THE RECOVERY OF TRYPTOPHAN FROM HYDROLYSIS OF PROTEINS WITH TRIFLUOROACETIC ACID AND HYDROCHLORIC ACID

being a thesis presented by

CHERUB ADEOLU AKIODE

to the University of St. Andrews, in application for the Degree of Master of Science

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a Higher Degree.

The research was carried out in the Department of Biochemistry and Microbiology in the United College of St. Salvator and St. Leonard, the University of St. Andrews, under the direction of Professor G.R. Tristram.

CERTIFICATE

I hereby certify that Cherub Adeolu Akiode has spent seven terms engaged on research work under my direction, and that he has fulfilled the Condition of No. 1974 No. 2 Resolution of the University Court (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Master of Science.

29th April 1980

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1962 and graduated with the Degree of Bachelor of Science (Ordinary) in Chemistry and Biochemistry in 1965. My subsidiary subjects were Physics, Zoology, Botany, and Psychology at first B.Sc. level. On returning to Nigeria, I started teaching Chemistry and Biochemistry in Yaba College of Technology, Lagos.

In October 1977, I matriculated in the University of Lagos, Nigeria, and passed the Postgraduate Diploma in Education (Science) examination in June/July 1978 in the following subjects at the grades written against each subject:

Education I:	Educational Psychology & Philosophy of Education	A
Education II:	Educational Administration & Educational Development in West Africa	B+
Education III:	Principles & Methods of Educational Teaching & Visual Aids	B
Education IV:	Curriculum Development and Educational Foundations	B+

(Key to Grades: A = 70% and above: B = 60-69%;
C = 50-59%; Below 50% = Failure)

In October 1978, I matriculated as a Research Student for the Degree of Master of Science in the Department of Biochemistry and Microbiology, University of St. Andrews, St. Andrews, Scotland, United Kingdom.

ACKNOWLEDGEMENT

I thank most sincerely Professor G.R. Tristram for his patience, sympathy, helpful advice, encouragement and guidance throughout this work. I highly appreciate the research opportunity and facilities made available for me to work in his personal laboratory.

I should also like to express my thanks to Messrs Jim Crawford, Jim Hunter, Angus Grieve, Bill Blyth, and Colin Armit for their technical assistance.

My thanks also go to Governing Council of Yaba College of Technology for granting me study leave and to my wife and children for their moral support.

ABSTRACT

A method has been developed for the determination of tryptophan in proteins. This technique modified the use of strong acid such as 6M HCl containing mercapto-acetic acid (thioglycollic acid). Tri-fluoro acetic acid has been used to dilute concentrated HCl and mercapto acetic acid was added to prevent the destruction of labile tryptophan during acid hydrolysis of lyophilized proteins in an evacuated sealed tube. Trifluoro-acetic acid and hydrochloric acid (1:1) mixture serves several advantages: (1) The two reagents are miscible, (2) Trifluoro acetic acid is a good solvent for peptides and in strong acid, amino groups are protonated and hence not acetylated, (3) Trifluoro acetate can be easily removed and the point of attack is probably trifluoro acetyl as well as carbonyl carbon. The activation of the β-carboxyl group of some amino acids could result in the formation of cyclic imide by displacement of tri-fluoro acetic acid. But this is doubtful, because if it occurs, there will be incomplete hydrolysis.

The sample proteins were lyophilized and suspended in freshly prepared Hydrochloric acid diluted to 6M with Trifluoro-acetic acid containing mercapto acetic acid. The tube was then placed in solid-carbon dioxide and ethanol. When frozen, the tube was evacuated to 0.1mm Hg and sealed under vacuum. Hydrolysis was conducted at a controlled temperature of 110° ± 1°C for 24, 36 and 48 hours in a DRI-BLOCK heater. The excess solvent was removed on a rotatory vacuum evaporator connected to Paxman Cooling System, water bath at 37°C and oil pump pressure within 20 minutes. The residue was quantitatively estimated using norleucine which is used as internal standard for JEOL JLC-5AH Amino Acid Analyzer. The values of tryptophan and other amino acids obtained were close to the expected integral values and recoveries of all other amino acids were comparable to those observed after hydrolysis with 6M HCl or 6M HCl containing 4% mercapto

acetic acid or p-toluene sulphonic acid. The method can also be used for protein containing about 5% ($^{w}/_{w}$) carbohydrate. The hydrolysate can also be used in Miller's colorimetric method for the determination of tryptophan in purified proteins. The procedure, however, is not yet tested with feedingstuffs.

Hence, a new method for the microanalysis of tryptophan by acid hydrolysis is proposed.

CONTENTS OF THE THESIS

1.1. INTRODUCTION

Amino acid analysis has been a subject of research for many years and will continue to be so as new methods are developed for satisfactory determination of amino acid composition of proteins in plants and animals. For Stein (1946) wrote "From the point of view of protein structure there can be no doubt that an accurate and complete knowledge of the amino acid composition of numerous different proteins is essential to further fundamental advances in protein chemistry".

Moore and Stein (1963) also reported thus "Since the amino acid composition is a primary characteristic of a given protein, accuracy in the analysis is of greater importance than the speed or convenience of the determination. The ease of the analysis increases in significance in the study of small peptides when less accurate data may suffice to define the composition".

Tristram (1949), Tristram and Smith (1963) carried out critical reviews of analytical procedures in order to obtain the highest degree of accuracy in amino acid analysis.

As the accurate analytical methods for the determination of amino acids have increased, the limiting factor in determining the amino acid composition of a protein has become the extent to which the composition of the hydrolysate is a true reflection of the composition of parent protein.

Warner (1942) carried out alkaline hydrolysis of protein for the determination of amino acids which are unstable in strong acid and also reported the course of the hydrolysis by acid, but remarked that the theory - "to account for the rate of formation of free amino acids as a function of the number of peptide bond hydrolysed - fails with alkaline hydrolysis". Warner (1942) proposed theoretical relationship between the number of free amino acids and the

number of bonds hydrolysed throughout the course of the reaction may be examined on the basis of the following assumptions:

(1) A molecule of protein X has a molecular weight A and consists of Y amino acids joined in a single chain by Y-1 peptide bonds.

(2) The intrinsic rate of hydrolysis of all the bonds is the same and is independent of the length of the chain, the position of the bonds in the chain and the particular amino acid forming the bond.

Then, the rate of hydrolysis of bonds should follow the FIRST ORDER LAW:-

$$K_t = \log (Y-1) \Big/ \{(Y-1) - x\}$$

where x is the number of bonds hydrolysed, Y amino acids joined in a single chain by Y-1 peptide bonds.

However, the two major problems associated with acid hydrolysis using 6M HCl are destruction of labile amino acids, Serine, threonine and tryptophan and the slow hydrolysis of some peptide linkages between bulky amino acids. Several methods (such as Stein and Moore (1960)) are used to solve the problems but the determination of tryptophan still presents a problem if acid hydrolysis is not avoided as in spectrophotometric procedure of Goodwin and Morton (1946), the colorimeter procedure of Spies and Chambers (1949), Barman and Koshland (1967), alkaline hydrolysis methods, Dreze and Reith (1956), Goswami (1974) and Datta (1977) and more recently Matsubara and Sasaki (1969).

Recently, mercapto-acetic acid (thio-glycolic acid) has been used to prevent the oxidation of methionine, tyrosine or Carboxymethyl cysteine during acid hydrolysis of peptides and proteins (Tupp and Kreel, 1962).

So, Matsubara and Sasaki (1969) used 6M HCl containing 4% mercapto-acetic acid to prevent the destruction of tryptophan during acid hydrolysis.

The objects of the present work were:

(1) to modify Matsubara and Sasaki's method by using a mixture of HCl and TFA (1:1) containing mercapto acetic acid (thioglycolic acid) for acid hydrolysis of proteins, glycoproteins, peptides and extracted gliadin;

(2) to investigate the recovery of tryptophan from the hydrolysates;

(3) by using JLC-SAH automatic ion-exchange chromatographic method;

(4) compare the recovery of tryptophan in protein hydrolysate with other methods. Since some researchers always omit tryptophan determination in their analysis of amino acid composition of proteins containing tryptophan because of the destruction of tryptophan by hydrolytic reagents; and

(5) to show that the reagent (HCl/TFA 1:1) is more effective and less destructive than 6M HCl containing 4% mercapto acetic acid.

1.2 LITERATURE REVIEW

Some modern analytical methods that are used for the amino acids are given in Tables I-III under the titles:

(i) Methods of amino acid analysis Table I

(ii) Methods of tryptophan determination in protein, food and feeding-stuffs Table II

(iii) Methods of tryptophan determination in serum and blood plasma Table III.

Two methods are generally used for the analysis of basic amino acids in protein, namely:-

(1) Direct Methods or Isolated Methods: such as enzymic, isotope dilution analysis, hydrolysis with bases and microbiological procedure.

(2) Indirect Methods: such as colorimetry or manometry or spectrophotometry.

The quantitative estimation of tryptophan has usually been carried out by methods that avoid acid hydrolysis such as

(a) The Spectrophotometric method of Goodwin and Morton (1946), fluorimetry, Buttery (1975)

(b) The Colorimetric methods of Spies and Chambers (1949), Miller (1967), and Goswami (1974).

One may say that as a rule, lower results have been obtained by direct than by indirect methods. This inconsistency is found in the methods themselves. The direct methods generally give a minimum figure since it requires a quantitative isolation of pure materials which may be subjected to unrecognised or underestimated losses. On the other hand, the indirect methods usually give a high figure because working losses are generally small and the working conditions can be specific. Anyway, under ideal conditions, the results of the two methods should agree within the limits of error of individual methods neglecting, of course, the chief difficulty such as a clean solution of acids/bases for hydrolysis or precipitating reagents or proper and perfect hydrolytic conditions.

Tristram (1946), Rees (1946), Tristram (1949), Lugg (1938), Tristram and Smith (1963), published articles/critical reviews about the methods of following

liberation and losses of amino acids. Buttery and Cole (1977) also published sources of error in estimating amino acids and microbiological assay.

At the symposium on amino acid analysis in clinical chemistry and medical research held in Edinburgh in June 1979, it was reported by the editor of Protein Evaluation Group, Williams(1979) that the delegates agreed that the main advantage of fluorescent detection over ninhydrin was the greater sensitivity, though it is difficult to measure proline and hydroxyproline with fluorescent reagents (now overcome by oxidation with NaOCl). And that other methods, such as the use of GLC, HPLC and automatic amino acid analyser of amino acids were discussed and most delegates preferred the use of Ion-exchange Chromatography.

TABLE I

(a) Methods of amino acid analysis

1. General: Blackburn 1978; Tristram 1979; Martin and Synge 1945; Evans et al 1978; Nicolet and Shinn 1941a,b; Rees 1946.

2. Gas Chromatography: Desgres et al 1979; Nair 1978.

3. Isotope dilution: Shermin and Foster 1946; Frank et al 1978; Brand and Edsall 1947.

4. Partition Chromatography of Acetylated Amino Acids: Martin and Synge 1941; Tristram 1946; Cannan 1946; Stein 1948· Moore and Stein 1949; Elsden and Synge 1944.

5. Ion-exchange Chromatography: Bech-Anderson et al 1979a,b: Ersser 1979; Mason et al 1979; Rudemo et al 1979; Tajima et al 1978; Welch 1978; Buttery et al 1977; Kraak et al 1977; Taka Hashi 1978; Moore and Stein 1951, 1954, 1958; Eastoe 1961; Hamilton and Anderson 1959; Spachman et al 1958.

6. High Performance Liquid Chromatography: Hancock et al 1979; Lefebure et al 1978: Kroeff et al 1978.

7. Microbiological Assay: Snell 1945, 1946; Shepherd et al 1977: Miller 1967.

8. Chromatography: Cannan 1946.

9. Enzymatic Methods: Archibald 1946· Holz 1972.

10. Electrodialysis of Basic Amino Acids: Albanese 1940; Theorell and Aheson 1942; Macpherson 1946.

TABLE II

(b) Methods of analysis for the determination of tryptophan in protein, food and feeding stuffs

1. Fluorimetry: Steinhart 1978; Escanda et al 1977; Escanda et al 1978; Mason et al 1978; Buttery 1975.

2. Ion-exchange Chromatography: Gaitonde 1979; Adriaens et al 1977; Datta 1977.

3. General Method: Westgarth and Williams 1974.
Silica gel Chromatographic Method: Gordon et al 1943; Tristram 1946.

4. Gas Chromatography: Danielson et al 1978; De Langa et al 1977.

5. Colorimetry: Miller 1967; Holz et al 1972; Spies and Chambers 1948, 1949; Eckert 1943; Barman and Koshland 1967; Goswami 1974.

6. Absorption Spectroscopic Methods: Holiday 1936; Goodwin and Morton 1946; Bencze and Schmid 1957; Edelhoch 1967.

7. Enzymic Hydrolysis: Hill and Konisberg 1962; Nomoto and Muralan 1960; Tower et al 1962; Gall 1945, 1946.

TABLE III

(c) Methods of analysis for the determination of tryptophan in serum plasma

1. Thin Layer Chromatography: Edvinsson et al 1972; Mondino 1969.

2. Ion-exchange Chromatography: Gehrke et al 1973; Felker 1976; Chilcote et al 1972; Berridge et al 1971; Wilkinson et al 1976; Spackman et al 1958; Arala et al 1977; Hashi 1978; Thornber et al 1978.

3. Gas-Chromatography: Gehrhe et al 1973; Felker 1976.
   Colorimetry: Dichman and Crockett 1956.

4. Ultraviolet Spectrophotometry: Hassan 1975; Isumi et al 1976; Wapnir et al 1969.

5. Fluorimetry: Lewis et al 1978; Eftink et al 1976; Peters et al 1970; Mason et al 1978.

6. High-Performance Liquid Chromatography: Krstulovic et al 1977.
   General Method: Wood et al 1977.

1.3. THE OBJECTIVES OF AMINO ACID ANALYSIS

Generally, amino acid analyses as reported in literature can be grouped into three categories.

The first category will be those amino acids analysed for the purpose of assessing the nutritive value of a protein and/or foodstuff, whether they are essential or non-essential amino acids.

The second category is related to those for the purpose of regulating amino acid fractionation in which comparative values and not absolute values are essential, eg. amino acids with specific groupings such as arginine, cystine and tyrosine.

The last group is concerned with the investigation of protein stoichiometry and of correlating physical and chemical properties with amino acid composition.

Consequently, the objectives of analysis are:

(a) to establish the amino acid composition of a protein and hence determine the minimum molecular weight of the protein. And if the molecular weight of a protein is known from the complex amino acid analysis, the number of residues of each amino acid within the molecule can be calculated.

The relationship such as follows can be used:-

(i) $M_o = M_m \times R$

where M_o = Observed molecular weight

M_m = Minimum molecular weight

R = The number of residues of an amino acid in the protein molecule

(ii)/

(ii) Minimal Molecular Weight = $\frac{\text{(amino acid molecular weight) X 100}}{\text{per cent of amino acid residue in protein}}$

(iii) Number of Residues = $\frac{\text{percent of anhydro amino acid} \times \text{Average molecular weight}}{\text{amino acid residue molecular weight}}$

(b) Sequence of amino acid in Protein molecule (Chemical structure of a protein molecule)

The amino acid composition gives an idea about the structure of protein molecules, and the nature of the approach to the structural study will be influenced by the presence or absence of certain amino acids such as tryptophan, cystine and cysteine.

Canfield and Liu (1965) determined the amino acid sequence of lysozyme, so also Nakashima et al (1966) determined the amino acid sequence of Bovine Heart Cytochrome C and hence reported the sequence of amino acid in the protein molecules. The elucidation of the sequence of amino acid residues in peptides isolated by fragmentation of the parent protein is therefore essential in order to avoid unambiguous results.

(c) Distribution of Specific Groupings

In order to understand the chemical, physical and biological properties such as active sites of an enzyme, of a protein, an accurate knowledge of the distribution of certain specific groups such as ionic, groups, polar groups but uncharged and non-polars groups is of great importance.

(d) Establishment of Protein Purity

Analysis of protein must be done on a pure sample of protein. Hence, the amino acid composition of a protein may serve as a check to establish the purity of the protein, because the amino acid composition of the protein in the solid phase should be identical with that of the supernatant solution with which the solid is in equilibrium. Hence, the analyses are use-

ful in evaluating the purity of protein preparation of each step in the Isolation procedure of large proteins when monitored by amino acid analysis, because once a substance is pure, there will be no change in amino acid composition, and this can be expressed in terms of integral numbers of amino acid residues per molecule.

