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ANALYSIS OF AMINO ACIDS BY GAS-LIQUID CHROMATOGRAPHY

THE DEVELOPMENT OF A METHOD AND IT'S APPLICATION

by

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Thesis presented for the degree of

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ABBREVIATIONS

The following abbreviations are used in the text:

ALA	-	Alanine
ASP	-	Aspartic Acid
ARG	-	Arginine
CYSH	-	Cysteine
CYS	-	Cystine
GLU	-	Glutamic Acid
GLY	-	Glycine
HIS	-	Histidine
HOPRO	-	Hydroxyproline
ILE	-	Isoleucine
LEU	-	Leucine
LYS	-	Lysine
MET	-	Methionine
PHE	-	Phenylalanine
PRO	-	Proline
SER	-	Serine
THR	-	Threonine
TRY	-	Tryptophan
TYR	-	Tyrosine
VAL	-	Valine
TFA	-	Trifluoroacetyl
PVP	-	Polyvinyl pyrrolidone
DNP	-	2,4- Dinitrofluorobenzene
PTH	-	Phenylthiohydantion
TLC	-	Thin Layer Chromatography

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INTRODUCTION

INTRODUCTION

I Proteins and their role in biology

(i) Protein: Organic chemistry was originally defined by the Swedish chemist Berzelius in 1806 as "the part of physiology which describes the composition of living bodies, and the chemical processes which occur in them." It was implicit in such a definition that the chemical substances found in living things were fundamentally different from the inorganic materials found in inanimate matter.

However, the advent of synthetic organic chemistry led to the view that no fundamental difference existed between inorganic and organic compounds, and organic chemistry thus came to be regarded as the chemistry of carbon compounds. The study of chemical processes in the organs of plants and animals, termed physiological chemistry, led to the discovery that many of the products isolated from these organisms contain carbon. Amongst these were certain nitrogenous compounds, common to both plants and animals, to which the Dutch chemist Mulder in 1839 gave the name protein and which he believed to be basic constituents of protoplasmic materials. The name comes from the Greek, signifying that which is first or of prime importance.

Justus von Leibig took up Mulder's proposition, and like him concluded that "the true starting point for all tissues is albumen; all nitrogenised articles of food, whether derived from the animal or from the vegetable kingdom, are converted to albumen before they can take part in the process of nutrition." (Leibig, 1840).

Like Mulder, he ascribed the formula $C_{48}H_{36}N_6O_{14}$ to protein, and albumen he wrote as $C_{48}H_{36}N_6O_{14}+P+S$.

In his further investigations in the field of protein chemistry, Leibig came to reject Mulder's concept of the nucleus of "protein", and recognised that chemical formulae for albumen, fibrin, etc. as compounds of protein with sulphur and phosphorus did not agree with the results of more precise analysis of these substances (Leibig, 1847). Indeed it is true to say that when we use the word protein we are commemorating an erroneous oversimplification of protein structure, and are using the word in a quite different sense from its original usage.

It was found that on hydrolysis proteins yield amino acids, the simplest of which, glycine, was also the first to be recognised as a product of protein hydrolysis by Braconnot in 1820. Some twenty five amino acids have since been found as products of protein breakdown. In 1902 Emil Fischer and Franz Hofmeister independently advanced the hypothesis that in proteins the α-amino group of one amino acid and the α-carboxyl group of another amino acid are joined, with the elimination of a molecule of water, to form an amide linkage. The peptide bond so formed serves to join amino acid residues in long poly-peptide chains - this is now termed the primary structure of proteins (Linderstrom Lang, 1952a). Thus the view that proteins consist of long chains of amino acids linked together by peptide bonds, sometimes associated with other complex materials, and sometimes on their own, has come to be generally accepted.

(ii) Biological Function of Proteins: In 1897 Buchner prepared an extract from yeast which, though free from cells, still carried out the fermentation of sugar

to alcohol. By this experiment he demonstrated that some "ferments" or enzymes could be separated from cells and still catalyse specific reactions which had earlier been thought to be inseparable from living matter. The crystallisation by Sumner in 1926 of the enzyme urease, and of various proteolytic enzymes by Northrop during the 1930's (Northrop, Kunitz & Herriott, 1948), and their identification as specific proteins of high molecular weight, led to the gradual realisation that enzymes are proteins. Indeed no enzyme is known which is not protein in nature.

Besides their function as biological catalysts, proteins have been shown to play an important structural role. Thus collagen forms the organic matrix of bone and keratin serves a similar function in hair. The contractile process in muscle is due to the interactions of specific proteins. Certain of the hormones of the pancreas, thyroid, parathyroid and pituitary glands are proteins, as are the antibodies formed by the body in response to infection, or introduction of foreign antigens into the circulation. Proteins such as haemoglobin and myoglobin serve a respiratory function as carriers and stores for oxygen, whilst other proteins serve as electron carriers. The deoxyribonucleic acid in the nucleus of cells is associated with basic protein in the form of nucleo-protein. In the membranes of the cell protein is found in association with lipid and carbohydrate. Though by no means exhaustive this gives some impression of just how proteins are of prime importance in the economy of living cells.

Nor are proteins stable in living systems. The concept of the dynamic equilibrium of body proteins, the early history of which has been summarised by a former Professor of Physiological Chemistry in this University (Cathcart, 1912) gained considerable support from the researches of Schoenheimer and his

associates (Schoenheimer, 1942), and information has continued to accumulate. Thus it has been found that in rabbits the turnover rate of the enzyme aldolase is about one per cent per day (Simpson & Velick, 1954). Most of the specific proteins of an organism are undergoing continuous synthesis and breakdown even in the "resting" state.

