FACTORS AFFECTING THE DEVELOPMENT OF FLUORESCENCE IN THE TRYPTOPHAN-DEXTROSE REACTION

by

GERALD DALE MILLER

B. S., University of Nebraska, 1924

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Flour and Feed Milling Industries

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1953

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INTRODUCTION

One of the chief sources of error in the analysis for tryptophan is the destruction of that amino acid during hydrolysis. This destruction is particularly marked when plant materials and foods are hydrolyzed and is due, presumably, to the Maillard (25) reaction of tryptophan with the aldehyde group of the carbohydrate fraction.

The ideal analytical method for tryptophan, and, in fact, for all amino acids, would be one that could be applied to the unhydrolyzed and unpurified material. Some progress has been made toward that goal for pure proteins, but little has been made for impure proteins. Some of the methods available for the anlaysis of tryptophan in purified unhydrolyzed proteins are the fluorometric method (10), spectrophotometric methods, (9), (15), the glyoxylic acid method (5), (38), the p-dimethylaminobenzaldehyde (p-DAB) method as modified by Spies and Chambers (39), and the microbiological procedure making use of the protozoan, Tetra hymena geleii (20), (34). Two of these methods (38) (39) may be used with hydrolysates of pure proteins. Only the latter method (20), (34) may be applicable also to the analysis of proteins contaminated with carbohydrate material.

Additional chemical methods which are suitable for the analysis of tryptophan in the hydrolysates of pure proteins make use of oxidizing agents which react with the indole group in the presence of acid to form colored products. Some of the oxidizing agents include sodium hypochlorite (27), (28), sodium nitrite (16), nitric acid and sulfuric acid (35), and ferric chloride in the presence of hydrochloric acid (43). Sodium nitrite and potassium persulfate in a solution of thymol also have been used to develop a colored product with tryptophan (1). Yoshida (49) made use of

a color produced by tryptophan with bromine in the presence of methyl orange.

The more important chemical methods for the analysis of tryptophan measure the color that is formed by the oxidation of the complex produced by the reaction of tryptophan with aldehydes (39), (46). Some of the aldehydes that undergo reaction with tryptophan include: formaldehyde (8); salicylic aldehyde (46); and vanillin (21). The aldehydes most frequently used are p-dimethylaminobenzaldehyde (18), (39), and glyoxylic acid (5), (38), both of which have been used for the analysis of tryptophan in intact proteins.

A unique chemical method for the analysis of tryptophan employing a diazo reaction was introduced by Nickols and Eckert (32). The resulting product was condensed with n-(1-napthyl)-ethylenediamine hydrochloride. This procedure was subsequently modified by Eckert (6) to make it suitable as a microphotometric method.

None of the above methods have been successful when applied to the analysis of tryptophan in food materials because of carbohydrate interference. Methods in which tryptophan is separated from the hydrolysate before the final measurement, should be better suited for this purpose. It must be recognized, however, that there is still destruction of tryptophan caused by reaction with carbohydrate material during alkaline hydrolysis.

Lugg (23) used mercury salts to precipitate tryptophan from a protein hydrolyzed with barium hydroxide. The mercury could have been removed from the precipitate and the estimation of tryptophan made by any one of several methods. Lugg chose to use the Millon reagent as modified by Abderhalden (23). The reaction is based on the formation of a red color by the action of sodium nitrite on the tryptophan that had been previously

heated with mercury salts in the presence of sulfuric acid. Although this method did not necessitate the removal of mercury, it is not specific for tryptophan. Interferring substances such as skatole, phenol, and indole were removed by extraction with ethyl ether prior to the precipitation with mercury. Tyrosine also interferes, but it is neither precipitated by the mercury salts nor extracted ethyl ether. The time for reading the maximum color development is very critical because it begins to fade rapidly 10 seconds after the nitrite has been added. Hence, this colorimetric method has serious limitations for the quantitative estimations of tryptophan. Lugg realized that the results obtained were low, and in 1938 (24) suggested a correction factor of 3 per cent when stannous chloride plus sodium hydroxide was used, and 6 per cent when sodium hydroxide alone was used for hydrolysis.

Several variations in chromatographic technic have been applied to the separation of amino acids in mixtures. One of these employs filter paper on which is placed a spot that contains the amino acid mixture. One end of the paper dips into a solvent or mixture of solvents and as the solvent moves past the spot, a difference in the rate of travel of the individual amino acids causes them to be spearated. When there is no difference in rate of travel, no separation results, however, by a judicious choice of solvents, separation can usually be attained. Tryptophan and related compounds are usually chromatographed with water-saturated-n-butanol. After identification of the area on the filter paper containing the tryptophan, any of several quantitive analytical tenchics may be applied. A red color may be developed by spraying the chromatograph with a solution of ninhydrin or p-dimethylaminobenzaldehyde (2). The color density can be measured with a densitometer. By measuring the color densities

of similarly treated spots containing known amounts of tryptophan, an estimate of the tryptophan in the unknown can be calculated. The red colored substance from the individual spots may be extracted and their extinctions measured in a photoelectric colorimeter (3). The assay levels are in the order of a few ppm., but because the effects of humidity and temperature during chromatographing are critical, the error may be as large as 15 per cent.

