THE ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO-ACIDS.

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The methods for isolating the amino-acids yielded by hydrolyzed proteins, as introduced by Kossel and Fischer, and further developed by other workers, have given us most of our present knowledge of the composition of proteins, and still constitute the means of attaining the most complete information concerning their chemical nature. These methods are still encumbered by two inherent difficulties, however, which limit their application in problems involving the chemical nature of proteins. (1) A large supply of material is required, 20 or 30 grams of protein for the determination of the bases by Kossel's method, while at least 100 grams, and preferably 300 or 400 grams, are used for the esterification. (2) The methods for determining most of the amino-acids are not quantitative. As a consequence, the most careful work leaves from a third to a half of the protein molecule still unaccounted for.

The analysis outlined below\(^1\) is designed to enable one to attain an insight into the composition of proteins by methods which require but small amounts of material and yield approximately quantitative results, indicating the nature of all the nitrogenous products yielded by complete acid hydrolysis.

The analysis is based, not on the isolation of the amino-acids, but on determinations of their characteristic chemical groups.

\(^1\) A brief account of the method as developed a year ago was published in the *Ber. d. d. chem. Ges.*, 1910, p. 3176. The method here published is different in many details from its previous form, which it supersedes.
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By precipitation with phosphotungstic acid the amino-acids are separated into two fractions, the "bases," which are precipitated, and the other acids, which are not. The proportions in which the different types of amino-acids are present in each fraction are then ascertained by determination of their characteristic chemical groups.

Phosphotungstic acid as a precipitant for the basic amino-acids obtained by hydrolysis of proteins was introduced by Drechsel,¹ who with its aid discovered lysine. Soon afterwards Hedin² discovered arginine among the bases precipitated by phosphotungstic acid, and then histidine.³ Kossel and Kutscher later devised the well known method for determining lysine, histidine, and arginine.⁴ It was observed that the phosphotungstic precipitate constituting the "lysine fraction" sometimes contained a nitrogenous substance other than lysine and Winterstein identified this as cystine.⁵ The large amount of work done on proteolytic products has discovered no amino-acids, other than the above four, which are present after complete acid hydrolysis of proteins and are precipitated in dilute solution by phosphotungstic acid. Positive evidence that the phosphotungstic precipitate is the entire precipitate was afforded by Osborne and his co-workers. Osborne and Harris⁶ ascertained the conditions necessary for determining quantitatively the proportion of "basic nitrogen," or that precipitated by phosphotungstic acid in dilute solutions of completely hydrolyzed proteins. Using the method of Osborne and Harris, Osborne, Leavenworth, and Brautlecht⁷ found that, in a large series of proteins analyzed, the percentage of nitrogen precipitated by phosphotungstic acid agreed within reasonably close limits with that of the histidine, arginine, and lysine, as determined by Kossel’s method. That these authors overlooked the cystine could easily occur from the fact that, as will be shown later, a large proportion of it is altered by the pro-

³ Ibid., xxii, p. 191, 1896.
⁴ Ibid., xxxi, p. 165.
⁵ Ibid., xxxiv, p. 153.
longed boiling with acid necessary to completely hydrolyze some proteins. Consequently, although cystine, is readily precipitated, the precipitate actually obtained from proteins contains half or less of the original cystine nitrogen.

The work above cited indicates that arginine, histidine, and lysine, may be separated by means of phosphotungstic acid with approximately quantitative completeness from the other amino-acids. This has been confirmed by our experience, the details of which appear later in this paper, and which show that the precipitation of the unaltered cystine is also almost complete.

The principle of the scheme of analysis is briefly outlined in the following paragraph.

**PRINCIPLE OF THE METHOD.**

After the removal of the ammonia by vacuum distillation, the arginine, histidine, lysine, and cystine are precipitated with phosphotungstic acid. The precipitate is redissolved, and these four "bases" are determined by utilization of their marked chemical differences. The determination of the amino nitrogen of this fraction at once divides it into pairs, lysine, and cystine, containing only amino nitrogen; and arginine and histidine, containing respectively three-fourths and two-thirds of their nitrogen in other forms. Of the first pair, the cystine is given by the sulfur content, the lysine by subtracting the cystine from the sum of the two. Of the second pair, the arginine is determined by decomposing it with alkali, which drives off half its nitrogen as ammonia, and the histidine is obtained by difference. In the filtrate from the bases the amino-acids are divided into two sub-fractions: (1) The acids containing only primary amino nitrogen (leucine, alanine, etc.); (2) Those containing nitrogen in pyrrolidine (proline, oxyproline) or indol (tryptophane) rings.

In order that this method of analysis may be applied, it is, of course, necessary that the protein shall be free from nitrogenous impurities, such as purine bases.

The scheme of analysis is represented diagrammatically by the table on page 19. That the sub-fraction of the filtrate, con-

\[ ^{1} \text{Van Slyke, This Journal, ix, p. 485, 1911.} \]
taining 100 per cent of amino nitrogen may contain some acids at present unknown is possible, because the chief losses in hydrolyses by the methods of isolation fall here. As, however, the ester method, by which all of these acids except glutaminic and tyrosine must be determined, is at present accompanied by unavoidable losses, the discrepancies may well be due to these losses, rather than to the presence of unknown α-amino-acids.

**METHODS IN DETAIL.**

**Hydrolysis.** A successful hydrolysis has been performed, using only 1 gram of protein (see hemoglobin hydrolysis), but for most satisfactory conditions 3 grams are required; and, if material suffices, it is well, as in all analyses, to perform duplicates, using 6 grams. The protein is dissolved in 10 or 20 parts of 20 per cent hydrochloric acid, and boiled in a tared flask under a reflux. At intervals of six or eight hours the hydrolysis is stopped, the solution cooled, and portions of 1.00 or 2.00 cc. (enough to contain about 0.1 gram of protein) are withdrawn by a pipette. These portions are diluted to 10 cc. and used for determination of the amino nitrogen. The different determinations of the series should all be run under the same conditions, as otherwise the ammonia from the amid nitrogen might cause an error in the determination. Ordinarily it is most satisfactory to run all the determination 6 minutes, the solutions of amino-acids and nitrous acid being mixed and allowed to stand for five minutes, then shaken for one minute. Under these conditions, room temperature being fairly constant, the same proportion of the ammonia (15–20 per cent at 20°) is decomposed in each case. After removal of each portion for amino determination, the flask in which the hydrolysis is performed is weighed; and the weight is again taken, after the hydrolysis has been continued 6 to 8 hours longer, before the next sample is withdrawn. These weights serve to detect concentration of the solution by loss of vapor. In case such concentration occurs, a correction for the percentage of volume decrease of the solution is made. The hydrolysis is continued until the amino nitrogen becomes constant. This shows when the hydrolysis is complete, and enables one to avoid the errors, which as Osborne has shown, may result from incomplete hydrolysis. (For an example
TABLE I.

<table>
<thead>
<tr>
<th>Precipitated by phosphotungstic acid</th>
<th>100 per cent of N as NH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S) Cystine</td>
<td>S-CH₄-CH,NH₂-COOH</td>
</tr>
<tr>
<td>(No S) Lysine</td>
<td>NH₃-(CH₄)₃-CH,NH₂-COOH</td>
</tr>
<tr>
<td>(Guanidine group) Arginine</td>
<td>NH=CH(C(NH₃)+) - NH</td>
</tr>
<tr>
<td>Non-Amino Nitrogen</td>
<td>*(CH₄)+-CH-(NH₃)COOH</td>
</tr>
<tr>
<td>Glutaminic acid</td>
<td>HOOC-CH₂-CH₂-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>HOOC-CH₂-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>OH-CH₄-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Phenyl alanine</td>
<td>CH₃-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Serine</td>
<td>OH-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Leucine</td>
<td>CH₃-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>CH₃₁-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Valine</td>
<td>CH₃₁-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Alanine</td>
<td>CH₃₁-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Glycocoll</td>
<td>CH₃₁-CH₁-CH(NH₃)+-COOH</td>
</tr>
<tr>
<td>Proline</td>
<td>CH₂-CH⁺</td>
</tr>
<tr>
<td>Oxyproline</td>
<td>CH₂-CH⁺-CH₁-CH(COOR)</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>CH₃₁-CH₁-CH(NH₃)+-COOH</td>
</tr>
</tbody>
</table>

*Non-amino nitrogen atoms (not reacting with nitrous acid. This includes the NH₃ group of the guanidin nucleus in arginin).
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of this method of following the hydrolysis, see the gliadin analysis described in the latter portion of this paper.)

Determination of Ammonia (Amid Nitrogen). The determination of ammonia yielded by hydrolyzed proteins has become especially significant since Osborne, Leavenworth, and Brautlecht have shown that the nitrogen of the ammonia is usually equal to that of the dicarboxylic acids, with which it is presumably combined in the protein molecule in the form of acid-amid radicles.

In order that the subsequent determinations may not be interfered with, it is necessary that every trace of ammonia be removed when it is determined, but that the treatment with alkali be so mild that neither the arginine nor the cystine is attacked. Denis has shown that boiling at 100° with even so weak an alkali as magnesium oxide attacks cystine and drives off a portion of its nitrogen as ammonia. We have noted the same behavior of cystine, although arginine is not attacked. As a result of the sensitivity of cystine one must drive off the ammonia at room temperature, using either the aeration method of Denis, or vacuum distillation. After trying various modifications of both principles, the following technique was adopted as the most convenient and certain.

The solution of hydrolyzed protein is placed in a small double-necked distilling flask, and concentrated under diminished pressure until all the hydrochloric acid possible has been driven off. The residue is taken up with warm water, and the solution is transferred to a measuring flask of 100 to 250 cc. capacity, according to the amount of protein hydrolyzed. Aliquot portions of sufficient size to contain about 0.2 gram of protein are withdrawn and used for Kjeldahl analyses, which give the total nitrogen, on the basis of which the other determinations are calculated.

For the determination of the ammonia, distillation under diminished pressure with lime is used. No special apparatus is required, a one-liter, double-necked distilling flask, an ordinary one-liter distilling flask, and a 200 cc. distilling flask being arranged as shown in Fig. 1. As indicator in the \( \frac{8}{5} \) acid, alizarine sulfonate is used. The solution, containing about 3 grams of hydrolyzed protein, is placed in the double-necked flask, and diluted to about

\[1\] This Journal, viii, p. 365.
200 cc. One hundred cubic centimeters of alcohol, to prevent foaming during distillation, is added, then a 10 per cent suspension of calcium hydrate until a slight excess is present, as shown by the turbidity and alkaline reaction of the solution. The apparatus is then joined together as shown in the figure and evacuated to a pressure of 30 mm. or less. The Claissen flask is then placed in a bath at 45°-50°, and the solution distilled for a half-hour. In case distillation starts too rapidly, a little air is let in from the stop-cock in one neck of the Claissen flask. When the distillation is finished the flask is lifted from the water bath, and the vacuum is released by opening this stop-cock. The $\frac{7}{6}$ acid from the receiving flask and the smaller guard flask is washed into a half liter beaker or Erlenmeyer and titrated back with $\frac{7}{6}$ NaOH. The amount of $\frac{7}{6}$ acid in the larger flask is usually 30 cc. when an animal protein is being analyzed, 60 cc. when the protein is of plant origin, as some plant proteins yield much ammonia.