II. Protein Structure

Protein structure can be conveniently divided into primary, secondary, and tertiary structure as suggested by Linderstrom-Lang (1952a), and extended to quaternary structure by Bernal (1958). As mentioned earlier, the _primary structure_ refers to the conventional chemical structural formula, expressed as the sequence of amino acids along the polypeptide chain, together with any other covalent bonding, such as through disulphide bridges. Present views on protein synthesis suggest that the genetic information encoded in deoxyribonucleic acid determines only the amino acid sequence - the three-dimensional conformation of the protein is derived _in vivo_ from the sequence. That this is indeed so receives strong support from the fact that many proteins can undergo the process of "reversible denaturation". The physical properties of such thoroughly denatured proteins indicate that no covalent structure exists apart from the amino acid sequence itself, and that the molecule is present in solution as a randomly oriented coil. The reversal of the unfolding process to regenerate the native three-dimensional conformation of the protein suggests that no information other than the sequence itself is required to determine the native conformation of the protein.

The spacial relationships between residues in the protein chain, particularly any patterns of regularity, such as the formation of helical structures, is referred to as the secondary structure. In the case of fibrous and globular proteins, two extreme configurations of the polypeptide chain have been proposed. These are the α- helix (Pauling, Corey and Branson, 1951) and the random coil (Flory, 1953). The α-helix involves hydrogen bonding between peptide amino and carboxyl groups, which are disrupted when the helix is converted to the random coil.

Superimposed on the helical configuration of the secondary structure is a higher level of structural order - the coiling of the protein into a compact three-dimensional conformation to give the tertiary structure. Here again a variety of interactions between side chain groups are possible including hydrophobic bonds, electrostatic interactions, hydrogen bonds, Van der Waal's forces, and steric effects. Since secondary and tertiary structure are both aspects of folding of the polypeptide chain, they are not always readily distinguishable.

The association of units, folded in three-dimensional conformations, occasionally by means of disulphide bridges, but in many cases by non covalent bonds, into relatively stable aggregates is termed the quaternary structure. Such units are normally capable of an independent existence. The first such evidence for quaternary structure was in the case of tobacco mosaic virus protein (Schramm and Zillig, 1955; Fraenkel-Conrat and Singer, 1959) where the protein coat surrounding the viral ribonucleic acid consists of 2,200 identical sub-units each containing 158 amino acid residues (Anderer, 1963; Caspar, 1963).

Another example of chains held together by non covalent bonding is haemoglobin (Steinhardt, 1938; Perutz, Rossmann, Cullis, Muirhead and Will, 1960), but in this case the chains are not identical. Certain enzymes such as glutamic dehydrogenase (Olson and Anfinsen, 1952) and aldolase (Grazi, Chen and Horecker, 1962) have also been shown to consist of aggregates.

III. Amino Acid Analysis in the Determination of Protein Structure

(i) Introduction: With the isolation of homogeneous proteins, the study of the structure of individual proteins became possible. Present concepts of protein structure are discussed in another section. For the moment it will suffice if we consider the first step in the determination of protein structure, namely the determination of the amino acids present in the hydrolysates of proteins. A highly purified protein, well characterised by a variety of methods, is hydrolysed to free amino acids, and the number of residues of each amino acid in the hydrolysate is determined. If the molar ratios approach integral values this may be taken as additional evidence for the homogeneity of the sample. The absence of one or more amino acids from the hydrolysate may also be a useful indication of homogeneity. Thus, for example, the complete lack of isoleucine in highly purified samples of human haemoglobin A (Stein, Kunkel, Cole, Spackman and Moore, 1957) served as evidence of its homogeneity.

The work of Sanger (1959) on insulin illustrates the advantages that can be gained from studying small peptides obtained after enzymic and partial acid hydrolysis of proteins. The sum of the amino acid residues present in the purified peptides must be equal to the amino acid composition of the whole protein.

Hirs, Moore and Stein (1956) showed from the study of peptides formed after digestion of ribonuclease with trypsin that the analysis of the protein was in error by two amino acid residues.

The determination of the sequence of amino acids in peptides usually requires several techniques, but common to all is the use of quantitative amino acid analysis. Thus at every stage in the determination of the primary structure of a protein, quantitative methods for the analysis of amino acids are essential.

At this point it may be well to outline the steps involved in obtaining an amino acid analysis of a peptide or protein (Martin & Synge (1945), Block (1945), Tristram (1949), Moore & Stein (1963)):

(i) preparation of sample for hydrolysis and estimation of ash, moisture, nitrogen and sulphur content.

(ii) Hydrolysis of the protein to free amino acids.

(iii) estimation of the free amino acids.

(iv) determination of tryptophan, cystine and cysteine by independent methods.

(v) calculation of amino acid composition as molar ratios.

(vi) calculation of weight and nitrogen recoveries.

Some of these steps will be discussed in detail, emphasis being placed on currently preferred procedures, and preference being given to quantitative methods suitable for the amino acids present in proteins.

(ii) <u>Preparation of Material for Analysis</u>: Before submitting a protein to analysis it must satisfy standard tests of protein homogeneity.

These include tests based on physical properties of which the main ones are ultracentrifugation, electrophoresis and solubility tests. In the case of enzymes there are additional tests based on catalytic activity which may serve to confirm the physical tests. The solubility test (Northrop, Kunitz and Herriott, 1948) is probably the most sensitive for detecting minor components. However, once satisfactory criteria of purity have been established it is necessary to account for the protein in terms of its components (nitrogen, sulphur, phosphorus etc.) and weight in order to evaluate the data from subsequent analyses for completeness of recovery of all components.

(iii) Hydrolysis of Proteins: The use of strong acid for protein hydrolysis dates from at least 1820 when Braconnot used sulphuric acid. It was found that losses of various amino acids during the course of the hydrolysis could be reduced by using a large excess of acid (Linderstrom-Lang, 1952b). Hydrolysis procedures in standard use are described by Kimmel, Markowitz & Brown (1959), Mahowald, Noltmann & Kuby (1962), and Moore & Stein (1963). Ikawa & Snell (1961) observed ninhydrin positive artefacts when the hydrolysate was evaporated over sodium hydroxide. Hindrance of hydrolysis of peptide bonds involving valine, leucine, alanine and tryptophan in simple peptides was observed by Synge (1945). Despite this, acid hydrolysis using 6N hydrochloric acid is the method of choice. Tryptophan completely decomposes on acid hydrolysis of most proteins, whilst serine, threonine, and certain other amino acids decompose slowly during prolonged acid hydrolysis. The amides, glutamine and asparagine are also destroyed in the course of acid hydrolysis.