Moore and Stein (30) employed a starch column to separate amino acids chromatographically. A known quantity of a protein hydrolysate was placed on top of a potato starch column, and the chromatogram developed by an organic solvent not quite saturated with water or dilute HCl. One-half ml. or larger portions of the eluate are taken with an automatic fraction collector and the quantity of amino acid determined by reaction with ninhydrin. On plotting the volume of eluate versus quantity of amino acid, a series of peaks is obtained. The amino acid concentration can be calculated from the area under the curve. The identity of the amino acids are established by use of known samples. By changing solvents during the development of the chromatogram, a better separation of the amino acids can be accomplished. Moore and Stein (31) point out that traces of carbohydrate, and the low capacity of these starch columns tends to interfer with the separation of pure amino acids.

Recently, Moore and Stein (31) have applied sulfonated polystyrene resins having a resolving power comparable with starch or paper to the chromatographic separation of amino acids. The use of buffers minimizes the decomposition of the amino acids during the development of the chromatogram, and it is easier to analyze amino acids in a more neutral effluent by the ninhydrin method. Essentially, the development of the chromatogram

is started by placing a measured amount of the hydrolysate on the column and then adding a low pH buffer at a controlled temperature. As the development progresses, the pH of the buffer is raised and higher temperatures may be used. The fraction collector, ninhydrin reagent, and the calculations are the same as for the starch-column method. By a slight modification of the technique, tryptophan can be separated without separating the entire series of amino acids. Paper and column chromatographic technics promise to be useful, but much research remains to be done to make them adaptable to the routine analysis of amino acids.

A method that has yielded much valuable information in the field of nutrition and one that has served as an excellent aid in verifying the analysis of amino acids by other methods has been the microbiological assay technic which is based upon the nutritional requirements of microorganisms. By using a media that contains all essential nutrients other than the one being analysed, various amounts of a solution or hydrolysate containing an unknown quantity of the constituent in question will produce growth which can be measured either by trubidometric methods or by titration with base. The quantities of unknown amino acids can be determined by reference to a standard curve established for the particular experimental conditions employed.

The inherent error of microbiological amino acid assay is in the order of ±15 per cent (41), but this may be reduced by careful attention to details and replicate determinations. Kuiken, et al (22), Stokes, et al, (42), and Wooley and Sebrell (48), have developed microbiological methods that are far superior to any method except the most elaborate types, such as the isotope dilution method and the better chromatographic technics.

Microbiological methods are subject to many more factors of variation

than are chemical and physical measurements. Only the natural forms of the amino acids are normally utilized (22), (41), however, Rydon (37) has pointed out that the d-amino acids may affect the accuracy of microbiological determinations while Kidder (20) has indicated that Tetra hymena geleii can utilize up to 75 per cent of dl-tryptophan. The presence or absence of vitamins (47), of lipid materials (47), of oxygen (4), and closely related materials (37), (40) all affect the rate of growth of the test organism. With few exceptions (34) microbiological amino acid assays require complete hydrolysis of the protein, and therefore are subject to the same hydrolytic losses which occur in other methods.

Liberation of tryptophan from the protein for assay purposes can be accomplished in several ways. Generally, enzymatic or alkaline hydrolysis technics are used to prevent excessive destruction of tryptophan. The stability of tryptophan, even in the presence of copper and iron ions and aldehyde groups is much greater in hot alkali than in hot acids. Recently, Underwood and Deatherage (45) introduced a method for protein hydrolysis in which the protein is refluxed in water in the presence of the hydrogen form of a sulfonated polystyrene resin. Unfortunately, tryptophan was destroyed in the process. Tryptophan destruction might be limited, however, by employing the sodium form of the resin and an alkaline medium.

When it is desirable to avoid destruction and/or racemization of tryptophan, hydrolysis by proteolytic enzymes is often used. Pancreatin, papain, pepsin, trypsin, hog and rat mucosa (12), (13), (18), (26), and combinations of these enzymes have been employed. Greenhut, et al (13) advised the use of a mixture of 50 mg. of pancreatin and 25 mg. of hog mucosa (Wilson) at pH 8.2 per gram of protein at 37° C. for 24 hours, while

Horn (17) recommends papain at pH 8 at 70° C. overnight, Shaw and McFarlane (38) recommend pepsin and trypsin or trypsin alone in lieu of alkaline hydrolysis. There are, however, several disadvantages associated with the use of proteolytic enzymes. The hydrolysis seldom goes to completion, and many days are often required to get satisfactory r esults. Enzymes, being proteins, often undergo autolysis resulting in the liberation of tryptophan from the enzyme itself. Carbohydrate is still present in the enzymatic hydrolysates of food materials, and the problem of interference by the "browning" reaction and/or darkening by contact with sulfuric acid is still a problem. Thus, the use of enzymatic methods of hydrolysis is but a compromise with the ideal for a precise method for estimating tryptophan in materials containing carbohydrates.

