platinum contents, differ distinctly from the chloroplatinate of natural muscarine in crystal forms, the latter crystallizing in octahedra which have no definite melting point.[1]

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[CONTRIBUTION FROM THE DIVISION OF AGRICULTURAL BIOCHEMISTRY, MINNESOTA AGRICULTURAL EXPERIMENT STATION.]

## THE COLORIMETRIC ESTIMATION OF TYROSINE BY THE METHOD OF FOLIN AND DENIS.[2]

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Folin and Macallum,[3] in 1912, noted that uric acid and phenols produced a deep blue color in solutions of phosphotungstic acid when alkali was subsequently added. This observation was rapidly followed by a paper by Folin and Denis[4] in which they give detailed directions for the preparation of 2 reagents, one of which contains phosphotungstic acid and the other both phosphotungstic and phosphomolybdic acids. The former reacts with uric acid but not with monohydric phenols including tyrosine, while the latter reagent produces a beautiful blue color with phenol solutions, the reaction, according to Folin and Denis, being unmistakably positive with 1 part of tyrosine in 1,000,000 parts of water.

In a third paper Folin and Denis[5] apply the phenol reagent to the colorimetric estimation of tyrosine in proteins, without, however, reporting any careful quantitative study of the reagent when pure tyrosine was used. In all some 27 proteins were tested for tyrosine content by the new reagent and in every instance more tyrosine was found than was recorded in the literature from gravimetric determinations. The average percentage of tyrosine found was 5.065% by the colorimetric method and 2.647% by the gravimetric method for the 20 proteins where both figures are available. Folin and Denis express their belief that tyrosine is the only component of the proteins which reacts with the phenol reagent and that the colorimetric method gives an accurate measure of the amount of tyrosine present.

Shortly after Folin's paper appeared Abderhalden and Fuchs[6] and Abderhalden[7] stated that tryptophane, oxytryptophane and oxyproline

[1] A. J. Ewins, *Biochem. J.*, 8, 209 (1914).

[2] Published with the approval of the Director as Paper No. 203, Journal Series of the Minnesota Agricultural Experiment Station. Presented before the Division of Biological Chemistry at the Spring Meeting of the American Chemical Society, St. Louis, April 12–16, 1920.

[3] *J. Biol. Chem.*, 11, 265–6 (1912).

[4] *Ibid.*, 12, 239–243 (1912).

[5] *Ibid.*, 12, 245–251 (1912).

[6] *Z. physiol. Chem.*, 83, 468 (1913).

[7] *Ibid.*, 85, 91 (1913).

likewise gave the blue color with Folin's phenol reagent and that, if these amino acids were present, reliable tyrosine values could not be obtained.

Only recently Johns and Jones[1] have reviewed the previous work. They point out that Abderhalden's criticism is based on qualitative tests. Consequently they proceed to make a quantitative study of the behavior of tryptophane. They state:

"It is true that tryptophane gives a blue color with the reagent of Folin and Denis, but the intensity of the color is much less than that given by an equivalent weight of tyrosine. It is well known that tryptophane is decomposed by acid hydrolysis. To ascertain whether this decomposition is complete and that the decomposition products do not give a color with the reagent of Folin and Denis, the following experiment was made: 5% of tryptophane was added to 0.5 g. of kafirin, and the mixture was boiled with 20% hydrochloric acid for 12 hours. The tyrosine in the hydrolysate was estimated by the method of Folin and Denis and was found to be 4.36%. A hydrolysis of kafirin, performed under similar conditions without the addition of tryptophane, gave 4.84% of tyrosine. Hence the tryptophane was completely decomposed and its decomposition products gave no blue color with the reagent of Folin and Denis. It is also to be expected that oxytryptophane, if present, would be decomposed by acid hydrolysis, since the presence of the hydroxyl group would probably render it less stable than tryptophane. A sample of gelatin to which tryptophane had been added was also hydrolyzed. The blue color obtained with the reagent of Folin and Denis was of the same intensity as that obtained by a control hydrolysis where no tryptophane was added. The faint blue color obtained from the hydrolysate of the gelatin was probably due to tyrosine since the gelatin gave a distinct test for tyrosine with Millon's reagent.

Abderhalden states that *l*-oxyproline gives a blue color with the reagent of Folin and Denis. On the other hand, Folin and Denis obtained only a faint blue color from the hydrolysate of gelatin which contains 3 to 6 % of oxyproline. This color was probably due to tyrosine. We tested a number of high-grade samples of gelatin and did not find one that did not respond to the test for tyrosine with Millon's reagent. Even gelatin prepared from carefully cleaned cartilaginous rings of ox trachea gave a decided test for tyrosine."

Johns and Jones, therefore, conclude that reliable values for the tyrosine content of a protein can be obtained by the use of Folin's phenol reagent.

We recently desired to estimate the amount of tyrosine remaining in a series of mixtures of pure amino acids which had been boiled for some time in the presence of hydrochloric acid, and in some instances in the presence of formaldehyde. Tyrosine was present in 3 of the mixtures, tryptophane was present in 2, while in one neither tyrosine nor tryptophane was present.[2] The samples in which the "tyrosine" content was measured were the solutions of the "bases" and the "filtrate from the bases" as separated by Van Slyke's method.[3]

In certain of the experiments our method of work was such that all

[1] *J. Biol. Chem.*, **36**, 319–322 (1918).