Melanin Nitrogen. During the distillation all of the black coloring matter, or melanin, which is formed during the hydrolysis of proteins, is adsorbed by the undissolved lime. The latter is filtered off on a folded filter and washed with water until the washings come free of chloride. The precipitate and paper are then submitted to Kjeldahl analysis, using 35 cc. of sulfuric acid to
digest the large amount of organic matter in the paper. In this determination, as in the ammonia distillation, the lime performs the function of the magnesia in the nitrogen distribution method of Osborne and Harris.  

Precipitation, Washing, and Redissolving of the Bases. The filtrate from the melanin is neutralized with hydrochloric acid, returned to the vacuum distilling flask, and concentrated to about 100 cc. It is then washed into a 200 cc. Erlenmeyer, and 18 cc. of concentrated hydrochloric acid and a solution containing 15 grams of phosphotungstic acid are added. The entire solution is then diluted with water up to 200 cc. and heated in a water bath until the precipitate of the bases is nearly or quite redissolved. The bases reprecipitate on cooling as crystalline or granular phosphotungstes which can be readily washed and filtered. The above conditions of precipitation are practically those of Osborne and Harris, with the exception that, in order to avoid precipitation of calcium sulfate, hydrochloric acid is used here instead of sulfuric. The solution is allowed to stand forty-eight hours for the precipitate to form; for in less time the precipitation of histidine may be incomplete.

The washing of the phosphotungstic precipitate has been one of the most troublesome points of the "nitrogen distribution" method of Hausmann. Against this method (the division of the aminoacids into the fraction precipitated by phosphotungstic acid and the fraction not precipitated) Kossel raised the objection that the results varied greatly according to the manner in which the precipitate was washed. Osborne and Harris, however, showed that when a moderate volume of a solution containing 2.5 per cent of phosphotungstic acid and 5 per cent of sulfuric was used to wash the precipitate the results were constant. These authors allowed the precipitate to drain on the filter, then suspended it in the washing solution and allowed it to drain again, washing it three times by this method. The slight amount of nitrogen in the mother liquor not washed out of the precipitate was approximately balanced by the slight amount of the bases dissolved in the mother liquors and washings.

For our purpose, however, it is necessary that the precipitate

shall be washed entirely free of the mother liquors and the amino-
acids of the unprecipitated fraction. It is also necessary that
as small an amount of the washing solution as possible shall be
used, in order that the precipitate, which is slightly soluble in the
washing solution, shall not dissolve to an appreciable extent during
the washing. These requirements are met by the following tech-
nique, which permits the quantitative washing of the precipitate
with 100–200 cc. of solution, and without dissolving appreciable
amounts. A hardened filter paper is cut to the proper size to fit
accurately against both the bottom and side walls of a 3-inch
Buchner funnel. The part of the filter paper which lies against
the sidewalls of the funnel is folded in about twenty plaits, so that
it fits the wall snugly all the way around. Into the pocket thus
formed by the filter paper the precipitate is poured, and the mother
liquors are drawn off as completely as possible by suction, the
precipitate being pressed down by a flattened rod. The filtrate
is transferred from the suction flask to a beaker. Onto the pre-
cipitate in the funnel are poured 10–12 cc. of a washing solution
containing 2.5 per cent phosphotungstic acid and 3.5 per cent of
hydrochloric acid, and the precipitate and solution are stirred up
together until a smooth suspension is formed. Care must be taken
that all the lumps are broken up, and the precipitate completely
reduced to a granular suspension. It is then sucked dry, as before.
The washing in this manner is repeated until the filtrate comes
free of calcium, from eight to fifteen washings being required, accord-
ing to the bulk of the precipitate. The first three or four portions
of washing solution are used to dislodge the last granules of the
precipitate from the flask in which the latter formed. The suc-
ceeding portions are added from a wash bottle or a pipette in a fine
stream around the edge of the filter paper, so that the latter is
washed from the edge downwards around its entire circumfer-
ence. In case the first four washings leave a few granules of the
precipitate still in the flask, they are allowed to remain there, as
they are already sufficiently washed, and the subsequent portions
of the washing solution are used to wash the filter paper as well as
the precipitate, in the manner just described. In case the later
washings run through somewhat turbid, as is often the case, they
are filtered through a small folded filter before being combined
with the main filtrate. The phosphotungstic acid used in the
precipitation and in the washing solution is purified by shaking with ether and water after the method of Winterstein.

In testing the washings for calcium, a solution of oxalic acid in 3 per cent sodium hydrate is used. To about 1 cc. of this solution one adds two or three drops of the filtrate, and shakes slightly until the upper layer, where the filtrate remains, becomes alkaline. The washings are continued until a sample of the filtrate gives no trace of turbidity after standing several minutes with the oxalate solution.

The washing being finished, the precipitate is removed as completely as possible by means of a spatula and a wash bottle with distilled water, to a beaker holding more than a liter. When the precipitate has been removed as completely as possible by mechanical means, the filter paper is spread out on the bottom of a dish and washed with water made alkaline by addition of a few drops of 20 per cent sodium hydrate. This dissolves the portions of precipitate imbedded in the fibers of the filter paper. The small folded filter paper used for filtration of the later washings is similarly freed of adherent precipitate. Also, in case any granules of the precipitate have remained in the flask in which the precipitate originally formed, these are either washed or dissolved out, and added to the other portions in the large beaker. To the suspension therein one adds a few drops of phenolphthalein solution, then 50 per cent NaOH solution drop by drop, with stirring. As soon as the solution becomes red, addition of the alkali is discontinued until the color fades again. All of the precipitate is soon brought into solution in this manner. The solution must be red at the end, but must not contain more than three or four drops of alkali in excess of the amount required to neutralize it. Greater excess of alkali is to be avoided because of the sensitiveness to it of cystine and arginine.

The solution is diluted to about 800 cc., and a 20 per cent solution of crystalline barium chloride is added in portions of a few cubic centimeters each, until a test with neutral sodium sulphate solution shows the presence of an excess of barium above the amount required to precipitate the phosphotungstic acid. In case the solution loses its red color during the precipitation, two or three drops more of the alkali solution are added, as the precipitation is not satisfactory unless the solution is slightly alkaline.
The barium solution must be added until the test results in an immediate granular precipitate of barium sulfate. A detectible turbidity can be obtained before enough barium chloride has been added to complete the precipitation of the phosphotungstate. On the other hand, more barium chloride than a few cubic centimeters excess is to be avoided, as a large excess of barium causes disagreeable bumping when the solution is boiled later in the arginine determination. The dilution which is directed before precipitation is necessary in order to avoid appreciable loss of the bases through adsorption by the barium phosphotungstate.

The precipitate of barium phosphotungstate is filtered and washed with water, the filtration and washing being performed in the manner described for the phosphotungstates of the bases, except that such small portions of wash water do not need to be used. The same funnel and piece of hardened filter paper can usually be used to advantage for both filtrations. The washing is continued until the filtrate comes free of chloride. The filtrate is concentrated in vacuo in the same double-necked distilling flask used for the determination of amid nitrogen, the concentration being continued until the volume of the solution is reduced to about 50 cc. During the concentration, a small amount of barium phosphotungstate which was not precipitated at first separates from the solution. The latter is filtered into a 200 cc. double-necked distilling flask, the filter being washed with water until chloride free. The solution is then concentrated, and transferred to a 50 cc. measuring flask.

**Determination of Arginine.** The determination of arginine is based on the fact, first noted by Osborne, Leavenworth, and Brautlecht, that arginine, when boiled with dilute alkali evolves half of its nitrogen in the form of ammonia. The explanation of the reaction is that, as shown by Schulze and Winterstein, arginine when heated with alkaline solutions decomposes into one molecule each of urea and ornithine. The urea then is decomposed into ammonia. Under the conditions described below the reaction is quantitative. Of the 50 cc. of solution containing the bases, 25 cc. are placed in the 200 cc. Jena Kjeldahl flask of the apparatus shown in Fig. 2. The Folin bulbs at the top of the condenser of the apparatus are connected with the condensing tube by a ground glass joint, as shown in the figure, or by a very heavy
piece of rubber tubing, which is less convenient but equally efficient. In the Folin tubes one places 15 cc. of \( \frac{1}{4} \) acid, with alizarin sulfonate as indicator. To the solution in the flask one adds 12.5 grams of solid KOH, and a bit of porous porcelain to prevent bumping. The solution is then boiled gently for exactly 6 hours. At the end of this time the Folin tube is disconnected from the condenser, and through the latter 100 cc. of water are poured into the Kjeldahl flask. The solution in the flask contains a slight amount of ammonia, although the greater part diffuses up into the Folin bulbs during the 6 hours boiling, and is absorbed by the acid. In order to obtain also the small amount remaining in the
flask, the latter is connected with the condenser of an ordinary Kjeldahl still and the ammonia driven off in the customary manner. The receiver contains the acid from the Folin bulbs, so that all the ammonia from the arginine determination is collected in one solution of $\text{H}^\text{+}$ acid. Care must be taken that not more than 100 cc. of water is boiled off during the distillation; for if the alkaline solution becomes too concentrated other decompositions besides the desired one will result. The excess of $\text{H}^\text{+}$ acid in the receiver is titrated back as usual. Each cubic centimeter neutralized by the ammonia indicates 0.0028 gram of arginine nitrogen in the solution decomposed, or 0.0056 gram in the total solution of the bases. In case cystine is present, 18 per cent of its nitrogen is evolved as ammonia during the arginine determination, and a corresponding correction to the arginine figures must be made. The correction is, however, practically negligible with all ordinary proteins except the keratins. As the behavior of the cystine under the conditions of the determination is quite constant, the accuracy of the arginine determination is not seriously affected even in analysis of the keratins. The cystine determination, from which the correction can be accurately made, is described below.

The 200 cc. Kjeldahl flasks should not be used for more than two or three arginine determinations, as the glass is attacked by the strong alkali. Unfortunately, copper flasks cannot be used because of the effect of the copper in oxidizing the cystine present.

**Determination of the Total Nitrogen of the Bases.** The solution used for the arginine determination is transferred from the 200 cc. Kjeldahl flask to one of 500 cc. Thirty-five cubic centimeters of concentrated sulfuric acid are cautiously added, with cooling, and 0.25 gram of copper sulfate. The solution is then digested as in an ordinary Kjeldahl determination, and its nitrogen content determined. The cubic centimeters of $\text{H}^\text{+}$ acid neutralized in this determination are added to those neutralized in the arginine determination. The sum multiplied by 0.0028 gives the total nitrogen content of the bases. The use of the same portion of solution for the determinations of both arginine and total nitrogen of the bases permits one to use half the solution for them, and obtain each more accurately than would be possible if separate and smaller portions were used for each determination.
Determination of Cystine. The amount of cystine present with the bases is obtained from the content of the solution in organic sulfur. For this determination the most accurate and convenient method is that based on Benedict's principle of oxidation by ignition with copper nitrate.\(^1\) We have used the modification of Denis.\(^2\) The oxidation is invariably complete, not a trace of carbon or other insoluble matter remaining. We have found, consequently, that one can safely proceed with the sulfur determination without first removing the barium chloride present in the solution of the bases. Ten cubic centimeters of the solution are placed in a porcelain evaporating dish of 7 to 10 cm. diameter with 5 cc. of Denis' solution. The mixture is concentrated to dryness on the water bath, then gradually heated to redness and maintained at that temperature for some minutes, as directed by Benedict. The residue is dissolved in 10 cc. of 10 per cent hydrochloric acid, and the solution diluted to about 150 cc. It is heated to boiling, and, in order to make sure that an excess of barium chloride is present, 10 cc. of a 5 per cent solution are added. The barium sulphate is washed and weighed as usual. Each milligram of barium sulfate indicates 0.06 mg. of cystine nitrogen in the portion of solution analyzed, or 0.3 mg. in the total solution of the bases. The weight of barium sulfate obtained must be corrected for the amount of sulfur found in the reagents by blank analyses. In the reagents used by us the correction was 1.5 mgs. of barium sulfate. Reagents requiring a much larger correction should not be used, as the cystine present often yields only a few milligrams of barium sulphate.

