The Photometric Microdetermination of Tryptophan in Biologic Materials

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A modification of the Fischl (6) glyoxylic acid method for tryptophan was developed which permits the reaction to occur quantitatively in aqueous solution by substitution of 70% HClO₄ (w/v) for concentrated H₂SO₄. Spectral analysis of the chromogen formed showed a strong adsorption band at 365 mµ for pure tryptophan which was 17 times more sensitive than that at 560 mµ. Factors were studied which influence the absorbance of the tryptophan chromogen at 365 mµ and the most favorable conditions for its formation.

A new method for hydrolyzing proteins and peptides was evolved using 3.5% HClO₄ (w/v) which results in complete rupture of the peptide bond while retaining intact the indole nucleus. Application of the modified glyoxylic acid method to perchloric acid hydrolyzates of various proteins and peptides of known tryptophan content gave experimental values in close agreement with those calculated from the structural formulae. The over-all reproducibility of the procedure for separate hydrolyzates, run in duplicate, of the same protein or peptide sample was about ±3.0%.

Comparison of the tryptophan values obtained for 19 different "purified" peptides and proteins checked rather well with some of the results reported in the literature for the same materials using other procedures, except for trypsin and ovomucoid.

The only natural amino acid with an indole ring, L-tryptophan, is not found in a free state in biologic materials with the possible exception of small amounts in urine and serum. Its concentration in animal proteins is relatively small, varying from about 0.2 to 3.0% (1). Analysis of the tryptophan content of proteins, peptides, and other biologic
materials presents a twofold problem. The first resides in the unusual difficulties encountered in attempting to isolate tryptophan from proteins by means of acid, alkaline, or enzymic hydrolysis (2). It is destroyed in toto by strong acid hydrolysis, racemized during alkaline hydrolysis and adsorbed on the insoluble salts formed by removing barium hydroxide as the sulfate or carbonate, and usually incompletely released by enzymic hydrolysis.

The second problem pertains to the lack of suitable specific and sensitive methods for determining the low levels of this amino acid present in protein hydrolyzates and other biologic materials. In 1874, Adamkiewicz (3) observed that the addition of concentrated sulfuric acid to a solution of albumin treated with glacial acetic acid yielded a violet-blue color. Hopkins and Cole (4) showed that the color produced was due to the presence of glyoxylic acid in the acetic acid and utilized this test to first isolate tryptophan from casein digests. For many years several modifications of the Hopkins-Cole procedure, especially that of Shaw and MacFarlane (5), have been used with moderate success for determining the tryptophan content of biologic substances. A more recent modification of the Hopkins-Cole reaction has been published by Fischl (6) for alkaline protein hydrolyzates. This investigator used persulfate to produce the tryptophan chromogen and thioglycolic acid to control the oxidation induced by the persulfate. Because of the difficulties encountered in obtaining reproducible results when utilizing concentrated sulfuric acid in the Fischl (6) method, we investigated the substitution of perchloric acid which had been previously suggested as a specific reagent for tryptophan and other indole compounds by Tauber (7). Since albumin contains about 0.2% tryptophan, whereas globulins contain about 3.0%, the quantitative analysis of the tryptophan content of proteins precipitated from various biologic fluids provides a measure of their total globulin content. In previous publications from this laboratory, a modification of the photometric Fischl (6) method has been employed for the quantitative determination of the total globulins of serum (8), cerebrospinal fluid (9, 10) and urine (11) and of the y-globulin content of serum (12).

It was noticed during the course of these experiments that, while a violet-blue color was obtained with an unhydrolyzed protein (containing tryptophan) with the glyoxylic acid reaction, a yellow color was produced with pure tryptophan standards or with some perchloric acid hydrolyzates. This confirmed the previous findings of Rieder
and Böhmer (13), who reported the presence of an absorption maximum at 370 mμ as well as at 555 mμ when the glyoxylic acid reaction, in the presence of concentrated sulfuric acid, was applied to tryptophan, tryptamine, and 5-hydroxyindole-3-acetic acid.