[2] For a composition of the mixture see Gortner and Holm, "The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. V," THIS JOURNAL, **42**, 821–27 (1920).

[3] *J. Biol. Chem.*, **10**, 15–55 (1911).

tyrosine had been removed from the soutions *before* testing with the phenol reagent.[1] What was our surprise, therefore, to find that both the "bases" and the "filtrate from the bases" gave, in every instance, a blue color with Folin's phenol reagent, this being the case even in the mixture where no tyrosine was present. We accordingly felt justified in regarding the color developed in this last instance as a "blank" and by subtracting the value of the color developed in the "blank" from the remaining experiments of the series we found that practically 100% of the tyrosine added was present in those solutions to which tyrosine but no aldehyde had been added, while in corresponding solutions to which aldehyde had been added no tyrosine remained. The added aldehyde, therefore, had quantitatively removed 100 mg. of tyrosine, or at least changed it so that it no longer gave a color with the phenol reagent, but besides the tyrosine there was still some compound present in each solution which reacted with the phenol reagent but which was not tyrosine. We therefore decided to make a careful quantitative study of the effect of various amino acids, and of various other factors upon the development of the blue color in solutions containing the phenol reagent.

## Experimental.

**The Reagent.**—A large quantity of Folin's phenol reagent was prepared exactly as outlined by Folin and Denis.[2] 100 g. of sodium tungstate, 20 g. of phosphomolybdic acid, and 50 cc. of 85% phosphoric acid were added to 750 cc. of distilled water and the mixture was refluxed for 2 hours and then diluted to one liter. Folin and Denis'[3] directions for the use of this reagent in the quantitative estimation of tyrosine in proteins are as follows.

"One gram of the dry protein is accurately weighed out and transferred to a 500-cc. Kjeldahl flask, 25 cc. of 20% hydrochloric acid is then added, the flask closed by means of a Hopkins condenser made from a large test-tube, and the contents of the flask boiled for twelve hours over a microburner. At the end of this time the flame is removed, the contents of the flask transferred on cooling to a 100 cc. volumetric flask and made up to volume. One or two cubic centimeters of this solution are then transferred to a 100-cc. volumetric flask, 5 cc. of the tyrosine reagent added, and after five minutes, 25 cc. of a saturated solution of sodium carbonate, and the mixture then made up to volume with cold tap water. The maximum color develops in about ten minutes. Therefore, the reading should not be made before this time has elapsed. Fading is very slow in the presence of the large excess of reagent used. As nearly at the same time as possible a standard is prepared by treating 1 mgm. of pure tyrosine with 5 cc. of the phosphotungstic-phosphomolybdic reagent, then adding 25 cc. of saturated sodium carbonate solution and making up to volume. The color comparison is made by means of a Duboscq colorimeter, the standard solution being placed at 20 mm.

[1] The tyrosine had been combined with formaldehyde to form "soluble humin." See Gortner and Holm, *loc. cit.*

[2] *J. Biol. Chem.*, **12**, 239–43 (1912)

[3] *Ibid.*, 12, 245–251 (1912).

As a standard solution, we use a solution of pure tyrosine in decinormal hydrochloric acid which is made of such a concentration that 5 cc. contain 1 mgm. of tyrosine."

We have followed these directions with the following exceptions. (1) Distilled water was used in ever instance instead of tap water. (2) 10 cc. of the reagent was used instead of the 5 cc. recommended by Folin and Denis. The reasons for this variation will be given later. (3) All readings were made with a Kober colorimeter, carefully adjusted and standardized.

**Qualitative Tests on Amino Acids.**—Our first tests were purely qualitative and we obtained, using 50 mg. of the amino acid, deep blue solutions from tyrosine, tryptophane, arginine, lysine hydrochloride and proline; light blue solutions from phenyl alanine, cystine, histidine and alanine; and pale blue solutions from serine (synth.) and valine, while leucine, glycine, aspartic acid and glutamic acid gave no color. We were aware that our arginine and proline were not absolutely pure and to date we have failed to obtain these amino acids in such a degree of purity that we fell justified in accepting the color values as final. We may say, however, that as purification has proceeded the color-producing power has, in some measure, decreased, indicating that at least a part of the color-producing power may have been due to an impurity. Likewise, our lysine dihydrochloride, while apparently pure, had been prepared from the picrate so that even though it gave no Millon's test, we have omitted this amino acid from our final studies. By careful manipulation we obtained a Millon's reaction with the phenylalanine and alanine so that their reaction was probably due, in part at least, to traces of tyrosine.

