CXXXIX. INVESTIGATIONS ON GELATIN. PART IV. THE PURIFICATION OF GELATIN BY FLOCCULATION IN AN ELECTRIC FIELD.

By JOHN KNAGGS and SAMUEL BARNETT SCHRYVER.

From the Biochemical Department of the Imperial College of Science and Technology.

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Introduction.

In a previous paper a method of purifying gelatin by separation from aqueous solution was described [Knaggs, Manning and Schryver, 1923]. In the present paper an alternative method is indicated. In carrying out a process such as this, it is essential to distinguish between the impurities due to the tissues from which the gelatin is derived, and those due to its thermal degradation. Most of the experiments, therefore, were carried out with a gelatin prepared in the laboratory from bone.

Preparation of Material.

Ossein, which was kindly supplied by Mr W. K. Beveridge, of Messrs Nelson Dale and Co., was used. The pieces of this material were approximately $\frac{1}{4}$ inch size. It was treated with 10 % brine and 0·5 % hydrochloric acid for 24 hours and well washed in running water to remove all the inorganic salts as far as possible. The ossein was then treated with 0·2 % sodium hydroxide solution, 100 g. of dry ossein to 1 litre of the alkali, for 60 days. At frequent intervals, the caustic soda solution was decanted off and a fresh batch added.

The prolonged treatment with 0·2 % caustic soda was found necessary to remove the last traces of the substances soluble in alkali present in the tissue, such as chondroitinsulphuric acid. The rate at which the latter substance was removed was followed by treating 100 g. of dry ossein with 1 litre of 0·2 % caustic soda; 25 cc. of the liquid were removed each day, and the amount of nitrogen determined by the Kjeldahl method. It will be seen from the following results that the nitrogen becomes nearly constant after eight days. Yet if the caustic soda solution is removed and a fresh quantity added, more of the chondroitinsulphuric acid is extracted; the solution gave a precipitate with 1 % acetic acid, showing the presence of a mucin, or some similar substance. It would seem from the results that the chondroitinsulphuric acid is in chemical combination with some other colloid present in the ossein and the above numbers give an indication of the rate at which such a complex is split up by dilute alkali.

Time in days	Mg. of N per 100 cc. NaOH solution
1	16·8
2	20·6
5	24·92
6	25·48
7	26·32
8	26·88
9	27·16
10	27·30

The ossein was therefore left in contact with the 0·2 % caustic soda solution for 60 days. Every third day the solution was changed, and tested for the presence of chondroitin by dilute acetic acid.

It was only after 51 days that no turbidity was observed when the soda solution was acidified.

This fact was also confirmed by the Hausmann numbers of gelatins which were extracted from ossein treated for varying lengths of time with 0·2 % caustic soda.

The following is the table, and the Hausmann numbers of a sample of a high-grade commercial bone gelatin are included for comparison.

Number of days ossein was treated with 0·2 % NaOH	Amide N	Humin N	Diamino N	Diamino N and humin N
1 day 1st extraction	3·3	1·3	19·9	21·2
2nd „	3·3	1·5	20·0	21·5
7 days	3·2	1·8	21·4	23·2
14 days 1st extraction	2·59	1·85	21·8	23·65
2nd „	3·0	·87	22·0	22·87
14 days 2nd „	2·48	1·6	22·8	24·4
(The gelatin was washed with *N*/1000 HCl and water for 10 days)				
30 days	1·76	1·48	23·4	24·88
60 days	1·54	1·28	24·09	25·37
Commercial bone gelatin	2·04	3·93	23·06	26·99
Commercial bone gelatin purified by washing with *N*/1000 HCl and water 10 days	1·7	3·35	23·05	26·40

Gelatin extracted from ossein which has had no treatment with soda has a percentage of amide nitrogen of 3·76. By treating with caustic soda the percentage of amide nitrogen is progressively reduced to 1·54 after 60 days, which is the minimum value which has been obtained for bone gelatin.

In the same way the percentage of diamino-nitrogen is increased from 19·9 after one day's treatment to 24·09 after 60 days' treatment with 0·2 % NaOH.

As the caustic soda extracts from the ossein chondroitinsulphuric acid, some of this compound was isolated from the alkaline extract by acidifying with acetic acid. The precipitate was collected and washed with dilute acetic acid on the filter. The Hausmann numbers were as follows:

Percentage of amide nitrogen	7·67
„ humin „	4·60
„ diamino „	15·48

This protein has a high percentage of amide N 7·67 and a low percentage of diamino N 15·48, and its presence in gelatin would appreciably alter the Hausmann numbers, as above results indicate.

The percentage of amide N in a sample of gelatin would be a good criterion as to its purity. It would give no indication of the products of thermal decomposition, but the presence and approximate amount of other protein impurities could be detected fairly accurately.

In the following experiments on flocculation a gelatin was used which was freed as far as possible from organic nitrogenous impurities such as chondroitin. The gelatin was extracted from the ossein at 80° to ensure that the percentage of heat degradation product was as low as possible.

