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 5 mg) are hydrolyzed at 110° or 135° 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-35 resin (column height 8 or 12 cm) has been accomplished in 30 to 50 min with pH 5.4 buffer, 0.21 N in Na+.

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 w-(DL-2-amino-2-ethyl) indole, 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 inability 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,...

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of these observations with improvements in the ion exchange
such a hydrolysate and upon the recent use by Oelshlegel et al.
recovery of tryptophan under various conditions of hydroly-
NaCl does not interfere with the chromatography.

The following experiments were designed to determine the
recovery of tryptophan under various conditions of hydroly-
sis in NaOH. We have drawn particularly upon the observa-
tion of Drèze (14) that starch is a convenient antioxidant in
acid hydrolysates than on the measurement of amino acids other
than tryptophan in the alkaline hydrolysate.

EXPERIMENTAL PROCEDURE

Materials—The L-tryptophan used was chromatographically
homogeneous on PA-55 resin (Decksman) 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 \( ^2 \) was synthe-
sized by Bohak (21) and was chromatographically pure as judged
by amino acid analysis. Defatted and gel filtered bovine serum
albumin prepared according to the method of King
was obtained from Dr. T. P. King. Half-cystinyl human serum albumin was obtained from Dr. E. T. Mertz. Hecker's commercial unbleached wheat
fatted maize meals, normal and opaque-2 (25), were provided
by Dr. W. F. Harrington. Pepsin, purified by the method
of Rajagopalan et al. (24), was prepared by Dr. T. A. A. Do-
phieide and Miss W. M. Jones. Chromatographically purified
pepsinogen (Lot 9A, Worthington) was a gift from Dr. G. E. Perlman and Dr. Y. Nakagawa. Bovine thymotropin was ob-
tained from Dr. J. G. Pierce and Dr. T. H. Liao. Recrystallized
bovine \( \alpha \)-chymotrypsinogen (CD541), bovine \( \alpha \)-chymotrypsin
(CD15G), bovine trypsin (TRI 6257), and bovine pancreatic RNase A (RAF OAB) were purchased from Worthington. De-
fatted maize meal, normal and opaque-2 (25), were provided
by Dr. E. T. Mertz. Hecker's commercial unleached 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 electro-
phoresis was purchased from Pierce Chemicals. All other chem-
icals were analytical reactant grade.

Partially Hydrolyzed Potato Starch—Potato starch was par-
tially hydrolyzed in acid in order to increase its solubility in the
hydrolysis medium for proteins (14); the hydrolysis was accom-
plished according to a method similar to that described by
Smithies (26). Approximately 50 g of potato starch were added to
90 ml of acetic acid 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 was washed with
acetone and dried. In each case, the starch was ground to a
fine powder. No detectable tryptophan was observed when a
25-kg sample of either starch was analyzed after alkaline hy-
drolysis.

Hydrolysis of Proteins—With the purified proteins, solutions
were prepared in dilute (0.005 \( \times \)) HCl or NaOH. In general, a
solution containing 0.1 to 0.5 \( \mu \)mole 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 \( \times 5 \) HCl for 22 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 com-
position 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 alka-
line hydrolysis. The protein solution was pipetted into a Nal-
gene 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 \( \times \) 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 evacuation by add-
ing \( \approx 1 \) ml of 1% 1-octanol in toluene as described by Spies (11).

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.\( ^5 \) 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 \( \mu \)m of mer-
cury.\( ^7 \) The stopcock adjacent to the top of the tube was re-

\( ^2 \) The abbreviation used is: Lysinoalanine, \( \text{N}^\text{L} \text{-amino-2-
carboxyethyl}-\text{L}-\text{lysine.} \)

