An important criterion used for the characterization of proteins is the amount of certain neutral salts that is required for the precipitation of proteins from their saline or aqueous solution. The amount of salt, such as ammonium sulfate, that is required to precipitate any given globulin is largely characteristic for the individual globulin. The precipitation limits of different globulins studied in this laboratory cover a wide range. Some of the α-globulins, such as arachin and those of the lima, navy, and mung beans, begin to precipitate from their solutions at concentrations of ammonium sulfate as low as 0.15 of saturation with this salt. Other globulins are known which require for their precipitation as much as 0.7 to 0.8 of saturation. Up to the present time there was not known a lower concentration limit for precipitating proteins with ammonium sulfate than that required for the globulins. Since there is a relationship between protein solubility in aqueous media and the quantity of ammonium sulfate needed for complete precipitation, it seemed probable that glutelins, which are less soluble proteins as a group than globulins and require the use of alkalies to bring them into solution, would need less ammonium sulfate for their precipitation, if they are at all precipitable, than the relatively low limit given above for globulins.

In a recent paper (1) on the isoelectric points of proteins it was pointed out that protein precipitation by ammonium sulfate is a

* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists held in Cleveland, December 28 to 30, 1925.
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Salting out effect, and that the precipitation is not brought about by a change of pH. Therefore, it was believed that the alkalinity of the glutelin solution would not necessarily interfere with our project. The correctness of this view was supported by the fact that a globulin could be precipitated from either a 0.2 per cent sodium hydroxide solution or a 10 per cent sodium chloride solution by addition of the same amount of ammonium sulfate. These considerations led us to study the possibility of separating glutelins from alkaline solution by precipitation with ammonium sulfate.

It was found that most of the wheat glutelin contained in a 0.2 per cent sodium hydroxide extract of the residue remaining after removal of gliadin from wheat gluten, could be precipitated from the alkaline extract by addition of small amounts of ammonium sulfate, corresponding to 0.018 to 0.02 of saturation. Results of work still in progress have shown that glutelins of other cereals, namely those of rice, corn, and oats, can also be similarly precipitated by correspondingly small quantities of ammonium sulfate. It appears, therefore, that we have here a method applicable not only for characterizing glutelins as a class, but also for a more satisfactory separation and preparation of a class of proteins concerning which but relatively little is known. Glutenin, the glutelin of wheat, is the only representative of this class of proteins that has been extensively studied, and only two others, the glutelins of rice and maize, can be considered as at all well defined. Glutelins from other sources have been reported but sufficient evidence has not been obtained to justify considering them as even approximately pure preparations.

Glutelins are generally prepared from seed residues that have been exhaustively extracted with neutral salt solutions and alcohol for removing albumins, globulins, and prolamins. Contact with the solvents during extraction frequently has a denaturing effect upon these proteins, rendering them insoluble in neutral solvents. When alkalies are used to extract glutelin from such residues, the denatured proteins will also go into solution. The glutelin preparation obtained by neutralizing the alkaline extract with acid will consequently consist of a mixture of proteins and not of glutelin alone. Such preparations may also contain, as Osborne has pointed out, small quantities of proteins which escaped extraction by neutral solvents, either on account of having
been contained in unruptured cells which were afterward disintegrated by the alkaline solution, or because they were retained in the seed residue in combination with substances such as nucleic acid or tannin, which rendered them insoluble in neutral solvents.

Inasmuch as the glutelins which we have studied thus far have been found to be precipitable from alkaline solutions by quantities of ammonium sulfate that come within rather narrow limits, and are far too small to precipitate globulins under similar conditions, this method makes possible the preparation of glutelins free from significant amounts of these other proteins.

The idea that wheat glutenin as generally prepared is not an individual protein, but consists of more than one glutelin has been previously advanced by several investigators, this view being based chiefly on the physical behavior of glutenin.