1.4. WHY QUANTITATIVE DETERMINATION OF TRYPTOPHAN?

The quantitative determination of tryptophan content in proteins and serum tryptophan content is very important in protein chemistry because it is useful in the evaluation of the nutritional values of the foods as well as in certain disease states. Patients with breast cancer had been found to have abnormally increased amounts of tryptophan, metabolites in their urine samples. The role of tryptophan has also been implicated in bladder cancer, schizophrenia and Parkinson's disease. McArthur et al (1969), Paoletti et al (1975) and McArthur (1971) have shown that acidic drugs such as salicyclic acid and indomethacin lower the association constant for the tryptophan-protein complex from 1 to 0.0007. Hence, the possibility that anti-rheumatic drugs displace tryptophan from the tryptophan-protein complex. So, they postulated that a decrease in total tryptophan and an increase in the free fraction might be part of the mechanism of action of drugs that would be effective against rheumatoid arthritis.

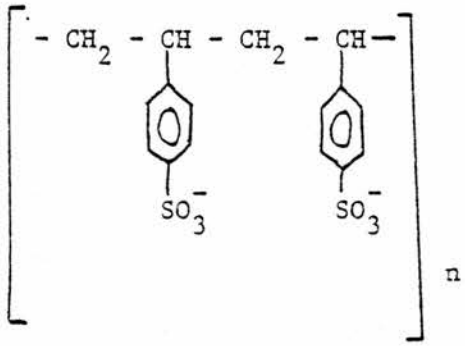
Many researchers are, therefore, still interested in the accurate determination of tryptophan in proteins, feedings stuffs (Agricultural Research Council, Protein Evaluation Group, Williams 1978, 1979) in order to establish more accurate evaluation of nutritional values of the foods with respect to tryptophan and other individual amino acids.

1.5 AUTOMATED ION-EXCHANGE AMINO ACID ANALYZERS

1.5.1 Historical and General: The knowledge of the acid-base properties of amino acids and peptides has been used to design separation procedures by Ion-exchange method in amino acid analysis of proteins.

SM. Partridge and his co-workers developed ion-exchange column chromatography with sulfonated polystyrene resins for separating mixtures of amino acids in protein hydrolyzases. Moore and Stein perfected the technique. The method passed from non-automatic to semi-automatic method (Hamilton and Anderson 1959; Moore and Stein 1951, 1954; Moore et al 1958) and finally to fully automatic procedure (Spachman et al 1958) for amino acid analysis by ion-exchange chromatography.

The amino acid mixture is applied to a column of the resin (sulphonic acid cation exchanger of polystyrene - structure given below -



and then eluted by passing a buffer of specified pH and ionic strength through the column. The positive charges of an amino acid are attracted to the resin by electrostatic forces, and the hydrophobic regions of amino acids interact with the non-polar benzene ring. At any pH, a certain fraction of any amino acid exists in positively charged forms. An amino acid with a higher $[AA^+]$ / $[AA^\circ]$ ratio will move through the column slower than one of equal non-polar character with a lower $[AA^+]$ / $[AA^\circ]$ ratio. In general, the amino acid, with the lower $[AA^+]$ / $[AA^\circ]$ ratio will elute before the one with a higher ratio, if they have equal non-polar attractions for the resin.

1.52 Methodology: In a series of articles published between 1949 and 1958, Spachman, Moore and Stein described analytical conditions suitable for separation of a wide range of amino acid mixtures on heated columns of cation-exchange resin coupled to a quantitative system of a continuous-effluent monitoring.

Modern amino acid analyzers have incorporated autosamplers of different capacities, sophisticated control and data handling systems. But there are certain factors that influence the modern columns and materials used in the design of present day instruments. Some of the factors are (1) the structure of the cation-exchange resin which fills the column, (2) the dimensions of the column, (3) the composition and flow rate of the eluting buffers which resolve the amino acids on their passage through the column and (4) the composition and reaction conditions of the chemical detection systems to give high sensitivity, greater resolution and decreased analysis time.

One of the traditional procedures for the determination of the amino acid content of protein involves the following general sequence:

(a) Hydrolysis of protein

(b) Removal of hydrolytic reagent such as acid under vacuum

(c) Dissolving the sample residue in an acidic buffer solution, and

(d) Chromatographic separation of the resulting amino acids by ion-exchange chromatography (preferred method as reported at Edinburgh Symposium for Protein Evaluation Group, in June 1979).

1.53 Ion-Exchange Resins: The resolving power of ion exchange resins has been studied. The studies involved the size, shape and structure of the polystyrene/divinyl benzene polymer lattice of the particles to which exchangeable sulphonic acid groups are attached (Hamilton 1966). Over the

years, the length of resin columns required for good separations has been reduced from 1.5m to a little over 0.5m, but the 9mm column diameter has remained standard until quite recently, and there are many 9mm columns in daily use all over the world, filled with crushed or spherical resins (20-30μm diameter). Hamilton (1968) showed that large columns of small beads are slow but yield a high resolution of complex mixtures and their more rapid exchange and improved mechanical strength had been argued by Ersser (1976) to be responsible for their relatively high elution flow rates on shorter columns.

The uniform size of resin and especially the degree of cross-linkage of the beads affect the rate of elution and the complexity of the amino acid mixture. Hence, fast chromatography will rely on high quality resins.

1.5.4. Column Elution Conditions: During the analysis of amino acid mixture to acidic, neutral and basic amino acids from the column as compact zones within an acceptable time-scale, the following conditions are necessary:-

(1) changes in pH and/or

(2) cation molarity of eluting buffers, and

(3) changes in column temperature.

Spackman et al (1958) used two sodium citrate buffers to resolve acidic and neutral amino acids on a long column of resin in the sodium form. The first buffer (pH 3.25, 0.2M Na^+) was abruptly replaced by the second (pH 4.25, 0.2M Na^+) at a temperature of 55°C for the amino acid mixtures present in protein hydrolysates. It was, however, suggested that a lower starting temperature (30°C) was superior for the first part of the separation of more complex amino acid mixtures (physiological fluids). A second sample was placed on a shorter column of resin which was eluted with a buffer of higher

sodium molarity (0.5M, pH4.5).

A single column can also be used. The advantage is that one will use less sample and avoid the danger of missing compounds emerging from the column in the area where elution profiles overlapped thus simplifying automation. But there are disadvantages in the rise in baseline, prolonged leaking of the ammonia from the column (ammonia plateau), the effects of high molarity buffers on resin beads yielding uncertain quantitation. Hamilton (1968), Piez and Morris (1960) described two techniques using a three buffer system.

Hare (1972) used a constant molarity buffer system and (Dionex or Pierce) 180-pH buffer systems have been used to improve baseline stability.

These suitable elution buffers concentrates are available commercially (Pierce Chem. Co.). But another method of eliminating the ammonia plateau and to give a rapid resolution of simple mixtures of basic amino acids is to use solely increases in pH rather than molarity as suggested by Ersser (1975). Or better still one can use alkaline buffers containing borate ions either as a gradient system (Murren et al 1975) or as a discrete system (Ersser 1975).

However, the twenty amino acids commonly found in protein hydrolysates can be well resolved by dual or single column techniques as in JLC-SAH with improvement in the speed and sensitivity of the analysis.

For the separation of amino acids in physiological fluids, lithium has replaced sodium as the counter ion. LKB4440 Amino acid analyser uses a single column technique coupled with latest buffer technology and completes a protein hydrolysis analysis in 65 minutes. Physiological fluid analysis takes less than 4½ hours (Lab. Equip. Digest, June 1979).

1.5.5. Elution Analysis: Ion-exchange chromatography depends largely but not entirely on interactions between the fixed charged groups on the ion-exchange resin and the charged groups on the amino acids. The chemical and physical

nature of the resin also has minor but often important but unpredictable effect.

Simply, a protein will stick to a cation exchange resin below its isoelectric point and be released when the pH approaches its isoelectric pH. The protein can also be released by competition from other ions (ie by raising the ionic strength).

The acid hydrolysate is applied to a cation exchange column (ie a column with replaceable positive ions). In acid solutions, the amino acids have a positive charge and, therefore, tend to displace some of the exchangeable cations.

The column is eluted with buffers of successively higher pHs causing the amino acids to move down the column at different rates to be collected in small fractions. The amino acid content of each fraction was automatically quantitatively analysed (ie in an automatic amino acid analyser) and an elution curve plotted. The position (or retention time) of each peak enabled the amount of each amino acid to be determined.

So, ion-exchange chromatography may be regarded as a special type of adsorption chromatography (ie the partition principle has been refined) in which coulombic (electrostatic) forces between charged stationary phase and oppositely charged solute molecules are involved. Partition of solute between mobile and stationary phases is dependent on competition for charged sites on the stationary phase by the solute and other ions in the mobile phase. Hence, ion-exchange chromatography is limited to aqueous solvents in which free ions can exist.

The solphonated polystyrene resin is equilibrated with 0.2M NaOH solution in order to charge its sulphonic acid groups with Na^+ and this is called sodium form of the resin. When this is washed with an acid, it is then protonated (ie in hydrogen form). If amino acid mixture is then added to the hydrogen form of the resin, say at pH 3.0, amino acids are largely cations with net positive charge. So the cationic amino acids tend to displace some of the

bound Na^+ ions from resin particles. The amount varies with differences in degree of ionization. Hence at pH 3.0, the most basic amino acid (tryptophan, lysine, histidine, arginine) are tightest bound to resin while glutamic acid and aspartic acid are least bound due to electrostatic force. As the pH and the Na^+ concentration of eluting aqueous medium are gradually increased, the amino acids move down the column at different rates and analysed by ninhydrin reaction. The most anionic amino acid, eg Lys, appears last.

But in JLC-5AH automatic amino acid analyser with two columns - Short and Long ion - exchange columns - the hydrolyzates of proteins are used and the eluting buffers pH 5.28, pH 4.25, pH 3.25, are forced through the column under pressure and analysed quantitatively after reaction with ninhydrin (see page 18). The order of elution is given below: (see page 18) – FIG B.

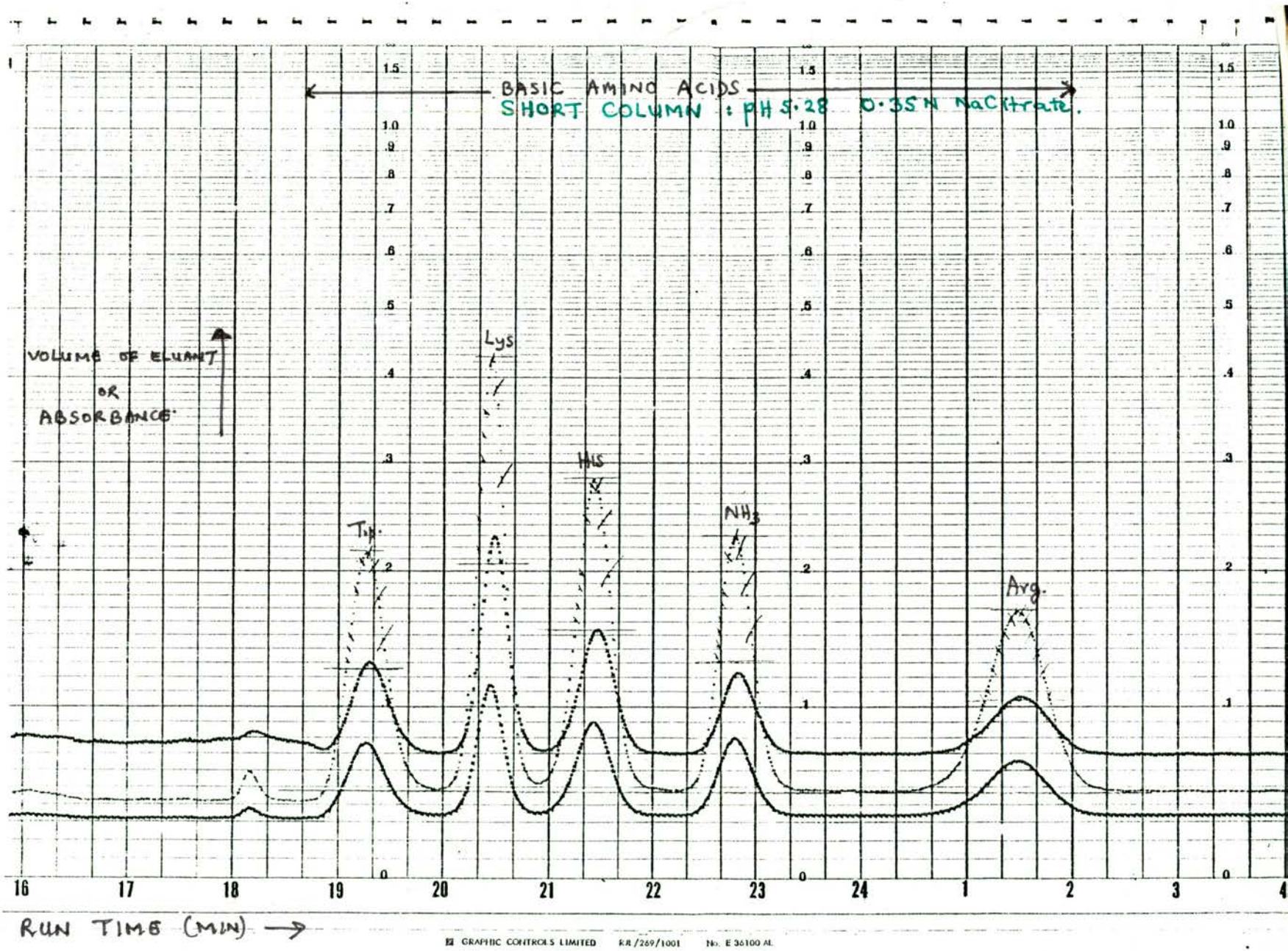
(1) Short Column: 15cm X 0.9cm. Buffer pH 5.25 $\pm$ 0.10, Na^+ 0.35N. Resin LCR1: Tryptophan, Lysine, histidine, ammonia, arginine.