**Preliminary Quantitative Experiments.**—The qualitative experiments were repeated using 100 mg. of each amino acid. In these experiments only 5 cc. of Folin and Denis' reagent was used. The color values were read against a standard of one mg. of tyrosine + 5 cc. of the reagent. The results are shown in Table I. *This table is of value only in indicating that amino acids analyzing 100% pure by our accepted methods of analysis may actually contain as much as 1% (as in the case of phenylalanine isolated from protein) of some other amino acid.*[1]

The table also indicated that any interference of the other amino acids with a tyrosine determination would be practically negligible. However, we could detect no tyrosine in our tryptophane preparation. Inasmuch as we had available 7 samples of typtophane which had been prepared by various workers at different times, we decided to see whether the color was due to tryptophane itself or to a tyrosine contamination, since it appeared improbable that all samples would be contaminated to an equal

[1] The high values for arginine and proline are ignored because, as stated above, we knew that these amino acids were somewhat impure. The color values for tryptophane, as will be shown later, are not due to an impurity but to the tryptophane itself.

TABLE I.

The Color Equivalent of the Various Amino Acids Tested. (100 mg. of amino acid + 5 cc. of Folin and Denis' Reagent to 100 cc. Volume.)

| Amino acid. | Color intensity equivalent to mg. tyrosine.[1] |
|---|---|
| Tryptophane | 2.5 |
| Arginine | 1.48 |
| Proline | 1.40 |
| Phenyl alanine | 1.17 |
| Phenyl alanine (synth.) | 0.075 |
| Alanine | 0.60 |
| Alanine (synth.) | None |
| Cystine | 0.21 |
| Histidine dihydrochloride | 0.16 |
| Serine (synth.) | 0.15 |
| Valine | 0.085 |
| Glycine | Trace |
| Leucine | None |
| Glutamic acid | None |
| Aspartic acid | None |

[1] See statement in text regarding these color values.

degree. In this new experiment we used quantities of tryptophane varying from 20 to 50 mg. with amounts of phenol reagent varying from 2.5 to 5 cc. The resulting solutions were made to a volume corresponding to a concentration of one mg. of the tryptophane in each cc. of final volume. The results are shown in Table II.

TABLE II.

Color Value of Different Samples of Tryptophane Under Various Experimental Conditions.

| Sample. | Tryptophane. Mg. | Vol. reagent. Cc. | Final vol. Cc. | Color value. 100 mg. tryptophane = mg. tyrosine. |
|---|---|---|---|---|
| 1 | 50 | 2.5 | 50 | 2.5 |
| 2 | 25 | 2.5 | 25 | 4.71 |
| 3 | 20 | 2.5 | 20 | 6.09 |
| 4 | 50 | 5.0 | 50 | 4.83 |
| 5 | 50 | 5.0 | 50 | 4.80 |
| 6 | 50 | 5.0 | 50 | 5.09 |
| 7 | 50 | 5.0 | 50 | 4.97 |

It will be noted that *Samples 2, 4, 5, 6 and 7 gave almost identical color values, that Sample 1, which gave only half of the color value, contained only* $^1/_2$ *as much phenol reagent, and that Sample 3, which had 25% excess of reagent, gave 24.8% more color.* In other words, all samples were apparently identical and the color developed was dependent on a ratio of amino acid to the phenol reagent. By doubling the amount of reagent the color increased approximately 100%. This suggested a quantitative study of the effect of concentration of the amino acid on the color produced when all other factors, such as volume of reagent, final volume, etc., were kept constant.

*The effect of concentration of reactive substance upon color depth.* Insofar as we are aware, no study of the relation of color intensity to concentration of reactive substance has been made, although the phenol reagent has been used by various workers. Folin and Denis[1] state, "two cubic centimeters will give the maximum color with 1 mg. of tyrosine or uric acid," but give no data to show exact relations between color and concentration of reagents.

We have, therefore, measured the depth of color produced in solutions of tyrosine, tryptophane, uric acid, ferrous iron, and α-methyl indole in the presence of the phenol reagent. In every instance 10 cc. of the phenol reagent, 25 cc. of sodium carbonate and a final volume of 100 cc. were used, the only variable being the reactive organic compound which was used in concentrations ranging from 0.5 mg. to 100 mg. The colors in every instance were read against a tyrosine standard of one mg. of tyrosine + 10 cc. of reagent in a final volume of 100 cc. Table III shows the colori-