The cystine actually present in the solution of the bases can be very accurately determined by the above method; for an error of 1.5 mg. in the barium sulphate weighed, which is as large a margin as duplicates usually differ by, introduces an error of only 0.1 per cent in the total percentage of cystine nitrogen calculated. As about half the cystine originally present is altered during the hydrolysis with acids, however, and an amount containing 0.5 per cent of the nitrogen of the protein remains in solution when the bases are precipitated, the amount of cystine obtained by the above method

\(^1\) This *Journal*, vi, p. 363, 1909.
represents less than half that actually present in the protein. The precipitate of the bases from proteins not unusually rich in cystine contains, therefore, but a small proportion of its nitrogen in the form of cystine. This explains why the presence of cystine in the phosphotungstate precipitate has been overlooked by most workers, with the exception of Winterstein.¹

_Determination of Amino Nitrogen of the Bases._ Ten cubic centimeters of the solution are used for this determination, which is performed in the usual manner. Because of relative slowness with which the ω-amino group of lysine reacts, the determination must be continued for a half-hour at 20°, or for a somewhat longer time if the temperature is lower. A blank determination of the reagents must be run for the same length of time. Cystine gives off gas equivalent to 107 per cent of the amount of nitrogen which it should yield, and therefore a corresponding correction for the cystine is to be made to the amino determination. Except in proteins like the keratins, which are unusually rich in cystine, this correction is negligible, however.

_Calculation of Histidine._ The non-amino nitrogen of the bases comes from the arginine, which contains three-fourths of its nitrogen in a form which does not react with nitrous acid, and from the histidine, which contains two-thirds of its nitrogen in non-amino form. Therefore to calculate the histidine nitrogen, we subtract three-fourths of the arginine nitrogen from the total non-amino nitrogen, and multiply the difference by 3/4.

Or, letting \( D \) represent the total non-amino nitrogen of the bases (difference between total nitrogen and amino nitrogen), and \( \text{Arg} \) represent the arginine nitrogen, determined as previously described, we have the formula:

\[
\text{Histidine N} = \frac{3}{4} (D - \frac{3}{4} \text{Arg.}) = 1.667D - 1.125 \text{Arg.}
\]

The histidine figure is more liable to error than that of any of the other three amino-acids of the basic fraction, because it is affected by error in the determination of either arginine total nitrogen, or amino nitrogen. In these determinations errors of + 1 per cent would cause errors of −1.125, + 1.5, and −1.5 per cent.

¹ _Loc. cit._
respectively in the histidine nitrogen. As, however, they can all be performed accurately, there is no reason why fairly constant results for the histidine should not be obtained. As a matter of fact our duplicates for histidine usually agree within a per cent.

Calculation of Lysine. The nitrogen of the other three amino-acids of this fraction having been calculated, the lysine is obtained by difference. Or:

\[ \text{Lysine N} = \text{Total N} - (\text{Arginine N} + \text{Cystine N} + \text{Histidine N}) \]

At first sight it would appear that the lysine determination, since it involves the figures for all the other three acids of the fraction, should be more subject to inaccuracy than the histidine determination. This is not the case, however. The accuracy of the lysine calculation depends chiefly upon that of the determinations of cystine and the amino nitrogen of the fraction, which are usually very accurate. An error of + 1 per cent in any of the four determinations made on the bases would cause an error in the lysine nitrogen indicated by one of the following figures: amino nitrogen, + 1.5 per cent; cystine nitrogen, − 1 per cent; total nitrogen, − ½ per cent; arginine nitrogen, + ½ per cent.

Determination of the Total Nitrogen in the Filtrate from the Bases. To the combined filtrate and washings from the phosphotungstate precipitate of the bases 50 per cent sodium hydrate solution is added until the solution turns slightly turbid by precipitation of lime. It is then cleared again by addition of a little acetic acid. In adding the alkali, it is essential that the neutral point should not be passed by more than a few drops, as otherwise a precipitate may form which will not redissolve. The solution is placed in a double-necked distilling flask and concentrated under diminished pressure until salt begins to crystallize. The solution is then washed into a 150 cc. measuring flask and diluted up to the mark. Duplicate portions of 25 cc. each are taken for Kjeldahl determinations. For each portion one uses 15 grams of potassium sulfate, 35 cc. of concentrated sulfuric acid, and 0.25 gram of copper sulfate. The sulfuric acid must be added carefully and under a hood, because of the vigorous evolution of hydrochloric acid gas. The digestion is continued for three hours after the solutions have become clear. Under these conditions the phosphotungstic acid does not interfere at all with the accuracy of the determination.
Determination of Amino Nitrogen in the Filtrate from the Bases. Ten cubic centimeter portions of the filtrate are used for the amino determinations, which are run in the usual manner for six to ten minutes. The volume of nitrogen gas given off by a given amount of amino nitrogen is 2.5 times the volume of $\frac{1}{20}$ acid neutralized if the same amount is determined by the Kjeldahl method. Therefore the portions (25 and 10 cc.) taken for total and amino nitrogen determinations give results of similar accuracy. As 25–35 cc. of gas or acid are usually measured, with an error not, as a rule, exceeding 0.2 cc., the percentage error of these determinations is small.

Purity of Reagents. Since some of the calculations are based on differences between determinations, it is imperative that the latter shall be accurate. Every reagent used for either Kjeldahl or amino determination must be tested by blank analyses, and a correction applied in case any nitrogen is detected. A slight correction is usually necessary for both the commercial alkali used in distilling off the ammonia in the Kjeldahl determinations and for the sodium nitrite used in the amino determinations. We have also found potassium sulfate imported from one of the best known firms, 10 grams of which gave off ammonia by Kjeldahl sufficient to neutralize 2 cc. of $\frac{1}{20}$ acid. The accuracy of the standard solutions and apparatus used for both Kjeldahl and amino determination should likewise be carefully checked by determinations performed on pure substances. It is, of course, essential that pipettes, measuring flasks, and burettes should be calibrated.

The phosphotungstic acid used is purified with ether and water, by Winterstein’s method.

Corrections for Solubilities of the Bases. The solubility corrections are made from the following table, which can be applied directly when the bases are precipitated in the prescribed manner from a solution of 200 cc. volume. It is true that the concentration of phosphotungstic acid left in solution, when the bases are precipitated, varies somewhat with the amount of the latter, so that the conditions of precipitation are not absolutely constant. It does not appear, however, that the variation is sufficient to cause significant change in the solubilities of the bases. When the latter are precipitated from solutions of greater or less volume than 200 cc., the solubility correction will, of course, vary in direct
Analysis of Proteins

proportion to the volume of the solution. The amount of nitrogen dissolved from the precipitate by the washing, performed in the manner described previously, is not, to judge from the control experiments, appreciable.

**TABLE II.**
Solubilities of the Bases in 200 cc. of the Solution in which they are Precipitated.

<table>
<thead>
<tr>
<th>Base</th>
<th>Total N (Add to the Individual Bases)</th>
<th>Amino N</th>
<th>Non-Amino N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine N</td>
<td>0.0032</td>
<td>0.0008</td>
<td>0.0024</td>
</tr>
<tr>
<td>Histidine N</td>
<td>0.0038</td>
<td>0.0005</td>
<td>0.0025</td>
</tr>
<tr>
<td>Lysine N</td>
<td>0.0005</td>
<td>0.0005</td>
<td>0.0000</td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.0026</td>
<td>0.0026</td>
<td>0.0000</td>
</tr>
<tr>
<td>Sum (subtract from figures for filtrate)</td>
<td>0.0052</td>
<td></td>
<td>0.0049</td>
</tr>
</tbody>
</table>

Limits of Accuracy of the Determinations. The maximum and average differences found between duplicates in analyses of gliadin, edestin, hair, gelatin, fibrin, and hemocyanin are shown in the following table, expressed in percentages of the total nitrogen of the proteins.

**TABLE III.**

<table>
<thead>
<tr>
<th>Base</th>
<th>Maximum Difference Between Duplicates</th>
<th>Average Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N</td>
<td>0.37</td>
<td>0.12</td>
</tr>
<tr>
<td>Melanin N</td>
<td>0.39</td>
<td>0.20</td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>Arginine N</td>
<td>1.27</td>
<td>0.73</td>
</tr>
<tr>
<td>Histidine N</td>
<td>2.14 (0.93)</td>
<td>0.79</td>
</tr>
<tr>
<td>Lysine N</td>
<td>1.23</td>
<td>0.61</td>
</tr>
<tr>
<td>Amino N in filtrate from bases</td>
<td>1.60 (0.60)</td>
<td>0.63</td>
</tr>
<tr>
<td>Non-amino N in filtrate</td>
<td>1.20</td>
<td>0.68</td>
</tr>
</tbody>
</table>

The maximum difference for the histidine (from edestin analysis) and that for amino nitrogen of the filtrate (from hair analysis) are more than twice as great as any other deviations found for these figures in the series. The next largest are given in parentheses, and represent as large differences between duplicates as are normally to be expected.
Tests of the Methods on Pure Amino-Acids.

Experiments showing the manner in which all the natural amino-acids react when analyzed for amino nitrogen have been reported in the paper on the amino determination method, and therefore require no discussion here.

The principle of ammonia determination by vacuum distillation is so well known that it likewise requires no especial discussion. The apparatus shown in Fig. 1 embodies no new principles, but is merely a simpler form which enables one to perform the determination without special apparatus. The 200 cc. guard flask, to prevent escape of the ammonia which is not condensed in the receiving flask, is the only addition required to the vacuum distillation apparatus used for merely concentrating water solutions. In control analyses with standard ammonium sulphate solutions the method gave absolutely accurate results; and the determinations of amid nitrogen in the protein analyses always checked closely. Control determinations with 0.2 gram portions of cystine and of arginine carbonate showed that neither of these amino-acids gives off any ammonia under the conditions of the determination.

Arginine. The two steps in the determination of arginine are:
1. the precipitation with phosphotungstic acid;
2. the decomposition with alkali.

Experiments were first undertaken to establish the conditions for the quantitative decomposition with alkali. Schulze and Winterstein have shown that arginine when boiled in alkaline solution decomposes into ornithine and urea:

\[
C_6H_{14}N_4O_2 + H_2O = C_6H_{12}N_2O_2 + CN_2H_4O
\]

Arginine ornithine urea

It is well known that urea, when boiled with either mineral acids or alkalis, is hydrolyzed into ammonia and carbon dioxide. Combining this reaction with the one written above, we have the following equation, which should represent the end products of the action of alkalis upon arginine if no side reactions occur:

\[
C_6H_{14}N_4O_2 + 2H_2O = C_6H_{12}N_2O_2 + 2NH_3 + CO_2
\]

1 Cf. Abderhalden: Biochemische Arbeitsmethoden.
That is, one-half of the arginine nitrogen is transformed into ammonia. The possibility that this reaction might serve for determination of arginine is apparent from the work of Osborne, Leavenworth, and Brautlecht.\(^1\) These authors found that when arginine was boiled down twelve to twenty times with \(\frac{9}{10}\) NaOH in a Kjeldahl flask it evolved ammonia at a gradually decreasing rate, the total amount representing the greater part of the ammonia indicated by the above equation. Also, when proteins were similarly treated they evolved amounts of ammonia approximately equivalent to the amid nitrogen of the proteins plus one-half the arginine nitrogen.