The best method for the liberation of tryptophan from protein employs stong alkalies (sodium hydroxide, potassium hydroxide or barium hydroxide) which results in the complete racemization of the amino acid. There is considerable difference of opinion concerning the merits of the various alkalies for the hydrolysis of proteins. Folin and Ciocalteu (7) claimed that hydrolysis of proteins with 1.4 per cent barium hydroxide gave low and erratic results, while Green and Black (12) reported that hydrolysis with 5N barium hydroxide for seven hours at 15 lbs. pressure was satisfactory for the microbiological determination of tryptophan. Horn (17) pointed out that alkaline hydrolysis in naturally occurring protein foods was unsatisfactory for colorimetric analysis because of the dark and colored solutions, while Greenhut, et al (13) reported erratic results and loss of tryptophan as a result of using 5N sodium hydroxide for the hydrolysis of foodstuffs when measured with L. arabinosus. Lugg (24) reported that the use of 5 per cent stannous chloride in 5.5 N sodium hydroxide reduced the hydrolytic

destruction of tryptophan in the presence of large quantities of carbohydrates.

Kuiken, et al (22) investigated the factors affecting the stability of tryptophan in alkali and concluded that a reaction between molecular oxygen and tryptophan resulted in a loss of the latter. After a survey of the advantages and disadvantages of the various methods of alkaline hydrolysis of proteins Spies and Chambers (39) made a critical study of sodium hydroxide as a hydrolyzing agent for proteins and evolved a method of hydrolysis based on the use of a reducing atmosphere of hydrogen within a sealed nickel Parr microbomb. The procedure as described (39) provides: (1) an inert vessel that can be used over a large range of temperatures; (2) protection of the tryptophan from oxidation by an inert atmosphere, thus making unnecessary the addition of antioxidants that may interfere with reactions being studied or require removal to prevent interference with chemical or microbiological analysis of hydrolysates; (3) prevention of sample loss on evacuation and filling with inert gas; (4) use of reproducible conditions; (5) preparation of sufficient hydrolysate for both chemical and microbiological tests; and (6) use of 20 to 50 mg. of protein sample.

Haney and Johnson (14), while studying the factors involved in the production of "browning" in baked products, observed that tryptophan, on being warmed in the presence of glucose, produced a product or products that had highly fluorescent properties in comparison to those produced by the other amino acids. The object of the present study was to further define the conditions affecting the development of fluorescent compounds resulting from the "browning" reaction and to apply the reaction to the quantitative estimation of tryptophan.

MATERIALS AND METHODS

Amino Acid Source

All the amino acids normally found in wheat flour hydrolysates were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, and were used without additional purification. One sample of analytically pure l-tryptophan from Nutritional Biochemicals Corporation and two samples of pure dl-tryptophan obtained from the Dow Chemical Company, Midland, Michigan, and Merck and Company, Rahway, New Jersey respectively, were studied. The sample from the Dow Chemical Company, recrystallized twice from water and ethanol followed by a rinse with anhydrous ethyl ether, was used as the standard.

Dextrose Source

Analytically pure anhydrous dextrose samples were obtained from Merck and Company, Rahway, New Jersey, Fisher Scientific Company, St. Louis, the Matheson Company, Joliet, Illinois, and Pfansteihl Chemical Company, Waukegan, Illinois. A commercial grade of dextrose (Cerelose) also was obtained from The Corn Products Sales Company, Kansas City, Missouri.

The buffer was prepared by mixing one part of a 0.40 M sodium citrate solution with seven parts of a 0.40N hydrochloric acid solution yielding a buffer with a pH of 1.2 ±.05.

Protein Hydrolysis Technic Applied to Tryptophan

The hydrogen-filling apparatus procedure, and the nickel Parr microbombs employed throughout this work were the same as described by Spies and Chambers (39). Samples of tryptophan were mixed with 1.0 ml. of a 5.0 N solution of sodium hydroxide in a nickel bomb. The bomb was closed in a hydrogen atmosphere and placed in a thermostatically controlled air oven for five hours at 100° C. After cooling to room temperature, the bomb was opened, and the contents washed into a beaker with hot water using a micro-wash bottle. This solution was neutralized with hydrochloric acid (10 ml.) and diluted to the desired volume with buffer.

A Coleman photofluorometer Model 12, with a Corning filter, No. 5684 for the 365 mμ mercury line and another filter made up with two Corning filters Nos. 3398 and 4308 which absorb below 425 mμ was used for the estimation of fluorescence. For higher tryptophan levels, another Corning filter, No. 5864, equipped with a screen that restricted all but 19 per cent of the incident light was used. The instrument had been modified by the manufacturer to increase the sensitivity about ten times.

