·17	0·91	1·30	59
5	12	2·0	0·74	0·56	43
6	12	1·54	0·28	0·28	50
7	13	1·54	0·28		
*8	12	1·84			
9	12	1·26	N in filtrate constant.		
10	13	1·33			
11	12	1·26			
12	12	1·26			
Totals		24·98	9·21		

Amount of albumose found at end of Exp. 153·93 mg. N.

* No. 8 is an obviously faulty observation.

The final precipitate was dissolved in water and the organic nitrogen estimated by Kjeldahl's method, the ammonia being got rid of by the magnesia method. The mean of three observations showed the amount to be 153·93 mg. N. The amount in the original impure albumose was 177·13 mg.: therefore the amount of N contained in the united filtrates should be 23·20 mg. Thus the total N in the filtrates estimated directly being 24·98 (see table) is 1·78 mg. N too high. If, however, we consider that the obviously faulty observation No. 8 should be 1·26 mg. then the total is only 1·2 mg. too high, which is an error of 5 %. It may be noted that the tendency is to make too high estimations. This is no doubt due to there being small amounts of ammonia left over in the liquid before the Kjeldahl is performed. This is why I give the direction to continue to evaporate half-an-hour after the test shows no more ammonia.

This method of estimating the organic N is quite accurate enough for the purpose, since, as is seen in the above table, 0·3 mg. N impurity was easily detected, which is only 0·2 % of the albumoses concerned.

In this series of experiments it is clearly demonstrated that in repeated precipitations at constant volume the amount of nitrogen in the filtrate becomes after a time constant. This shows that all nitrogenous impurities are got rid of, the only matter in the last filtrates being albumose. The separation is thus proved to be complete since it

can be safely assumed that the non-nitrogenous constituents, if any exist, are separated as well.

Besides this main result there are various other points of importance in regard to the salting out process which are for the first time shown with some degree of exactitude. It is seen that working with Witte's peptone in a 10 % solution it was necessary to salt out no less than twelve times (five times before the quantitative experiments were commenced it will be remembered) before the separation was complete. This is far in excess, so far as I am aware, of any procedure recommended by any investigator. It plainly shows the absolute necessity of testing the purity of preparations, if indeed further proof is needed.

In the fourth column the figures denote the amount of nitrogen in the impurities. These are arrived at on the assumption that in each filtrate 1·26 mg. N are from albumose, while the rest is from the impurities. (It will be seen later that this assumption is not quite correct: it is correct enough for my present purpose however.) In the fifth column are the figures which denote the nitrogen of the impurity retained in the precipitate, while in the sixth is the percentage which this bears to the whole impurity present before that precipitation.

From these it is seen that in any precipitation the amount of impurity retained in the precipitate is generally actually greater than that left in the filtrate. Only in one case is less than 50 % retained. The "carrying down" property of albumose which is the cause of all the difficulty in this separation is thus shown in a most striking way. It is clearly a property that must be constantly remembered when essaying these and similar separations.

Perhaps I may here remark on the inutility of washing albumose precipitates. This is very generally recognised now, most authors relying on repeated precipitations for purifying; but one occasionally sees the direction wash till the wash water no longer contains impurity. If an albumose precipitate containing impurity be washed, after a few repetitions the wash water will be found to contain only very small traces of organic matter. If, however, the precipitate be then redissolved and again salted out, the filtrate will be found to contain large amounts of impurity. This may be readily explained, I think, partly by the physical nature of the albumose precipitate, which is often in sticky masses sometimes quite large, of which only the outsides could be affected by the washing, and partly by the singular tenacity with which as we have seen albumoses retain the other substances of the digestive mixture.

It is obvious that ammonium sulphate is a most inconvenient salt for testing the purity of albumose preparations in the manner I suggest. The getting rid of the ammonia nearly doubles the time that would otherwise be required. I therefore sought to replace this salt by another not containing nitrogen. I selected sodium sulphate as used by Pinkus[1].

In order to make an exact comparison I separated albumoses in some quantity from other nitrogenous bodies by means of repeated salting out (14 times) with ammonium sulphate and tested the purity of the preparation in the manner described. Finding the N value in two successive filtrates under the same conditions of volume constant, I got rid of the bulk of the salt by dialysis and the last small amounts by careful addition of baryta, filtering and evaporating. It was then dried by alcohol as usual. About 4 grams were taken and dissolved in water, the solution made to 80 c.c. and the nitrogen estimated by means of two Kjeldahls. Three quantities, each of 15 c.c. and each containing 173·74 mg. N, were then taken. The first was evaporated to 11 c.c. so as to be of 10 % strength and saturated with ammonium sulphate, allowed to stand 24 hours, the precipitate filtered off, the volume of the filtrate estimated and also the amount of organic N. The other two quantities were treated in a similar way except that sodium sulphate was the salt used and the operations were all conducted at a temperature of 37° C. as is necessary in using this salt for proteid precipitations. Instead of being allowed to stand 24 hours they remained in the incubator 3½ and 4½ hours respectively. (If allowed to stand 24 hours too much evaporation takes place: I found that 3—4 hours at 37° C. with this salt is about equivalent to 24 hours standing with ammonium sulphate.)

I give the results in the following table.

	Amount of albumose	Vol. of solution	Vol. of filtrate	N in filtrate	Percentage N in filtrate
$(NH_4)_2SO_4$	173·74	11 c.c.	13 c.c.	1·61	12·5
Na_2SO_4	173·74	11	10	1·26	12·6
Na_2SO_4	173·74	12	12	1·47	12·25

From this it is clear that sodium sulphate at 37° C. has the same salting out capacity as ammonium sulphate. In the last column is shown the percentage of nitrogen in the filtrates of saturated salt solutions, and in the case of the two salts it is practically identical.

Since in sodium sulphate I had found the salt required I did not investigate the action of any others. But it may be remarked that with these quantitative methods we are in possession of means to accurately test the salting out capacity of different substances.

In using Na_2SO_4 I found it best to adopt Pinkus' recommendation of carrying out all the operations (with the exception of the actual salting out which is done in a water-bath) in the incubator. Working on a small scale this scarcely presents any inconvenience. The salt has another advantage in that it is much easier to get rid of, especially

[1] Pinkus. This *Journal*, xxvii. p. 57. 1901.

in saturated solutions, than $(NH_4)_2SO_4$. By crystallisation a saturated solution can easily be freed from all but a small quantity. Estimations of nitrogen by Kjeldahl's method can easily be carried out on quantities of saturated salt solution up to about 50 c.c. No K_2SO_4 should be added but rather more H_2SO_4 than usual: for the distillation also more water than usual should be added.

By means of this salt I again proceeded to the quantitative study of the separation of mixed albumoses from the digestive mixture. Instead of Witte's peptone I used a mixture obtained by the peptic digestion of commercial casein. My main object was again to demonstrate the possibility of completing the separation and of proving it complete. But I also desired to shed further light on the salting out process itself, which though much used has been very little studied.

