A LABORATORY MANUAL OF ANALYTICAL METHODS OF PROTEIN CHEMISTRY

(INCLUDING POLYPEPTIDES)

Editors

P. ALEXANDER

R. J. BLOCK

A LABORATORY MANUAL OF

ANALYTICAL METHODS OF PROTEIN CHEMISTRY

(INCLUDING POLYPEPTIDES)

VOLUME 2

THE COMPOSITION, STRUCTURE AND REACTIVITY OF PROTEINS

EDITORS

P. ALEXANDER

CHESTER BEATTY RESEARCH INSTITUTE

R. J. BLOCK

BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH

PERGAMON PRESS LTD.
Headington Hill Hall, Oxford
4 & 5 *Fitzroy Square, London, W.*1

PERGAMON PRESS INC.
122 *East* 55*th Street, New York* 22, *N.Y.*
P.O. Box 47715, *Los Angeles, California*

PERGAMON PRESS S.A.R.L.
24 *Rue des Écoles, Paris V^e^*

PERGAMON PRESS G.m.b.H.
Kaiserstrasse 75, Frankfurt am Main

PRINTED IN GREAT BRITAIN BY THE PITMAN PRESS, BATH

The Editors will gratefully receive comments and criticisms by the Readers and will appreciate the Readers calling to their attention factual and typographical errors.

PREFACE

IN the last fifteen years there has been a revolution in the techniques available for the analysis and isolation of proteins. Every time a new technique has been introduced, numerous papers have appeared describing modifications to it and the research worker who wishes to employ these methods is faced with a very serious problem in deciding which particular variant to use. These volumes are intended to provide the fullest practical detail so that any scientist can follow the procedure by using this book alone and without having recourse to the original literature. No attempt has been made by the contributing authors to describe all the variants. The techniques which are described in full are ones in which all the authors have had first-hand experience and as a result the descriptions contain those small, but important, points of techniques which are often omitted from the scientific papers, but which save so much time if known. Where the techniques require a large instrument such as the ultra-centrifuge or the electron microscope, no attempt has been made to describe the working of these instruments in detail, since this is provided in the manufacturers' manuals. However, the authors have attempted to give full details of the preparation of samples before they can be used in these techniques and for the evaluation of the data. For methods which do not require large instruments or which require instruments which must, in general, be made by the investigator himself, more detailed working details are given. In each of the articles a short discussion of the background and theoretical principle is given and a more detailed description of the difficulties in interpretation. It is our hope that workers who find that they have a problem in protein chemistry will be able to turn to these volumes and, by looking through the chapters, decide which of the techniques is the most suitable for their purpose and then be able to follow this technique from the instructions provided.

In the first volume, separation and isolation procedures are discussed; the second volume concerns its analysis and reactivity, and the third volume with the measurement of the macromolecular properties of proteins.

The contents of Volumes 1 and 3 are given overleaf.

R. BLOCK
P. ALEXANDER

October 1960

Contents List: Volume 1

Contents List: Volume 3

CONTENTS

1

AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES

By RICHARD J. BLOCK

from

The Boyce-Thompson Institute for Plant Research, Yonkers 3, N.Y., and the Department of Biochemistry, New York Medical College, New York 29, N.Y.

CONTENTS

1

AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES

By RICHARD J. BLOCK

from

The Boyce-Thompson Institute for Plant Research, Yonkers 3, N.Y., and the Department of Biochemistry, New York Medical College, New York 29, N.Y.

A. PREPARATION OF THE SAMPLE FOR ANALYSIS

General Procedure

As a preliminary step in the amino acid analysis of any material it is recommended that its protein content (nitrogen × 6·25) be determined. If the percentage of nitrogen is 13·5 or above, the material can be considered relatively pure protein and can be hydrolysed without further treatment. However, if the nitrogen content falls below the indicated limit, it is essential to remove non-protein substances. The success of the analysis and the accuracy of the results depend in part on the removal of interfering substances.

