XLII. STUDIES ON GELATIN.

XI. DIAMINO-NITROGEN OF GELATIN.

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THE problem of the inherent stability of the protein molecule is one which has often been discussed, but upon which very little definite evidence is forthcoming.

When the colloid protein particle is envisaged as a complex amino-acid structure, there emerges the possibility that this structure may be subject to change. Changes in the surrounding conditions may produce changes in the internal structure, which in their turn would lead to alterations in the external behaviour of the molecule.

The fact that such external changes do occur may be instanced by the fall in viscosity, and ultimate loss of gelling power, of gelatin solutions on heating, but the occurrence of internal changes, whether related or not to external behaviour, is not so readily observed. This is to be ascribed largely to the lack of suitable analytical methods.

In recent years, however, Linderstrøm-Lang [1929] claims to have shown for caseinogen, and Schryver and his co-workers for gelatin, that there is evidence of an almost continuous variation in chemical composition, dependent upon conditions of treatment of the protein. An analogous series of changes has been shown to occur in the denaturation of ovalbumin by Mastin and Schryver [1926].

One of the analytical methods available is the precipitation of a portion of the nitrogen of the hydrolysed protein with phosphotungstic acid. This nitrogen is generally ascribed to the diamino-acids, but it will be shown that this involves something of an assumption, and it is desirable provisionally to consider this fraction simply as the nitrogen precipitated by phosphotungstic acid, without assigning to it any theoretical basis.

It was shown by Knaggs [1923] that if gelatin be allowed to stand in cold acid before hydrolysis, the amount of nitrogen which is precipitated by phosphotungstic acid in the hydrolysate increases. Increases varying from 4 to 10 % on a total of 20.4 % were obtained by treating the gelatin with cold HCl for 48 hours and afterwards throwing into boiling 20 % HCl.

Schryver and Thimann [1927] found that the soluble gelatin produced when the purified protein is flocculated from alkaline solutions does not show this effect, while the insoluble form—produced at the same time—does. In the same way, gelatin which has been brought permanently to the sol form by heating with water shows no rise in the nitrogen of the precipitate when it is similarly treated with cold acid before hydrolysis. The rise in the nitrogen precipitated by phosphotungstic acid, brought about by treatment with acid before hydrolysis, is therefore not constant but varies with the type of protein and with the conditions.

Thornley [1927] showed that the amount of increase varied with the strength of cold acid with which the gelatin was treated, and obtained a curve with a maximum rise of 5.8 %, corresponding to 2.5*N* cold HCl. In very strong acid a slight decrease took the place of an increase. Schryver and Buston [1927] traced the increase in this phosphotungstic precipitate to quantities of additional lysine; they found an increase of 1.6 % of ordinary lysine and 4.1 % of *dl*-lysine over the amounts present in gelatin hydrolysed in the normal way.

Daft [1929], however, published results which disagreed with the above and which apparently showed that no rise was produced by treatment of gelatin with cold acid before hydrolysis. In his experiments the gelatin was hydrolysed with and without pre-treatment with cold acid, and the nitrogen precipitated by phosphotungstic acid was shown to be constant. In the course of these experiments, however, the gelatin was heated with water (for a length of time which is not stated), before treatment with cold acid, and this may have seriously modified the results. It has, in fact, been observed by Knaggs and Schryver [1924] that boiling with water increases the non-amino-nitrogen of gelatin. Daft has therefore compared the value for the nonamino-nitrogen of the heated gelatin used in his experiments with that of the original gelatin before heating, and has shown that there is no great difference in the figures; he has thereupon assumed that his gelatin could not have been materially altered by this treatment. On the other hand, his value for the nonamino-nitrogen, 27 % as determined by the Van Slyke method, does not agree with the 12 % quoted by Knaggs and Schryver, and it is therefore possible that his gelatin had already been heated with water in some way before use, so as to increase the nonamino-nitrogen to a value which would remain unchanged on further heating. Now it has been shown [Thornley, 1927] that gelatin which has been boiled with water for 24 hours no longer gives a rise in phosphotungstic acid precipitate after standing in cold acid. Daft's gelatin may therefore have been boiled with water for a sufficient time to produce this effect. No information is given as to the source of his gelatin, or as to the methods adopted for its purification, so that it is impossible to determine how far this criticism affects his figures. Another point with reference to this work is discussed later (p. 366).