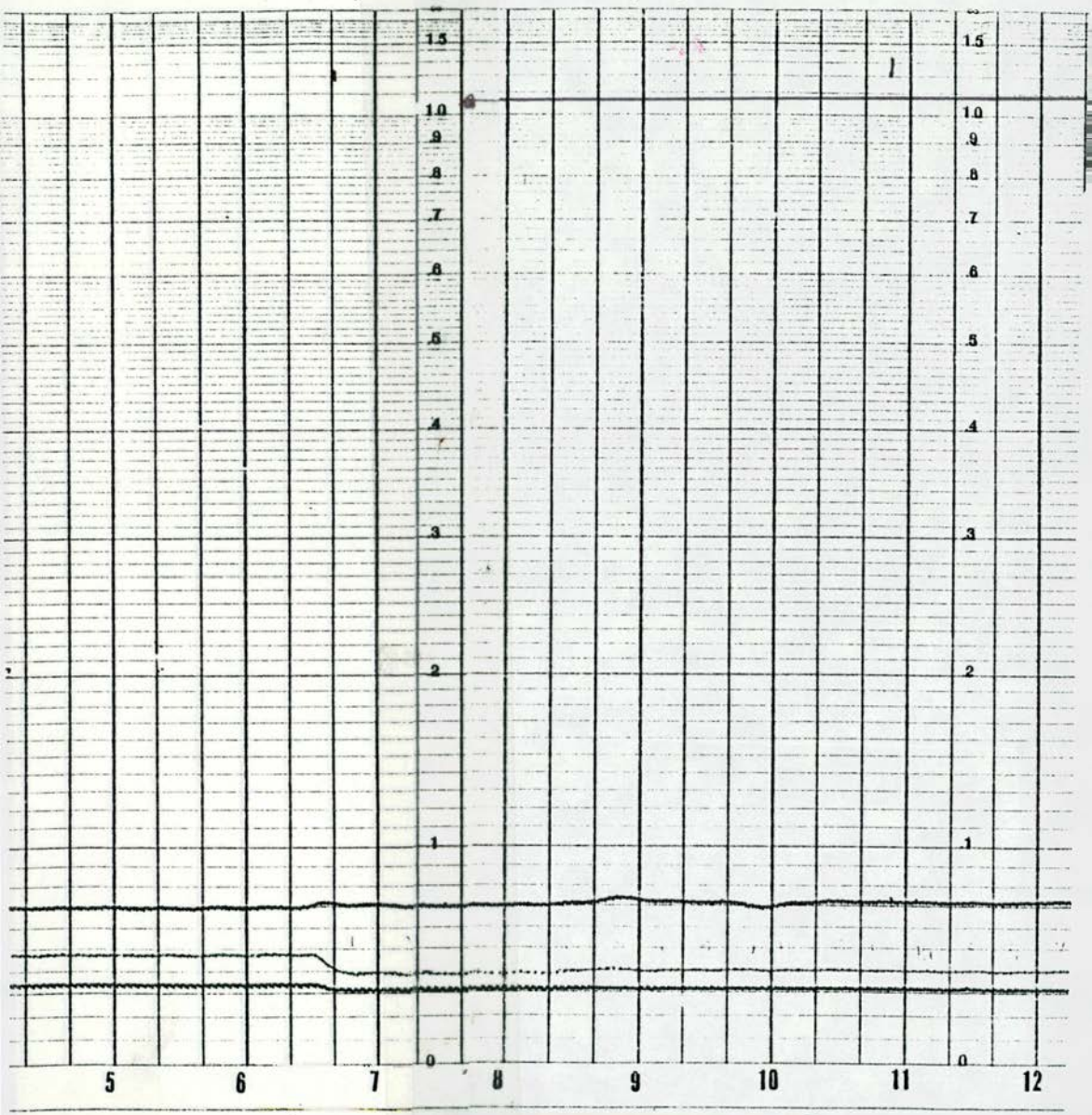
(2) Long Column: 70cm X 0.9cm. Resin LCR1

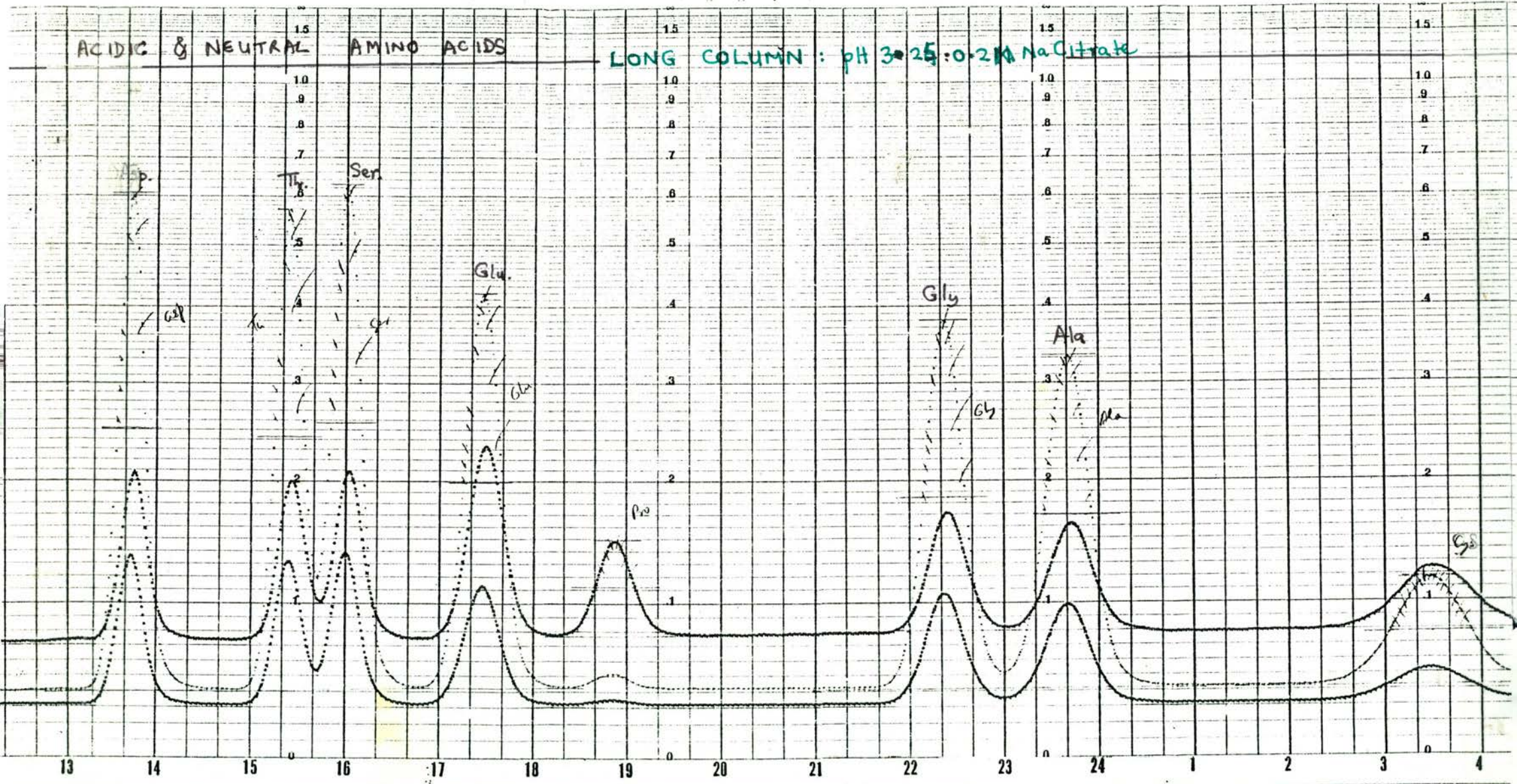
(a) pH 3.25 $\pm$ 0.08, Na^+ 0.2N:- Cysteic Acid, Aspartic Acid, Threonine, Serine, Glutamic Acid, Proline, Glycine, Alanine, Half-Cystine, Valine.

(b) pH 4.25 $\pm$ 0.10, Na^+ 0.2N:- Methionine, Iso-leucine, Leucine, nor-leucine (internal standard), Tyrosine and phenylalanine.

FIGA — ORDER OF ELUTION






ACIDIC & NEUTRAL AMINO ACIDS
LONG COLUMN : pH 3·25 : 0·2M NaCitrate
Asp.
Thr.
Ser.
Glu.
Pro
Gly
Ala
Cys
13
14
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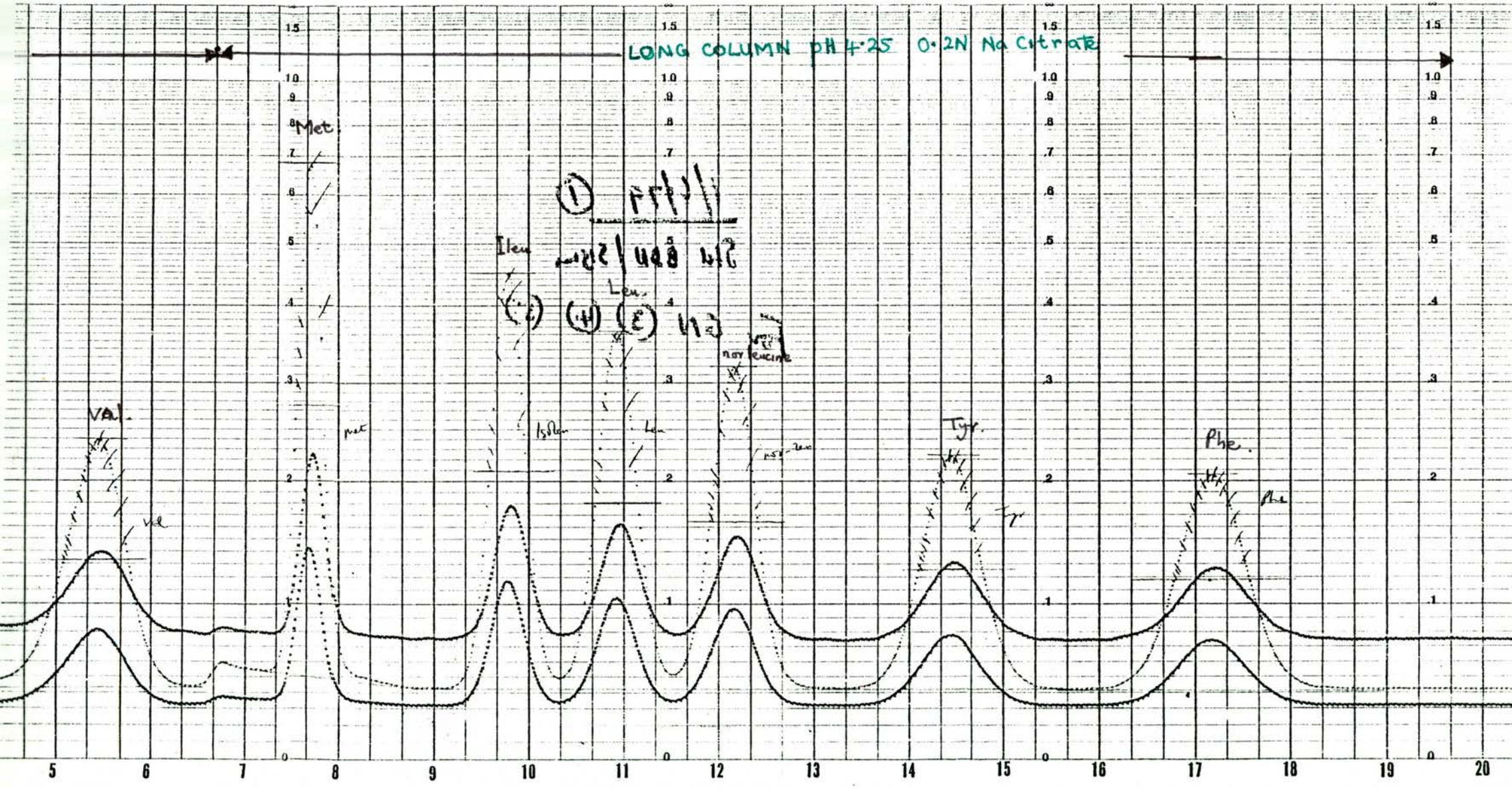


JLC-5AH FULL-AUTOMATIC AMINO ACID ANALYZER.

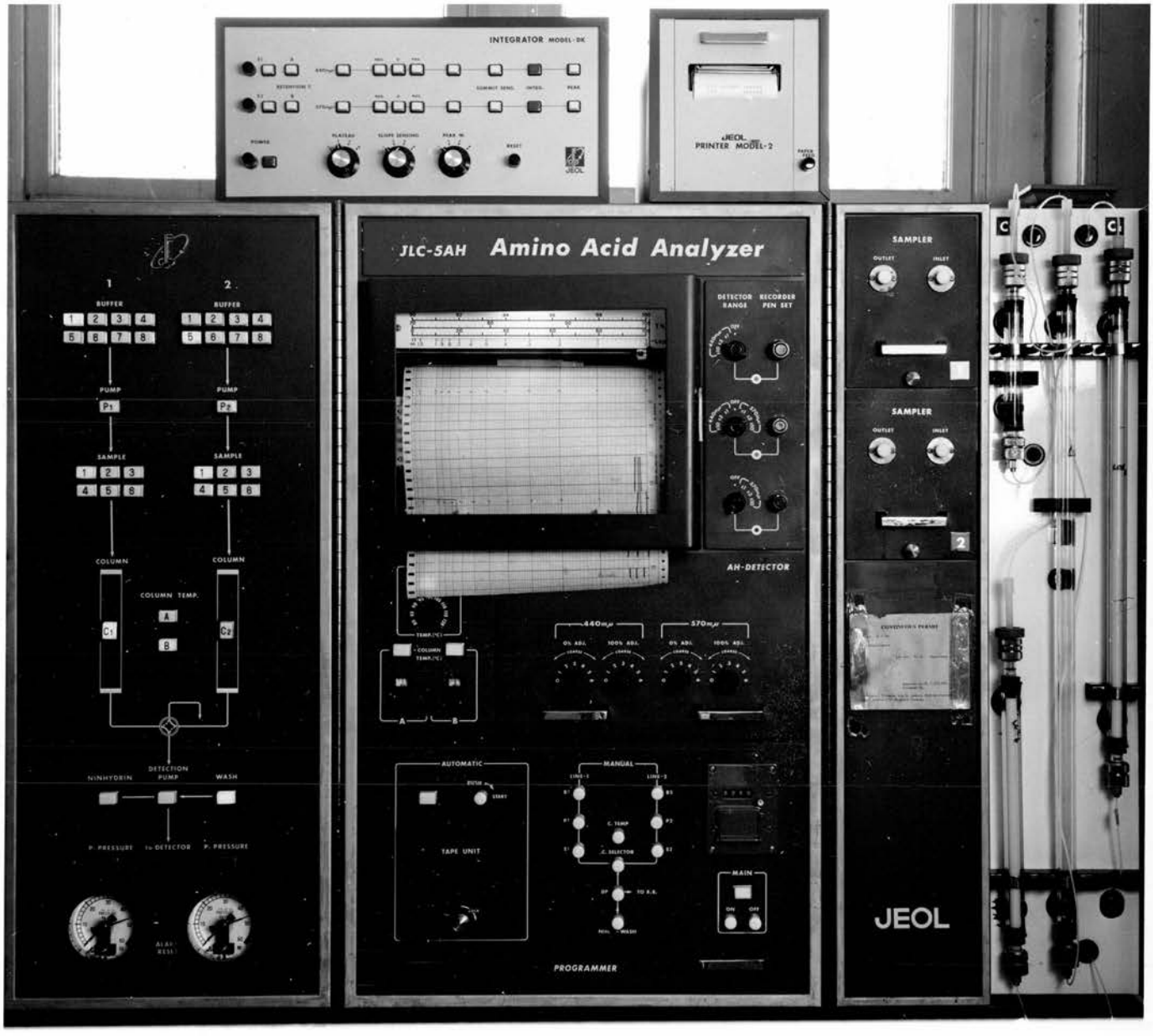


FIG I

1.6.4 JLC-5AH FULL-AUTOMATIC AMINO ACID ANALYZER

See Fig. 1: Block Diagram, and Fig. 2: Flow Diagram.

The operating parameters for optimal chromatographic performance are:

(1) Column Temperature

(2) Buffer pH, and

(3) Salt concentration.

Increases in buffer pH or ionic strength (Na or Li ion concentration) or an increase in temperature will accelerate amino acid elution from the Column. Conversely, a decrease in buffer pH or lowering of column temperature will delay peaks on the chromatogram.

The problems of dissimilarity of peak may be due to

(i) Incorrect buffer installation or connection

(ii) Contamination of resin

(iii) Incorrect buffer pH

(iv) Ninhydrin - usage and age

(v) Column temperature.

In general, the following effects could be noticed:

1.6.4.(I) (a) Effect of variations in Resin: The resin with the highest degree of cross-linking compatible with the separation to be achieved must be used. Joel 5AH amino acid analyzer employed the use of Joel resin LC-R-1 for Short and Long Columns for non-physiological analysis.

(b) Height of Resin in the Column: The height of resin in the column also affects the separation of amino acids. It was observed that in order to obtain good separation on the short column a height of 13.5cm is desirable, if tryptophan is to be fully recovered. The height of resin as well as pH of buffer solution in the Long Column also affect the separation of serine and threonine, while the pH of buffer solution only affects the separation of

FIG 2.