A solution of 0.01 μg per ml. of quinine sulfate in 0.1 N sulfuric acid was used to adjust the sensitivity of the instrument to read 40 units. For use with the filter equipped with the screen, the concentration of the quinine sulfate solution was increased ten times and the instrument adjusted to read 80 units. Dilutions of the reaction mixture were made with buffer in order to adjust them to the reading range of the photofluorometer.

One ounce prescription bottles, with aluminum foil inserts in the caps, were used as containers for the solutions that were heated in the autoclave.

EXPERIMENTAL

In order to adapt the well-known Maillard or "browning" reaction to the quantitative estimation of tryptophan, a critical evaluation of the effects of pH, temperature, time, sugar and tryptophan concentrations was made. The variation in the reaction of the biologically active or l-form and the racemic or dl-form of tryptophan, the purity of the tryptophan sample used for standard, the effect of grades of dextrose, and the interference of the other amino acids normally found in foods were also investigated.

The Effect of pH on the Development of Fluorescence

Roxas (36) was one of the first workers to report that humin was formed by boiling tryptophan with dextrose. When the reaction was carried out in a 20 per cent solution of hydrochloric acid the quantity of humin accounted for 71 per cent of the nitrogen in the tryptophan. Tarassak and Simonson (44) pointed out later that browning and fluorescence develop simultaneously during the sterilization of evaporated milk, but that the materials were not necessarily identical. Olcott (Mohammed, et al (29)), studied the reaction between glucose and proteins, and found that the rate of browning was a function of the hydrogen-ion concentration.

A study of the effect of pH was made employing solutions containing 8.0 mg. of tryptophan and 10 g. of dextrose diluted to 100 ml. with various concentrations of hydrochloric acid and sodium hydroxide. Since fluorescence was inhibited at a pH above 8.0, studies on the effect of pH were limited to the range between 1.0 and 7.0. To develop fluorescence, the solutions were placed in a preheated autoclave and heated to 119° C. for 2.5 hours.

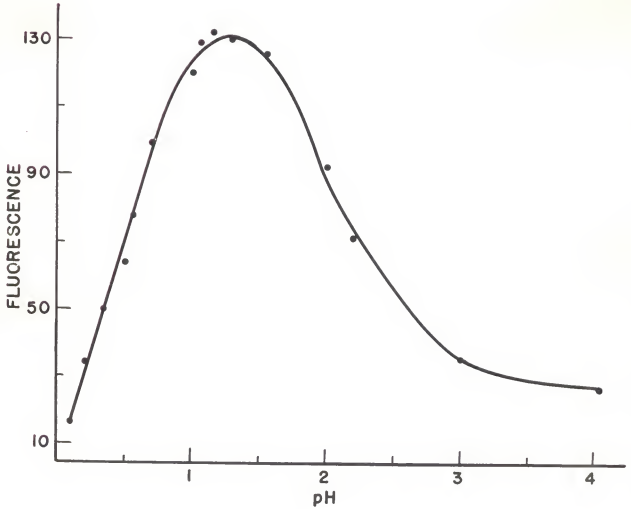

Fig. 1. The effect of pH on the development of fluorescence.

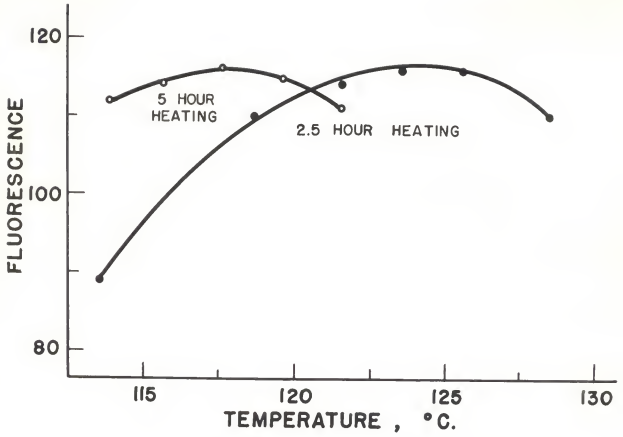

Fig. 2. The effect of temperature on the development of fluorescence.

Simultaneously, solutions of glucose were heated to provide blank determinations. The data indicating that maximum fluorescence was produced between pH 1.15 and 1.30 are shown in Fig. 1. In view of these data, a sodium citrate-hydrochloric acid buffer (pH 1.20 ± 0.05) was chosen for the study of other factors which influence fluorescence of these products. Experiments established that the presence of buffer salts did not affect the development of fluorescence.

The Effect of Temperature and Time on the Development of Fluorescence

Temperatures above 100° C. were chosen for this study in order to shorten the reaction time. A solution containing 15 grams dextrose and 6.0 mg. of tryptophan was diluted to 100 ml. with buffer. Aliquots of these solutions were heated for 2.5 and 5 hours in a preheated autoclave at various temperatures ranging from 114° to 126° C. These data, shown in Fig. 2, indicate that the development of fluorescence in the glucose-tryptophan reaction is a function of both time and temperature. A temperature of 119° C. was selected for further study.

