

CONTRIBUTIONS TO THE CHEMISTRY OF TRYPTOPHANE

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The amino acid, tryptophane, is a protein "building block", indispensable in animal nutrition and is characterized by reactivity and fragility. It is rapidly destroyed by boiling with concentrated mineral acids to give humin substances and by strong alkalies and by certain fecal bacteria to yield indol.

A number of methods have been proposed for the quantitative determination of tryptophane in proteins. In nearly every instance there is prescribed a different, specific way in which the protein hydrolysis is to be carried out. The final result is perhaps quite as dependent upon this factor as upon the method of analysis itself.

Dakin², using active pancreas extract as the hydrolyzing agent, isolated and weighed the tryptophane in casein and reported 1.7%. Other methods applied to casein produced results which range from 0.5%, as found by Herzfeld,³ who digested the protein with pancreatin, to 2.26% as reported by Furth and Nobel.⁴ The method of Sanders and May,⁵ based upon the estimation of indole produced by the action of fecal bacteria on casein, yielded 1.6%.

The present investigation is concerned with further study of the phenol-hypochlorite color reaction of tryptophane previously reported by the authors⁶ and the extension of its application to certain phases of tryptophane chemistry, as follows:

(1) Use of the color test for tryptophane as a means of studying the extent of protein cleavage based upon tryptophane production;

(2) Improvement of the phenol-sodium hypochlorite method for tryptophane estimation by increasing its sensitivity, simplicity, speed, and ease of application, so that it may be applied directly to protein hydrolysates without decolorizing, concentrating, or additional procedures of any kind other than neutralizing and making up to volume;

(3) Use of the improved tryptophane test as a means of studying the resistance of the liberated tryptophane in protein hydrolysates when subjected to the continued action of strong hydrolyzing agents;

(4) Use of the improved test as a means of studying the efficiency of some hydrolyzing agents in the cleavage of tryptophane from casein.

Experimental

The reagents used in this work are the same as those described in the above mentioned paper.

To determine if the phenol-hypochlorite color test is applicable directly to proteins, i.e. without previous hydrolysis, egg albumin "solu-

¹Condensed form of a thesis submitted in partial fulfillment of requirements for the degree Ph.D.

²Dakin, H. D., 1918. *Biochem. J.* 12:290.

³Herzfeld, E., 1913. *Biochem. Z.* 56:258.

⁴Furth, O. and Nobel, E., 1920. *Biochem. Z.* 109:103.

⁵Sanders, J. A. and May, C. E., 1912-3. *Biochem. Bull.* 2:373.

⁶Miller, F. M. and Lyons, R. E., 1934. *Proc. Ind. Acad. Sci.* 43:132.

tions" of various concentrations were subjected to the test with results as follows:

	NaOCl .26%
	Na ₂ O .009%
Tryptophane.....	rose
M/100 egg albumin.....	no color
M/200 egg albumin.....	no color
More dilute egg albumin-	blue-green
Very dilute egg albumin-	blue-green

The rose color reaction of tryptophane is not obtained when the test is applied to the unhydrolyzed protein molecule, but the blue-green color characteristic of glycine is given by egg albumin under the described conditions.

Numerous attempts were made to execute the necessary hydrolysis of protein in such a manner as to yield a colorless hydrolysate suitable for the tryptophane test. The hydrolysates of casein produced by refluxing with 10, 15 and 20% hydrochloric and sulphuric acids were, without exception, highly colored. Hydrolysates, when neutralized or when freed from excess of acid by distillation under reduced pressure, could not be obtained sufficiently color-free by subjecting casein or egg albumin to the action of formic or acetic acids in 10% to 50% concentration in an autoclave at 150° C., as recommended by Zelinsky and Ssadikow⁷.

It was also our observation that pure tryptophane heated for 7 hours with 25% acetic acid in the autoclave at 150° C. yields some indole as evidenced by odor, positive Konton's test, and positive p-dimethyl amino benzaldehyde test.

The baryta hydrolysis of casein was undertaken in sealed flasks at 150° C. for 18-22 hours in an autoclave with the idea that both tryptophane and any indole, resulting from continued action of the agents of alkaline proteolysis upon tryptophane, could be determined in the hydrolysate after removal of the barium by precipitation with sulphuric acid.

The slightly acid filtrates from barium sulphate were colored or acquired objectionable color after a few hours. Indole determinations by the Bergeim method⁸ in the distillates from seven hydrolysates of casein showed that the amount of indole produced under these conditions is but .004% to .008%.

In the attempts to apply the phenol-sodium hypochlorite color test as a quantitative procedure to protein hydrolysates the unavoidable dark color and the difficulty of obtaining a perfectly neutral solution proved to be serious obstacles, and the necessity for application of the color test to highly acid or highly alkaline solutions was apparent.

