FRACTIONATION OF GELATIN.

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It has been shown by the writers that such phenomena as the swelling of electrolyte-free gelatin blocks, the high viscosity of gelatin solutions, the decrease of the pH effect on viscosity with increase in the concentration of gelatin solutions, and, finally, the peculiar phenomenon of shrinking of dilute gels when placed in cold water, are explainable on the assumption that gelatin consists of a mixture of at least two substances, one of which is soluble in cold water, while the other is insoluble even in warm water. Actually, it has been found possible to isolate from gelatin by means of alcohol precipitation two fractions which differ enormously in their physical properties. One of the fractions has properties similar to those of albumin; it is soluble even in ice water, does not set to a gel, has a low viscosity, and does not give rise to the pH-viscosity curve characteristic for gelatin. Besides, it has an osmotic pressure at its isoelectric point (pH 4.7) considerably higher than that of isoelectric gelatin, from which it has been isolated. The second fraction has properties opposite to those of the first one; it is insoluble in cold water, sets to a gel at a low concentration, swells much less, and has a higher viscosity and lower osmotic pressure than ordinary gelatin. But this fraction is still “soluble” in warm water.

It had been previously found by Schryver and his associates that a soluble material could be obtained from gelatin by allowing isoelectric salt-free gelatin to stand at 14–16°C. Under these conditions the

gelatin separates out as a clot leaving some soluble protein material in
the supernatant fluid. If the precipitate is then suspended in water a
lower concentration of the soluble material is found in the supernatant
solution. This solution moreover did not have the properties of ordi-
nary gelatin, so that, as these workers point out, it can not be consid-
ered as a saturated solution of gelatin. These experiments were re-
peated and confirmed by the present writers, but it was found that if the
gelatin were allowed to stand at 23° instead of 15° a very much larger
amount of the soluble material was obtained and this fact was used as
the basis of the method of separation used in the present paper.

Experimental Procedure.

A stock of isoelectric gelatin is prepared from Cooper's non-bleached gelatin
by the modified Loeb's method as described elsewhere. A solution of 60 gm.
air-dried isoelectric gelatin (85 per cent dry weight) is made in 3000 cc. of distilled
water, which has been adjusted to pH 4.7 by means of acetic acid, filtered through
cotton wool into Pyrex flasks, and put into a water bath kept at a constant tem-
perature of 23°C. A layer of toluene is added to the gelatin to prevent mould
and bacterial growth. The gelatin solution gradually turns milky white, thickens,
and after several days the thick white gel begins to break up into a gelatinous
precipitate which settles slowly. The precipitation may be accelerated by stirring
up the gel occasionally. After 5 or 6 days the flasks are removed from the bath,
the supernatant solution is decanted as much as possible, and the rest is cen-
trifuged, care being taken not to heat the gelatin in the centrifuge. The centrifuged
precipitate is stirred up once or twice in the centrifuge bottles with cold water of
pH 4.7 and recentrifuged. It is then melted and made up to about 80 per cent
of the original volume of the 2 per cent solution with water of pH 4.7, and allowed
to set at 23°C. for the second precipitation. The process is repeated 12 to 15
times until a precipitate is left which does not dissolve even at 50°C. Care has
to be taken during the whole process that the pH should remain at 4.7. The
final precipitate is washed several times in the centrifuge bottles with hot water,
and transferred into 95 per cent alcohol, where it is allowed to remain overnight.
The alcohol is then decanted off and the gelatin is ground up in a mortar with
ether, which is allowed finally to volatilize.

4 Schryver and Thimann were also able to obtain a soluble and insoluble frac-
tion by means of electrolysis of gelatin solutions.
prepared in this way is practically free from diffusible nitrogenous material,
cf. Knaggs, Manning, and Schryver.
The air-dried precipitate is a fine powder of a light chocolate appearance. The yield is about 1 per cent of the original amount of gelatin.