Solutions containing 6.0 mg. of tryptophan and 15 g. of dextrose diluted to 100 ml. with buffer were heated for various time intervals at 119° C. The relationship of time of reaction and fluorescence development is shown in Figure 3. These data suggest that the development of fluorescence had reached its maximum after 180 minutes of heating. In further experiments solutions were heated for 180 minutes.

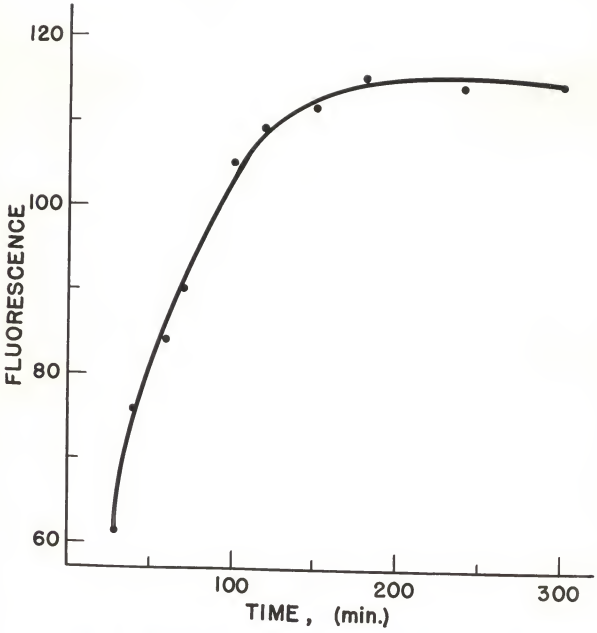

Fig. 3. The effect of time on the development of fluorescence.

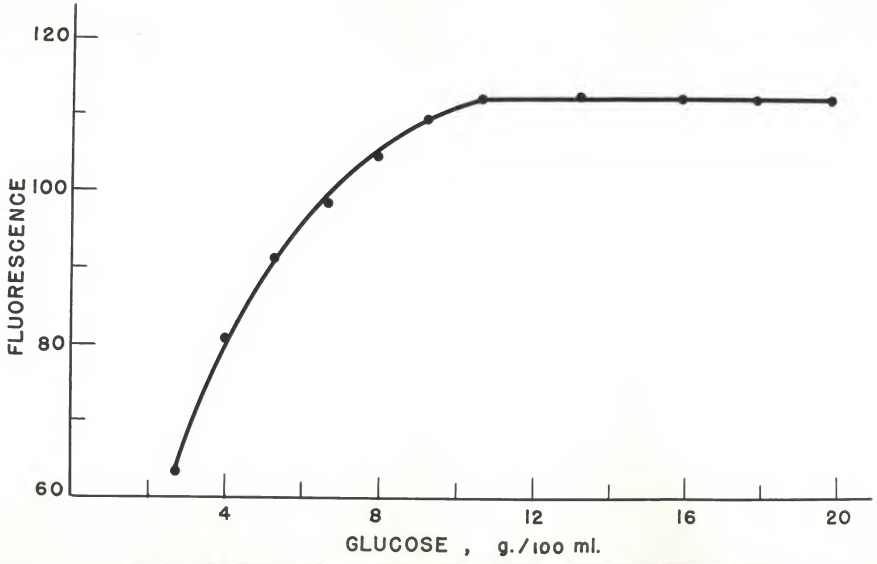

Fig. 1. The effect of glucose concentration on the development of fluorescence.

The Effect of Dextrose Concentration on the Development of Fluorescence

The relationship between dextrose concentration and fluorescence developed when heating 6.0 mg. of tryptophan with from 2.7 to 20.0 g. of dextrose per 100 ml. of solution at pH 1.2 is shown in Fig. 4. These experiments showed that a minimum of 11.5 g. of dextrose were required for 6.0 mg. of tryptophan. As the result of these experiments 15 g. of dextrose was adopted as an adequate amount provided the concentration of tryptophan does not exceed 6.0 mg.

After testing several different dextrose samples including one of commercial grade (cerelose), it was found that only the analytical grades were satisfactory for use as a reagent in the dextrose-tryptophan reaction.

Preparation of the Standard Curve

A standard curve was prepared by reacting 15 grams of dextrose with from 1 to 6 mg. of tryptophan made up to 100 ml. with buffer pH 1.20 for 180 minutes at 119° C. All samples were diluted 1 to 1250 before reading in the fluorophotometer. The data plotted in Fig. 5 indicate that there is direct proportionality between the tryptophan concentration and the amount of fluorescence developed under standard conditions.

