XIX. A STUDY OF FACTORS SAID TO INFLUENCE THE NITROGEN DISTRIBUTION OF GELATIN.

By FLOYD SHELTON DAFT (*Cheney Fellow, Yale University*).

From the Carlsberg Laboratory, Copenhagen.

(Received December 12th, 1928.)

WITHIN the past few years, several publications have appeared from the Biochemical Department of the Imperial College of Science and Technology, London, concerning the effect on the nitrogen distribution of gelatin of certain preliminary treatments of the protein. In discussing these publications and in describing experimental procedures, terminologies which have been well established by custom will be employed. By the percentage of a type of nitrogen is indicated that part of the total nitrogen of the protein which, in the hydrolysate or in a designated fraction of it, appears in this form; the basic nitrogen refers to the nitrogen of those compounds which are precipitated, under the defined conditions, by phosphotungstic acid.

According to Knaggs [1923], if gelatin is allowed, prior to the hydrolysis proper, to stand at laboratory temperature with 20 % HCl, a marked increase in the percentage of basic nitrogen takes place; the percentage of amino-nitrogen is, however, unchanged. He attributes the increase in the basic nitrogen to the formation of difficultly hydrolysable polypeptides.

Knaggs and Schryver [1924] report that while the percentage of basic nitrogen remains constant, the percentage of non-amino-nitrogen of the filtrate is increased by certain treatments of this protein. In their experiments, before hydrolysis proper, gelatin was allowed to stand at laboratory temperature with *N* HCl or *N* NaOH or was heated at 105° with water. The effect of the three treatments on the nitrogen distribution of the protein is said by the authors to be, in general, the same. In regard to the treatment with water, the percentage of non-amino-nitrogen of the filtrate is shown to increase gradually with the time of heating until it reaches a fairly constant maximum.

These two sets of results appear to be somewhat inconsistent. Moreover, the results of Thornley [1927], coming from the same laboratory, are in serious disagreement with both of them. On the authority of Thornley's experimental results and the curve that he constructed from them, the preliminary treatment of the protein with *N* HCl or *N* NaOH causes a large increase in the percentage of basic nitrogen; preliminary treatment with 20 % HCl, on the other hand, causes a smaller increase in this fraction. The former findings are contrary to

those of Knaggs and Schryver, the latter are contrary to those of Knaggs. In agreement with Knaggs and Schryver, however, Thornley finds that heating gelatin with water causes practically no change in the basic nitrogen fraction. He finds, further, that subsequent treatment of the same material with HCl does not cause an increase of this percentage. Figures for non-amino-nitrogen are not included in his published results.

Finally, Schryver and Buston [1927] attribute the increase in the percentage of basic nitrogen, which they observed, to *dl*-lysine. The details of their isolation of this amino-acid are reported.

In many cases, we are unable to deduce from these publications the number of duplicates which have contributed to the results and the degree of agreement of these duplicates. There are, in addition, various inconsistencies in the conclusions drawn from the experimental work. The investigators agree, however, that the nitrogen distribution of gelatin is altered by preliminary treatment of the protein with acid or alkali. Were this fully established, it would be necessary to conclude that the protein molecule is much more labile than has been hitherto supposed. The figures appearing in the literature as nitrogen distribution numbers could not, moreover, be regarded as definite for the proteins in question. In view of the importance of these data, Professor Sørensen suggested to the writer that an investigation of the subject be carried out. The results obtained by this investigation show no change in nitrogen distribution due to preliminary treatment of gelatin with acid or alkali, and fail, accordingly, to support the principal contention of the investigators mentioned.

EXPERIMENTAL.

In order to become thoroughly familiar with the methods of analysis and to investigate certain parts of the procedure, we carried out twenty preliminary nitrogen distribution experiments. The samples of gelatin used for these analyses were divided into groups on the basis of the treatment given them prior to the hydrolysis proper. Of the results, only those which pointed the way to the experimental method adopted are mentioned. The nitrogen distribution numbers recorded in Table IV under their group headings (A, B, C and D) are derived from twelve later analyses of gelatin. The details of the method used for obtaining and classifying them are given below. The conclusions to be drawn from the two sets of experiments are the same but the earlier ones were carried out on smaller amounts of material.