**TABLE I.**

*Precipitation of Isoelectric Gelatin at 23°C.*

<table>
<thead>
<tr>
<th></th>
<th>Volume (cc.)</th>
<th>Dry gelatin (gm.)</th>
<th>Strength of solution (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>3000</td>
<td>51</td>
<td>1.66</td>
</tr>
<tr>
<td>1st precipitate</td>
<td>2100</td>
<td>43.5</td>
<td>2.07</td>
</tr>
<tr>
<td>1st supernatant solution</td>
<td>900</td>
<td>6.9</td>
<td>0.77</td>
</tr>
</tbody>
</table>

**TABLE II.**

*Influence of Initial Concentration of Gelatin Solution on Concentration of Gelatin in the Supernatant Liquid after 1st Precipitation at 23°C.*

<table>
<thead>
<tr>
<th>Initial concentration in gm. dry gelatin per 100 gm. solution</th>
<th>Gm. dry gelatin per 100 gm. supernatant liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>0.19</td>
</tr>
<tr>
<td>0.19</td>
<td>0.19</td>
</tr>
</tbody>
</table>

**TABLE III.**

*Gelatin Found in Supernatant Liquid on Repeated Precipitation of a Gelatin Solution at 23°C. Original Amount of Gelatin 51 Gm. by Dry Weight.*

<table>
<thead>
<tr>
<th>Precipitation</th>
<th>Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution</th>
<th>Gm. gelatin in the supernatant liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution</td>
<td>0.77</td>
<td>0.55</td>
</tr>
<tr>
<td>Gm. gelatin in the supernatant liquid</td>
<td>6.9</td>
<td>9.5</td>
</tr>
</tbody>
</table>

**TABLE IV.**

*Influence of Initial Concentration of Gelatin Solution on Concentration of Gelatin in the Supernatant Liquid after 1st Precipitation at 23°C.*

<table>
<thead>
<tr>
<th>Precipitation</th>
<th>Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution</th>
<th>Gm. gelatin in the supernatant liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Gm. gelatin in the supernatant liquid</td>
<td>1.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The supernatant solutions may be used for preparation of the soluble fraction by means of alcohol, as described elsewhere. If the precipitation is carried out at a lower temperature than 20°C, practically
all of the gelatin comes out as a precipitate with only a trace remaining in the supernatant solution. On the other hand, when the precipitation is done at 23°C, 14 to 18 per cent of the original gelatin remains in the supernatant solution. The distribution of gelatin between the precipitate and the supernatant solution after the first precipitation at 23°C. is shown in Table I.

The concentration of soluble material increases with the total concentration of gelatin, as shown in Table II.

On further precipitation the amount of gelatin in the supernatant solution gradually decreases, as shown in Table III.

The Physical Properties of the Separated Fractions of Gelatin.

Solubility.—The difference in the solubility of the various fractions of gelatin appears even after the first precipitation. Thus, in one of the experiments a solution of the first precipitate and of the first supernatant solution was completely precipitated by means of alcohol, washed with ether, and dried in the air. Suspensions were then made of 0.1 gm. dry powder of the two fractions in 10 cc. of water each, at 25°C. The fraction from the supernatant solution dissolved immediately in the water producing a clear solution, while the fraction from the first precipitate swelled but did not dissolve unless it was heated. After repeated precipitation the solubility of the precipitate becomes less until finally it does not dissolve even in hot water. The insoluble fraction appears only on repeated fractionation. Simple reheating of gelatin without separation of the precipitate does not produce any visible effect on the gelatin. A 2 per cent solution remained unchanged after heating to 50° and cooling to 23° more than 20 times during a period of 2 months.

Swelling of Powdered Gelatin.

The final precipitate does not swell when suspended in cold water. Its bulk is the same whether it is suspended in water, alcohol, or toluene. This is shown in Table IV. The bulk was determined by centrifuging suspensions of 0.1 gm. of the dry powder in 10 cc. of various liquids to constant volume of sediment. As a comparison suspensions were also made of ordinary isoelectric gelatin which had been precipi-
tated and dried by means of alcohol and ether in the same way as the fractionated gelatin, and also of the precipitate from the first precipitation.

Thus it is seen that when 0.1 gm. of ordinary gelatin is suspended in water it has a bulk of 0.9 cc. when centrifuged as compared with the bulk of 0.25-0.30 cc. when it is suspended in absolute alcohol or toluene; on the other hand, the insoluble fraction of gelatin from the final precipitation has practically the same bulk, 0.35-0.40 cc., no matter whether water, alcohol, or toluene is used. It is to be noted that the insoluble gelatin which had been precipitated only once gives higher swelling in water than ordinary gelatin, which is partly soluble at 25°C.