Whether this be the correct explanation or not of the disagreement

between the results of Daft and those previously published, it was obviously desirable to reinvestigate the whole matter, for the existence of similar discrepancies has already been noted by other workers. Thus, Knaggs and Schryver [1924] found in one experiment that treating gelatin with cold *N* HCl produced no rise in the nitrogen precipitated by phosphotungstic acid, but only a rise in the nonamino-nitrogen. Thornley, on the other hand, obtained a considerable rise by treatment with cold *N* HCl. A careful examination of the method of analysis brought to light certain causes for variation in the amount of nitrogen precipitated by phosphotungstic acid.

(a) Different samples of phosphotungstic acid were found to produce slightly different amounts of precipitate; the crystalline acid of Kahlbaum yielded higher figures than the amorphous compound prepared in the laboratory both for gelatin and for pure arginine.

(b) Heating in the autoclave before precipitation, as recommended by Knaggs, was shown to cause a steady fall in the amount of nitrogen in the precipitate, varying with the time of autoclaving.

(c) The temperature at which the amide-nitrogen is determined has also a slight effect.

These factors are dealt with below.

None of the differences caused by these experimental variations was, however, great enough to account for the discrepancies observed, and attention was therefore directed to the concentration of nitrogen in the solution. The influence of this factor has long ago been noted [Thimann, 1926; Kernot and Knaggs, 1928], but early experiments had indicated that, if the concentration were kept within certain limits, constant values could be obtained. Those experiments were carried out, however, at relatively high concentrations, using the macro-method. The more convenient micro-method involves more dilute solutions of the amino-acids, and investigation of the effect of changes in the nitrogen concentration showed that under these conditions the amount of phosphotungstic acid precipitate varied almost continuously with the concentration of nitrogen. A single determination of this phosphotungstic precipitate therefore means nothing, and it is essential to plot the curve of "nitrogen concentration" against "percentage nitrogen precipitated" in order to obtain figures of any value. If this is done, curves are obtained which are characteristic of the protein used and are highly sensitive to differences in the treatment of the protein before hydrolysis. They therefore form a valuable means for comparison, and further afford definite evidence of the ready occurrence of intramolecular changes in gelatin under various treatments.

METHOD.

The micro-method adopted has already been described [Thimann, 1926], and the only modification introduced is in regard to the amide-nitrogen (see p. 360). The gelatin gel was warmed to 40°, made up to 15 cc. with water,

and poured into 15 cc. boiling conc. HCl, so as not to interrupt the boiling. Hydrolysis was continued for 21 hours under a hang-in reflux condenser, the liquid rapidly cooled, 10 cc. samples were pipetted off and total nitrogen was determined on the remainder. The 10 cc. samples were evaporated *in vacuo* to remove acid, and diluted.

Determination of the amide-nitrogen.

2 cc. 10 % NaOH and 1 drop 1 % phenolphthalein were added, 1 : 1 H₂SO₄ was run in drop by drop till the solution was just acid, and 2 % NaOH added till just alkaline, as previously described. The liquid was transferred with washing to the Parnas and Wagner distillation apparatus and the ammonia distilled off in steam into N/70 H₂SO₄. Four minutes' distillation, followed by half a minute's steaming with the N/70 acid lowered from the nozzle were sufficient. The liquid was allowed to suck back into the trap and the apparatus washed out three times with vigorous shaking. The liquid was finally re-acidified with one drop H₂SO₄, evaporated down, and precipitated with phosphotungstic acid at 0°, as previously described.

Comparison was made with the aspiration method, both in regard to the amide-nitrogen and the phosphotungstic precipitate, in case the treatment with boiling alkali should have affected the latter. Gelatins of high amide content, and also gliadin, were used, to obtain better comparison (Table I).

Table I.

	Amide-N determined at 100° (4 min.).		Amide-N determined at 40° (90 min.).	
	Amide-N %	N in phosphotungstic ppt.* %	Amide-N %	N in phosphotungstic ppt.* %
Gelatin 1	3.20	14.98	2.58	15.80
Gelatin 2	4.39	14.90	3.70	15.24
Gliadin	26.75	—	26.30	—

All values represent the mean of two determinations.

* Low diamino-figures because dilute solutions used. See latter half of paper.

The boiling method therefore yields a higher value for the amide-nitrogen, lowering the N in the phosphotungstic acid precipitate by approximately the same amount. There is no reason, however, to believe that the lower figure is the more correct. If boiling with alkali drives off any nonamide-nitrogen, the amino-acids so decomposed should be arginine and cystine. Since gelatin contains no cystine, experiments were carried out with pure arginine. Distillation by boiling for 4 minutes, even with 40 % NaOH, however, yielded an amount of ammonia scarcely higher than the blank value. (The possibility of the partial scission of arginine into ornithine and urea, however, should not be overlooked (cf. following communication).) It is therefore safe to assume that no true diamino-nitrogen is distilled off under these conditions, and since the boiling method is quicker and provides closer agreement between duplicates it was used throughout.