Fleurent (2) in 1896 published a method for the quantitative determination of prolamins and glutelins in different cereals. This method consisted of extracting these proteins with a solution of potassium hydroxide in alcohol (0.3 gm. of potassium hydroxide per 100 cc. of 70 per cent alcohol). In the case of wheat, he extracted the gluten, prepared by washing out the starch from the flour with water, with the alcoholic potassium hydroxide solution. By saturating the alkaline extract with carbon dioxide, a precipitate was obtained which he designated as glutenin, a name originally given to wheat glutelin by Osborne. When the filtrate was acidified with sulfuric acid a second precipitate was obtained which he named conglutin. Fleurent states that the yield obtained by the second precipitation does not represent more than 2 to 8 per cent of the gluten and therefore plays only a secondary part in the physical character of gluten. Because no analytical data are given for these glutelin preparations, comparison of them with our preparations cannot be made. Blish and Sandstedt (3) published a quantitative method for the determination of glutenin using the principle introduced by Fleurent of extracting with an alcoholic solution of alkali. They, however, found methyl alcohol preferable to ethyl alcohol, and used hydrochloric acid instead of sulfuric acid for neutralizing the alkali. This procedure would give them both the glutenin and conglutin of Fleurent.

Halton (4) claimed that by fractional precipitation he obtained two glutenins differing in optical rotation. Blish (5) applying
Halton's method to purified glutenin preparations, obtained from flours of different sources, could not confirm the latter's results. In no instance was more than one fraction obtained. Blish offers as an explanation for Halton's results that a partial racemization of the glutenin had occurred before fractionation, which might have produced fractions of the same protein having different isoelectric points. That this was possible was actually shown experimentally.

Woodman (6) determined the racemization curves of glutenins prepared from strong and weak flours. Since the curves differed he concluded that these glutenin preparations were not identical. On the other hand, several investigators (7–11) found little or no difference in the chemical composition of glutenin preparations obtained from different varieties of wheat.

By fractional precipitation with ammonium sulfate, we have obtained from a 0.2 per cent sodium hydroxide extract of the residue remaining after exhaustively extracting wheat gluten with alcohol to remove gliadin, two fractions, the properties and composition of which indicate that they are two different glutelins. The first fraction, which we have designated α-glutelin, separated as a flocculent precipitate when the alkaline extract was made 0.018 to 0.02 saturated with ammonium sulfate. More ammonium sulfate added to the filtrate from this precipitate caused no further precipitation until a quantity had been added sufficient to make the solution 0.16 to 0.18 saturated. This fraction is referred to as the β-glutelin. The α-glutelin was obtained in the greater quantity, the yield being very nearly 7 times that of the β-glutelin obtained.

That we are dealing here with two different glutelins is shown not only by the sharp line of demarcation in their precipitation limits with ammonium sulfate, but also in their nitrogen content and the distribution of nitrogen as determined by the Van Slyke method (Table III). The most striking differences found between these two glutelins are shown in their content of amide nitrogen, arginine, and lysine.

In view of the fact that the yield of the β-glutelin was relatively small it might be urged that this fraction represented the small amount of globulin that is usually present in wheat flour. That this is not the case is indicated by the fact that a third fraction
separated when ammonium sulfate was added to the filtrate from the \( \beta \)-glutelin to 0.3 of saturation. The amount of this fraction corresponded roughly to that of the globulin usually found in flour, and it was precipitated at a concentration of ammonium sulfate well within the limits at which globulins are frequently precipitated. Furthermore, when the gluten had been exhaustively extracted with sodium chloride solution previous to the extraction with alcohol and alkali, thereby removing any globulin that may have been present, no trace of a precipitate was obtained by addition of ammonium sulfate to the filtrate from the \( \beta \)-glutelin. The fact that the \( \beta \)-glutelin contained only 16.1 per cent of nitrogen while the globulin isolated by Osborne and Voorhees (12) contained 18.39 per cent, furthermore renders it improbable that our second fraction consisted of a globulin.

