A METHOD FOR THE FRACTIONAL ANALYSIS OF INCOMPLETE PROTEIN HYDROLYSATES.

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The constituents of an enzymatic hydrolysate of protein can be divided according to their complexity into six fractions; protein, metaprotein, proteose, peptone, subpeptones, and amino acids. A method for the quantitative estimation of these fractions was devised in order to secure more definite information regarding the changes occurring during hydrolysis than is obtained by the usual free amino nitrogen determinations. The method has stood the test of continued use by different workers for over a year, and has given consistently accurate results.

Briefly, the method involves the precipitation of the protein by trichloroacetic acid, of the metaprotein by careful adjustment of the reaction, of the proteoses by Na_2SO_4 at 33°C., of the peptones by tannic acid under definitely fixed conditions; and the determination of the residual amino acids and simple peptides by a slight modification of the alcohol precipitation methods of Folin and Denis (1) and Van Slyke and Meyer (2). No new principle is introduced, but quantitative results with these reagents were found to be obtainable only under rigidly fixed conditions. The metaprotein fraction is determined on a separate aliquot of the original solution. For the assay of the other five fractions 100 cc. are required, containing not less than 200 mg. of nitrogen. The amount of each fraction is estimated by the nitrogen content, obtained by difference before and after filtration.

I.

Method of Fractional Analysis of Hydrolysate.

The total nitrogen of the hydrolysate is first determined on 5 cc. samples in triplicate. Then, to 40 cc. are added 10 cc. of

10 per cent trichloroacetic acid, and the mixture is allowed to stand for 1 hour. The solution is filtered and the total nitrogen determined on two 5 cc. portions of the filtrate. The difference between the first and second total nitrogen estimations is the sum of the protein and metaprotein.

To estimate the amount of metaprotein, a portion of the original solution of which the total nitrogen is known is brought to pH 6.0. At this hydrogen ion concentration the insolubility of albumin acid metaprotein was found to be greatest. The solution is filtered and the total nitrogen of the filtrate determined. After allowing for dilution, the difference between the total nitrogen of the original solution and of this filtrate is taken as a measure of the metaprotein content. The difference between the metaprotein nitrogen and the total trichloroacetic acid precipitable nitrogen is the protein nitrogen.

The remainder of the filtrate from the trichloroacetic acid precipitate, approximately 35 cc., is poured into a 35 cc. volumetric flask, provided with a long, wide neck, and graduations above and below the 35 cc. mark. It is then boiled in a water bath for 3 hours to decompose the trichloroacetic acid, and to drive off the resulting carbon dioxide and chloroform, cooled to room temperature, and made up to the original volume before boiling. Approximately 30 cc. are poured into a 50 cc. Erlenmeyer flask and 20 gm. of Na_2SO_4 are added. The excess of salt is advisedly as little as possible, in order to minimize the possibility of error due to abstraction of water by the hydrated crystals. The mixture of salt and solution is kept at 33°C. for $\frac{1}{2}$ hour, instead of 32.5°, which is the temperature of maximum solubility, because the transition point from the hydrated to the anhydrous form of Na_2SO_4 is at 32.75°; and when anhydrous salt is employed at 33°C., no hydration of the undissolved salt, and hence no error due to concentration of the solution, can occur. The difference in solubility at the two temperatures is negligible. In a water-jacketed filter, maintained at 33°, the solution is then filtered up to the mark into a 25 cc. volumetric flask without allowing it to cool, it is then washed quantitatively into a 50 cc. volumetric flask, and made up to volume. Two 10 cc. aliquots are taken for total nitrogen. The difference between this total nitrogen after allowing for the dilution, and the nitrogen of the trichloroacetic acid filtrate, is the proteose nitrogen.

25 cc. of the filtrate are pipetted into a 200 cc. Erlenmeyer flask; 25 cc. of 2.21 N sodium hydroxide and 125 cc. of 20 per cent tannic acid dissolved in 0.1 N H_2SO_4, containing 20 per cent Na_2SO_4, are then added in that order. The mixture is thoroughly shaken and kept at 20°C. for 4 hours. It is important to maintain the temperature near 20°C. because at temperatures only slightly higher the precipitation of peptone by tannic acid is not complete (see Table III); and at lower temperatures salt begins to crystallize out of solution, concentrating it by abstracting water of crystallization, and thereby introducing a small error. The solution is filtered, and two 50 cc. portions of this tannic acid filtrate are taken for total nitrogen. The difference between this and the previous total nitrogen gives the peptone nitrogen.