Some casein was digested with pepsin and HCl for 24 hours, when the mixture gave a strong biuret reaction. The clear liquid was poured off from the remaining insoluble casein, was neutralised, boiled and filtered. The liquid was then evaporated to smaller bulk, poured into a measuring flask and made up to 500 c.c. The total nitrogen was then estimated in four portions each of 5 c.c. The mean of these was 24·08 mg. N. 60 c.c. containing, therefore, 288·96 mg. N was evaporated to 40 c.c. and placed in a graduated beaker. The liquid was then placed in a water-bath at 40° C. and carefully saturated with dry powdered Na_2SO_4. The whole was allowed to stand in incubator at 37° C. for three hours. It was then filtered and the precipitate twice washed in order to collect portions of the filtrate remaining in the beaker and filter-paper. The total N was then estimated in the filtrate. The precipitate was dissolved, again salted out and after standing filtered off. On this occasion and subsequently the precipitate was not washed. I have already pointed out that simple washing only serves to get rid of that portion of filtrate taken up in the filter-paper: it has no effect on the impurity intimately connected with the albumose, as may be shown by direct experiment. On this occasion therefore I rubbed the precipitate in a saturated salt solution. This rubbing is carried out best as follows. The previous filtration is so arranged that nearly all the precipitate remains in the beaker. (This is quite easy with Na_2SO_4 precipitation.) A quantity of saturated solution is then put in the beaker and the latter is placed in a water-bath at a temperature of 45°—50° C. At this temperature the albumose becomes quite soft and is easily rubbed so that all parts of it come in contact with the liquid. Care must be taken that there is always small excess of salt to ensure complete saturation. (Subsequently I showed by direct experiment that albumoses suffered no decomposition by being heated to 60° C. with saturated Na_2SO_4.) The rubbing is continued some 15—20 minutes. The salt solution is then poured on to the filter-paper, practically the whole of the precipitate remaining in the beaker as before. The rubbing may then be repeated, the wash water again being poured into the filter. In the case of experiments 2, 3, and 6 the nitrogen in these washings was estimated in order to see the exact effect of the process, in the case of exp. 6 two rubbings being undertaken. The precipitate adherent to the filter-paper is dissolved off in the manner before described, added to that in the beaker which is also dissolved, the whole made to given volume and again saturated with salt. Before the nitrogen in the filtrates became constant, showing complete separation, the albumoses were precipitated six times and washed by rubbing 14 times. The results are shown in the table.

TABLE II. *Amount of* N *in mixture operated on* 288·96 *mg.*

Precipitations	Vol. of filtrate	Vol. of wash water	N in filtrate in mg.	N in wash water in mg.	Calculated impurity in filtrate	Calculated impurity retained in ppt.	Calculated percentage impurity retained in ppt.
1st	46 c.c.		42·25		39·45 mg. N	35·04 mg. N	48
2nd	45	14 c.c.	19·77	3·57	17·03	18·01	51
3rd	38	11	9·25	1·61	6·94	6·04	47
4th	40	9	6·93				
5th	39	15	5·88				
6th	40		3·29				
		41		2·8			
		24		1·05			
7th	40		2·45	N value constant.			
8th	40		2·59				
9th	40		2·45				
Totals	368	114	103·89				

482 c.c. Total in filtrates and washings.

Albumose found at end of experiment	178·46 mg. N
Total N in filtrates and washings	103·89 mg. N
Total N found	282·35

Loss of substance in experiment 6·61 mg. N = 2·3 %.

It is again clearly demonstrated that in repeated precipitations at constant volume the amount of nitrogen in the filtrate becomes after a time constant, proving the separation to be complete.

In the case of the first three precipitations I have again calculated the amounts of impurity in filtrate and precipitate. It is seen that about half the impurity present is retained in the precipitate at each precipitation. As in the previous series the calculations are based on the assumption that the amount of albumose in the filtrates is the same as it would be were no impurities present. This is not in reality the case, as there is more albumose left over in the filtrate when impurities are present than when there are none. I have already noticed this property of albumoses and other proteids, and it will be shown more clearly later on. If, however, we allow for this it would make the percentage of impurity retained in the precipitate even greater than shown in the table.

If the amount of albumose calculated to be in the total filtrates be added to the amount found at the end of the experiment, a total is arrived at which represents with some degree of accuracy the amount of albumose in the original digestive mixture. In this experiment counting albumose as containing 17 % N it would be about 1·22 grams. Thus the strength of the solution would be about 3 %. In the previous

series it was about 10 %. On comparing the two tables it will be seen that this difference in the strength of the solutions only makes a small difference in the "carrying down" property of the precipitates. Still, the proportion of the impurity retained in the precipitate is distinctly less in the more dilute solution, so that for salting out dilute solutions are to be preferred. It is also to be noted that in this experiment it was only requisite to salt out 6 as against 12 times in the preceding, so that working with a dilute solution together with washing by rubbing the precipitates diminishes the need for salting out so many times.

The difficulty of effecting a complete separation in this instance is well illustrated by noting that 362 c.c. of saturated solution were required in preparing about 1 grm. of albumose.

Sensitiveness of the test. Since a higher proportion of impurity is retained in the precipitate on salting out in the more concentrated solutions, it is clear that the more dilute the solution the greater will be the difference in the amount of nitrogen in two successive filtrates. Consequently the test of purity will be more sensitive if dilute solutions are employed.

In Table I it is seen that filtrates 6 and 7 have the same N value, although the albumose is not freed from impurity, and between filtrates 2 and 3 there is only a small difference. In this case I set all doubt at rest by continuing precipitation until constant N value was found in four successive filtrates. If however dilute solutions are used there is never need on a constant N value being obtained in two successive filtrates to further continue the precipitations.

Subsequent experience with this test has shown that it is best performed at a dilution 1—2 %. Reference to Table III clearly shows this. Two successive precipitations in solution of about 1½ % quite clearly show the last amount of impurity present; the first filtrate giving 1·54 mg. N, the second 1·05 mg. N, a well-marked difference considering the smallness of the amount of impurity 0·5 %.

A rough test. It will have been noted that the test for completion of separation that I propose takes some time to carry out. Using sodium sulphate two successive precipitations with N estimations cannot be done under two days. The investigator, therefore, will not desire to put the test in operation until he has a reasonable certainty that the separation is complete. To meet this I have devised a rough test by means of which the progress of the separation may be followed without having recourse to actual estimations of nitrogen.

