LVII. INVESTIGATIONS ON GELATIN. PART II. RESEARCHES ON THE METHODS OF PURIFYING GELATIN.

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*Report to the Adhesives Committee of the Department
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(Received June 6th, 1923.)

INTRODUCTION.

IN spite of the fact that a very large literature exists dealing with the physical and chemical properties of gelatin, no definite criterion seems to have been established up to the present time by means of which the purity of the material investigated can be defined. The necessity for defining the state of purity is an urgent one, when the origin of a sample of gelatin is considered. In the first place, it is derived from animal tissues, which, in addition to the actual precursors of gelatin, contain other complex nitrogenous substances, which may contaminate the finished product, and in the second place, it is itself produced from these precursors by the chemical action of hot water, undergoing during the process degradation into simpler products, the nature of which has not yet been definitely established. It follows, therefore, that every sample of gelatin is a mixture of gelatin with other nitrogenous substances, even when the greatest care has been taken in its preparation.

The following investigations were undertaken with two main objects in view. It was desired in the first instance to establish some physical criterion, by means of which the state of purity of a given sample could be defined; in the second instance it was necessary to discover processes by means of which the nitrogenous contaminations of gelatin could be removed, so as to yield a product conforming with the established physical criterion, which should remain unchanged when these processes were repeated an indefinite number of times.

An ordinary high grade commercial preparation, when allowed to soak in water, yields to the aqueous phase certain amounts of diffusible nitrogenous substances, the amounts of which vary from preparation to preparation, when the same conditions of experiment are chosen. These substances comprise, for the most part, the contaminating substances to which reference has been

already made. If a gelatin preparation is free from these, then a definite equilibrium of some kind should exist between the nitrogenous contents of an aqueous phase and a gelatin phase (either in the form of a jelly or otherwise) which are in contact with one another. If this equilibrium can be ascertained, then a criterion for the definition of the state of purity of a sample of gelatin will have been established.

As a result of the investigations described in this communication, preparations of gelatin have been obtained in which the desired nitrogenous equilibrium has been found to exist, and which must be taken, therefore, to be free from diffusible nitrogenous contaminations.

The researches started with the determination of the amount of nitrogen which diffused out from a given surface of a gelatin jelly, prepared under certain specified conditions, into a given volume of water in contact with it within 20 hours at the temperature of 20°. This method of investigation yielded valuable information as to the character of commercial samples of gelatin and glues (to which reference has been made in the *Bulletin on Adhesives* issued by the Department of Scientific and Industrial Research in 1922) and the determination of the so-called "diffusible nitrogen" is suggested as a routine method for the examination of these products. If the water in contact with the jelly is removed daily, and the nitrogen is estimated in each portion, it will be found that this diminishes rapidly until it reaches a small quantity, which remains constant over a prolonged period. Similar experiments were carried out with relatively large amounts of gelatin jelly with large surfaces in contact with water, and it was found that even after several months of contact, the amount of nitrogen in the water phase was continually increasing. The experiments illustrating these statements are given in Section I of this paper.

A detailed examination was then undertaken of certain properties of the nitrogenous diffusate, and also of a solution of gelatin (10 %) which had been heated for several hours, as it was assumed that the diffusate consisted largely of the products of thorough decomposition of gelatin. When these products were submitted to dialysis through parchment paper, it was found that at first relatively large amounts of nitrogenous substances diffused through in one day, but these somewhat rapidly diminished, until, after a certain time, the quantities passing the dialyser in a day became practically constant. This apparent constancy was maintained for several weeks. A diminution of the rate of diffusion could then only be ascertained when the diffusate of several days was estimated together. The rate of diffusion was so slow, that the results originally suggested that there were products present within the dialysers which were undergoing continual degradation in the presence of water at ordinary room temperature. These results are described in Section II.

The general result of the experiments given in Sections I and II was to indicate the great difficulty, if not the impossibility of freeing gelatin from the nitrogenous substances contaminating it by the method of washing or dialysis.

Incidentally, an examination was made of the products which passed the dialysers and those which remained within. The differences in the "Hausmann numbers" indicate that gelatin on heating does not break down into smaller aggregates of the same chemical character. The nature of the thermal decomposition of gelatin has not yet been definitely established.

