THE SPECTROPHOTOFUOROMETRIC DETERMINATION
OF TRYPTOPHAN IN PLASMA AND OF TRYPTOPHAN
AND TYROSINE IN PROTEIN HYDROLYSATES

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A spectrophotofluorometer capable of continuous activation of solutions
and measurement of resultant fluorescence throughout the quartz-ultra-
violet and visible regions of the spectrum has been previously described (1).
The ability of such an instrument to deliver high intensity monochromatic
light at the absorption maxima of tryptophan and tyrosine, and to measure
the resulting ultraviolet fluorescence spectra, provides the basis for a
simple and extremely sensitive method for the determination of these amino
acids in tissues and in protein hydrolysates.

EXPERIMENTAL

Spectrophotofluorometer—The design and operation of this instrument
are described in detail elsewhere (1). It consists essentially of a high
intensity xenon arc source which emits continuously throughout the ultra-
violet and visible regions and two monochromators, one to isolate mono-
chromatic light for activation and the other at right angles to the first to
analyze the emitted fluorescence; a photomultiplier tube is used to measure
the intensity of the fluorescent light. Both activation and fluorescence
spectra may be rapidly displayed on the screen of a cathode ray oscilloscope
or the chart of a pen and ink recorder. Activation and fluorescence spectra
for tryptophan and tyrosine are given in Figs. 1 and 2. The intensity of
fluorescence of both amino acids is proportional to concentration through-
out the range of concentration employed in these studies.

Tryptophan and Tyrosine in Protein Hydrolysates

A survey of all of the naturally occurring amino acids found in proteins
revealed that only tyrosine and tryptophan exhibit detectable fluorescence
in aqueous media. Fig. 3 shows the fluorescence spectra of pure samples

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1 The experimental instrument (1) has been used interchangeably with commer-
cial spectrophotofluorometers obtained from the American Instrument Company
and the Farrand Optical Company, Inc.

RCA 1P28 photomultiplier.
of these two amino acids and of mixtures of the two at various pH values. At pH 11, the intensity of the tryptophan fluorescence is approximately 100 times greater than that of a corresponding concentration of tyrosine, and the ability of a spectral instrument to resolve the two fluorescence bands whose maxima occur 50 m\( \mu \) apart makes it possible to measure tryptophan in the presence of large excesses of tyrosine. Since tryptophan is destroyed by acid hydrolysis, the tyrosine content of proteins containing any amount of tryptophan may be readily determined without interference.

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Activation and fluorescence spectra of tryptophan. Curve A, activation spectrum for pure tryptophan (0.1 \( \gamma \) per ml.) in 0.5 M Na\( \text{2} \)CO\( \text{3} \) obtained by setting fluorescence monochromator constant at 360 m\( \mu \) and scanning with activating monochromator. Curve B, fluorescence spectrum for same sample with activation monochromator set at 280 m\( \mu \) while scanning with fluorescence monochromator.

**Fig. 2.** Activation and fluorescence spectra of tyrosine. Curve A, activation spectrum of pure tyrosine in phosphate buffer, pH 8.0; Curve B, fluorescence spectrum of same. The activation and fluorescence spectra were obtained as for tryptophan with monochromators set at 310 and 275 m\( \mu \), respectively.

*Materials*-Pure crystalline samples of reference proteins were employed; zinc insulin, lot No. 535664 was obtained from Eli Lilly and Company; bovine serum albumin, lot No. 370-295-B was obtained from Armour and Company; crystalline \( \beta \)-lactoglobulin was made available by Dr. Thomas L. McMeekin of the Eastern Utilization Research Branch, United States Department of Agriculture.

Tryptophan and tyrosine standards were prepared daily by dilution of stock solutions\(^3\) of the L-amino acids obtained from the Nutritional Biochemicals Corporation.

\(^3\) Stock solutions of 1 mg. of amino acid per ml. of 0.1 N NH\(_3\) were employed. These were kept refrigerated and were freshly prepared each week.
Basic Hydrolysis—The protein is dissolved in 1.0 ml. of 5.0 N NaOH and heated in an open tube\(^4\) in an autoclave for 20 hours at 2 atmospheres. The amount of protein taken (20 to 50 mg.)\(^6\) is corrected for moisture content as determined by drying a separate sample in vacuo at 100\(^\circ\). The cooled hydrolysate is acidified with 1.5 ml. of 5 N \(\text{H}_2\text{SO}_4\), transferred quantitatively to a 25 ml. volumetric flask, and made up to the mark with water. The acidified solution is clarified by centrifugation. A tryptophan standard (1 to 2 \(\gamma\)) and a blank are carried through the hydrolysis procedure.\(^6\)

Acid Hydrolysis—The protein sample is heated as above in 1.0 ml. of 7 N \(\text{H}_2\text{SO}_4\), neutralized by the dropwise addition of ammonium hydroxide, and diluted to 25 ml. with water.

\[
\begin{align*}
\text{pH 1.0} & : \text{A (i) TYROSINE, B (2) TRYPtopHAN, C A+B} \\
\text{pH 7.0} & : \text{A, B, C} \\
\text{pH 11.0} & : \text{A, B, C}
\end{align*}
\]

**Fig. 3.** Variation of tryptophan and tyrosine fluorescence with pH. Activating wave length = 280 m\(\mu\) in each case. pH 1.0 = 0.1 N \(\text{H}_2\text{SO}_4\); pH 7.0 = phosphate buffer; pH 11.0 = 0.09 N \(\text{NH}_3\).