To 2—3 c.c. of the filtrate an equal volume of concentrated sulphuric

acid is added and the whole heated to boiling point. This causes a brown or yellow colour to appear, which varies in intensity according to the amount of organic material present. If a series of filtrates be examined in this way it will be found that a gradation of tints is observable, the earlier ones being deeply coloured, while the later are only slightly tinted. The test can be used with solutions of ammonium or sodium sulphate of any concentration: it is especially useful in dealing with the former salt, since the biuret is not then practical. It is very useful when employed as follows. Two successive precipitations at constant volumes are performed, the filtrates are tested in this way, and if any difference is observed there is no need to proceed with the estimation of nitrogen.

Working with dilute solutions as explained above (1—2 %) and taking care that the conditions of testing are alike in each case a fair accuracy can be attained: such an amount of impurity as is shown in the experiment of Table III, that is about 0·5 %, being capable of detection. It is thus able to replace the actual N estimations except, of course, the final ones.

Washing by rubbing. In order to test this method more closely I sought to separate out the mixed albumoses from the digestive mixture almost entirely by its means.

A digestive mixture (pepsin and casein) was neutralised, boiled and filtered. It was then saturated with Na_2SO_4. The precipitate was then rubbed with successive portions of saturated solution. These rubbings were conducted at a temperature of 50°—55° C.; rather higher than before since the albumose is softer at this temperature. Each rubbing was continued about twenty minutes and the wash water then poured away, the small amount of albumose it contained being neglected.

The precipitate was washed in this way nine times; it was then redissolved, again salted out and again washed once. The progress of this separation was followed throughout by means of the sulphuric acid test above described. A small portion of the albumose was then tested. About 0·75 grams were dissolved in 52 c.c. water and salted out and the process continued as before.

TABLE III.

	Vol. of filtrate	N in filtrate
1	48 c.c.	1·54 mg.
2	55	1·05
3	54	0·98

Comparison of the N values of the first two filtrates shows that impurity was still present. In order to see what amount of impurity was present I salted out a third time and the N value being practically the same as in No. 2 and also very small, I concluded that nearly all the impurity present was got rid of in the first precipitation; the amount

being about 0·5 % of the albumose tested. The main portion of the albumose was then rubbed three times more to get rid of this last amount of impurity and again tested.

About 0·75 gm. taken and dissolved in 50 c.c.

1st filtrate vol. 48 c.c. N value 0·98 mg.

2nd ,, ,, 52 c.c. N value 0·98 mg.

Allowing for the difference in volume of the filtrates the N value is sufficiently constant to show the separation to be complete.

It may be here noted that when this test is employed in dilute solutions such as the above (which was about 0·5 %) the N of the second filtrate is expected to be rather less than that of the first, since an appreciable proportion of the albumose is lost in the first filtrate and there is less substance to be precipitated a second time, and so less in the filtrate. This will be noted in most subsequent experiments in dilute solutions.

In all, the albumoses were salted out twice and washed by rubbing 13 times. It is clear, therefore, that the latter process is very effective; it is also more rapid than repeatedly dissolving and reprecipitating.

In rubbing also less albumose passes into solution in the wash water than would be dissolved were it precipitated from the same volume of water by salting out.

Some mixed albumoses from casein were dissolved in 42 c.c. of water and salted out with Na_2SO_4, and allowed to stand 4 hours. The precipitate was filtered off and then well rubbed for fifteen minutes at a temperature of 45° C. with 20 c.c. of saturated solution. The wash water was then filtered.

	Vol.	N	% strength of solution in mg. N
Filtrate from precipitation	40 c.c.	2·24 mg.	5·6
Wash water	18	0·63	3·5

A second comparative experiment was performed in the same way except that the albumose was rubbed in a saturated solution at 60° C. (when it was very soft) on and off for 2 hours.

	Vol.	N	% strength of solution in mg. N
Filtrate from precipitation	36·5 c.c.	1·47 mg.	4·0
Wash water	40	1·05	2·5

The percentages of strengths of the solutions have, curiously, the same ratio to each other as in the preceding case; the sample of albumose is less soluble however.

From the second experiment it is seen that long-continued rubbing at higher temperature does not cause any more albumose to go into solution than when rubbed a shorter time at lower temperature; this proves that in both cases the wash water was saturated with albumose (saturated, that is to say, in the conditions prevailing), and that much less albumose is capable of going into solution by being rubbed in the

salt solution than is left in solution after a salting out. The experiment also shows that albumoses may be heated to 60° C. with saturated Na_2SO_4 solution without decomposition.

Effect of precipitating in dilute solutions. We have seen already that in dilute solutions the carrying down property of albumoses is less marked and that therefore it is best to salt out in 1—2 % solutions. Now the filtrates from solutions of varying strength do not all contain the same proportion of albumose. The concentration of the albumose in the filtrate varies with that in the original solution; the filtrate from the more dilute solution containing a smaller proportion of albumose than that from the more concentrated solution. So that in diluting a given solution of albumose to twice its volume and then precipitating, the albumose left in the filtrate is not twice as great as it would have been if precipitated undiluted.

I give the following experiment on 1 gram of albumose salted out with ammonium sulphate.

	Strength of solution salted out	Vol. of filtrate	N in filtrate	% of albumose in filtrate
1	10 %	12	1·26 mg.	0·0675 gms.
2	4	29	1·96	0·0434

This can be shown in a more simple and striking way by making a greater difference in the dilutions, say 10 % and 1 %, when a marked difference in the two filtrates can be shown by the sulphuric acid test. In exceedingly dilute solutions also (0·01 %) while a precipitate may be shown the amount left in solution may be hardly enough to detect.

Solubility of albumose. The above experiments and those in the preceding paragraph raise some interesting questions regarding the solubility of albumose in saturated salt solutions and the nature of the salting out process. No exact work has yet been done on this subject so far as I know. It would appear that albumose has no definite solubility in saturated salt solutions comparable to the solubility of a salt in water; but that saturated salt solutions will take up different quantities of albumose in different conditions. The more dilute the solution that is salted out the lower the concentration of the albumose left in the filtrate. It must be remembered, of course, that we are here dealing with a mixture of albumoses: but that does not explain the peculiarities noted.

Summary. In this section we have seen that the first test of purity proposed can be successfully applied to the separation of total albumoses

from the digestive mixture, and that by observing the proper conditions it is an extremely sensitive test.

We have also seen that the "carrying down" property of albumoses, hitherto under-estimated, is less in dilute than in more concentrated solutions, and that the quickest way of getting rid of impurities is to rub the albumose in the saturated solution at a temperature (45°—55° C.), at which it is quite soft, ordinary washing being ineffectual. We have further seen that ammonium sulphate can be replaced by sodium sulphate at 37° C., the two salts having almost exactly equivalent precipitating powers: that the nitrogen of small amounts of organic matter can be estimated in the saturated solutions of either of these salts: and that the more accurate quantitative method can be largely replaced by a simple colorimetric test.

Section II. Estimation of albumoses.

