Determination of the Tryptophan Content of Proteins by Ion Exchange Chromatography of Alkaline Hydrolysates*

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SUMMARY

A study of the variables in techniques for alkaline hydrolysis of proteins and for chromatographic analysis of the products has led to a method for the accurate determination of tryptophan. Quantitative recoveries of tryptophan are obtained when proteins (1 to 3 mg) are hydrolyzed at 110°C or 135°C in 0.6 ml of 4.2 N NaOH containing 25 mg of starch. The hydrolysis is performed in polypropylene liners sealed inside glass tubes evacuated to below 50 μm of mercury. Ion exchange chromatography of tryptophan on Beckman PA-55 resin (column height 8 or 12 cm) has been accomplished in 30 to 50 min with pH 5.4 buffer, 0.21 N in NaNO₃.

The details in the procedure which make possible complete recovery of tryptophan include: (a) addition of the sample at pH 4.25 instead of at pH 2.2 in order to avoid loss of tryptophan in citrate buffer at acid pH; (b) the use of NaOH instead of Ba(OH)₂, to avoid loss of tryptophan by adsorption on BaSO₄ or BaCO₃; (c) the inclusion of starch as the most effective antioxidant tested; and (d) chromatography with a buffer which separates tryptophan from N'-[(1-L-2-amino-2-carboxyethyl)-L-lysine, which can be formed in significant quantities during alkaline hydrolysis. Molar calculations of protein concentrations are based on the results from analysis of an acid hydrolysate run parallel with the alkaline hydrolysate.

Integral values (100 ± 3%) have been obtained for the expected number of tryptophan residues in tryptophyl-leucine, human serum albumin, porcine pepsin, sperm whale apomyoglobin, and in bovine α-chymotrypsin, trypsin, deoxyribonuclease, and serum albumin. Since carbohydrate does not interfere, the procedure is applicable to foods and has been tested on normal and opaque-2 maize meals and on wheat flours.

With the advent of chromatographic methods for the accurate determination of most of the amino acids of proteins, analysis for tryptophan has remained a special problem. The lability of tryptophan under the conditions usually used for the acid hydrolysis of proteins, particularly in the presence of carbohydrate (1), has led, over the years, to the study of various approaches for the determination of this amino acid.

Spectrophotometric measurement of tryptophan in the intact protein has the advantage of avoiding the problems of hydrolysis. The procedures of Goodwin and Morton (2) and of Edelhoch (3) have been practical for soluble proteins when the tyrosine to tryptophan ratio is not too high. Recently the use of magnetic circular dichroic absorbance at 293 nm (4) has given accurate estimates of tryptophan in several proteins, but this method requires highly specialized equipment. These procedures have the disadvantage of being inapplicable when the protein is insoluble, as is often the case in the analysis of foods. The same limitation applies to methods designed to give a colored derivative of tryptophan in the intact protein (5-7).

If some method of hydrolysis is to be used, the most convenient approach might be to modify acid hydrolysis to render tryptophan stable. Matsubara and Sasaki (8) have found that the addition of mercaptans to 6 N HCl in the absence of oxygen improves the recovery of tryptophan when carbohydrate is absent. Liu and Chang (9) have recently made the valuable observation that p-toluenesulfonic acid containing 3-(2-aminoethyl) indole is much preferable to HCl in terms of the stability of tryptophan. Their procedure is capable of giving about 90% recovery of tryptophan in a 22-hour hydrolysate of a purified protein; when the values observed after 22, 48, and 72 hours were extrapolated to zero time, good results were obtained with 10 proteins. However, if the carbohydrate content of the sample is appreciable, the recovery is decreased.