The method of purifying gelatin by passing a current through a 20 % jelly [Knaggs, Manning and Schryver, 1923] was only suitable for removing soluble inorganic salts. The protein impurities and insoluble inorganic salts were only partly removed.

This was proved to be the case by submitting a gelatin known to contain these impurities to this process. The gelatin used was extracted from ossein treated for only 5 days with 0·2 % caustic soda. The gelatin was washed with *N*/1000 HCl and then with frequent changes of water for 10 days. The percentage of ash in the washed sample was found.

A 20 % gel of the gelatin was made up, and electrolysed, and after several days the gelatin was examined quantitatively.

The Hausmann numbers of each fraction are given below.

	Amide N	Humin N	Diamino N	Diamino N and humin N
A. Gelatin from ossein treated for 5 days with 0·2 % caustic soda	3·26	2·53	21·76	24·29
A was washed with *N*/1000 hydrochloric acid and then with water for 10 days	2·83	2·41	22·00	24·41
The washed gelatin was electrolysed as a 20 % gel	2·98	2·54	21·95	24·49

The process of washing removed some of the organic impurities, shown by the change in the percentage of amide N from 3·26 to 2·83. The electrolysis did not further reduce the values, for the Hausmann numbers of the washed gelatin and electrolysed gelatin are practically the same.

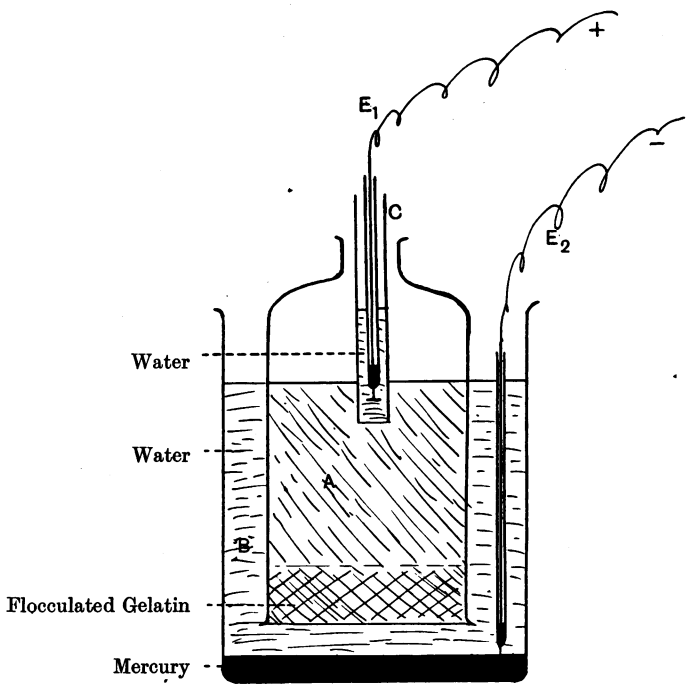
During electrolysis of gelatins obtained from ossein which had not been treated for a sufficient length of time by alkali, the jelly became very turbid, but this turbidity could not be removed by filtration of the hot solution through paper pulp. This was probably due to the chondroitinsulphuric acid being split up under the influence of the current into chondroitin and sulphuric acid, the chondroitin being held in suspension in the gel. The Hausmann numbers would not indicate such a change as the percentages of each group are calculated from the total nitrogen present in the protein.

The ash in the washed gelatin amounted to 0·35 % and consisted almost entirely of calcium phosphate. After electrolysis this value was 0·32 % and the gelatin could readily be "recrystallised" from a 1 % solution.

In carrying out the experiment on purification by flocculation, it was found advisable, therefore, to use a gelatin made as free as possible from chondroitin, etc., by prolonged treatment of the ossein with alkali, and to free it, finally, from electrolytes by the electrolysis of a 20 % gel.

Flocculation of gelatin from electrolytes and tissue impurities.

Method of working. The apparatus which was used is shown graphically in the accompanying figure.



A is a bell-jar internal diameter 7·8 cm. and height 30 cm. across the bottom of which is stretched muslin firmly bound by means of string. The muslin is rendered semi-permeable by a coating of collodion.

B is a bucket or glass vessel, the base of which is covered with a layer of mercury to act as cathode E_2. *B* is filled with a known volume of water and the base of *A* dips into the water as shown.

C is a piece of glass tube internal diameter 1 inch and length 8 inches, the end which dips into the liquid being also closed by a collodion membrane. The tube *C* is filled with water and the anode E_1 is immersed in the liquid. By separating the anode in this way with a membrane, any acid which is liberated during electrolysis is isolated from the liquid in *A*.

In the experiment about to be described, a gelatin was employed which had been prepared from an ossein which had been treated for 6 weeks with 0·2 % NaOH solution. 6 litres of a 2 % solution were placed in *A*. The poles were 30 cm. apart and a P.D. of 150 volts was established between them. In this experiment the electrolytes had not been completely removed by a preliminary electrolysis of the gel.