\( ^3 \) 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 proce-
dure 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-cm polypropylene tube can be cut for 1 ml of NaOH or a
15.8-mm tube for 2 ml; the latter is sealed in 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.
pipette and rubber bulb. The solution was then transferred
mixture stirred by repeated suction and delivery with a Pasteur
ysate is viscous, 0.5 ml of 0.20 s sodium citrate buffer at pH
about 0.5.ml portions of the buffer. The HCl was first chilled
in a beaker with Dry Ice. Since the starch-containing hydrol-
repeatedly opened and closed while the tube was constantly tapped
by hand to keep air bubbles moving upwards through the solution.
After vacuum is thus applied several times, the pressure
should be stabilized between 25 and 50 pm of mercury. If foam
rises too high in the plastic liner, the vacuum can be broken
and the tube recooled again before completing the evacuation.
With the vacuum below 50 pm the tube was sealed. The samples
were heated in a forced-draft oven at 110° i 1° or in a
heating block (model 2128-A, Warner Chilcott Laboratories,
Morris Plains, ?i. J.) at 135°. The tubes were carefully re-
portedly opened and closed while the tube was constantly tapped
in order to prevent any loss of tryptophan from warming of the
acidic mixture during neutralization of the alkali. The poly-
propylene liners were recovered and reused indefinitely.
Tryptophan analyses of insoluble protein-containing ma-
were obtained on a dry weight basis. The samples were
hydrolyzed in 4.2 x NaOH (sample plus 0.1 ml of H2O plus 0.5
ml of 5 x NaOH) with the amount of starch added depending
on the carbohydrate content of the sample.
Even though the neutralized 110° hydrolysates of purified
proteins were slightly cloudy, it was not necessary to treat them
further before applying them to the ion exchange column.
However, all of the 135° hydrolysates and the hydrolysates of
corn and wheat meals were highly turbid and were centrifuged
at 40,000 x g for 30 min at 2° in order to remove degraded ma-
terials that could accumulate on the top of the column.
Chromatography—A 1-ml sample of the neutralized hydrolys-
ate at pH 4.25 was added to a column (0.9 x 8 cm or 0.9 x 12
amino acid analyzer (19) to use a pH 2.2 citrate buffer (29) to
dilute the sample for analysis. Since tryptophan may be lable
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 trypto-
phane 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 analy-
sis is a buffer at pH 4.25 in which the loss of tryptophan over-
night is negligible (less than 1%; Fig. 1). This stability is im-
portant when samples are to be stored in automatic sample injectors.*

\* Stock solutions of pure tryptophan (in the absence of other
amino acids) in 0.1 x HCl are remarkably stable; no loss of trypto-
phane 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 de-
creases significantly with time; losses were also observed when a
solution of pure tryptophan in 0.005 x NaOH was stored in the
presence of air.

Fig. 1. The rate of destruction of free L-tryptophan under various conditions. A dilute (0.05 pmole 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. ●—●, L-tryptophan stored at room temperature (23 to 25°) in 0.2 N sodium citrate buffer at pH 2.2; ▲—▲, L-tryptophan stored at -20° in 0.2 N sodium citrate buffer at pH 2.2; ■—■, L-tryptophan stored at room temperature in 0.25 N sodium citrate buffer at pH 4.25.

RESULTS

pH of Buffer for Sample Addition—It is customary with an
amino acid analyzer (19) to use a pH 2.2 citrate buffer (29) to
dilute the sample for analysis. Since tryptophan may be lable
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%;
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night is negligible (less than 1%; Fig. 1). This stability is im-
portant 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 con-
tains 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.
This procedure avoided the possibility that the relatively high
conzentration of breakthrough acidic and neutral amino acids
may interfere with the tryptophan peak.
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 e-amino group of a lysyl residue to the double bond of a dehydroalanyl 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 Uppsala 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 x 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 x 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. Hydroxysine, 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 when chromatographed on either the 8- or 12-cm column. The most slowly hydrolyzed tryptophan-containing dipeptides, isoleucylisoleucyl and valylvaline, were eluted well after the tryptophan position.

Effectiveness of Starch in Protecting Tryptophan under Alkaline 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 Oelshlegel 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 ± 3%), the recoveries of tryptophan after hydrolysis at 110° for 16 hours in the presence of starch are quantitative.

### Table I

| Hydrolysis conditions | Additives | Tryptophan recovered |
|-----------------------|-----------|----------------------|
| L-Tryptophyl-L-leucine | None      | 91.0                 |
| 4.2 N NaOH for 16 hours | None      | 91.5                 |
| 4.2 N NaOH for 24 hours | Thiodiglycol | 97.5                |
| 4.2 N NaOH for 24 hours | Starch    | 98.2                 |
| RNase + tryptophan*   | Thiodiglycol | 94.6                |
| 4.2 N NaOH for 16 hours | Starch    | 100.2                |

* Average of duplicate runs.