<table>
<thead>
<tr>
<th>TABLE IV.</th>
<th>Comparison of Swelling of Various Types of Gelatin at 25°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>Insoluble gelatin from final precipitation</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.40</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>0.35</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Effect of Acid or Alkali on the Swelling of the Insoluble Fraction of Gelatin.

Addition of dilute HCl or NaOH to a suspension of the insoluble fraction of gelatin in water brings about a considerable swelling of the particles of gelatin, owing to the unequal distribution of the diffusible ions between the particles and the outside solution in accordance with the Donnan equilibrium. The swelling increases with increase in the amount of acid or alkali until a pH about 3.0 on the acid side or 9.0 on the alkaline is reached when the material begins to dissolve on heating, forming a viscous solution. On bringing the solution back to pH 4.7 the material rapidly precipitates out in a flocculent state.

Swelling of Gels Made of Mixtures of the Soluble and the Insoluble Fractions of Gelatin.

The mechanism of swelling of gels of isoelectric gelatin has been studied extensively by the writers. The final conclusions reached may
be summarized as follows. There are three forces which affect the swelling of isoelectric gels:

1. The osmotic force due to the presence of a soluble ingredient of gelatin even at temperatures as low as 5°C. This force causes the block of gel to take up water, and is the significant factor in gels of high gelatin content.

2. The residual elastic force in the micelle, which originates while the gelatin is still in the sol state. This force causes the block of gel to lose water and shrink. It is the significant factor in dilute gels.

3. Finally the elastic force of the block of gel as a whole which resists both the swelling and the shrinking of gels.

According to this viewpoint a concentrated gel made up entirely of the insoluble ingredient should not swell at all in the absence of acid,
alkali, or salt, since there are no soluble molecules to produce an osmotic force. Addition of increasing amounts of the soluble fraction to a gel of the insoluble fraction of gelatin gives rise to osmotic pressures, and increased swelling should be expected. Fig. 1 shows what actually takes place when gels containing various mixtures of the insoluble and soluble ingredients were placed in a m/30 acetate buffer pH 4.7. The experiment was done as follows:

A series of solutions containing 30 gm./100 gm. of solvent was made up of various proportions of soluble and insoluble fractions in warm 0.075 N NaOH. 2 cc. of each solution was poured into a mould of glass tubing, mounted on a paraffin block, and allowed to set for 24 hrs. in the refrigerator at 5°C. The blocks of gel were then removed from the glass tubing, weighed, and put into 200 cc. of m/30 acetate buffer pH 4.7 which had been previously cooled to 5°C. The blocks were weighed at various intervals of time in a refrigerating room which was kept at constant temperature of 5°C. A block of 30 per cent ordinary isoelectric gelatin dissolved in 0.075 N NaOH was prepared and put into 200 cc. of m/30 acetate buffer pH 4.7 as a control. The blocks remained at 5°C. for 8 days, and then were transferred with the outside solutions to a constant temperature water bath of 20°C.

The plotted curves for the rate of swelling of the gels show that at 5°C. the gel consisting of 100 per cent insoluble fraction of gelatin swells slightly if at all, while the addition of the soluble ingredient brings about swelling. The swelling increases rapidly as the proportion of the soluble material increases. Gels consisting of more than equal parts of soluble and insoluble fractions swell more than the normal gelatin control. When transferred to 20°C. all gels begin to swell faster, and those that have been swelling more than the control at 5°C. swell at 20°C. so rapidly that in a day or so they dissolve completely leaving a residue of insoluble gelatin. The gel consisting entirely of the soluble fraction melts immediately when the temperature is raised to 20°C. That the fractionation is not complete yet is shown by the fact that even the 100 per cent insoluble gel swells somewhat at 20°C. which indicates that there is still left in it some of the fractions which are soluble at 20°C. It is also possible that the presence of the electrolytes of the buffer affect the solubility of the gelatin as was found before in the case of the swelling of ordinary gelatin. The fractionation of the soluble fraction used in this experiment was purposely stopped at this
point so that a 30 per cent gel at 5°C. would be obtained. By means of further fractionation of the soluble ingredient with alcohol it is very easy to obtain a gelatin fraction which does not set even at 5°C. no matter how concentrated the gel is.