Alkaline hydrolysis, using sodium hydroxide or saturated baryta, leads to racemisation of amino acids, and partial or complete destruction of arginine, cysteine, threonine and certain other amino acids (Block, 1945).

However, tryptophan is not destroyed by alkali, and procedures for complete hydrolysis using 4N barium hydroxide, which may be used for its determination, have been described (for example, Noltmann, Mahowald & Kuby, 1962).

In order to circumvent the difficulties caused by the decomposition of certain amino acids during hydrolysis by acid or alkali, some workers have suggested the use of enzymic hydrolysis. To achieve a complete breakdown of a protein by enzymic means is a difficult and time consuming business. Hill and Schmidt (1962) carried out a hydrolysis of a number of proteins using papain followed by treatment with leucine aminopeptidase and prolidase. They suggest that from considerations of experimental convenience, it cannot replace acid hydrolysis in protein analysis. Tower, Peters & Wherrett (1962) obtained a partial hydrolysate of protein with the aid of a mixture of enzymes produced by activation of desiccated, defatted pancreas (Viokase). This partial hydrolysate is suitable for microbiological assays, by means of which asparagine, glutamine, glutamic acid and aspartic acid may be determined. The technique was applied to a number of peptides and proteins with quite satisfactory results. However, it appears that neither of these procedures are successful in completely hydrolysing the protein to amino acids. From the results obtained on insulin and ribonuclease, it seems that it is difficult to attain complete liberation of amino acid residues adjacent to cystine in native proteins. Another obstacle to the use of enzymic hydrolysis is the very real danger of autolysis, leading to liberation of amino acids in the hydrolysate from the enzymes themselves as well as from their substrate.

In conclusion, it is fair to say that acid hydrolysis is the most satisfactory general method of hydrolysis. It is now usual to study the yield of different amino acids liberated from a protein after different periods of time.

If destruction of an amino acid occurs, then the concentration can, assuming first order kinetics, be determined by extrapolation to zero time. If there is no evidence of destruction a mean value is obtained. Finally if an amino acid is difficult to liberate maximal values will be obtained after long periods of hydrolysis. All recent work on amino acid analysis (as for example, Tristram and Smith, 1963) shows that accurate results can be obtained if attention is paid to conditions of hydrolysis; if hydrolyses are carried out for varying times and the amino acids known to be unstable are estimated by extrapolation to zero time; and if separate estimations of certain amino acids (tryptophan, cysteine and cystine) are carried out on the intact protein, or on protein that has been hydrolysed by an alternative procedure.

IV. Quantitative Amino Acid Analysis

When Mitchell and Hamilton surveyed the subject of amino acid analysis in 1929 they observed that only half of the then known amino acids could be determined with any accuracy. Yet today the analysis of amino acids in protein and peptide hydrolysates is a routine procedure in most Biochemistry Departments. Before proceeding to a discussion of methods in current use it will be useful to observe the way in which amino acid analysis has proceeded in the past.

(i) Gravimetric Methods: In Emil Fischer's study of amino acid derivatives (1901) he observed that the ethyl esters of certain of the amino acids could be separated by distillation in *vacuo*. The fractions secured in this way could then be hydrolysed and the individual amino acids separated by a process of fractional crystallisation. Brazier (1930) and Damodaran (1931), working in Schryver's laboratory, established procedures for the fractionation of amino acids in hydrolysates of zein and glutenin using fractional crystallisation after

group separation of the copper and zinc salts. Previously Kossel and Kutscher (1900) had suggested a method whereby arginine, histidine and lysine could be estimated. The amino acid content of a protein was simply based on the yield of isolated material and the analytical procedures required up to 100 g. of protein.

The accuracy of such gravimetric methods was considerably improved by introduction of the isotope dilution method. If an amino acid containing an excess of a particular isotope is added to a mixture containing that amino acid with normal isotopic proportions, the quantity of the amino acid in a mixture can be inferred from the proportion of the isotope in the amino acid after isolation and purification. Rittenberg and Foster (1940) analysed amino acid mixtures by this isotope dilution method using ^{15}N amino acids. For this procedure complete recovery of amino acid is not required but complete purification of the isolated compound is essential. The method is capable of analysis with errors of one per cent, better than most methods in use at present. However this advantage is considerably outweighed by the fact that the materials required are expensive and that the method is cumbersome and time consuming.

(ii) Enzymic and Microbiological Methods: Enzymes specific for certain amino acids have been useful for the direct determination of these amino acids in protein hydrolysates without the necessity of prior isolation. Thus, for example, Hunter and Dauphinee (1930) estimated arginine by conversion to ornithine and urea through the action of arginase. Tower, Peters and Wherrett (1962) have used an enzyme preparation from *Clostridium perefringens* for the assay of dicarboxylic acids and their amides.

Microbiological methods for amino acid analysis (Snell, 1945) depend on knowledge of the nutritional requirements of bacteria, and are successful with organisms having an absolute requirement for a particular amino acid to support normal growth. An assay consists of allowing bacteria to grow on a medium deficient in the amino acid under study, and observing the rate of growth when graded amounts of the test material are added.

The disadvantage of this latter method is that care must be taken to ensure that the strains of microorganism used do not change in their nutritional requirements. Microbiological assays have the advantage of permitting multiple analyses in a relatively short time by very simple techniques.

(iii) Colorimetric and Spectrophotometric Procedures: Colorimetric assays have been extensively used for estimation of amino acids. The basic principle is the formation of a coloured compound, the intensity of the colour being related in some regular way to the concentration of the substance under assay. The colour reaction is specific to the amino acid to be estimated and so other amino acids present either in the intact protein, or in a hydrolysate do not interfere.