FLOW LINE OF JLC-SAH

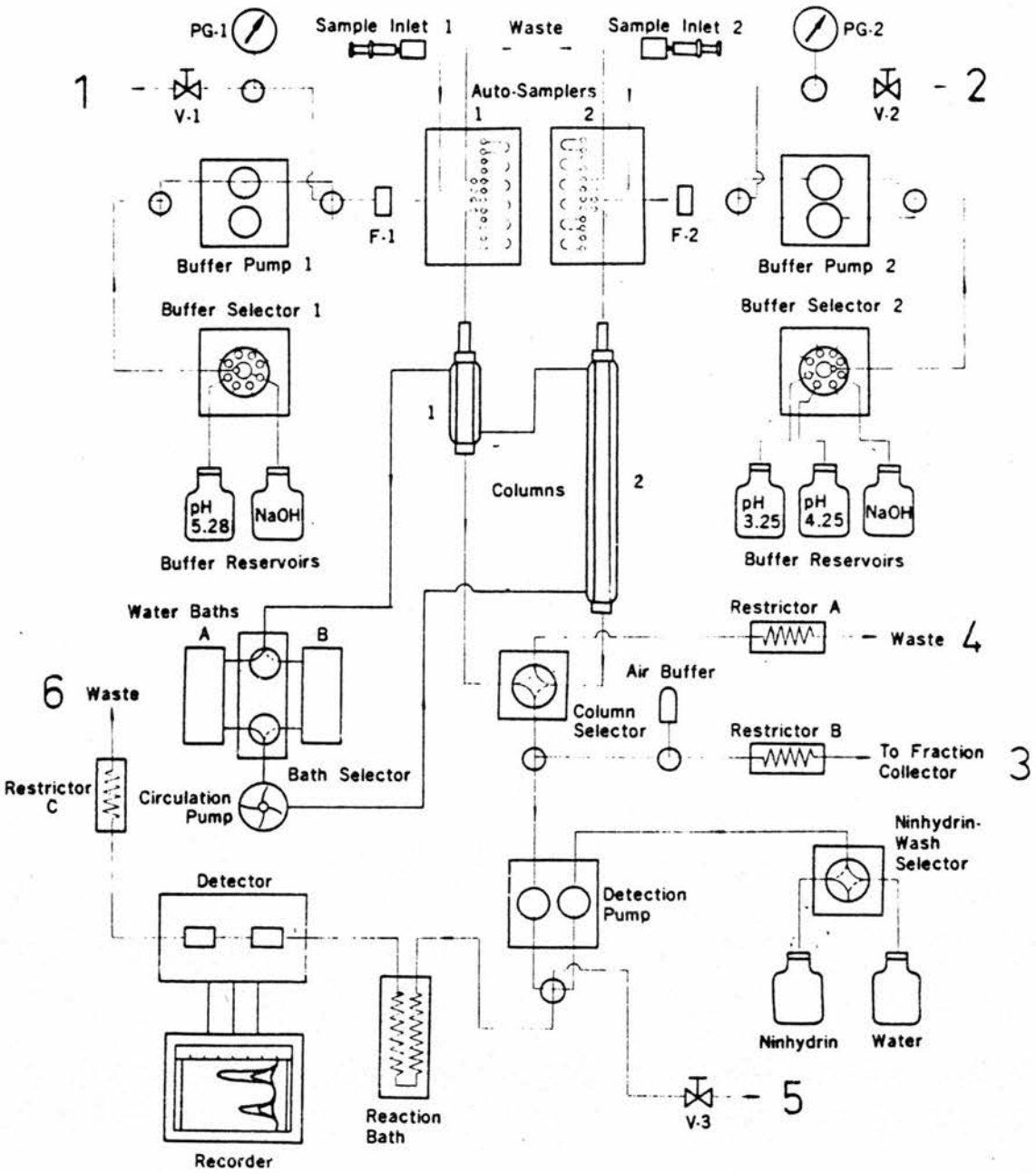


FIG. 2.

cystein and hydroxy proline.

Re-packing the column is essential when the peak profiles becomes smaller especially standard peaks of trp and proline

1.6.4 (II) Effects of variations in Eluant:

These effects are:-

(a) pH of column eluant - It has been found that low pH values of eluting buffers decrease the rate of travel of certain amino acids while high pH values increase the rate of travel of certain amino acids. For example, aspartic acid, glutamic acid, cystine and proline are relatively the most sensitive to pH when buffers between pH3 to 5 are employed.

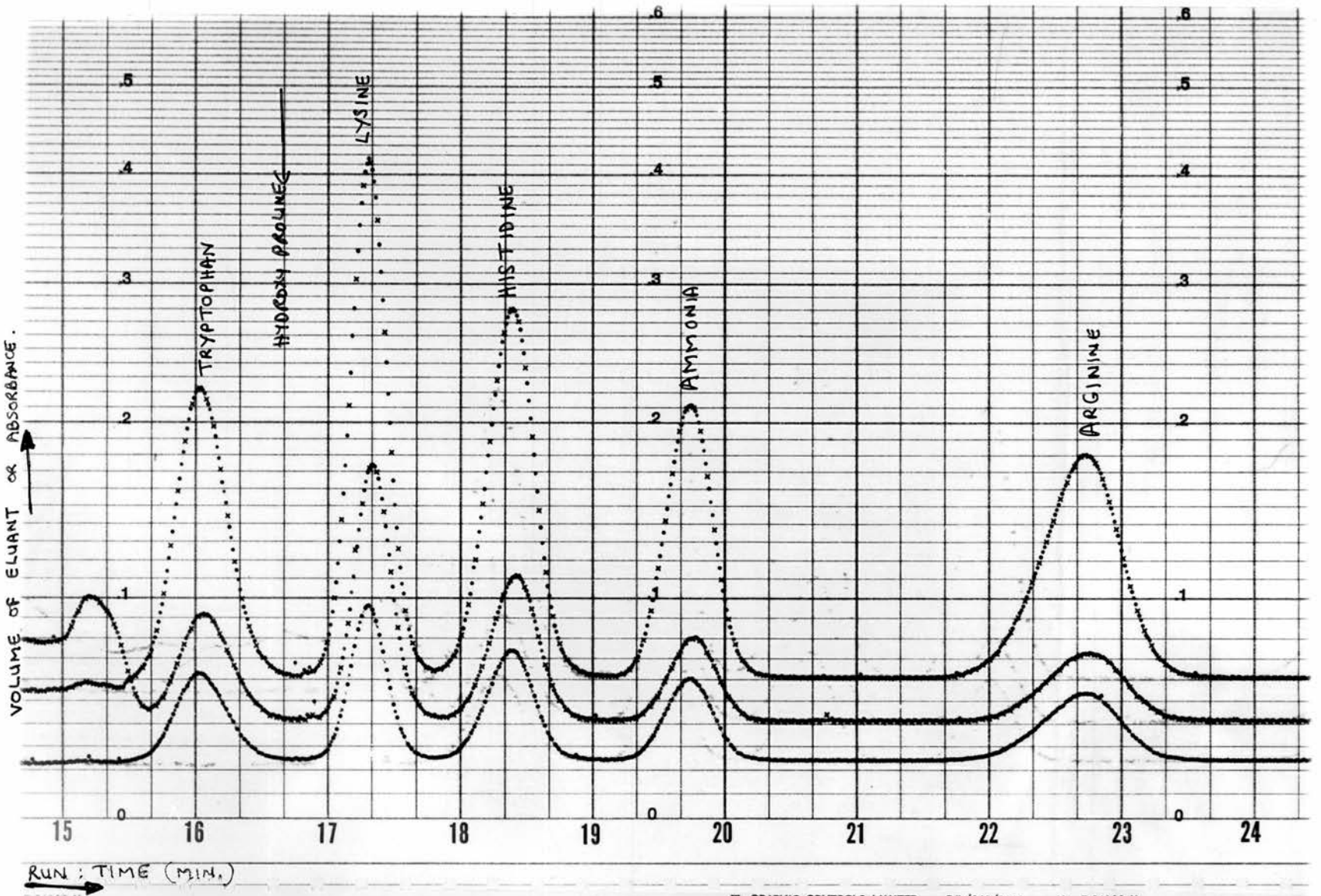
(b) Ionic strength - By raising the Na^+ concentration (that is, citrate buffer molar variation), changes are produced which are similar to those induced by raising the pH.

(c) Added organic solvents to column buffers - The property of eluant can be changed by addition of organic solvents such as

(i) Propanol: Hubbard (1965) modified sodium citrate buffer pH5.25 $\pm$ 0.02 for basic amino acids eluted by adding Na^+ 0.35M and 5% ($^{v}/_{v}$) propanol to the buffer used in Beckman Amino Acid Analyzer for separation of tryptophan. He reported that propanol in the buffer increased peak sharpness of the basic amino acids and moved arginine peak closer to ammonia, and tryptophan forward (ie away from lysine). Further increase in propanol accentuates this peak movement as the concentration is increased to the 10% level. He argued further that the use of propanol and citric acid buffer mixtures keep for a long period (about 6 months) and that, there was no ion-exchange column degradation in the analyzer for periods as great as 3 months.

(ii) Methanol: The use of methanol in citrate buffers (Thompson and Miles, 1964) produced no improvement in resolution but caused a retardation of peaks. As the methanol content was increased from the 5% to 100%, retardation

FIG 3



BASIC AMINO ACIDS ANALYSIS : SHORT COLUMN : PROTEIN HYDROLYZATE

of the peaks became worse. Production of gas bubbles in the analyzer was observed as a result of the low boiling point of methanol. It was decided that 7% $^{v}/_{v}$ methanol added to pH 3.25 $\pm$ 0.08 would give the best resolution.

(iii) Benzyl alcohol: Moore and Stein (1951) and Hubbard (1951) added 0.5% benzyl alcohol to sodium citrate buffer, although the resolution of tryptophan peak was restored, the peaks were not sharply defined. However, addition of 1% $^{v}/_{v}$ benzyl alcohol to sodium citrate buffer pH 5.28 $\pm$ 0.1 in the eluant has been found to (a) have a sharpening effect on the peaks of aromatic amino acids and allow tryptophan peak to emerge before others, (b) tends to increase their rates of travel relative to others.
For example, 1% $^{v}/_{v}$ benzyl alcohol added to sodium citrate buffer will not show the hydroxy-lysine peak, but addition of 2% $^{v}/_{v}$ benzyl alcohol will produce hydroxy lysine peak after tryptophan.

So, in general, organic solvents accelerate preferentially the amino acids possessing the long non-polar side chains, thus, in effect, tending to reverse the normal order of emergence for glycine through leucine groups of amino acids.

The order of elution of the amino acids are:

Tryptophan pH 5.28 $\pm$ 0.1 (1% $^{v}/_{v}$ or 2% $^{v}/_{v}$ Benzyl alcohol)
Hydroxy-lysine
Lysine
Histidine
Ammonia
Arginine

See Partial elution diagram attached. — FIG. 3.

(d) Effect of temperature on the column: Column temperature - In general, increasing the temperature of the citrate-buffered columns increases the rate

of movement of all the amino acids. Hence, Moore (1951) reported that tyrosine and phenyl-alanine were completely resolved at 60°C whereas at 25°C and 37°C they emerge together.

1.6.4(III) Length of Column

In JEOL JLC-5AH full automated amino acid analyzer, there are two columns - Short Column: length 15cm X 0.9cm and Long Column: length 70cm X 0.9cm. The short column is used for analysis of amino acid mixture containing tryptophan, lysine, histidine, ammonia and arginine, and the long column for separation of the mixture of cysteic acid, aspartic acid, theronine, serine, glutamic acid, proline, glycine, alanine, valine (buffer solution pH 3.25 ± 0.08, $0.2Na^+$ and methionine, iso-leucine, leucine, nor-leucine (internal standard) tyrosine and phenylalanine (buffer solution 4.25 ± 0.10, 0.2N Na^+).

Table 5 shows the effect of the length of the short column upon the recovery of tryptophan.

1.6.4(IV) Thiodiglycol in Column Buffers

The presence of thiodiglycol in column buffers as an anti-oxidant (or of versene to remove traces of metal ions) helps to protect the sulphur completely from oxidation or of slight impurities in the sample. Example of this is, methionine being protected by thiodiglycol. It has no effect upon the recovery of other amino acids.

Summary of principle:

Separations attained can, therefore, be ascribed to:

(i) differences in the ionic natures of the amino acids (this occurs in most sulphonated polystyrene resins but not in Dowex 50).

(ii) the rate of travel on a column (eg of a sulphonated polystyrene resin) is a function both of the charge possessed by an amino acid and the nature of its side chain, and

(iii) it is a result of the affinity of the resin for both the ionic and non-

ionic portions of the molecule.

It was noted, however, that the rate of the exchange of amino acid ion between solution and resin is probably increased at higher temperatures. For example, tryptophan cannot be separated completely at a temperature lower than 55°/60°C using JEOL JLC-5AH amino acid analyzer. So, faster rates of solvent flow can also be used under certain conditions without a concomitant broadening of the amino acid peaks.

1.6.4(v) Optimising Detection Reagents

The most widely employed detection system, is based on the reaction of amino acids in column effluent with a heavily buffered solution (pH 5.5) of ninhydrin in the presence of reducing agents such as stannous chloride (now being replaced by titanous chloride: James, 1971), which does not cause precipitation in flow lines or hydrindantin added directly. The absorbance of the resulting complex (Ruthmann's purple) is measured at a wavelength of 570nm (440nm for the yellow product with proline line). Elias and Garcia (1966) showed that a wavelength of 410nm all amino acid/ninhydrin adducts could be detected. Ninhydrin reacts with a wide range of amino compounds but at a slow rate, even when heated (95°C) and oxygen excluded from the system. The ninhydrin was dissolved in a mixture of sodium acetate buffer and 2-methoxy ethanol (b.pt 124°C) (which is to keep hydrindantin - i.e. reduced ninhydrin - in solution) or ethylene glycol (b.pt 196°C less volatile and less toxic). Moore (1968) recommended an alternative mixture of dimethyl sulphoxide, lithium acetate and addition of hydrindantin for increased reagent stability.

1.6.4.5 PURITY OF SAMPLES OF REAGENTS

Modern amino acid analysis requires high purity reagents, and chemical companies such as Sigma, BDH, etc, provide suitable tested chemicals, solutions and calibration standards.

Modern techniques also provide a method of checking the purity of samples obtained after extraction or preparations, etc.

1.6.4.6 Qualitative Analysis

The position (i.e. retention time) of a peak is used to identify the substances separated. In the JEOL 5AH analyzer, the short column is used for the basic amino acids (pH 5.28 ± 0.10) and the long column for acidic and neutral amino acids pH 3.25 ± 0.08 and pH 4.25 ± 0.10 buffers respectively. The peaks of an unknown component are identified by comparing the chromatogram with that of the standard sample eluted under the same conditions.

1.6.4.7 Quantitative Analysis

Since the quantity of each component of amino acid in a sample is proportional to the peak areas of the chromatogram, quantitative analysis is carried out by comparing the peak areas of chromatogram of standard samples which have been weighed or prepared as amino acid standards.

Generally, for measuring the peak areas, one can use either of the following methods:-

(i) Absorbance method which accumulates absorbance dots at either 570mμ for most amino acids and 440mμ for proline.

Or (ii) HW method, which is calculating the product HW of the peak height (H)

and the peak width W (half-width). The product (HW) is proportional to the peak area.

1.6.4.8. Calculation by HW method:

(i) The procedure for calculating HW using Joel Ruler is used for absorbance characteristics of amino acid ninhydrin colouration at 570mμ and 440mμ for proline.

(ii) HWs with respect to all amino acids and nor-leucine (internal standard) in the standard sample (0.1μm/ml) and in unknown sample are calculated:

$$\text{Ratio} = \frac{\text{HW of each amino acid component}}{\text{HW nor-leucine}}$$

for Standard sample and unknown sample

from this the nanomoles of each amino acid is calculated which is equal to

$$\frac{\text{Ratio of unknown amino acid}}{\text{Ratio of Standard amino acid}} \times 80$$

Since the sampler capacity = $0.8cm^3$ and $0.1\mu Mole/cm^3$ of internal standard nor-leucine is equivalent to 80 nanomoles. The number of moles in $1cm^3$ can be calculated from the colour yield.

Hence, the percent of the anhydro amino acid and the number of Residues per molecule can be calculated (ie no. of Residues of amino acid =

$$\frac{\text{percent of anhydro amino acid}}{\text{mol.wt. of anhydro amino acid}} \times \text{Min. mol.wt. of protein}$$

1.6.4.9. Compensation for sample pipe capacity

The compensation coefficient for the pipe capacity is assumed to be constant and may be determined prior to some of the analysis.

1.7 The Precision of the Method

Tristram 1949, 1947 and other co-workers suggested criteria for the establishment of accuracy and specificity of numerous methods based upon different principles of amino acid analysis. The suggested criteria are

(1) The analysis of ad hoc mixtures of amino acids simulating the protein hydrolyzate under examination,

(2) The comparison of the values obtained by the various methods of analysis, and

(3) Any method of analysis should be capable of indicating the presence of unknown amino acids or alternatively, that prior to the analysis of any protein, the amino acid constituents should be characterised by some general qualitative method.