**Negative Swelling of Dilute Gels of Insoluble Gelatin.**

When gels containing less than 10 per cent gelatin are placed in cold water or dilute buffer pH 4.7 the gels not only do not swell but actually shrink and lose water. It has been shown by one of the writers** that the force causing the dilute gels to lose water is the elasticity in the micelle of gelatin. This elastic force, which is brought about by the hydration of the micelle in the gelatin solution, is due, first, to the presence of soluble gelatin inside of the micelle and, second, to an excess of diffusible ions inside of the micelle over the outside solution. The osmotic pressure in the micelle is therefore higher than in the surrounding solution and water enters until the elastic force is equal to the osmotic pressure. When the gelatin solution sets to a gel the soluble material precipitates and the ions diffuse out when the gel is placed in water. The osmotic pressure is thereby destroyed and the water is forced out by the elasticity of the micelle, thus bringing about a gradual shrinking and loss of water by the block of gel as a whole. This contraction is greater the greater the amount of water originally held by the micelle. The amount of water held in them is large in gelatin sols made up in acid or alkali. In ordinary gelatin the micelle form only a part of the total gelatin, the rest of which is found in solution outside of the micelle. A solution of the insoluble fraction of gelatin in dilute NaOH is not a perfect solution but rather a colloidal suspension of swollen micelle, as shown by the high viscosity of the mixture. The number of micelle per gm. of substance then is greater in the case of the insoluble fraction of gelatin than in the case of ordinary gelatin. Hence it is to be expected that when dilute gels are made up of both materials the one consisting of the insoluble component of gelatin should lose more water when put in dilute buffer pH 4.7 than the gel which was made up from ordinary gelatin. That this is actually what happens is shown by the following experiment.

Two solutions were made up each containing 0.1 gm. gelatin + 2 cc.
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\( \frac{m}{10} \) NaOH. One was made up of ordinary isoelectric gelatin, while in the other the insoluble fraction was used. Both solutions were allowed to set for 24 hours at 5°C. and then the gels were weighed and each put into 250 cc. \( \frac{m}{30} \) acetate buffer pH 4.7 at 5°C. where they remained for about a month. The weight of the gels was determined at various intervals of time. Fig. 2 shows the results. It is seen that while the block of gel which was made up of ordinary gelatin has lost finally about 22 per cent of its original weight, the one that was made up of the insoluble fraction has lost as much as 68 per cent under the same conditions. The dry weight of the gel was unchanged so that the result is not simply due to solution.

![Graph showing negative swelling of 5 per cent gels in \( \frac{m}{30} \) acetate buffer pH 4.7 at 5°C.](image)

**Fig. 2.** Negative swelling of 5 per cent gels in \( \frac{m}{30} \) acetate buffer pH 4.7 at 5°C.

**The Irreversibility of Gelatin Fractionation.**

According to the mechanism of swelling outlined above the soluble ingredient of ordinary gelatin is present both inside and outside of the micelles. Each micelle may be compared to a cell, filled with soluble gelatin; the cell wall, which is impermeable to the soluble gelatin, consists of the insoluble fraction of gelatin. The number of micelles in a gelatin solution increases with the decrease in temperature until the setting point is reached. When a dilute solution of salt-free isoelectric gelatin is left at a temperature of about 23°C. the micelles together with the contained soluble fraction of gelatin in them gradually settle down as a gelatinous precipitate, while the supernatant liquid consists of a
solution of practically pure soluble material. The process of removal of the soluble fraction of gelatin from inside of the micelles is a difficult one owing to the impermeability of the micella wall. Only through a long series of reprecipitations accompanied by repeated heating of the solution is it possible to obtain an insoluble fraction which is apparently free from the soluble component. The liberation of the soluble material is probably due to a rupture of the micelle by osmotic pressure. In a gelatin solution some soluble material exists both inside and outside of the micelle. When the gelatin is allowed to set in dilute solution some of the soluble material escapes and is removed. On remelting the gelatin the osmotic pressure difference between the inside and outside of the micelle is greater than before and they therefore swell more. That this actually happens is shown by the fact already mentioned that a partially purified fraction has a higher viscosity than the original gelatin. As the purification proceeds the swelling increases until the micelle rupture and liberate the soluble material. The ruptured micelle thus give rise to the insoluble fraction of gelatin. This fraction is not only insoluble in hot or cold water in the absence of acid or alkali, but it will not mix even with the soluble fraction or with ordinary gelatin to form a homogeneous solution unless it is first dissolved in acid or alkali; on bringing the solution back to pH 4.7, however, the insoluble fraction precipitates out again. In order to resynthesize gelatin from its final fractions it is not enough to mix the fractions in the right proportions but it is necessary to reintroduce the molecules of the soluble material into the insoluble particles; or, in other words, to fill again the micelle cells with a substance for which the cell wall is impermeable. This may be partially accomplished in the case of a solid block since the soluble material is held in the network of micelles forming the block but it has not been possible to introduce the soluble material into the individual micelles. It is probable that in the manufacture of gelatin the soluble material is actually formed inside the micelles.