Treatment and hydrolysis of gelatin.

The different treatments with acid and alkali accorded to gelatin by Knaggs and by Schryver were duplicated as closely as was compatible with obtaining uniformity of samples. To obtain the desired uniformity, the protein was dissolved in hot water, care being taken to obtain a homogeneous solution. The ratio by weight of gelatin to water was approximately 11 : 100. 100 cc.

portions of the hot solution were pipetted into flasks and weighed. Upon the cooling of the material, gels formed.

The total nitrogen per gram of solution was determined on a separate sample. 20 cc. were diluted to a volume of 500 cc., and portions of the dilute solution were analysed. Here, and wherever possible in subsequent steps of the procedure, solutions and the parts of them used for analysis were weighed. Where this was not feasible, a method of calibration was employed.

The preliminary treatments of the 100 cc. samples are described in the following paragraphs. The concentration of HCl in each of the solutions was brought to 20 % before the hydrolysis proper of the gelatin, which was accomplished by boiling the acid solution for 24 hours. Hang-in reflux condensers were used.

Group A. No preliminary treatment. The gel was liquefied by heat and the resulting solution heated almost to boiling. A hot solution of HCl was added and the gelatin immediately hydrolysed.

Group B. Treatment with N NaOH. The gel was liquefied by heat and a solution, containing the calculated amount of NaOH, was added; the flask was thoroughly shaken and at once plunged into a cooling-bath. The mixture was allowed to stand for 24 hours at room temperature. At the expiration of this time, a hot solution of HCl was added and the gelatin was at once hydrolysed.

Group C. Treatment with N HCl. The gel was liquefied by heat and a solution containing the calculated amount of HCl was added. The procedure given for Group B was then followed except that the time of standing was 48 hours.

Group D. Treatment with 20 % HCl. The gel was liquefied by heat, a solution of HCl of proper strength was added and the flask was plunged into a cooling-bath. As soon as the mixture had stood for 24 hours at room temperature, the gelatin was hydrolysed.

Group A, then, consisted of controls, approximately 10 g. of protein being hydrolysed by some 25 times its weight of 20 % HCl. The members of Group B differed in that, prior to the hydrolysis proper, they were allowed to stand for 24 hours with N NaOH and in that the hydrolysis was accomplished in the presence of NaCl. The members of Group C differed from the controls in that they were allowed to stand for 48 hours with N HCl, and the members of Group D in that they were allowed to stand for 24 hours with the acid used for hydrolysis.

Comparison with the publications of Knaggs and of Knaggs and Schryver will show the similarity of the experimental conditions. The chief point of difference is that, in our experiments, the gelatin was first heated for a short time with water. According to Knaggs and Schryver, any noteworthy change in the protein, due to this treatment with water, would make itself apparent by an increase in the percentage of the non-amino-nitrogen of the filtrate. It is extremely unlikely that such an increase would be compensated by

a corresponding decrease in the non-amino-nitrogen of the precipitate, particularly since the total nitrogen of the latter fraction was reported by Knaggs and Schryver as remaining unchanged. A change in the protein should, consequently, be accompanied by an increase in the percentage of total non-amino-nitrogen.

To make certain that such a change had not occurred, a control determination was carried out. Gelatin was dropped into boiling HCl and hydrolysed and the ammonia and humin were removed from the hydrolysate. Portions of the residue were used for determinations of total nitrogen and of amino-nitrogen. By the formaldehyde titration, 17.81 % and by the nitrous acid method, 27.80 % of the total nitrogen of the hydrolysate was in the non-amino-form in this residue. Corresponding figures for the members of Group A average 17.82 % and 26.97 % respectively. From the non-amino-nitrogen test of two of the above investigators, then, it would seem that our treatment with water did not greatly alter the protein material. Our preliminary treatments of gelatin should, consequently, be justly comparable to theirs.