The large amount of carbon to be digested necessitates modification of the usual procedure of the Kjeldahl method. The 50 cc. of tannic acid filtrate are pipetted into a 500 cc. Kjeldahl flask and a piece of copper wire and 1 cc. of concentrated H_2SO_4 are added. The flask is then heated on the digestion rack until salt begins to crystallize out of the hot solution. It is cooled, 7 gm. of K_2SO_4 are added, and then 24 cc. of concentrated H_2SO_4. The digestion is allowed to continue until all frothing has ceased and the solution has become freed of all suspended particles. The solution, still a dense dark brown, almost black, is cooled, 5 cc. of 10 per cent mercuric acetate are pipetted in, and the digestion is then carried to completion in the usual manner.

A portion of the tannic acid filtrate is adjusted to pH 5.0, and then pipetted into nine times its volume of 95 per cent alcohol. It is then allowed to stand for 24 hours. This hydrogen ion concentration was shown by electrometric titrations to be in the region of the isoelectric points of both proteose and peptone, including subpeptone, obtained from albumin, Witte's peptone, and Parke, Davis and Co.'s peptone. At hydrogen ion concentrations on either side of this point the precipitation of proteose, and to a greater extent of peptone, falls increasingly short of completion. After standing 24 hours the solution is filtered. To the filtrate 2 or 3 drops of saturated alcoholic solution of zinc chloride are added. After standing for a few minutes the mixture is again filtered through a dry paper. A measured amount of the second alcoholic filtrate is concentrated down on a water bath,

or, if the amounts of alcohol are too large to be lost, in Claisen flasks according to the procedure of Van Slyke. The residue is taken up again with distilled water, made up to known volume, and the total nitrogen determined.

The nitrogenous material remaining in solution, consisting probably of amino acids and the simpler peptides, has been termed the residual nitrogen.

The nitrogen in the alcohol precipitate is the subpeptone nitrogen.

II. DISCUSSION.

Trichloroacetic acid for the precipitation of protein has been employed for years; but its failure to precipitate proteoses is not so widely known. Hiller and Van Slyke (3) are of the opinion that intermediate products are precipitated by trichloroacetic acid, and Shonle and Waldo (4) have recently employed precipitability by this reagent as a criterion for proteose. In our hands, carefully purified proteose from such widely varying sources as Witte's peptone, Merck's pepsin, Eimer and Amend's pepsin, Parke, Davis and Co.'s peptone, and a peptic digest of egg albumin, failed entirely of precipitation by trichloroacetic acid. Even a derived protein, so little changed as gelatin, is not precipitated by as high a concentration of trichloroacetic acid as 30 per cent. Our results are in accordance with those of Shin Shima (5).

The conditions in which the tannic acid precipitation of peptones gives quantitative yields required careful and exact definition. The methods described in the literature, Simon (6), Henriques and Gjaldbäk (7), and Plimmer (8), were found by trial to be unsatisfactory. Tannic acid was chosen because it precipitates most of the polypeptides known to be precipitable (9); and because histidine, tyrosine, and cystine, which are precipitated by otherwise suitable reagents, such as sodium tungstate, are not precipitated by tannic acid. Alcohol precipitation is undesirable because as Van Slyke and others have pointed out, alcohol precipitates are inextricably contaminated with other non-precipitable contents of the solution. Tannic acid under optimum conditions precipitated about 51 per cent of the nitrogenous material originally precipitated by alcohol.

The estimation of the amino acids was at first attempted by the usual Van Slyke amino nitrogen determination on the tannic acid filtrate before and after acid hydrolysis. Unexpectedly a reaction was found to occur between the tannic acid employed and the nitrous acid, which liberated a large amount of gas not absorbable by the alkaline permanganate. 5 cc. of tannic acid solution, containing no protein material, gave 18 cc. of gas. The nitrous acid method was clearly not feasible. Direct determination by alcohol precipitation was then resorted to. The final procedure employed was a slight modification of the methods of Folin and Denis, and Van Slyke and Meyer, for the determination of amino acids in blood.

The results for the residual nitrogen fraction by this method are probably too low, but it is the only ready method available, and for comparative work gives results, as the work of Folin and Van Slyke has shown, sufficiently indicative of the actual figures.

III. EXPERIMENTAL.

The procedure described for the complete analysis of a digest was arrived at after investigation and testing of methods for the precipitation of each of the fractions separately.

Protein Fraction.