As it had been shown that gelatin could not be freed from the contaminating nitrogenous substances even by months of continuous washing and dialysis, the effect of electrolysis on their removal was next investigated. It was found that by this means simple electrolytes such as salts were readily removed. Electrolytes of colloidal character and non-electrolytes remained, however, in the gelatin, when submitted to electrolysis. A simple ampholyte, such as glycine, which has weak acidic and basic dissociation constants, was removed only very slowly under the influence of an electric current. The result of these experiments, which are described in Section III, was to indicate, that, even by electrolysis, the nitrogenous contaminating products could not be completely removed.

Electrolysis, however, removes very readily the last traces of salts and other strong electrolytes from gelatin, which can thus be obtained practically ash-free. The electrolysis process, as described in this paper, is best applied to gelatin, from which the greater part of the salts have been already removed by washing the jellies with dilute acid and water.

When freed from electrolytes, gelatin possesses the property of separating out as a hydrated insoluble precipitate when solutions of 2 % concentration or less are cooled. Advantage has been taken of this fact to purify gelatin still further by a process conveniently termed "recrystallisation." The nitrogen content of the clear liquid after separation of the insoluble hydrate has been determined after successive "recrystallisations." This was found to fall to a certain constant value which remained the same whether the gelatin separated from a concentration of 0.1 or 2 %. Such a condition should hold when a substance is in equilibrium with its saturated solution, and the actual experiments indicate that such a saturated solution contains about 0.056 % of gelatin¹. This result is in close accord with that found by Fairbrother and Swan [1922] although obtained by an entirely different method. Some of the results of these investigators are not, however, in agreement with those obtained by the authors of the present paper. It has been found impossible to obtain nitrogenous equilibrium between the aqueous phase and the gelatin phase in the short time suggested by Fairbrother and Swan; such an equilibrium was not attained with a well-washed gelatin sample even after several months. The gelatin was estimated by those authors in the aqueous phase by precipitation with tannic acid. This reagent will, however, precipitate, in addition to gelatin, a great part of the degradation products which are present as contaminants. The method employed by Fairbrother and Swan appears to the authors of this paper to be affected by two sources of error, which will

¹ See however, p. 486.

tend to compensate one another, and the accordance found in the determination of the solubility of gelatin in water by the two sets of investigators must be to a large extent accidental. The experiments on the "recrystallisation" of gelatin are described in Section IV.

According to the researches herein described, gelatin free from soluble nitrogenous contaminants can be obtained by submitting it to the following successive processes: (i) washing the jelly first with weak acid and then with water, (ii) submitting the washed concentrated jelly to electrolysis, and (iii) recrystallising the electrolysed gelatin.

Certain samples of gelatin thus purified still appear to contain a very small amount of an insoluble nitrogenous contaminant, which imparts to the jellies an opalescent appearance. Investigations on the separation of this are still in progress. Some gelatins appear to be nearly free from it. It is, however, only present in very small quantities and does not influence to any large degree the properties of gelatin, as do the water-soluble contaminants.

EXPERIMENTAL.

SECTION I. THE "DIFFUSIBLE NITROGEN."

When gelatin is placed in contact with water, a certain amount of nitrogenous material passes out from it. If the experiment is carried out under conditions defined as regards strength of gelatin, volume of gelatin and water, area of contact of phases, time of contact and temperature, the number of milligrams of nitrogen which pass into solution is termed the "diffusible nitrogen."

