CXLII. INVESTIGATIONS ON GELATIN. PART VII. THE NON-AMINO NITROGEN OF GELATIN.

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IN the purification of gelatin by flocculation in an electric field, the endeavour was made to separate gelatin from the products of its thermal degradation, produced in the course of its preparation from its precursors. To amplify this work, a series of experiments was undertaken with the object of following in some detail the process of separation of the products of thermal degradation from a gelatin which had been highly purified by repeated flocculation and subsequently heated with water. During the course of these experiments a certain chemical change in the protein molecule was noted which does not appear to have been previously recorded. If a freshly prepared purified gelatin is submitted to rapid hydrolysis by acids, and the Hausmann numbers are determined, it will be generally found that the non-amino nitrogen is relatively low. If the same gelatin be heated, and the degradation products afterwards separated by the method of flocculation in an electric field, a product will be obtained, of which (after rapid hydrolysis by acids) the Hausmann numbers will be found to differ markedly from those of the original gelatin in only one factor. It will be found that there has taken place a marked increase in the non-amino nitrogen. This phenomenon may be most plausibly explained by assuming that the addition of hot water produces a molecular re-arrangement in the protein molecule (or molecules) with the formation of closed rings of the nature of proline or ketopiperazines in which an amino-group takes part, which are resistant to hydrolysis with acids and do not yield nitrogen on treatment with nitrous acid as did the amino groups which were responsible for this ring formation.

These observations also offer a partial explanation of another fact. Although gelatin, on heating with water, rapidly undergoes an irreversible change, yielding simpler products which rapidly diffuse, no increase in the amino nitrogen could be detected by the methods of Sørensen or Van Slyke in the degraded gelatin, as would be expected, had any scission of peptide linkages taken place. The formation of stable ring compounds from amino groups would effectively mask an increase in such groups due to this scission.

It was also found that an increase in the non-amino nitrogen of the hydrolysis products took place when gelatin was repeatedly flocculated, or when it was dissolved in cold solutions of acids and alkalis.

EXPERIMENTAL.

Flocculation of a purified gelatin which had been subjected to the action of heat.

A 2 % solution of a bone gelatin which had been purified by four flocculations was heated in a quartz vessel under a reflux for 5 hours. The solution was then flocculated in an apparatus similar to that already described [Knaggs and Schryver, 1924]. A control experiment was carried out in an exactly similar apparatus, and as nearly as possible under the same conditions with a 2 % solution of the same gelatin which had not been subjected to heat. 500 cc. were introduced into the flocculating tube in each case, and 500 cc. of water into the beaker which contained the mercury cathode. At intervals 25 cc. were pipetted out of both the supernatant fluid and the liquid round the cathode (which contained substances which had passed through the collo-dion membrane), from both sets of apparatus, and the nitrogen was determined in these samples. The results are recorded in the following table:

Table I.

Time in hrs.	Heated gelatin solution		Unheated gelatin solution	
	Supernatant fluid mg. N/100 cc.	Cathode liquid mg. N/100 cc.	Supernatant fluid mg. N/100 cc.	Cathode liquid mg. N/100 cc.
0	230.6	—	230.6	—
28	51.56	9.92	60.8	1.13
53	34.28	10.9	22.36	1.56
100	25.16	14.1	10.2	1.41
197	22.36	18.7	5.5	1.42
250	21.2	19.2	3.7	1.50
315	20.94	20.68	3.5	1.59

It will be noticed that the nitrogen of the supernatant fluid from the heated sample becomes constant after about 250 hours, and amounts to 21 mg./100 cc. which is in great contrast to the corresponding figure when constancy has been obtained of the unheated sample, which is only 3.5 mg./100 cc. The following are the Hausmann numbers of the various samples:

Table II.

	Amide N	Humin N	Diamino N	Mono- amino N	Non- amino N
A. Original gelatin purified by 2 flocculations	2.06	1.93	21.59	61.37	12.32
B. Original gelatin, purified by 2 flocculations, of which a 2 % solution had been heated for 5 hours	2.08	3.81	22.64	54.2	17.27
C. Gelatin produced from B by flocculation	1.78	1.63	22.2	56.1	18.29
D. C re-flocculated	1.85	2.98	21.42	56.60	22.15

It will be noticed from the table above that, with the exception of the non-amino nitrogen, the variations are small and irregular, and these are due to

the somewhat limited accuracy of the experimental methods. There is, however, a marked change in the non-amino nitrogen.

A similar experiment was carried out with another sample of gelatin which had been flocculated seven times. In this case the non-amino nitrogen of the unheated gelatin was high to start with and was 18.05 %¹. When it was heated for 5 hours in a 2 % solution, and the solution was then placed in an electric field, a gelatin was flocculated which gave a non-amino nitrogen of 20.87 %. Again a distinct increase produced by heating.