The three amino acids phenylalanine, tyrosine and tryptophan display characteristic absorption bands in the ultraviolet region of the spectrum between 250 and 300 m.u. (Beaven and Holliday, 1952) and so may be determined by a spectrophotometric assay procedure. Although these techniques are very sensitive they are nowadays generally only used for the estimation of tryptophan (Harrison and Hofmann, (1961) describe a colorimetric assay, and Henning, Helinski Chao and Yanofsky (1962) a spectrophotometric estimation)& very occasionally for cysteine (Boyer 1954) in proteins and protein hydrolysates.

Brand and his associates combined a variety of techniques, involving colorimetric, gravimetric and microbiological procedures to effect a complete analysis of β-lactoglobulin and of several other proteins (Brand, 1946). However, the development of chromatographic and electrophoretic methods for the separation of the amino acids in a mixture and the application of the ninhydrin reaction for their detection and quantitative estimation has led to these earlier methods falling into disuse, and amino acid analysis is now almost exclusively carried out by chromatographic methods.

(iv) Electrophoresis: Electrophoresis at high voltages has been applied by a number of workers for the separation and estimation of amino acids. Atfield and Morris (1961) were able to carry out a complete separation of all the protein amino acids using electrophoresis at three different pH values. The method has proved of particular value in investigations of amino acid sequence, where a limiting factor may be the rate at which quantitative amino acid analyses can be carried out. Thus Ambler (1963) used high voltage electrophoresis for amino acid analysis of peptides from *Pseudomonas fluorescens* cytochrome C_{551} since the automatic amino acid analyser could only measure seven samples per week, and the number of samples requiring analysis was often more than this. Electrophoresis of amino acids has recently been reviewed by Blackburn (1965).

(v) Paper and Thin Layer Chromatography: The introduction of partition chromatography in 1941 by Martin and Synge was followed in 1944 by the classical paper of Consden, Gordon and Martin who combined the technique of chromatography on paper with a distribution method based mainly on liquid-liquid partition.

Earlier investigation of chromatography on paper had been essentially due to adsorption by the paper. These workers also introduced the technique of two dimensional chromatography on filter paper sheets. This method was applied in the first instance to amino acids and has subsequently been developed for their quantitative analysis. A number of procedures for quantitative determination of amino acids by paper chromatography have been described, one such being the method of Tigane, Wade, Wong and Hanes (1961).

Chromatography on thin layers of silica gel or kieselguhr has been shown to be a convenient method for the separation of mixtures of hydrophilic compounds. It possesses two main advantages over chromatography on paper, namely that there is less broadening of the spots produced and that there can be much greater speed of analysis. Because of this reduction in diffusion smaller amounts of material can be detected on thin layer chromatograms, and separations may be achieved in a shorter length of time. The application of TLC for analysis of amino acids has recently been reviewed by Brenner, Niederwieser and Pataki (1964).

(vi) Estimation of Amino Acid Derivatives: Sanger (1945) introduced 2, 4 - dinitro-fluorobenzene as a reagent for the identification and analysis of the amino terminal residues of proteins. Levy (1954) applied the method to amino acids in protein hydrolysates and developed a two dimensional separation by paper chromatography. This method can be used to estimate 0.1 μmole of each amino acid per chromatogram from protein hydrolysates. The sensitivity of the method has been increased by Whitehead (1961) who applied the isotope dilution technique using tritium labelled 2, 4-dinitro-fluorobenzene and known quantities of dinitro-phenyl ^{14}C derivatives of the amino acids added before separation of the dinitro-phenyl amino acids.

Brenner, Niederwieser and Pataki (1964) have discussed the separation of dinitrophenyl amino acids by TLC, whilst Satake, Matsuo and Take (1965) have described a procedure for the automatic analysis of amino acids and peptides with dinitro-fluorobenzene using a Technicon autoanalyser.

Sjoquist (1960) applied the phenylthiohydantoin procedure of Edman (1950) to the analysis of amino acid mixtures, using paper chromatography or column partition chromatography to separate and estimate these derivatives. The precision of analysis with 0.5 to 1 mg. of protein is quite good. Laver (1961) has discussed the use of the method for determination of the amino terminal amino acid of peptides and proteins using ^{35}S labelled phenylisothiocyanate. As little as 10^{-4} μmole of amino acid could be detected.

(vii) <u>Column Chromatographic Methods</u>:

A. <u>Partition Chromatography</u>: Partition chromatography of N-acetyl amino acids on silica gel columns was developed by Martin and Synge (1941) and by Tristram (1946). A separation of free amino acids on a starch column was achieved by Moore & Stein at the Rockefeller Institute in New York (Stein & Moore, 1948; Moore & Stein, 1948a, 1949) and these workers were also responsible for the development of a quantitative procedure for analysis of amino acids with ninhydrin (Moore & Stein, 1948b). The separation was carried out on a column of potato starch using butanol/water for prior treatment of the column and n-butanol/n-propanol/0.1N HCl (1:2:1) and n-propanol/0.5N HCl (2:1) as solvents and an automatic fraction collector (Stein & Moore, 1948) was used for collecting the large number of small fractions required by the procedure. The fractions collected in this way were then processed by hand, treated with ninhydrin and their optical density determined.

A complete separation using these conditions required 2.5 mg. of protein and took 8 days. The analysis of two proteins by this method was reported - B-lactoglobulin and bovine serum albumin. (Moore and Stein, 1949). The resolution of a protein hydrolysate on a single column was inadequate and three starch column procedures were required for a complete analysis.

(B) Ion Exchange Chromatography: The groups of S.M. Partridge and his collaborators at Cambridge and R. Consden, A.H. Gordon and A.J.P. Martin working at Leeds, deserve much of the credit for the development of ion exchange chromatography for separating mixtures of amino acids from protein hydrolysates. The latter developed a method for separating glutamic acid from aspartic acid and for their separation together from a protein hydrolysate (Consden, Gordon and Martin, 1947). Ion exchange resins were also used to separate amino acid mixtures into basic, acidic and neutral fractions (Block, 1949). However, it fell to Moore & Stein to perfect ion exchange chromatography as the method for amino acid analysis. A complete resolution of the amino acids commonly found in proteins was achieved by Moore & Stein (1951) using columns of sulphonated polystyrene resin, Dowex 50, with 8% cross linking. A single column of resin in the sodium form was used with buffers of pH 3.4 to 11 and a temperature range of 25° to 70°. This gave quantitative recoveries for all of the amino acids with the exception of the basic amino acids and tryptophan, which could however be estimated using a short column operated in the pH range 6 to 6.8.