We found that by using stronger solutions of alkali the reaction could be made sharply quantitative. 0.2127 gram of the acid silver nitrate salt, \(\text{C}_6\text{H}_4\text{N}_4\text{O}_6\cdot\text{AgNO}_3\cdot\text{HNO}_3\), was placed in a Kjeldahl flask with 50 cc. of 50 per cent NaOH and 300 cc. of water. The solution was boiled down to about 100 cc., then diluted and boiled down again. This process, performed five times, resulted in the evolution of the following amounts of \(\frac{9}{10}\) ammonia: 6.95, 2.00, 0.75, 0.48, and 0.20 cc., making in all 10.38 cc.; while the theoretical amount is 10.45 cc. Repetition of the experiment gave similar results.

The method was, however, prolonged and inconvenient because of the number of times which the distillation had to be repeated, and because the solutions required constant watching in order to prevent too great concentration of the alkaline mixture and resultant destruction of the ornithine. The apparatus shown in Fig. 2 was accordingly tried, with entirely satisfactory results.

\(\text{Experiment 1.}\) Of the acid silver nitrate salt of arginine 0.2145 gram was dissolved in 25 cc. of water in the Kjeldahl flask of the apparatus. Twenty-five cc. of 50 per cent sodium hydrate solution was added, and the solution was kept boiling gently for five hours. The ammonia which collected in the Folin bulbs during this time neutralized 10.00 cc. of \(\frac{9}{10}\) acid. One hundred cc. of water were added to the solution, and the remainder of the ammonia distilled off as described in the directions for arginine determination. 0.35 cc. of \(\frac{9}{10}\) acid was neutralized, making 10.35 cc. in all, while the theoretical amount is 10.53 cc.

\(\text{Experiment 2.}\) Of the same arginine salt 0.2031 gram was treated in the same manner, except that the boiling was continued for six hours. The ammonia collected in the Folin bulbs neutralized 9.75 cc. of \(\frac{9}{10}\) acid, and the

subsequent distillation yielded an amount neutralizing 0.25 cc. more, making 10.00 cc. in all, the theoretical amount being 9.98 cc.

Experiment 3. To a solution of 0.1758 gram of arginine carbonate and 30 cc. of water, 15 grams of solid potassium hydrate were added, and the solution was boiled in the usual manner for five hours. The ammonia collected in the Folin bulbs neutralized 14.45 cc. of HCl acid, that by the subsequent distillation 0.48 cc. more, making in all 14.93 cc., the theoretical amount being 15.02 cc.

In order to determine whether the other amino-acids precipitated by phosphotungstic acid were stable under the conditions of the arginine determination, portions of lysine, histidine, and cystine were treated in the same manner as the arginine. The results in detail will be given in the sections devoted to the experiments with these acids. The lysine and histidine were found to give off no ammonia, while the cystine gave off regularly 17-18 per cent of its nitrogen in this form.

Gulewitsch has found that the solubility of arginine phosphotungstate, under practically the same conditions used in the precipitation and washing of the bases here, is such that 2.2 mg. of arginine nitrogen are dissolved in 100 cc. of the phosphotungstate solution. In the following experiment we obtained the slightly smaller figure of 1.6 mg.

0.215 gram of the acid silver nitrate salt of arginine was dissolved in water and precipitated at 200 cc. dilution, in the presence of 5 per cent of sulphuric acid, with 12 grams of phosphotungstic acid. The solution was allowed to stand twenty-four hours at 20°, then filtered on a folded filter and allowed to drain. 175 cc. of the filtrate were submitted to Kjeldahl determination, and neutralized 2.00 cc. of HCl acid, indicating 2.8 mg. of nitrogen in the 175 cc. of filtrate, or 1.6 mg. in 100 cc. As a check on the determination, the precipitate and the remainder of the filtrate were placed in a Kjeldahl flask with 300 cc. of water and 50 cc. of 50 per cent sodium hydrate solution. The mixture was boiled down repeatedly, as described in the first experiment on the arginine determination, and gave off during the successive distillations 6.86, 1.45, 0.73, and 0.30 cc. of HCl ammonia, making in all 9.44 cc. This indicates 0.0264 gram of nitrogen, while the amount present, as calculated by difference from the total nitrogen content of the arginine and the nitrogen found in the filtrate, should be 0.0268 gram.

HISTIDINE. 0.2 gram of pure histidine dichloride was precipitated at 20° in the same manner as the arginine. The histidine did not begin to precipi-
tate for some time after the phosphotungstic acid was added, but finally appeared in the form of shining crystals, which grew slowly on the bottom of the flask. After standing over night, the compact precipitate was filtered off. 100 cc. of the filtrate were freed from phosphotungstic acid with barium hydrate, and the filtrate from the barium phosphotungstate was concentrated and submitted to Kjeldahl determination. The amount of N acid neutralized was 2.68 cc., indicating 3.68 mg. of nitrogen in the 100 cc. of filtrate. The remainder of the filtrate was allowed to stand another twenty-four hours, however, and a little more histidine phosphotungstate crystallized from it. This was filtered, without washing, and the filtrate, amounting to 95 cc., was treated in the same manner as the former portion. The amount of N acid neutralized was 1.30 cc., indicating 1.9 mg. of nitrogen in 100 cc. of the filtrate.

The above results show that at least forty-eight hours must be allowed for the complete precipitation of the histidine and that the solubility of the histidine under the conditions of the precipitation is about 1.9 mg. of histidine nitrogen per 100 cc. of solution.

The histidine precipitates were combined and boiled with alkali in the same manner as the arginine, but gave off no ammonia. Negative results were also obtained when 0.1 gram of histidine dichloride was boiled with alkali under a reflux as in the regular arginine determination.

**LYSINE.** 0.2 gram of lysine picrate was precipitated at 200 cc. volume in the same manner as the arginine and histidine. The lysine phosphotungstate, even in so dilute a solution, forms immediately, and the solubility determination shows that the lysine phosphotungstate is the most insoluble formed by any of the bases. 190 cc. of the filtrate were freed from phosphotungstic acid with barium hydrate, and from excess of barium with an equivalent of sulfuric acid. The solution was then concentrated to a volume of a few cc., and used for determination of amino nitrogen. 0.9 cc. of nitrogen gas at 751 mm., 23°, was given off. This indicates the presence of only 0.24 mg. of lysine nitrogen in 100 cc. of the filtrate.

The experiment was repeated in the same manner, except that the precipitation was performed in the presence of an equivalent concentration (3.5 per cent) of hydrochloric acid instead of 5 per cent sulphuric. 193 cc. of the filtrate was freed from phosphotungstic acid by extraction with ether, according to the method of Winterstein,¹ and the solution was concentrated to dryness to drive off the hydrochloric acid. The residue was redissolved in a few cc. of dilute sodium hydrate solution, and made up to 10 cc. volume. 9.67 cc. of this solution gave, on amino determination,

0.84 cc. of nitrogen gas at 768 mm. and 20°. This indicates, as did the former
determination, 0.24 mg. of lysine nitrogen dissolved in 100 cc. of the filtrate.

In order to test the stability of lysine under the conditions of
the arginine determination, 0.2 gram of lysine picrate was dis-
solved in dilute sulfuric acid and freed from picric acid by extrac-
tion with ether. The solution was brought to 20 cc. volume, and
placed in the flask of the apparatus for arginine determination
(Fig. 2). An equal volume of 50 per cent sodium hydrate was
added, and the solution was boiled for 8 hours. No ammonia
whatever was evolved.

**Cystine. Experiment 1.** 0.1 gram of cystine was dissolved in water con-
taining 10 cc. of concentrated hydrochloric acid, a solution containing 5
grams of phosphotungstic acid was added, and the entire solution was
diluted to 100 cc. In a few minutes the cystine phosphotungstate began
to separate in crystals similar to those formed by histidine. After twenty-
four hours the precipitate was filtered on a small hardened filter paper and
washed with suction several times, exactly in the manner described for
treating the precipitate of the bases in the protein analysis. The precipi-
tate was removed from the filter, dissolved in the requisite amount of dilute
sodium hydrate, as described for the protein analysis, and the solution
was freed from phosphotungstic acid with barium chloride. It was then
brought to 50 cc. volume, and determinations of the sulfur, amino nitro-
gen, and total nitrogen were made upon aliquot portions.

**Sulfur.** Ten cc. duplicate portions. The weights of BaSO₄ were 0.0333
and 0.0332 gram, the average of the two indicating 10.0 milligrams of cystine
nitrogen in the entire 50 cc. of solution.

**Nitrogen.** Twenty cc. of the solution were used for Kjeldahl determina-
tion. The ammonia from the latter neutralized 2.95 cc. of H₃O⁺ acid, indicat-
ing 10.3 mgs. of nitrogen in the total 50 cc.

**Amino Nitrogen.** From 9.60 cc. of the solution 3.80 cc. of nitrogen gas
at 22°, 760 mm. were obtained. Multiplying this by the factor 0.92 to correct
for the abnormal behavior of cystine with nitrous acid, gives 3.50 cc. of nitro-
gen from the cystine, indicating 10.1 cc. of cystine nitrogen in the entire
50 cc.

**Nitrogen in the Filtrate.** The filtrate and washings were digested together
for Kjeldahl determination, air being drawn through the solutions, as
suggested by Denis, to prevent the bumping usually caused by the presence
of a large amount of phosphotungstic acid during the digestion. The nitro-
gen present neutralized 0.95 cc. of H₃O⁺ acid, indicating 1.3 mg. of nitrogen in
the filtrate and washings from the cystine phosphotungstate precipitate.
Added to the amount of cystine nitrogen in the precipitate, as determined

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1 This Journal, ix, p. 199, 1911.
2 This Journal, viii, p. 365.
respectively by the three above methods, this gives 11.3, 11.6, or 11.4 mgs of cystine nitrogen regained out of the 11.66 mgs. present in the 0.1 gram of cystine used. It also indicates that the amount of cystine nitrogen dissolved in 100 cc. of solution under the conditions of the precipitation is not more than 1.3 mgs. A repetition of the solubility determination under the same conditions, except that only the filtrate, not the washings, was used for the Kjeldahl, gave the same result, 1.3 mg., indicating that the washing did not dissolve appreciable amounts of the precipitate. Determinations under the same conditions, except that they were made in the presence of 5 per cent sulfuric instead of 3.5 per cent hydrochloric acid gave the slightly higher solubility figure of 1.85 - 1.88 mg. per 100 cc.