The precision of ion-exchange method (automatic or non-automatic) in amino acid analysis of proteins was established by series of experiments performed by Moore and Stein (1951, 1954b); Spachman et al (1958); and Hamilton and Anderson (1959). Tristram and Smith (1963) published a critical review of the precision of the ion-exchange methods of amino acid analysis but failed to report about the accuracy of tryptophan content of the proteins examined. But with the tremendous progress made in the resolving power of ion-exchange resins, optimum column elution conditions, optimum detection reagents, high purity reagents, suitable tested chemicals, internal standard solutions, calibration standards and integrator for working out peak areas, the precision of the method can be regarded as very good and reasonably accurate results can be obtained by the method. The testimony for the use of this method was given at the Symposium of Protein Evaluation Group held in Edinburgh in June 1979 as reported by the editor, Williams (Sept 1979) thus: "... most people would still use IEC". (IEC = Ion-exchange chromatography.)

2. GENERAL METHODS OF HYDROLYSIS OF PROTEINS

2.1. Introduction

Although the precision of analytical methods for the determination of amino acids has increased, the limiting factor in obtaining the highest degree of accuracy of the amino acid composition of a protein as a true reflection of the composition of the parent protein is still the problem of hydrolysis as determined from the composition of the hydrolyzate. The problems are (1) destruction or decomposition of certain labile amino acids during hydrolysis; (2) conditions for perfect hydrolytic procedures as designed by Kimmel et al (1959) and Mahowald et al (1962); (3) period of hydrolysis (ie Hydrolysis time) in order to have complete hydrolysis eg. isoleucine was found to hydrolyse slowly in ribonuclease because of the presence of an 180 leucyl-isoleucyl sequence (Hirs et al 1956). Tristram and Smith (1963) suggested hydrolysis time of 20, 40, 70 and 140 hr; and (4) then the amino acids known to be unstable are estimated by extrapolation to zero time of hydrolysis, Tristram and Smith (1963) then suggested that the losses of certain amino acids might be a function of the amino acid composition and sequence.

However, the general methods that are used for the hydrolysis of proteins are:

(1) Acid Hydrolysis

(2) Alkaline Hydrolysis

(3) Enzymatic Hydrolysis.

2.2. Acid Hydrolysis: Chibnall and his co-workers (Macpherson 1946) used 11M HCl to dissolve the protein by heating it in a water-bath and then diluted the solution with water to 6M for carrying out hydrolysis under reflux for 24 hours. Linderstrom-Lang (1952) reported that losses of amino acids obtained

during hydrolysis by Chibnall conditions could be reduced by using large excess of 6M HCl prepared by distillation in an all-glass apparatus. Smith and Stockell (1954) also hydrolysed crystalline carboxy-peptidase with large excess of 6M HCl (obtained after re-distillation three times in glass) in sealed pyrex tubes at 105°C. Other workers had to evacuate the hydrolysis tubes to get rid of residual air before sealing the tube under vacuum and remove excess HCl by repeated evaporation in vacuo at 40°-50°C. Moore and Stein (1963) carried out hydrolysis of protein (5mg) with 1ml of 6M HCl at 110° ± 1. for 20 hours or 70 hours in a sealed heavy walled pyrex test tube having air-dried or lyophilized protein to be hydrolysed and noted that tryptophan presented a special problem because it is markedly labile during hydrolysis. Matsubara and Sasaki (1969) reported a new method for acid hydrolysis of protein (0.1-0.2mg) by using 6M HCl (0.2cm^3) containing 0.5-6% $^v/_v$ mercapto-ethanoic acid (Thioglycolic acid) in an evacuated sealed tube (25 to 50 microns) for 24 to 64 hours at 108-110°C. The hydrolysates were dried in a rotary evaporator at 60°C within 15-20 minutes. The recovery of tryptophan was reported as 84-91% (against 60% recovery without mercaptoethanoic acid) by using mercaproethanoic acid (thioglycolic acid) to prevent the destruction of tryptophan. Blake and Liu (1968) also used β-mercaptoethanol to protect tryptophan from destruction during the synthesis of a tryptophan peptide. Liu and Chang (1971) carried out hydrolysis of protein (2 to 3mg) in evacuated sealed tubes at 110°C for 22, 48, and 72 hours using 1cm^3 3N 4-toluene-sulphonic acid containing 0.2% 3-(2-amino ethyl) indole as scavanger for tryptophan. The solvent was not removed before the hydrolysate was placed on the ion-exchange column. Liu and Chang (1971) reported the recovery of tryptophan to be 90 ± 3% in a 22-hour hydrolysate. The yield when extrapolated to zero time hydrolysis was about 98-88% for different proteins. Borders et al (1972) reported that tryptophan modified with dimethyl-(2-hydroxy-5-nitrobenzyl) sulphonium bromide was stable to conditions of acid hydrolysis with 3N-4-toluene-sulphonic acid. The recovery was not, however, reported.

Spitz (1973) developed a method based on partial neutralization of 6M HCl instead of removing the excess solvent after hydrolysis, he used NaOH solution, which he referred to as "buffer-neutralizer solution". However, the recovery of tryptophan was only 80%.

Ewart (1977) measured tryptophan content on the analyzer after hydrolysis of 0.5mg of protein with 4M methane - sulphonic acid, 0.2% in 3-(2-amino ethyl) indole ($0.1cm^3$) in a sealed evacuated tube for 23 hr at 110°C. The hydrolysate was neutralized with $0.18cm^3$ of 2M NaOH solution, and after adding $0.8cm^3$ of pH 2.5 citrate buffer. The recovery of tryptophan was omitted from the report.

In the present work, it is intended to use a modified work based on Matsubara and Sasaki (1969).

2. 3. Alkaline Hydrolysis: Tristram (1946), Tristram and Smith (1963) reported that certain amino acids were more stable to alkaline hydrolysis than to acids and that tryptophan should be determined on independent samples of the protein after hydrolysis with alkali. The chief problems of alkaline hydrolysis is racemization of amino acids, eg tryptophan, destruction of cysteine, serine and threonine when boiled with strong NaOH, and the neutralisation of the alkali after hydrolysis. Noltmann et al (1962) used 4N $Ba(OH)_2$ at 110°C to hydrolyse ATP transphosphorylases and obtained complete hydrolysis with respect to tryptophan after 50-70 hr.

Dreze and Reith (1956) used the method of Moore and Stein (1949) for the quantitative determination of tryptophan under various conditions of protein hydrolysis, and found a considerable destruction of tryptophan (0.01mM) when heated in a sealed glass tube (20hr, 120°C) in NaOH (5M, $5cm^3$) solution. Dreze and Reith (1949) also reported that the destruction of tryptophan could be prevented by the addition of a reducing agent such as stanuous chloride ($5\%^{w}/_{v}$). They recommended the hydrolytic conditions (100mg protein, 15ml 6N $Ba(OH)_2$, 500mg starch) to be more suitable than (100mg protein, 5ml 5N NaOH). Miller (1967) reported (microbiological assay) hydrolytic conditions (0.2 -

2g crude protein, 15.4g of powdered $Ba(OH)_2$, .8 H_2O; 9ml water, autoclaved at 1516/in^2 for 7 hr) and the precipitation of barium sulphate from an acid solution and colorimetric analysis using 4-dimethyl-amino-benzaldehyde for the determination of the tryptophan content of feedingstuffs with particular reference to cereals. Goswami (1974) used hydrolytic conditions (100mg protein, 10cm^3 of 5M NaOH solm. at 110°C for 18 hrs in a sealed tube) for the determination of tryptophan and Indole substances by colorimetric diazotisation method (N-1-naphthyl-ethylene-diamine-dihydrochloride / HNO_2). He reported recovery of 97-102%.

Datta (1977) carried out alkaline hydrolysis (100mg finely ground, defatted sample, 400mg acid-washed starch and 1.5g $Ba(OH)_2$.$8H_2O$ in 50ml flask made of alkali-resistant glass) in his method for the microdetermination of tryptophan in compounded poultry rations using N-1-naphthyl 2-ethyl-enediamine (NED) for the quantitative estimation of tryptophan in purified feed hydrolysate at 530nm.

2.4 Enzymatic Hydrolysis: Hill and Schmidt (1962) carried out complete enzymatic hydrolysis of the proteins, corticotropin, carboxypeptidase, enolase, papain and ribonuclease with papain, leucine, aminopeptidase and prolidase, and recommended that enzymatic hydrolysis despite its advantages, should not replace acid hydrolysis in protein analysis.

2.5. SOURCES OF ERROR AND CORRECTION FOR THE DESTRUCTION OF AMINO ACIDS DURING HYDROLYSIS

Three possible sources of variation in amino acid analysis are:

(i) the purity of the sample

(ii) the hydrolytic procedure, and

(iii) the accuracy of the chromatographic analysis itself.

(i) and (iii) had already been discussed. However, Lugg (1938) considered the sources of error in the estimation of tyrosine and tryptophan in complex materials when subjected to hydrolysis and put them into three classes, namely:

(a) incomplete liberation of amino acids from the original sample by the hydrolytic reagent

(b) destruction or modification of the amino acids by the hydrolysing reagent or by other substances during liberation, and

(c) destruction or modification of the amino acids subsequent to their liberation. Lugg, therefore, recommended different methods to be used to eliminate these errors. But still loss of tryptophan, serine and threonine, by destruction of different hydrolytic reagents is reported frequently in the literature.

Moore and Stein (1963) suggested that to obtain more accurate figures, the results of a companion hydrolysate heated for 70 hours should be made. They found that the decomposition of threonine, cystine, and tyrosine was about 50% in 20 hours and that of serine was about 10%. Hence, they reported that equation:

$$\log A_o = \left(\frac{t_2}{t_2-t_1}\right) \log A_1 - \left(\frac{t_1}{t_2-t_1}\right) \log A_2$$

where A_1, A_2 and A_o are the quantity of amino acids present after t_1, t_2 and zero hours of hydrolysis respectively, could be applied by assuming (a) first-order kinetics and (b) rate of decomposition of free amino acids may differ from one protein sample to another as well as from one laboratory to another. Or by plotting the values obtained during hydrolysis of the labile amino acids at the two times of hydrolysis (20°C and 70°C) and extrapolate this to zero time. For amino acids that are liberated unusually slowly by acid hydrolysis it was suggested that a longer time of hydrolysis would be necessary in order to obtain accurate values.

Tristram and Smith (1963) in their critical review of the methods of following liberation and losses of amino acids agreed with the view expressed by Moore and Stein (1963) and suggested 70hr or 140hr hydrolysis for amino acids which were difficult to liberate and that where there is no evidence of destruction a mean or maximal value could be obtained after different periods of hydrolysis. It was reported that protein which contains carbohydrase may accelerate the decomposition of amino acids and affect the recovery of amino acids, hence the precaution stated above was necessary.

2.6. THE IDEAL ANALYSIS

Tristram and Smith (1963) pointed out that the accuracy with which the amino acid composition may be determined is

(1) the precision with which the free amino acids may be determined in the hydrolysate

(2) difficulties associated with protein hydrolysis such as (a) the resistance of certain peptides (eg those with isoleucine and valine) to hydrolysis with respect to period of complex hydrolysis of all proteins, and (b) the destruction of amino acids during protein hydrolysis.

Hence, Tristram and Smith (1963) suggested that to obtain a satisfactory amino acid analysis of a protein by the ion-exchange method, one must satisfy the following criteria - I quote:

> "(a) The ion-exchange analyzer should be carefully calibrated.
> (b) The weight of hydrolysate and the amount of N, determined analytically, applied to the column should be compared, respectively, with the weight of anhydroamino acids recovered from the column and with their total, calculated, N content.
> (c) Acid hydrolysis of the protein should be carried out for periods of 20, 40, 70, and 140 hrs., and the analytical results averaged

or extrapolated to provide the best results for amino acids which are either labile or difficult to hydrolyze.
(d) Independent analyses should be carried out for tryptophan, cystine/cysteine, and for amide N."

The above criteria would be followed in the work carried out except that the JEOL-5AH automatic amino acid analyzer had been calibrated and one has to work with standards, the amount of N would not be determined, and the period of hydrolysis would be 24, 36 and 48 hours.

3. COLORIMETRY

(Specific Analytical Reactions for Tryptophan)

3.1. INTRODUCTION

The indole nucleus of tryptophan residue can undergo colour-forming reactions with certain numbers of chemical reagents such as

(i) oxidizing agents like Iron III chloride or bromide, Copper II sulphate, $NaNO_2$ or sodium - hypochlorite in acidic solution.

(ii) KNO_3 and an aliphatic or aromatic aldehyde in concentrated HCl or H_2SO_4. Or

(iii) certain aromatic aldehydes like 4-dimethyl-amino-benzaldehyde (DMAB), 4-nitro-benzaldehyde or vanillin in 10% H_2SO_4 containing concentrated HCl.

Hence, the colour intensities developed by such reactions led to quantative determination of amino acid present. So, tryptophan whether in peptide linkage or free gives coloured products with a number of reagents such as N-bromosuccinimide, 4-toluene-sulphonic acid, 4-dimethyl-amino-benzeldehyde, etc. Such coloured products have formed the basis of photometric measurements of tryptophan in soluble or intact proteins.

3.2. LITERATURE REVIEW

The loss of tryptophan by destruction of different hydrolytic reagents as frequently reported in the literature led to chemical methods of measuring

trytophan in the intanct protein.

Spies and Chambers (1948) allowed tryptophan to react with the Ehrlich reagent (4-dimethyl-amino-benzaldehyde) in acid media (H_2SO_4) to form a blue colour after oxidation with sodium nitrite. This procedure can be used directly on peptides and proteins and is suitable in the range of 5-120µg of tryptophan (Spies and Chambers, 1949). The presence of carbohydrate as glucose or fructose in 50-fold excess over the quantity of tryptophan had no influence on the tryptophan determination. The method of Spies and Chambers (1949) was probably the most reliable procedure at that time for the analysis of tryptophan content of proteins, but it does have the disadvantage of showing a variation of the absorption maximum for different proteins (from a normal value of 590-600mµ to 545-600mµ). Harrison and Hofman (1961) eliminated the variations among proteins by applying the Spies and Chambers method to a partial digest of the protein obtained by the action of trypsin and chymotrypsin.

Tryptophan, in the range of 10-80 µ g, was determined after reaction with xanthydrol in hot concentrated HCl with a standard error of 2% (Dickman and Westcott, 1954; Dickman and Crockett, 1956). The product, xanthydrol-tryptophan, has a purple colour, and the absorbance is measured at 510mµ. Tryptophan in proteins reacts with xanthydrol, but the time of reaction must be increased to obtain maximum tryptophan values. This procedure has received limited application because under these conditions a partial reaction with tyrosine occurs, and a correction must be applied, by measurement of the absorbancy of the solution at two wavelengths.

Goswami (1974) developed a colorimetric method for estimating tryptophan at absorbance 530nm in protein hydrolysates by its conversion into nitrosamine with nitrous acid followed by diazotisation with N-1-naphthyl-ethylene-diamine dihydrochloride (NED). He reported a recovery of 97 to 102 per cent. Datta (1977) used this coloured reaction on samples hydrolysed with $Ba(OH)_2$ and modified Miller method (1967) - using DMAB and measuring the absorbance at 530mn - to

estimate tryptophan in compounded poultry rations because he argued that Miller did not separate soluble carbohydrates which interfered. Hence, Datta used a Dowex-50 column to separate various interfering component and this as reported might have led to the higher tryptophan content (97-99 per cent recovery) in different rations determined by his method. Datta (1977), however, used $Ba(OH)_2$, instead of NaOH to hydrolyse feeds because Holler (1958) stated that NaOH destroyed substantial amounts of tryptophan. Moreover, Goswami (1974) stated that the colour in tensity of NED-treated tryptophan solution would be reduced in presence of sodium chloride.

The tryptophan content of feedstuffs is now generally carried out by a colorimetric assay after digestion of the sample with pronase (Holz et al, 1972). The method is modified by allowing the released tryptophan which reacts with dimethyl-amin-cinnamaldehyde reagent to be determined by automation of the colorimeteric reaction.

Williams (1979) reported in the Protein Evaluation Group News Sheet No. 5, September 1979, that at the present time, the preferred method of tryptophan determination in feedingstuffs is by Miller's (1967) method.

Miller's colorimetric method is used in this work after hydrolysis with acid.