The dependence of the non-amino nitrogen on the length of the treatment of the gelatin with hot water. In the following experiment a sample of Coignet's gold label gelatin was used, which had been freed from electrolytes by electrolysis, and had been subsequently "recrystallised" once [Knaggs, Manning and Schryver, 1923]. This yielded on analysis the following numbers:

Table III.

	Amino N by		Amide N	Humin N	Diamino N	Mono-amino N	Non-amino N
	Van Slyke's method	Sørensen's method					
	5.1	5.1					
Coignet's ash-free gold label gelatin, "recrystallised" once	Determined before hydrolysis		1.02	0.74	22.34	64.19	11.71
			Determined after hydrolysis				

A 5 % solution of this gelatin was heated in a quartz vessel in an oil-bath at 105° under a reflux. A sample was removed at intervals, hydrolysed and the Hausmann numbers determined. The humin and diamino N showed the usual slight irregular variations, but the non-amino N continuously increased till it became constant. The results are indicated in the accompanying curve (Fig. 1).

Fig. 1. Rate of change in the percentage of non-amino nitrogen when a 5 % solution of ash-free Coignet's gold label gelatin is heated at 100° C.

¹ For explanation of this high number see remarks in introduction and in sequel.

The solution of the heated gelatin would no longer flocculate in an electric field and the liquid round the cathode containing the portion readily dialysable gave a high value for the diamino nitrogen [cf. Knaggs, Manning and Schryver, 1923, p. 4]. When the solution was tested both by the Van Slyke and Sørensen methods, it showed the same percentage of amino N (5.1 %) as the unheated solution. This same figure was obtained even when solution had been heated 100 hours. The percentage of amide nitrogen remained constant at 1.1 % after 40 hours of heating at 100°.

Effect of flocculation on the non-amino N.

As noticed in the introduction, the non-amino nitrogen increases with successive flocculations. The following numbers taken from another paper will illustrate this point. Only these figures are quoted as there are only slight irregular variations in the other Hausmann numbers.

Table IV.

	Gelatin								Non-amino N
Original gelatin (ash-free) ...	...	...	...	...	...	...	...	...	9.34
The same, once flocculated ...	...	...	...	...	...	...	...	...	12.32
The same, twice flocculated ...	...	...	...	...	...	...	...	...	13.05
The same, after 7 flocculations ...	...	...	...	...	...	...	...	...	18.05
The same, after 2 flocculations and heating the flocculated gel in 2 % solution 5 hours ...	...	...	...	...	...	...	...	...	17.27

The action of cold dilute acids and alkalis on the non-amino N.

A gelatin prepared from ossein, which had been rendered ash-free by electrolysis and once flocculated, was used for the experiments. A 3 % solution of this was treated with an equal volume of 2 *N* hydrochloric acid; and the mixture was allowed to stand for 48 hours. After this interval all the gelatin had gone into solution. The Hausmann numbers were then determined, after hydrolysis. A similar experiment was carried out by diluting the 3 % gelatin solution with an equal volume of 2 *N* sodium hydroxide. In this case the mixture was allowed to stand for 24 hours only before hydrolysis. The Hausmann numbers of the original and the acid- and alkali-treated gelatins are shown in the following table.

The gelatin which had been treated with acid formed a gel when the acid was removed. This was not the case with gelatin which had been treated with alkali.

Table V.

	Amide N	Humin N	Diamino N	Mono-amino N	Non-amino N
A. Gelatin electrically flocculated once	2.06	3.53	21.60	60.49	12.32
A was treated with <i>N</i> HCl for 48 hours, i.e. until all gelatin was in solution, and immediately hydrolysed with 20 % HCl ...	2.25	1.23	20.91	55.12	20.49
A was treated with <i>N</i> NaOH for 24 hours, neutralised, and quickly hydrolysed ...	1.93	1.94	20.94	52.30	22.89

CONCLUSIONS.

When a gelatin purified by flocculation is heated with water and then hydrolysed, a marked increase in the nitrogen of the hydrolysis products which will not react with nitrous acid to give gaseous nitrogen is observed. This indicates in all probability a molecular re-arrangement in the molecule with the formation of stable ring compounds. A similar result is produced by repeated flocculation in an electric field and also when gelatin is treated with cold dilute acids and alkalis. The results indicate furthermore the variations which can exist in different samples of gelatin derived from the same precursors. It is not yet possible to consider gelatin as a definite chemical entity even after submission to purification by the methods which have been discussed in earlier papers.

REFERENCES.

- Knaggs, Manning and Schryver (1923). *Biochem. J.* **17**, 473.
Knaggs and Schryver (1924). *Biochem. J.* **18**, 1079.