Temperature of precipitation with phosphotungstic acid.

Daft states that precipitation in the cold yields a precipitate difficult to manipulate, and that the results were erratic, and recommends precipitation in the hot, followed by cooling. Comparison of the two methods, however, failed to yield any evidence for this statement, since the precipitates obtained in the two pairs of experiments carried out were identical in appearance and consistency, so far as could be ascertained, and also in nitrogen content (Table II).

Table II.

N	Precipitated hot %	Precipitated at 0° %
Amide	1.75	1.73
Phosphotungstic ppt.	20.49	20.58
Filtrate	78.57	78.57
Total	100.81	98.88

All values represent the mean of two determinations.

The discussion of Daft on the experimental procedure introduces no other fresh considerations, the effect of salts having been already pointed out [Thimann, 1926].

Effect of autoclaving before precipitation.

The rise in phosphotungstic acid-nitrogen produced by treating gelatin with acid before hydrolysis was ascribed by Knaggs [1923] to formation of a difficultly hydrolysable peptide. This peptide was said to be destroyed by heating under pressure at 120° with 5 % H_2SO_4 for 2-3 hours before precipitation. Since the bulk of the increase has been shown to be due to lysine and not a peptide, the effect of autoclaving seemed to call for explanation, particularly as it undoubtedly does lower the amount of nitrogen in the phosphotungstic acid precipitate. Check experiments were therefore carried out on a synthetic mixture of amino-acids which contained arginine, proline, and glycine. 10 cc. samples containing 5 % of H_2SO_4 were pipetted into short tubes, plugged with cotton wool, and autoclaved at 130° (Table III).

Table III.

Time of autoclaving (hrs.)	0		2.5		5.5	
	mg.	%	mg.	%	mg.	%
N in precipitate	1.22	20.58	1.16	19.68	1.04	17.63
N in filtrate	4.71	79.47	4.72	80.07	4.82	81.70
Total N	5.91	100.05	5.88	99.75	5.88	99.33

All values represent the mean of two determinations.

These experiments indicate that, quite apart from peptide-nitrogen, precipitable nitrogen of the true diamino-type is slowly destroyed by heating in the autoclave with 5 % acid. The use of this process is therefore not desirable, though the point might with value be subjected to a more complete investigation.

Effect of differences in the phosphotungstic acid used.

Three samples were investigated.

Sample A, prepared in the laboratory some time ago.

Sample B, freshly prepared. Both *A* and *B* had been prepared according to the standard method and thoroughly extracted with ether. They were amorphous white powders, whose solutions rapidly turned purple on standing. *Sample A* also yielded a slight deposit on long standing.

Sample C (Kahlbaum), was greenish and crystalline; its solutions became only faintly purple on standing.

The nitrogen content of the solutions was very low, being almost entirely due to the sulphuric acid used. For the 10 cc. of 2.5 % wash-liquid a blank of 0.12 cc. *N*/70 acid was obtained, while for the 3 cc. of 20 % precipitant the value was negligible.

10 cc. of a solution of pure arginine, containing 5 % H_2SO_4 , were used for comparison. In duplicate experiments the precipitates were (a) centrifuged and then incinerated directly; (b) washed on the centrifuge four times with a total of 10 cc. of 2.5 % wash-liquid. The first gives a measure of the maximum precipitation possible, and the second the amount of precipitation which would be obtained under standard experimental conditions, since this is the usual procedure (Table IV).

Table IV. *Arginine solutions.*

Precipitates centrifuged without washing.				
	Sample A			
		3-months-old solution containing deposit		
	Fresh solution		Sample B	Sample C
% N in ppt.	86.4	83.7	89.6	90.1
% N in filtrate	13.6	16.2	10.4	9.9
Precipitates washed with 10 cc. wash-liquid.				
% N in ppt.	68.3	61.3	74.3	74.6
% N in filtrate	31.7	38.7	25.6	25.4

All values represent the mean of two determinations.