The conditions employed in the experiments described below were as follows: 20 g. of gelatin and 100 cc. of water were placed in a wide-mouthed stoppered bottle of $9\frac{1}{2}$ cm. internal diameter. The gelatin was dissolved in the water at 37° , and the solution allowed to set for 24 hours at room temperature. 100 cc. of water were then poured gently on to the surface of the gelatin, and the bottle was placed in a thermostat at 20° for 24 hours. The water was then poured off through a filter, and the surface of the gelatin and sides of the bottle were washed once or twice with distilled water, which was also poured through the filter. The nitrogen in the filtrate was then determined. (The "diffusible nitrogen.") In nearly all the experiments described in this paper, Coignet's "gold-label" gelatin was employed. In a few experiments other samples of gelatin were also occasionally employed, and these yielded the same results as Coignet's. For the latter, the "diffusible nitrogen," determined under the conditions mentioned above, gave values varying from 10–20 mg. If, after the first determination of the diffusible nitrogen, the process is repeated on the same gelatin by the addition each time of 100 cc. of water, the value falls to 2 mg. per 24 hours, at which it remains sensibly constant (with occasionally higher values, owing to the swelling and increase

of surface of the gelatin). As an illustration, the results of one experiment are given below:

"Diffusible nitrogen" in Coignet's "gold-label" gelatin.

	Period of contact	Diffusible nitrogen mg.	Diffusible nitrogen in 24 hours mg.
1.	24 hours	11.6	11.6
2.	24 "	5.8	5.8
3.	48 "	8.65	4.32
4.	48 "	7.01	3.50
5.	48 "	6.46	3.23
6.	72 "	9.16	3.05
7.	8 days	20.6	2.57
8.	72 hours	9.37	3.12

Fresh water was used in each experiment.

The process cannot be carried on indefinitely, since the gelatin ultimately swells and the surface breaks up. If the gelatin is washed until the approximately constant nitrogen value is reached, and then remelted at 37°, and allowed to set, it is found that the "diffusible nitrogen" has risen again nearly to its original value. For example, a sample giving the initial value of 15.3 was washed till the value fell to 1.96. On remelting it rose to 12.1. It appears probable that the diffusible nitrogen comes from the surface layer or its immediate neighbourhood only. This statement is confirmed by experiments in which different areas of contact between jelly and water were used, the other conditions being exactly similar. Thus, when the ratio of the two surfaces was as 2.2:1, the diffusible nitrogens were 27.2 and 14.

The ultimate small value (2 to 3) obtained by continuous washing may be due to (a) simple solubility, (b) slow diffusion from the lower layers, (c) slow degradation of the gelatin in contact with water. The "diffusible nitrogen" is not exactly proportional to the concentration of the gelatin. Thus

Concentration of gelatin	"Diffusible nitrogen"
5 g. gelatin + 100 cc. water	7.4
10 " "	10.0
20 " "	12.6

Washing the gelatin with dilute hydrochloric acid and then with water (Loeb) produced a marked lowering of the diffusible nitrogen; thus, for example:

First	24 hours.	Diffusible nitrogen	2.5
Second	"	"	1.3
Third	"	"	1.25

Fresh water was used each time.

This case was somewhat exceptional, other samples giving the higher values of 4 to 6 for the first 24 hours.

The use of very dilute hydrochloric acid ($N/1000$ or less) for making up the gelatin and washing it makes no appreciable difference to the diffusible

nitrogen. The presence of neutral salts, on the other hand, increases it, as the following experiments (some of which were carried out by Mrs Horne) show:

Salt used	Concentration of salt in jelly and water	First four days	Next six days	Next eight days
NaCl	0.0	15.3	12.3	17.4
	N/10	18.6	14.7	20.3
	N/4	19.2	15.8	21.7
	N/2	21.9	18.2	23.3
CaCl ₂	0.0	17.4	15.6	16.5
	N/10	21.5	19.4	19.7
	N/4	24.9	—	19.5
	N/2	25.8	—	20.3

Fresh salt solutions were employed for each of the periods of experiment given in the above table.

The "diffusible nitrogen" is derived to a large extent, if not wholly, from the products of thermal degradation of gelatin, as is shown from the following experiment. A 20 % gelatin was made up in a series of wide-mouthed bottles, as described above, and each bottle was heated for a given period in a hot-water oven. After removal, the gelatin was allowed to set for at least 24 hours, and the "diffusible nitrogen" determined in the usual manner. The results are indicated in the accompanying table and curve (Fig. 1). After 16 hours the jelly is so weak, that the determination of the diffusible nitrogen is unreliable. After 29 hours' heating the gelatin would not set at all. The curve for the first 16 hours is a regular one.