To make sure that no errors would arise from incomplete hydrolysis of the protein, the method adopted was compared with hydrolysis for 1½ hours in the autoclave at approximately 150°. Samples hydrolysed in the autoclave gave no greater figures for amino-nitrogen but higher figures for ammonia-nitrogen than those hydrolysed at ordinary pressures. Hydrolysis at ordinary pressures for 24 hours was, therefore, deemed sufficient. Since the figures for ammonia-nitrogen obtained by these experiments are needed for a part of the discussion in the next section, they are given in Table I.

Table I.

Hydrolysis	% of total N as ammonia-N
Ordinary pressure: 20 % HCl	1.49 (average of 8 determinations)
Autoclave: 10 % HCl	2.27 (average of 6 determinations)
Autoclave: 20 % HCl	2.81 (average of 6 determinations)

Maximum deviation of an individual figure from any average: 0.36 % of total nitrogen.

Determination of ammonia-nitrogen.

For the removal of ammonia, MgO and boiling at ordinary pressures, CaO and boiling at low pressures, and NaOH and aeration have been used by different investigators. The first and second of these methods, however, have been severely criticised. As noted by Jodidi and Moulton [1919], MgO, in excess of that required to release the ammonia and saturate the solution, occludes some nitrogen-containing substances other than those correctly given the name of humin. The amount of nitrogen occluded is roughly proportional to the amount of excess MgO and the substances are not removed by washing the oxide with hot water. Our results confirm this observation. The larger the amounts of MgO used, the higher are the apparent values for humin-nitrogen. Although it has not been tested, it is likely that CaO as well causes

an error at this point. Treatment of the excess oxide with HCl before filtering should, by giving soluble products, eliminate this error.

The MgO and CaO procedures, as originally presented, must be criticised on another count. As will appear in the section on precipitation with phosphotungstic acid, the amount of inorganic salt introduced into the solution of amino-acids should be known. The original oxide methods are unsatisfactory in this respect. Thimann [1926] gives a thorough discussion of this point. Although his conclusions are to the contrary, it seems to us that this offers a second important reason for treating the excess oxide with HCl. If a known amount of oxide is added to the hydrolysate and this oxide is completely changed to the soluble chloride, it is self-evident that the amount of salt introduced into the solution can quickly be calculated.

Owing to the fact that cystine is not present in the hydrolysate from gelatin, the question of its decomposition by heating to 100° in alkaline solution need not be considered. Either of the oxide methods, altered as indicated, should be adequate to the purpose at hand. For this particular investigation, however, it was not judged advisable to make use of either of them. NaCl was earlier introduced into the members of Group B by the preliminary treatment of the protein samples with NaOH and the subsequent acidification. Complication of matters by the introduction of a second salt was not desired. This consideration limited us to a procedure involving the use of NaOH.

In the MgO method, the hydrolysate is heated to 100° in alkaline solution. Such a practice should be as permissible in any other method, provided that the p_H of the solution be no higher, a factor which may be controlled by the use of the proper indicator. The procedure finally adopted entails the use of NaOH and boiling at ordinary pressures, the p_H being controlled by titration of the solution with phenolphthalein as the indicator. Before its adoption, it was compared to the MgO method. The results of this comparison are given in Table II. From them, it may be seen that the amounts of ammonia obtained by the two methods do not greatly differ and that the addition of several millimols of NaOH in excess of the proper amount does not markedly affect the results.

Table II.

MgO (g.)	% of total N as ammonia-N (average of duplicates)
3	1.32
4	1.35
1.6 N NaOH (cc.)	
54	1.32
55	1.34
56	1.36
57	1.36
60	1.39
65	1.40

Maximum deviation from any average: 0.02 % of total nitrogen.