It will have been noted that in the experiments shown in Tables I and II I have been able to make an estimation of the amount of albumose present in the mixtures dealt with. On that basis I have endeavoured to found a method for the estimation of the total albumoses in the digestive mixture. There are several methods already in existence. These, however, are dependent on a single precipitation of the albumoses, followed by simple washing, so that from what has gone before it is evident that they must be liable to enormous errors, and I do not propose to discuss them further[1].

A consideration of the experiments set forth in Tables I and II shows that the bulk of albumose is obtained free from impurity at the end of the experiment and can be estimated directly by Kjeldahl's method; while the very experiment that proves the purity of the albumose gives the indication of how much albumose has been lost in the filtrates. The last two filtrates containing nothing but albumose give the solubility, and from this the albumose in the other filtrates is determined. It is not necessary, of course, to estimate the N of the filtrates except in the last two, the sulphuric acid test being used as an indicator of the progress of the separation.

In order to test the accuracy of this method I mixed known

[1] An account of these methods will be found in a paper by Baumann and Bömer in *Zeitsch. f. Untersuchung. d. Nahrung und Genussmitel.* 1. p. 107. 1898.

quantities of albumose with varying quantities of other nitrogenous substances.

The first experiment was performed with mixed albumoses from Witte's peptone (previously, of course, proved to be freed from impurity) and some products obtained by the decomposition of Witte's peptone by boiling with sulphuric acid till the biuret reaction had entirely disappeared. The sulphuric acid was got rid of by baryta, and the little ammonia formed was evaporated off. The mixture so obtained gave no biuret reaction and no trace of precipitate on being saturated with ammonium sulphate. It was therefore free from albumose (and also peptones). A portion of a solution of albumose was taken of which the N was carefully estimated by Kjeldahl's method, three estimations being carried out. This was mixed with a quantity of the mixture described in which there were 532·0 mg. N at a rough estimate. The whole was then saturated with Na_2SO_4 at a volume of 30 c.c. The further course of this experiment is seen in the following table. I estimated the N in the earlier filtrates not because it was necessary for the main object of the experiment but for the purpose of studying the course of the separation.

TABLE IV.

Precipitation	Vol. of filtrate	N in filtrate in mg.
1	24 c.c.	446·6
2	27	65·8
3	29	11·06
4	26	2·94
5	26	1·61
6	27	1·68
Totals	159	

Amount of albumose in mixture (mean of 3 determinations) = 215·6 mg. N

Albumose found (mean of 3 determinations) = 198·8 mg. N

Albumose estimated in filtrates = 9·8 ,,

Estimated albumose 208·6 mg. N

Error 3·5 %

Thus the estimate is 7 mg. N less than the true value. Now the error in the determinations by Kjeldahl's method, both of the albumose put in the mixture and that found at the end, could scarcely be more than ½ %. Therefore the greater part of the error (which is over 3 %) must be in the estimation of the albumose remaining in the filtrates. This was estimated on the supposition that the filtrates 1 to 4 contained the same percentage of albumose as the last two filtrates, which contain nothing but albumose. This estimate gives 9·8 mg. N. This, however, is much too low, since by deducting the amount of albumose found at the end of the experiment from that at the beginning we arrive at the figure 16·8 mg. The error, therefore, in the estimation of the albumose in the filtrates is very large; considering that the albumose in the last two filtrates is estimated to about 5 % it would appear that the main

error must be in estimating the albumose in the first four filtrates. The estimated amount is 6·51 mg. N, the real amount 13·51—an error of 50 %! It is clear that the albumose in the earlier filtrates cannot be properly estimated in this way and that there is more albumose left over in the saturated solution in the presence of other substances than when these are absent.

In order to test this point further I endeavoured to extract what albumose there was from the first filtrate. The Na_2SO_4 was got rid of by crystallisation and the liquid saturated with ammonium sulphate, when a small precipitate appeared. I then added Siegfried's iron alum solution, when a further precipitate appeared. (This was albumose, since it will be recollected there was no peptone present, and moreover the original mixture of nitrogenous bodies gave no precipitate when tested in this way.) This was filtered off, the iron got rid of and the albumose precipitated by saturation with ammonium sulphate, the precipitation now occurring easily. It was reprecipitated four times in great dilution, the last two filtrates showing practically no organic matter. The organic N was then estimated and gave 3·64 mg. This is more than double the albumose in the last two filtrates, which contain no impurity. This then is conclusive proof that in precipitation of albumoses by salting out more albumose is left over in the filtrate when impurity is present than when there is none.

This is also the case in the precipitation of primary albumoses, whether by sodium chloride or ammonium sulphate ("half saturation"). It may be simply demonstrated as follows. Some Witte's peptone at 10 % solution is saturated with NaCl: to the filtrate is added some acetic acid till a fair precipitate appears: this is filtered off, dissolved in water, neutralised, and brought to exactly the same volume as was the original solution. On again saturating with NaCl a well-marked precipitate results, showing that considerably more primary albumose is left in solution after precipitation when secondary albumoses and other substances are present in large quantity than when they are present in smaller quantity. If the primary albumoses are precipitated by the addition of an equal volume of saturated ammonium sulphate the same thing can be shown, the precipitation of the primary albumose in the filtrate being brought about by the addition of a little more ammonium sulphate solution (instead of acetic acid) as described in the introduction. The same phenomenon can also be demonstrated in the same way in the case of serum globulin and albumin. It would thus seem that this is a

general property of proteids on being salted out. By a single salting out only a very imperfect separation is effected: in the precipitate are carried down large quantities of the soluble substances belonging to the filtrate: while in the filtrate is left over an unexpectedly large amount of the insoluble substance of the precipitate.

It would appear also from the above experiments that the amount of precipitable matter left in solution in this way varies with the quantity of soluble matter in the filtrate. I also came to the conclusion from these and other more extensive experiments to be described later on that the different albumoses or proteids of serum dissolve much more extensively in each other than they do in non-proteid bodies; which was to be expected.

It is obvious that this property must considerably affect the method of estimation I propose, the estimate of the albumose in the filtrates being always too low.

In the preceding section I showed that by washing by rubbing much less albumose passes into the saturated solution than in precipitation. If, therefore, rubbing replaced precipitation in the experiment much less albumose would be lost in the filtrates. And I thought that if this amount were calculated in the same way from the amount in the two last filtrates from the final test of purity a more exact result might be obtained.

To test this I performed an experiment similar to the first except that after the first precipitation the separation was carried out by rubbing.