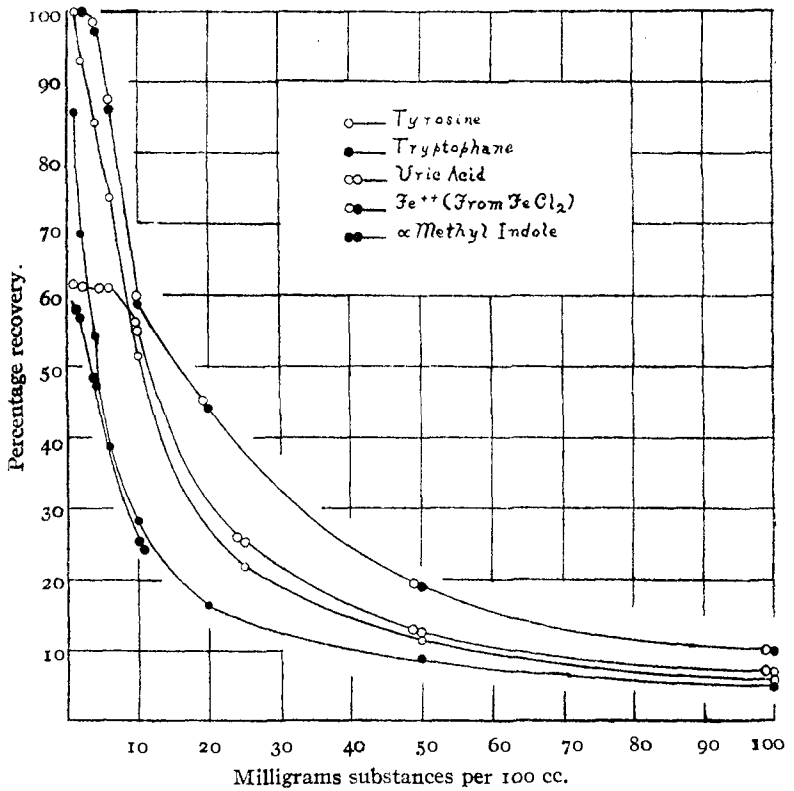


Fig. 1.—Showing the relation of color developed by the Folin-Denis phenol reagent to the concentration of reactive material.

[1] *J. Biol. Chem.*, **12**, 240 (1912).

metric ratios which were obtained. These data were recalculated so as to show the percentage of material which would have been indicated on the basis of the colorimetric data, using a tyrosine standard. These figures are given in Table IV and in the form of curves in Fig. 1.

TABLE III.

Showing the Relation of Final Color Depth to Concentration of Reactive Materials.

| Concentration of substance in 100 cc. of final volume. | Ratio of color developed in solutions of the following substances at different concentrations to the color of the standard tyrosine solution. | | | | | |
|---|---|---|---|---|---|---|
| | | | | | α-Methyl indole.[1] | |
| Mg. | Tyrosine. | Tryptophane. | Uric acid. | Fe″. | Mg. indole. | Color ratio. |
| 1 | 1.00 | 0.858 | 0.614 | 0.995 | 0.44 | 0.272 |
| 2 | 1.859 | 1.370 | 1.157 | 2.072 | 0.88 | 0.515 |
| 4 | 3.365 | 2.162 | 2.427 | 3.885 | 1.32 | 0.760 |
| 6 | 4.404 | 2.326 | 3.649 | 5.162 | 2.20 | 1.250 |
| 10 | 5.150 | 2.798 | 5.492 | 5.883 | 4.40 | 2.075 |
| 20 | ... | 3.278 | ... | 8.800 | 11.00 | 2.631 |
| 25 | 5.460 | ... | 6.290 | .... | ... | ... |
| 50 | 5.741 | 4.520 | 6.410 | 9.526 | ... | ... |
| 100 | 5.741 | 4.900 | 7.092 | 10.000 | ... | ... |

[1] The α-methyl indole has a maximum solubility of 22 mg. per 100 cc. of solution. Therefore, no more than 11 mg. could be used in our work (50 cc. of indole solution, 10 cc. of reagent, 25 cc. of $Na_2CO_3$ solution and water to 100 cc.).

TABLE IV.

Showing the Percentage of the Reactive Substance which would have been Indicated by the Color Depth, Using a Tyrosine Standard.

| Concentration of substance in 100 cc. of final volume. | Percentage of reactive substance "recovered" by colorimetric measurement. | | | | | |
|---|---|---|---|---|---|---|
| | | | | | α-Methyl indole. | |
| Mg. | Tyrosine. | Tryptophane. | Uric acid. | Fe″. | Mg. indole. | %. |
| 1 | 100.00 | 85.84 | 61.40 | 99.5 | 0.44 | 61.83 |
| 2 | 92.95 | 68.50 | 57.90 | 103.6 | 0.88 | 58.57 |
| 4 | 84.13 | 54.05 | 60.70 | 97.1 | 1.32 | 57.57 |
| 6 | 73.40 | 38.76 | 60.80 | 86.0 | 2.20 | 56.83 |
| 10 | 51.50 | 27.98 | 54.92 | 58.8 | 4.40 | 47.15 |
| 20 | .... | 16.39 | ... | 44.0 | 11.00 | 23.92 |
| 25 | 21.84 | ... | 25.16 | ... | ... | ... |
| 50 | 11.48 | 9.00 | 12.82 | 19.0 | ... | ... |
| 100 | 5.74 | 4.90 | 7.09 | 10.0 | ... | ... |

It will be noted that the curves in Fig. 1 are remarkably alike and that the percentage of the material which reacts to produce the blue color falls extremely rapidly as the concentration of reactive substance in the solution is increased. *These curves show conclusively that if tryptophane or any other easily oxidizable substance is present, color values will be obtained almost, if not quite, as great as those given by tyrosine itself, and that, therefore, tyrosine can be estimated colorimetrically only if all other easily oxidizable substances, such as tryptophane, ferrous iron, etc., are known to be absent.* In addition, it is unsafe to assume that the color given by

an aliquot is an accurate measure of the reactive material which is present, for if an aliquot actually contained 25 mg. of tyrosine a colorimetric reading, under the conditions of our experiments, would record the presence of only 5.46 mg. In our experiments we used 10 cc. of the reagents which, according to Folin and Denis, should be sufficient to give the maximum color with 5 mg. of tyrosine, but according to our curves, if 5 mg. had been present the color developed would have indicated the presence of only 3.90 mg.