Although cystine is precipitated as completely as either arginine or histidine the amounts obtained from hydrolyzed proteins fell far short of what would be expected from the sulfur contents. It appeared possible that the cystine is partially destroyed during the hydrolysis. The results in the following table show that this is the case,—the cystine is gradually altered during acid hydrolysis into a substance or substances which are not precipitated by phosphotungstic acid.†

<table>
<thead>
<tr>
<th>HOURS BOILED WITH HCl</th>
<th>N IN 100 CC. FILTRATE</th>
<th>N IN PRECIPITATE</th>
<th>METHOD OF DETERMINATION OF N IN PRECIPITATE</th>
<th>N IN FILTRATE CORRECTED FOR SOLUBILITY</th>
<th>PER CENT CYSTINE DESTROYED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>mg. 1.3</td>
<td>mg. 10.2</td>
<td>S, N, NH₂</td>
<td>mg. 0.0</td>
<td>per cent 0</td>
</tr>
<tr>
<td>8</td>
<td>mg. 4.6</td>
<td>mg. 7.0</td>
<td>Difference</td>
<td>mg. 3.3</td>
<td>29</td>
</tr>
<tr>
<td>16</td>
<td>mg. 6.0</td>
<td>mg. 5.6</td>
<td>Difference</td>
<td>mg. 4.7</td>
<td>41</td>
</tr>
<tr>
<td>24</td>
<td>mg. 7.1</td>
<td>mg. 4.53</td>
<td>N</td>
<td>mg. 5.8</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.55)</td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.65)</td>
<td>NH₂</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiment 2. One-tenth gram portions of cystine were boiled under a reflux condenser with 20 cc. portions of 20 per cent hydrochloric acid for the lengths of time indicated in the table. The solutions were then transferred to 100 cc. flasks, and 5 grams of phosphotungstic acid in solution added to each. The solutions were diluted with water to 100 cc. each, and the cystine allowed to precipitate, as before, under practically the same conditions under which the bases precipitate in the protein analysis. The amount of phosphotungstic acid, 5 grams per 100 cc., was less than that used

† Mörner found that boiling cystine 102 hours with 10 per cent hydrochloric acid reduced its specific rotation from $-223^\circ$ to $-134^\circ$, and changed part of it into a form apparently more soluble than the natural cystine. (Zeitschr. physiol. Chem., xxxiv, p. 207.)
Donald D. Van Slyke

in the protein analysis (7.5 grams) in order to allow for the fact that in the latter a portion of the phosphotungstic acid is withdrawn from solution by the other bases which are precipitated.

It remains to test the behavior of cystine when boiled with alkali under the conditions of the arginine determination. The following tests were carried out under the exact conditions of the arginine determination, samples of cystine being boiled 6 hours with either 25 per cent sodium hydrate or 33 per cent potassium hydrate, the solutions being then diluted, and the residual ammonia boiled off on a Kjeldahl still.

Experiment 3. (a). One-tenth gram of cystine was boiled with 50 cc. of 25 per cent "reagent" sodium hydrate, as described. The ammonia evolved neutralized 1.50 cc. of $\frac{N}{10}$ acid, equivalent to 2.10 mg. of nitrogen, or 18.0 per cent of that present in the cystine.

(b). The same experiment repeated yielded ammonia neutralizing 1.55 cc. of $\frac{N}{10}$ acid, equivalent to 18.6 per cent of the cystine nitrogen.

(c). The same conditions were repeated, except that 0.2 gram of cystine was used. The ammonia neutralized 2.75 cc. of $\frac{N}{10}$ acid, equivalent to 16.5 per cent of the cystine nitrogen.

(d). One-tenth of a gram of cystine was boiled six hours with a solution of 15 grams of potassium hydrate in 30 cc. of water. The ammonia evolved neutralized 1.50 cc. of $\frac{N}{10}$ acid, equivalent to 18 per cent of the nitrogen present in the cystine.

(e). One-tenth gram portions of cystine were boiled in the same manner with 50 cc. portions of 25 per cent sodium hydrate, but in a copper flask. The amounts of $\frac{N}{10}$ acid neutralized in three successive experiments were 5.03, 5.80, and 5.55 cc., equivalent to 60.3, 69.6, and 66.6 per cent, respectively, of the cystine nitrogen. Because of the effect of the copper in catalysing the reaction that results in the evolution of ammonia from cystine copper flasks can not be used in the arginine determination.

The cystine used in the above experiments was obtained from a bladder stone and purified by repeated solution in water, to which a minimum amount of sodium hydrate was added, followed by precipitation with acetic acid. The purity was controlled by determinations of the nitrogen, carbon, hydrogen, and sulfur.

TRYPTOPHANE. Tryptophane is known to be precipitated partially with phosphotungstic acid, even from fairly dilute solutions. When it is boiled with mineral acids, however, it is, to a large extent at least, destroyed, the nature and fate of the products being unknown. In order to ascertain the behavior of tryptophane under the conditions of the protein hydrolysis described in this paper, the following experiment was performed.
Nine-tenths of a gram of tryptophane was boiled for twelve hours with 100 cc. of 20 per cent hydrochloric acid. The solution was concentrated as dry as possible in vacuo, then redisolved in water. The solution was clear, free from insoluble matter, and but very slightly colored. It is evident that the melanin formed when proteins are hydrolyzed is not a decomposition product of tryptophane.

The ammonia determination also was negative.

After the latter, the solution was brought to 100 cc. and determinations of the total and amino nitrogen made on portions of 10 cc. each.

Amino nitrogen. 11.00 cc. of nitrogen gas at 21°, 756 mm., indicating 0.0620 gram of amino nitrogen in the total 100 cc. of solution.

Total nitrogen. In the Kjeldahl determination 8.90 cc. of $\frac{N}{2}$ acid were neutralized, indicating 0.1246 gram of nitrogen in the total solution.

The remaining 80 cc. of solution were treated with phosphotungstic acid, in the regular manner for precipitating the bases, the solution being at 200 cc. volume. A precipitate formed, even while the phosphotungstic acid was being added. When it was filtered, after 48 hours, the filtrate was not diluted with the washings, but portions of 49 and 25 cc. respectively were used for Kjeldahl and amino nitrogen determinations. Before withdrawing the portions for determination of amino nitrogen, the solution was freed from air by shaking it in an evacuated flask, as the air in 25 cc. of solution would necessitate a correction of 0.4 cc. to the nitrogen gas. The results were:

Amino nitrogen. 5.25 cc. of nitrogen gas at 22.5°, 758 mm., indicating 0.0300 gram of amino nitrogen in the total filtrate (from 0.72 gram of tryptophane).

Total nitrogen. 7.00 - 6.95, average, 6.98 cc. of $\frac{N}{2}$ acid, indicating 0.0611 gram of nitrogen in the total filtrate.

Both filtrate and precipitate, when portions of them were freed from phosphotungstic acid with barium hydrate and tested for tryptophane with glyoxylic acid, gave positive tests.

The main portion of the precipitate was boiled with 25 per cent sodium hydrate solution in the apparatus for arginine determination; it gave off no ammonia.

The above facts lead to the following conclusion.

1. The tryptophane is responsible for none of the nitrogen estimated as ammonia, arginine, or melanin.

2. Boiling with 20 per cent hydrochloric acid does not alter the ratio 2:1 of total nitrogen to amino nitrogen in tryptophane.

3. Tryptophane, under the conditions of the protein analysis, can be precipitated with the "base" fraction. The amount of tryptophane taken, however, was equivalent to 30 per cent of the total amount of protein used in an analysis. Even of this large amount, only 38.7 per cent was precipitated by the phospho-
tungstic acid; that is, an amount equal to about 20 per cent of a protein, as ordinarily analyzed, remained unprecipitated. Of tryptophane which is precipitated three-fourths would, in the protein analysis, be calculated as histidine, the other fourth as lysine. In order to determine whether tryptophane were actually present in the phosphotungstic precipitated from any of the proteins analyzed (cf. latter portion of this paper), a portion of the solution of the precipitated amino-acids was tested in each case for tryptophane with glyoxylic acid. Although the control experiment cited above indicates that the tryptophane or products thereof precipitated gives the glyoxylic test, none of the precipitates yielded by the proteins gave any trace of it. It appears improbable that tryptophane affects the composition of the phosphotungstic precipitate under the usual condition of the analysis, but it is advisable in the latter, as a precaution, to test a few drops of the solution of the bases for tryptophane.

**Analysis of an Artificial Mixture of Amino-acids.** In order to obtain a mixture of complexity similar to that obtained by hydrolyzing proteins, the following were dissolved together: of aspartic acid, glutaminic acid, proline, oxyproline, phenylalanine, tyrosine, alanine, glycocoll, valine, leucine, cystine, histidine dichloride, and ammonium sulfate, each 0.200 gram. Of arginine carbonate, 0.2137 gram was taken. For the lysine, 0.75 gram of lysine picrate was dissolved in dilute hydrochloric acid and freed from picric acid by extracting with ether. The solution was then concentrated and brought to 50 cc. volume. For a Kjeldahl determination 9.50 cc. were used, and 6.73 cc. of H₂SO₄ acid were neutralized. Forty cubic centimeters of the solution, containing 0.0395 gram of lysine nitrogen were added to the mixture of amino-acids. The nitrogen contents of the substances present add up to 0.4266 gram of nitrogen. The solution was brought to 100 cc. and aliquot parts of 5 cc. each were used for Kjeldahl determinations. The amounts of H₂SO₄ acid neutralized were 15.24 and 15.39 cc., indicating 0.4267 and 0.431 gram of nitrogen respectively in the entire solution.

The solution was analyzed in exactly the manner described for protein analysis. Ninety cubic centimeters of the original 100 cc. of solution were used, the nitrogen results being calculated to apply to the total 100 cc.
Ammonia. 27.8 cc. of $\frac{N}{10}$ acid were neutralized, indicating 0.0432 gram of ammonia nitrogen in the original total 100 cc. of solution.

Cystine. The BaSO$_4$ weighed 0.0618 gram, indicating 0.0206 gram of cystine nitrogen in the total solution.

Arginine. 8.89 cc. of $\frac{N}{10}$ acid were neutralized. A correction of 1.00 cc. must be applied for the cystine, leaving 7.80 cc. for the arginine, indicating 0.0485 gram of arginine nitrogen.

Total Nitrogen of the Phosphotungstate Precipitate or "Bases." The solution which had been used for the arginine determination neutralized, by Kjeldahl method, 35.78 cc. of $\frac{N}{10}$ acid. This, added to the 8.89 cc. of the arginine determination, gives 44.67 cc., indicating 0.1390 gram of nitrogen in the amino-acids precipitated by phosphotungstic.

Amino Nitrogen of the Phosphotungstate Precipitate, or "Bases." The amino determination yielded 20.3 cc. of nitrogen gas at 19°, 768 mm., indicating 0.0845 gram of amino nitrogen. The cystine introduces a correction of 0.0014 gram, leaving 0.0831 gram.

Amino Nitrogen of the Filtrate from the Bases. The determinations gave:
(I) 20.8 cc. of nitrogen gas at 21°, 766 mm., indicating 0.1975 gram of amino nitrogen in the filtrate.
(II) 20.7 cc. of nitrogen gas at 22°, 766 mm., indicating 0.1960 gram of amino nitrogen in the filtrate. The average is 0.1968 gram.

Total Nitrogen in the Filtrate. The duplicate Kjeldahl determinations neutralized 26.27 and 26.20 cc. of $\frac{N}{10}$ acid, the average indicating 0.2450 gram of nitrogen in the filtrate.

The results are summarized in the following table, the histidine and lysine being calculated as described previously.