A Study of the Precision of Replications

A series of tryptophan-glucose buffered solutions was prepared in triplicate, consisting of four replications and four tryptophan levels and containing from 1.0 to 6.0 mg. of tryptophan and 15 g. of dextrose per 100 ml. Replicate solutions were heated at 119° C. for 3 hours on four consecutive days. Results from individual assays from all tryptophan

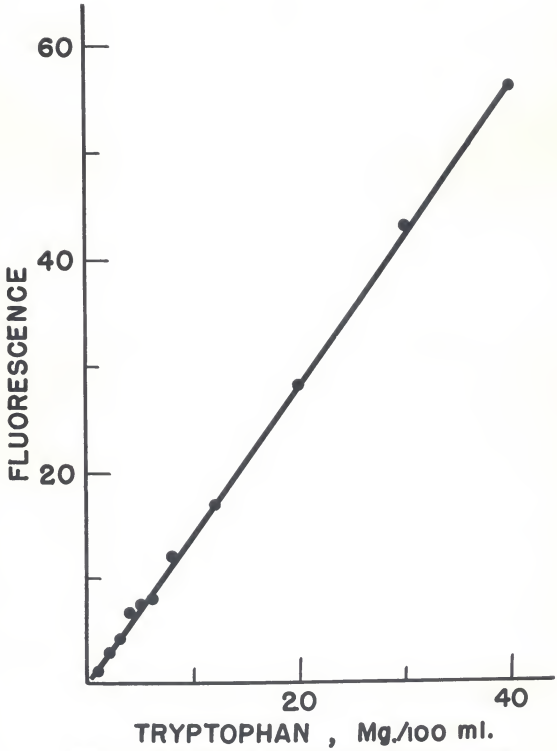

Fig. 5. The effect of tryptophan concentration on the development of fluorescence.

levels and an analysis of variance are reported in Table I. No significant difference was found between days and the level of tryptophan accounted for most of the variation.

Table 1. The effects of tryptophan concentration on fluorescence.

Levels of tryptophan mg/100 ml.	Fluorescence produced by the reaction Days 1	2	3	4	Average
1.0	15.0	15.0	15.5	15.6	
	15.5	16.0	15.5	15.6	
	16.0	16.0	15.5	15.1	15.5
2.0	32.0	32.0	31.5	31.6	
	32.0	32.0	31.5	31.6	
	31.0	31.5	32.0	34.1	31.9
4.0	61.0	62.5	61.5	61.6	
	61.0	62.5	61.5	61.6	
	61.0	62.5	63.0	62.6	61.9
6.0	91.0	96.0	92.6	94.9	
	91.0	95.0	92.1	94.9	
	91.0	97.0	92.6	96.9	93.4

Analysis of variance

Sources of variation	Degrees of freedom	Mean Squares
Days	3	0.07 ns
Levels	3	55,039.38 ***
Error (within levels)	41	1.748

***Significance exceeds the 0.1% level.

DISCUSSION

The problem of assay for tryptophan always has been difficult. The numerous limitations of available methods have suggested that a new approach might be profitable. Since tryptophan was known to react with reducing carbohydrates (25) to produce a fluorescent material (11),(14), this reaction appeared to offer several advantages over other methods. Normally, losses occurred during hydrolysis of proteins and this loss is usually augmented by the presence of reducing sugars. It was anticipated that the utilization of the reaction between tryptophan and dextrose for purposes of measurement might recover some of the apparent losses which are not measureable by other methods.

Amino acids other than tryptophan undergo a similar reaction to produce "melanoidan" substances. It was observed by Haney and Johnson (14) that fluorescent substances also are produced by some amino acids other than tryptophan, however, it had not been established whether sufficient fluorescence is contributed by them to interfere with the estimation of tryptophan. The factors that affect the development of fluorescence when amino acids are heated with glucose were unknown, hence, this investigation was designed to study these conditions.

It has been known for a long time that variations in pH influence solubilities, reaction rates, and biological activities. Changes in pH also may quench or irreversibly destroy the fluorescent properties of a substance. Figure 1 indicates that, under the conditions used, the maximum production of the fluorescent material in the tryptophan-dextrose reaction occurred in the range of pH 1.15 to 1.30. Unless some undesirable product is produced with fluorescent properties at or near this pH range, a reaction pH of 1.15 to 1.30 would be the logical choice for the reaction to be conducted

for maximal differentiation between quantities of tryptophan. This is especially true when low levels of tryptophan are being measured because the largest amount of fluorescent products is produced at this pH. The method is most sensitive and critical in this range.

Hydrogen-ion concentration, however, not only affects the development of fluorescent compounds, but may have a direct affect on the intensity of fluorescence. The reading-pH fluorescence curve is a complex function of the influence of acidity upon the absorption spectrum and upon fluorescent efficiency. These curves have been considered by many to be at least as useful as melting points, elementary analysis and absorption spectra in the confirmation of a few compounds (19). The color of the fluorescent light, as well as the rate of photodecomposition, often changes with a shift in pH. Occasionally, the measured fluorescence increases with the wave length of irridation, and often the fluorescence is completely quenched by a change in pH. A preliminary investigation of the affect of the reading-pH on the intensity of the fluorescent product from the tryptophan-dextrose reaction revealed that the maximum was not identical with that of the reaction-pH maximum. Although the reading-pH for maximum intensity has not been established definitely for this substance it appears to pass through a maximum slightly above pH 4 and falls rapidly as a pH of 6 is approached. The fluorescent material is irreversibly quenched above pH 7.0. Concomitant with the disappearance of the fluorescence there is a development of a brown color. These observations suggest that the compounds responsible for fluorescence are probably precursors of the browning reaction products. Apparently, condensation of the tryptophan-dextrose compounds follows the initial reaction.