Our study of the reaction of α-methyl indole was undertaken to ascertain whether or not the color was due to the presence of the indole nucleus. Folin and Denis[1] state that indole and its derivatives do not react with the reagent. This is certainly an error. We did not have sufficient pure indole to justify our taking our entire supply to make a quantitative study of the color values at the various concentrations, but a single quantitative test of a one mg. sample of pure white crystalline material gave a color value equal to 0.71 mg. of tyrosine, or approximately equal to the color value of tryptophane. We did have a considerable amount of pure α-methyl indole and, therefore, ran our quantitative color curve with that material, with the result that we have conclusively demonstrated that the indole nucleus will react with the phenol reagent and give relatively high color values.

In view of the results which we obtained, it seemed remarkable that Johns and Jones should have arrived at a conclusion diametrically opposed to that indicated by our data. In looking over their paper for some reason why they obtained the results upon which they base their conclusions, we observed that *they decolorized their hydrolysate with carbon (Norite)*, whereas Folin and Denis do not mention the use of carbon or bone black. We therefore decided to repeat and amplify Johns and Jones' experiment.

**Colorimetric Studies of Gelatin with and without the Addition of Tyrosine and Tryptophane and with and without Previous Decolorizing with Bone Black.**—The following solutions were prepared for this work.

Expt. 1. One g. of gelatin was hydrolyzed by boiling with 25 cc. of 20% hydrochloric acid for 12 hours. The acid was then removed by evaporation *in vacuo* and the solution made to 100 cc. volume.

Expt. 2. Expt. 1 was repeated with the exception that 20 mg. of tryptophane was added before hydrolysis.

Expt. 3. Expt. 2 was repeated with the exception that the hydrolysate was decolorized with one g. of acid-washed bone black after the excess of hydrochloric acid had been removed and before making to the final volume.

Expt. 4. Expt. 2 was repeated, using 20 mg. of tyrosine instead of 20 mg. of tryptophane.

[1] *J. Biol. Chem.*, **12**, 241 (1912).

Expt. 5. This experiment was a duplication of Expt. 3 with the exception that tyrosine was used instead of tryptophane.

Expt. 6. This experiment was a duplication of Expt. 5 with the exception that untreated bone black was used, *i. e.*, the calcium phosphate was not previously removed by washing the bone black with hydrochloric acid.

The results of the colorimetric study are given in Table V, the concentration of reagents being the same as we have used above. The data of Table V are shown graphically in the form of curves in Fig. 2. In Fig. 3 are shown the curves for percentage recovery of the added tyrosine

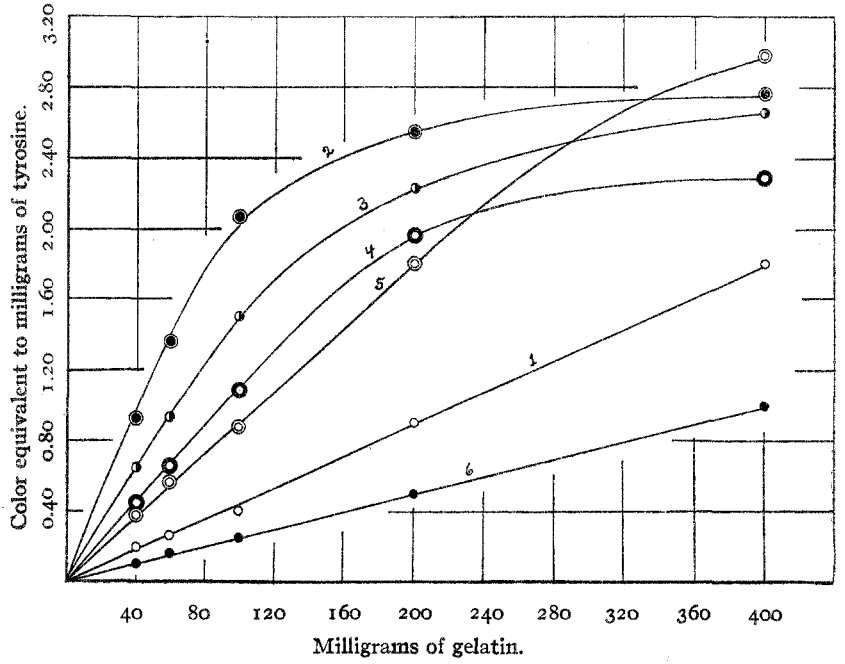


Fig. 2.—Showing quantitative changes in color produced by Folin's phenol reagent in hydrolyzates of gelatin and of gelatin + tyrosine and tryptophane, respectively. The curves also show the diminution of color values when the hydrolyzates have been treated with bone black.