<table>
<thead>
<tr>
<th>TABLE V.</th>
<th>PRESENT</th>
<th>FOUND (UNCORRECTED)</th>
<th>FOUND (CORRECTED FOR THE SOLUBILITY OF THE BASES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total N</td>
<td>0.4266</td>
<td>0.4289 (Kjeldahl)</td>
<td></td>
</tr>
<tr>
<td>Ammonia N</td>
<td>0.0424</td>
<td>0.0432</td>
<td>0.0432</td>
</tr>
<tr>
<td>Arginine N</td>
<td>0.0511</td>
<td>0.0485</td>
<td>0.0517</td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.0233</td>
<td>0.0206</td>
<td>0.0232</td>
</tr>
<tr>
<td>Histidine N</td>
<td>0.0369</td>
<td>0.0294</td>
<td>0.0332</td>
</tr>
<tr>
<td>Lysine N</td>
<td>0.0396</td>
<td>0.0405</td>
<td>0.0410</td>
</tr>
<tr>
<td>Amino Nitrogen of the filtrate from the bases</td>
<td>0.1867</td>
<td>0.1968</td>
<td>0.1916</td>
</tr>
<tr>
<td>Non-amino N of the filtrate (proline and oxyproline)</td>
<td>0.0457</td>
<td>0.0482</td>
<td>0.0433</td>
</tr>
<tr>
<td>Total N regained (sum)</td>
<td>0.4266</td>
<td>0.4272</td>
<td>0.4272</td>
</tr>
</tbody>
</table>
The agreement between the nitrogen figures found and those calculated from the amounts of amino-acids present is fairly satisfactory, especially when the solubility corrections for the bases are applied. The mixture contained all of the amino-acids known to occur commonly in proteins except tryptophane. The results indicate about the same degree of accuracy as the agreement between the duplicates in the protein analyses reported in the latter portion of this paper. The test of the method would be made more complete by analysis of a mixture like the above, but containing tryptophane and boiled 24 hours with 20 per cent hydrochloric acid. Unfortunately the completion of this experiment was prevented by the expiration of the period available for the work, which can not be taken up again for a number of months. The consistency of the results obtained, however, both in experiments with pure amino-acids and in analyses of proteins, indicates, with a fair degree of conclusiveness, the reliability of the method.

ANALYSES OF PROTEINS.

Gliadin.

Ten grams of gliadin from wheat were dissolved in about 60 cc. of 20 per cent hydrochloric acid and boiled under a reflux in a tared flask. After twenty hours a sample of 1 cc. was withdrawn, diluted to 10 cc., and used for determination of amino nitrogen. 9.65 cc. of this solution yielded 20.0 cc. of nitrogen gas at 17°, 763 mm., indicating 23.81 mg. of nitrogen gas from the entire 10 cc. The weight of the hydrolyzing solution after the sample was withdrawn was 81.7 grams. After eight hours more boiling the solution had concentrated to 79.5 grams. If the amino nitrogen had remained constant, 1 cc. should now yield 24.45 mg. of nitrogen gas. The amount obtained was 26.45 mg., showing that additional hydrolysis had occurred since the preceding determination. The hydrolysis was continued for ten hours more, during which the weight of the solution decreased from 78.4 to 77.6 grams. The amount of nitrogen gas to be expected from 1 cc. of this solution, if no further hydrolysis had occurred, was 26.72 mg. The amount obtained was 26.92 mg., showing that the hydrolysis had been complete at the end of 28 hours.
Analysis of Proteins

The solution was diluted to 250 cc. and 10 cc. samples taken for Kjeldahl determinations. The amounts of \( H \) acid neutralized were 43.00 and 43.05 cc., the average indicating a nitrogen content of 0.452 gram in the 75 cc. portions of the solution used for the subsequent analyses.

The analyses were performed on duplicate solutions, the details corresponding in all respects to the description of the method in the early part of the paper. The results follow.

Ammonia. The amounts of \( H \) acid neutralized were 82.78 and 81.9 cc., indicating 0.116 and 0.1147 gram of amid nitrogen.

Melanin. The amounts of \( H \) acid neutralized were 2.80 and 2.80 cc., indicating 0.0039 gram of nitrogen.

Cystine. The \( BaSO_4 \) weighed 0.0086 and 0.0097 gram, indicating 0.0026 and 0.0029 gram of cystine nitrogen. The amount of cystine here, as in most of the proteins analyzed, is too small to affect significantly the figures for the arginine or amino nitrogen.

Arginine. The amounts of \( H \) acid neutralized were 4.11 and 3.97 cc., indicating 0.0230 and 0.0223 gram of arginine nitrogen.

Total Nitrogen of the Bases. The amounts of \( H \) acid neutralized by the Kjeldahl determinations were 13.39 and 12.75 cc. Added to the amounts neutralized by the ammonia evolved in the arginine determinations, these give 17.50 and 16.72 cc., indicating 0.0490 and 0.0468 gram of nitrogen.

Amino Nitrogen of the Bases. The amounts of nitrogen gas evolved were: 6.50 cc. at 23°, 760, and 6.00 cc. at 21°, 760 mm. These indicate 0.0183 and 0.0170 gram of amino nitrogen.

Amino Nitrogen of the Filtrate from the Bases. The amounts of nitrogen gas were: (I) 28.8 - 28.7 cc. at 23°, 748 mm., indicating 0.2399 gram of amino nitrogen in the filtrate; (II) 29.0 - 29.1 cc. of gas at 24°, 748 mm., indicating 0.2414 gram of nitrogen.

Total Nitrogen of the Filtrate. The amounts of \( H \) acid neutralized were: (I) 33.43 - 33.63 cc., indicating 0.2820 gram of nitrogen; (II) 33.93 - 34.13 cc., indicating 0.2860 gram of nitrogen.

The results calculated from these data are collected in the table on the following page.

The figures for the amino and non-amino nitrogen of the filtrate under "results by other methods" are calculated from the isolated amounts of the amino-acids which are listed in Table I as belonging to these fractions. The methods of isolation are known to be accompanied by unavoidable losses. Their extent is indicated by comparison with the results of the group determination method, which furnishes a criterion for the completeness of the isolations,
### Table VI.
Results of Gliadin Analysis in Percentages of Total Nitrogen.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>Average</th>
<th>AVERAGE CORRECTED FOR SOLUBILITY OF BASES</th>
<th>RESULTS BY OTHER METHODS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N</td>
<td>25.66</td>
<td>25.37</td>
<td>25.52</td>
<td>25.2†, 19.51†</td>
<td></td>
</tr>
<tr>
<td>Melanin N</td>
<td>0.86</td>
<td>0.86</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.72</td>
<td>0.63</td>
<td>0.68</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>Arginine N</td>
<td>5.10</td>
<td>11.1</td>
<td>10.3</td>
<td>5.01, 10.7</td>
<td>5.71, 12.9</td>
</tr>
<tr>
<td>Histidine N</td>
<td>4.42</td>
<td>4.28</td>
<td>4.36</td>
<td>5.20</td>
<td></td>
</tr>
<tr>
<td>Lysine N</td>
<td>0.82</td>
<td>0.49</td>
<td>0.64</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Amino N in filtrate</td>
<td>52.85</td>
<td>53.40</td>
<td>53.13</td>
<td>51.98</td>
<td></td>
</tr>
<tr>
<td>Non-amino N in filtrate</td>
<td>62.1</td>
<td>63.3</td>
<td>62.7</td>
<td>60.5</td>
<td></td>
</tr>
<tr>
<td>Total regained</td>
<td>99.72</td>
<td>99.76</td>
<td>99.78</td>
<td>28.5, 29.9†</td>
<td></td>
</tr>
</tbody>
</table>


[Osborne and Clapp: Amer. Journ. of physiol., xvii, p. 331.]


That a part of the large deficit in the latter may be due to unknown α-amino-acids is, of course, not impossible.

### Edestin.

Two separate portions of edestin were hydrolyzed, the course of the hydrolysis being followed as in the case of gliadin, by amino determinations until the latter showed constant results, which occurred after about 15 hours. The solutions used for the following analyses contained 0.3848 and 0.3305 gram of nitrogen respectively.

**Ammonia.** The amounts of $\text{NH}_3$ acid neutralized were 26.93 and 24.03 cc., indicating 0.0377 and 0.0336 gram of nitrogen.

**Melanin.** The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 4.24 and 5.23 cc., indicating 0.0064 and 0.0073 gram of nitrogen.

**Cystine.** The BaSO$_4$ weighed 0.0060 gram in each case, indicating 0.0027 gram of cystine nitrogen.

**Arginine.** The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 18.53 and 15.02 cc., indicating 0.1033 and 0.0841 gram of arginine nitrogen.
Analysis of Proteins

Total Nitrogen of the Bases. The amounts of acid neutralized by the Kjeldahl determinations were 29.22 and 27.25 cc. Added to the amounts neutralized by the ammonia in the arginine determination, these give 47.75 and 42.27 cc. indicating 0.1337 and 0.1184 gram of nitrogen.

Amino Nitrogen of the Bases. The amounts of nitrogen gas measured were: (I) 16.4 cc. at 19°, 762 mm., indicating 0.0470 gram of nitrogen; (II) 15.27 cc. at 22°, 758 mm., indicating 0.0429 gram of nitrogen.

Amino Nitrogen of the Filtrate. The amounts of nitrogen gas measured were: (I) 22.0 cc. at 18°, 762 mm., indicating 0.1896 gram of nitrogen, and 22.3 cc., at 20°, 762 mm. indicating 0.1909 gram; (II) 18.80 cc. at 19°, 762 mm., indicating 0.1616 gram of nitrogen, and 18.90 cc. at 22°, 758 mm., indicating 0.1594 gram.

Total Nitrogen of the Filtrate. The amounts of acid neutralized were: (I) 24.22 cc., indicating 0.2034 gram of nitrogen; (II) 20.1 and 20.3 cc., the average, 20.2, indicating 0.1698 gram of nitrogen.

| TABLE VII. |
| Results of Edestin Analysis in Percentages of the Total Nitrogen. |

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>AVERAGE OF CORRECTED RESULTS</th>
<th>RESULTS BY OTHER METHODS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLAUCONIC</td>
<td>CORRECTED FOR ALKALI</td>
<td>GLAUCONIC</td>
<td>CORRECTED FOR ALKALI</td>
</tr>
<tr>
<td>Ammonia N</td>
<td>9.80</td>
<td>10.17</td>
<td>9.99</td>
<td>1.6†</td>
</tr>
<tr>
<td>Melanin N</td>
<td>1.83</td>
<td>2.12</td>
<td>1.98</td>
<td></td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.71</td>
<td>1.38</td>
<td>0.82</td>
<td>1.60</td>
</tr>
<tr>
<td>Arginine N</td>
<td>26.85</td>
<td>34.8</td>
<td>25.45</td>
<td>26.41</td>
</tr>
<tr>
<td>Histidine N</td>
<td>3.63</td>
<td>4.63</td>
<td>5.63</td>
<td>6.77</td>
</tr>
<tr>
<td>Lysine N</td>
<td>3.58</td>
<td>3.72</td>
<td>3.93</td>
<td>4.01</td>
</tr>
<tr>
<td>Amino N of</td>
<td>49.4</td>
<td>43.1</td>
<td>48.6</td>
<td>47.0</td>
</tr>
<tr>
<td>the Filtrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-amino</td>
<td>52.9</td>
<td>51.4</td>
<td></td>
<td>49.2</td>
</tr>
<tr>
<td>N in Filtrate</td>
<td>3.5</td>
<td>2.1</td>
<td>2.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Sum</td>
<td>99.30</td>
<td>99.52</td>
<td>99.37</td>
<td></td>
</tr>
</tbody>
</table>

Hair.