**Determination of Tryptophan**—An aliquot of the acidified alkaline hydrolysate (8.0 ml.) is diluted to 50 ml. with N sodium carbonate. The final solution (133 to 333 \(\gamma\) of protein per ml.) is activated at 280 m\(\mu\) and its fluorescence at 360 m\(\mu\) is compared to that of an appropriate standard.

**Determination of Tyrosine**—An aliquot of the neutralized acid hydrolysate is diluted 10-fold with phosphate buffer, pH 8.0, and activated at

\(^4\) Test tubes of Corning brand No. 7280, alkali-resistant glass are used. The use of standard Pyrex tubes for alkaline hydrolysis results in a high degree of light scattering in the final solution owing to the presence of colloidal silicate. Scattering of the fluorescent light may lead to low results, as much as 20 per cent in error. High speed centrifugation (about 30,000 r.p.m.) can be used to minimize light scattering.

\(^6\) Quantities of this magnitude were used in these studies because of the availability of the protein employed. The sensitivity of the method is such, however, that samples as small as 100 \(\gamma\) may be carried through the same dilution process.

\(^6\) The tryptophan standard is subjected to hydrolysis conditions to correct for losses (5 to 10 per cent) during alkaline hydrolysis.
TRYPTOPHAN AND TYROSINE ASSAYS

275 mμ, and its fluorescence at 310 mμ is compared to that of a standard solution of tyrosine (1 to 2 γ per ml.).

Specificity—Full activation and fluorescence spectra of the diluted hydrolysates proved identical with those of authentic samples of the respective amino acids determined under the same conditions. The ease with which full spectra may be obtained makes possible their application to each

**Table I**

Tyrosine and Tryptophan Contents of Known Amino Acid Mixtures

The values are given in gm. per 100 gm. of mixture.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Tyrosine</th>
<th>Tryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>I</td>
<td>11.8</td>
<td>12.1</td>
</tr>
<tr>
<td>II</td>
<td>6.31</td>
<td>6.22</td>
</tr>
<tr>
<td>III</td>
<td>0.80</td>
<td>9.85</td>
</tr>
<tr>
<td>IV</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Table II**

Tyrosine and Tryptophan Contents of Protein

The values are given in gm. per 100 gm. of protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tyrosine</th>
<th>Tryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Given in literature</td>
</tr>
<tr>
<td>Insulin</td>
<td>11.6</td>
<td>12.25 (2)*</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>6.0</td>
<td>5.5 (4)</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>3.75</td>
<td>3.69 (5)</td>
</tr>
</tbody>
</table>

* Bibliographic reference.

individual analysis. Thus the presence of extraneous fluorescent or absorbing materials may be readily detected from the shape of the curve.

Results—Known amounts of tyrosine and tryptophan when added to mixtures of amino acids7 and subjected to hydrolytic conditions were quantitatively recovered (Table I). In addition, the tryptophan and tyrosine contents of crystalline samples of insulin, bovine serum albumin, and β-lactoglobulin were determined and found to be in good agreement with values cited by other workers (Table II).

7 Synthetic mixtures of pure amino acids were prepared which contained approximately 5 per cent by weight of each of the following: glycine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, histidine, lysine, serine, threonine, proline, hydroxyproline, phenylalanine, methionine, cysteine, asparagine, and glutamine. Known amounts of tryptophan, tyrosine, or both were then added.
Tryptophan in Plasma

Plasma proteins are precipitated according to the procedure of Dunn et al. (6). The supernatant solution is brought to pH 11 and activated at 280 μm. The resultant single fluorescence band which specificity studies showed to arise solely from tryptophan is measured at 360 μm.

Method—A 1.0 ml. sample of plasma is diluted with 4 volumes of water and acidified with 0.5 ml. of 0.6 N H₂SO₄. Protein is precipitated by the addition, with constant shaking, of 0.5 ml. of 10 per cent sodium tungstate and is removed by centrifugation. A 3.0 ml. aliquot of the clear supernatant fluid is transferred to a small centrifuge tube containing 1.0 ml. of 0.25 M barium chloride and the precipitate of barium sulfate and barium tungstate is removed by centrifugation.* A 2.0 ml. aliquot of the solution is treated with 0.5 ml. of 2 M sodium carbonate to precipitate excess barium ion and bring the pH of the sample to the optimal value for tryptophan fluorescence. After centrifugation for 10 minutes at 3000 r.p.m., a small volume (1 to 1.5 ml.), which should be clear and colorless,10 is carefully withdrawn and its fluorescence is measured as described for protein hydrolysates.