The current was passed for several days. When all the electrolytes were removed, the gelatin solution became cloudy, and the protein after a time was precipitated in *A* as a white flocculent mass. After 5 days the solution became quite clear, and the precipitated gel was very similar to one which had been recrystallised from water.

The distribution of the nitrogen in the various layers was found to be as follows:

Round anode	negligible
Supernatant liquid	17·38 %
Precipitated gelatin	64·33 %
Diffused through membrane to cathode *B*	18·18 %

The strength of the precipitated gelatin was found to be 7·5 %; and the mg. of N per 100 cc. of the supernatant liquid, and the liquid round the cathode, were 34·12 and 32·1 respectively.

Experiments were carried out to determine the rate at which the gelatin was precipitated.

The same sample of gelatin as above was used, but it was first washed with water for 4 days, and the last trace of ionisable salts removed by electrolysing a 20 % gel. Experience showed that purification by electric flocculation can only be conveniently carried out with a gelatin freed from chondroitin by the method described above, and subsequently purified by washing and the electrolysis of a strong solution.

The apparatus was the same as described above, except that the tube *C* was removed so that the anode dipped directly into the gelatin solution. At known intervals of time 50 cc. of the supernatant liquid in *A* and the cathode liquid in *B* were pipetted out, and the amount of nitrogen determined by Kjeldahl's method.

The current was allowed to pass until the value mg. N/100 cc. in both the supernatant liquid and cathode liquid became constant.

The following table gives the readings for the first and second flocculations:

	First flocculation			Second flocculation	
Time in hrs	Supernatant liquid mg. N/100 cc.	Cathode liquid mg. N/100 cc.	Time in hrs	Supernatant liquid mg. N/100 cc.	Cathode liquid mg. N/100 cc.
0	240·8	0	0	207·2	0
24	97·4	3·11	24	53·34	2·1
54	42·98	2·90	48	26·35	2·55
100	29·77	3·11	70	13·31	2·27
192	15·15	3·11	120·5	11·05	2·55
220	14·8	3·0	145·5	3·97	1·71
244	15·1	2·9	169·0	5·38	1·98
268	14·7	3·20	193·0	5·52	1·70
			217	5·67	1·98
			234	5·52	2·27
			286·5	5·52	2·58

The gelatin which was precipitated in the first flocculation was redissolved and reflocculated by the electric current. The readings under "Second flocculation" refer to this.

First flocculation. After the current has been passing for 200 hours the nitrogen remaining in the supernatant liquid becomes constant at 15 mg., but in the liquid round the cathode this value becomes constant within the first 24 hours at 3·0 mg. Therefore the portion which diffuses through the parchment to the cathode must be of low molecular weight and carry a relatively high electric charge.

Second flocculation. The readings are very similar to those of the first flocculation except that when the amount of nitrogen becomes constant in the supernatant liquid, the value is 5·5 mg., which is much lower. The liquid round the cathode reaches a constant nitrogen value within the first 24 hours of 2·0 mg. per 100 cc.

A third flocculation was carried out, and it was found that when equilibrium was established nitrogen remaining in the supernatant liquid was 6·3 mg. per 100 cc., and that which diffused through the membrane to the cathode was 1·1 mg. per 100 cc.

The quantity of nitrogen in the supernatant liquid in the third flocculation is higher than in the second flocculation, and the precipitated gelatin when "recrystallised" from water did not separate properly on standing, but always gave a turbid liquid. The turbidity could be readily removed by electrolysing the solution. The only way this could happen would be by the solution containing a trace of electrolyte. The experiments described above were carried out using an enamelled bucket to contain the cathode liquid. Under the influence of an electric current some of the salts from the enamel would most likely find their way into the flocculated colloid. The experiments were therefore repeated using glass vessels, and the following results show the above consideration to be correct:

Experiment carried out in glass vessels	mg. N remaining in the supernatant liquid when constant
Second flocculation	5·09
Third ,,	4·53
Fourth ,,	3·41

The value 3·4 mg. N per 100 cc. appears to be a constant for the particular gelatin, for even after seven flocculations the value does not alter.

SUMMARY.

1. It is shown that the Hausmann numbers of bone gelatin can be influenced by the amount of chondroitinsulphuric acid present. The compound may be removed from ossein by treating it for 60 days with 0·2 % caustic soda.

2. Gelatin may be freed from the products of thermal degradation by flocculating several times in an electric field a 2 % solution of gelatin which is free from electrolytes, chondroitin, etc.

3. After a certain number of flocculations, under the conditions of experiment specified, the amount of nitrogen remaining in the supernatant fluid becomes constant, and is not diminished on further flocculation.

REFERENCE.

Knaggs, Manning and Schryver (1923). *Biochem. J.* **17**, 473.