Enzymatic hydrolysis is an attractive alternative (10), but as yet it has not proved capable of giving complete hydrolysis in all instances. Spies (11) has studied the colorimetric determination of tryptophan after partial enzymatic hydrolysis. If complete hydrolysis is desired for a chromatographic determination of tryptophan, alkaline hydrolysis has been shown to be the most likely means of obtaining a quantitative recovery. The early observations on the relative stability of tryptophan to alkali (12), subsequently extended to the study of the effectiveness of added reducing agents (1) and other protectants (11, 13), have been carefully examined by various current methods of chromatographic analysis (13-18). The aim in the present experiments has been to develop a simple procedure which would give strictly quantitative recoveries of tryptophan by ion exchange chromatography on an automatic analyzer (19, 20).
20. Since the use of Ba(OH)₂ requires the precipitation of Ba⁺⁺ by SO₄²⁻ or CO₃²⁻, with the problem of adsorption of tryptophan to the precipitate (16), we have preferred to study the use of NaOH; this base can be neutralized and the resulting NaCl does not interfere with the chromatography.¹

The following experiments were designed to determine the recovery of tryptophan under various conditions of hydrolysis in NaOH. We have drawn particularly upon the observations of Drèze (14) that starch is a convenient antioxidant in the recovery of tryptophan under various conditions of hydrolysis. The combination of these observations with improvements in the ion exchange chromatography has led to a general procedure which has been tested on a variety of purified proteins and on corn and wheat as examples of foods.

EXPERIMENTAL PROCEDURE

Materials—The L-tryptophan used was chromatographically homogeneous on PA-35 resin (Deckeran) and gave values for carbon, hydrogen, and nitrogen that were within 1.0% of the calculated values. L-Tryptophyl-L-leucine obtained from Mann and L-isoleucyl-L-tryptophan obtained from Fox Chemicals gave the correct elementary analyses. Lysinoalanine was synthesized by Bohak (21) and was chromatographically pure as judged by amino acid analysis. Defatted and gel filtered bovine serum albumin was obtained from Dr. T. P. King. Half cysteinyl human serum albumin serum albumin prepared according to the method of King and Szencsi (22) was a gift from Dr. H-C. Chen. Bovine pancreatic DNase A was prepared according to the method of Salnikow et al. (23). Sperm whale myoglobin (Seravac, Lot B3), which had the heme removed, was a gift from Dr. R. Feinberg. Recrystallized (6 times) egg white lysozyme was a sample prepared by Dr. W. F. Harrington. Pepsin, purified by the method of Rajagopalan et al. (24), was prepared by Dr. T. A. A. Doppleide and Miss W. M. Jones. Chromatographically purified pepsinogen (Lot 9AA, Worthington) was a gift from Dr. G. E. Perlman and Dr. Y. Nakagawa. Bovine thymus was obtained from Dr. J. G. Pierce and Dr. T.-H. Liao. Recrystallized bovine a-chymotrypsinogen (CD541), bovine a-chymotrypsin (CD1 5GA), bovine trypsin (TRI 6257), and bovine pancreatic RNAase A (RAF OAB) were purchased from Worthington. Defatted maize flour, normal and opaque-2 (25), were provided by Dr. E. T. Mertz. Hecker’s commercial unbleached wheat flour and Gold Medal commercial bleached flour were analyzed. Unmodified potato starch was obtained from the Amend Drug Co., and Connaught hydrolyzed potato starch for gel electrophoresis was purchased from Pierce Chemicals. All other chemicals were analytical reagent grade.

Partially Hydrolyzed Potato Starch—Potato starch was partially hydrolyzed in acid in order to increase its solubility in the hydrolysis medium for proteins (14); the hydrolysis was accomplished according to a method similar to that described by Smithies (26). Approximately 50 g of potato starch were added to 90 ml of acetone to which 1 ml of concentrated HCl was added. This mixture was held at 50° for 2 hours; 25 ml of 1 M sodium acetate were added to neutralize the hydrolysate, and the slurry was poured into a chromatograph tube and washed with 2 liters of distilled water and with acetone. The product was thoroughly dried in a desiccator. Commercial partially hydrolyzed starch was equally suitable; 50 g were washed with acetone and dried. In each case, the starch was ground to a fine powder. No detectable tryptophan was observed when a 25-mg sample of either starch was analyzed after alkaline hydrolysis.