The albumose used was from casein, while the products artificially mixed with it were obtained also from casein but from a tryptic digestion. I thought that with such products a fairer test could be made. The tryptic digestion was carried on 5 days, when only a small precipitate was given on saturation with ammonium sulphate. The mixture was then evaporated to dryness and extracted with a saturated solution of Na_2SO_4 at 40° C.: this was then filtered and the bulk of the salt removed by crystallisation: it was then again saturated with Na_2SO_4 when a faint precipitate appeared: this, after standing, was filtered off and the bulk of the salt again removed. The liquid then only gave a trace of albumose on salting out, while a distinct biuret showed the presence of peptone. An amount of this containing 78·4 mg. N was mixed with some albumose containing 139·3 mg. N. The volume of the mixture was 46 c.c., it was saturated with Na_2SO_4, allowed to stand 5 hours and filtered. The precipitate was then rubbed with successive quantities of the saturated solution at 50° C., the wash water in every case being filtered to collect the small amounts of solid matter it contained. Seven such washings were performed and by the sulphuric acid test the last showed only a little organic matter. The albumose on the filter-papers was then dissolved off as described in the preceding section, together with the albumose that was on the sides of the beaker and on the spatula used for rubbing, the solution placed in the beaker and salted out; this was done to collect and purify these small

amounts. The whole was then further rubbed three times, the last wash water showing scarcely any colour with sulphuric acid.

The albumose was then dissolved in 35 c.c. water and salted out, allowed to stand 4 hours and filtered; it was then redissolved in 35 c.c. water, again salted out, standing 4 hours.

1st filtrate vol. 34 c.c. contained 1·33 mg. N,
2nd ,, 35 c.c. ,, 1·26 mg. N,

which showed the separation to be complete.

The albumose was then dissolved in water made up to 50 c.c. in a measuring flask and the N estimated, two observations being made.

The total volume of the wash water together with the filtrate from the first precipitation was 182 c.c. The amount of albumose in this was calculated on the basis that it was on the average at the same strength as in the last filtrate, viz. 1·26 mg. in 35 c.c.

Albumose put in mixture = 139·3 mg. N	Albumose found	129·5 mg. N
	Albumose in last two filtrates	2·59 ,,
	Albumose estimated to be in 182 c.c. washings	6·55 ,,
	Total estimated albumose	138·64 mg. N

It will be seen that a highly accurate result was obtained. I do not, however, claim that in estimating the albumoses of a digestive mixture anything like this accuracy is attainable.

It will no doubt have been noted that in the case of these artificial mixtures the separation is much more easy and rapid than in the original digestive mixture. This is especially marked in the experiment shown in Table IV. In this case working with a 5% solution of albumose the large excess of impurity is removed in only four precipitations.

The "carrying down" property of the albumose is scarcely at all marked, only about 15% of the impurity present being retained in the precipitate (in first two saltings out) as compared with 50% in the case of the digestive mixture. At first I thought that this striking difference might be due to the products used in Table IV being artificially prepared, and it was partly on this account that I used products from a tryptic digestion in the second experiment. There is no doubt, however, that in that experiment also the separation was much more easy than in a digestive mixture. The total volume of the filtrates and washings was 182 c.c.; in the experiment in the preceding section conducted by rubbing the total volume of filtrate and washings was 750 c.c.; and as more than three times the quantity of albumose was concerned the figure for comparison would be 250 c.c. Again, the former was precipitated once and rubbed ten times while the later was precipitated twice and rubbed thirteen times before the separation was complete. The ease with which the separation of Table IV was

conducted, therefore, could not be due entirely, if at all, to the products being artificial, though perhaps the absence of peptone had something to do with it. Perhaps it is that in the digestive mixture there is some sort of loose combination between the various substances. The phenomenon, however, whatever may be the cause, prevents a complete test of this method of estimating albumoses, and since in the case of the digestive mixture the volume of the wash water is greater than in these artificial mixtures, and since the principal source of error lies in estimating the albumose contained therein, it is clear that the error in the case of the digestive mixture will be the greater.

And furthermore the degree of error in the case of estimations in the digestive mixture would also depend on the proportion of albumose present, since the greater the quantity of peptones and other substances present, the more albumose will be dissolved in the washings, so that the higher the proportion of albumose present in any given liquid the less will be the error in the estimation. The high accuracy of the last experiment no doubt partly depends on the fact that there was a fairly large proportion of albumose present, about 64 % of the total nitrogen in the experiment belonging to the albumose. In this experiment the error is about 0·5 %. If there had been 10 times the amount of impurity, possibly 10 times the amount of albumose might have been carried into the wash water, and then the error would be 5 %. I do not think, therefore, in view of the unknown quantity of albumose that may be lost in this way, that the method can be relied on when there is only quite a small proportion of albumose in relation to other substances in the solution to be examined. The estimations would always be too low. In mixtures, however, containing a larger proportion of albumose, say 20 % and over, the method should give results of fair accuracy, and where there is a still higher proportion, say 60 % and over, considerable accuracy may be expected, and the error should not exceed 2·5 %.

Section III. The primary albumoses.

Having completely separated albumoses from the other constituents of the digestive mixture I now proceed to consider the separation of one albumose from another. Kühne divided albumoses into four different bodies, dys-, hetero-, proto-, and deutero-albumose, the three former being

separated out of the digestive mixture by complete saturation with sodium chloride. It has since been supposed that dys-albumose is an insoluble modification of hetero-albumose. Pick, using his fractional method separated out hetero- and proto-albumoses, primary albumoses, by means of the addition to the albumose mixture of an equal volume of saturated solution of ammonium sulphate. He furthermore separated hetero- from proto-albumose by means of adding to their solution an equal volume of alcohol. Kühne, on the other hand, effects this separation by means of dialysis, defining hetero-albumose as insoluble in water, proto-albumose as soluble. Folin[1] also has introduced another method of separating proto- from the secondary albumoses by means of copper acetate which produces a precipitate in the digestive mixture. As I have pointed out in the introduction, these authors (with the exception of Kühne, in the case of hetero-albumose) do not offer any adequate evidence in favour of the purity of their preparations. The probability is therefore that these bodies have not been completely separated from each other or from other substances. Furthermore the different methods have not been adequately correlated to each other. In the case, for example, of proto-albumose it has not been shown that the three different methods of Kühne, Pick, and Folin produce or tend to produce the same substance. Perhaps I should also mention the methods of Schrötter[2] who seeks to make separations by means of acetyl and benzoyl compounds: but here again there is no correlation to other methods.

In attempting to apply more exact methods to the study of these separations, I began by precipitating the primary albumoses with sodium chloride after Kühne.

40 grms. of Witte's peptone were dissolved in 400 c.c. of water, the solution completely saturated with NaCl and allowed to stand 24 hours. The precipitate was then filtered off and well rubbed in saturated salt solution eight successive times, it was then redissolved and reprecipitated and again rubbed in 10 successive portions of salt solution. I then tested as to whether the separation was complete, by two successive precipitations in the manner before described.

1st filtrate vol. 127 c.c. Of this 60 c.c. contained 19·32 mg. N.

2nd filtrate vol. 126 c.c. Of this 60 c.c. contained 11·76 mg. N.

It was thus clear that since the first filtrate contained more N than the second the separation was not complete.

