XII. NOTE ON THE DETERMINATION OF TRYPTOPHAN BY MEANS OF *p*-DIMETHYL-AMINOBENZALDEHYDE.

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Tryptophan reacts with aldehydes in the presence of acids to give coloured solutions varying in colour according to the aldehyde used.

It is well known that in some cases the reaction requires the presence of an oxidising agent. Thus the colour reaction of formaldehyde with proteins in presence of sulphuric acid is promoted by the addition of a trace of ferric sulphate. Oxidising agents in general have a similar effect on the reaction of tryptophan with benzaldehyde and vanillin respectively, and it appears that this applies generally. Dakin [1906], however, states that the glyoxylic acid reaction (Adamkiewicz test) of tryptophan in presence of sulphuric acid does not require an oxidising agent, but this is true in measure of the reaction with other aldehydes. It can be shown that the presence of reducing substances such as hydrogen sulphide or formaldehyde retards the development of the glyoxylic acid coloration as well as that with other aldehydes.

In carrying out some work on the tryptophan content of fish-muscle protein by means of the method of May and Rose [1922] certain facts came to light which are of considerable importance in the application of this test since large errors may arise if they are ignored.

The May and Rose method is carried out as follows: 0·1 g. protein is added to a mixture of 50 cc. concentrated hydrochloric acid, 50 cc. water and 1 cc. of a 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulphuric acid. The mixture is incubated at 36° for 24 hours and then allowed to stand for 24 hours or longer at room temperature. When tryptophan is present a blue coloured solution is obtained. The colour is compared by means of a colorimeter with the colour given by caseinogen under the same conditions, and the tryptophan content is calculated on the assumption that caseinogen contains 1·5 % of tryptophan. Jones, Gersdorff and Moeller [1924] have pointed out that different proteins are hydrolysed under these conditions at different rates and that a longer time must be given for development of the colour.

Holmes and Greenbank [1923] have studied the influence of temperature on the reaction of tryptophan, free and combined in proteins, with *p*-dimethyl-aminobenzaldehyde. They found that the development of the colour is much more rapid and complete at 37° than at room temperature and also that fading goes on concurrently with colour production. The colour is very stable at

room temperature but fades rapidly at 42°. They chose 37° as a suitable temperature and kept the mixture till a maximum intensity of coloration was obtained. This method gave in their hands good results for solutions of pure tryptophan but they do not state what standard was used. The conditions with proteins are very different since hydrolysis, colour development, and colour fading are all going on at once. For this reason a standard solution of pure tryptophan is not permissible in determining the tryptophan content of proteins if a high temperature is used.

Holmes and Greenbank have also found difficulty in the varying rate of hydrolysis of different proteins and favour the use of enzyme digests.

Some experiments were carried out with a view to explaining large variations in colour intensity obtained using equal volumes of the same trypsin digest of caseinogen or equal weights of the same sample of dry caseinogen. The relative intensities were sometimes as 7 : 10 and it was also found that the order might be reversed, that which at first was less intense becoming the stronger. These variations were traced to accidental differences in the illumination of the two tests. Also, sometimes delay occurred in the development of the colour in solutions of other proteins which caused it to be assumed that only a trace of tryptophan was present, an assumption subsequently disproved by development of considerable coloration. This was traced to the presence of small quantities of reducing substances. It was shown that development of the colour in solutions of pure tryptophan is retarded by (1) the presence of hydrogen sulphide or formaldehyde, (2) a layer of toluene over the reacting mixture, (3) absence of light. It was also found that addition of traces of an oxidising agent brings about rapid development of the colour at room temperature, and that exposure to bright light has a similar effect. The presence of pure isinglass does not hinder the development of colour, it is neither hindered by bubbling carbon dioxide through the reacting mixture in daylight, nor hastened by bubbling oxygen through the reacting mixture.

The following factors will now be considered in turn: (1) effect of reducing substances; (2) effect of oxidising agents; (3) effect of light.

Effect of reducing substances.

Herter [1905] has shown that the colour reaction of urine with *p*-dimethylaminobenzaldehyde (due to indole derivatives) is prevented by the presence of formaldehyde. Acree [1906] and Dakin [1906] also mention that excess of formaldehyde prevents the development of the coloration given by milk with formaldehyde in presence of sulphuric acid. In the course of work on cod-muscle protein it was found that, if this purified protein is soaked in a dilute solution of formaldehyde and then thoroughly washed with cold water, the Adamkiewicz reaction with glyoxylic acid in presence of sulphuric acid and the *p*-dimethylaminobenzaldehyde reaction in presence of hydrochloric acid become faint. The material appears to recover the power of giving positive tests with these reagents on prolonged storage.

These observations, together with the experiment with hydrogen sulphide already mentioned, show that the presence of reducing substances is a disturbing factor in the determination of tryptophan by the method of May and Rose and similar methods using other aldehydes. It follows too that excess of the aldehyde reagent will hinder the development of the colour and may cause colorimetric readings at a given time to be not truly proportional to the tryptophan present. In this connection it may be noted that Holmes and Greenbank found little difference in the results obtained, using 1 mol. of reagent for every mol. of tryptophan and using 10 mols. of reagent for every mol. of tryptophan, but they obtained the best results with 2 mols. It is evident that if only 1 mol. is present the aldehyde reagent will be gradually oxidised so that by the time all the tryptophan has been liberated there will not be the equivalent quantity of aldehyde to combine with it. On the other hand, too great an excess of reagent may retard the oxidative development of colour. Probably at the higher temperature, 37°, this is not so evident as at lower temperatures.