As a preliminary, a mixture of 2 m.l. M/100 aqueous tryptophane solution, 8 drops 5% aqueous phenol and 2 m.l. 0.15% sodium hypochlorite solution was prepared in each of five test tubes. Of these, two are made alkaline and two acid with results as follow:

6 drops	3 drops	Blank	3 drops	6 drops
.4N. NaOH	.4N. NaOH		.4N. HCl	.4N. HCl
No Color	No Color	Pink	Deep Red	Deep Red

⁷A. C. S. Monograph 48:98. 1929.

⁸Hawk and Bergeim, Phys. Chem. (10th Ed.) p. 371. 1931.

Repetition of the above without the presence of phenol showed that the latter was not essential for the production of the red color with tryptophane in acid medium. It follows, in the light of previous work, that phenol probably serves only in creating a slightly acid medium and does not enter into the molecule of the tryptophane color complex.

To determine whether or not the accentuation of the color in the test solution due to the addition of mineral acid was specifically characteristic for tryptophane, the test was applied to 1/50 and 1/100 M. solutions of twelve amino acids and to indole under the same conditions, i. e. 2 m.l. amino acid solution, 2 m.l. 0.15% sodium hypochlorite and varying amounts of 0.4N. acids.

	M/50 Blank	M/50 3 dr. HCl	M/50 8 dr. HCl	M/100 3 dr. HCl	M/100 8 dr. HCl	M/100 4 dr. H ₂ SO ₄
Glycine.....						
Glutamic acid.....						
Aspartic acid.....						
p-NH ₂ phenylacetic...	brown	brown	brown	brown	brown	brown
Alanine.....						
Proline.....						
α -amino caproic.....						
Histidine.2HCl.....						
Iso-leucine.....						
Phenylalanine.....						
Leucine.....						
Tryptophane.....	pink	red	red	red	red	red
Indole.....	turb.	gr-yl	gr-yl	gr-br	gr-br	gr-trub

The intense deep red color is specific for tryptophane. The faint pink color yielded by indole changes quickly to greenish yellow.

The effect of the strength of the hypochlorite solution upon the color formation was determined on 1 m.l. portions of 1/100 M. tryptophane.

Drops	"A"	"B"	"C"	"D"
0.4N HCl	0.31% NaOCl	0.15% NaOCl	0.10% NaOCl	0.08% NaOCl
1	dark	very good	very good	fair
4	dark	very good	very good	fair
7	dark	very good	very good	fair

In series "A" the colored compound precipitated. Series "B" and "C" were brilliant cherry red in color and showed no precipitation of the pigment.

About equally satisfactory color was obtained when 1 to 10 drops of 0.4N. hydrochloric acid were used; more induced fading of the color.

Satisfactory check results were obtained only when the hydrochloric acid was added last. Under those conditions the cherry red color formed at once and exhibited marked stability for weeks.

Sensitivity of the Improved Test.—Two m.l. portions of very dilute tryptophane solutions were mixed with 2 m.l. of 0.1% sodium hypochlorite and 1 to 2 drops of 0.4N. hydrochloric acid added with results as follow:

M/50 tryptophane: Very intense red color. Too intense for quantitative comparisons; M/1250 showed good red color; M/3750 good pink; M/6250 nice pink color suitable for quantitative work. This amounts to 0.0000313 gr. tryptophane per ml. or one part in 31900 parts. M/12500 immediately distinct pink sufficient for identification test. This dilution expresses about the lower limit of the sensitivity and corresponds to one part in 63000 parts of solution.

Solution and Extraction of the Reaction Color.—A number of solvents, benzene, toluene, chloroform, ether, n-butyl alcohol and iso-amyl alcohol, were tried with the idea of extracting the pink or red color which is formed by the action of sodium hypochlorite on tryptophane in an acid medium. Iso-amyl alcohol only was found satisfactory. By it the pink color is extracted quantitatively from the reaction mixture. The alcoholic solution of the pigment may be kept unchanged for weeks.

Advantages of the Reaction Color Extraction.—1. The iso-amyl alcohol color extraction enables the analyst to make quantitative determinations on hydrolysates otherwise too highly colored for successful colorimetric work.

2. It enhances the sensitivity of the test; a 10 ml. portion of amyl alcohol will, for example, extract all of the color from a volume of 50 ml. of reaction mixture which would in itself be too weak in color for direct colorimetric work. This increases the normal sensitivity of the test many times.

3. The test becomes very flexible. Either standard or unknown color extract may be diluted with additional alcohol for purposes of approximating color intensities. This does away with the need of having several standards, since only one is needed.

4. It does away with the necessity of keeping constant volumes in the reaction mixtures as compared to standards, involving quantities of reagents.