In developing a method for the determination of tryptophan it was found

that the effect of changes of reading-pH of the dextrose blank was about equal to the effect of changes of reading-pH on the fluorescence of the reaction products. Therefore, the fluorescence measurements were made at the same reading-pH as the reaction-pH. The buffer employed in these studies was found to have no quenching effect on the fluorescence.

Time and temperature are so closely related that it would be difficult to c onsider them separately. Any reaction is an equilibrium between the products and the reactants and a measure of the reaction rate can be obtained by plotting the concentration of reaction by-products vs. time. At the temperature selected, the rate of the reaction as indicated by the time vs. fluorescence curve (Fig. 2) proceeded rapidly for the first two hours after which a change in slope indicated a decrease in this rate. After a period of about three hours the reaction appeared to have been completed. When the reaction time was considerably longer than is indicated by the curve in Fig. 2, the fluorescent compound disappeared and "melanoidan" pigments were rapidly produced.

An inverse relationship exists between time and temperature, when applied to a reaction. The same end result can be obtained by varying time and temperature in the proper ratio. In Fig. 3, the same maximum fluorescence was produced by heating the reactants at 119° C. for 2.5 hours as was produced by heating them at 125° C. for 5 hours. In the interest of the economy of time, the three hour heating period and a temperature within the operating range (119° C.) of most autoclaves was selected. Many other combinations of time and temperature would achieve the same end result, however, these should be selected to permit reasonable control and to assure the absence of pyrolysis.

Selection of the proper concentration of glucose depends upon the amount of tryptophan to be assayed. Sufficient glucose must be present

to completely utilize all of the tryptophan and still not contribute sufficient by-products to hamper seriously the passage of light through the solution during the photo-optical measurements. A ratio of 11.5 g. of dextrose to 6.0 mg. of tryptophan in 100 ml. was found to be sufficient to complete the reaction. It was observed during this study that excessively high ratios of tryptophan to dextrose (in the order of 1:50,000 as compared to 1:2500 as used) caused low results. When low assay levels of tryptophan are used, the dextrose concentration must be lowered proportionately.

When 2.0 mg. of tryptophan per 100 ml. was reacted with glucose, it was necessary to make a dilution of 1:500 for measurement in the middle of the reading range of the photofluorometer. This is an estimated reading level of 4.0 µg. of tryptophan per 100 ml. (4×10^{-2} ppm.). One can then assume that the tryptophan concentration may be lowered to this same level (4×10^{-2} ppm.) in the reaction mixture if the concentration of glucose is correspondingly decreased. The fluorescence of the reacted solutions then could be measured directly without dilution.

Since the assay level for tryptophan by the microbiological method (41) is in the order of 100 ug. per 100 ml. (1 ppm.) the sensitivity of the present method is about 25 times higher than that for the microbiological method. A standard deviation of 0.3 fluorescent units was calculated for the present method and the error is estimated to be in the order of 0.5 per cent when based upon the analysis of pure tryptophan.

All of the amino acids other than tryptophan which are found in protein hydrolysates were tested for their contribution to the production of fluorescent materials when reacted with dextrose under the outlined standard conditions. No one amino acid contributed much fluorescence but

when these individual contributions were pooled, the total was in the order of 20 per cent of that produced by the tryptophan-glucose reaction. This source of error cannot be ignored and it is proposed to use an internal standard of tryptophan to be added or superimposed upon the aliquot of hydrolysate to be assayed. Another like sample without the added tryptophan would be run concurrently and the difference in amount of fluorescence would be used as a standard.

It is suggested that the tryptophan-dextrose or "browning" reaction, carried out under the standard conditions as outlined, can be applied to the estimation of tryptophan in amino acid hydrolysates. The standard conditions include: (1) a ratio of tryptophan to dextrose in the order of 1:2500; (2) a reaction pH of 1.15 to 1.30; (3) a reaction time of 3 hours; (4) a reaction temperature of 119° C. in a preheated autoclave, or some other combination of time and temperature that may be suitable for the reaction; and (5) a reading pH of 1.20. A standard curve for tryptophan may be prepared by using an internal standard consisting of known amounts of tryptophan added to the substance to be assayed. The differential or delta fluorescence may be plotted vs. added tryptophan concentrations. It may be necessary to employ the Moore and Stein procedure (31) for removing interfering amino acids before reaction with glucose.