1. Gelatin only.
2. Gelatin + 20 mg. of tyrosine per g. of gelatin.
3. Gelatin + tyrosine, hydrolyzate cleared with untreated bone black.
4. Gelatin + tyrosine, hydrolyzate cleared with acid-washed bone black.
5. Gelatin + 20 mg. of tryptophane per g. of gelatin.
6. Gelatin + tryptophane, hydrolyzate cleared with acid-washed bone black.

and tryptophane. These figures were arrived at by subtracting the values of the color present in the hydrolysate where only gelatin was present from the corresponding values obtained in the hydrolysates when tyrosine or tryptophane had been added.

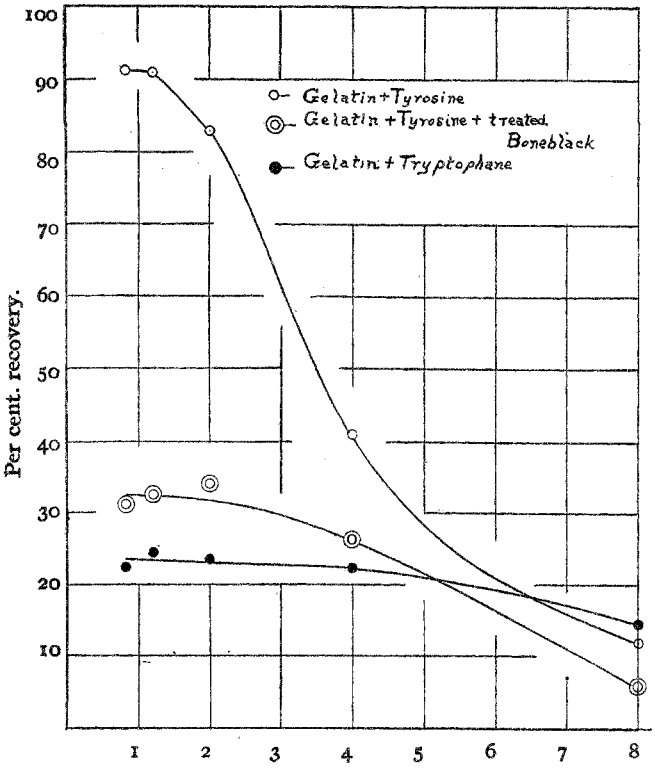


Fig. 3.—The percentage recovery, measured colorimetrically, of tyrosine and tryptophane when added to gelatin. Correction has been made for the color produced by gelatin alone.

TABLE V.

The Color Intensities of Various Dilutions of the Solutions in Expts. 1 to 6 Inclusive, as Compared with a Standard Tyrosine Solution Containing 1 mg. of Tyrosine (1 mg. Tyrosine = 1).

| Aliquot taken contained gelatin. Mg. | Expt. 1. Gelatin only. Color intensity. | Expt. 2. 1 g. of gelatin + 20 mg. of tryptophane. Color intensity. | Expt. 3. 1 g. of gelatin + 20 mg. of tyrosine + acid-washed bone black. Color intensity. | Expt. 4. 1 g. of gelatin + 20 mg. of tyrosine. Color intensity. | Expt. 5. 1 g. of gelatin + 20 mg. of tyrosine + acid-washed bone black. Color intensity. | Expt. 6. 1 g. of gelatin 20 mg. of tyrosine + untreated bone black. Color intensity. |
|---|---|---|---|---|---|---|
| 40 | 0.20 | 0.38 | 0.13 | 0.93 | 0.45 | 0.65 |
| 60 | 0.268 | 0.562 | 0.164 | 1.36 | 0.66 | 0.94 |
| 100 | 0.408 | 0.879 | 0.250 | 2.07 | 1.09 | 1.50 |
| 200 | 0.907 | 1.801 | 0.508 | 2.55 | 1.96 | 2.23 |
| 400 | 1.810 | 2.980 | 1.040 | 2.77 | 2.29 | 2.66 |

This study makes it apparent why Johns and Jones arrived at the conclusions which they published. It will be noted that the color values for

gelatin alone when plotted form a straight line, indicating that with 400 mg. of gelatin present there is still a direct proportionality of color produced. This is a strong indication that the compounds which react with the reagent to produce color are present in gelatin in minimal amounts. We have recently shown[1] that in all probability our sample of gelatin contains approximately 0.035% of tryptophane and 0.47% of tyrosine, figures which are in excellent agreement with the present color values where a content of 0.45% of tyrosine + tryptophane is indicated. These values are also a strong indication that proline or oxyproline does not produce appreciable color values with the phenol reagent, contrary to the belief of Abderhalden and Fuchs.[2] When, however, tryptophane is added, *and the solution is not treated with carbon or bone black*, there is a sharp rise in the color values up to approximately 25% of the color value of the added tryptophane. The reason why 100% of the added tryptophane was not recovered is perfectly obvious if our previous publications on humin formation are kept in mind. We have shown[3] that the black acid insoluble humin of a protein hydrolysate is due to the presence of tryptophane in the protein. We have also shown that proteins apparently contain some unknown factor, perhaps an aldehyde, which induces humin formation. This unknown factor is present in small quantities in our sample of gelatin.[4] Therefore, in the present experiment, although we added 20 mg. of tryptophane, a part of the tryptophane was converted into insoluble humin and only the remaining portion reacted with the phenol reagent. However, in our calculations we used the 20 mg. as 100 %, whereas probably 15 mg. had been converted into insoluble humin and only 4.5 mg. (basing our calculation on the color values at 200 mg. of gelatin) or 22.5% of the added tryptophane remained to react with the phenol reagent. If this reasoning be true, our color values actually record quantitatively the amount of tryptophane which was not converted into insoluble humin. At any rate, the data show that tryptophane which we added does increase the color values practically 100%.