The resolution achieved with ion exchange columns was much greater than with starch columns and gradual improvements of the method, such as the use of fine mesh, 4% cross-linked resin, increased length of column and the use of a discontinuous gradient elution, enabled Moore & Stein to separate a synthetic mixture of fifty components on a 150 cm. column (Moore & Stein, 1954).

The use of small particle size resin separated by a hydraulic method (Hamilton, 1958) permits faster flow rates and gives higher resolution. Spackman, Stein and Moore (1958) adapted the system to automatic operation and used a recorder to obtain a trace of the amino acid concentration in the column effluent. Buffer is pumped through the column at a fixed rate, the column effluent is mixed with a stream of ninhydrin reagent, and the colour developed by passing through a reaction coil at 100°. Thestream from the coil passes through a colorimeter; the photocell potential is plotted on an automatic recorder. Usually three colorimeters are used in series, one at 570 m.μ. the wavelength at which the product of the reaction between amino acids and ninhydrin has its absorption maximum; one at 440 m.μ. for estimation of the yellow colour produced by proline and hydroxyproline, and the third at 570 m.μ. in a cuvette of one third the normal width. This last trace enables calculation of the concentration of amino acids that are off scale on the first trace. An analysis using the 100 cm. column by this procedure, along with a short column analysis for the basic amino acids and tryptophan takes about 22 hours.

Automatic analysis of all the amino acids using a single column of Dowex 50-X12 resin with a continuous gradient produced by using a Varigrad (Peterson and Sober 1959) was described by Piez and Morris (1960). The time of the analysis has been cut, generally by employing smaller and smaller resin particles and faster and faster buffer flow rates through resin columns. Thus, the method was cut to six hours by Spackman (1963) and to four hours by Benson & Paterson (1964) and by Spackman (1964).

Recently, Hubbard (1965) has reduced the time required for a complete analysis to two hours, and by the use of high sensitivity cuvettes (Hubbard & Kremen 1964) was able to obtain a chromatogram by this method using 0.1 mg. of ribonuclease hydrolysate. This enables five analyses to be carried out per working day. The sensitivity of the method has been greatly increased by Dus, Dekker and Smith (1965) who have obtained an analysis of up to six samples simultaneously; four of these samples may contain as little as 1-10 nanomoles of each amino acid. This same group in San Diego (Dus, Lindroth, Pabst and Smith, 1966) have recently described the application of a simple and inexpensive rotary valve mechanism which is used in two different steps in the column chromatography procedure: the stepwise four buffer elution programme and the sequential selection of columns. The system can be used for uninterrupted and unattended sequential operation of a series of columns, since sample application and column regeneration are integral parts of the elution programme. With this arrangement six complete amino acid analyses can be carried out automatically each day, using a sample application procedure similar to that described by Crestfield (1963).

(vii) Conclusion: In conclusion it can be seen that:

1. a wide variety of methods for analysis of amino acids are available. Qualitative paper chromatography, TLC and colorimetric procedures are very useful for certain problems.

2. for reasons of speed, convenience and precision, it is clear that automatic amino acid analysis by ion-exchange chromatography is the most widely applied method for routine work in association with studies on protein structure.

3. a serious disadvantage of automatic amino acid analysers is, however, that the apparatus is expensive, and the running costs are quite high.

In this context, the possibilities of application of GLC to amino acid analysis were clearly worth investigating since:

(1.) when this investigation was begun amino acid analysis required 22 hours, and GLC was potentially much faster than this. Even though accelerated analyses have been developed in the past three years to reduce the analysis time to 2 hours, GLC is still potentially faster than ion-exchange chromatography.

(2.) the apparatus required for GLC is of much more general applicability than the amino acid analyser.

(3.) the capital cost and the running costs of a gas chromatograph are low compared to the amino acid analyser.

It was for these reasons that the investigation of the application of GLC to amino acid analysis was begun.

V. Gas-Liquid Chromatography

Although the use of an inert gas as the mobile phase in partition chromatography was envisaged by Martin and Synge in 1941, it was not until 1952 that the practical application of this form of chromatography (gas-liquid chromatography, GLC) was described by James and Martin for the separation of fatty acids. Since that time the area of application of GLC has expanded enormously and its value in analytical chemistry is attested by the large and rapidly expanding literature on the subject.

The characteristic feature of gas-liquid partition chromatography is the use of an inert gas such as nitrogen, argon, or helium as the mobile phase in a partition column which is frequently operated at an elevated temperature. The solutes are transported through the column in the vapour state. The stationary phase is usually a high boiling point liquid supported on a relatively inert porous material such as kieselguhr (Celite, or ground firebrick). Resolution of the components of the mixture occurs as a result of their varying distribution ratios between the gaseous and the liquid phase. The distribution mechanism is basically similar to that of liquid-liquid partition chromatography, but owing to the inert character of the mobile phase and the elevated operating temperature, interactions between mobile and stationary phases and between the solutes and the mobile phase are relatively unimportant.

The advantages of using a gaseous mobile phase as compared with liquid phases are:

1. The low viscosity of the mobile phase permits the use of very long columns (up to 150 ft. with packed columns and upwards of 1,000 ft. with unpacked capillary columns) of narrow diameter, while retaining high flow rates. Also the column geometry does not materially affect the resolving power of the column so that tightly coiled spirals, or columns bent into any shape required to fit the oven being used can be constructed.
2. Equilibrium between mobile and stationary phase is rapidly attained, and this results in high column efficiencies.
3. A variety of special methods are available for the detection and estimation of organic compounds in the vapour state in the presence of an inert carrier gas.