4. - EXPERIMENTAL

Materials and Methods

4.1. MATERIALS

The following materials were purchased from:

(a) Sigma Chemical Co. Ltd. :- Amino Acid Standard Solution Stock No. AAS18, Lot no. 27C-7380; α-chymo-trypsinogen A from Bovine Pancreas Type II No. C 4879; Cytochrome C from Horse Heart, Type III, No. C 2506; Insulin from Bovine Pancreas; Edestin from Hemp Seed No. E-0376; Trypsin from Bovine Pancreas Type I, No T-8003; Lysozyme from Egg White, Grade I, No L-6876; L-Tryptophan Sigma Grade: β-Lactoglobulin A, Edestin 1.

(b) E. Merck, Darmstadt :- $SnCl_2.2H_2O$; Thiodiglycol; Thioglycolic acid (mercapto-acetic acid); Ninhydrin (2,2 dihydroxy-indandione-1,3)

(c) BDH Chemical Ltd., Poole, England :- Amino Acid Calibration Standard B; Ninhydrin (Analar Grade) in-danetrione hydrate; Trifluoroacetic acid special for spectroscopy; n-octoic acid.

(d) M & B: Proanalysis Grade :- Sodium citrate dihydrate; Glacial Acetic acid; Hydrochloric acid: Methanol; Sodium acetate trihydrate; Sulphyric acid.

(e) Johnson of Hendon, London :- Benzyl alcohol (pure).

(f) FISONS, Loughborough, Leicestershire :- Starch; Iron II sulphate ($FeSO_4.7H_2O$).

(g) Eastman Organic Chemicals, Lodak Ltd., London :- DL nor-leucine.

(h) Distillers Co (CO_2) Ltd., Stirling :- Cardice (solid CO_2).

4.2 APPARATUS

JLC-5AH Amino Acid Analyser and Integrator Model DK and Joel Printer Model 2, from Joel House, Colindale, London; Tecam Dri-Block DB-3H Heating Block drilled to hold test-tubes sizes 10-75mm and 16x150mm and connected to Tecam Laboratory Temperature Controller; Thermometer E-MIL, Perman Line Pat No 1120773, Great Britain; Genevac Rotatory Piston Vacuum Pump, Radcliffe, Lancs., England; Exelo 6mm T-bone High Vacuum Stopcock; Portable Jencons Oxygen torch (Jencons, Hertfordshire, England); Oxygen cylinder from British Oxygen Ltd; Cooling System Rotarty Regavolt Paxman from Stuart Murray, Henley-on-Thames, England, connected to Rotatory Evaporator (Buchi, Switzerland); Water-bath temeperature controlled; and Vacuum Pressure (Leybold Trivan 16044 AEG); pH meter 26 from Radiometer, Copenhagen.

4.3 REAGENTS

4.3.1. Preparations of Solutions for JLC-5AH Amino Acid Analyzer

(a) Buffer solutions:

The three buffer solutions were prepared from analytical reagents as shown in the table below:

Buffer Materials	pH 5.28±0.10 at 20°C±5°C	pH 3.25±0.08 (0.2NNa+) at 20 ± 5°C	pH 4.25±0.10 (0.2NNa+) at 20 ± 5°C
Sodium citrate dihydrate	171.5g	98.5g	98.5g
Benzyl alcohol (1%$^{v}/_{v}$) cm^3	50.0	-	-
Concentrated HCl cm^3	32.5	61.5	42.0
n-Octoic Acid cm^3	0.5	0.5	0.5
Thiodiglycol	-	25.0	25.0
Methanol (7%$^{v}/_{v}$) cm^3	-	350.0	-
Final volume (with de-ionized water)	5litres	5litres	5litres

(b) Sodium Acetate Ninhydrin Reagent:

One or two litres were generally prepared. The figures quoted below were for 1 litre Ninhydrin reagent preparation.

(i) Sodium Acetate solution for Ninhydrin:

Reagents:			
	Sodium Acetate trihydrate	136.1g	Analytical Reagent
	Glacial Acetic Acid	25.0cm^3	Pronalysis
	Final volume with de-ionised water	250.0cm^3	

Preparation: 136.1g of sodium acetate tri-hydrate ($CH_3COONa.3H_2O$) were dissolved in about 150cm^3 de-ionized water and gently heated for about 15 minutes to expel carbon-dioxide. The solution was then filtered when warm into 250cm^3-volumetric standard flash and allowed to cool. 25cm^3 glacial ethanoic acid (acetic acid) was added and the mixture after cooling to room temperature was made up to 250cm^3-mark with de-ionized water.

(ii) Re-distilled 2-methoxy-ethanol for Ninhydrin Reagent:

2methoxy-ethanol is to keep hydrindantin-reduced ninhydrin - in solution.

The solvent for ninhydrin should be free from peroxides, hence 2-methoxy ethanol purchased was re-distilled to obtain 2-methoxy ethanol free from peroxides.

Solution A was prepared by adding 3cm^3 conc. H_2SO_4 (pronalysis) to 30g Iron II sulphate ($FeSO_4.7H_2O$) Ferrous Sulphate and then 55cm^3 de-ionized water.

1% $^v/_v$ of this solution A was added to 6 litres of 2-methoxy ethanol and distilled. The first, 5% $^v/_v$ of the distillate at 125°C (768mm pressure) was discarded as well as the final 10% $^v/_v$ left undistilled. The distillate was tested to be free from peroxides, according to the method of Eastoe and Courts (1972) or by adding 2ml of the solvent to 1ml of freshly prepared 4% aqueous KI. A colourless to light straw-yellow test is satisfactory.

(iii) Preparation of the Ninhydrin Reagent for the Analyzer:

	Ninhydrin Reagent
Sodium acetate buffer solution	250 cm^3
Redistilled 2 methoxy ethanol	750 cm^3
Ninhydrin	20 g
$SnCl_2.2H_2O$	0.6 g
Final volume	1 litre

250cm^3 of sodium acetate buffer solution (b(i)) was placed in ninhydrin reservoir pressure bottle covered with dish cloth and 750cm^3 redistilled 2-methoxy-ethanol (b(ii)) were added, and continuously stirred with the magnetic stirrer. Under pressure, nitrogen gas (free from oxygen) was passed into the mixture for about 20 minutes. Then 20g of 2,2,di-hydroxy-indandion-1,3 (ninhydrin analytical grade from Merck) were added to the mixture, with stirring. The nitrogen gas was bubbled into the mixture until all the ninhydrin had dissolved. The reducing agent, 0.6g stannous chloride ($SnCl_2.2H_2O$)

was added to the solution and nitrogen gas passed into the ninhydrin reagent with stirring till all dissolved to form a red-wine colour.

(iv) Age and Stability of Ninhydrin Reagent:

Because of the ageing of ninhydrin reagent with time when ninhydrin solutions were prepared, standard measurements were made before and after running samples for analysis. The solution was stored in the ninhydrin reservoir pressure bottle under N_2 covered completely with dark cloth.

The colour yield from proline (ie recovery of proline) as well as the recovery from standard amino acid runs were used to find out the ageing of the ninhydrin. If the colour yeild of proline remains relatively constant while the colour production from the amino acids is decreased, the problem then comes from access of oxygen to the ninhydrin reagent, and hence new ninhydrin reagent will be prepared. Because of ageing of ninhydrin reagent it is advisable to run out standard sample regularly during the life of the reagent and check the area peak of each amino acid from the standard peaks.

4.3.2. 0.1µm/ml Amino Acid Mixture Standards

0.1µm/ml BDH or Sigma amino acid mixture. Standards containing 2.5µmoles of each amino acid were prepared with 0.1µm/ml nor-leucine internal standard with pH 2.2 buffer. The solution is stored in a refrigerator.

4.3.3. Preparation of reagents for Liu and Chang method

(i) P-Toluene-Sulphonic Acid, monohydrate was purchased from Sigma Chemical Co. It was re-crystallized as described by Liu and Chang (1971) and the material was shown to be HCl free.

(ii) 3-(2-Amino-ethyl)-indole was purchased from Sigma Chemical Co. in its salt from (Tryptamine HCl). The reagent 3-(2-Amino-ethyl) indole was prepared from its HCl by the method described by Liu and Chang (1971).

4.4. EXTRACTION OF GLIADIN BY OSBORNE (1912) METHOD

Plain Flour (Wheat) was purchased from J & T Rodgers, The Meal Shop, St Andrews (Millers Burnside Mills, Cupar, Fife).

Duplicate samples of the wheat flour were extracted successively with large quantity of cold distilled water. The water soluble portion contained starch and some protein. But the water insoluble proteins subjected to successive extraction of 70% alcohol. The protein insoluble in 70% alcohol was concentrated in alcohol, re-extracted with large colume of cold water and the water insoluble protein, Gliadin, was washed with fresh absolute alcohol, (three times) and ether and finally reduced to powder form. It was then placed in air to dry - snow white powder and found to be soluble in 70% alcohol. All other details were as previously described by Osborne (1912) and in Alexander and Black (1960).

5. HYDROLYSIS

The hydrolysis was carried out on gliadin using (i) Matsubara and Sasaki (1969) method; (ii) Liu and Chang (1971) method, and (iii) Present method.

5.1. PRELIMINARY INVESTIGATION: HYDROLYSIS OF STANDARD AMINO ACID MIXTURES

Hydrolysis was carried out in 10x75mm medium-walled rimless pyrex test tube (Corning Cat.No. 1672/02) which had been washed with H_2SO_4-HNO_3 (3:1 $^{v}/_{v}$), rinsed in de-ionized water and dried in oven.

Lyophilized 100ccm (micropipette from HE Pedersen, Denmark) of BDH Standard Amino acids mixture containing 2.5 μmoles of each amino acid was placed in clean, dried test tube. 0.2ml of concentrated HCl diluted with ice cold trifluoro-acetic acid containing 0.6M to 1.8M cold mercapto-acetic acid (thioglycolic acid) freshly prepared was added to amino acid mixture in the tube. The tube was then placed in a bath of cardice - solid carbon dioxide and ethanol. When the sample is frozen, the tube was connected via a short and narrow sleeve of tygon tubing to a vacuum system through an exelo T-bore high vacuum stopcock (6mm) or Rotoflow TF6/18. The system was evacuated with an oil vacuum pump for about 15-20 minutes. The tube which was still in a bath of solid carbon-dioxide and ethanol was sealed under vacuum. The tube was then removed from the bath and the solution was allowed to thaw at room temperature. Other details as described by Moore and Stein (1963).

The hydrolysis was conducted at constant temperature 87°C to 110° ± 1°C,

for 24, 36, and 48 hours in Tecam Dri-Block DB-3H heating block with a thermometer and temperature controller. The maintenance of constant temperature was ensured and accurately controlled.

After hydrolysis and the tube was cooled to room temperature, any liquid on the walls of the tube was spun down by gentle centriguation. If the sample was not to be analysed immediately, the hydrolysis tube was stored in deep-freeze. Below the top tapered end of the hydrolysis tube was marked with a sharp file (Griffin & George Ltd.), moistened and cracked by the use of a hot glass rod.

The hydrolysate was then quantitatively transferred to a quick-fit pyrex round bottom conical flask after rinsing the tube with about 0.5ml de-ionized water and connected to rotatory vacuum evaporator (Leybold 160 ee AEG Type ADEA 71N4746) with Paxman Cooling System (Type 42A) and water-bath at 37°C for 20-25 minutes to remove the excess acid and to obtain complete dryness. The residue was then dissolved in 0.1µm/ml nor-leucine standard to give amino acid mixture of 0.1µm/ml. This was kept in the refrigerator if not loaded into the analyzer immediately.

5.2. HYDROLYSIS OF INSULIN IN PRESENCE OR ABSENCE OF L-TRYPTOPHAN

Lyophilized 10mg (or 1mg sample without L-tryptophan) of protein and 0.4mg of L-Tryptophan were hydrolyzed with 2ml of the mixture of the acid as mentioned in (i) above. In this case the sample was placed in 16-150mm medium walled clean, dry, rimless pyrex test tube. When the sample was frozen, the tube was drawn to about 1mm bore about 3cm from the top and the tube was connected to a vacuum system, as previously described in (i). Other details as described by Moore and Stein (1963) and as in (i) above).

5.3. HYDROLYSIS OF OTHER PROTEINS

Trypsin + 5% $^{w}/_{w}$ starch lyophilisized proteins (5mg or 1mg) were hydrolysed in an evacuated, sealed tube with concentrated HCl diluted with cold trifluoro-acetic acid (3ml or 1ml) containing 1.0m mercapto-acetic acid (thio-glycolic acid). The solution was freshly prepared before use and hydrolysis was carried out at $110^{\circ} \pm 1^{\circ}C$ for 24, 36, and 48 hours. Other details as described in (i) above and Moore and Stein (1963).

5.4. HYDROLYSIS OF GLIADIN AND SOME PROTEINS were carried out as detailed by Liu and Chang (1971) using 3N p-Toluene-Sulphonic Acid containing 0.2% 3-(2-amino-ethyl)-indole in evacuated sealed tubes at 22, 36 and 48 hours.

6. JLC-5AH AMINO ACID ANALYZER USED FOR SEPARATION AND IDENTIFICATION OF THE COMPONENTS OF THE HYDROLYSATES OF PROTEINS

6.1. A JEOL JLC-5AH Amino Acid Analyzer was used for the separation and identification of the components of the hydrolysates of proteins. The three buffer solutions, 0.2M NaOH and 50% $^{v}/_{v}$ 2-methyl ethanol used by the Analyzer were prepared as described at pages 39-42. The Analyzer has two buffer pumps, P_1 for short column (15cm x 0.9cm) and P_2 for long column (70cm x 0.9cm) and each pump system was programmed for an automatic buffer change and regeneration of column with 0.2M NaOH and "wash" with 50% $^{v}/_{v}$ 2-methoxy-ethanol after each cycle.

Conditions used for analysis are:

			Column 1 S.C.	Column 2 L.C.
	Stationary Phase:		JEOL Resin LC-R-1	JEOL Resin LC-R-1
	Mobile Phase:		Sodium citrate	Sodium citrate
(i)	Flow Rate buffer pump		1.22ml/min	0.93ml/min
(ii)	Flow Rate of Detection Pump	(a) Sample pump		0.42ml/min
		(b) Ninhydrin pump		0.21ml/min
		Total		0.63ml/min
(iii)	Column temperature		55°C	60°C
(iv)	Reaction Bath Temperature	95°C		
(v)	Chart speed: 12 cms/hr			
(vi)	Pump Pressures		4-6kg/cm^2	8-10kg/cm^2
(vii)	Range		570mμ Blue 100∿70% fs(X3) Green 100∿0% fs(X1)	440mμ Red 100∿70% fs(X3) Light path length 2.0mm

(b)/

6.2. Resin Heights of the Columns:

The short column was packed with Resin LCR, and tryptophan estimation were carried out at different resin heights of

(i) 9.00cm

(ii) 10.00cm

(iii) 12.00cm

(iv) 13.5cm

The height of resin LCR, on long column was 66.2cm and the height affects the separation of threonine and serine (re-base line).

6.3. BDH Amino Acid Calibration Standard B

It contains 18 amino acids and ammonia-mixture of concentration 2.5 micromoles per ml of each component of the amino acid in 0.05M HCl (mercury II chloride was used as a preservative).

1ml of 2.5µm/ml mixture of the BDH amino acid mixture and 1ml of 2.5 µm/ml of nor-leucine (internal standard) were made to 25.0ml in a standard volumetric flask with buffer solution of pH2.2 (HCl in de-ionized water). Hence 0.1 micromoles per ml BDH Standard amino acid containing 0.1micromole per ml of nor-leucine as internal standard was prepared.

The BDH amino acid calibration standard solution has no cystine but it contains tryptophan and cysteic acid.

6.4. Sigma Amino Acid Standard Solution of concentration 2.5micromoles per ml contains 17 amino acids and ammonium chloride solution in 0.1M HCl. 0.1 micromoles per ml of the standard solution containing 0.1micromole per ml nor-leucine (used as internal standard) was prepared with pH 2.2 HCl buffer solution.

The Sigma amino acid standard solution has no tryptophan but it contains cystine.

5. Protein Hydrolysates

0.1micromole per ml of the hydrolysates containing 0.1micromole per ml nor-leucine (internal standard) were prepared for loading into the analyzer.