Hydrolysis of Proteins—With the purified proteins, solutions were prepared in dithiothreitol (0.005 x) HCl or NaOH. In general, a solution containing 0.1 to 0.5 μmole of tryptophan per ml in a total volume of approximately 0.5 ml was used. Aliquots of 0.100 ml were taken and two such samples were hydrolyzed with 1 ml of 6 N HCl for 2 hours at 110° (27). The acid-hydrolyzed samples (evaporated to dryness and then diluted to 5 ml and 1 ml analyzed) served to establish the relative amino acid composition and the concentration of protein. The calculations of molar ratios were usually based on the average of the results for glutamic acid, aspartic acid, glycine, alanine, and leucine.³ Duplicate samples of 0.100 ml of protein were taken for alkaline hydrolysis. The protein solution was pipetted into a Nalgene polypropylene centrifuge tube (10.9 x 50 mm) (cut to size with a razor blade from a 10.9 x 77 mm tube), and 25 mg of the partially hydrolyzed starch were added. Exactly 0.5 ml of 5 N NaOH, freshly prepared from 50% NaOH, was added and the polypropylene tube with its contents was placed in a thin walled Pyrex test tube (16 x 150 mm). ⁴ It was observed that foaming was greatly suppressed during evaporation by adding 0.5 ml of 1% I-octanol in toluene as described by Spies (11). ⁵ The Pyrex tube was constricted near the middle over a gas-oxygen flame and drawn to about 2 mm; with a thin walled tube this constriction can be accomplished without overheating the top of the plastic insert. ⁶ The lower portion of the tube was cooled in an acetone-Dry ice bath for exactly 90 s to chill, but not to freeze, the solution. The tube was connected through a rubber stopper and a two-way stopcock to a high vacuum line equipped with a Dry ice trap and operating below 25 μm of mercury. ⁷ The stopcock adjacent to the top of the tube was re-

¹ Preliminary experiments on the chromatographic procedure were performed in this laboratory by Dr. George R. Stark in 1962.
² The abbreviation used is: Lysinoalanine, N ε-(pI-2-amino-2-carboxyethyl)-L-lysine.
³ Since alkaline hydrolysis can give rise to artifacts that overlap the peaks of some of the stable amino acids, we find it more accurate to base all calculations of molar ratios on the analysis of acid hydrolysates than on the measurement of amino acids other than tryptophan in the alkaline hydrolysate.
⁴ Oelshlegel et al. (13) employed a re-usable glass vessel with an O-ring seal and a Teflon stopcock. They mention, and we also find, that such equipment occasionally leaks at 110°. In order to eliminate this variable, we have used only sealed glass tubes.
⁵ In most of the present experiments, the protein concentration was low (less than 1 mg of protein per 0.6 ml), and foaming of the solution was not a major problem during desorption by the procedure described. However, the addition of octanol is recommended for it does not appear to interfere with the tryptophan recovery under the present experimental conditions (see Table II) and makes the sealing process simple in all cases.
⁶ If a thick walled ignition tube (18 X 150 cm) is preferred, the constriction can be made about 3 cm from the top (28) with a glass rod handle (bent to be coaxial) sealed to the outside rather than to the inside of the tube. For use with larger volumes of NaOH, a 12.5-mm polypropylene tube can be cut for 1 ml of NaOH or for a 15.8-mm tube for 2 ml; the latter is sealed to a Pyrex ignition tube (20 X 150 mm).
⁷ Most conveniently measured on a Pirani gauge, type GP-110, Consolidated Vacuum Corporation, Rochester, New York.
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Fig. 1. The rate of destruction of free L-tryptophan under various conditions. A dilute (0.05 μmole per ml) solution of L-tryptophan was stored in both pH 2.2 and 4.25 buffers and the tryptophan recovery versus time was determined.