Most of these methods can be adapted to continuous recording, so that after the introduction of the analytical sample, subsequent operations can be made automatic, and a permanent record of the separation can be obtained. The detectors used in GLC possess a greater sensitivity than most chemical analytical methods. The generality of detection is in sharp contrast to most liquid partition systems, where detection is usually colorimetric and is more or less specific.

VI. GLC of Amino Acids

(i) Introduction: Early applications of GLC were restricted to volatile compounds but it soon became apparent that the method could be applied to non-volatile compounds by making them more volatile and reducing their polarity prior to GLC, through removing or masking functional groups by oxidation, acylation, alkylation, or through other means. Once this has been done, quite as much information can be gathered about these compounds by chromatography in the vapour phase as is obtainable with more volatile compounds.

Progress in the analysis of amino acids by this technique has been slow, principally because they are not volatile owing to their zwitterion structures. Thus, alanine with a molecular weight of only 89, melts at 297°, and on more intense heating chars. Obviously such compounds must be converted to derivatives in which the carboxyl group, the amino group, or both, are removed or masked, before they can be chromatographed in the gas phase.

This will eliminate charges, reduce hydrogen bonding, and so render the molecules more volatile, and indeed there are a number of potentially useful methods available for this purpose.

The application of GLC to analysis of amino acids has recently been reviewed by Potteau (1965), by Karmen and Saroff (1964) and by Weinstein (1966).

(ii) GLC in quantitative Amino Acid Analysis: So far as quantitative analysis of amino acids is concerned, it appears that unless the following conditions are met, the method will not be suitable for quantitative work:

(1) it is essential that reproducible yields of derivative be obtained. and preferable that the yields should be high.

(2) the losses of certain amino acids (by decomposition or fixation on the column support) should be reduced to a minimum and be constant.

(3) the detector response should be linear over the range of concentrations employed, or if not it must be accurately evaluated.

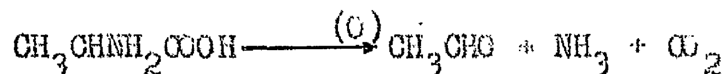
(4) the general applicability of the method must be considered - whilst no difficulties may result from work on made up amino acid mixtures, there may be interference from volatile constituents in mixtures of biological origin.

In fact, as will be seen from the subsequent review it is doubtful whether any of the methods currently described in the literature meets all of these criteria.

(iii) Preparation of Volatile Derivatives and Resolution by GLC: Essentially, two main approaches have been made to this problem. On the one hand there are procedures which produce compounds amenable to gas chromatography through degradative reactions. On the other, there are methods which rely on the synthesis of amino acid derivatives having higher molecular weights than their precursors, but with increased volatility owing to their lower polarity. We shall consider each of these in turn.

A. Derivatives Produced by Degradative Procedures:

Foremost among the methods which rely on producing suitable compounds through degradative reactions is the oxidation of amino acids to aldehydes having one less carbon atom. Deamination accompanies oxidation. The product from alanine, for example, is acetaldehyde.



$$CH_3CHNH_2COOH \xrightarrow{(O)} CH_3CHO + NH_3 + CO_2$$

Langheld (1909) showed that this reaction could be carried out by treatment with alkaline hypochlorite. This was the reagent used by Bayer (1958) to oxidise alanine, 2 - amino-butyric acid, norvaline, norleucine and leucine. He obtained a good separation of the resulting aldehydes by chromatography on a dinonyl phthalate column at 92°.

Oxidation of amino acids with ninhydrin is one of the most extensively studied chemical reactions of amino acids and is frequently employed for the quantitative estimation of total amino nitrogen in hydrolysates, either colorimetrically or by measurement of the carbon dioxide liberated. Virtanen and Rautanen (1947) have shown that the formation of aldehydes is quantitative by determining the aldehydes obtained by distillation of the volatile reaction products by titration. This result has been confirmed by Hunter and Potter (1958) who also employed a distillation procedure. However, Bier and Teitelbaum (1959) found that only about eight amino acids were amenable to gas chromatographic analysis by this method, due to lack of volatility of the aldehydes. Hunter, Dimick and Corse (1956) used a modification of the method of Virtanen and Rautanen to prepare aldehydes derived from valine, leucine, isoleucine and alanine. The aldehydes derived from leucine and isoleucine could be separated on a silicone-celite column at 69°.

Zlatkis and Oro (1958) and Murato and Tackenishi (1961) have likewise carried out work on the aldehydes produced by the action of ninhydrin on amino acids, whilst Baraud (1960) distilled the volatile aldehydes into alkaline permanganate and oxidised them to their corresponding carboxylic acids. These he recovered as their sodium salts and after esterification separated them by GLC on a poly(propylene glycol adipate) column at 150°. Under the conditions of preparation, only amino acids that yield volatile aldehydes can be detected, and this clearly eliminates the use of these methods for amino acid analysis.

Yet another method of obtaining suitable derivatives for GLC is to decarboxylate the amino acids. There are available specific decarboxylating enzymes which will convert certain amino acids to the corresponding amines possessing one less carbon atom but for general application a chemical method is required. Abderhalden and Gebelein (1926) found that by heating tyrosine with diphenylmethane at 240°, a 95% yield of tyramine was obtained. Bier and Teitelbaum (1959) studied the decarboxylation of amino acids by p - dimethyl - aminobenzaldehyde and by diphenylmethane; although the yields of amine were not quantitative the amines could be separated by gas chromatography. The decarboxylation method was applied to sixteen amino acids, but cysteine, arginine, histidine, tryptophan, aspartic and glutamic acids could not be analysed by this method.

Liberti (1958) deaminated the amino acids with sodium nitrate and, after methylating the resulting hydroxy acids, ran these on columns of sterchamol coated with 30% of silicone. The system works well, but only for these amino acids which react with sodium nitrate i.e. the α-amino acids.