Again, the isoelectric point of the \( \beta \)-glutelin was found to be pH 6.45, identical with that found for the \( \alpha \)-glutelin. In the light of the results we obtained when working on the isoelectric points of proteins (I), an isoelectric point of pH 6.5 is much too high for a globulin. It was shown that the isoelectric points of the proteins of any one group fall within rather narrow limits, and that the more soluble proteins, as the albumins, have the lower isoelectric points, while less soluble proteins precipitate with ammonium sulfate at a lower concentration and have higher isoelectric points. The isoelectric points of albumins were found to range in general from pH 4 to pH 5; those of globulins from pH 5 to 5.5, and those of prolamins from pH 6 to 6.5.

The amount of ammonium sulfate required to precipitate the glutelins was found to vary to a certain extent both with the concentration of the alkali solution used and with the concentration of the protein in the extract (Table I). The higher the concentration of alkali, the more ammonium sulfate was required to precipitate the glutelins.

Addition of ammonium sulfate to the alkaline extract containing the glutelins lowers the pH of the solution. However, it still remains on the alkaline side when the precipitation of the glutelins occurs.

It is of interest to note that gliadin can also be precipitated from a 0.2 per cent aqueous sodium hydroxide solution by the addition of even a smaller amount of ammonium sulfate than is required
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to precipitate the $\alpha$-glutelin. At 0.01 of saturation with ammonium sulfate gliadin separates from a 0.2 per cent sodium hydroxide solution as a sticky precipitate.

**Preparation of the $\alpha$- and $\beta$-Glutelins from Wheat Flour.**

2 kilos of wheat flour were kneaded to a stiff dough. After standing for an hour, the starch was washed out with tap water, during which process the gluten was loosely contained in a cheesecloth bag, in order to avoid loss. The gliadin was then removed by successively extracting the gluten with about 1000 cc. portions of 70 per cent (by volume) alcohol. The ninth extract contained 0.45 gm. of solids which, judging from the small amount of nitrogen (14 mg.), was only partly protein material. To hasten the extraction, the gluten was first cut up into small pieces in a meat grinder and allowed to stand in the alcohol for 1 to 2 days with occasional stirring.

After the gliadin had been removed, the residue was stirred for 2 hours with 2 liters of 0.2 per cent sodium hydroxide, then centrifuged, and the supernatant liquid filtered clear through paper pulp. Centrifugation aids materially in the subsequent filtration, as it separates starch and undissolved protein which would clog the filter. To the clear filtrate (1900 cc.) 50 cc. of saturated ammonium sulfate solution were added with constant stirring, whereupon the $\alpha$-glutelin separated in a flocculent form and settled

### TABLE I.

**Influence of Concentrations of Protein and Alkali upon Amount of Ammonium Sulfate Required for Precipitation of the $\alpha$-Glutelin of Wheat.**

<table>
<thead>
<tr>
<th>Concentration of NaOH (per cent)</th>
<th>Concentration of glutelin: 0.2 per cent</th>
<th>1.0 per cent</th>
<th>2.0 per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate required for precipitation.*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.04</td>
<td>0.018</td>
<td>0.018</td>
</tr>
<tr>
<td>1.2</td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>0.073</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Expressed in terms of saturation.
quickly. As much as possible of the supernatant liquid (A) was siphoned off and the residue centrifuged, in order to separate more thoroughly the precipitate from the liquid. The precipitate was washed by centrifugation twice with 500 cc. of 0.04 saturated ammonium sulfate solution. It was then dissolved in 0.2 per cent sodium hydroxide solution, and reprecipitated and washed as before. After the last washing with ammonium sulfate the precipitate was washed once with distilled water to which a few drops of dilute hydrochloric acid were added—just enough to bring the liquid in which the precipitate was suspended and well mixed to a pH of 6.4. By this manipulation most of the inorganic salts were removed. The precipitate was finally washed twice with 60 per cent alcohol followed by two washings with 95 per cent alcohol.

After dehydration with absolute alcohol and ether in the usual way, 20 gm. of the glutelin were obtained as a fine, grayish white powder.

The β-glutelin was obtained from the liquid (A) which had been siphoned off from the precipitate representing the α-glutelin. The liquid was filtered through a mat of paper pulp and enough saturated ammonium sulfate solution was added to the clear filtrate to make it 0.18 saturated. The resulting precipitate was then purified by reprecipitation and treated in the same manner as the α-glutelin, with the exception that 0.2 saturated ammonium sulfate solution was used for the first two washings. A yield of 1.35 gm. of air-dried material was obtained.