5. Since the amount of color extracted, and not the quantity of hypochlorite absorbed, is a measure of the tryptophane value, it follows that the presence of other substances which also absorb sodium hypochlorite does not interfere with the accuracy of the test.

Application of the Test to Protein Hydrolysates.—1. Successful quantitative application of the improved test to an unknown requires that the correct amount of sodium hypochlorite should be added to produce the maximum intensity of color. To determine this the following scheme has been adopted:

1	2	3	4	5	6	7
5.0 ml. sol. 0.2 ml. NaOCl 10 drops HCl no color	5.0 ml. sol. 0.4 ml. NaOCl 5 drops HCl weak color	5 ml. sol. 0.6 ml. NaOCl 10 drops HCl good color	5.0 ml. sol. 0.8 ml. NaOCl 5 drops HCl good color	5.0 ml. sol. 1.0 ml. NaOCl 10 drops HCl good color	5.0 ml. sol. 1.4 ml. NaOCl 10 drops HCl good color	5.0 ml. sol. 1.8 ml. NaOCl 10 drops HCl dark color

The conditions as described for No. 3 were found the best for color development.

2. The hydrochloric acid should be the last reagent, and the entire quantity used should be introduced at one time. A fast rate of fading of color is indicative of excessive acidity.

3. The reaction mixture is extracted with iso-amyl alcohol as soon as possible after the addition of the hydrochloric acid. Color comparisons of the clear extracts are then made by use of a Dubosq colorimeter against a standard prepared from pure tryptophane. A 2 ml. portion containing 0.00015 gr. per ml. is placed in a test tube containing about 3 ml. of water. To this is added 0.1 ml. of 0.5% sodium hypochlorite solution and one or two drops of 0.4N. hydrochloric acid. The amyl alcohol extraction may be made at any time within about ten minutes after the last reagent is added.

Effect of Strong Hydrolyzing Agents.—It is our observation that a sample of casein which had been hydrolyzed with 15% sulphuric acid for a period of 30 hours at reflux temperature showed no tryptophane present. If the same conditions are maintained except that the heating period is reduced to 15 hours, there is obtained a test which may indicate a trace of tryptophane present. If the heating period is cut to five hours and the same test is applied, a good amount of tryptophane is indicated by the color. This shows that the long heating period and the continued action of the sulphuric acid are destroying the tryptophane previously liberated. One is not surprised to find, in view of the above observations, that a casein sample when refluxed with 20% hydrochloric acid for 25 hours should fail also to give a positive test.

The hydrolysate obtained from the use of a strong solution of barium hydroxide upon casein at 150° C. for 30 hours and neutralized with hydrochloric acid contained 0.44% tryptophane. If the heating period is cut to 20 hours and to 10 hours, values of 0.48% and 0.63% respectively were obtained.

The following table shows the results obtained when 2.5 gr. portions of casein (technical) were heated with 100 gr. each of the indicated barium hydroxide solution.

Ba(OH) ₂	10 Hours	20 Hours	30 Hours
10%.....	0.3%	0.4%	0.7%
20%.....	0.6%	0.8%	0.8%
30%.....	0.6%	0.7%	0.9%
40%.....	0.7%	0.9%	0.7%
50%.....	1.0%	1.0%	0.7%

An aliquot part of a solution of pure tryptophane refluxed for 30 minutes with 20% barium hydroxide gave but 84% of the amount present in the part not treated with barium hydroxide. Since the strong mineral acids and alkalies possess as casein-hydrolyzing agents the ability to split off tryptophane and also to decompose this amino acid, they are not satisfactory. The tryptophane value so obtained is merely the difference between the amount released and the amount decomposed.

Conclusions

1. The phenol-sodium hypochlorite color test for tryptophane, when applied to the usually discolored protein hydrolysates, was found unsatisfactory.
2. In ordinary casein hydrolysis very little tryptophane is converted to indole. The addition of some inorganic salts aids slightly in indole production. Pure tryptophane is unable to withstand the continued action of barium hydroxide and yields considerable indole.
3. A very specific, sensitive, speedy, and reliable color test for the qualitative and quantitative determination of tryptophane, resulting from the addition of a small amount of sodium hypochlorite previous to slight acidification, was developed. The test is dependent upon a cherry red color which develops immediately, is very stable, and is quantitatively extractable with iso-amyl alcohol; it is applicable to protein hydrolysates, regardless of discoloration, and is so flexible as to require only one tryptophane solution as a standard over a wide range of concentrations of unknown samples.
4. The continued action of strong sulphuric and hydrochloric acids as hydrolyzing agents has been found to be so destructive to the tryptophane contained in protein hydrolysates that not a trace could be detected.
5. Low concentrations of barium hydroxide as hydrolyzing agents are slow in splitting tryptophane from the casein molecule, while higher are more effective and also more destructive to the tryptophane previously set free.