Determination of Nitrogen

1. Semi-micro Kjeldahl Method

Reagents

Digestion mixture. 100 g of K_2SO_4 + 10 g $CuSO_4$. Grind in a mortar so that lumps of $CuSO_4$ are powdered. Mix well.

Selenium granules. Se-coated chips (Hengar Co., Philadelphia).

Concentrated sulphuric acid. H_2SO_4, AnalaR.

Hydrogen peroxide. 30% H_2O_2, AnalaR, nitrogen-free.

Boric acid solution. 2% H_3BO_3 in distilled water.

Indicator for titration. 200 mg methyl red + 100 mg methylene blue. Make up to 200 ml with absolute ethyl alcohol (keep cold).

Sodium hydroxide. NaOH pellets, AnalaR.

Standard acid solution. 0·02 N HCl or $KHIO_3$ prepared as follows: make an approximately 0·02 N solution of the acid of choice. Weigh samples of dried glycine and ammonium sulphate to contain 1–3 mg N. Digest and distil according to the procedure given below. Titrate with acid solution to be standardized.

$$\text{Factor} = \frac{\text{mg N}}{\text{ml acid}}$$

Apparatus

100 ml Kjeldahl flasks.

Pyrex air-cooled condensers for distillation, bent in U-shape (Folin and Wright, 1919).

Procedure. Weigh the sample, transfer quantitatively to Kjeldahl flask, or pipette aliquot of liquid into flask. The sample should contain 1–3 mg of nitrogen. Add approximately ¼ teaspoonful of digestion mixture, 1 Se-coated granule, and 2·5 ml of concentrated H_2SO_4. Place on digestion rack and heat for 15–20 min. Add 0·5 ml of H_2O_2 carefully. Return to heat and add 0·5–1·0 ml more of H_2O_2 in 0·5 ml portions as it is needed. Turn flasks occasionally to aid complete digestion of all particles. When mixture is clear green, heat for 30–60 min longer or if necessary, heat 7 hr longer. Samples containing much carbohydrate tend to foam considerably and must be watched until foaming subsides. It may be necessary to wash down the neck of the flask with distilled water if excessive foaming has caused the sample to move up the neck of the flask. When digestion is complete, allow flasks to cool and add 35–40 ml of distilled water, mixing until the salts are all in solution. Cork flasks and cool in refrigerator or place flasks in ice water until they are cold. Meanwhile prepare receiving flasks for distillation; into each place 30 ml of the boric acid solution and 3–4 drops of the indicator. Rinse the inside of the condensers with distilled water and place the receiving flasks under the end of the condenser so that the condenser end is just under the surface of the boric acid solution but does not touch the bottom of the flask. When the contents of the digestion flasks are well-chilled, add approximately ¼ teaspoonful of NaOH pellets. Add them quickly and do not shake the mixture. Attach the flask to the rubber connexion on the condenser as quickly as possible and then shake the mixture. Heat gently. Distil for 15 min, then lower the receiving flasks so that the end of the condenser is out of the solution and continue distilling for 5 min longer. The condenser above the receiving flask should be hot near the top but not at the bottom of the arm. If the distillate is hot when it reaches the boric acid, the acid thus becomes warm and loses its ability to retain the ammonia. Titrate the ammonia with the standard acid and calculate as follows:

$$\text{ml acid} \times \text{factor} = \text{mg nitrogen in sample}$$

2. Micro Kjeldahl Method for 0·5–15 V of Nitrogen

This procedure (Boissonnas and Haselbach, 1953) is essentially the same as described above except that the ammonia is measured colorimetrically with ninhydrin (see below) and smaller quantities of sample and reagents are used.

3. Rapid Kjeldahl Method

This was developed by Perrin (1953).

Reagents

Silica granules. Not selenized.

Mercuric oxide. HgO, AnalaR.
Boric acid. 4% H_3BO_3.
Zinc. 20 mesh Zn.
Indicator. 5 parts of 0·2% bromocresol green + 1 part of 0·02% methyl red in ethanol.