The present paper deals with the application of dilute (3.5% w/v) perchloric acid as a medium for the hydrolysis of tryptophan-containing proteins during which there is no destruction of the indole nucleus although deamination occurs. The acid hydrolyzate thus obtained can be utilized directly for the analysis of its tryptophan content with a specific and sensitive photometric glyoxylic acid reaction for the indole nucleus of tryptophan in which the yellow color is read at 365 mμ. The method has been found applicable for the determination of the tryptophan content of a wide variety of peptides and proteins.

Experimental

A. Reagents and Apparatus

1. Perchloric acid 7% (w/v).
2. Various purified protein and peptide solutions whose nitrogen content was determined with a micro-Kjeldahl procedure.
3. Autoclave 15 lb. pressure, 120°.
4. Hydrolysis tubes or flasks
5. Nitrogen gas
6. Glyoxylic acid 1.0% (w/v) solution in glacial acetic acid
7. Perchloric acid 70% (w/v).
8. Potassium persulfate, saturated solution Add 25 ml. distilled water to 2 gm. potassium persulfate (reagent grade) and shake well for 5 min. Keep the solution refrigerated when not in use and prepare fresh weekly.
9. Thioglycolic acid (a) Stock solution: Add 1 ml. thioglycolic acid (kept refrigerated) to 19 ml. glacial acetic acid and prepare fresh weekly. (b) Working solution: Dilute 1 ml. of the stock solution with 9 ml. of glacial acetic acid. Prepare fresh for each day’s run.
10. Standard solution L-Tryptophan (10 mg./100 ml.).
11. Spectrophotometer Beckman DU or other instrument suitable for reading in the near-UV range, i.e., 365 mμ.

B. Method for Photometric Determination of Tryptophan

The method, as finally evolved, for the determination of solutions of pure tryptophan in the range of 0.006 to 0.150 mg. per final volume of 5.7 ml., is as follows: Various amounts of the tryptophan standard
solution are pipetted into 13 × 100-mm. test tubes and made up to 1.0 ml. with water; 1 ml. water is used as a blank. Add 2.5 ml. of the glyoxylic acid solution and mix the contents. Add 2.0 ml. of 70% perchloric acid and mix. Let stand at room temperature for 5 min. Add 0.1 ml. of the potassium persulfate solution. Mix and let stand at room temperature for 5 min. Add 0.1 ml. of thioglycolic acid solution. Mix and let stand at room temperature for 25 min. Centrifuge the tubes at 3000 rpm for 5 min. Read in a spectrophotometer at 365 mμ against the reagent blank set at zero.

C. Studies of Factors Affecting Color Development

1. Effect of Variation of Glyoxylic Acid Concentration. Two concentrations of tryptophan (0.025 and 0.125 mg.) were employed in these experiments. The glyoxylic acid concentration in glacial acetic acid was varied from zero to 5.0%. The tryptophan procedure was carried out exactly as described in Section B above and the results obtained are plotted in Fig. 1. A marked increase in the sensitivity of the reaction occurs with increasing glyoxylic acid concentration up to about 1%. Only a slight additional rise in absorbance occurs between 1.0 and 5.0%. A 1.0% glyoxylic acid solution in glacial acetic acid was employed in all subsequent runs.

2. Effect of Varying the Ratio of Glacial Acetic Acid to Perchloric Acid. Using two concentrations of tryptophan (0.025 and 0.062 mg.) the ratio of acetic acid to perchloric acid was varied over the range shown in Fig. 2. All other factors were kept constant and the tryptophan reaction was performed exactly as described in Section B above. Maximum absorbance values and stoichiometry were obtained only over a narrow range, i.e., between 3.0/1.5 and 2.0/2.5. A ratio
value of 2.5/2.0 (acetic to perchloric acids) was therefore employed in all subsequent runs.