12 The hydrolysates contained 250 \( \mu g \) of RNase and 30 \( \mu g \) of tryptophan.

14 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*. 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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TABLE II

Tryptophan recovery 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.

| Materials                                      | Tryptophan observed with additive | Thiodiglycol | Starch | Nearest integera |
|------------------------------------------------|----------------------------------|--------------|--------|------------------|
| Bovine θ-chymotrypsin                         | 7.44                             | 7.75         | 8      |                  |
| Bovine θ-chymotrypsinogen                     | 7.73                             | 8            |        |                  |
| Bovine deoxyribonuclease                      | 2.77                             | 2.92         | 3      |                  |
| Bovine serum albumin                         | 1.94                             | 2.03         | 2      |                  |
| Human serum albumin                          | 1.00                             | 1            |        |                  |
| Bovine trypsin                               | 3.80                             | 3.97         | 4      |                  |
| Porcine peptin                               | 4.40                             | 4.87         | 5      |                  |
| Porcine peptin                               | 5.02                             |              | 5      |                  |
| Hen egg white lysozyme                       | 5.55                             | 5.75         | 6      |                  |
| Bovine thyrotropin                           | 0.03                             |              | 0      |                  |
| Sperm whale apomyoglobin                     | 1.20                             |              | 2      |                  |

a 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 peptin.

b Hydrolyzed in the presence of 5 m NaOH of a 1% 1-octanol in toluene.

c Hydrolyzed for 98 hours at 110° (see text for discussion).

d Hydrolyzed for 48 hours at 135° (see text for discussion).

TABLE III

Determination of tryptophan in defatted maize and wheat flour samples after alkaline hydrolysis

With pepsin and peptinogen, spectrophotometric measurements (30, 40) indicated 6 residues. Dopheide and Jones (41) could find only 4 tryptophan-containing peptides, but Kostka et al. (42) found evidence for a fifth. Snies (11) and Knox et al. (18) have obtained a value of 5 for pepsin by their procedures; the present value of 5 is in agreement with the recent results by alkaline hydrolysis and the current structural data.

**Rates of Hydrolysis in 4.2 N Sodium Hydroxide for Peptides and Proteins Containing Tryptophan Residues**—For the first 10 proteins analyzed for Table II, hydrolysis at 110° for 16 hours was adequate. But none of these proteins is known to contain the -Ile-Trp- or -Val-Trp- sequences which are likely to be the most slowly hydrolyzed linkages with tryptophan. The present method was therefore 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 obtained 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, glycylylvaline and glycylglycylvaline are hydrolyzed at least 10 times more rapidly than are valglycine and isoglycine (43, 44). 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-leucine 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 study at 110° (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-Ise- 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 tryptophalvaline. All of these sequences occur in egg white lysozyme. Recovery of 95% 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 Λ, 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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**Materials**

- Maize meal (Normal)
- Maize meal (Opaque-2)
- Wheat flour (Unbleached)
- Wheat flour (Bleached)

**Grains of tryptophan per 16 g of nitrogen**

| Materials          | Present result | Literature values |
|--------------------|----------------|-------------------|
| Maize meal (Normal)| 11.56          | 0.69 ± 0.01 (0.72) |
| Maize meal (Opaque-2)| 12.00        | 1.03 ± 0.01 (1.08) |
| Wheat flour (Unbleached) | 13.50      | 1.07 ± 0.01 (1.13 ± 0.01) |
| Wheat flour (Bleached)  | 12.50       | 1.05 ± 0.005 (1.11 ± 0.005) |

a Averages of duplicate analyses.
b Colorimetric determination (47).
c Corrected for loss to pellet after centrifugation (see text).
d Microbiological assay (47).
The ratio observed for the tryptophan contents of normal and opaque-2 maize agrees with that found by Dr. E. T. Mertz.\textsuperscript{13}

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\(^\circ\). However, in 0.2 M H\textsubscript{2}SO\textsubscript{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\textsubscript{2}SO\textsubscript{4}, the same derivative shows an 11\% decrease in its content of unaltered tryptophan.

These results show that soluble DNase 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 acidic 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 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 \(\mu\)m, 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 \(\beta\)-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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