RESULTS

pH of Buffer for Sample Addition—It is customary with an amino acid analyzer (19) to use a pH 2.2 citrate buffer (20) to dilute the sample for analysis. Since tryptophan may be labile in acid solution, the recovery of the amino acid was checked after different periods of standing in the pH 2.2 buffer (Fig. 1). At room temperature, the loss of tryptophan is significant (7%; in 20 hours). Since the ion exchange column used for tryptophan analysis is buffered at pH 5.4, it is not necessary to add the sample at pH 2.2 in order to meet the need for adding the sample in a solution about 1 unit lower in pH than the column. Therefore, the diluent for the hydrolysate for tryptophan analysis is a buffer at pH 4.25 in which the loss of tryptophan overnight is negligible (less than 1%; Fig. 1). This stability is important when samples are to be stored in automatic sample injectors.

Samples containing very little tryptophan may require special attention during chromatographic analysis. For instance, the single tryptophan residue in human serum albumin, which contains more than 570 residues, was easily determined on the column (0.9 X 12 cm) by waiting 20 min after the sample was injected before turning the eluent into the coil of the amino acid analyzer.

Stock solutions of pure tryptophan (in the absence of other amino acids) in 0.1 N HCl are remarkably stable; no loss of tryptophan was detected after 11 days at room temperature. However, when a mixture of other amino acids, starch, or citrate buffer is present in the solution the tryptophan content of the solution decreases significantly with time; losses were also observed when a solution of pure tryptophan in 0.005 N NaOH was stored in the presence of air.
Possible Interference by Lysinoalanine, Amino Sugars, and Peptides—When an alkaline hydrolysate of a protein is analyzed for tryptophan with the 0.35 \( N \) sodium citrate buffer at pH 5.28 (19) normally used for analysis of an acid hydrolysate on a short column, high results can be obtained. For example, when we first analyzed an alkaline hydrolysate of bovine serum albumin in this manner, we obtained a peak at the tryptophan position which integrated for 5.8 residues of tryptophan. Since bovine serum albumin is known to have only 2 residues of tryptophan per molecule (30), it was clear that another ninhydrin-positive compound was co-chromatographing with tryptophan under these conditions. Since the peak is not present in a chromatogram of an acid hydrolysate of the protein, the compound was apparently one that is formed only under alkaline conditions.

The most likely candidate was lysinoalanine which Bohak (21) has shown to be synthesized under alkaline conditions through the addition of the \( \epsilon \)-amino group of a lysyl residue to the double bond of a dehydroalanine residue, which can be formed by a \( \beta \)-elimination reaction in a half-cystine or a serine residue. When we added lysinoalanine to the alkaline hydrolysate, the amino acid peak coincided with the position of tryptophan. The most convenient way around this difficulty came from a conversation with Dr. David Eaker of the University of Upsala who has observed that the simplest means of resolving the lysinoalanine and tryptophan peaks is to dilute the 0.35 \( N \) pH 5.28 buffer to 0.2 \( N \). The analysis of a synthetic mixture on a column (0.9 \( \times \) 8 cm) is shown in Fig. 2. When samples of low tryptophan content are analyzed on this column, the peak of the neutral and acidic amino acids may not reach base-line before tryptophan emerges. For such samples a column (0.9 \( \times \) 12 cm) is preferred. Tryptophan is eluted in about 35 min, and, for serial analyses, 1 ml of 1 \( N \) NaOH can be applied immediately after tryptophan is eluted and the next sample added 5 min later. If two short columns are used, multiple samples can be analyzed at less than 30 min per hydrolysate.

Possible interference from amino sugars must also be considered, since they elute near tryptophan (19). When amino sugars are hydrolyzed under the alkaline conditions, no peaks are observed in the positions of glucosamine, mannosamine, or galactosamine. A small amount of a ninhydrin-positive degradation product is eluted in the range of the neutral and acidic amino acids. Hydroxylysine, as Knox et al. (18) have shown, is eluted well after tryptophan when a 0.20 to 0.25 \( N \) buffer is used. Ornithine, which is formed from arginine during alkaline hydrolysis, is eluted just preceding lysine and is not close to tryptophan with the 0.2 \( N \) buffer.