3. Effect of Variation of Water Content. Two concentrations of tryptophan (0.05 and 0.10 mg.) were used in experiments in which the water content was varied from 0.30 to 1.7 ml. As the water content was increased, the total acid volume (acetic/perchloric = 2.5 ml./2.0 ml.) was correspondingly decreased so that the final volume was kept constant. The results obtained, using the tryptophan procedure described in Section B above, are illustrated in Fig. 3. The experimental data show that the greatest sensitivity and good stoichiometry are obtained when 1.0 to 1.2 ml. of water was present in the system. The water content of all subsequent experimental runs was maintained between these limits.

4. Effect of Variation of Persulfate Concentration. Two concen-
trations of tryptophan (0.05 and 0.10 mg.) were employed in these experiments in which the volume of saturated potassium persulfate solution was varied from 0.025 to 1.0 ml. The tryptophan reaction was run as described under Section B above and the results obtained in these runs are shown in Fig. 4. It was concluded from these data that the amount of persulfate used above 0.10 ml. has little or no effect on the absorbance values obtained. Therefore 0.10 ml. of this reagent was used in all subsequent experiments.

5. Effect of Variation of Thioglycolic Acid Concentration. In these runs the volume of the glyoxylic acid solution (0.5% in glacial acetic acid) was varied between 0.025 and 1.0 ml. The total volume and other factors were kept constant except that two tryptophan concentrations (0.05 and 0.10 mg.) were run in order to check the stoichiometry of the color reaction. The results obtained are illustrated in Fig. 5 and it is evident that the amount of thioglycolic acid has little effect on the absorbance values obtained; 0.10 ml. of this solution was employed in all subsequent runs.

6. Effect of Varying Time Interval between Addition of Perchloric Acid and Persulfate. The results obtained in these runs at tryptophan concentrations of 0.05 and 0.10 mg. are illustrated in Fig. 6. They indicate that no further change in absorbance occurs, after perchloric acid addition, beyond a time interval of 5 min.

7. Effect of Varying Time Interval between Addition of Persulfate and Thioglycolic Acid. The results obtained in these runs at the two tryptophan concentrations (0.05 and 0.10 mg.) are shown in Fig. 7.
They demonstrate that no significant changes in absorbance values occur after a time interval of 5 min. following addition of persulfate.

8. **Effect of Variation of Time of Reading on Absorbance Values.**

Two sets of tryptophan standards (0.05 and 0.10 mg.) were run with

![Fig. 5. Effect of variation of thioglycolic acid concentration (0.025 to 1.0 ml.) on absorbance values of tryptophan standards (0.05 to 0.10 mg.) obtained with modified glyoxylic acid.](image)

![Fig. 6. Effect of variation of the time interval (0 to 60 min.) between addition of 70% perchloric acid and saturated persulfate solution on absorbance values of tryptophan standards (0.05 and 0.10 mg.) obtained with modified glyoxylic acid reaction.](image)

the photometric procedure, exactly as described in Section B above, and the color read at 365 μm at the various time intervals following thioglycolic acid addition as is noted in Fig. 8. The results obtained show significant increases in absorbance values up to a 30-min. interval beyond which no further change occurs over a 2.5-hr. period. All subsequent readings were taken after a 30-min. period to insure full color development.
D. Spectral Curves for Unhydrolyzed and Hydrolyzed Tryptophan-Containing Compounds

Solutions of pure tryptophan, a tryptophan-containing peptide (tyrocidine), and a protein (γ-globulin) were subjected to the color reaction as described under Section B. The spectral transmittance curves of each of these substances were determined with a recording spectrophotometer and the results obtained are illustrated in Fig. 9. These substances show relatively broad absorption bands at 560 and 365 m\(\mu\), with an even wider absorption band between 400 and 475 m\(\mu\). The substance with the highest tryptophan content, i.e., pure tryptophan, gave the smallest absorbance value at 560 m\(\mu\) but the largest value at 375 m\(\mu\), whereas, γ-globulin showed the greatest absorbance value at 560 m\(\mu\) although its tryptophan content was about one-half that of the pure tryptophan solution. When these samples were hydrolyzed with 3.5% HCl for 24 hr., the shape of the spectral curves for both tyrocidine and γ-globulin became identical with that of pure
tryptophan. Absorbance values for the three substances were directly related to tryptophan content at 365 m\( \mu \) but not at 560 m\( \mu \).