6. Loading of the Sample into the Analyzer

The inlet and outlet of the system were rinsed with distilled water injected from 10ml or 5ml Sabre disposable syringe and excess water and air were removed with sabre disposable syringe. The sample load which was 0.8ml for each column was slowly injected with 2ml sabre sterile disposable syringe. The sample selector was changed to the next position before the syringe was removed. The inlet and outlet of the system leading to the second sample and so on were rinsed, dried and loaded as before. The sample selector was finally left at position 6 before the syringe was removed.

Usually, BDH amino acid standard solution was first loaded into the short column and sigma amino acid standard solution into the long column. This was followed by the second sample (usually 24-hour hydrolysates) being loaded into the short and long columns. Then, 36-hour hydrolysates and 48-hour hydrolysates were loaded into the analyzer.

The auto-sampler capacity is 0.8ml.

7. Elution Programmes

The elution of each amino acid component of the standard/hydrolysates was programmed according to the Instruction Manual, Bulletin no. 306-1 for Model E536A(12) NIWO-L-NS(JE). The basic amino acids were first eluted from the short column (15cm) in this order - try, lys, hist, NH_3, and arg. Other amino acids were first eluted as follows: pH 3.25$\pm$0.08 buffer solution - Asp,

Thr, Ser, Glu, Pro, Gly, Ala, Cys and Valine.

pH4.25 ± 0.1 buffer solution - metheonine, isoleucine, leucine, nor-leucine, tyrosine and phenylalemine.

One single run and regeneration of column would take 6½ hours. The temperatures of the column baths are 55°C for Short Column and 60°C for Long Column and the Reaction Bath 95°C, Flow rate 0.64ml/min.

Pump pressure 1 (Short Column) 4-6 kg/cm²

Pump pressure 2 (Long Column) 8-10 kg/cm²

6.8. INTEGRATOR - JLC-DK digital Intergrator attached to the Analyzer was used and the results compared with the calculations by HW method.

7. RESULTS AND DISCUSSION

7.1. Preliminary Investigation:-

The yield of tryptophan was observed to increase with the height of the resin in short column as shown in Table 5, as well as the age or period of use of resin. This shows that the recovery of tryptophan was between 98-100% if the resin column height was above 13.5cm. In contrast, Matsubara and Sasaki (1969) found a 7cm column height to give high yield. The resin height in the long column also affected the separation and the yield of serine and threonine. Hence, regular repacking, after 24 to 30 runs, washing and regeneration of resin contributed to a better yield of tryptophan, serine and threonine.

The following hydrolytic reagents were tried during a preliminary investigation on Standard amino acid mixtures (BDH and Sigma) (see Table 4).

(a) 6M HCL containing 4% mercaptoacetic acid (thioglycolic acid) added to lyophilized mixture in an evacuated and sealed tube and hydrolysed for 24 hours at $110^{\circ}C \pm 1^{\circ}C$.

(b) Standard amino acid mixtures were hydrolysed in an evacuated sealed tube using a 1:1 mixture of HCl/Trifluoro-acetic acid containing 0.6M - 1.2M mercapto-acetic acid (final concentration of HCl 6M) for 24 hours at $110^{\circ}C \pm 1^{\circ}C$.

(c) A number of hydrolytic reagents were tried during the preliminary investigation using BDH and Sigma amino acid mixtures. Details of these reagents, the temperature used for hydrolysis and the yield of tryptophan can be found in Table 4.

It can be seen that yields using HCl and mercapto-acetic acid mixtures and Trifluoro-acetic acid and mercapto-acetic acid mixtures are low whereas for HCl and Trifluoro-acetic acid (1:1) mixtures containing 1.0M mercapto-acetic acid, a maximum yield is obtained. With the exception of cysteine, the recoveries of other amino acids in the hydrolysate were not affected.

RESULTS

TABLE 4

Preliminary investigation: Hydrolysis of Standard BDH Amino Acid Mixtures

The results are the average of determinations of tryptophan from 24, 36 and 48 hours hydrolysed as described in Section .

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The boiling point of Tri-fluoro acetic acid (TFA) 72.4°C
The boiling point of mercapto acetic acid TGA 120°C
The boiling point of 6M HCl (conc:water 1:1) 110°C

	Each Hydrolytic Reagent used on each sample	Temperature of Hydrolysis °C	Recovery of Tryptophan %
(a)	6M HCl + 4% TGA	110 ± 1°	84 ±2
1.	6M HCl + 0.6M TGA	110 ± 1°	86 ± 2
2.	6M HCl + 1.2M TGA	110 ± 1°	84 ± 1
3.	6M HCl + 1.8M TGA	110 ± 1°	92 ± 2
4.	100% TFA + 0.6M TGA	85 ± 1°	75 ± 1
5.	100% TFA + 1.2M TGA	83 ± 1°	96 ± 1
6.	100% TFA + 1.8M TGA	93 ± 1°	96 ± 2
7.	100% TFA + 6M HCl (1:1) + 1.2M TGA	110 ± 1°	95 ± 1
8.	Conc. HCl diluted to 6M with 100% TFA + 1.2M TGA	93 ± 1°	97 ± 2
9.	Conc. HCl diluted to 6M with 100% TFA + 1.0M TGA	110 ± 1°	99 ± 1

This reagent has, therefore, been used in all subsequent experiments.

It appears that HCl and Trifluoro-acetic acid mixtures have several advantages:

(i) The two reagents are mixable; (ii) Trifluoro-acetic acid is a very good solvent for peptides and in strong acid, amino groups are protonated and are hence not acylated; (iii) mercapto-acetic acid protects the destruction of tryptophan, and (iv) the solvent can be removed after hydrolysis using a rotary evaporator to which a cooling system has been attached (air/water condenser) at 40°C under pressure control within 15-20 minutes. The addition of ether facilitates the removal of mercapto-acetic acid which is otherwise a bit difficult.

7.2. Recovery of Tryptophan added to Insulin (Bovine)

The recovery of tryptophan in the presence of insulin was examined. Insulin was chosen since it contains no tryptophan and therefore an unequivocal estimate of recovered tryptophan can be made since the amount present is exactly known.

(a) 0.26µmoles L-Tryptophan was added to a sample of bovine insulin (100mgs) and the recovery of added tryptophan from the hydrolysate by using the acid hydrolytic reagent was found to be 90% using the JEOL Amino Acid Analyser.

(b) 5.1mg/100 L-Tryptophan was also added to bovine insulin and the recovery of added tryptophan by Miller (1967) colorimetric method was 98%. The method of Matsubara and Sasaki (1969) hydrolysate when determined by Miller (1967) colorimetric method gave 94.1% recovery of free tryptophan.

7.3 Determination of tryptophan content of several proteins using a TFA/HCl (1:1) mixture containing TGA for hydrolysis

The present method has been applied to bovine insulin, trypsin (Table 7), lysozyme (Table 8), cytochrome C (Table 9), Edestin (Table 11), Trypsinogen (Table 10), α-chymotrypsinogen A (Table 11), and β-Lactoglobulin A (Table 11). In each case, the recovery of tryptophan was encouraging, for instance, examination of a 24-hour hydrolysis showed recoveries of, in the case of lysozyme, 95%, in the case of cytochrome C in the order of 100%.

To obtain more accurate figures, the values obtained for 24, 36, and 48 hours were extrapolated to zero time assuming first order kinetics. It would be better to obtain readings up to 72 hours and more to ensure complete hydrolysis and obtain more satisfactory extrapolations.

It is clear that better results would have been obtained by extending hydrolysis times to 72 hours. Nevertheless, it is clearly demonstrated that the method used provides results which have been by comparison with the best results for tryptophan found in the literature. Tables 7-9 also clearly show that the results for other amino acids such as lysine, glutamic acid, tyrosine, are also comparable with accepted literature values.

The high concentration of mercapto acetic acid used to protect tryptophan during hydrolysis could not be completely removed at the rotary evaporator temperature, because of its boiling point of 120°C. Since it is soluble in ether, it was removed by mixing with ether and residue obtained. The residue hydrolysate which is quantitatively prepared with nor-leucine (internal standard for JEOL Amino Acid Analyzer) produces a high peak between cysteic acid and aspartic acid - this was also reported by Matsubara and Sasaki (1969) - and a small peak at carboxymethyl cysteine position.

It is also observed that the position of the cystine peak is very sensitive to the pH of the buffer. At pH 3.47, cystine emerges always with alanine, while at pH 3.37 its peak overlaps that of valine. So pH of the buffer is adjusted to that cystine peak is about mid-way between the neighbouring alanine

and valine peaks, pH 3.47 ± 0.01. The influence of cystine, cysteine, serine, threonine, methionine and valine when present in protein containing tryptophan requires special attention for the buffer pH 3.47. Buffer pH 3.25 ± 0.08 may be used to solve part of the problems.

7.4 Added Carbohydrate to Protein

It is reported in the literature that large amounts of carbohydrates present associated with the broken structure or otherwise interfere with the estimation of tryptophan present and experiment was carried out when starch was added to the sample. This experiment has particular significance to the estimation of tryptophan present in samples of food, feedstuffs when large amounts of carbohydrates may be present.

It was found that the addition of 5% $^{w}/_{w}$ starch to trypsin before hydrolysis does not affect the recovery of tryptophan after 24 hour hydrolysis with the present method (see Table 10). Addition of starch after hydrolysis was also found to have no effect.

The yield of recovery of Tryptophan with 5% starch was 97.5% while trypsin without starch, the recovery was 99.3% (see Table 11).

7.5 Gliadin

Gliadin is used as a model compound because it is a common constituent of foodstuffs and therefore was chosen as a model compound.

The results at Table 10 showed the comparison of the present method with that of Matsubara and Sasaki (1969) and Liu and Chang (1971). The values (uncorrected) of amino acids obtained were closer to those in the literature.

Each value as reported in Table 10 was derived from the average of two

hydrolysates of two different samples of extracted and purified Gliadin.

7.6 Millet

The result obtained from millet (extracts) another constituent of foodstuffs, is not reported because it is inconclusive.

TABLE 6

Amino Acid Composition of Insulin (Bovine) after Acid Hydrolysis with Concentrated HCl diluted to 6M with 100% Trifluoro acetic acid (1:1 $^{v}/_{v}$) in presence of Mercapto acetic Acid (Thioglycolic Acid) for 24 Hours at $110^{\circ} \pm 1^{\circ}C$

Three analyses from two different samples JLC-5AH Amino Acid Analyzer, Norleucine as internal standard.

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	Anhydrous amino acid per 100 g protein	No. of Residues for Molecular Weight 5732	Literature values Takahashi (1977)	µmoles added Tryptophan	µmoles Tryptophan Recovered after hydrolysis	% Recovery
Tryptophan	0	0	0	0.25	0.225	90
Lysine	2.75±0.15	1.08	1			
Histidine	4.80±0.2	1.88	2			
Arginine	2.99±0.1	0.98	1			
Aspartic Acid	2.56±0.05	1.1	1			
Threonine	1.69±0.2	0.81	1			
Serine	1.56±0.1	0.85	1			
Glutamic Acid	8.20±0.3	3.2	3			
Proline	2.60±0.2	1.3	1			
Glycine	3.75±0.3	2.86	3			
Alanine	3.45±0.2	2.22	2			
Half-cystine	2.52±0.3	1.2	(2)			
Valine	5.83±0.1	2.85	3			
Methionine	0	0	0			
Iso-leucine	0.73±0.3	0.32	0			
Leucine	8.65±0.2	3.78	4			
Tyrosine	6.63±0.15	2.1	2			
Phenyl alanine	7.95±0.1	2.76	3			

TABLE 5

Variation of Length of Resin on Short Column with Buffer pH 5.28;

Length of Short Column 15.0cm

Length of Resin LCR1 in the Column	FACTORS 1 Effect on Separation of Tryptophan	FACTORS 2 Effect on the Recovery of Tryptophan
8.00cm	Not separated from lysine	Very poor recovery
9.00cm	Poor separation	Poor recovery
10.00cm	Fair separation	Low recovery
11.00cm	Very fair separation	Fair recovery
11.4cm	Good separation	Fairly good recovery
12.5cm	Better separation	Better recovery
13.5cm	Best separation	Much better recovery

TABLE 7

Amino Acid Composition of Trypsin[1] after Acid Hydrolysis (HCl + TFA : 1 : 1) in presence of Mercapto Acetic Acid (Thioglycolic Acid) at 110° + 1°C

	Calculated number of Residues per molecule[2]			Extrapolated Value	Recovery %	Literature Values Takahashi (1977)
	24hr (a)	36hr (b)	48hr (c)			
Tryptophan	3.33	3.84	3.78	3.97	99.3	4
Lysine	13.63	13.71	13.70	13.95	99.6	14
Histidine	3.06	2.92	2.95	3.0	100	3
Arginine	1.38	1.88	1.96	1.95	98	2
Aspartic Acid	21.39	21.74	21.64	21.98	99.9	22
Threonine	9.24	8.89	8.67	9.8	98	10
Serine	29.41	27.80	26.56	32.5	98	33
Glutamic Acid	13.86	13.93	13.81	13.9	99	14
Proline	8.85	8.81	8.75	8.98	99.8	9
Glycine	24.59	25.03	24.96	25.0	100	25
Alanine	13.89	13.81	13.69	13.9	99	14
Valine	14.69	14.95	14.60	16.8	98.8	17
Methionine	1.61	1.97	1.88	2.0	100	2
Iso-leucine	13.68	13.20	12.64	14.75	98	15
Leucine	13.89	14.03	13.92	13.98	99.9	14
Tyrosine	9.55	9.73	9.20	9.9	99	10
Phenyl alanine	2.79	2.75	2.70	2.9	96	3

1 Minimal Molecular Weight 24,000

2 Calculated number of Residues per molecule was made from g anhydro amino acid per 100g protein (a) (b) (c) average of duplicate determinations from 24, 36, 48 hours hydrolysates. (d) Calculations based on BDH/Sigma amino acid standards and nor-leucine internal standard.

TABLE 8 Amino Acid Composition of Lysozyme[1] after Acid Hydrolysis in Presence of Thioglycolic Acid (Mercapto acetic Acid) at $110^{o} \pm 1^{o}C$

	Calculated Number of Residues per molecule[2]			Extrapolated value	Recovery %	Literature values Cornfield & Liu, 1965
	24hr	36hr (a)&(b)	48hr	Zero Hydrolysis or Average (x)		
Tryptophan	5.68	5.45	5.54	5.95	99	6
Lysine	5.94	5.91	5.95	6.0	100	6
Histidine	1.23	1.02	0.99	1.08(x)	108	1
Arginine	10.73	10.65	10.62	10.9	99	11
Aspartic acid	20.14	20.8	21.2	20.7(x)	99	21
Threonine	6.98	6.87	6.85	6.9	98.6	7
Serine	9.65	8.94	8.7	9.9	99	10
Glutamic Acid	4.77	4.92	5.03	4.95	99	5
Proline	6.68	7.04	6.80	6.84(x)	-	2
Glycine	11.68	11.98	12.06	11.9	99	12
Alanine	11.09	12.35	12.26	11.9(x)	99	12
Valine	5.73	6.07	6.05	5.95	99	6
Methionine	2.13	2.08	2.1	2.05	103	2
Iso-leucine	5.21	5.85	6.14	5.73(x)	96	6
Leucine	7.43	7.88	7.92	7.74(x)	97	8
Tyrosine	2,80	3.06	3.04	2.96(x)	99	3
Phenyl alanine	2.65	2.95	2.98	2.95	98	3

1 Minimal molecular weight 14,700

2 Calculated number of Residues per molecule was made from g,anhydro amino acid per 100g protein.

(a) Average of duplicate determination from 24,36,48 hrs hydrolysate. (b) Standard BDH/Sigma & Nor-leucine used as internal standard.