It could be assumed that the soluble fraction was formed from the gelatin by an irreversible chemical reaction and hence mixing the fractions would not give the original gelatin. This assumption appears improbable since, as was shown, repeated heating and cooling does not result in the separation of the two fractions. It has also been found by
Alsberg and Griffing\textsuperscript{7} that gelatin becomes partly soluble in cold water after prolonged grinding. If the grinding were sufficient to rupture the micelle, the soluble material would be liberated.

**Isolation of an Insoluble Substance from Gelatin by Partial Acid Hydrolysis.**

A substance similar in many respects to the insoluble ingredient of gelatin described above is obtained when a 5 per cent solution of gelatin is partially hydrolyzed in \(\text{m}/10\) HCl at 90\(^\circ\)C. for about 2 hours. At this stage the gelatin no longer sets when cooled. On neutralization of the solution a flocculent precipitate appears, which can be centrifuged off and washed with water. On drying with alcohol and ether it gives a light brown powder similar to the one obtained by fractional precipitation. The yield is about 0.2 per cent. The powder is insoluble in hot or cold water, and swells considerably on addition of acid or alkali to its suspension in water until it is finally dissolved, as is the case with the insoluble component of gelatin from fractional precipitation. The only difference between the two substances is their behavior with regard to setting. It has been mentioned before that when an alkaline solution containing 5 per cent or more of the insoluble fraction of gelatin is left to cool to 5\(^\circ\)C. it gradually sets to a gel. This does not happen with the insoluble substance obtained by acid hydrolysis of gelatin. A solution of it in dilute NaOH is very viscous, but it does not set even at a concentration of 10 gm. per 100 cc. solution. It is possible that the setting of gelatin depends on the presence of an ingredient which still exists in the material obtained by fractional precipitation but which is destroyed on hydrolysis with acid.

**Summary.**

1. It is possible to fractionate gelatin by means of reprecipitation at 23\(^\circ\)C. of a salt-free solution of pH 4.7 into two fractions, one of which is soluble in water at any temperature, and a second one which does not dissolve in water even when heated to 80\(^\circ\)C.
2. The proportion of the soluble fraction in gelatin is much greater than of the insoluble one.
3. The insoluble fraction of gelatin does not swell when mixed with water, but it does swell in the presence of acid and alkali which finally dissolve it.
4. Blocks of concentrated gel made by dissolving various mixtures of

the soluble and insoluble fractions of gelatin in dilute NaOH swell differently when placed in large volumes of dilute buffer solution pH 4.7 at 5°C. The gel consisting of the insoluble material shows only a trace of swelling, while those containing a mixture of soluble and insoluble swell considerably. The swelling increases rapidly as the proportion of the soluble fraction increases.

5. A 5 per cent gel made up by dissolving the insoluble fraction of gelatin in dilute NaOH loses about 70 per cent of its weight when placed in dilute buffer pH 4.7 at 5°C. A similar gel made up of ordinary gelatin loses only about 20 per cent of its weight under the same conditions.

6. It was not found possible to resynthesize isoelectric gelatin from its components.

7. An insoluble substance similar in many respects to the one obtained by reprecipitation of gelatin is produced on partial hydrolysis of gelatin in dilute hydrochloric acid at 90°C.