That the type of spectral curve obtained bears no direct relationship to tryptophan content, but rather is determined by the manner in which tryptophan is linked to other amino acids, is best illustrated by the spectral curves for L-alanyl-L-tryptophan and L-tryptophyl-L-alanine shown in Fig. 10. When the amino group of tryptophan is involved in the peptide linkage and the carboxyl group is free, as in the case of L-alanyl-L-tryptophan, the formation of the blue compound which absorbs at 560 m\( \mu \) is favored. Whereas, when the amino group is free as in L-tryptophyl-L-alanine the formation of the compound absorbing at 365 m\( \mu \) is favored. The spectral curve obtained for the latter compound bears a close resemblance to that of tryptophan itself. When these dipeptides are hydrolyzed by heating with 3.5% \( \text{HClO}_4 \) for 24 hr., the color reactions yield almost identical spectral curves, which are the same as that of pure tryptophan.

It can be concluded from these data that, in order to determine the tryptophan content of a peptide or protein, it is first necessary to break it down to its constituent amino acids. While in the past it has

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**Fig. 9.** Spectral transmittance curves for unhydrolyzed tryptophan (0.25 mg. = 50 mg./100 ml.), tyrocidine (0.03 mg. = 6 mg./100 ml. tryptophan) and \( \gamma \)-globulin (0.14 mg. = 27 mg./100 ml. tryptophan) solutions subsequent to color development with modified glyoxylic acid reaction.

**Fig. 10.** Spectral transmittance curves for unhydrolyzed L-tryptophyl-L-alanine and L-alanyl-L-tryptophan and for these same dipeptides after a 24-hr. hydrolysis in 3.5% \( \text{HClO}_4 \) solution subsequent to color development with modified glyoxylic acid reaction.
been customary to hydrolyze proteins in alkaline solution in order to
determine tryptophan quantitatively, we have found the use of dilute
(3.5%) perchloric acid as a simple and convenient means of both hy-
drolyzing the peptide linkage and preserving the indole structure. The
modified glyoxylic acid reaction could then be utilized to determine the
tryptophan content of the hydrolyzate.

E. Concentration Curves for Tryptophan

Amounts of tryptophan ranging from 6 to 150 μg were determined
with the glyoxylic acid method and the absorbance readings plotted
as a function of concentration at three wavelengths: 365, 440, and 560
mμ (Fig. 11). Stoichiometric readings were obtained at each wave-
length although the sensitivity of the method at 440 mμ is 6 times
greater than at 560 mμ, and 17 times more sensitive at 365 mμ. How-
ever, in the case of an actual protein hydrolyzate, in which other amino
acids are present, the sensitivity of the readings at 440 and 365 mμ are
twofold and sixfold respectively, of those at 560 mμ. The absolute

![Fig. 11. Concentration curves for tryptophan standards ranging from 6 to 150 μg. Colors were read at 365, 440, and 560 mμ after color development with modified glyoxylic acid reaction.](image)

values of the readings obtained at 365 mμ are the same per milligram
of tryptophan in pure solution as in a mixture of amino acids similar
to that found in protein hydrolyzates.

F. Application of Tryptophan Procedure to Hydrolyzates of Peptides and Proteins

An amount of a protein, or peptide, was taken for analysis so that
the final hydrolyzate should contain between 0.025 and 0.125 mg. of
tryptophan per milliliter 3.5% HClO₄ solution. At the beginning of
these experiments substances such as tryptophan, γ-globulin, casein,
and lysozyme were subjected to hydrolysis in sealed tubes, previously
flushed out with N₂ gas, for various time intervals in an autoclave at
120° (15 lb. pressure). These hydrolyzates were analyzed for their
tryptophan content with the colorimetric procedure described under
Section B using 0.50- and 1.00-ml. aliquots of the hydrolyzates. The re-
sults obtained in these experiments are given in Fig. 12. These data
demonstrate that, while complete hydrolysis usually occurs between
3 and 24 hr., the exact time is quite variable with different proteins or
peptides. In practice, protein or peptide samples were hydrolyzed for
5, 8, 16, and 24 hr., together with suitable tryptophan standards, and
only those results utilized in calculating tryptophan content which
gave the maximal absorbance readings. If maximal values were ob-
tained at more than one hydrolysis time, the results were averaged.
When the perchloric acid hydrolysis procedure was applied to a num-
ber of peptides and “purified” proteins of known tryptophan content
ranging from 1.0 to 100%, excellent agreement was found between the
calculated and the experimentally determined values, as given in Table