Note. The Kjeldahl process can be carried out as usual in the presence of excess of NaCl. It is best to dilute the saturated salt solution before adding sulphuric acid.

At this point of the experiment a large proportion of the albumose was found to be

[1] Folin, *Zeitsch. f. physiol. Chem.* xxv. p. 152. 1898.

[2] Schrötter, *Monatschriften f. Chemie*, xiv., xvi., xvii., xix. 1893—1896.

insoluble: this was filtered off and the liquid dialysed for 24 hours in order to separate out some further portion of the hetero-albumose. The total nitrogen in a small portion of the clear filtrate was estimated, and it was found from this that rather more than a gram of albumose was present. The liquid was then evaporated to 25 c.c. and again saturated with NaCl. Owing to the failure of the rubbing method in this instance to effect the separation I continued to redissolve and reprecipitate, only occasionally rubbing. As a rough method for testing the progress of the separation I used the biuret reaction, adding the equal quantities of $CuSO_4$ solution and KOH to equal volumes of successive filtrates and observing the difference of tint. I reprecipitated the albumose 10 times before the N value of the filtrate became constant. Several precipitations were conducted at a 2 % solution, others at 3 or 4 %.

I give the last five filtrates.

	Vol. of filtrates	N in filtrate
10	23·5 c.c.	2·52 mg.
11	22·5	2·03
12	23·25	—
13	23·5	2·03
14	23·0	1·96

From this it is clear that the N value of the last four filtrates is constant and the separation therefore complete. By the biuret test I could detect a difference between filtrates 8 and 9, but not between 9 and 10.

From a comparison of this experiment with the previous ones it is clear that it is much more difficult to separate one albumose (or one group of albumoses) from another than to separate total albumoses from peptones and other bodies. This, however, is only what we should expect since it is generally found that the more nearly alike bodies are the more difficult it is to separate them. In this case the rubbing method only effected separation very slowly, while in the preceding cases it was comparatively rapid. In the previous cases also a 2 % strength of solution for precipitation was found fairly effective, but in this case 2 % dilution was not much better than 3 or 4 %.

In the first test experiment at a dilution of 0·5 to 0·75 % a fairly well-marked difference in the N of the filtrates is seen, so that had the experiment been continued at this dilution the separation would have been more quickly effected: I feared, however, that I should lose too much material.

Kühne in order to separate his proto- from his deutero-albumose precipitated the former with sodium chloride from 2—4 times: it is obvious, therefore, that his preparations must have been highly impure.

I next proceeded to compare the precipitating capacity of NaCl (complete saturation) with that by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution.

To do this effectually I first got rid of the greater part of the NaCl that was mixed with the albumose obtained as above, by precipitating it by the addition of about two volumes of saturated $(NH_4)_2SO_4$ solution, so that while most of the NaCl remained in solution, practically all the albumose present must have been precipitated. The albumose was then dissolved in a given volume of water and precipitated by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. This process was repeated and the two filtrates examined as to their organic nitrogen by the method explained.

1st filtrate vol. 22 c.c., N 0·56 mg.
2nd filtrate vol. 21·5 c.c., N 0·56 mg.

From which it is plain that all the albumoses precipitated by saturation with NaCl are also precipitated by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution, and further that the latter is a more effective precipitant than NaCl. (Compare previous table.)

I now proceed to the examination of Pick's method of preparing hetero-albumose by means of precipitating it by the addition of alcohol to the solution of albumoses: he claims that it is much better than Kühne's method. The method is certainly a careful and laborious one, involving no less than seven precipitations, and the substance so prepared must certainly be regarded as one of the purest albumoses yet obtained. I followed as exactly as possible his method of preparation as given on pp. 238, 239[1], and which consists (put briefly) of 3 precipitations with ammonium sulphate and 4 with alcohol.

I examined the resulting substance in the way I have proposed. It was dissolved as far as possible in hot water (150 c.c.) and precipitated by an equal volume of alcohol and allowed to stand 24 hours. The precipitate was then filtered off and the whole process exactly repeated. The N in the two filtrates thus obtained was estimated by Kjeldahl's method after the alcohol had been evaporated off and any traces of ammonia got rid of.

1st filtrate vol. 230 c.c., of this 40 c.c. gave 13·16 mg. N, in whole 75·67 mg. N.

2nd filtrate vol. 240 c.c., of this 40 c.c. gave 1·89 mg. N, in whole 11·34 mg. N.

From this it is clear that the preparation was not pure, the substance not being yet separated from those albumoses which are soluble in equal volumes of water and alcohol. And further examinations of the first filtrate showed the presence of secondary albumoses and a small quantity of an albumose easily soluble in equal volumes of alcohol and water, but precipitated by an equal volume of saturated $(NH_4)_2SO_4$

[1] Pick, *Zeitsch. f. physiol. Chemie*, XXVIII. 1899.

solution. The impurity amounted to about 10 %. I then carried the separation of the albumose precipitated by an equal vol. of alcohol to completion by a further precipitation.

Finally, two successive filtrates were tested as follows:

4th filtrate vol. 242 c.c. gave 8·12 mg. N.
5th „ „ 244 „ 9·1 „

Since the second filtrate actually contained more N than the first it was obvious that the separation was complete, since the excess must have been due to more of the substance of the precipitate being left over in solution. I subsequently found that this was due to the effect of temperature, the solubility of albumoses in alcohol and water being much affected by temperature.

It may be noted that in this experiment the impurity was almost entirely got rid of in the first filtrate, the difference between the N in the filtrates 1 and 2 being very striking, while there is very little difference between filtrates 2 and 4.

The amount of albumose obtained was about 5 grams, so that the dilution was rather less than 2 %. This is a striking contrast to the behaviour of albumoses with sodium chloride.

I then examined the behaviour of this fraction of albumose in water. During the precipitations it was apparent that a considerable part of it was insoluble in water at room temperature, and that it did not completely dissolve in hot water. The completely separated substance was very largely insoluble, both in hot and cold water; at the same time it was apparent that part of it was soluble.

The whole was well rubbed in hot water for some time and then allowed to cool. After standing some hours it was filtered, the clear filtrate being of a yellow colour and containing plenty of albumose. The insoluble portion was further rubbed in hot water, allowed to cool and again filtered: the filtrate on this occasion containing much less albumose. This process was repeated until the filtrate only contained quite a small quantity of albumose and was colourless, there being still plenty of insoluble matter. The filtrates were then united.

It is clear from this experiment that the albumose precipitable by the addition of an equal volume of alcohol can be further separated into two substances, one nearly insoluble in water, the other soluble. Now Kühne describes his hetero-albumose as insoluble or nearly so in water, and as I see no reason at present for changing this definition I propose to continue to speak of this water-insoluble fraction as hetero-albumose.