The experiments were carried out with a gelatin that had been well-washed beforehand.

Fig. 1

		Diffusible nitrogen
Unheated gelatin		1.9
Heated 5 hours		6.9
" 9 "		13.1
" 12 "		21.4
" 16 "		32.6
" 22 "		59.9
" 29 "		Gelatin did not set

The question next arose as to whether, on leaving gelatin a sufficiently long time in contact with water, an equilibrium value for the nitrogen content of the water could be reached. To test this 112 g. of gelatin, previously washed carefully with dilute hydrochloric acid and water, were immersed in the form of thin sheets in 3 litres of water, a little chloroform having been added to keep the gelatin sterile. The whole was kept in a tightly stoppered bottle and left for prolonged periods during which, at intervals, 50 to 100 cc. of the aqueous phase were removed, filtered and submitted to an estimation of nitrogen. No indication of an equilibrium was ever found, as the following numbers indicate:

Period of contact	Milligrams of nitrogen per 100 cc. of aqueous phase
2 months	43.0
3 "	45.5
4 "	48.9

The water after this time was poured off, the gelatin was washed, and fresh water was added. The nitrogen in portions of 50–100 cc. was determined at intervals with the following results:

Period of contact	Milligrams of N per 100 cc. aqueous phase
7 days	11.2
14 "	12.6
21 "	14.6
28 "	16.0
53 "	20.3
4 months	35.3

Thus, after even 8 months of washing, nitrogen appeared to be still coming out from the gelatin into the water. These results are at variance with those of Fairbrother and Swan [1922]. They suggest that gelatin itself undergoes some degradation in contact with water. It was therefore considered advisable to obtain some idea as to the diffusibility of the nitrogenous products which could be washed out of the gelatin, and experiments dealing with this factor are given in the next section.

SECTION II. THE DIFFUSIBILITY THROUGH PARCHMENT OF THE NITROGENOUS PRODUCTS WHICH CAN BE EXTRACTED FROM GELATIN BY WATER.

Two main series of experiments were carried out. (a) With the washings of gelatin. (b) With a sample of gelatin, which had been previously well washed till nearly salt-free and then heated for a long period, so that it no longer set in concentrated solutions.

(a) *Experiment with the washings.* The following experiments carried out by Mrs Horne, were made with a sample of Nelson Dale and Co.'s bone gelatin, which was kindly provided by Mr W. K. Beveridge. Similar results were got with Coignet's "gold-label" gelatin.

Three extracts (I, II and III) were made. Extract I was made by extracting 2 lbs. of gelatin first with 10 litres of water for 4 days, then after pouring off the water which was not absorbed by the gelatin, and which amounted to about $2\frac{1}{2}$ litres, with 10 litres of water for 2 days, this extraction being followed by another extraction by 10 litres of water for 2 days. The washings from these extractions, which lasted altogether for 8 days, were combined to form extract I.

The second extract (II) was made by treating the washed gelatin with 10 litres of water for 13 days, whilst extract III was made by a subsequent extraction with the same amount of water for 24 days. The three extracts were concentrated under diminished pressure until they all contained the same amount of nitrogen, viz. 246.6 mg. per 100 cc. The concentrated solutions were then introduced into parchment dialysers and submitted to dialysis under as nearly as possible identical conditions, *i.e.* the size of the parchment bags, the size of the containing vessels, the amounts introduced into the dialysers, and the water outside were the same in all cases. All the experiments were carried out under bell-jars under which were placed also open dishes containing chloroform so as to keep the solutions sterile throughout the whole course of the experiments. 144 cc. of solution were introduced into the dialysers and 600 cc. of water were placed outside. The exterior water was changed from time to time and the nitrogen content thereof estimated. The period of dialysis into the same exterior liquid (600 cc.) is indicated in the second column.