![Graph](image)

**Fig. 12.** Effect of variation of time of hydrolysis (0.5 to 48 hr.) on absorbance values for human γ-globulin (2.9% tryp.),
lysozyme (7.3% tryp.), casein (1.1% tryp.), and tryptophan (25 mg./100 ml.) obtained with modified glyoxylic acid color re-
aaction.

1. Application of the method to a series of “purified” proteins, as
listed in Table 2, also shows good agreement between tryptophan
values found experimentally and those reported in the literature, ex-
cept in a few instances, as for trypsin and ovomucoid.
Table I. Tryptophan Values Obtained with Spectrophotometric Method as Compared to Calculated Values

<table>
<thead>
<tr>
<th>Materials*</th>
<th>Tryptophan (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated</td>
</tr>
<tr>
<td>N-Acetyl-DL-tryptophan (M)</td>
<td>83.0</td>
</tr>
<tr>
<td>L-Leucyl-l-tryptophan (M)</td>
<td>64.2</td>
</tr>
<tr>
<td>Glycyl-l-tryptophan (Y)</td>
<td>78.2</td>
</tr>
<tr>
<td>L-Tryptophyl-glycine (Y)</td>
<td>78.2</td>
</tr>
<tr>
<td>L-Phenylalanyl-l-tryptophan-H2O (Y)</td>
<td>55.3</td>
</tr>
<tr>
<td>L-Tryptophyl-l-phenylalanine-H2O (Y)</td>
<td>55.3</td>
</tr>
<tr>
<td>L-Alanyl-l-tryptophan-H2O (Y)</td>
<td>69.6</td>
</tr>
<tr>
<td>L-Tryptophyl-l-alanine (Y)</td>
<td>74.2</td>
</tr>
<tr>
<td>L-Tryptophyl-l-tryptophan (Y)</td>
<td>100.0</td>
</tr>
<tr>
<td>Z-Try-Lys-OH† (A)</td>
<td>33.0</td>
</tr>
<tr>
<td>Human hemoglobin</td>
<td></td>
</tr>
<tr>
<td>(α-chain) (K)</td>
<td>1.04</td>
</tr>
<tr>
<td>(β-chain) (K)</td>
<td>2.00</td>
</tr>
<tr>
<td>Glucagon (B)</td>
<td>5.97</td>
</tr>
</tbody>
</table>

†Z = carbobenzoxy. Try = tryptophan. L = lysine.

Discussion

In 1926, Looney (14) summarized the current state of knowledge with respect to then available methods for the quantitative determination of tryptophan in biologic fluids as follows: "The multiplicity of the methods proposed for the estimation of tryptophan and the inconsistency of the values given are sufficient evidence of the importance attached to the subject and the worthlessness of most of the methods." That the situation with respect to tryptophan analyses of proteins has not changed markedly for the better over the past 25 years is exemplified by the statement in the textbook of Fox and Foster (15) that "some amino acids are more reliably determined than others, tryptophan particularly giving various values." These statements receive ample experimental support from the extensive data on the amino acid composition of proteins compiled by Block and co-workers (1, 16), who found a wide divergence of tryptophan values for the same protein when different colorimetric procedures were employed.