Method. Mix in a 500 ml Kjeldahl flask the following: 500–1000 mg of sample, 6 silica granules, 1·3–1·5 g of H_2O, 12 ± 0·5 g of K_2SO_4 and 15 ml concentrated H_2SO_4. Digest at a low heat for 5 min or until frothing stops. Then increase heat to "full" for 8–12 min. Cool, add 200 ml of water, 25 g of NaOH, 5 g of $Na_2S_2O_3$, and 500 mg of Zn. Distil. Determine ammonia in any convenient fashion.

Removal of Lipids

The lipid-containing material is extracted with approximately 10 times its weight of the following solvents in succession; acetone (twice), hot benzene: absolute ethanol = 95:5 v/v (twice), hot ethanol and ether. Each solvent is removed from the proteinaceous residue by filtration (or centrifugation) and drying in air.

Removal of Nucleic Acids

Tissue nucleic acids may be removed from lipid-free tissues by digestion with 10% sodium chloride at 85°C for 6 hr (Weiss *et al.*), the residue is washed with hot water and dried with acetone.

Removal of Carbohydrates

The presence of carbohydrates results in from negligible to very large losses of amino acids during hydrolysis, especially with mineral acids. It is therefore necessary to separate as much of the contaminating carbohydrates as possible from the protein. Unfortunately, there is no procedure that can be applied under all conditions which will result in the desired goal; however, one of the most useful methods is: Ten grammes of grain is ground to a fine flour, if necessary, and triturated with 90 ml of hot water until no lumps remain. The pH is adjusted to 4·5 with a little 5N acetic acid and the suspension is heated in boiling water for 1 hr. Then 180 ml of cold water are added to the suspension and the pH is adjusted to 7·0 with 5N NaOH. After cooling to 37°C, 10–25 ml of fresh human saliva clarified by centrifugation, or a few milligrammes of crystalline bacterial amylase (Nagase & Co., Ltd., Amagasaki, Japan) are added. The starch is digested at 37–40°C over-night in the presence of toluene as the preservative. At the end of the digestion period, the insoluble protein residue is removed by centrifugation and thoroughly washed with hot water, acetone, hot benzene: ethanol = 95:5, ethanol and ether. The increase in nitrogen achieved by this procedure is from three- to five-fold.

Sahyun (1949) has used proteolytic enzymes to separate protein from insoluble carbohydrates. The soluble peptides are then completely hydrolysed with acid.

Removal of Protein from Amino Acid Solutions

Where an investigation of the free amino acids and peptides is to be made, as many of the other constituents as possible must be removed. Dialysis or precipitation with trichloroacetic acid, tungstic acid, ferric hydroxide, zinc sulphate:barium hydroxide at pH 7·2–7·6, perchloric acid, acetone, and ethanol have been used (cf. Block and Weiss, 1956).

One method is to grind the tissues with sufficient absolute ethanol in a Waring Blendor so that the final concentration of alcohol is 80% by volume. The insoluble material is removed by filtration and washed with 80% ethanol. Then, three volumes of chloroform are added to each volume of the ethanol extract. After thorough shaking the resulting aqueous layer (upper) is removed and concentrated to the desired volume.

If trichloroacetic acid is used as the protein precipitant, the excess acid may be neutralized to pH 6 with N-trioctylamine or the like, and the salt extracted with chloroform and ether (Smith, E. L., 1948).

Desalting of Amino Acid Solutions

Preparation of Cation Exchange Resin

Dowex 50-X8 (H^+), 200–400 mesh, is washed with water four times and the fines are removed. The resin is then treated with 10% w/v NaCl in a column until the pH is approximately 7. The excess NaCl is washed out with water and then the column is treated with 2N HCl in slight excess. The excess acid is removed by washing with water. After 3 or 4 such cycles, the H^+ resin is washed with 80% v/v ethanol and stored in this solvent until used (Plaisted, 1958).