*Rate of dialysis of nitrogenous substance from three washings
of the same portion of gelatin.*

Time from commencement of experiment Days	Period of dialysis Hours	Extract I mg. N	Extract II mg. N	Extract III mg. N
1	24	29.07	16.88	13.45
2	"	15.01	9.2	10.26
3	"	10.53	5.93	6.21
4	"	9.09	5.64	4.56
7	72	16.5	8.25	9.22
8	24	5.65	3.36	3.06
9	"	4.95	2.83	2.74
10	"	5.54	3.4	—
11	"	4.49	2.94	—
14	72	9.9	6.6	—
15	24	4.04	3.36	—
16	"	4.04	3.25	2.6
17	"	—	3.2	2.59
18	"	4.34	3.28	2.59
24	144	15.5	11.3	8.89
30	"	14.2	11.84	—
36	"	12.28	9.95	7.2
42	"	7.79	7.22	5.88

It will be noticed from the above table that even the third extract of the gelatin, after as long an interval of dialysis as 6 weeks, still contains nitrogenous substances which can pass the dialyser, the amount of nitrogen thus passing amounting to the quite appreciable quantity of nearly 1 mg. per day. About 30 % of the nitrogen of extract I and 18 % of extract II had diffused through the parchment in 14 days.

(b) *Experiment with heated gelatin solution.* 200 g. of gelatin, which had been previously washed till it was nearly ash-free, were dissolved in 4 litres of water, and the solution was heated for 48 hours in an enamelled iron digester with a reflux condenser in a glycerol bath at 110°. A dark-coloured insoluble compound was formed, which was filtered off. The filtrate was concentrated to a thick syrup under reduced pressure at 40°. This syrup was then dissolved in water and 200 cc. of a solution containing 1200 mg. of nitrogen were introduced into a parchment dialyser. The water on the outside of the dialyser was 500 cc. and this was changed daily and the nitrogen estimated therein. The results are given in the accompanying table. The second column of the table gives the amount of nitrogen in each sample of the dialysate, and the third column the total amount which had diffused through after the period of dialysis indicated in the first column. The rate of dialysis is rapid at first, but quickly diminishes, till after about 21 days it becomes constant at 7 mg. a day, which is equivalent to 0.58 % of the total of the nitrogen originally in the dialyser and 1.6 % of that in the dialyser at this period. These experiments were continued for 60 days without any apparent diminution in the daily rate. After 3 months, however, when the exterior fluid was changed only once every 6 days, a small diminution of the rate of dialysis could be detected. The rate of diffusion is indicated on the curve (Fig. 2). This, it will be observed, becomes practically a straight line after 20 days. These experiments indicate quite clearly the intensely slow rate of diffusion of the degradation products of gelatin, and the impracticability of freeing gelatin from them by any process of washing or dialysis.

The results for the rate of diffusion through parchment of the sample of Coignet's Gold Label gelatin (ash-free) which had been heated for 48 hours at 100°.

Time Days	Quantity of nitrogen diffused in mg.	Total amount of nitrogen diffused in mg.	Percentage of nitrogen diffused through parchment
1	116.0	116.0	9.68
2	68.2	184.2	15.4
3	40.4	224.6	18.6
4	35.5	260.1	21.6
5	29.3	289.4	24.0
6	—	—	—
7	43.1	332.5	27.6
8	22.5	355.0	29.6
9	22.0	377.0	31.4
10	20.3	397.3	33.0
11	18.6	415.9	34.6
12	18.9	434.8	36.2
13	—	—	—
14	24.6	459.4	38.2
15	14.4	473.8	39.5
16	15.0	488.8	40.6
17	9.5	498.3	41.5
18	8.6	506.9	42.2
19	8.5	515.4	42.9
20	—	—	—
21	12.6	528.0	43.8
22	7.8	535.8	44.5
23	7.7	543.5	45.2
24	7.8	551.3	45.9
25	7.8	559.1	46.4
26	7.8	566.9	47.2

Fig. 2.