When, however, we investigate the color values for the corresponding hydrolysate which has been treated with bone black, we find that not only is there no added color value, due to the added tryptophane, but that *the color depth is less in the decolorized solution of the hydrolysate of gelatin + tryptophane than it is in the hydrolysate of gelatin alone.* In other words, the carbon which Johns and Jones used removed not only all of the tryptophane which they added but also some of the tyrosine and tryptophane

[1] Holm and Gortner, THIS JOURNAL, 42, 637 (1920).

[2] *Loc. cit.*

[3] Gortner and Blish, THIS JOURNAL, 37, 1630–1636 (1915); Gortner, *J. Biol. Chem.*, 26, 177–204 (1916); Gortner and Holm, THIS JOURNAL, 38, 2477–2501 (1917); 42, 632–640, 821–827 (1920).

[4] Holm and Gortner, *ibid.*, 42, 639 (1920).

originally present in their proteins, so that the color values which they obtained were less in the decolorized hydrolysate to which tryptophane was added than in the original protein hydrolysate to which no tryptophane was added. They conclude from their findings that "tryptophane is completely decomposed during the hydrolysis of proteins and the decomposition products do not interfere with the determination of tyrosine by the method of Folin and Denis." As a matter of fact, (1) tryptophane is not[1] completely decomposed during the hydrolysis of proteins; (2) certain of the decomposition products do give colors with the reagent of Folin and Denis, but the explanation of Johns and Jones' findings lies in the fact that the carbon which they used adsorbed both tyrosine and tryptophane, together with decomposition products, so that their color values were not the true color values for the hydrolysates under investigation.

This adsorption of amino acids by bone black is shown very markedly by Curves 2, 3 and 4 of Fig. 2. It is interesting to note that untreated bone black apparently adsorbs less tyrosine than does bone black which has been washed with hydrochloric acid to remove calcium phosphate. Such, however, is not necessarily the case and only illustrates the ease with which a false conclusion may be reached. The reason why the untreated bone black gives more intense color values lies in the fact that the *untreated bone black contains some substance capable of giving an intense blue color with the phenol reagent.* The acid washnigs of bone black react with the phenol reagent to form blue solutions. This is probably due to ferrous iron inasmuch as the acid washings decolorize permanganate and also give a blue color with Folin and Denis' uric acid reagent just as does ferrous iron. Probably the iron originally present in the bones was reduced to metallic iron by ignition in the presence of the carbon and this reduced iron dissolves in acid solutions to give ferrous ions. Because of the presence of this ferrous iron the unwashed bone black, Curve 3, Fig. 2, represents the balance between the adsorption of amino acids which reduces the color intensity, and the addition of ferrous salts which intensifies the color. We have not thought it worth while to investigate the quantitative relationships of these 2 opposing factors. The fact, however, that when one adds bone black to an acid solution he adds at the same time ferrous iron should be taken into account in future work. Perhaps some of the properties of "active" bone black are in reality due to the presence of reduced iron.

## Discussion.

From the foregoing data it becomes perfectly obvious that the phenol reagent of Folin and Denis cannot be used to estimate colorimetrically the tyrosine content of a protein unless it is absolutely certain that no

[1] A study detailing additional evidence on these points will be published as a subsequent paper.

other substances are present which will react with the phenol reagent. Inasmuch as tryptophane and certain tryptophane decomposition products will react to produce almost as much color depth as an equal weight of tyrosine, and inasmuch as we know that tryptophane is a normal constituent of most proteins, it is not surprising that Folin and Denis found values for the tyrosine content of proteins, measured colorimetrically, far in excess of the values obtained by gravimetric determinations.

Whether or not there are other constituents of the protein molecule which react analogously to tyrosine and tryptophane, we are unable to state with certainty. We believe, however, that such is the case, for we have on hand at the present time fractions of protein hydrolysates which certainly contain no tyrosine and which as certainly contain no unaltered tryptophane, but which, nevertheless, give intensive colorations with the phenol reagent. However, we know that these fractions do not consists of a single pure amino acid, and until we have investigated them further we are unwilling to commit ourselves as to the nature of the substance which causes the reaction.