Albumins and globulins were employed as the sources of protein. Hiller and Van Slyke found that concentrations of trichloroacetic acid above 5 per cent have some slight hydrolyzing power, and, accordingly, amounts of this reagent were used such that the final concentration did not exceed 2 per cent.

In order to test the thoroughness of this mode of precipitation, a 3 per cent solution of egg albumin (Baker and Adamson) was filtered and precipitated with trichloroacetic acid. The experiment was carried out in triplicate. 96 per cent of the protein nitrogen was found to have been precipitated, and the triplicates were in quite satisfactory agreement.

The albumin on subsequent analysis was found to contain 94.5 per cent protein, 4.4 per cent proteose, and 1.1 per cent peptone.

In order to determine whether temperature variations over a normal range have any effect on the degree of precipitation, a 5 per cent solution of egg albumin (Merck) was precipitated in 2 per cent trichloroacetic acid at 10°, 15°, 20°, and 25° (± 0.5°). Again, approximately 96 per cent was

precipitated and the results showed that temperature changes, within the above range, are without effect on the degree of precipitation of protein by trichloroacetic acid.

Metaprotein Fraction.

The following experiments indicate that metaprotein is also precipitated quantitatively by trichloroacetic acid.

A solution of Lieberkühns jelly was made in dilute HCl and filtered, giving a clear filtrate, containing 10.4 mg. of metaprotein N in 10 cc. This filtrate was neutralized to litmus, producing a filterable suspension, which was divided into two portions, *A* and *B*. *A* was filtered and to the filtrate sufficient 20 per cent trichloroacetic acid was added to give a final concentration of 2 per cent. It was allowed to stand for 5 minutes, filtered, and the total nitrogen of the filtrate determined. To *B*, with no previous filtration, trichloroacetic acid was added directly and it was then allowed to stand for 5 minutes, after which it was filtered and the total nitrogen of the filtrate determined. In *A*, metaprotein was removed by filtration, after neutralization, before the addition of the trichloroacetic acid, leaving a considerable residue of protein, as shown by the subsequent precipitate with trichloroacetic acid. On comparing the total nitrogen of the final filtrates from *A* and *B* they were found to be identical.

Proteose Fraction.

The quantitative precipitation of proteose involves inconveniences not encountered in the trichloroacetic acid precipitation of protein. Ammonium sulfate cannot be used on account of its nitrogen content; and Na_2SO_4, which is employed instead, for quantitative precipitation, involves filtration at 32.75°C. (10). It is desirable also to use a specimen of salt with as low and as constant a water of crystallization as possible. The commercial C.P. anhydrous salt is quite suitable, and the volume changes due to the addition of the Na_2SO_4 can be determined in controls and the necessary corrections estimated.

To test the possibility of quantitative precipitation of proteose in this way, 5 cc. of 5 per cent proteose, obtained from Parke, Davis and Cos'. peptone by salting-out with Na_2SO_4, were pipetted into each of three test-tubes, to which were added 3 gm. of Na_2SO_4, and kept at 32–34° for 1 hour, with frequent stirring. The solutions were then filtered directly into 500 cc. Kjeldahl flasks and the precipitates washed three times in the funnels with saturated Na_2SO_4 at 32.5°C. Total nitrogen estimations were carried out in the usual way on these filtrates and on the original solution.

The precipitation was practically complete, 99.2 per cent of the proteose was precipitated, and the checks were in good agreement.

In the actual working routine the procedure is somewhat different. The solution saturated with Na_2SO_4 at 33°C. is filtered at the same temperature, using a filtration box (see below), into a 25 cc. volumetric flask also maintained at 33°C. When 25 cc. of filtrate have been obtained the flask is removed and its contents are immediately washed quantitatively into a 50 cc. volumetric flask and made up to volume. At this dilution the solution is just under saturation at room temperature (20°) and can be pipetted without clogging the point of the pipette. With this procedure, after allowing for the dilution, 99.1 per cent of the proteose was removed.

For the convenient carrying out of this filtration a piece of apparatus, the filtration box mentioned above, was constructed. This consists essentially of two copper boxes, one above the other, connected by two side walls and a back of sheet copper. The upper box is a battery of four hot water

TABLE I.