A few experiments were carried out with the object of throwing some light on the nature of the thermal degradation of gelatin. No very definite scission of the peptide linkage could be ascertained by either the formalin titration method, or by the estimation of the nitrogen set free by nitrous acid by the method of van Slyke. (There were certain difficulties in applying this method, which will not be discussed in this place.) It was thought that heat might bring about a simple disaggregation of a large molecule into simpler aggregates of the same type. If this were the case, the part of the degraded gelatin which passes the dialyser should yield the same hydrolysis products as the part that remained within. This, however, was not found to be the case as the following determinations of the Hausmann numbers show:

	Amide N	Humin N	Diamino N	Humin + diamino N
Ash-free Coignet's "gold-label" gelatin	1.1	—	22.3	—
A. The same, of which a solution had been heated for 48 hours at 100°	1.2	2.2	21.5	23.5
Dialysis of A through parchment for 21 days:				
(i) Portion which diffused through	1.25	1.3	26.5	27.8
(ii) Portion which did not diffuse	1.1	2.6	20.5	23.1

The diamino-nitrogen of the diffusible nitrogen was distinctly higher than that of the non-diffusible.

SECTION III. EXPERIMENTS ON THE PURIFICATION OF GELATIN BY ELECTROLYSIS.

Loeb's method of purifying gelatin [1922], based on the consideration of its amphoteric character, consists in treating the material first with a solution of hydrochloric acid with a hydron concentration just greater than that of iso-

electric gelatin and then with water until it is free from acid. It was found, however, that only by very prolonged treatment by this method was it possible to remove the last traces of electrolytes, and it was considered advisable to supplement it by a method of electrolysis. The presence of very small amounts of electrolytes modifies very considerably the properties of gelatin, and an efficient method for obtaining a gelatin which is practically ash-free is, therefore, very essential. It was also necessary to ascertain the efficiency of an electrolytic method for removing the nitrogenous contaminations. The apparatus employed is shown in the accompanying illustration (Fig. 3).

Fig. 3.

One to 1.5 litres of a gelatin solution of 10–20 % were allowed to set in the lower part of a bell-jar of 5 cm. diameter. Both the upper and lower surfaces of the gelatin were in contact with water and a current was passed through the system from a platinum anode to a mercury cathode. The distance from anode to cathode was about 20 cm. The initial voltage employed was usually 100 which was afterwards increased to a maximum of 220. The water was changed once a day at least, and the process was continued until there was no further appearance of alkali at the cathode or of acid at the anode. The gelatin was then removed and cut into slices which were dried in a current of filtered air at ordinary room temperature. The properties of the gelatin thus prepared are referred to in greater detail in the next section.

The gelatin used as the starting material in these experiments was Coignet's "gold-label." It had an ash content of about 1.5 %. The initial washings with dilute acid and water brought this down to 0.4–0.1 %, which was further reduced by the electrolysis to 0.02 % or less. It will be noticed that no membranes were employed for the process. This was found to be possible when the apparatus described above was employed, if the greater part of

the ash content had been first removed. If a sample containing the original amount of ash (1.5 %) was submitted to electrolysis by this method without any previous treatment, it was found that acid gelatin formed towards the anode and alkali gelatin towards the cathode. These underwent swelling, and particles of the jelly escaped into the water, and often the whole jelly broke away from the bell-jar. The method was quite satisfactory, however, for gelatin which had been submitted to a preliminary purification by acid.

Efficiency of the process of electrolysis. A number of experiments were carried out with a view to determine the efficiency of the electrolytic process in removing contaminations other than the inorganic.

The first experiments were made with aniline dyes, and these were carried out in a smaller apparatus than the one described above. This consisted of a simple glass tube of about 5 cm. diameter, which was used instead of a bell-jar; the remainder of the apparatus was practically the same. Into the tube were introduced 50 cc. of a solution of electrolyte-free gelatin (made up from 20 g. gelatin to 100 cc. water), to which the dye was added. The solution was then allowed to set. Methylene blue and methyl red were readily removed from the gelatin by electrolysis, the former to the cathode, the latter to the anode. The colloidal dyes, Congo-red and night-blue were, however, not removed, under the highest potential gradient available (about 25 volts/cm.). Sugars were not removed at all, whilst glycine, an ampholyte with both acid and basic dissociation constants very weak, was only removed very slowly.