A study of the curves in Fig. 1 indicates another unfortunate characteristic of the phenol reagent, *i. e.*, that the color intensity is not directly proportional to the concentration of the reacting substance, but that the color value is constantly changing with the concentration of the tyrosine, tryptophane, etc. If this be the case, we fail to see how the colorimetric data can be correctly interpreted in the case of an unknown solution unless the investigator runs a color curve similar to our curves in Figs. 1 and 2 and ascertains whether or not the color which is developed is the maximum possible, *i. e.*, whether the concentration of the reactive material is low enough so that 100% of the reactive substance reacts with the phenol reagent. At extremely low concentrations of tyrosine, tryptophane, uric acid, etc., where the concentration lies between approximately 0.4 and 1.8 mg. of reactive material, the color intensities when plotted form approximately a straight line and are directly proportional to the concentration of the reactive substance and it is only in this region of the curve that accurate quantitative measurements of the reactive material in an unknown solution are possible.

However, the most serious objection to the use of the phenol reagent lies in the fact that apparently any substance which is easily oxidized will react with the reagent and cause the production of the blue color. Folin and Wu[1] have recently applied the phenol reagent to blood analysis, using it to estimate the sugar content of the blood. In this method the sugar is oxidized by a weakly alkaline copper tartrate solution and the cuprous copper formed is estimated colorimetrically by the phenol re-

[1] *J. Biol. Chem.*, **38**, 106 (1919).

agent.[1] Although we have not investigated the form of the cuprous copper curve, it appears extremely improbable that it is different in general form from our curve for ferrous iron (Fig. 1), and if that should prove to be the case our criticism as to color intensity and concentration would hold for the sugar work.

During our study of the ferrous iron curve we made quantitative studies on both ferrous sulfate solutions and ferrous chloride solutions, checking the content of ferrous salt in each instance by a permanganate titration. We were much surprised to find that the 2 curves did not coincide at identical concentrations of ferrous iron, but that the ferrous sulfate curve was consistently lower than the ferrous chloride curve which is given in Fig. 1. Obviously more factors were involved than simply the concentration of the ferrous iron, but whether the discrepancies were due to hydrogen ion concentration, or to the nature of the anion, or to other unknown factors, we are unable to state, inasmuch as we did not pursue our observations further. We further noted that ferrous iron produced the same blue coloration in solutions where Folin and Denis' uric acid reagent replaced the phenol reagent.

That accurate colorimetric data cannot be obtained if 2 reacting substances are present because of different reactivity of different amino acids at the same concentration is illustrated by reference to the tyrosine and tryptophane curves of Fig. 2. Here at a low concentration of amino acids the color intensity of the tyrosine-containing solution is far greater per milligram of tyrosine than is that of the tryptophane solution; nevertheless, at a higher concentration of amino acid the tyrosine color intensity falls off rapidly while the tryptophane color intensity decreases only slightly so that at 8 mg. of added amino acid the tryptophane color curve has crossed the tyrosine color curve.

As a result of our study we are firmly convinced that a quantitative measurement of the blue color produced by the phenol reagent in a solution is only an indication that easily oxidizable substances are present in that solution, and that accurate quantitative data can only be had when one knows that only one such substance is present in the solution, and even then the approximate concentration of the reacting material must be known or else a series of colorimetric determinations must be made so as to ascertain whether or not the maximum color in proportion to concentration is being developed.

## Summary.

As the results of a study of the various factors influencing the color intensity of protein hydrolysates to which have been added the phenol

[1] Folin and Wu, *J. Biol. Chem.*, 41, 367–374 (1920), have recently published the formula of a similar phosphotungstic-phosphomolybdic acid reagent which they state is preferable to the phenol reagent for this purpose.

reagent of Folin and Denis, according to their directions for the quantitative estimation of tyrosine, we are forced to conclude that:

1. Tyrosine cannot be quantitatively estimated in a protein hydrolysate by the use of the phenol reagent because

2. Tryptophane, if present, will also produce intense colors with the reagent, the color produced by one mg. being approximately 85% of that produced by tyrosine at an equivalent concentration.

3. Indole and indole derivatives, contrary to the statement of Folin and Denis, react strongly with the phenol reagent to produce the blue color.

4. Ferrous iron, and apparently any other easily oxidizable material, also reacts with the reagent.

5. There is considerable evidence that tyrosine and tryptophane are not the only protein constituents which produce blue colors with the phenol reagent.

6. The amount of color which is developed in a solution is not a linear function of the concentration of the reactive material, but the color values fall off sharply as concentration increases until only an insignificant fraction of the reactive material is indicated by a measurement of the color values of the solutions containing any considerable amount of the reactive substance.

7. Because of the peculiar form of the color curves in relation to concentration, it becomes necessary for one to know the approximate concentration of reactive material in advance of the colorimetric determination so that the colors may be developed and read at such a concentration that the maximum color values will be developed.

8. Because of the fact that solutions of tyrosine and tryptophane do not give the same color values at equivalent concentrations, it is impossible to measure accurately the sum of these amino acids in a mixture which contains no other reactive substances.

9. Protein hydrolysates must not be decolorized with carbon or bone black if they are to be used subsequently for a quantitative determination of amino acid content, for the bone black adsorbs at least tyrosine, tryptophane and tryptophane decompositions products in appreciable amounts, Whether or not other amino acids were adsorbed was not determined.

10. Bone black contains some easily oxidizable material, probably reduced iron, which dissolves in acid solutions. These acid solutions give the blue color with the phenol reagent.

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