The use of stronger solutions of phosphotungstic acid, or larger volume of precipitant, gave in general lower values, though the differences were slight; the above amount, 3 cc. of 20 % solution, was therefore adhered to. It will be seen that the Kahlbaum acid and the freshly prepared laboratory material are identical within the ordinary limits of error, while the loss of efficiency in the old sample is considerable. Further comparison with gelatin-hydrolysates showed a distinct balance in favour of the Kahlbaum acid, which was accordingly employed hereafter.

Table V. *Gelatin hydrolysate.*

	N	Sample B %	Sample C %
Amide		2.06	2.07
Phosphotungstic ppt.		16.34	18.20
Filtrate		81.21	78.82
Total		99.61	99.09

It is not easy to assign reasons for the varying efficiency as precipitants of samples of phosphotungstic acid, but no doubt in such a complex molecule considerable differences in composition are possible, and even an ageing effect such as is observed above is not without precedent in inorganic materials.

Importance of the concentration of nitrogen.

It was stated above that earlier (unpublished) experiments showed that within certain limits the amount of nitrogen precipitated by phosphotungstic acid was independent of the concentration of nitrogen. These experiments were, however, carried out with Coignet's Gold Label gelatin, the use of which was afterwards discontinued on account of its uncertain origin. Further, the experiments were conducted under slightly different conditions and without the precautions shown since (and herein) to be of importance. The present experiments were carried out with a gelatin guaranteed to be prepared only from bone, and kindly supplied by Messrs Nelson, Dale and Co., of Warwick, to whom I wish to express my thanks.

As soon as precipitations had been carried out with this gelatin at different nitrogen concentrations, it became obvious that the principal source of disagreements lay therein. The curve, Fig. 1, shows the precipitation characteristic of this gelatin, compared with that of Coignet's Gold Label gelatin purified by flocculation, and it is clear that the former yields no flat range within the limits of concentration used (Table VI).

Table VI.

Mg. N in 10 cc. solution	N. D. and Co., Bone gelatin						Coignet gelatin (purified)			
	4.67	7.97	16.77	27.19	38.86	42.07	5.45	9.32	16.28	31.16
Amide-N (%)	1.60	2.18	1.89	1.65	2.01	1.74	2.19	1.77	1.58	1.59
"Diamino"-N (%)	15.89	18.72	23.34	24.53	22.48	20.54	16.61	19.66	22.38	22.75
Filtrate-N (%)	83.71	78.29	75.35	73.20	75.85	77.57	81.49	78.64	76.72	74.70
Total N (%)	101.20	99.19	100.58	99.38	100.34	99.85	100.29	100.07	100.68	99.04

EFFECT OF ACID ON GELATIN.

It was shown in the previous communication of this series [Schryver and Thimann, 1927] that if gelatin be dissolved in water, the solution made acid, and a potential difference applied across the solution, the gelatin is precipitated from solution as a gel instead of a white flocculum. Gelatin so treated has therefore been actually dissolved in acid and reprecipitated therefrom, and it is to be expected that the effect of acid applied in this way would be more complete than that produced by merely placing the dry solid in acid solutions for a corresponding length of time. In the latter case the effect will be modified by the relative ease with which the acid diffuses into the gelatin, *i.e.* probably by the water content of the gelatin. The effect may, of course, not be identical with that produced by the mere contact of the gelatin with

acid; if, however a change is produced at all, it would be reasonably anticipated to occur in this case. Experiments were therefore carried out on the flocculation of gelatin from acid solutions.

The gel so produced was shown in the paper quoted above to possess properties differing from those of the flocculum obtained from neutral or alkaline solutions. Chemical differences were therefore to be expected.

Fig. 1. Precipitation curves of gelatin.

Experimental.

The general procedure was as follows. 500 cc. of 1.5 % solution of the bone gelatin in water at 40° were brought to p_H 3 by addition of 60–70 cc. N HCl and electrolysed between collodion-muslin membranes. The outer vessels were supplied with slowly running freshly boiled distilled water, into which 1-inch square platinum foil electrodes dipped. A P.D. of 50 volts, *i.e.* about 2 volts per cm., was applied until the current began to fall from the original 5 to 2–3 milliamp. The P.D. was then slowly raised to 200 volts. After 10–12 days the current had fallen to 1 milliamp., and the p_H of the anode liquor had returned to 6–7. The stiff gel was removed, dissolved at 40° in 50–100 cc. water according to the dilution required, and cooled. For each hydrolysis the gel was warmed quickly to 40°, the required volume poured into 15 cc. boiling concentrated HCl, and water added if necessary to bring the volume of hydrolysate to 30 cc. The gelatin was preserved cold, under a layer of toluene. Samples having thus been removed for analysis, the remainder of the gel was redissolved in water at 40°, again acidified to p_H 3 and the flocculation repeated. On the second and subsequent occasions the resistance of the now salt-free gelatin solutions was considerably higher, and after passage of 1000 coulombs the final current fell below 0.2 milliamp. at 200 volts. In each case the gelatin was precipitated at the cathode membrane in the form of a stiff 10 % gel.