Conversion of the amino acids to α-chloro-acids has been carried out with some success by Renard and his collaborators (Melamed and Renard, 1960; Mathieu and Renard, 1961). The action of hydrochloric acid and nitric acid on α-amino acids is to produce the corresponding α-chloro-acid, which can be used for GLC, although better results are obtained by converting this to the methyl ester with diazomethane. The method has been applied to hydrolysates of casein, edestin, fibrinogen, gelatin, gliadin, gluten, ovalbumen and zein, using columns of polyethylene glycol or silicone oil-stearic acid. Only a small number of amino acids may be analysed in this way, and no evidence for quantitative estimation is presented.

The application of GLC has made possible the isolation and quantitative determination of the nitriles formed in the reaction between N-bromosuccinimide and amino acids (Stevenson and Luck, 1961; Konigsberg, Stevenson and Luck, 1960). The volatile products that resulted from the reaction of N-bromosuccinimide with alanine, 2-amino butyric acid, norvaline, valine, leucine and phenylalanine were quantitatively analysed. However, the method has not been applied to the other sixteen protein amino acids.

In connection with mass spectrometric studies of peptides, Biemann and Vetter (1960) have analysed mixtures of di-and tripeptides by converting them to amino alcohols prior to GLC. N-acetyl ethyl esters were prepared first, and these were reduced by overnight treatment with lithium aluminium hydride. The peptides were chromatographed on a column coated with 8% of Apiezon L at a temperature of 260°. Weinstein and Fenselau (1964) have reduced amino acids to the corresponding amino alcohols with lithium aluminium hydride and using either methylsilicone fluid (SF-96) or methyl phenyl silicone gum (SE-52) have separated seven different amino alcohols in a very short time period. It is unlikely that this could be extended to all of the protein amino acids.

In conclusion, these degradative procedures have not been extensively studied and are unlikely to be of use in quantitative analysis of the protein amino acids. The methods are not sufficiently general to be applied to all of the amino acids, and it seems unlikely that any of these methods would give results that could be relied upon to give the required volatile derivative in quantitative or reproducible yields. For these reasons they have been abandoned by most workers in favour of the technique of masking polar groups, to which subject attention is now directed.

B. Synthetic Procedures

(i) Esterification of the carboxyl function: We now turn our attention to those methods which rely on the preparation of volatile derivatives of amino acids in which either one or both of the functional groups is masked by a suitable substituent. The earliest attempt was that of Bayer (Bayer, Reuther and Born, 1957; Bayer, 1958) who converted the amino acids to their methyl esters, as was done by Fischer (1901) in his classic work on their separation by fractional distillation. Bayer achieved the separation of the methyl esters of valine, norvaline, leucine and norleucine on a silicone column at 138°. On this same column at 191° he obtained good resolution of the methyl esters of glutamic acid and phenylalanine. In the methanolysate of albumin he was able to show the presence of esters of alanine, proline, valine, leucine and isoleucine at 140° and of aspartic and glutamic acids at 187°. The silicone grease used as stationary phase contained 10% of sodium caproate to reduce tailing. Bayer states that the esters of aliphatic, aromatic, sulphur-containing and heterocylic amino acids are well separated rapidly. Whilst this is true in specific cases, esters of many of these amino acids do not appear on the chromatograms.

Thus, under certain conditions the Bayer technique can be used to separate alanine, proline, valine, leucine, isoleucine, methionine, phenylalanine, aspartic and glutamic acids. However, threonine, lysine, arginine, tyrosine, hydroxyproline, tryptophan and histidine are not accounted for. It is possible that additional esters could be recovered by modifying operating conditions; for example, some of the derivatives might not be sufficiently volatile to be eluted at the column temperatures used. Another modification would be to increase the temperature so that the aliphatic esters are eluted at low temperatures, and aromatic and heterocyclic esters at higher temperatures - the disparity in molecular weight between amino acids such as glycine and tryptophan would indicate that such a step would be desirable.

Subsequent to this work of Bayer which appeared to promise at best only a partial analysis of amino acids, two papers have been published by Saroff and his collaboraters (Saroff, Karmen and Healy, 1962; Nicholls, Makisumi and Saroff, 1963). Although earlier attempts to apply the principle of dissociation of ammonium salts of the esters of amino acids to gas phase analysis had resulted either in failure, or in very poor results, subsequent re-investigation enabled derivatives of all of the common amino acids except histidine, tryptophan and tyrosine to be analysed by GLC. The free basic form of the methyl esters were either generated by the use of anion exchange resins and subjected directly to analysis or by allowing the acetate salts of the amino acid esters to dissociate into the free base and acetic acid on the gas chromatographic column under the influence of temperatures varying from 120° to 200°. The free bases, as well as the salts, were chromatographed on columns coated with 2% of neopentyl glycol succinate (NPGS). The methyl esters were very stable in solution and exhibited equivalent stability during the course of gas phase analysis.

The possibility of diketopiperazine formation in the column was ruled out in the case of glycine by comparing the behaviour of the methyl ester of glycine and glycine anhydride. Table 1 shows the retention data for the methyl esters as the free base, acetate and chloride salts. Whether this method could be developed to include all of the common amino acids is not certain since no data for tyrosine, tryptophan and histidine are given, but clearly it has the advantage that only one step is required to prepare the amino acids for GLC, and that step can be carried out quantitatively.

(ii) Trimethylsilyl Derivatives: Yet another derivative which can be prepared by means of a single step process is the trimethylsilyl derivative. Ruhlmann has been responsible for this approach (Ruhlmann 1961; Ruhlmann and Geiseke, 1961; Ruhlmann and Michael, 1963). The compounds are prepared by treating amino acid salts with trimethylchlorosilane, or by the reaction of N-trimethylsilyldialkylamines with free amino acids. Both amino and carboxyl groups react. The N-trimethylsilyl trimethylsilyl esters of glycine, alanine, leucine, isoleucine, valine, glutamic acid and phenylalanine can be separated on a 30% column of silicone oil at 165°. The phenylalanine derivative is eluted after 28 to 30 minutes. On a column of 19% silicone oil at 200°, aspartic acid, hydroxyproline, glutamic acid and methionine all have the same retention time. Results are claimed to be reproducible to within 0.5% using peak areas with a thermal conductivity detector. It is stated that all available amino acids could be silylated almost quantitatively, but no data are given for chromatography of such amino acids as histidine, arginine and tryptophan. The N-silyl esters are extremely sensitive to minute traces of moisture, and very great precautions must be taken, in order to avoid their destruction to some extent.