Slowly hydrolyzed dipeptides such as isoleucylisoleucine and valylvaline, which are often present in short term alkaline hydrolysates, were eluted well ahead of the tryptophan position. L-tryptophyl-L-leucine was hydrolyzed in 4.2 \( N \) NaOH for 16 hours, and, for accurate analysis of alkaline hydrolysates, the stability of tryptophan residues was examined under different conditions. When L-tryptophyl-L-leucine was hydrolyzed in 4.2 \( N \) NaOH (Table I), the presence of starch (25 mg) raised the recovery from 91\% to 98\%. Thiodiglycol (20 \( \mu l \)), which has been employed as the antioxidant by Oelschlegel et al. (13), was equally effective in this case. When free tryptophan was added to bovine ribonuclease, which contains no tryptophan, the recovery with thiodiglycol was 95\% and with starch it was 100\%. Our experience, documented further in Table II, is that when all of the protein amino acids are present, starch is the preferable antioxidant.

Recoveries of Tryptophan from Purified Proteins—The results in Table II show that within the precision of the chromatography (100 \( \pm \) 3\%), the recoveries of tryptophan after hydrolysis at 110\° for 16 hours in the presence of starch are quantitative.

![Fig. 2. Chromatographic separation of tryptophan from other amino acids by ion exchange chromatography. A column (0.9 \( \times \) 8.0 cm) of Beckman PA-35 resin was developed at a buffer flow rate of 50 ml per hour at 52\° with 0.21 \( N \) sodium citrate buffer at pH 5.4.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Hydrolysate conditions</th>
<th>Additives</th>
<th>Tryptophan recovered&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tryptophyl-L-leucine</td>
<td>None</td>
<td>91.0</td>
</tr>
<tr>
<td>4.2 ( N ) NaOH for 16 hours</td>
<td>None</td>
<td>91.0</td>
</tr>
<tr>
<td>4.2 ( N ) NaOH for 48 hours</td>
<td>None</td>
<td>91.5</td>
</tr>
<tr>
<td>4.2 ( N ) NaOH for 24 hours</td>
<td>Thiodiglycol</td>
<td>97.5</td>
</tr>
<tr>
<td>4.2 ( N ) NaOH for 24 hours</td>
<td>Starch</td>
<td>98.2</td>
</tr>
<tr>
<td>RNase + tryptophan&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Thiodiglycol</td>
<td>94.6</td>
</tr>
<tr>
<td>4.2 ( N ) NaOH for 16 hours</td>
<td>Starch</td>
<td>100.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of duplicate runs.

<sup>b</sup> The hydrolysate tubes contained 230 \( \mu g \) of RNase and 30 \( \mu g \) of tryptophan.

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<sup>14</sup> Lysinoalanine would be a possible interferent in some earlier methods (15, 16), but not in the procedure of Knox et al. (18) who used a buffer 0.25 \( N \) in Na\(^+-\).<br>

<sup>11</sup> Since ammonia is eluted well after tryptophan, the base-line remains constant through the tryptophan peak without having to wait for an equilibration of the resin with the ammonia in the buffer.

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line Hydrolytic Conditions—With the chromatographic system for accurate analysis of alkaline hydrolysates, the stability of tryptophan residues was examined under different conditions. When L-tryptophyl-L-leucine was hydrolyzed in 4.2 \( N \) NaOH (Table I), the presence of starch (25 mg) raised the recovery from 91\% to 98\%. Thiodiglycol (20 \( \mu l \)), which has been employed as the antioxidant by Oelschlegel et al. (13), was equally effective in this case. When free tryptophan was added to bovine ribonuclease, which contains no tryptophan, the recovery with thiodiglycol was 95\% and with starch it was 100\%. Our experience, documented further in Table II, is that when all of the protein amino acids are present, starch is the preferable antioxidant.