The Hausmann numbers of the gels were determined at several nitrogen concentrations. The amide-nitrogen varied irregularly between 1.5 and 2.0 %, although agreement between duplicates was close. The variation in the nitrogen precipitated by phosphotungstic acid¹ is shown by curve B in Fig. 2. Curves C, D and E give the variation for the same gelatin after the second, third and fourth flocculation respectively from acid solution.

Fig. 2. Precipitation curves of acid-flocculated gelatin.

Some attempt to arrive at an interpretation of these curves has been made (see following communication), but at this point it will be sufficient to note that they indicate a continuous change in the amount of nitrogen precipitated, and a considerable difference from the original bone gelatin, the curve of which (A) is given for comparison.

When gelatin is flocculated from neutral or alkaline solutions it is obtained as a white hydrated flocculum, and it was thought to be of interest to compare

¹ An explanation of the unusually low values shown on curve B is given in the following paper.

this with the gels obtained above. A quantity of the crude bone gelatin was therefore dissolved to 1.5 % solution, brought to p_H 9 with KOH, and flocculated in the same way as the acid solutions but with the polarity reversed. Fig. 3 shows the curve (*F*) obtained with this preparation, compared with that given by the gelatin flocculated once from acid (*B*).

Comparison with other workers.

A word must be added here with regard to the concentrations of nitrogen used by other workers. The amount of gelatin used by Thornley [1927] corresponds to 5.6 mg. N per 10 cc., and it will be seen that although this falls on the steeply rising portion of the curve, yet even at this point quite definite differences should be experimentally observable between ordinary gelatin and that treated with acid before hydrolysis. The amount used by Daft, however, corresponds to 76.5 mg. per 10 cc., which lies altogether

Fig. 3. Precipitated curves of acid- and alkali-flocculated gelatin.

outside the scale of magnitudes possible for the micro-method. It should, however, be noted, that the slope of the curves of Fig. 3, if extrapolated, indicates for this concentration an approximate value for the nitrogen precipitated by phosphotungstic acid of 20 %: the value given in Daft's experiments averages 16.5 %, precipitated at 18°. This figure must be corrected to 0°, to correspond with those quoted here and this may be done by using his own figure of 2.5 % as the difference between the precipitates obtained at 0° and 18°. We thus obtain 19 % as the corresponding value at this concentration. It is therefore clear that Daft's figures lie close to the general curve, and, since these curves all appear to follow the same course at these high concentrations, no differences due to acid pretreatment in analyses carried out at these concentrations would be expected. This constitutes yet another

reason for believing that this worker's results in no way disagree with those obtained by Schryver and others, previously quoted.

As was pointed out above, it has not been possible to repeat the analyses quoted by Daft, since no indication is given by him as to the nature and source of the gelatin used or its purification. Since the values obtained by him probably coincide with the curves as mentioned above, however, such repetition is scarcely necessary.

SUMMARY.

1. An investigation has been made of the reasons for the discrepancies between the results of different workers in regard to the amount of nitrogen precipitated from gelatin hydrolysates by phosphotungstic acid.

2. Differences in the precipitating power of different samples of phosphotungstic acid are shown to be responsible for subsidiary variations in this nitrogen.

3. Heating with 5 % sulphuric acid in the autoclave before precipitation destroys part of the precipitable diamino-nitrogen of arginine, and is therefore to be avoided as a step in the analysis of protein hydrolysates.

4. The amount of nitrogen precipitated by phosphotungstic acid varies almost continuously with the concentration of nitrogen in the solution, and in this fact lies the principal source of the discrepancies.

5. Curves showing the variation in the nitrogen precipitated with the concentration of nitrogen used must be substituted for single determinations in order to characterise the protein investigated.

6. Such curves clearly prove that treating gelatin with acid before hydrolysis profoundly affects the amount of nitrogen precipitated by phosphotungstic acid.

7. The protein molecule must, therefore, be regarded as an extremely labile structure.

8. A rapid method for the determination of the amide-nitrogen is described, and its effect on the subsequent precipitation with phosphotungstic acid is determined.

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