*Removal of Impurities from an Amino Acid Solution**

The amino acids are dissolved in 80% v/v ethanol and passed through a resin column 5 × 0·9 cm at the rate of about 3 ml/min. The unabsorbed materials are washed from the resin with 15 ml of 80% ethanol. The amino acids are eluted by successive applications of 40 ml of 0·4N NH_4OH in 80% ethanol; 15 ml of 80% ethanol, 15 ml of water, 4N aqueous NH_4OH, and finally 15 ml of water.

The combined elutriates are concentrated *in vacuo* and the residue is dissolved in 10% v/v aqueous *iso*propanol.

Comments. Glutamic acid is converted, in limited amounts, to its γ-monoethyl ether. The bulk of the basic amino acids are eluted by the aqueous 4N NH_4OH.

B. HYDROLYSIS OF PROTEINS

Acids

Hydrochloric, sulphuric, hydriodic, hydrochloric, formic and other acids have been used to hydrolyse proteins to their constituent amino acids. In

* Busson and Guth (1956) absorbed the amino acids and cations on Amberlite IR-120-X8 by shaking for 1 hr, the unexchanged substances are washed off with water and the amino acids are eluted with 4N NH_4OH by shaking for 15 min. Buchanan (1957) employed Dowex 50-X4 (H^+), 200–400 mesh and eluted the amino acids with 0·1 M piperidine.

general, the concentration of hydrolysing acid used is inversely proportional to the time and temperature of the reaction.

Hydrochloric acid. It is customary to boil the protein with from 2·5 to 5000 times its weight of 6N HCl under reflux for 18–24 hr. When only small amounts of the protein are to be hydrolysed it is advisable to use 6N HCl equal to 1000 to 5000 times the weight of the protein. This large excess of hydrolysing acid reduces amino acid losses.*

For many purposes, especially for analysis by microbiological methods, 3N HCl at 15 lb pressure for 5–10 hr is also widely employed but it is not as satisfactory as 6N HCl under reflux when crude proteins are to be analysed.

It is well recognized (Block and Weiss, 1956) that the rate of liberation of some amino acids from peptide linkage is faster than others; furthermore, it is known that the amount of destruction during hydrolysis increases with the length of heating. Thus, for the most accurate work, it is advisable to hydrolyse samples of the protein for different lengths of time, e.g., 20, 40, 80, and 120 hr, and to determine the amino acid composition of each hydrolysate. Then if it is observed that one or more amino acids (e.g., cystine, methionine, serine, threonine) shows a progressive decrease, then the true value may be assumed to be that found at zero time from a plot of the values obtained at different periods of hydrolysis. Likewise, if the quantity of an amino acid (e.g., *iso*leucine, valine) gradually rises to a peak value, the assumed maximal value may be obtained graphically, These refinements are only valuable when the methods employed have the highest accuracy, and must be applied empirically for each separate protein. When investigating highly purified proteins it is also advisable to redistil the HCl several times in glass.

Chromatographic assay methods require complete hydrolysis to amino acids for obvious reasons. Complete hydrolysis is also necessary when microbiological assay methods are employed because the growth response of many organisms is different (it may be greater or less) for the free amino acid than for the same amino acid in peptide linkage.

A point too often forgotten in many laboratories is that destruction of amino acids can take place even after the hydrolysis is ended, especially when the hydrolysate contains free acid and humin. It is therefore advisable to remove the excess HCl as soon as possible after hydrolysis, filter off the humin, decolourize with a small quantity of charcoal (Darco G-60), if necessary, and after evaporation to dryness, dissolve the amino acid hydrochlorides in 10% 2-propanol and store in the cold.

Hydrochloric acid: formic acid. From 10 to 1000 times the weight of protein is used of the following acid solutions; 38% HCl:88–90% HCOOH = 1:1 v/v or 6N HCl:88–90% HCOOH = 1:1 v/v. This mixture has been claimed to be preferable to 6N HCl for the analysis of cystine in insulin and nucleoproteins. However, for the majority of proteins it is not superior to hydrochloric acid.

Sulphuric acid. Although 8N H_2SO_4 at atmospheric pressure is usually employed, other concentrations of acid and pressures may be used (Block and

* Five per cent w/v phenylhydrazine added to 6 N HCl is reported to reduce losses during hydrolysis (Nakajima, 1958; Watsuji, 1958).