In recent years, the use of automatic amino acid analyzers, based on
Table 2. Tryptophan Content of Various Proteins (Comparison of Present Spectrophotometric and Previous Literature Values)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Literature value (%)</th>
<th>Present value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>1.19, 1.50</td>
<td>1.14</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>5.7</td>
<td>5.37</td>
</tr>
<tr>
<td>Human γ-globulin Red Cross</td>
<td>2.74, 2.9</td>
<td>2.88</td>
</tr>
<tr>
<td>Squibb</td>
<td>2.74, 2.9</td>
<td>2.86</td>
</tr>
<tr>
<td>Pentex</td>
<td>2.74, 2.9</td>
<td>2.62</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>7.2, 7.8</td>
<td>7.31</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>1.99-2.3</td>
<td>2.01</td>
</tr>
<tr>
<td>Bovine fibrinogen</td>
<td>3.5, 3.3</td>
<td>3.38</td>
</tr>
<tr>
<td>Bovine hemoglobin</td>
<td>1.0</td>
<td>1.63</td>
</tr>
<tr>
<td>Human hemoglobin</td>
<td>1.1, 1.3, 2.0, 1.9</td>
<td>1.95</td>
</tr>
<tr>
<td>Edestin</td>
<td>1.3, 1.22</td>
<td>1.31</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>5.7</td>
<td>5.18</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>2.0, 2.2</td>
<td>1.88</td>
</tr>
<tr>
<td>Tyrocidine</td>
<td>6.2</td>
<td>6.09</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>0</td>
<td>0.86</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>1.5, 1.3, 1.8</td>
<td>1.24</td>
</tr>
<tr>
<td>Zein</td>
<td>0.1</td>
<td>0.18</td>
</tr>
<tr>
<td>Papain† (crystalline)</td>
<td>4.2</td>
<td>4.04</td>
</tr>
<tr>
<td>Papain-PM 613</td>
<td>2.3</td>
<td>2.84</td>
</tr>
<tr>
<td>Papain-PM 637</td>
<td>2.3</td>
<td>2.82</td>
</tr>
<tr>
<td>Pepainogen #109§ (purified)</td>
<td>3.0</td>
<td>2.72</td>
</tr>
<tr>
<td>Pepainogen #6022§ (purified)</td>
<td>3.0</td>
<td>2.76</td>
</tr>
<tr>
<td>Trypsin‡</td>
<td>1.0</td>
<td>2.25</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>2.3, trace</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*Unless otherwise noted all values in this column were obtained from data listed in Block and Weiss (1).
†A value of 2.70 gm./100 ml. (average of five determinations) was reported in a recent paper by Opińska-Blauth et al. (30).
‡See Smith et al. (41). Obtained gratis from Dr. E. L. Smith, Biochemistry Department, University of Utah, Salt Lake City, Utah.
§See Aron and Perlmann (32). Obtained gratis from Dr. G. E. Perlmann, The Rockefeller Institute, New York, N. Y.
||Only one value of the tryptophan content of this enzyme was found in the literature.

The ion-exchange column chromatographic techniques of Moore and Stein (17, 18), have greatly facilitated the compilation of voluminous and precise data on the amino acid composition of many 'purified' proteins, e.g., hemoglobin (19, 20), papain (21), pepsinogen (22), glucagon (23), ribonuclease (24), albumin (25), etc. Unfortunately, this major technical advance in amino acid analysis has not remedied the situation with regard to obtaining accurate tryptophan values. This amino acid is almost entirely destroyed by the customary acid hydrolysis of proteins or peptides in 6N HCl. Tryptophan content must
then be determined by either performing a separate alkaline hydrolysis of the protein or peptide again followed by automatic analysis or by a spectrophotometric procedure which is presumably specific for tryptophan using either the basic hydrolyzate or a solution of the unhydrolyzed protein using absorption measurements in the UV region (26).