Ten grams of dog's hair were hydrolyzed, in the same manner as the gliadin, and the solution brought to 250 cc. Of this, 75 cc. portions, containing, according to Kjeldahl determinations 0.4495 gram of nitrogen each, (10 cc. of solution neutralized 35.64–35.72 cc. of $\frac{N}{10}$ acid) were used for the following duplicates.

Ammonia. The ammonia neutralized 32.07 and 32.05 cc. of $\frac{N}{10}$ acid, indicating 0.0449 and 0.0449 gram of amid nitrogen.

Melanin. The amounts of $\frac{N}{10}$ acid neutralized were 23.98 and 23.58 cc., indicating 0.0336 and 0.0330 gram of nitrogen.

Cystine. The weights of BaSO$_4$ were 0.0668 and 0.0910 gram, indicating 0.0269 and 0.0273 gram of cystine nitrogen.

Arginine. The amounts of $\frac{N}{10}$ acid neutralized were 13.39 and 13.37 cc., or, subtracting the corrections for the amounts of ammonia evolved by the unusually large amount of cystine, 11.76 and 11.71 cc., indicating 0.0659 and 0.0656 gram of arginine nitrogen.

Total Nitrogen of the Bases. The amounts of $\frac{N}{10}$ acid neutralized by the Kjeldahl determinations were 32.34 and 32.53 cc., which, added to the amounts previously neutralized by the ammonia from the arginine and cystine, give 45.73 and 45.39 cc., indicating 0.1281 and 0.1236 gram of nitrogen.

Amino Nitrogen of the Bases. The amounts of nitrogen gas evolved were: (I) 26.20 cc. at 21°, 776 mm., (II) 25.30 cc. at 20°, 760 mm. These indicate 0.0740 and 0.0718 grams of amino nitrogen, or 0.0722 and 0.0700 grams when corrected for the abnormal behavior of cystine with nitrous acid.

Amino Nitrogen of the Filtrate. The amounts of nitrogen gas were: (I) 25.7 – 25.4 cc. at 20°, 770 mm., indicating 0.2207 gram of nitrogen; (II) 25.50 cc. at 19°, 760 mm., and 25.10 cc. at 20°, 760 mm., indicating 0.2157 and 0.2162 gram of nitrogen.

Total Nitrogen of the Filtrate. The amounts of $\frac{N}{10}$ acid neutralized were 28.38 – 28.31 and 28.25 – 28.20 cc., indicating 0.2382 and 0.2373 gram of nitrogen.
Analysis of Proteins

TABLE VIII.

Results of Dog's Hair Analysis in Percentages of the Total Nitrogen.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>AVERAGE</th>
<th>AVERAGE CORRECTED FOR SOLUBILITY OF BASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N.</td>
<td>10.10</td>
<td>9.99</td>
<td>10.05</td>
<td></td>
</tr>
<tr>
<td>Melanin N.</td>
<td>7.35</td>
<td>7.48</td>
<td>7.32</td>
<td></td>
</tr>
<tr>
<td>Cystine N.</td>
<td>6.08</td>
<td>6.09</td>
<td>6.04</td>
<td>6.00</td>
</tr>
<tr>
<td>Histidine N.</td>
<td>2.20</td>
<td>3.13</td>
<td>2.67</td>
<td>3.48</td>
</tr>
<tr>
<td>Lysine N.</td>
<td>5.72</td>
<td>5.26</td>
<td>5.50</td>
<td>5.70</td>
</tr>
<tr>
<td>Amino N of filtrate</td>
<td>49.5</td>
<td>53.1</td>
<td>52.9</td>
<td>52.9</td>
</tr>
<tr>
<td>Non-amino N of filtrate</td>
<td>4.8</td>
<td>4.2</td>
<td>4.2</td>
<td>3.1</td>
</tr>
<tr>
<td>Total regained</td>
<td>99.23</td>
<td>98.46</td>
<td>98.96</td>
<td></td>
</tr>
</tbody>
</table>

Gelatin.

Ten grams of gelatin were hydrolyzed, as before described, and the solution brought to 250 cc. Ten cubic centimeter portions were used for Kjeldahl determinations. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 40.95 and 40.50 cc., indicating 0.428 gram of nitrogen in each of the 75 cc. portions used for the following duplicate determinations.

Ammonia. The ammonia neutralized 7.00 and 6.85 cc., of $\text{H}_2\text{SO}_4$ acid, indicating 0.0098 and 0.0096 gram of ammonia nitrogen.

Melanin. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 2.0 cc. in each case, indicating 0.0028 gram of nitrogen.

Cystine. The sulfur determinations were negative.

Arginine. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 11.10 and 10.30 cc., indicating 0.0621 and 0.0577 gram of arginine nitrogen.

Total Nitrogen of the Bases. The Kjeldahl determinations neutralized 25.55 and 25.60 cc. of $\text{H}_2\text{SO}_4$ acid. Added to the amounts neutralized in the arginine determinations, these give 36.65 and 35.90 cc.; indicating 0.1027 and 0.1096 gram of nitrogen.

Amino Nitrogen of the Bases. The amounts of nitrogen evolved were 16.1 cc. at 17°, 758 mm., and 16.3 cc. at 19°, 758 mm., indicating 0.0464 and 0.0467 gram of amino nitrogen.

Amino Nitrogen of the Filtrate. The amounts of nitrogen evolved were: (I) 29.25 — 29.45 cc. at 24°, 750 mm., the average indicating 0.2428 gram of amino nitrogen; (II) 29.50 at 23°, 750 mm. and 29.40 cc. at 22°, 750 mm., both indicating 0.245 gram of amino nitrogen.
Total Nitrogen of the Filtrate. The amounts of $\text{NH}_3$ acid neutralized were 37.20 - 37.00 and 37.28 - 37.28 cc., indicating respectively 0.3118 and 0.3130 gram of nitrogen.

**TABLE IX.**

Results of Gelatin Analysis in Percentages of the Total Nitrogen.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>Average</th>
<th>Average Corrected for Solubility of Bases</th>
<th>Results by Other Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N</td>
<td>2.30</td>
<td>2.20</td>
<td>2.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanin N</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine N</td>
<td>14.52</td>
<td>13.48</td>
<td>14.00</td>
<td>23.6</td>
<td>14.74</td>
</tr>
<tr>
<td>Histidine N</td>
<td>3.39</td>
<td>3.79</td>
<td>3.59</td>
<td></td>
<td>4.48</td>
</tr>
<tr>
<td>Lysine N</td>
<td>6.10</td>
<td>6.31</td>
<td>6.20</td>
<td></td>
<td>6.32</td>
</tr>
<tr>
<td>Amino N of the Filtrate</td>
<td>56.7</td>
<td>57.3</td>
<td>57.0</td>
<td>73.0</td>
<td>55.8</td>
</tr>
<tr>
<td>Non-amino N of Filtrate</td>
<td>16.1</td>
<td>15.9</td>
<td>16.0</td>
<td>14.9</td>
<td>70.7</td>
</tr>
</tbody>
</table>


The unusually high percentage of non-amino nitrogen in the filtrate indicates an exceptional amount of proline and oxyproline, which have, in fact, been isolated in previous hydrolyses by Levene and Beatty, and by Fischer and Boehner.

**Fibrin.**

Ten grams of Merck's fibrin were hydrolyzed in the usual manner, and the solution was brought to 250 cc. volume. Ten cubic centimeter portions taken for Kjeldahl determinations neutralized 42.00-42.15 cc. of $\text{NH}_3$ acid, indicating 0.4415 gram of nitrogen in each of the 75 cc. samples used for the following determinations.

**Ammonia.** The amounts of $\text{NH}_3$ acid neutralized were 26.0 and 26.4 cc., indicating 0.0364 and 0.0370 gram of ammonia nitrogen.
Melanin. The amounts of $\frac{N}{10}$ acid neutralized were 10.8 and 9.08 cc., indicating 0.0151 and 0.0127 gram of nitrogen.

Cystine. The amounts of BaSO$_4$ were 0.0059 and 0.0070 gram, indicating 0.0018 and 0.0021 gram of cystine nitrogen.

Arginine. The amounts of $\frac{N}{10}$ acid neutralized were 10.72 and 9.83 cc., indicating 0.0600 and 0.0551 gram of arginine nitrogen.

Total Nitrogen of the Bases. The amounts of $\frac{N}{10}$ acid neutralized were 34.3 and 36.0 cc. When added to the amounts neutralized in the arginine determinations, these give 45.0 and 45.8 cc., indicating total basic nitrogen of 0.1261 and 0.1284 gram.

Amino Nitrogen of the Bases. The amounts of nitrogen gas were 24.1 and 25.0 cc. at 21°, 772 mm., indicating 0.0693 and 0.0719 gram of amino nitrogen.

Amino Nitrogen of the Filtrate from the Bases. The amounts of nitrogen were: (I) 28.2 - 28.1 cc. at 17°, 760 mm., indicating 0.244 gram of amino nitrogen; (II) 27.7 - 27.9 cc. at 19°, 768 mm., indicating 0.2452 gram of amino nitrogen.

Total Nitrogen of the Filtrate. The amounts of $\frac{N}{10}$ acid neutralized were 31.3 - 31.5 and 30.9 - 31.0 cc., indicating respectively 0.2638 and 0.2597 gram of nitrogen.

**TABLE X.**

Results of Fibrin Analysis in Percentages of the Total Nitrogen.

<table>
<thead>
<tr>
<th>Ammonia N</th>
<th>Melanin N</th>
<th>Cystine N</th>
<th>Arginine N</th>
<th>Histidine N</th>
<th>Lysine N</th>
<th>Amino N in filtrate</th>
<th>Non-amino N in filtrate</th>
<th>Total N recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.24</td>
<td>3.43</td>
<td>0.41</td>
<td>13.60</td>
<td>3.78</td>
<td>10.78</td>
<td>55.3 59.7</td>
<td>4.4 59.7</td>
<td>99.94 99.13</td>
</tr>
<tr>
<td>8.38</td>
<td>2.87</td>
<td>0.45</td>
<td>12.48</td>
<td>4.14</td>
<td>12.01</td>
<td>55.5 58.8</td>
<td>3.3 59.3</td>
<td>99.13 99.67</td>
</tr>
<tr>
<td>8.32</td>
<td>3.17</td>
<td>0.43</td>
<td>29.1</td>
<td>3.96</td>
<td>11.40</td>
<td>55.4 59.3</td>
<td>3.85 59.3</td>
<td>99.67 99.67</td>
</tr>
</tbody>
</table>

Hemocyanin of Limulus.

The hemocyanin in the blood of Limulus, the horse-shoe crab, takes the place of hemoglobin in the blood of mammals. The specimen analyzed was kindly furnished by Dr. C. L. Alsberg, from material prepared at Woods Hole. Seven grams of the protein were hydrolyzed, and the solution made up to 250 cc.
tions of 20 cc. were taken for Kjeldahl determinations. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 64.1 and 64.0 cc., indicating 0.449 gram of nitrogen in each of the samples of 100 cc. used in the following determinations.

Ammonia. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 19.10 and 19.05 cc., indicating in both duplicates 0.0267 gram of ammonia nitrogen.