The method was applied as follows. A hydrolysate, after being twice concentrated to small volume on the steam-bath to remove HCl, was introduced into a 1.5 litre flat-bottomed flask and a few drops of phenolphthalein were added. The solution was then titrated with NaOH to a distinct pink. Additional NaOH, sufficient in amount to react with the ammonium chloride present, was added and the ammonia boiled off. Immediately after the removal of the ammonia, the solution was acidified with HCl, a procedure designed to stop any decomposition of amino-acids. It is unlikely, however, that with either this or the MgO method there is a complete absence of amino-acid decomposition. In a series of eight determinations with MgO, water was added to the residue from the usual distillation and a second distillate was collected and analysed. The ammonia-nitrogen, obtained from the second distillation, averaged 0.07 % of the total nitrogen of the protein. Although these results imply that the treatment is too drastic, the error indicated is small when compared to the total error of the determination.

As may be seen from Tables I and IV, the figures obtained for the ammonia-nitrogen of analogous solutions vary greatly. On the other hand, duplicate analyses of a single solution, as given in Table II, show no such variation. From Table I, it may further be seen that different conditions of hydrolysis give rise to ammonia-nitrogen values of different magnitude. This was remarked for a number of proteins by Henderson [1899] and for gelatin by Denis [1910]. These observations, taken together, suggest that a lack of constancy of the ammonia-nitrogen figures in duplicate nitrogen distribution experiments is due to differences at present uncontrollable in the conditions of hydrolysis of the protein samples.

Determination of humin-nitrogen.

The residue, after the distillation of ammonia and the subsequent acidification, was filtered into a 250 cc. volumetric flask and the filter-paper was thoroughly washed. The precipitate was analysed and the result recorded as humin-nitrogen.

The filtrate was made up to a volume of 250 cc. and parts of it were used for determinations of amino-nitrogen and of total nitrogen and for the precipitation of the bases with phosphotungstic acid; one 5 cc. portion was titrated to neutrality. The figure obtained by this titration is of value in adjusting any sample to a definite acid concentration. This adjustment is necessary before the decolorisation for the formaldehyde titration and in the preparation of the solution for the precipitation of the bases.

Determination of amino-nitrogen¹.

By the nitrous acid method. This was carried out on 5 cc. samples according to the method of Van Slyke [1912], except that the sample was introduced

¹ When this investigation was carried out (spring 1927), the method of Linderstrøm-Lang [1927, 1928] was not elaborated.

by means of a calibrated pipette as recommended by Plimmer [1924]. The temperature was always 20° or more, so a reaction time of 30 minutes was allowed.

By the formaldehyde titration. This was carried out according to the directions of Jessen-Hansen [1923], with a modification, used in the Carlsberg Laboratory and involving the substitution of bromothymol blue for litmus paper as the indicator in the neutralisation. The use of separate samples for neutralisation and for titration with formaldehyde is thus necessitated.

A 70 cc. portion of the solution of amino-acids was used for the decolorisation and, of the resulting 100 cc., 40 cc. were used for the treatment to remove CO₂. Of the 50 cc. of CO₂-free filtrate, two 20 cc. portions were used for the analysis itself.

Precipitation with phosphotungstic acid.

Thimann [1926] reported that the precipitation of the bases with phosphotungstic acid is influenced by the concentrations of salts and of the mineral acid. Our results confirm these observations but were obtained under somewhat different experimental conditions and are, consequently, of a different magnitude. The results of these control experiments are given in Table III.

Table III.

Approximate molarity in			N (mg.) in precipitate (average of duplicates)
NaCl	MgCl ₂	HCl	
0.01	—	1.2	13.6
0.44	—	1.2	15.2
0.87	—	1.2	16.5
0.01	0.25	1.2	14.7
0.01	0.50	1.2	15.5
0.01	—	2.3	14.4

Total nitrogen of each sample: 87.2 mg.