Bolling, 1951). The advantage of H_2SO_4 lies in the ease with which the excess acid can be removed with $Ba(OH)_2$. The humin is also largely precipitated along with the $BaSO_4$. H_2SO_4 is less effective, at equal concentrations, than HCl.

Comment. Other methods of hydrolysis are given in Block and Weiss (1956).

Bases

Sodium hydroxide, 5N, or 14% w/v barium hydroxide under reflux for 18–20 hr must be employed for liberating tryptophan from the protein. The excess barium ions are removed by neutralizing the diluted hydrolysate with gaseous CO_2 or "dry ice."

Enzymes

Proteolytic enzymes are usually not practical for the preparation of hydrolysates for chromatographic methods and not advised for microbiological procedures (Block and Bolling, 1951) because they contribute some amino acids to the final solution.

However, if enzymatic hydrolysis is desired,* it is advisable first to denature the protein by heat coagulation at pH 4 (dilute acetic acid) and then to digest at 40°C at pH 8·6 in 0·01 M borate buffer for at least 24 hr with one-tenth the weight of the protein of pancreatin powder (Viokase, Viobin Corp., Monticello, Ill.); the enzyme is then inactivated by boiling and removed by filtration. After cooling the solution, the resulting peptides are hydrolysed with an amount of erepsin equal in weight to the pancreatin.

Losses During Hydrolysis

Block and Weiss (1956) have reviewed this subject.

Completeness of Hydrolysis

The liberation of carboxyl and amino groups from peptide linkage is the object of protein hydrolysis. Hydrolysis is considered complete when a maximum number of —COOH and —NH_2 groups have been liberated. The carboxyl groups can be readily estimated by one of the modifications of the Schiff–Sørensen formal titration method or by oxidation with ninhydrin.

Reaction of Amino Acids with Ninhydrin

Ammonia resulting from the oxidative deamination of amino acids with ninhydrin is aerated or steam distilled† into boric acid and titrated (Sobel, *et al.*, 1945).

Reagents

Caprylic alcohol. Saturate with thymol.
Ninhydrin (solid).

* Enzymatic hydrolysis is required for the liberation of iodoamino acids from iodoproteins.

† The Conway microdiffusion method may also be used (McConnell, 1952).

Citrate buffer, pH 2·5. Grind 2·06 g of trisodium citrate and 19·15 g of citric acid to a fine powder.

Hydrogen peroxide, 30%.

Potassium hydroxide, saturated. To a cylinder containing water under mineral oil 1 in. thick, add solid KOH to saturation. Preserve under oil.

Indicator. Add 10 parts of 0·1% bromocresol green to 1 or 2 parts of 0·1% methyl red in 95% ethanol.

Boric acid. Dilute 20 g of H_3BO_3 to 1 l. Add 20 ml of indicator per litre.

Hydrochloric acid. Standard 0·0714 N HCl.

Method. Add 1 ml of solution containing 20–100 μg of carboxyl nitrogen to an aeration tube containing 0·3 mg of buffer and 50 mg of ninhydrin. The pH should be 2·4–2·6. Shake to mix and place in boiling water for 10 min. At the end of 2 min heating, shake to dissolve the ninhydrin. After 10 min heating, add 3 drops of 30% H_2O_2, shake and heat for 3 min longer. Set up the tubes for aeration or distillation. Add 1 ml of saturated KOH and aerate into H_3BO_3 for 40 min using 1·5 ml of 2% boric acid to trap the ammonia. Titrate* with HCl or $KHIO_3$. Calculate as follows:

$$\text{ml of } 0{\cdot}0714\,\text{N HCl} \times 1000 = \text{mg of amino acid N}$$

Colorimetric Estimation of Amino Groups

Harding and MacLean's (1916) *method.* To 1 ml of neutralized unknown (0·01–0·08 mg of amino nitrogen) add 1 ml of 10% aqueous pyridine and 1 ml of 2% aqueous ninhydrin. Stopper the test-tube lightly with a cotton plug and place in a boiling water bath for 20 min. Cool in water, dilute to 50 ml and read at 570 mμ. Leucine may be used to prepare the standard curve.