(x) Average of the three values

TABLE 9

Amino Acid Composition of Cytochrome C[1] after Acid Hydrolysis with HCl and TFA (1:1) in presence of Mercapto Acetic Acid (thioglycolic acid) at 110° ± 1°C

	Calculated number of Residues per molecule[2]			Extrapolated values or Average (a)	Recovery %	Literature values Nakashima et al 1966
	24hr	36hr	48hr			
Tryptophan	0.998	0.81	0.88	0.98	98	1
Lysine	17.89	18.02	17.98	17.99	100	18
Histidine	3.26	2.99	3.19	3.14(a)	104	3
Arginine	2.01	2.04	2.1	2.05(a)	103	2
Aspartic Acid	8.28	8.11	8.29	8.1	101	8
Threonine	7.72	7.43	7.20	7.9	98.8	8
Serine	0.88	1.01	1.22	0.9	90	1
Glutamic Acid	12.03	12.07	12.1	12.0	100	12
Proline	4.43	4.11	5.23	4.59(a)	115	4
Glycine	13.96	14.03	13.9	14.0	100	14
Alanine	6.15	6.0	6.06	6.07(a)	101	6
Valine	2.85	2.52	2.44	3.0	100	3
Methionine	1.92	1.85	1.90	1.95	98	2
Iso-leucine	5.86	5.41	5.70	5.98	99.6	6
Leucine	5.94	5.78	5.92	5.99	99.8	6
Tyrosine	3.86	3.75	3.91	3.98	99.5	4
Phenyl alanine	3.72	3.48	3.78	3.9	98	4

1 Minimal Molecular Weight 12,750
2 Calculated number of residues per molecule was based on g anhydrous amino acid per 100g protein
3 Average of duplicate determinations from 24, 36, 48 hours hydrolsates
(a) Average determinations

TABLE 10 Comparison of different methods for estimation of amino acid composition of Gliadin (Gramineae) by acid hydrolysis

Hydrolysis Time: 24 hours A.M. Wt. 1990 Temperature $100^{o} \pm 1^{o}$ (0.5mg/ml)

	Present Method	Matsubara & Sasaki 1969 Method	Liu & Chang 1971 Method	Literature Values		
				Tristram & Smith 1963	Bietz & Roth 1970	Chibnall 1946
					Molar basis	
Tryptophan	5.1	3.1	1.68	6.02	-	0.6
Lysine	1.12	1.46	1.00	0.57	0.7	0.65
Histidine	3.98	3.59	2.52	1.61	1.8	1.82
Arginine	4.77	4.92	2.87	2.46	1.9	2.74
Aspartic acid	2.05	1.97	2.12	1.16	3.2	1.34
Threonine	1.52	2.04	1.62	1.78	2.5	2.10
Serine	3.03	3.40	3.75	4.06	4.1	4.9
Glutamic Acid	40.97	40.10	38.18	40.11	37.0	45.9
Proline	12.85	11.48	12.18	11.43	14.7	13.35
Glycine	1.52	2.42	1.57	nd	3.7	nd
Alanine	1.55	1.63	1.52	1.70	3.3	2.13
Half-Cystine	0.88	1.85	0.91	2.21	2.0	2.58
Valine	2.61	3.17	2.72	2.25	4.6	2.66
Methionine	1.53	1.0	1.07	1.45	1.2	1.69
Iso-leucine	3.91	3.34	3.10	10.27	5.0	11.90
Leucine	6.38	5.81	6.06		8.2	
Tyrosine	2.87	3.73	2.71	2.88	2.3	3.2
Phenyl alanine	5.83	4.62	5.53	5.74	3.9	6.44

1 Values are in gram anyhdro amino acid per 100g protein
2 Each value was derived from the average of two hydrolysates of two different samples of extracted and purified Gliadin
3 The figures were uncorrected

TABLE 11 Analyses of Tryptophan in Protein by Acid Hydrolysis with HCl/TFA containing 1.0 Mercapto acetic acid

SAMPLE	Gram of Anhydrous amino acid per 100gm protein HYDROLYSIS TIME Hours 24	36	48	Calculated Number of Residues per Minimum Molecular Weight HYDROLYSIS TIME Hours 24	36	48	Extrapolated Value Zero Time	Literature Values	Yield %
1. Insulin	0	0	0	0	0	0	0	0	0
2. Trypsin	2.83	3.26	3.21	3.33	3.84	3.78	3.97	4	99.3
3. Lysozyme	7.86	7.57	7.68	5.68	5.45	5.54	5.95	6	99
4. Cytochrome C	1.6	1.3	1.41	0.998	0.81	0.88	0.98	1	98
5. Bovine Trypsinogen	3.3	3.17	3.08	3.85	3.70	3.60	3.85	4	96
6. Trypsin + 5% Starch	3.18	3.10	3.00	3.74	3.64	3.58	3.90	4	97.5
7. α-Chymotrypsinogen A	5.57	5.43	5.29	6.82	6.65	6.48	7.2	8	90
8. Edestin	1.59	1.79	1.57	3.82	4.3	3.77	3.96(a)	4	99
9. β-Lactoglobulin A	2.03	2.02	1.97	3.68	3.66	3.57	3.80	4	95

1 Minimum molecular weight 5732
2&6 Minimum molecular weight 24,000
3 Minimum molecular weight .14,700
4 Minimum molecular weight 12,750
5 Minimum molecular weight 23,800
7 Minimum molecular weight 49,000
8 Minimum molecular weight 37,000
9 Literature value for Edestin 1.65-1.60% (Colorimetric determination) Spies & Chambers, 1949
(a) " " " β-Lactoglobulin 2.64-2.57% (- " - " -)

SEE FIGS 4 & 5.

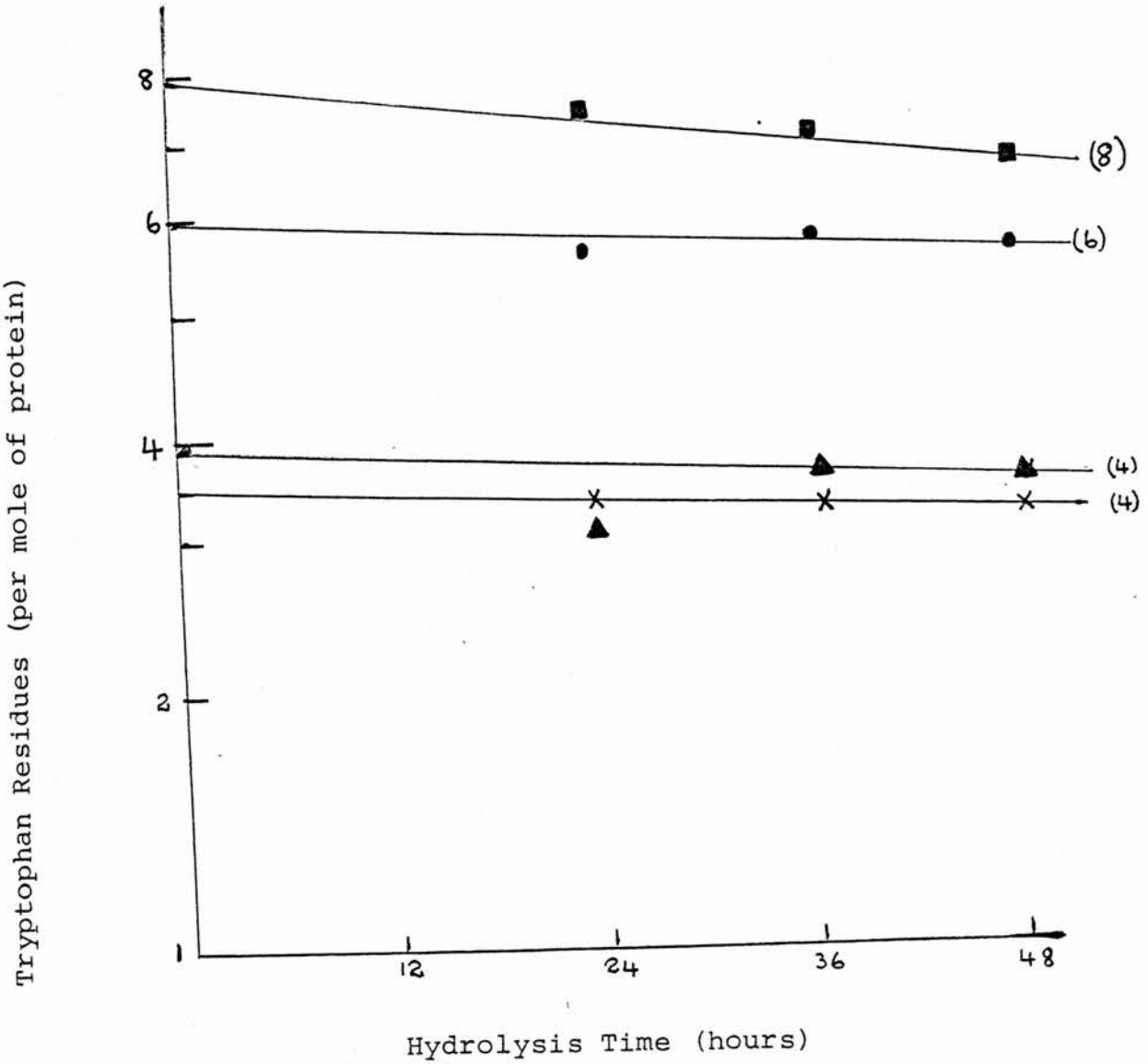

Fig. 4. Rate of decomposition of tryptophan in proteins during acid hydrolyses with hydrochloric acid diluted with trifluoroacetic acid (1:1 v/v) containing 1.0m mercaptoacetic acid.

▲ Trypsin
● Lysozyme
X β-Lactoglobulin A
■ α-Chymotrypsinogen A.

The time of hydrolysis is plotted against the residues of tryptophan found per mole of protein on a semilogarithmic scale. Literature values are shown in parentheses.

Fig. 4

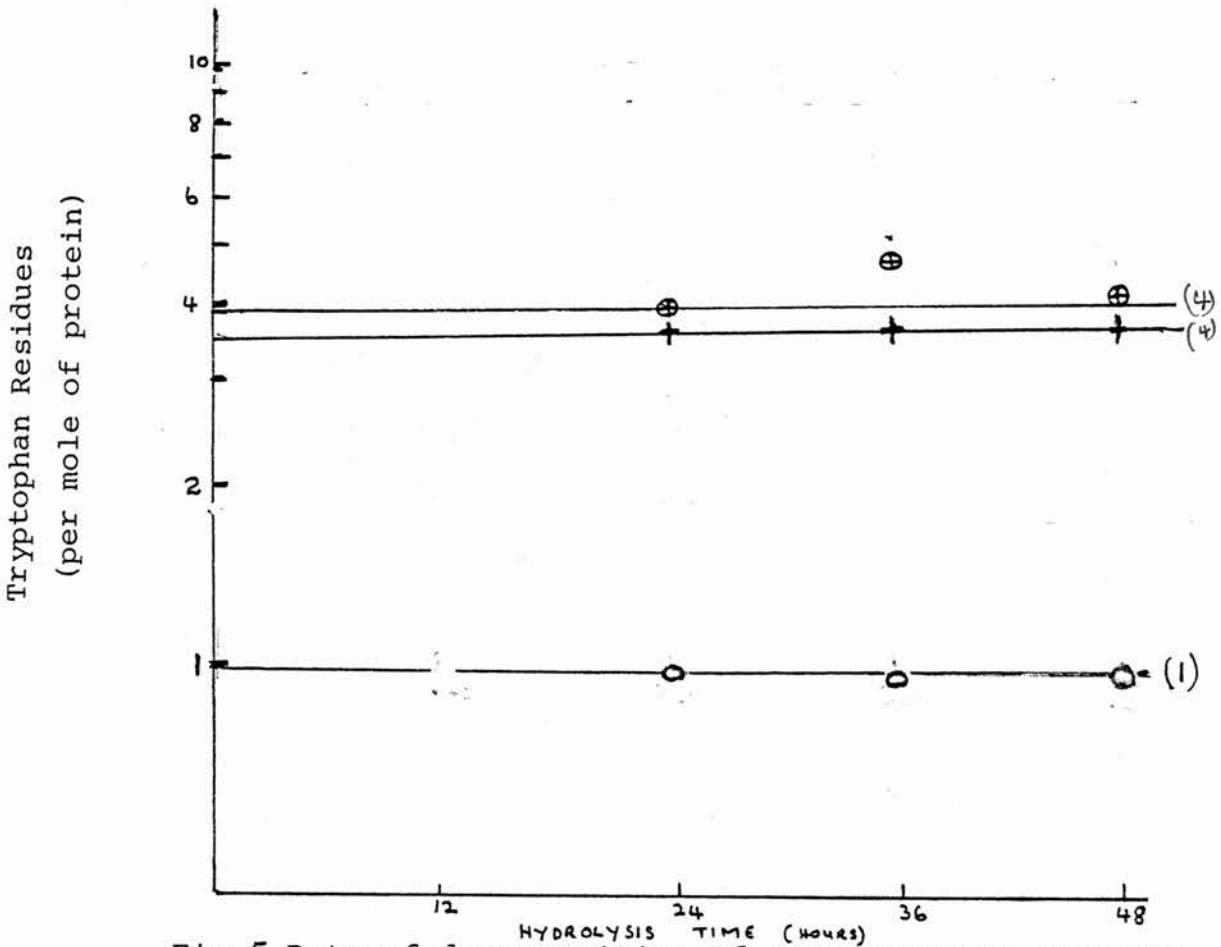

Fig.5 Rate of decomposition of tryptophan in proteins during acid hydrolysis with hydrochloric acid diluted with trifluoroacetic acid (1:1; v/v) containing mercaptoacetic acid

⊕ Edestin
\+ α-Bovine trypsinogen
O Cytochrome C

The time of hydrolysis is plotted against the residues of tryptophan found per mole of protein on a semilogarithmic scale. Literature values are shown in parentheses.

Fig. 5

8. CONCLUSION

The present procedure provides a new method for the determination of tryptophan in proteins and glycoproteins. Attempts have been made to apply the hydrolytic reagent to the extracted samples of proteins from millet, though that of the extracted and purified gliadin from flour was done. The millet extract did not give particularly successful results, however, the method as applied to extracted and purified gliadin appeared to give good results in comparison with the highest values found in the literature although considerable differences are found in literature results. The present method gives tryptophan values for trypsin, lysozyme, cytochrome C, trypsin + 5% starch, edestin, α-chymotrypsin and chymotrypsinogen A, in good agreement with those obtained by other methods reported in the literature that do not use acid hydrolysis. In addition, the recovery of all other amino acids with the exception of cystine, was comparable to those obtained from (1) 6M HCl hydrolysate (Moore and Stein, 1963), (2) 6M HCl hydrolysate containing 4% thioglycolic acid (Matsubara and Sasaki, 1969), (3) 3N-4 toluene sulphonic acid containing 0.2% 3-(2-amino-ethyl) indole hydrolsyate (Liu and Change, 1971), (4) 6M HCl "buffer neutralizatin solution" hydrolysate of Spitz (1973), and (5) 4M methane-sulphonic acid containing 0.2% in 3-(2-amino-ethyl) indole hydrolysate of Ewart (1977), and (6) the recovery of added tryptophan from the acid hydrolysates of insulin with the present method using Miller's (1967) colorimetric method was 98 $\pm$ 2%.

Since this present method used simple techniques coupled with nor-leucine as an internal standard for the amino acid analyzer, it should be more convenient than the other methods for routine amino acid determination and is best for tryptophan analysis. The high recovery 99 $\pm$ 1% of tryptophan compared with those obtained by others as shown below:

Table II

Protein	Number of Analysis	Present Method	Liu & Chang 1971	Matsubara & Sasaki 1969
1. Lysozyme	4	99 ± 1	98%	91%
2. Cytochrome C	4	98 ± 2	-	86%
3. Trypsin	3	99 ± 1	-	-
4. α-Chymotrypsinogen A	4	90 ± 2	98	-
5. Bovine Trypsinogen	6	96 ± 3	95	-
6. Edestin	4	99 ± 1	-	-
7. β-Lactoglobulin A	4	95 ± 2	-	-
8. Trypsin + 5% Starch	4	97.5 ± 2	-	-
9. Insulin, bovine	3	0	0	0

shows that the hydrolytic reagent mixture HCl and Trifluoroacetic acid (1:1) containing mercapto-acetic acid is more effective for the hydrolysis of proteins, glycoproteins and causes less destruction of tryptophan and other labile amino acid.

The behaviour of other acids (6M HCl + 4% TGA), p-Toluene-sulphonic acid as substitutes for conc. HCl diluted to 6M with 100% $^{v}/_{v}$ trifluoro-acetic acid containing mercapto-acetic acid has been studied with respect to the hydrolysis of extracted and purified gliadin and the recovery of tryptophan and other amino acids as shown on Table II proves that the present procedure provides an improved new method for the determination of tryptophan in protein and glyco-proteins.

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