For the basic figures of two series of nitrogen distribution numbers to be strictly comparable to each other, then, the concentrations of acid and of salts in the solutions used for precipitation with phosphotungstic acid must be the same. The concentration of acid may easily be controlled by the determination of the acidity of each solution and by the addition of an amount of acid sufficient to bring the total concentration to the required figure. Control of the salt concentration may be accomplished by the use for hydrolysis of protein samples of equal or of known salt content, by ascertaining the amounts of salt introduced into the solution at any point in the procedure, and by a final adjustment of each solution to a definite salt concentration. The magnitude of the fluctuations in the basic fraction to be expected from a failure to control the concentrations of acid and of salt may be calculated from the figures in Table III. For this investigation, they would have been perceptible. The concentrations of NaCl in the 200 cc. precipitating solutions, arising from the NaOH used for boiling off the ammonia, varied from 0.33 *N* to 0.38 *N*. The acidity of the solutions varied from 0.07 *N* to 0.14 *N*.

The concentration of nitrogen, also, in each of the solutions used for precipitation of the bases should be kept at a constant value. This is amply demonstrated by the work of Kernot and Knaggs [1928], which is well supported by theoretical considerations. Van Slyke [1911] recognised that the phosphotungstates of the bases have solubilities great enough to be of importance. These solubilities have not been determined in the presence of known amounts of salts.

Lastly, the solution in which the precipitation of phosphotungstates is taking place should be kept at a definite temperature and a definite time should be allowed for the precipitation. A variation in the solubility of phosphotungstates with temperature was reported by Gortner and Hoffman [1925] and this variation was confirmed by Kernot and Knaggs [1928]. Our results, also, afford a confirmation. Two filtrates from 48-hour precipitations at 18° were allowed to stand an additional 48 hours at 4°. Additional precipitates, the nitrogen of which averaged 2.45 % of the total nitrogen of the protein, were obtained. Plimmer and Rosedale [1925] reported that the percentage of basic nitrogen is influenced by the time allowed for the precipitation to take place.

For our experiments, the volume in which each precipitation was allowed to take place was 200 cc., the concentrations of HCl and NaCl were 1.0 *N* and 0.75 *N* respectively; and the nitrogen in the solution before precipitation was 900–908 mg. Forty-eight hours were allowed for each precipitation to take place and the temperature of the solutions during this period was 18°.

In preparation for this precipitation, 150 cc. of the solution of amino-acids were introduced into a 300 cc. Erlenmeyer flask and the calculated amounts of HCl, NaCl and water were added. The solution, the volume of which at this point was 190 cc., was now heated almost to boiling and 15 g. of phosphotungstic acid in 10 cc. of solution were added. The flask was then allowed to stand for 48 hours in a thermostat at 18°. The precipitate of phosphotungstates appeared only after the solution began to cool. If the precipitate was allowed to form in the cold, it was found almost impossible to redissolve it by heating. Further, its manipulation was difficult and the results were erratic.

Filtration and washing of precipitate. The precipitate was collected and washed according to the method of Van Slyke [1915], except that the amount of solution used for washing was carefully controlled, as recommended by Plimmer and Rosedale [1925]. For the amount of precipitate with which we were dealing, five 20 cc. portions of the wash liquid recommended by Van Slyke, cooled to about 0°, appeared to be satisfactory, and this amount was accordingly adopted as a standard.

Treatment of the precipitate. The precipitate was dissolved in approximately *N* NaOH as directed by Plimmer and Rosedale. Of the resulting 100 cc., 10 or 20 cc. portions were used for the determination of total nitrogen. The remainder was used for the determination of amino-nitrogen by one of the methods mentioned above. For the nitrous acid method, 10 cc. samples were

used. For formaldehyde titrations it was necessary to remove the phosphotungstic acid and to make sure that the solution was free from CO_2 . From 60 to 80 cc. the phosphotungstic acid was removed by the BaCl_2 method of Van Slyke [1911], after which the solution was acidified and evaporated to a small volume on the steam-bath. It was then given the treatment outlined by Jessen-Hansen for the removal of CO_2 , by which the last traces of phosphotungstic acid also were removed. Finally, the solution was filtered into a 50 cc. volumetric flask and two 20 cc. portions were used for the analysis.