As stated in the introduction, the problem of obtaining accurate tryptophan values cannot be divorced from its partial or complete destruction during the hydrolysis of proteins or peptides into their constituent amino acids. The literature in the field has been adequately reviewed by Block and Bolling (16). About the only general conclusion that can be drawn from the many publications on this subject is that the stability of tryptophan to hot alkali is considerably greater than to acids. What is meant by the term "increased stability" is the preservation of the intact amino acid including its ninhydrin-reactive amino group. However, if one should employ a technic of hydrolysis that would preserve the indole nucleus intact, then application of a color reaction specific for this structure would also constitute an accurate measure of the tryptophan content of the hydrolyzate. This possibility arose from the report by Hakim (27) that cancerous sera subjected to mild (5% HCl) acid hydrolysis showed an increase in absorbance at 270 to 275 m\(\mu\) as compared to normal sera, and the finding by Brackenridge (28) that this increase could be accounted for by the raised level of the tryptophan content of such sera. We chose to utilize dilute (3.5%) perchloric acid, since we had shown in previous publications (8, 9, 12) that 70% HClO\(_4\) could be substituted for concentrated sulfuric acid in carrying out the glyoxylic acid reaction in an aqueous medium. The results obtained in these studies (Fig. 12) show that complete release of tryptophan from proteins occurs between hydrolysis times of 5 to 24 hr. and that there is but little or no change in the absorbance of tryptophan itself between 5- through a 72-hr. period of hydrolysis. The hydrolysis product formed from tryptophan gives a negative ninhydrin reaction and thus cannot be used for determining this amino acid in the Moore and Stein (18) system. When the modified glyoxylic acid reaction is applied to hydrolyzed tryptophan, it gives the same spectral curve as does unhydrolyzed tryptophan, with the major absorption band at 365 m\(\mu\) and a considerably smaller one at 560 m\(\mu\) (Fig. 9). While the presence of this band was noted for tryptophan and some related compounds by Rieder and Böhmer (13), it has not to our knowledge been previously employed for the quantitative
determination of tryptophan. Investigation of the various factors which influence the amount of yellow chromogen formed were then systematically investigated (Fig. 1 through 8) and the method finally evolved gave absorbance values which followed Beer's law at tryptophan concentrations between 5 and 150 μg in 5.7 ml. of solution (Fig. 11).

Application of the modified glyoxylic acid method to an unhydrolyzed protein, e.g., γ-globulin, produced mainly the blue chromogen with the main absorption band at 560 mμ. It was first thought that the blue chromogen was characteristic of tryptophan present in a polypeptide linkage since hydrolysis in 3.5% HClO₄ changed its spectral curve (Fig. 9) to one similar to that of pure tryptophan. Application of the color reaction to tryptophan-containing dipeptides gave some surprising results. L-Alanyl-L-tryptophan yielded mainly the blue chromogen, whereas L-tryptophyl-L-alanine gave the yellow chromogen absorbing at 365 mμ. Only after hydrolysis did their spectral curves become identical and equivalent to that of free tryptophan (Fig. 10). Even more surprising was the fact that increasing amounts of the blue chromogen could be formed by adding increments of a mixture of the other common amino acids to a constant amount of tryptophan. Under these conditions only the absorbance readings at 365 mμ remained constant and were consistent with the known tryptophan content of the sample. The finding by Boyd (29) that different proteins produce colors of different tints with the glyoxylic acid reaction is in accord with our present data. When proteins or peptides of known tryptophan content were hydrolyzed to completion with 3.5% HClO₄ and their absorbance values at 365 mμ calculated against tryptophan standards treated in the same manner, excellent checks were obtained between the calculated and the experimentally determined values (Table 1). Similarly, when the method was applied to a whole series of tryptophan-containing proteins, the results (Table 2) check rather well with those reported in the literature for the same protein except for trypsin, in which a higher value (2.26 gm./100 gm.) was obtained, and for ovomucoid (1.15 gm./100 gm.), in which the result was lower. The precision of the method is ±3.0% for the over-all procedure including the hydrolysis step. The excellent correlation between tryptophan values obtained for different preparations of the same "purified" protein are shown in Table 2 for human γ-globulin, pepsin, and pepsinogen.

Work is now in progress on the application of the method to plasma
protein fractions in various chronic diseases and to similar proteins of different animal species. The chemical nature of the chromogen absorbing at 365 m, is also being investigated.

References