I found this substance to be very little soluble in water, either hot

or cold. After heating a portion with water to 60° C. for some time and filtering hot, the filtrate, which was clear and colourless, only showed a slight cloud after cooling and standing. This water-insoluble portion consisted of rather less than half the fraction insoluble in equal parts water and alcohol.

Thus we have a body clearly and sharply defined from other albumoses.

Fractional precipitation. The soluble substance I propose, provisionally, to call *a*-proto-albumose. The solution of *a*-proto-albumose obtained as above is, as we have seen, completely separated from all more soluble albumoses; we should expect, however, to find some considerable amount of hetero-albumose dissolved in it, since we have seen how the less soluble albumoses are always retained in solution by the more soluble. I therefore examined this solution for hetero- or water-insoluble albumose by the method of fractionation proposed in the introduction.

Saturated ammonium sulphate solution was slowly added till a small precipitate appeared. This precipitate on being filtered off and well pressed between filter-papers was found to be nearly entirely insoluble in water, hot or cold. It was clear, therefore, that hetero-albumose was present in some quantity. To get rid of it I proceeded to take further fractions by the addition of further small quantities of the saturated solution. The earlier fractions all showed themselves partly insoluble in water. After a time, however, a fraction was obtained which was completely soluble in water: this did not show that no hetero- was present but simply that there was sufficient *a*-proto-albumose and salt to hold what there was of hetero- in solution. On adding to this solution a small quantity of alcohol or salt solutions, *i.e.* taking a sub-fraction, a small precipitate was obtained which was partially insoluble in water; thus demonstrating the presence of hetero-albumose. Continuing the process the main liquid was entirely divided into small fractions in each of which hetero-albumose could be demonstrated in decreasing quantity, the last (obtained by nearly completely saturating the solution with salt) showing only a very small quantity.

Each of the soluble fractions had to be sub-fractioned in order to test for hetero-albumose, and this process was continued until a series of end fractions was obtained, each containing only a very little hetero-. Many of the middle fractions were then united and further fractioned. All the end fractions were then added together and the whole process repeated; this time alcohol being used as a precipitant. Finally, a small quantity of *a*-proto-albumose was obtained nearly free from hetero-albumose.

In this particular experiment the presence of the salt introduced a slight complication, since on testing the fractions it tended always to hold the hetero- in solution; so that in the later fractions it always had to be got rid of by further fractionation or precipitation by alcohol. It would, of course, have been much simpler to have done the whole fractionation with alcohol: but I found on attempting it that the precipitates were so difficult to filter that the ammonium sulphate process was quicker. In the sub-fractioning with alcohol that I spoke of I found no

difficulty, the presence of salt apparently influencing the precipitates and making them easier of filtration.

I also found that, after most of the hetero- had been got rid of, the fractionation with alcohol was much easier: hence I used it instead of salt in the repetition of the process.

The nearly pure substance obtained in this way I found to be fairly easily soluble in water, solutions of syrupy consistence being obtainable. It is thus sharply distinguished from hetero-albumose. It is nearly completely precipitated from its solutions by rather less than an equal volume of alcohol or by an equal volume of saturated $(NH_4)_2SO_4$ solution.

I next proceeded to compare the effects of precipitation by equal volumes of saturated $(NH_4)_2SO_4$ solution and alcohol.

Fifty grams of Witte's peptone were dissolved in 500 c.c. of water and precipitated by the addition of an equal volume of saturated ammonium sulphate solution. This was repeated eight times, when two successive filtrates were tested as follows:

8th filtrate vol. 940 c.c., of this 50 c.c. gave 1·88 mg. N,
9th „ „ „ „ „ 1·82 mg. N,

which showed the separation to be complete.

In this experiment the strength of the solution of the primary albumoses was about 0·5 % (that is after the addition of the salt solution). This would account for the separation being more rapid than in the experiment with NaCl. I did not attempt any rubbing.

It would appear that the best strength of solution at which to conduct precipitations varies with the substance to be precipitated and the nature of the precipitant. On the whole very much more dilute solutions should be used than those generally recommended by workers in albumoses.

As a simple test of purity during the progress of the experiment I employed the following process. 200—300 c.c. of the filtrate were completely saturated with the salt: the resulting precipitate was dissolved in quite a small quantity of water, 10 c.c., and an equal volume of the saturated salt solution added. If nearly the whole of the albumose present was precipitated it was clear that there could only be a very small percentage of secondary albumose present. The test is the more delicate the larger the quantity taken of the filtrate to be tested.

The sulphuric acid test is not applicable here since the primary albumoses, especially hetero-, give a much darker tint than secondary albumoses, so that in comparative experiments the presence of the latter is too much obscured.

Having thus entirely freed the primary fraction from all more soluble albumoses I got rid of the salt that was present, with the exception of the last small amount, by carefully pressing between filter-papers and then by dialysis. An equal volume of alcohol was then added to the mixture and the whole allowed to stand 24 hours. The filtrate from this evidently contained some amount of albumose. The precipitate was again as far as possible dissolved in water and reprecipitated a second time. The filtrate from this was united with the first filtrate and the alcohol evaporated off at 37° C.: the small quantity of $(NH_4)_2SO_4$ remaining was then got rid of by the careful addition of barium hydrate and further evaporation. The albumose was then precipitated by addition of excess of alcohol, filtered off and redissolved in water; being then entirely free from salt. The addition of an equal volume of alcohol now gave only quite a small precipitate, the bulk of the albumose remaining in solution.

The existence, therefore, was to be inferred of an albumose precipitable by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution, but soluble in equal parts alcohol and water. I will call this body, provisionally, β-proto-albumose. The proto-albumose prepared by Pick must no doubt have consisted principally or largely of this substance.

I then proceeded by means of the process of fractional precipitation as described above to free this substance from the hetero-albumose and a-proto-albumose dissolved in it.

In this case I used alcohol as a precipitant; I found that the precipitates came down readily and could be easily filtered: very often they sank and formed a sticky mass at the bottom of the beaker so that the clear liquid had only to be poured off. This rendered the process much simpler than in the case of the purification of a-proto-albumose. The first few fractions disclosed both the last-mentioned substance and hetero-albumose. I continued this process some time, fractions and sub-fractions being taken as before described, until at length I obtained a small quantity of β-proto-albumose that was nearly pure.

It was easily soluble in water and precipitable by the addition of an equal volume of saturated solution of $(NH_4)_2SO_4$. At room temperature it was not precipitable by the addition to its solution of an equal volume of alcohol. I found, however, that in precipitating this substance with alcohol much depended on the strength of the solution and on the temperature. Concentrated solutions in the cold 0°—5° C. were partially precipitated by an equal volume, while in the case of more dilute solutions at room temperature the substance did not begin to fall out of solution until two or perhaps three volumes of alcohol had been added. Even after the addition of several volumes of alcohol a considerable quantity of the substance always remained in solution. The substance may, therefore, be described as very soluble in equal parts alcohol and water, though this solubility varies with the tempera-

ture. It is thus clearly distinguished from *a*-proto-albumose, which is almost completely thrown out of solution by less than an equal volume of alcohol, even at 40° C. (It may be noted that the presence of $(NH_4)_2SO_4$ in a solution retards the precipitation of these bodies by alcohol and of course must be excluded in comparative experiments.)