Treatment of the filtrate. The filtrate and washings were transferred to a 500 cc. volumetric flask and diluted to volume. 20 cc. samples were used for the determination of total nitrogen. From 250 cc. phosphotungstic acid and CO_2 were removed in the same manner as above and the solution of amino-acids was filtered into a 100 cc. volumetric flask. Of this, two 20 cc. portions were used for the formaldehyde titration and 5 cc. samples were used for the nitrous acid treatment.

DISCUSSION OF RESULTS.

The results of these experiments are given in Table IV. From them, it can hardly be concluded that preliminary treatment of gelatin with acid or alkali affects the nitrogen distribution. The possible exception is that the increase in the percentage of ammonia-nitrogen observable in the members of Group B may be due to the preliminary treatment of the protein with alkali. That this may not be the correct interpretation, however, is indicated by the statements of Hart [1901] and Kossel and Patten [1903] that the percentage of ammonia-nitrogen is increased by the addition of NaCl to the hydrolysing acid.

As has been stated, the solutions and the parts of them used for the various analyses were, whenever possible, weighed. Since no attempt was made to use exactly aliquot portions, it might be misleading to record the actual values of the determinations. The approximate magnitude of each analysis may easily be calculated from the figures for total nitrogen and the records of volume scattered throughout the discussion.

It is worthy of note that the values for total amino-nitrogen obtained by direct analysis are considerably higher than those obtained by adding the values for the amino-nitrogen of the precipitate to the figures for the amino-nitrogen of the filtrate. In the case of the nitrous acid method it is not difficult to assign the reason for this anomaly. Van Slyke [1911] advocates a reaction time of 10 minutes when lysine is absent from the solution to be tested and a reaction time of 30 minutes when lysine is present. This advice was followed. The filtrate from the bases was thus allowed a reaction time of 10 minutes while the other solutions were each allowed 30 minutes. In preliminary experiments it was found that four filtrates, which gave an average of 60.9 % amino-nitrogen for 10 minutes' reaction, gave an average of 65.8 %

Table IV.

A comparison of the nitrogen distribution numbers of treated and untreated gelatin.

Group	Total N found mg.	NH ₃ -N found (a) %	Humin-N found (b) %	Total amino-N found		Total non-amino-N calculated		Total amino- acid-N found (c) %	Total N (a)+(b)+(c) %
				Form. titration %	HNO ₃ method %	Form. titration %	HNO ₃ method %		
A	1529	1.60	0.06	80.57	71.45	18.11	27.23	98.68	100.34
A	1532	1.63	0.05	80.34	71.71	18.15	26.78	98.49	100.17
A	1532	1.39	0.04	81.45	71.75	17.22	26.92	98.67	100.10
B	1529	1.83	0.06	80.79	72.55	17.82	26.06	98.61	100.50
B	1532	1.68	0.05	80.92	71.18	17.52	27.26	98.44	100.17
B	1532	1.65	0.05	81.19	71.24	16.79	26.74	97.98	99.68
C	1530	1.58	0.05	80.05	71.45	18.50	27.10	98.55	100.18
C	1530	1.62	0.05	80.50	72.16	17.96	26.30	98.46	100.13
C	1531	1.52	0.04	80.92	71.84	17.71	26.79	98.63	100.19
D	1530	1.54	0.04	80.74	71.17	17.89	27.46	98.63	100.21
D	1530	1.78	0.07	79.98	71.33	18.19	26.84	98.17	100.02
D	1532	1.31	0.05	80.88	71.95	17.75	26.98	98.63	99.99
Av. A	—	1.54	0.05	80.79	71.64	17.82	26.97	98.61	100.20
Av. B	—	1.72	0.05	80.97	71.66	17.37	26.68	98.34	100.11
Av. C	—	1.57	0.05	80.49	71.82	18.06	26.73	98.55	100.17
Av. D	—	1.54	0.05	80.53	71.48	17.95	27.00	98.48	100.07
Av. of all	—	1.59	0.05	80.70	71.65	17.80	26.85	98.50	100.14