Recoveries of Tryptophan from Purified Proteins—The results in Table II show that within the precision of the chromatography (100 \( \pm \) 3\%), the recoveries of tryptophan after hydrolysis at 110\° for 16 hours in the presence of starch are quantitative.

<sup>13</sup> Spies (11) used histidine and basic lead acetate to protect tryptophan during alkaline hydrolysis. We have not tested this combination since its composition is inconvenient when a chromatographic analysis is being used.
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TABLE I

**Tryptophan recoveries from alkaline hydrolysates of purified proteins**

Residues of tryptophan per molecule were obtained from an average of duplicate determinations after 16 hours of hydrolysis in 4.2 N NaOH at 110° unless otherwise indicated.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Tryptophan observed with additive</th>
<th>Nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thiodiglycol</td>
<td>Starch</td>
</tr>
<tr>
<td>Bovine α-chymotrypsin</td>
<td>7.44</td>
<td>8</td>
</tr>
<tr>
<td>Bovine α-chymotrypsinogen</td>
<td>7.73</td>
<td>8</td>
</tr>
<tr>
<td>Bovine deoxyribonuclease</td>
<td>7.87</td>
<td>8</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>7.92</td>
<td>8</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>8.03</td>
<td>8</td>
</tr>
<tr>
<td>Bovine trypsin</td>
<td>8.30</td>
<td>8</td>
</tr>
<tr>
<td>Porcine pepsin</td>
<td>8.40</td>
<td>8</td>
</tr>
<tr>
<td>Porcine pepsinogen</td>
<td>8.40</td>
<td>8</td>
</tr>
<tr>
<td>Hen egg white lysozyme</td>
<td>8.55</td>
<td>8</td>
</tr>
<tr>
<td>Bovine thyrotropin</td>
<td>8.72</td>
<td>8</td>
</tr>
<tr>
<td>Sperm whale apomyoglobin</td>
<td>8.89</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>8.94</td>
<td>8</td>
</tr>
</tbody>
</table>

* The literature values are 8 for chymotrypsin and chymotrypsinogen (31), 2 for bovine serum albumin (30), 1 for human serum albumin (32), 4 for trypsin (33), 6 for lysozyme (34), 2 for sperm whale apomyoglobin (35), and 0 for thyrotropin (36). See text for discussion of values for deoxyribonuclease and pepsin.

 reproducible recovery of tryptophan from alkaline hydrolysates of purified proteins. The present method was used to examine the rates of hydrolysis at 110° in 4.2 N NaOH of peptides containing isoleucine and valine, which are known to be particularly resistant to hydrolysis by acid or alkali (43, 44). The rates for L-isoleucyl-L-isoleucine and L-valyl-L-valine hydrolyzed at 110° in 4.2 N NaOH were approximately one-half of the rates obtained from hydrolysis in 6 N HCl at 110°. The order of the residues is important; for example, glycylvaline and glycylisoleucine are hydrolyzed at least 10 times more rapidly than are valylglycine and isoleucylglycine in 6 N HCl at 110° (43). The half-time for hydrolysis of the dipeptide L-isoleucyl-L-tryptophan in the alkaline system was found to be 40 hours, indicating the relative resistance of this bond to alkaline hydrolysis. By comparison, the tryptophan was quantitatively recovered in 24 hours under the same conditions when L-tryptophyl-L-isoleucine was hydrolyzed (Table I).