Standards—The above procedure involves an over-all dilution of 1:10. Since the normal plasma tryptophan level in fasting subjects is quite constant at about 10 μg per ml., a standard solution of 1.0 μg of pure tryptophan per ml. in 0.3 M sodium carbonate will, therefore, give a reading close to that of the sample. As an additional check, internal standards are routinely used. A sample in which pure water is substituted for plasma is carried through the entire procedure to serve as a fluorescence blank. A typical determination in which full fluorescence spectra of sample, internal and absolute standards, and blank are employed is illustrated in Fig. 4.

Specificity—That tryptophan is the only component normally present in these plasma filtrates in concentrations high enough to show a measurable fluorescence under the conditions employed was indicated by a number of experiments. A 1:10 protein-free filtrate and a 1.0 μg per ml. solution of pure tryptophan, each buffered at pH 4.0, were subjected to a nine plate countercurrent distribution, with n-butanol as the organic phase. The aqueous phases from each tube were made alkaline by the addition of 1 volume of 2 N ammonium hydroxide, and the fluorescence was measured as described above. The distribution of the plasma fluorophor closely

* Reagent grade Na₂WO₄·2H₂O.
10 Tungstate ion has a slight quenching effect upon tryptophan fluorescence and must be removed.
10 If the blood sample has suffered extensive hemolysis, varying amounts of pigments absorbing in the region of tryptophan fluorescence will carry through the procedure and result in low readings. Although the use of internal standards will correct for this source of error, such samples should be avoided whenever possible.
approximated that of pure tryptophan, with a maximal concentration of fluorescent material appearing in the fifth tube in each case.

As is the case with many ampholytic fluorophors, the intensity of tryptophan fluorescence varies markedly with pH. The fluorescence and activation spectra of the filtrate were, therefore, compared to those of authentic tryptophan samples at various pH values. A sharp peak fluorescence was observed in each case at pH 11. Although several other indole derivatives have the same activation and fluorescence maxima as tryptophan, the variations in fluorescence intensity with changing pH are markedly different for each compound. Finally, the fluorescence of both authentic tryptophan and the plasma fluorophor was quantitatively quenched by ascorbic acid, inorganic nitrate, thiosulfate, and peroxide.

**Results**

Recovery of added amounts of tryptophan from plasma was found to be excellent. When 2.5 to 20 \( \gamma \) of tryptophan were added to 1.0 ml. aliquots of plasma, the mean recovery was 98.7 per cent with a standard deviation of 1.1 per cent.

The levels of plasma tryptophan of ten fasting normal subjects were determined, duplicate runs being made on each blood sample on 2 successive days. The mean value on the 1st day was 1.13 mg. per 100 ml. with an

![Diagram](http://www.jbc.org/)

Fig. 4. Curve A, absolute standard, 10 \( \gamma \) per ml.; Curve B, plasma; Curve C, \( \frac{1}{2} \) Curve A; Curve D, plasma + \( \frac{1}{4} \) Curve A; Curve E, blank.
average deviation of 0.04; on the 2nd day 1.11 ± 0.05. The average variation in the values obtained for individual samples was 4.7 per cent.

**DISCUSSION**

Fluorometric assay, when applicable, offers a number of advantages over other methods of assay. Its sensitivity is much higher than that of spectrophotometry and its specificity is greater since it is based on two spectral requirements, activation and emission.

The spectrophotofluorometric assay of tryptophan and tyrosine in protein hydrolysates is a rapid, simple, and specific one and yields values which agree with those obtained by other accepted procedures. The light scattering by suspended silicates formed from the action of alkali on glass during alkaline hydrolysis of proteins illustrates an important interference in this type of assay. Fortunately light scattering occurs at the wave length of activation and may, therefore, be distinguished from fluorescence. When scattering is unduly large, it will be immediately evident upon inspection of the spectrum.

The procedure for determining tryptophan in plasma is obviously simpler than the bioassay methods now in use. The availability of spectrophotofluorometer type instruments should make plasma tryptophan a useful clinical assay. Preliminary studies in this laboratory indicate that, in patients with malignant carcinoid, levels of fasting plasma tryptophan are frequently below normal. This is consistent with the huge excretion of the tryptophan metabolite, 5-hydroxyindoleacetic acid, previously reported in patients with this disorder (7). Tryptophan tolerance studies in humans are also being carried out by using the spectrophotofluorometric assay.

**SUMMARY**

1. Ultraviolet fluorescent spectra of tryptophan and tyrosine have been used for the determination of these amino acids in protein hydrolysates.
2. A spectrophotofluorometric method for the determination of free tryptophan in plasma is described. Levels of plasma tryptophan in normal fasting subjects were determined and found to be in good agreement with the results of microbiological determinations cited by other workers.

**BIBLIOGRAPHY**

THE
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