Effect of Varying C_{H^+} on the Precipitation of Proteose by Sodium Sulfate.

pH	Total N.	N after filtration.	Removal.
	mg.	*mg.*	*per cent*
3.0	87.2	2.0	97.7
4.0	87.2	2.0	97.7
5.0	87.2	2.0	97.7
6.0	87.2	2.0	97.7
6.6	87.2	2.0	97.7
9.2	79.3	1.8	97.7

funnels of suitable dimensions, maintained at 33°C. by a micro burner, with a steady stream of air bubbling through the water for mixing purposes. The flasks into which the liquid is filtered, stand in the lower box, in water, also maintained at 33°C. The depth of this box is such that the bulb of the 25 cc. volumetric flask may be kept under water. The flasks are held in position by a lid of sheet copper, which has four narrow slots, each just wide enough to admit the neck of a flask. The stems of the funnels project for about 1.5 inches into the necks of the flasks.

It was necessary to ascertain the influence, if any, of the hydrogen ion concentration on the degree of completeness of precipitation of proteose by Na_2SO_4. Proteose from Parke, Davis and Co.'s peptone, thrice precipitated and washed, was dissolved in distilled water and brought to a number of different hydrogen ion concentrations, maintaining the same dilution in all. The precipitation was carried out as described above. The results are given in Table I.

The degree of precipitation by Na_2SO_4 is clearly independent of the hydrogen ion concentration. This result was expected because it is unlikely that "salting-out" is either an isoelectric point phenomenon, or a precipitation as an insoluble salt. The consistency of the nitrogen content of the filtrate indicates probably an impurity. However, even if it represents a soluble residue, the smallness of the amount, and its constancy, testify to the serviceability of the method, with proper controls, for quantitative work.

The allowance for the dilution, and for the increase of volume due to heating to 33°C., is made by noting the increase in volume of water at 20°C., saturated with Na_2SO_4 at 33°C. This is carried out in a 50 cc. volumetric flask, with a long graduated neck, containing 50 cc. to the base of the neck.

The following is a typical example of the order of magnitude of the increase. 50 cc. of water at 20°C., saturated with Na_2SO_4 at 33°C., occupied 53.5 cc. Since only 25 cc. are employed in the analysis, and are diluted to 50 cc., the value of 1 cc. of diluted filtrate is $\frac{50.0}{53.5} \times \frac{1}{2} = 0.467$ cc.

Peptone Fraction.

For the determination of the optimum conditions for peptone estimation by tannic acid, after preliminary experiments, the various factors which might affect the degree of precipitation were separately investigated. After each precipitation, total nitrogen estimation was carried out on the filtrate. The first factor investigated was the influence of the hydrogen ion concentration. The varying conditions and their results are summarized in Table II.

The pH in every case was estimated electrometrically in duplicate. The mixtures were allowed to stand for 24 hours at room temperature before filtration. The results show that there is a definite optimum pH in the neighborhood of pH 7.0.

The experiment was repeated with twice the final concentration of tannic acid and optimum precipitation was again found to occur at exactly the same reaction, *viz.* pH 7.0, the results being similar to those shown in Table II.

Incidentally a curious phenomenon was observed, which seems worth noting; *viz.*, that on exposure to air, the tannic acid solutions became darker and curiously more acid, not unlike the behavior of hemoglobin on oxidation.

It is desirable, for several reasons, to use as little tannic acid as possible in this operation and accordingly the minimum concentration of tannic acid necessary for maximum precipitation was next explored. The minimum concentration was found to be about 14 per cent which effects a removal of 44 per cent of the nitrogen of Parke, Davis and Co.'s peptone remaining in

TABLE II.

Effect of C_{H^+} on Precipitation of Peptone by Tannic Acid.

Peptone solution.	20 per cent tannic acid in 0.1 N H_2SO_4.	0.221 N NaOH.	H_2O	pH	N in filtrate.
cc.	*cc.*	*cc.*	*cc.*		*mg.*
5					31.2
5	25	0	70	2.7	30.8
5	25	10	60	3.7	30.8
5	25	20	50	4.7	29.4
5	25	30	40	5.9	27.9
5	25	40	30	6.6	27.1
5	25	50	20	7.1	26.7
5	25	66	14	7.6	29.4
5	25	70	0	7.6	29.6

solution after precipitation of the proteoses by Na_2SO_4. A precipitation carried out simultaneously according to the procedure of Henriques and Gjaldbäk (7) removed only 20 per cent.

The experiments were all carried out in the summer at room temperature. One hot evening it was found, that under conditions so far considered optimal, very little precipitate was obtained. Cooling under the tap caused immediate precipitation. Evidently temperature is an important factor that cannot be disregarded.

