[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE DAIRY DIVISION, UNITED STATES DEPARTMENT OF AGRICULTURE]

THE QUANTITATIVE DETERMINATION OF TRYPTOPHAN

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Many methods, chiefly colorimetric, have been used for the quantitative determination of the tryptophan content of proteins, but few have given results accurate and concordant enough to warrant their general use where absolutely quantitative results are desired.

The bromine absorption test, the Adamkiewicz reaction, the nitroso-indole reaction, and others have been used with little real success.

Voisenet's[1] test for tryptophan, using formaldehyde in the presence of potassium nitrite and concd. hydrochloric acid, was made the basis for its quantitative determination by Fürth[2] and his associates, and excellent results were obtained. Their data indicate that the reaction of tryptophan with formaldehyde in the presence of sodium nitrite is rapid and while the color reaches a quite constant maximum, it may permit an error of from 10 to 20%. They also found that the color produced was very sensitive to an excess of hydrochloric acid, to excess of sodium nitrite and formaldehyde concentrations, to reducing and oxidizing substances and to metals. Various methods were tried for dissolving the proteins but the results obtained with these solutions are all within experimental error of the method.

The observation of Rhode,[3] that tryptophan gives a blue color with *p*-dimethylaminobenzaldehyde, has found the most general application and has been used by Herzfeld,[4] Kurchin,[5] Thomas,[6] and by May and Rose.[7] While the methods employing this aldehyde are capable of very accurate results, the most general error in the method used by these workers has been due to the fact that the reactions in the standard or the unknown protein solutions have not been brought to a maximum color intensity.

The work of Gortner and Holm[8] proved that the "humin" formed upon the acid hydrolysis of proteins in the presence of aldehydes was due to the presence of tryptophan. In the presence of a definite *optimum* amount of a certain aldehyde, the amount of "humin" formed, as measured by the nitrogen content, is a fairly accurate estimate of the amount of tryptophan. When a protein is allowed to stand at room temperature in the presence of benzaldehyde or some other aromatic aldehyde and 20% hydrochloric acid, a color develops which is peculiar to the aldehyde used. At lower temperatures this color is quite stable, but as soon as the solution is heated a dark "humin" is formed. The use of aliphatic aldehydes produces a dark condensation product immediately. Further unpublished work showed that the dark "humin" product formed when indole derivatives are condensed with aldehydes probably consists of a condensation of 1 molecule of the indole derivative with 2 or more molecules of the aldehyde. When the reaction is

[1] Voisenet, *Bull. soc. chim.*, [3] **33**, 1198 (1905).

[2] Fürth and Nobel, *Biochem. Z.*, **109**, 103 (1920). Fürth and Lieben, *ibid.*, **109**, 124 (1920).

[3] Rhode, *Z. physiol. Chem.*, **44**, 161 (1905).

[4] Herzfeld, *Biochem. Z.*, **56**, 256 (1915).

[5] Kurchin, *ibid.*, **65**, 451 (1914).

[6] Thomas, *Ann. Inst. Pasteur*, **34**, 701 (1920).

[7] May and Rose, *J. Biol. Chem.*, **54**, 213 (1922).

[8] Gortner and Holm, THIS JOURNAL, **39**, 2485 (1917).

carried out in a molecular ratio of 1:1 the product is chiefly a soluble, deeply colored compound. These results indicate that if the reaction could be controlled, and if a reaction of a molecular ratio of 1:1 only could be obtained, the color produced would be a direct measure of the amount of an indole derivative present.

These observations emphasize the fact that in all cases where aldehydes are used care must be taken that conditions are such as to prevent the secondary reaction, as far as this can be done. The sensitiveness of the reaction used by Fürth and his associates is evident, since formaldehyde condenses very readily with tryptophan even at relatively low temperatures. The aldehyde used by Herzfeld, and by Thomas and more recently by May and Rose, *p*-dimethylaminobenzaldehyde, is very suitable, in that at lower temperatures very little or none of the secondary condensation products is formed. Herzfeld's error was evidently due to the fact that the reacting mixtures of either standards or unknowns were not allowed to react long enough to produce a maximum color, and consequently his results are low in some cases. Thomas digested his proteins completely before applying the test and therefore his results are more nearly correct, although it is doubtful whether time enough was given for the development of maximum color. May and Rose used casein as a standard and calculated their results on the basis that the tryptophan content of casein is 1.50%, which is undoubtedly too low. Figures given later will show that the time allowed for the completion of the reaction in their standards and the unknowns was too short.