Amino-N of filtrate found		Non-amino-N of filtrate calculated		Total N of filtrate found (d) %	Amino-N of precipitate found		Non-amino-N of precipitate calculated		Total N of precipitate found (e) %	Total N (a)+(b)+(d)+(e) %
Form. titration %	HNO ₃ method %	Form. titration %	HNO ₃ method %		Form. titration %	HNO ₃ method %	Form. titration %	HNO ₃ method %		
69.52	58.46	12.26	23.32	81.78	—	8.00	—	8.66	16.66	100.10
69.85	58.76	12.37	23.46	82.22	8.07	—	8.45	—	16.52	100.42
70.99	59.34	11.26	22.91	82.25	—	—	—	—	16.14	99.82
68.63	57.87	13.12	23.88	81.75	—	7.74	—	8.75	16.49	100.13
70.53	58.80	10.80	22.53	81.33	7.93	—	8.73	—	16.66	99.72
71.49	59.73	10.32	22.08	81.81	—	—	—	—	16.43	99.94
70.11	57.93	11.31	23.49	81.42	—	7.89	—	8.98	16.87	99.92
69.11	58.03	12.54	23.62	81.65	7.96	—	8.59	—	16.55	99.87
70.61	58.34	11.38	23.65	81.99	—	—	—	—	16.22	99.77
70.62	58.65	11.44	23.41	82.06	—	7.95	—	8.40	16.35	99.99
69.35	58.06	12.44	23.73	81.79	7.87	—	8.54	—	16.41	100.05
70.68	59.15	11.20	22.73	81.88	—	—	—	—	16.52	99.76
70.12	58.85	11.96	23.23	82.08	—	—	—	—	16.44	100.11
70.22	58.80	11.41	22.83	81.63	—	—	—	—	16.53	99.93
69.94	58.10	11.75	23.59	81.69	—	—	—	—	16.55	99.86
70.22	58.62	11.69	23.29	81.91	—	—	—	—	16.43	99.93
70.13	58.59	11.70	23.24	81.83	—	—	—	—	16.49	99.96

amino-nitrogen for 30 minutes' reaction. This accounts for the greater part of the discrepancy. The temperature at which these reactions were carried out varied from 20° to 23°.

An attempt was made to discover the cause of the loss of formaldehyde-titratable nitrogen. Since there was no loss in total nitrogen, this must have occurred in preparing the solutions for the titration. In experiments carried through for this purpose, all precipitates arising from the addition of BaCl₂ to the solutions containing phosphotungstic acid were analysed for nitrogen. Despite a thorough washing of these precipitates, the analyses invariably showed the presence of considerable and nearly constant amounts of nitrogen. The amounts were higher in the precipitates from the mono-amino-fraction than

in those from the basic fraction, probably owing to the larger part of the phosphotungstic acid being associated with the first fraction. To the solutions from which the phosphotungstic acid had been removed, additional amounts of this acid were added and the BaCl_2 treatment was repeated. Additional amounts of nitrogen were removed from the solutions by this treatment. When the precipitation of 7.5 g. of phosphotungstic acid was carried out in a volume of approximately 800 cc. an amount of nitrogen, roughly corresponding to 1.5 % of the nitrogen present in the solution, was removed. This held true whether the solution in question was one of basic amino-acids or one of mono-amino-acids. Although insufficient data have been accumulated to permit of any definite statement, the indications are that a considerable loss of nitrogen is unavoidable when phosphotungstic acid is precipitated from a solution of amino-acids by means of BaCl_2 and that this phenomenon is not a selective one but depends on the concentrations of the amino-acids and of the phosphotungstic acid in the solution.

For future work, the more recent amyl alcohol-ether method of Van Slyke [1915] will be used for the removal of phosphotungstic acid. From test experiments carried out on gelatin, the separation of the amino-acids from phosphotungstic acid by this method seems to be very nearly quantitative. A small amount of the latter acid remains with the amino-acids and is precipitated upon treatment of the solution with BaCl_2 for the removal of CO_2 . However, the amount is so small that less than 0.1 % of the total nitrogen is removed at this point.