Five portions of the peptone solution were precipitated with the optimum concentrations of tannic acid and alkali at 10°, 15°, 20°, 25°, and 29° (± 0.5°). The solutions were maintained at these temperatures for 3 hours and then filtered.

The N in the filtrate was determined with the results recorded in Table III.

It is obvious from these results, that the tannic acid precipitation should not be carried out at temperatures higher than 20°C. It was found that 3 hours standing was only very slightly, if any, less effective than 24 hours. With 24 hours standing the nitrogen content of the filtrate from a precipitation carried out in the same manner as the 20°C. experiment above, was 34.7 mg.; with 3 hours standing, 35.2 mg.

To test if precipitation had been complete, solutions of peptone of various concentrations were precipitated with tannic acid solutions under the optimum conditions so far worked out, with

TABLE III.

Effect of Temperature on Precipitation of Peptone by Tannic Acid.

Temperature.	Total N in filtrate.	Removal.
C.	*mg.*	*per cent*
Control.	79.2	
10	32.9	58.4
15	32.7	58.4
20	33.0	58.3
25	28.4	51.5
29	79.2	0.0

the expectation that the same percentage of the total nitrogen would be precipitated out of each solution. The most concentrated solution of peptone was saturated with Na_2SO_4 at room temperature, and the other solutions were made up from it by dilution with water. The results are given in Table IV.

The difference in the percentage removal between the last two samples with a salt concentration of 145 gm. per liter was probably due to the dehydration caused by the crystallization of salt which occurred in the second of these and which, therefore, would increase the concentration of the dissolved substances there. The results shown in Table IV compelled investigation of the effect of salt concentration.

Peptone was prepared from Parke, Davis and Co.'s peptone, in the usual manner, by salting-out with Na_2SO_4 at 33°C. The concentrations of salt were varied by adding varying amounts of Na_2SO_4 to the 0.1 N H_2SO_4

in which 20 per cent of tannic acid was dissolved. Optimum precipitation was found to occur when the tannic acid solution employed contained 20 gm. of Na_2SO_4 in 100 cc. The degree of precipitation was then independent of the peptone concentration.

After this experiment it was thought that possibly a lower concentration of tannic acid might be employed, especially as a great deal of sodium tannate in addition to peptone tannate is precipitated when 14 per cent is employed.

With peptone solution from Parke, Davis and Co.'s peptone, and 20 per cent tannic acid dissolved in 0.1 N H_2SO_4 containing 20 per cent Na_2SO_4,

TABLE IV.

Effect of Salt Concentration on Degree of Precipitation of Peptone by Tannic Acid.

Salt concentration.	Total N.	N in filtrate.	Removal.
gm. per l.	*mg.*	*mg.*	*per cent*
120	94.5	41.6	56.0
60	72.1	32.7	54.6
30	47.0	24.9	46.9
15	24.7	15.3	38.1
145	94.5	35.6	62.3
145	24.7	9.7	60.9

TABLE V.

Fractional Analysis of a Known Mixture of Products of Peptic Hydrolysis.

	Calculated per cent of total N.	Experimental. Per cent of total N.			
		1	2	3	4
Protein	9.7	9.9	9.7		
Proteose	18.3	18.3	18.3		
Peptone	40.4	39.4	40.4	39.8	39.6
Subpeptone	31.6	32.4	31.6	31.3	31.6

mixtures were made up with the final tannic acid concentration varying from 7 to 14 per cent, and the precipitation was carried through under optimal conditions.

The results showed that on the whole the conditions for optimum precipitation were those arrived at previously.

With the completion of this experiment, all the possible factors had been taken into account: reaction, concentration of tannic acid, temperature, time, concentration of salt, and concentration of peptone.

A mixture was then made containing measured amounts of protein, proteose, peptone, and subpeptone, and analyzed by the method described on page 1, which is based upon the methods worked out for each of the fractions separately. The results are given in Table V.

Nos. 1, 2, and 3 were carried out on a total nitrogen content of 237.6 mg.; No. 4, on 207.9 mg.

TABLE VI.

Fractional Analysis of Peptic Hydrolysates in Presence of Varying Amounts of Pepsin of Known Fractional Composition.

	Pepsin.	Total N of digest after pepsin N was subtracted.	Per cent of total nitrogen.			
			Protein.	Proteose.	Peptone.	Sub-peptone.
	gm.	*mg.*				
A		269.1	8.4	31.7	34.7	25.3
B	0.51	294.5	8.4	31.8	35.9	23.7
A		345.5		38.1	32.1	30.3
B	1.03	345.5		38.1	32.4	30.3
A		470.0	1.3	32.0	35.6	31.0
B	1.1	536.0	1.0	32.0	35.9	31.3
A		307.1		38.1	32.1	29.8
B	1.0	337.8		38.3	30.5	31.4

TABLE VII.