Cocking and Yemm's (1954) *method.* Mix 1 ml of an amino acid solution containing 0·05–2·8 μg amino nitrogen with 0·5 ml citrate buffer of pH 5 (0·2 M). Add ninhydrin in methyl cellosolve (0·02 ml, 5% w/v) and KCN in methyl cellosolve (1 ml 2% w/v) to this solution either separately or as a single solution. Separately these reagents are stable for at least 1 month, and mixed, for at least 1 week. Heat the well-mixed solution for at least 15 min at 100°C and cool for 5 min in running tap water. The b.p. of the water:methyl cellosolve mixture is greater than 100°C and using tubes stoppered with a glass marble, evaporation losses during the heating period are negligible. Make the solution up to a convenient volume with ethanol (60% w/v) and determine the optical density at 570 mμ. The colours are quite stable for at least 1 hr at room temperature. Most of the common amino acids give colours equivalent to 100 ± 1% of that of pure dioxohydrindylidene-dioxohydrindamine (DYDA) except tryptophan (80%) and lysine (110%). Ammonia reacts yielding a colour equivalent to only 33% of that of pure DYDA.

* The quantity of ammonia may be also estimated colorimetrically (Brown, R. H. *et al.*, 1957).

C. PAPER CHROMATOGRAPHY

Qualitative Paper Chromatography

Present-day paper chromatography may be considered to have started with the report of Martin and Synge in 1944. Because of the extensive familiarity with this method and its adequate coverage in many books and review papers (cf. Block, Durrum and Zweig, 1958), a description of only a few useful procedures will be given.

1. Quantities of Amino Acids Used

The amount of each amino acid required to give a visible spot on a two-dimensional chromatogram is dependent on: type of colour reagent, size of spot initially applied, length of develc pment, type of paper, solvents employed and the nature of the amino acid. The approximate amounts in 5 μl of solution of each amino acid to be applied to a large (18 × 22 in.) two-dimensional chromatogram are given in Table I.

TABLE I. DESIRABLE QUANTITIES OF EACH AMINO ACID TO BE APPLIED TO A TWO-DIMENSIONAL CHROMATOGRAM IN 5 μl OF SOLUTION

Arg.	4 μg	Tyr.	4 μg	Cys.	5 μg	Ser.	1 μg
His.	10 μg	Try.	4 μg	Cysteic ac.	1 μg	The.	1 μg
Lys.	3 μg	Phe.	4 μg	Met.	2 μg		
Leu.	1 μg	Glu.	0·5 μg	Gly.	0·5 μg	Pro.	3 μg
Iso	1 μg	Asp.	1 μg	Ala.	0·5 μg	Hop.	3 μg
Val.	1 μg						

2. Paper

Whatman No. 1 or No. 3 or Schleicher and Schuell No. 598 are most widely used. The latter is especially valuable for cystine, methionine, histidine and tyrosine when one-dimensional chromatography and specific colour tests are employed.

3. Solvents*

(*a*) *Phenol.* One hundred millilitres of metal-free water are dissolved in 500 ml of Mallinckrodt Gilt Label liquid phenol by gentle warming. Add 25–50 mg of 8-hydroxyquinoline. The solvent is stored in a dark bottle in the refrigerator where the cold causes separation into two layers. When it is to be used, the bottle is vigorously shaken and the desired quantity of the emulsion is removed and gently warmed to effect solution.

A beaker containing 100 mg of NaCN in 4–6 ml of water and one containing 50 ml of 3–4% NH_3 are commonly placed in the chamber.

The relative distance (R_f) travelled by the more basic amino acids (arginine,

* The addition of a drop of 0·1% w/v bromocresol purple indicator to the amino solution is often helpful in enabling the investigator to follow the length of the solvent development, especially if the solvent is allowed to flow off the paper.