Experiments were also carried out to determine the rate of removal of the ordinary nitrogenous contaminants of gelatin. Two 50 cc. portions of an electrolyte-free gelatin (20 g. gelatin + 100 cc. water) were introduced into tubes 5 cm. diameter. One was submitted to electrolysis under a P.D. 60 volts with a distance of 8 cm. between the electrodes. The other tube, used as a control, was not submitted to electrolysis. From time to time the water round the electrodes was removed, and the nitrogen therein estimated. From the results, which are given in the following table, it will be seen that more nitrogenous matter passes out from the electrolysed than from the non-electrolysed gelatin. The experiment indicates the difficulty of complete removal of the nitrogenous contaminants by electrolysis.

		Electrolysed gelatin		Control	
		+	-	+	-
First	4 days	28.3	34.0	4.8	4.6 mg. N
Next	4 "	9.3	11.3	3.0	2.8 "
"	5 "	6.1	6.5	2.4	2.6 "
"	5 "	5.1	5.8	2.0	2.3 "
"	10 "	6.3	6.5	3.5	4.0 "

The electrolyte-free gelatin was found to be extremely resistant to putrefaction.

SECTION IV. THE "RECRYSTALLISATION" OF GELATIN.

A gelatin, which had been purified by the washing and electrolytic processes described above, was found to have properties markedly different from the unpurified. A solution of 2 % or less of the air-dried purified material (which corresponds to about 1.6 % when calculated on the true dry weight) is clear at 40°, but on cooling becomes white and opaque. The gel or solution formed is unstable and on standing for 24–48 hours, the hydrated gelatin separates out in the form of a bulky precipitate, which can be separated from the clear supernatant fluid by centrifuging in the case of the more concentrated solutions or by simple filtration in that of the more dilute solutions. The "recrystallisation" of gelatin, if so it may be termed, can be repeated an indefinite number of times, and results in a further purification of the gelatin.

Solutions of 3 % and higher concentrations set to stable gels on cooling. The 3 % gel is white and opaque, but with increasing concentrations the gels become more and more transparent. The gels of 10 % strength and higher, are, as regards transparency, indistinguishable from those made from ordinary commercial gelatin.

To investigate the recrystallisation process as a means of purification, the nitrogen content of the supernatant fluid obtained by centrifuging and filtering was investigated. The experiments were usually carried out with a litre of solution. After cooling this was generally allowed to stand at room temperature (14–16°) for two or three days, before centrifuging. From a litre of 2 % (air-dried) gelatin, 300 cc. of a clear fluid were usually obtained. After the first "recrystallisation" this contained nitrogen varying from 10–25 mg. per 100 cc. If the separated gelatin is redissolved at 40° and the bulk of the solution is made up to 1 litre, and allowed to cool, the separated clear solution has a nitrogen content of 9–11 mg. per 100 cc. Further "recrystallisations" fail to reduce this amount. Moreover, a solution which had been allowed to stand at room temperature for 6 weeks gave practically the same value for the nitrogen in the supernatant fluid as one which had been allowed to stand for only 48 hours (9.8 and 9.2 mg./100 cc. respectively). This result is in marked contrast with those found for unpurified gelatins.

Another quantity of the "recrystallised" gelatin was washed by decantation with large quantities of water, then separated by centrifuging and again made up to the original volume at 46°. After cooling, the nitrogen content of the supernatant fluid was found to be 10.4 mg./100 cc.

There appears, therefore, to be an equilibrium value of about 10 mg./100 cc. for the nitrogen content of the clear liquid which separates after melting and cooling. This value is not attained unless the solution is heated. When some of the precipitated gelatin was made up to the original volume with cold water without remelting and allowed to stand for 3 days with occasional shaking the supernatant fluid contained only 5.0 mg./100 cc.

When the gelatin is not melted the equilibrium value is only attained

slowly. The following experiments were carried out with a gelatin which had been purified by electrolysis, but not by recrystallisation. 40 g. of gelatin in sheets were allowed to soak in 1 litre of water. After $2\frac{1}{2}$ months, the aqueous phase contained 8.3 mg./100 cc. and after $4\frac{1}{2}$ months 16.2 mg./100 cc. In another case 20 g. gelatin as a 10 % gel was broken up finely and immersed in 800 cc. water. After 2 months the water contained 11.9 mg./100 cc. Experiments on similar lines are being carried out with "recrystallised" gelatin but are not yet completed.