Relation of C_{H^+} to Amount of Material Precipitated in 95 Per Cent Alcohol.

Proteose.		Peptone.	
pH	Weight precipitated from 10 cc. solution.	pH	Weight precipitated from 10 cc. solution.
	mg.		*mg.*
2.1	139	1.5	6
2.4	144	1.7	16
2.9	156	1.9	53
3.4	159	2.5	81
4.3	167	3.4	102
5.3	167	4.2	129
7.6	159	5.0	140
8.3	156	6.9	122
9.5	155	8.4	99

More complete confirmation of the accuracy of the method was provided by the following series of analyses. Eimer and Amend pepsin was analyzed, and it was found that 2.00 gm. contained 259.9 mg. of nitrogen; that it was free of protein nitrogen, and contained 31.0 per cent as proteose, 42.2 per cent as peptone, and 26.8 per cent as subpeptone. A number of samples from several prolonged and concentrated peptic digests were ana-

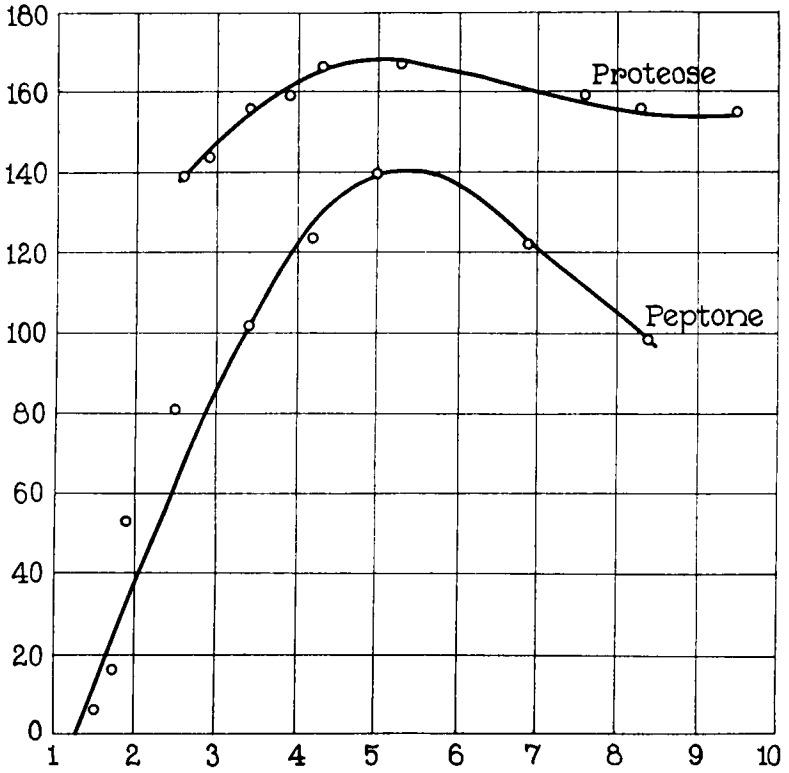

FIG. 1. Relation between pH and amount of proteose and peptone precipitated from 10 cc. of 2 per cent solution in 95 per cent alcohol. pH given as abscissæ; ordinates, amount precipitated in mg. of dry weight.

lyzed, half of which, *A*, contained no additional pepsin, and the other half, *B*, varying amounts. The values of pepsin corresponding to the above were subtracted from the analytical results obtained with *B*, and the final data compared with the results of *A*. The correspondence, as Table VI shows, is quite good. The widest varying pair, out of eighteen such analyses, carried out over a period of 5 months, is the last two given. The largest

error is in the peptone and subpeptone fractions where the titration result is multiplied by approximately 16, while the results for protein and proteose are multiplied only by 8; and all four are expressed in terms of percentage of the same original total nitrogen.

Residual Nitrogen.

The dependence of the degree of precipitation of proteose and of peptone by alcohol upon the hydrogen ion concentration is shown in Table VII and in Fig. 1. The phenomenon is similar to the precipitation of protein in alcoholic solution by small amounts of salt, which, as Loeb (11) has pointed out, is strongly indicative of the existence of a Donnan equilibrium.

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