SUMMARY.

1. The experimental results force us to the conclusion that, within the limits of accuracy of the method described, preliminary treatment of gelatin with acid or alkali has no effect on the percentages of non-amino-nitrogen and of basic nitrogen.

2. Several other factors have been reported by various workers as influencing the figures obtained as nitrogen distribution numbers of proteins. A study of several of these factors has been made. From the results of this study, certain modifications of the method of analysis of proteins are advocated. It is believed that the numbers obtained by the modified method will be, within certain limits of experimental error, fairly comparable to each other. The following paragraph gives a brief summary of the procedure as altered, particular stress being laid on the alterations.

The protein is hydrolysed by boiling with 20 % HCl . After removal of as much of the HCl as is possible by evaporation, the hydrolysate is rendered alkaline by the addition of a known amount of MgO , CaO or NaOH and the ammonia is removed under the proper conditions. The residue is acidified and the humin is filtered off. The filtrate, of which the acidity is determined, is divided into aliquot portions; some of these are used for determinations of amino-nitrogen and some for total nitrogen. In one portion the amino-acids

are divided into two groups by a precipitation with phosphotungstic acid. The concentrations of salt or of salts, of acid and of nitrogen are always brought to definite values in this latter portion before the precipitation is allowed to take place. The solution of amino-acids is heated almost to boiling before the phosphotungstic acid is added, and the resulting mixture is kept at a definite temperature during the definite time allowed for precipitation. The phosphotungstates are filtered off and are washed according to a rigidly defined technique. If determinations of the amino-nitrogen of the filtrate and of the precipitate are to be made by the nitrous acid method only, no preparation of the filtrate is necessary and the precipitate may be obtained in a suitable form by its solution in *N* NaOH. Should it be desirable to make use of the formaldehyde or alcohol titrations, the phosphotungstic acid is removed from each of the amino-acid fractions by the amyl alcohol-ether method. The amino-nitrogen and the total nitrogen of the filtrate and of the precipitate are determined.

In conclusion, I wish to thank Professor S. P. L. Sørensen for the suggestion of this problem and for his kind and helpful criticism and advice during the execution of the work. To Cheney Bros. of South Manchester, Conn., I am indebted for the Fellowship which permitted the research to be undertaken.

REFERENCES.

- Denis (1910). *J. Biol. Chem.* **8**, 427.
Gortner and Hoffman (1925). *J. Amer. Chem. Soc.* **47**, 580.
Hart (1901). *Z. physiol. Chem.* **33**, 347.
Henderson (1899). *Z. physiol. Chem.* **29**, 47.
Jessen-Hansen (1923). *Handb. Biol. Arbeitsmeth. (Abderhalden)*. Abt. I, **7**, 245.
Jodidi and Moulton (1919). *J. Amer. Chem. Soc.* **41**, 1526.
Kernot and Knaggs (1928). *Biochem. J.* **22**, 528.
Knaggs (1923). *Biochem. J.* **17**, 488.
— and Schryver (1924). *Biochem. J.* **18**, 1102.
Kossel and Patten (1903). *Z. physiol. Chem.* **38**, 39.
Linderstrøm-Lang (1927). *Compt. Rend. Lab. Carlsberg*, **17**, No. 4.
— — (1928). *Z. physiol. Chem.* **173**, 32.
Plimmer (1924). *Biochem. J.* **18**, 105.
— and Rosedale (1925). *Biochem. J.* **19**, 1004.
Schryver and Buston (1927). *Proc. Roy. Soc. Lond.* B **101**, 519.
Thimann (1926). *Biochem. J.* **20**, 1190.
Thornley (1927). *Biochem. J.* **21**, 1302.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.
— (1912). *J. Biol. Chem.* **12**, 275.
— (1915). *J. Biol. Chem.* **22**, 281.