Since sperm whale myoglobin contains a -Val-Trp- sequence (35), a long term hydrolysis study at 110° was performed. The results indicated that essentially quantitative recovery of tryptophan could be obtained only after 98 hours of hydrolysis (Table II). Estimation of the tryptophan content in materials of unknown sequence or composition should therefore include a long term hydrolysis at 110° or a hydrolysis at 135° (see below) for comparison with the result obtained in 16 hours at 110°. This procedure is analogous to that required in acid hydrolysis in order to obtain values for isoleucine when the -Ile-Ile- sequence is present, as in ribonuclease (45, 46). Tryptophan is stable during long term hydrolysis in NaOH when starch is present, and any significant increase with time in the tryptophan recovered would indicate the presence of either a valyl- or isoleucyltryptophan linkage.

The long term hydrolysis required at 110° when valyl- or isoleucyltryptophan bonds are present may be avoided if the temperature is increased to 135°. A tryptophan recovery of 95% was obtained after 48 hours of hydrolysis at 135° from isoleucyltryptophan in 4.2 N NaOH. Quantitative recovery from a protein can also be obtained under these conditions, as demonstrated for sperm whale apomyoglobin (Table II).

Other bonds which might be considered particularly resistant to hydrolysis in alkali include tryptophyltryptophan, tryptophylisoleucine, and tryptophylvaline. All of these sequences occur in egg white lysozyme. Recovery of 98% of the tryptophan known to be present in this protein after just 16 hours of hydrolysis clearly indicates that these bonds are adequately hydrolyzed by the short term treatment in 4.2 N alkali at 110°.

**Estimation of Tryptophan in Samples of Maize and Wheat**-

In order to test the procedure on insoluble samples, 20- to 30-mg portions of corn meal and wheat flour were hydrolyzed

For six proteins of known tryptophan content. With bovine pancreatic DNase A, a value of 4 residues per molecule had been obtained by spectral analysis (37, 38). The present results of alkaline hydrolysis, the recent results of hydrolysis in p-toluenesulfonic acid by Liu and Chang (9), and a spectral analysis quoted by the latter authors are in agreement on 3 residues.

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Recovery of tryptophan from acid-treated DNase A

Tryptophan was estimated following alkaline hydrolysis of the various samples in 4.2 N NaOH for 16 hours at 110° in the presence of starch.

<table>
<thead>
<tr>
<th>Material</th>
<th>Time at 25°</th>
<th>Micromoles of tryptophan determined</th>
<th>Relative per cent recovery</th>
<th>Estimated residues of tryptophan per molecule DNase A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase A in 0.5 m acetic acid</td>
<td>h2</td>
<td>0.0156</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>(soluble)</td>
<td>24</td>
<td>0.0157</td>
<td>100.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.0155</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>DNase A in 0.2 m sulfuric acid</td>
<td>h2</td>
<td>0.0145</td>
<td>100.0</td>
<td>3.00</td>
</tr>
<tr>
<td>(cloudy suspension)</td>
<td>60</td>
<td>0.0148</td>
<td>96.0</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.0133</td>
<td>93.2</td>
<td>2.32</td>
</tr>
<tr>
<td>Reduced and carboxymethylated</td>
<td>h2</td>
<td>0.0127</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>DNase A in 0.5 m acetic acid</td>
<td>120</td>
<td>0.0129</td>
<td>94.1</td>
<td></td>
</tr>
<tr>
<td>(cloudy suspension)</td>
<td>24</td>
<td>0.0120</td>
<td>94.5</td>
<td></td>
</tr>
<tr>
<td>Reduced and carboxymethylated</td>
<td>120</td>
<td>0.0113</td>
<td>89.0</td>
<td></td>
</tr>
<tr>
<td>DNase A in 0.2 m sulfuric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Based on the protein concentration as measured after acid hydrolysis (38).

Since these particular products are rich in carbohydrate, no additional starch was included. Centrifugation of the 5 ml of buffered solution was necessary to avoid the accumulation of suspended material on the surface of the ion exchange column. When one sample was analyzed without centrifugation, the resulting value was 4% higher. When a known amount of tryptophan was added to the flour sample after hydrolysis, only 9% of the amino acid was recovered after centrifugation. Hence a small amount of tryptophan is adsorbed on the pellet during centrifugation. The results in Table III are corrected for this loss, but for many practical purposes such a correction would not be necessary.