SUMMARY

A survey of the literature dealing with the available methods for the estimation of tryptophan has been made. Deficiencies which limited the use of most of these methods to the analysis of tryptophan in foods have been cited. These observations suggested the need for another approach to the problem of the estimation of tryptophan.

Fluorescent products were formed during the Maillard reaction. The fluorescent products resulting from the reaction between tryptophan and dextrose suggest that this reaction can be used as a measurement of tryptophan. The conclusions resulting from a study of the factors affecting the rate of formation of these fluorescent substances were summarized as follows:

(1) The optimum pH for the formation of fluorescent products was in the range of pH 1.15 to 1.30. During the measurement of fluorescence, above pH 7 a brown colored product was formed which was suggestive of a "melanoidan" pigment. Beyond pH 7.0 an irreversible quenching of fluorescence results.

(2) Time and temperature of reaction were interrelated. By the proper choice of different combinations of these two factors, the same net end result can be obtained. A temperature within the normal operating range of most autoclaves (119° C.) and a time of 3 hours had been suggested.

(3) Dextrose concentration was dependent upon tryptophan levels. A concentration of 11.5 g. of dextrose was sufficient to completely utilize 6.0 mg. of tryptophan. The use of 15 grams of dextrose was suggested for 6.0 mg. of tryptophan. Lower assay levels of tryptophan with correspondingly lower concentrations of dextrose were suggested as a micro-technic for the estimation of tryptophan.

(4) The measurement of the amounts of fluorescent substances formed under standard conditions was proposed as a method for the estimation of tryptophan. The error of measurement was in the order of ± 0.5 per cent.

(5) An internal standard for the preparation of the tryptophan standard curve was suggested. The removal of interferring amino acids before reaction with dextrose may be necessary.

ACKNOWLEDGMENTS

The author wishes to acknowledge his indebtedness and gratitude to Dr. Byron S. Miller for his generous contributions of time and effort in the construction of apparatus and his encouraging supervision and aid during the experimental phases and preparation of this thesis.

Special appreciation is extended to Prof. John A. Johnson for his helpful suggestions, gracious consideration and interest extended during the maturation of the problem and preparation of this thesis.

Acknowledgment is extended also to Dr. John A. Shellenberger for the provision of experimental facilities and his interest in this work, and to the other members of the Department of Flour and Feed Milling Industries who in many ways have aided in this work.

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FACTORS AFFECTING THE DEVELOPMENT OF FLUORESCENCE IN THE TRYPTOPHAN-DEXTROSE REACTION

by

GERALD DALE MILLER

B. S., University of Nebraska, 1924

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Flour and Feed Milling Industries

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1953

Since a review of the literature revealed deficiencies in all of the known methods for the estimation of tryptophan in foods, this study was undertaken to develop a new method for the assay of this amino acid. It has been observed that substances with fluorescent properties are produced during the Maillard or "browning" reaction. Since the conditions affecting the rate of formation of these compounds during the reaction between dextrose and tryptophan are not known, several factors including the influence of pH, time, temperature and the concentration of tryptophan and dextrose were investigated. The formation of fluorescent substances by amino acids, other than tryptophan, during the dextrose-tryptophan reaction also were studied.

A photofluorometer was used for the estimation of the amounts of these fluorescent materials. A dextrose blank was run concurrently with the tryptophan-dextrose reaction and the difference, or delta fluorescence, was calculated as a measure of the amount of fluorescence produced by the dextrose-tryptophan reaction.

Data showed that the maximum production of substances with fluorescent properties during the dextrose-tryptophan reaction, occurred in the pH range of 1.15 to 1.30. The hydrochloric acid used in the preliminary studies was replaced with a sodium citrate-hydrochloric acid buffer (pH 1.20 ± 0.5) which had no adverse effects on the tryptophan-dextrose reaction.

An inverse relationship was observed between the effects of time and temperature upon the formation of fluorescent substances. As temperature was increased the reaction time for the formation of the same quantity of fluorescent material was decreased. A combination of 3 hours of heating

time and a temperature of 119° C. was chosen. Other combinations of time and temperature also are possible.

Data also showed that 11.5 g. of dextrose was sufficient to completely utilize 6.0 mg. of tryptophan. It was observed, however, that an extremely high ratio (1:50,000) of tryptophan to dextrose had a deleterious effect on the reaction. When the concentration of dextrose was not the limiting factor in the reaction between dextrose and tryptophan, there was a straight line relationship between tryptophan concentration (up to 6.0 mg.) and fluorescence.

Amino acids, other than tryptophan, were found to contribute individually very little fluorescence when reacted with dextrose under standard conditions. When their individual fluorescent effects were pooled, however, their combined effects amounted to about 20 per cent of the amount formed by tryptophan. The ratio of amino acids used was that normally found in flour hydrolysates.

The measurement of the amounts of fluorescent substances formed under standard conditions is proposed as a quantitative means for the estimation of tryptophan. An internal standard, together with the use of the Moore and Stein (31) procedure for removing interfering amino acids, is suggested for the preparation of the tryptophan standard curve.