A somewhat better yield of the β-glutelin was obtained by a modified method wherein the extraction with 70 per cent alcohol to remove the gliadin was omitted. The gluten prepared from 1 kilo of flour was dispersed in 3 liters of 0.2 per cent sodium hydroxide and 60 to 80 cc. of saturated ammonium sulfate solution added. This precipitated both the gliadin and the α-glutelin. From the supernatant liquid the β-glutelin was separated and prepared as described above. A yield of 1.54 gm. was obtained.

The composition and distribution of the nitrogen of the two glutelins are shown in Tables II and III. The figures given represent the average of duplicate determinations. Inasmuch as our preparations represent two different glutelins, these analytical figures obviously differ from those found in the literature for
TABLE II.
Composition of Wheat Glutelins.

<table>
<thead>
<tr>
<th></th>
<th>α-Glutelin.</th>
<th>β-Glutelin.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>Sample 2.*</td>
</tr>
<tr>
<td>Nitrogen (moisture- and ash-free basis)</td>
<td>17.14</td>
<td>16.10</td>
</tr>
<tr>
<td>Sulfur (&quot;&quot;&quot;&quot;&quot;&quot;&quot;)</td>
<td>1.59</td>
<td></td>
</tr>
</tbody>
</table>
| Ash (moisture-free basis)                | 0.142       | 0.034       | 0.530       

* This preparation was obtained from gluten which had not been previously extracted with alcohol to remove gliadin.

TABLE III.
Distribution of Nitrogen in Wheat Glutelins as Determined by the Van Slyke Method.*

<table>
<thead>
<tr>
<th></th>
<th>α-Glutelin</th>
<th>β-Glutelin</th>
<th>Amino acids expressed in percentages of the ash- and moisture-free proteins.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td>α-Glutelin.</td>
</tr>
<tr>
<td>Amide N</td>
<td>17.8</td>
<td>11.06</td>
<td>Cystine</td>
</tr>
<tr>
<td>Humin&quot;</td>
<td>1.05</td>
<td>1.32</td>
<td>Arginine</td>
</tr>
<tr>
<td>Cystine N</td>
<td>1.76</td>
<td>5.43</td>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine N</td>
<td>10.95</td>
<td>6.10</td>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine &quot;</td>
<td>5.50</td>
<td>6.17</td>
<td></td>
</tr>
<tr>
<td>Lysine N</td>
<td>3.09</td>
<td>6.83</td>
<td></td>
</tr>
<tr>
<td>Amino &quot; of filtrate</td>
<td>45.4</td>
<td>49.13</td>
<td></td>
</tr>
<tr>
<td>Non-amino N of filtrate</td>
<td>13.0</td>
<td>14.9</td>
<td></td>
</tr>
</tbody>
</table>

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

It has been found that glutelins can be precipitated from a 0.2 per cent sodium hydroxide solution by addition of small amounts of ammonium sulfate. By this method two glutelin frac-
tions have been isolated from wheat gluten. The α-glutelin separates as a flocculent precipitate by making the alkaline solution 0.018 to 0.02 saturated with ammonium sulfate. The β-glutelin does not begin to precipitate at a concentration of ammonium sulfate less than 0.16 of saturation, and requires about 0.18 of saturation for complete precipitation.

The two glutelins differ also in their chemical composition. The α-glutelin contains 17.14 per cent nitrogen, and the β-glutelin 16.06 per cent. Analysis of the glutelins by the Van Slyke method gave the following results expressed as percentages of the total nitrogen: α-glutelin: amide N 17.8, cystine N 1.76, arginine N 10.95, histidine N 5.50, lysine N 3.09; β-glutelin: amide N 11.06, cystine N 5.43, arginine N 6.1, histidine N 6.17, lysine N 6.85.

Both glutelins have the same isoelectric point, namely pH 6.45.

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