I have not as yet had sufficient experience of the method of *fractional precipitation* employed in these two instances to enable me to form a final opinion as to the degree of purity attained by its means, but it is obviously a very efficient method when pushed far enough.

Primary albumoses are thus shown to consist of at least three different bodies:

1. Hetero-albumose.
2. *a*-proto-albumose.
3. *β*-proto-albumose.

I have not yet completed my investigations of these substances. It is possible they may be further split up. This question will be dealt with in my next paper, in which I shall also describe the secondary albumoses. Until this is done and we are enabled to get a general view of the series of substances into which albumose may be divided, I do not propose to make any detailed study of the constitution or properties of the bodies at present obtained.

Careful comparative experiments, however, have been made as to their behaviour with Millon's reagent which show an interesting difference between the two proto-albumoses. Hetero- and *a*-proto-albumose give only a deep yellow colour on boiling with Millon's reagent, while *β*-proto- gives a well-marked red colour, showing that the tyrosin group is present to a much greater extent in the latter than in the two former.

All three bodies give the glyoxylic reaction showing the presence of the tryptophane group.

In regard to Folin's copper method of preparing proto-albumose I found that the behaviour of albumoses is the same when precipitated by copper acetate (or sulphate) as when salts or alcohol are used, that is to say, large quantities of other albumoses are carried down. The two successive precipitations at about 10 % strength recommended by Folin are utterly inadequate to bring about anything like a complete separation. In two different preparations (one by another observer) I found secondary albumoses to the extent of 20 and 30 %. Hetero-albumose was also present in large quantity.

This indicates that the behaviour of these bodies is the same in

whatever way they may be precipitated, and that their separation is always very difficult.

In concluding I will describe two experiments very instructive as to the behaviour of nearly related proteid bodies towards each other on the addition of precipitating reagents.

An equal volume of saturated $(NH_4)_2SO_4$ was added to some serum; the precipitate after standing was filtered off. The filtrate was then by the gradual addition of the saturated salt solution divided into a series of fractions, the last being obtained by completely saturating the solution. In each of the fractions, some six in all, there was detected the presence of globulin, that is to say, of proteid insoluble in water, soluble in salt solution, precipitable by the addition to its solution of an equal volume of saturated salt solution.

In a similar fractionation by means of alcohol of a mixture of albumoses I was able to demonstrate the presence of hetero-albumose in every fraction, even in the last filtrate, which consisted of 90% alcohol.

SUMMARY AND GENERAL REMARKS.

1. In the foregoing Paper exact methods are proposed for the separation of proteids by precipitation.

(1) Repeated precipitation at constant volume till the organic nitrogen in filtrate is constant in quantity.

(2) Fractional precipitation. (To be carefully distinguished from Pick's method of taking fractions.)

2. The application of these methods to proteids of serum and the digestive mixture shows that the various procedures (crystallisation excepted) of previous authors in dealing with these complicated mixtures are quite inadequate to produce pure substances.

3. Albumoses have been completely separated from peptones and other bodies. On this separation is based a method of estimating total mixed albumoses.

4. Primary albumoses are shown to consist of at least three substances:

1. Hetero-albumose.
2. α-proto-albumose.
3. β-proto-albumose.

The last two substances have been obtained nearly pure and show an important difference in their constitutions.

5. The application of the above methods has advanced far enough to enable us, I think, to draw certain general conclusions.

It is abundantly clear that it is only by the rigid application of the chemical principle referred to at the outset that any real advance can be made, and that the difficulty of the separation of these bodies is very much greater than has hitherto been supposed.

We have seen that on a particular proteid (or group of proteids) being precipitated, it carries down with it large quantities of other proteids and also other substances: while large quantities of the proteid desired to be precipitated remain in the filtrate.

In the case of serum or the digestive mixture let us suppose that a fraction is taken: it will be found to contain some of the bodies soluble in saturated ammonium sulphate solution: it is to be inferred therefore that it contains a portion of every proteid present, since if still more soluble bodies are retained, how much more will the proteids be retained.

We have seen also that if a series of such fractions be taken, the last will contain small quantities of the most insoluble proteid present. We may therefore infer that in the last fraction there is also a portion of every proteid present, since if it contain the most insoluble, how much more must it contain the others. But if the first and last fractions each contain a portion of every proteid present, it is obvious that the intermediate fractions must also contain every proteid. Thus we arrive at the general conclusion that in a series of fractions every fraction must contain every proteid present.

There would not, of course, be equal quantities of the various bodies in each fraction: the first would contain a large quantity of the most insoluble, less quantities of the others, and only quite a small quantity of the most soluble. Each fraction would thus contain varying quantities of the different substances. Probably the relative amounts of the different proteids present in any one fraction would depend among other things on the dilution at which the precipitation was carried out.

We have seen that as the dilution increased the "carrying down" property becomes less; so that in great dilution it is possible that it may become quite small and that fractions thus obtained would only contain quite small amounts of the more soluble substances. I have no direct experiments on the point. Great dilution, however, and on this point I have some experience, does not prevent the substances properly belonging to the precipitate from being left over in the filtrate: so

that in all probability the conclusion I have drawn holds good at great dilution.

In regard to the method of fractionation as developed by Pick, I do not see that the fact, that, during the gradual addition of the precipitant to the proteid solution, at certain moments larger quantities and at others smaller or even very small quantities come down, can affect this conclusion. These natural boundaries, as we may term them, to certain fractions do not necessarily prove that the substances within them are pure; though it is not an unnatural inference to make. We have constantly seen in the case of the best marked of these divisions that between the globulins and albumins and between the primary and secondary albumoses such inference is highly erroneous. These "natural boundaries," then, can only be taken as showing that the proteid operated on is not homogeneous, and as indicating the existence of the various substances or groups of substances into which it can be split.

I think the best explanation of the tenacity with which these bodies cling to each other is to be found in the supposition that there is some sort of loose chemical combination among them. This hypothesis would also explain the fact that once a complete separation has been effected, if the products are artificially mixed again a second separation is not so difficult. It is also in harmony with our general knowledge of the chemical condition of the living organism. When the chemical equilibrium of such a system of proteids in loose combination is upset by the addition of a precipitant, only a partial separation is effected, some of the soluble proteids going into the precipitate and some of the insoluble remaining in the filtrate, the loose combination in these two cases being maintained.

It is only by extensive and exact methods such as I have described that we can hope to effect the isolation of such bodies.

This research was conducted during my tenure of the John Lucas Walker Studentship.