Melanin. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 5.1 and 5.5 cc., indicating 0.0071 and 0.0077 gram of nitrogen.

Cystine. The weights of barium sulphate were 0.0062 and 0.0035 gram, indicating in each case 0.0010 gram of cystine nitrogen in the precipitate of the bases.

Arginine. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized were 11.80 and 12.30 cc., indicating 0.0661 and 0.0688 gram of arginine nitrogen.

Total Nitrogen of the Bases. The amounts of $\text{H}_2\text{SO}_4$ acid neutralized in the Kjeldahl determinations were 45.35 and 46.0 cc. These, added to the amounts neutralized in the arginine determinations, give 57.15 and 58.3 cc., indicating 0.1601 and 0.1632 gram of nitrogen in the precipitate of the bases.

Amino Nitrogen of the Bases. The amounts of nitrogen gas obtained were 25.8 and 25.4 cc. at 20° and 772 mm., indicating 0.0746 and 0.0734 gram of amino nitrogen.

Amino Nitrogen of the Filtrate. The amounts of nitrogen gas obtained were 27.4 — 27.6 cc. at 21°, 768 mm., indicating 0.2570 and 0.2580 gram of nitrogen in the filtrate from the bases.

### TABLE XI.

Results of Hemocyanin Analysis in Percentages of the Total Nitrogen.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>AVERAGE</th>
<th>AVERAGE CORRECTED FOR SOLUBILITY OF BASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
</tr>
<tr>
<td>Melanin N</td>
<td>1.59</td>
<td>1.71</td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>0.80</td>
</tr>
<tr>
<td>Arginine N</td>
<td>14.72</td>
<td>15.33</td>
<td>15.03</td>
<td>15.73</td>
</tr>
<tr>
<td>Histidine N</td>
<td>12.00</td>
<td>12.77</td>
<td>12.39</td>
<td>13.23</td>
</tr>
<tr>
<td>Lysine N</td>
<td>8.71</td>
<td>8.94</td>
<td>8.82</td>
<td>8.49</td>
</tr>
<tr>
<td>Amino N in filtrate</td>
<td>52.6</td>
<td>52.3</td>
<td>52.4</td>
<td>51.3</td>
</tr>
<tr>
<td>Non-amino N in filtrate</td>
<td>4.6</td>
<td>5.2</td>
<td>4.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Total N regained</td>
<td>100.39</td>
<td>101.52</td>
<td>100.92</td>
<td></td>
</tr>
</tbody>
</table>
Analysis of Proteins

Ox Hemoglobin.

The specimen of pure, crystallized hemoglobin for this analysis was kindly furnished me by Dr. Butterfield of this Institute. Because of the relatively small amount of the material the details of the analysis had to be somewhat modified, in that the bases were precipitated from a solution of 100 cc. instead of 200 cc. volume, and the filtrate from the bases was brought to 100 cc. instead of 150 before the aliquot portions for nitrogen determinations were taken. Also, the total nitrogen, 0.1740 gram, on the basis of which the percentages are calculated, was obtained by addition of the ammonia, melanin, basic nitrogen, and nitrogen of the filtrate, instead of taking a separate portion of the solution for a Kjeldahl determination at the beginning of the analysis. In other respects the details did not differ from those of the preceding analyses.

The hemoglobin, which weighed a little more than a gram air-dried, was hydrolyzed for 28 hours.

Ammonia. The ammonia neutralized 6.50 cc. of $\text{HNO}_3$ acid, indicating 0.0091 gram of ammonia nitrogen.

Melanin. The amount of $\text{HNO}_3$ acid neutralized was 4.53 cc., indicating 0.0063 gram of nitrogen.

Cysteine. The weight of barium sulphate was less than 1 mg., which is considered a negative result.

Arginine. The amount of $\text{HNO}_3$ acid neutralized was 2.12 cc., indicating 0.0118 gram of arginine nitrogen.

Total Nitrogen of the Bases. The amount of $\text{HNO}_3$ acid neutralized in the Kjeldahl determination was 15.87 cc. Added to the amount neutralized in the arginine determination, this gives 17.99 cc., indicating 0.0504 gram of nitrogen in the precipitate of the bases.

Amino Nitrogen of the Bases. The amount of nitrogen gas obtained was 10.06 cc. at 21°, 752 mm., indicating 0.028 gram of nitrogen.

Amino Nitrogen of the Filtrate. The amounts of nitrogen gas obtained were 18.23 cc. at 21°, 749 mm., and 18.99 cc. at 21°, 749 mm. These indicate 0.1017 and 0.0995 gram of amino nitrogen, or an average of 0.1006.

Total Nitrogen of the Filtrate. The duplicates each neutralized 19.35 cc. of $\text{HNO}_3$ acid, indicating 0.1083 gram of nitrogen.
Donald D. Van Slyke

TABLE XII.

Results of Ox Hemoglobin Analysis in Percentages of the Total Nitrogen.

<table>
<thead>
<tr>
<th></th>
<th>UNCORRECTED</th>
<th>CORRECTED FOR SOLUBILITY OF BASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N</td>
<td>5.24</td>
<td>7.7</td>
</tr>
<tr>
<td>Melanin N</td>
<td>3.6</td>
<td>7</td>
</tr>
<tr>
<td>Arginine N</td>
<td>6.8</td>
<td>31.3</td>
</tr>
<tr>
<td>Cystine N</td>
<td>0.00</td>
<td>10.9</td>
</tr>
<tr>
<td>Histidine N</td>
<td>11.6</td>
<td>57.0</td>
</tr>
<tr>
<td>Lysine N</td>
<td>10.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Amino N in filtrate</td>
<td>57.8</td>
<td>60.9</td>
</tr>
<tr>
<td>Non-amino N in filtrate</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Total N*</td>
<td>(99.9)</td>
<td></td>
</tr>
</tbody>
</table>

*The total nitrogen in this case necessarily adds up to 100 ± 0.1 per cent because the total nitrogen obtained by addition is used as the basis for calculating the other percentages. It does not therefore serve as a control for the accuracy of the hemoglobin analysis.

From hydrolysis of horse hemoglobin Abderhalden obtained 9.8 per cent of arginine nitrogen, 16.7 per cent of histidine nitrogen, 4.6 per cent of lysine nitrogen, 27.4 per cent of primary mono-amino-acid nitrogen, and 2.2 per cent of proline and oxyproline nitrogen.1 The high histidine content is a striking characteristic of both ox and horse hemoglobin, as well as of Limulus hemocyanin.

In the following table the average results of the protein analyses, corrected for the solubilities of the bases, are collected.

1 Zeitschr. f. physiol Chem., xxxvii, p. 492, 1903.
Analysis of Proteins

**TABLE XIII.**

Summary of Corrected Results in Percentages of the Total Nitrogen of the Proteins.

<table>
<thead>
<tr>
<th></th>
<th>Gliadin</th>
<th>Egg-sal-</th>
<th>Hair (Dog)</th>
<th>Gela-</th>
<th>Fibri-</th>
<th>Hemo-</th>
<th>Ox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia N</td>
<td>25.52</td>
<td>9.99</td>
<td>10.05</td>
<td>2.25</td>
<td>8.32</td>
<td>5.95</td>
<td>5.24</td>
</tr>
<tr>
<td>Melanine N</td>
<td>0.86</td>
<td>1.98</td>
<td>7.42</td>
<td>0.07</td>
<td>3.17</td>
<td>1.65</td>
<td>3.6</td>
</tr>
<tr>
<td>Cystine N</td>
<td>1.25</td>
<td>1.49</td>
<td>0.60</td>
<td>0.7</td>
<td>0.99</td>
<td>0.60</td>
<td>0.7</td>
</tr>
<tr>
<td>Arginine N</td>
<td>5.71</td>
<td>27.05</td>
<td>7.33</td>
<td>14.70</td>
<td>13.86</td>
<td>15.73</td>
<td>7.7</td>
</tr>
<tr>
<td>Histidine N</td>
<td>5.20</td>
<td>5.75</td>
<td>3.48</td>
<td>4.48</td>
<td>4.83</td>
<td>13.23</td>
<td>12.7</td>
</tr>
<tr>
<td>Lysine N</td>
<td>0.75</td>
<td>3.86</td>
<td>5.37</td>
<td>0.32</td>
<td>11.51</td>
<td>8.49</td>
<td>10.9</td>
</tr>
<tr>
<td>Amino N of the filtrate</td>
<td>51.98</td>
<td>47.55</td>
<td>47.5</td>
<td>56.3</td>
<td>54.3</td>
<td>51.3</td>
<td>57.0</td>
</tr>
<tr>
<td>Non-amino N of the filtrate (proline, oxi-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proline, 1/3 trypto-</td>
<td>3.50</td>
<td>1.7</td>
<td>3.1</td>
<td>14.9</td>
<td>2.7</td>
<td>3.8</td>
<td>2.9</td>
</tr>
<tr>
<td>phane)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td>99.77</td>
<td>99.37</td>
<td>98.85</td>
<td>99.02</td>
<td>99.58</td>
<td>100.95</td>
<td>(100.0)*</td>
</tr>
</tbody>
</table>

*Cf. note to preceding table.

The question marks in the decimal places for the figures on the cystine of gelatin and hemoglobin indicate that no cystine was found in the precipitate of the bases, but that a fraction of a percent may have been present in the filtrate. The small amount of lysine obtained in the analysis of gliadin practically falls within the limit of experimental error, and is not to be regarded as contradictory to the negative results of previous authors. The marked individual peculiarities of the different proteins, such as the high ammonia and low lysine of gliadin, the low ammonia and high non-amino nitrogen of the filtrate of gelatin, the high histidine of hemoglobin and hemocyanin, are apparent on inspection of the above table.

**SUMMARY.**

Methods are developed for following the course of protein hydrolysis and detecting the time at which it is complete, and for determining the ammonia (amid nitrogen), arginine, histidine, lysine, cystine, amino nitrogen, not precipitated by phosphotungstic acid

1 Loc. cit.
(the group of ordinary primary amino-acids, leucine, alanine, etc.), and the non-amino nitrogen not precipitated by phosphotungstic acid (proline, oxyproline, one-half the tryptophane nitrogen). The scheme of analysis is outlined on pages 17–18.

The analysis require but 2.5 to 3 grams of protein, and indicates the nature of 98 to 100 per cent of the nitrogenous products of hydrolysis. The results of hydrolyses of wheat gliadin, edestin from hemp seed, dog's hair, gelatin, fibrin, hemocyanin (the protein from the blood of the king-crab corresponding to the hemoglobin of higher animals), and of ox hemoglobin are summarized in the last table. The limits of accuracy of the determinations are indicated by the figures in Table III (page 32).

Comparison of the amounts of amino-acids of the different groups present in proteins, found by the quantitative group determination method, with the amounts isolated in previous hydrolyses, indicates that the losses of isolation have fallen chiefly on the primary mono-α-amino-acids, containing all their nitrogen in the $-\text{CH(NH}_2\text{)} - \text{COOH}$ group. So far as can be judged from the analyses available for comparison, the presence of unknown amino-acids outside of this group seems improbable. Whether the losses of isolating those of this group arise from the presence of new, as yet unrecognized mono-amino-acids, or from the unavoidable losses connected with the methods of isolation, can not at present be stated.
THE ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO-ACIDS

Donald D. Van Slyke

J. Biol. Chem. 1911, 10:15-55.

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