In all the experiments mentioned above, dealing with the supernatant liquid from the "crystallised" gelatin, 2 % solutions of the air-dried material were used. In the following experiments gelatin was allowed to crystallise from solutions of varying strengths. From even the smallest concentrations used the gelatin separates as a hydrate. The amounts of nitrogen in the supernatant liquid are given in the following table.

Initial concentration	N content of clear fluid mg. N per 100 cc.
1.39	11.1
0.697	9.57
0.348	9.38
0.139	11.7

The initial concentrations are here based on the nitrogen determinations in the solutions, it being assumed that pure dry gelatin contains 18 % N. These experiments were not carried out in a thermostat, and the materials used in the determination of the nitrogen contained minute quantities of nitrogenous impurities for which allowance has not been made.

It seems, therefore, that purified gelatin has a real but small solubility in water at the temperatures 14–16° corresponding to about 10 mg./100 cc. N or 0.056 % gelatin.

Unfortunately, further experiment throws some doubt on this explanation of the nature of the apparent equilibrium observed. The filtrate from a solution of the "recrystallised" gelatin was compared with a solution of the gelatin made up directly to the same nitrogen content. On the assumption that we are dealing with a true solubility phenomenon these solutions should be identical. Their viscosity, surface tension and diffusibility through parchment were determined and, within the limits of experimental error, were found to be the same for both solutions.

A litre of each solution was then concentrated under reduced pressure at 40° until the volume was brought to 200 cc. These solutions, which should now correspond to a 0.28 % solution of gelatin, were cooled. *No separation of solid gelatin* occurred from the concentrated filtrate, which was only slightly turbid, whereas from the solution made up directly gelatin separated as from an ordinary 0.28 % solution. This failure to reverse the process and obtain the solid hydrated gelatin from the filtrate on concentration throws doubt not only on the hypothesis of a simple solubility effect but also on the assumption that the results recorded in Section IV above represent an equilibrium. It is

difficult to conceive of any true equilibrium, whether based on solubility, adsorption or disaggregation effects, which would not be reversed by the procedure described. The non-reversibility is not due to non-appearance of the solid phase, since addition of small amounts of the solid does not affect it. In spite then of the agreement of the results of Section IV the existence of a definite equilibrium cannot yet be taken as proved. It is conceivable that gelatin is not a simple molecule but an aggregate. Its molecular weight as determined by Biltz was found to be about 17,000. Repetition of the determination by other methods in this laboratory gave numbers varying between 17,000 and 19,000. It is difficult to conceive that these numbers represent the weight of a simple molecule. The behaviour of such aggregates when dispersed in water cannot yet be predicted.

CONCLUSIONS.

(1) In ordinary high-grade commercial gelatin, the nitrogenous contaminations are not removed even by washing for several months.

(2) The nitrogenous contaminations, which are derived mostly from the thermal decomposition of gelatin during the course of its preparation, contain products which diffuse only very slowly. After several weeks of dialysis of the products obtained from gelatin by diffusion, or of the products obtained by heating purified gelatin in water, in a parchment membrane, diffusible matter is still found to be passing the parchment. The conclusion is drawn that it is practically impossible to free gelatin from its nitrogenous contaminants by washing or dialysis.

(3) It is difficult to free gelatin entirely from electrolytes by washing with acid and water by the method of Loeb. Gelatin purified in this manner, can, however, be readily freed from almost the last traces of stronger electrolytes by submission to electrolysis without the use of membranes. Electrolysis is not, however, efficient in removing substances of colloidal character and non-electrolytes or even weak electrolytes.

(4) Gelatin when purified by washing and electrolysis separates out from solutions of less than 2 % as an insoluble hydrate. When this process of "recrystallisation" is repeated, a gelatin is produced which separates from a supernatant fluid which always contains about 10 mg. per 100 cc. This quantity remains constant, from whatever strength of solution the gelatin separates. When a gelatin with this property is obtained, it indicates that it is free from the soluble nitrogenous contaminants. The gelatin which separates behaves in many respects like a pure substance in equilibrium with a saturated solution, which solution contains 0.056 % gelatin.

REFERENCES.

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