Effect of oxidising agents.

The addition of an oxidising agent at the same time as the reagent is added will obviously alter the aldehyde rapidly before it has time to combine with the tryptophan, especially as the latter is only slowly liberated from the protein. This is confirmed in practice when quantities of the order employed by May and Rose are used. The oxidising agent must be added after hydrolysis is completed.

In making qualitative tests in small volumes of liquid the coloration can be produced at once on addition of an oxidising agent. Small pieces of tissue can be quickly stained for tryptophan in this way. In these instances, however, probably only a small fraction of the tryptophan present is transformed.

In order to compare the coloration obtained with and without addition of an oxidising agent, tests were carried out by the method of May and Rose, using 0·1 g. of caseinogen and cod-muscle protein respectively. Duplicate tests were carried out similarly but 3 drops of 0·5 % sodium nitrite solution were added after 3 days and again after 3 days more. The colorimetric readings were made on the following day using a Duboscq colorimeter and a blue glass screen of the correct tint as standard. They are given in Table I.

Table I. *Comparison of the intensity of coloration obtained with and without addition of sodium nitrite solution.*

Substance	Time at room temp. after 1 day at 38°	Without addition of sodium nitrite	Sodium nitrite added
Caseinogen	7 days	22·9 mm.	15·0 mm.
	31 days	17·4 mm.	
Cod-muscle protein	7 days	34·8 mm.	17·6 mm.

The quantity of tryptophan present is in inverse proportion to the colorimetric reading.

From these results it is evident that the development of colour, without addition of sodium nitrite, is so slow that it does not in 31 days attain to the intensity obtained in 8 days on addition of sodium nitrite. Also it appears that if caseinogen is taken as a standard in order to determine the tryptophan content of cod-muscle protein, a higher result is obtained with sodium nitrite than without it.

If caseinogen is assumed to have 2·2 % tryptophan [cf. Jones, Gersdorff and Moeller, 1924] the values for cod-muscle obtained by the two methods are 1·45 % and 1·87 %. As already explained, variations in the intensity of the illumination in the one case may give variable results.

Similarly for edestin a tryptophan content of 3·50 % was found, which is considerably higher than the value obtained by May and Rose [1922] and by Jones, Gersdorff and Moeller [1924] without addition of an oxidising agent, the same tryptophan content of the caseinogen being assumed in each case.

Addition of a trace of nitric acid or of hydrogen peroxide has a similar effect to that of sodium nitrite.

Effect of light.

It has been mentioned already that the coloration produced in the May and Rose test varies according to the degree of illumination of the solution.

The following experiment illustrates the effect of light in promoting the reaction of tryptophan with *p*-dimethylaminobenzaldehyde. 0·1 g. edestin was added to a mixture of 100 cc. 18 % hydrochloric acid with 1 cc. of a 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulphuric acid. This was allowed to stand at room temperature (10°) in the dark for 24 hours. A little gelatinous edestin remained undissolved and was removed by means of a glass rod. Equal portions of the clear colourless solution were decanted into three beakers of 5 cm. diameter. One of these was returned to the dark room, one was placed just inside a closed window and the third outside on the sill. The sky was cloudy but fairly bright. Within 1 minute the solution outside was distinctly blue, whilst no trace of blue colour could be detected in the other portions. In 30 minutes the solution outside was deep blue, that inside the window was faintly blue, whereas that in the dark was quite colourless. A drop of hydrogen peroxide rapidly developed colour in the last-mentioned solution. It is probable that the ultra-violet rays are the active agents since the interposition of glass or toluene makes a considerable difference in the rate of colour development.

From these considerations it appears that the best way to avoid the disturbing effects of varying illumination, and of reducing substances, is to add a little sodium nitrite, nitric acid or hydrogen peroxide to the test after hydrolysis is completed. For example 3 drops of 0·5 % sodium nitrite may be added to the test after incubation for 24 hours at 36° and 3 days at room temperature, and again after 3 days more, the colorimetric comparison being made next day.

The addition of sodium nitrite has been adopted by Fürth and Lieben [1920], using formaldehyde instead of *p*-dimethylaminobenzaldehyde. This addition is now shown to be advisable with the latter reagent.

Undoubtedly the ideal procedure would be to carry out the test on an enzyme-digest of the protein if the tints obtained with these digests were not so variable for different proteins. From some tests made on these lines it appears likely that the tryptophan content of cod-muscle is higher than the value given on page 81, and higher than that of caseinogen, but owing to the difficulty mentioned the results are uncertain.

Summary.

Errors can arise in the determination of tryptophan in proteins by the method of May and Rose (1) through unequal illumination of the reacting mixtures, and (2) through the presence of reducing substances such as hydrogen sulphide or aldehydes.

The development of the colour is an oxidation process which goes on slowly in dull light and more rapidly in bright light. It is not nearly complete in a period of 4 weeks in ordinary diffuse daylight in the laboratory. It can conveniently be hastened by adding a trace of an oxygen carrier or oxidising agent after hydrolysis of the protein.

In carrying out the test 3 drops of 0·5 % sodium nitrite solution should be added to the reaction mixture after 24 hours' incubation at 36° and 3 days at room temperature and again after a further 3 days, the colorimetric comparison being made next day or later.

By this modified method higher values for the tryptophan content of cod-muscle protein and edestin are obtained than by the unmodified method of May and Rose.

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