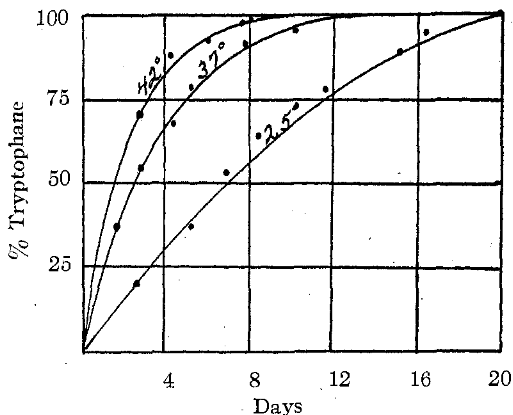

Fig. 1—Effect of temperature and time upon the rate of reaction of tryptophan with *p*-dimethylaminobenzaldehyde in 20% HCl

Experimental Part

Herzfeld's method, slightly modified, has been used by the authors for some time, and has been found to be reliable when certain precautions are taken with regard to the conditions under which the determinations are made. It has also been applied to samples of proteins without previous digestion with enzymes, and reliable results have been obtained.

The effect of temperature and time upon the reactions involved when a pure sample of tryptophan is treated with *p*-dimethyl-aminobenzaldehyde in the presence of 20% hydrochloric acid is shown by the curves given in Fig 1.

At 25° the reaction is slow, but there is little danger of a great amount of the secondary condensation product forming at that temperature, as shown by the fact that in every case the reaction reaches completion though the time factor is very large. At 37° and 42° the reactions are much more rapid but do not show maximum color development until the sixth or eighth day in the case of the experiment at 42°, and not until the tenth or twelfth day in the case of the experiment at 37°. At 42° the color is not permanent for any length of time. Under these conditions, therefore, when working at about 37°, as did May and Rose, it is necessary to allow approximately 8 days for the standard to develop maximum color. Assuming that the color of their standard developed according to the curve in Fig. 1, it was approximately 70% developed. Inasmuch, however, as the color of the unknowns was proportionately developed, the results are good in most cases. As soon as maximum color has been developed in a standard or unknown, it is best maintained if the solution is kept at a temperature of 25° or lower and if, in addition, it is diluted. Under such conditions standards have been found to maintain their color for 8 or 10 days and in some cases even longer.

Some of the results obtained by applying the facts illustrated in Fig. 1 are shown in Table I.

Several 2 to 5 mg. samples of tryptophan were weighed out and each was placed in 100 cc. of 20% hydrochloric acid solution to which had been added an excess of the aldehyde. From time to time readings were made against standards by means of a Duboscq colorimeter, until the readings of the unknowns became constant.

TABLE I

RESULTS OBTAINED WITH PURE TRYPTOPHAN

Expts.	Temperatures used °C.	Amount of tryptophan Calc. Mg.	Found Mg.	Recovered %
1. (Av. of 5 detns.)	25, 37, one detn. at 42	5.00	4.985	99.7
2. (Av. of 5 detns.)	25, 37, one detn. at 42	2.00	1.976	98.8
A	25	2.70	2.77	102.6
B	25	1.20	1.17	97.5

A and B were samples of unknown concentration prepared by J. M. S.

The results obtained in these experiments indicate that pure tryptophane in solution can be accurately estimated by this method under the conditions given.

The effect of various concentrations of the aldehyde was determined and it was found that there was practically no difference in the rate of the reaction at 25° whether 1 mol. or 10 mols. of the aldehyde were used to each mol. of tryptophan. Two mols. seemed to give the best results.

For the determination of pure tryptophan in solution or in a mixture of amino acids the authors recommend the use of approximately 2 mols. of aldehyde to each mol. of tryptophan present. The reaction may be carried out at 25° or 37°. The latter is preferable.

The method has been tried upon proteins that have not been previously digested with enzymes. In these cases the method is dependent upon the rate of hydrolysis of the protein by the acid used, which is quite rapid in concd. acid solution and much slower in dil. acid. Inasmuch as the color is less stable in concd. than in dil. acid solution, it has been found that a 20% hydrochloric acid solution is most satisfactory. Sulfuric acid has not been tried.

Several samples of proteins were ground very fine and 0.10 g. of each was suspended in 100 cc. of 20% hydrochloric acid containing an excess of *p*-dimethyl-aminobenzaldehyde. Reactions were carried out at both 25° and 37°. Readings were made from time to time against standards containing 2 mg. and 5 mg. of pure tryptophan, using a Duboscq colorimeter, until the colors of the unknowns reached maximum intensity.