The data in Table III show a value for normal maize which is consistent with the literature values (47). The high-lysine maize, opaque-2 (25), contains a higher content of tryptophan. The ratio observed for the tryptophan contents of normal and opaque-2 maize agrees with that found by Dr. E. T. Mertz. Commercial unbleached wheat flour gave a tryptophan value comparable to the published estimates (47). A sample of bleached wheat flour showed no drop in tryptophan content. Extension of the method to a variety of foods will require research on the use of larger volumes of NaOH in order to accommodate larger samples for analysis.

Stability of Tryptophan Residues in Proteins in Acidic Media—

Since the indole side chain of tryptophan is labile under some acidic conditions, proteins that have been exposed to acid during their preparation may have undergone alteration of a detectable percentage of their tryptophan residues. The precision of the present method makes it practical to study this question. The results in Table IV show that DNase A in 0.5 m acetic acid undergoes no alteration of its tryptophan residues during 120 hours at 25°. However, in 0.2 m H$_2$SO$_4$, which has a sufficient denaturing effect on the protein to cause it to precipitate, there is a 7% loss of tryptophan in 120 hours. A similar loss is obtained when reduced and carboxymethylated DNase A is exposed to 0.5 m acetic acid, a medium in which the protein derivative is insoluble. In 0.2 m H$_2$SO$_4$, the same derivative shows an 11% decrease in its content of unaltered tryptophan.

These results show that soluble DNase A has little tendency to undergo alteration of its tryptophan residues in acidic solutions. If the conditions are such that major unfolding of the natural conformation occurs, the exposed tryptophan residues are subject to alteration, but the reaction is a slow one. The encouraging conclusion from Tables II and III is that most proteins, even those from the pancreas that are usually exposed to acidic conditions during preparation, do not undergo detectable alteration of the indole side chains of their tryptophan residues. In general, however, long term exposure to a denaturing acid media is to be avoided.

**Discussion**

Most of the steps in this procedure are documented in terms of chemical parameters which contribute to the quantitative recovery of the recovery of tryptophan. The very effective protective action of starch is somewhat unexpected and cannot be explained in detail. Starch undergoes oxidative degradation in alkaline solution in the presence of oxygen (46), apparently the polysaccharide is capable of removing the residual traces of oxygen in the hydrolysis mixture by incorporating the oxygen into products that do not react with tryptophan. Thiodiglycol, for example, combines with oxygen to give the sulfoxide which is a mild oxidizing agent. The protective effect of starch is not sufficient, however, to permit the hydrolysis to be conducted without the removal of most of the oxygen by evacuation. If the tubes are not brought down to below 50 μm, the recovery of tryptophan falls off; with chymotrypsinogen, a tube that was sealed without any evacuation gave about a 75% recovery of tryptophan.

Acid hydrolysis and alkaline hydrolysis will both have uses in the determination of tryptophan. In structure work with proteins and peptides of low carbohydrate content, where about 90% recovery of tryptophan can be obtained in 20 hours with the p-toluenesulfonic acid or methanesulfonic acid procedures of Liu and Chang (9), acid hydrolysis can frequently serve to define the number of tryptophan residues with sufficient precision. The method is advantageous in that tryptophan is measured in the same hydrolysate used for the determination of the other amino acids. The ability to obtain quantitative recovery of tryptophan by alkaline hydrolysis will be useful in experiments on peptide synthesis when it is desired to determine the yield in the incorporation with maximum precision. Similarly, the exact tryptophan content of a newly isolated protein can best be examined by a method which gives quantitative recovery of tryptophan. Alkaline hydrolysis is essential if the carbohydrate content of the sample is appreciable, which includes the analysis of foods.

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Determination of the Tryptophan Content of Proteins by Ion Exchange Chromatography of Alkaline Hydrolysates

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