Results indicate that 37° is preferable to 25° in this experiment.

The results for casein, fibrin and Witte's peptone are embodied in Table II, which also gives results obtained by the same general method and by other methods. Some of the difficulties and errors of other authors who have used the same general method have been pointed out elsewhere in the paper.

TABLE II

TRYPTOPHAN CONTENT OF CASEIN, FIBRIN AND WITTE'S PEPTONE

	Authors %	Folin and Looney[9] %	Fürth and associates[2] %[a]	May[7] and Rose %	Herzfeld[4] %	Thomas[6] %	Hopkins and Cole[10] %
Casein	2.24	1.54	2.02	1.50	.51	1.7–1.8	1.50
Fibrin from blood	5.00	2.90	5.30	..	1.05	.....	..
Witte's Peptone	5.40	3.03	5.30	..	1.25	.....	..

[a] By formaldehyde-nitrite method.

With some proteins a sharp maximum in color intensity was produced, which extended over but 1 day. It is doubtful, therefore, whether results with all proteins are reliable when this method is used. This sharp maximum would indicate that a secondary reaction takes place to some extent before the primary color-producing reaction has been completed. The difficulty of hydrolysis of some proteins in the medium used may be a factor concerned here. From the results obtained upon pure tryptophan it seems that in general the best results might be obtained with the use of samples of proteins previously digested by enzymes.

In this connection it is interesting to call attention to the tryptophan content of fibrin as determined by Gortner and Holm.[8] The fibrin used in the determinations reported in this paper was taken from the same sample as that used in their work upon "humin" formation. They estimated that in the presence of an optimum amount of formaldehyde 95% of the tryptophan was removed as "humin." Their figures indicate that

[9] By phenol regent method. Folin and Looney, *J. Biol. Chem.*, **51**, 421 (1922).

[10] By direct isolation, Hopkins and Cole, *J. Physiol.*, **27**, 418 (1901–2).

the tryptophan content of this fibrin, calculated upon the basis of the humin nitrogen was 4.8%. The total tryptophan content would therefore be 5.05% which is in excellent agreement with the value obtained by the colorimetric method.

Summary

1. Some of the difficulties and errors in the methods used by various authors are pointed out.

2. The effect of temperature and time upon the reaction of *p*-dimethylaminobenzaldehyde in 20% hydrochloric acid has been studied. In this concentration of hydrochloric acid the reaction requires greater time for completion than is generally supposed. The higher the temperature, the greater the instability of the color produced.

3. Pure tryptophan in solution can be accurately determined by use of this method. The tryptophan content of proteins can also be accurately determined without previous hydrolysis of the protein, but from observations and from general considerations it seems that an enzyme-digested protein is better suited for this determination than is undigested protein.

4. There is excellent agreement between the figures for the tryptophan content of fibrin obtained by the "humin" formation method of Gortner and Holm[8] and those obtained by the colorimetric method.

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SOME NEW DERIVATIVES OF SYNTHETIC ADRENALINE (SUPRARENINE)

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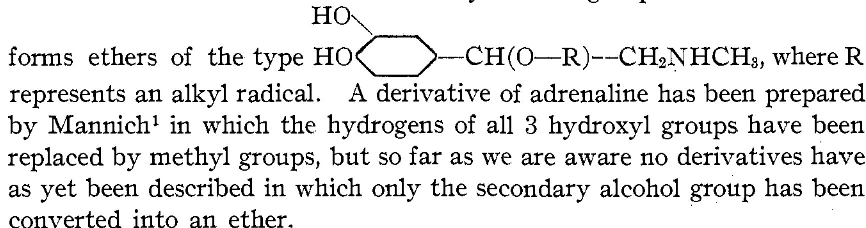
In the manufacture of synthetic adrenaline, and especially in its purification, we have observed the formation of certain derivatives which are of unusual chemical interest, and which may prove to have valuable pharmacological properties.

It has been found that the secondary alcohol group in the side chain forms ethers of the type



HO(HO)C_6H_3—CH(O—R)—CH_2NHCH_3, where R represents an alkyl radical. A derivative of adrenaline has been prepared by Mannich[1] in which the hydrogens of all 3 hydroxyl groups have been replaced by methyl groups, but so far as we are aware no derivatives have as yet been described in which only the secondary alcohol group has been converted into an ether.

We have succeeded in preparing the methyl and ethyl ethers of this type, but have failed so far to prepare the purified propyl and benzyl

[1] Mannich, *Arch. Pharm.*, **248**, 127, 154 (1910).