TABLE I

Gas chromatographic behaviour of Amino Acid Methyl Esters

onditions: 6ft.,3/16in.I.D., packed with 2% neopentyl glycol succinate on Fluoropak 0. Gas flow 60 ml./min. Sample size 0.25 umole. The recorded temperature was that f the preheater and column.

	Free Base [a]		Acetate Salt [a]		Hydrochloride Salt	
	Temp. ^{0}C	Retention time(min)	Temp. ^{0}C	Retention time(min)	Temp. ^{0}C	Retention time (min)
eucine [b]	120	4.3	120	4.3	120	4.7
	175				175	0.1
roline	115	5.2	115	5.2	115	5.5
	175	0.6	175	0.6	175	0.7
erine [c]	150	3.6	150	3.6	150	no peak
	175	2.4	175	2.4	175	2.9
spartic Acid [d]	150	4.8	150	4.8	150	6.7
	175	2.0	175	2.0	175	2.3
henylalanine [e]	155	9.0	155	9.0	155	11.0
	180	6.9	180	6.9	180	7.1
ydroxyproline	175	5.7	175	5.7	175	6.5
	195	2.9	195	2.9		
ysteine	175	negative result	175	1.9	175	2.0
ystine	180	6.7	180	6.7	180	6.7
ysine [f]	180	3.5	180	negative result	180	negative result

Except for cystine, lysine and arginine, all esters gave similar peak areas for both free base and acetate salt.
Alanine, glycine, valine and isoleucine gave results as for leucine.
Threonine gave results as for serine.
Glutamic acid gave results as for aspartic acid.
Methionine gave results as for phenylalanine.
Arginine gave results as for lysine.

From the data available it is difficult to assess the potential of this method, but it seems that the technical difficulties involved may be too great to allow its use routinely.

(iii) Phenylthiohydantoins and Dinitrophenyl Derivatives: Pisano, Vanden Heuvel and Horning (1963) have separated the phenylthiohydantoin (PTH) and dinitrophenyl (DNP) derivatives of the amino acids by GLC using thin film packings of several silicone polymers. (SE-30, QF-1 and PhSi) Most of the PTH derivatives chromatographed well, but serine, threonine, asparagine, glutamine, arginine and lysine were not eluted. All of the amino acids chromatographed except leucine and isoleucine could be separated on the SE-30 column and this separation could be achieved on the QF-1 column. Table 2 presents the relative retention times.

DNP derivatives were separated after conversion to methyl esters; good results were obtained for the simple neutral and acidic amino acids. However, serine, threonine, tryptophan, tyrosine, histidine and derivatives of the basic amino acids were not chromatographed with any success.

Ishii and Witkop (1963, 1964) used a combination of enzymatic degradation and quantitative gas chromatography (using methyl esters of DNP amino acids on columns of SE-30 at 175°) to assign the configuration of four of the six amino acids found in hydrolysates of gramicidin A and to demonstrate the heterogeneity of the preparation. They found the method rapid, sensitive and quantitative. A further possibility which makes the DNP method rather attractive is that Landowne and Lipsky (1963) were able to detect DNP amino acid methyl esters using an electron capture detector in amounts of the order of 3×10^{-16} moles/sec. in the effluents from columns coated with polyester or silicone phases at 220°.

TABLE 2

Relative Retention Times of PTH and DNP Amino Acids

Conditions: 1% of phase on 100-140 mesh Gas Chrom P; 6 ft x 3.4-5.0 cm. glass columns; argon ionisation detector.

Amino Acid	1% SE-30 PTH	1% SE-30 DNP°	1% QF-1 PTH	1% QF-1 DNP°	1% PhSi PTH	1% PhSi DNP°
	175°	175°	175°	194°	200°	200°
Alanine	0.81	1.93	0.63	2.33	0.35	0.44
Glycine	1.03	2.24	0.79	3.05	0.54	0.67
Proline	1.18	3.76	1.14	4.15	0.59	1.16
Valine	1.46	2.63	0.80	2.67	0.40	0.49
Leucine	1.80	3.23	1.21	3.30	0.53	0.56
Isoleucine	1.80	3.61	1.10	3.37	0.55	0.62
(Androstane)	1.00 9.3min	1.00 3.3min				
(Cholestane)			1.00 11.7min	1.00 4.7min	1.00 9.7min	1.00 9.7min
	200°	202°				
Aspartic acid	2.12°	5.14	1.89°	6.95		1.47
Glutamic acid	3.19°	7.88	2.31°	10.8		2.01
Methionine	3.95	8.80	3.04	6.77	2.03	2.32
Phenylalanine	5.38	12.4	3.52	11.6	3.10	3.94
(Androstane)	1.00 4.2min	1.00 3.3min				
(Cholestane)			1.00 2.7min	1.00 4.7min	1.00 9.7min	1.00 9.7min
	250°		255°		220°	
Tyrosine	1.12		2.77		9.67	
Histidine	1.29		4.07			
Tryptophan	2.84		5.46			
(Cholestane)	1.00 8.2min					
(Cholesterol)			1.00 1.3min		1.00 1.3min	

° Chromatographed as methyl esters.

The advantages of the use of an electron capture detector are:

(a) the minute quantities of material which can be detected.

(b) the uniform response of the detector to all of the DNP derivatives tested.

(c) the inherent selectivity of the detector eliminates interference from many impurities.

The advantages that would accrue from the determination of DNP and PTH amino acids by GLC in end group and sequence analysis of peptides are considerable.

(iv) N-formyl methyl esters: Losse, Losse and Stock (1962) have carried out some studies on N-formyl amino acid methyl esters. The formylation was carried out according to the method of Du Vigneaud, Dorfmann and Loring (1932) and the methylation using diazomethane. Derivatives of alanine, valine, proline, glutamic acid, methionine and phenylalanine were separated in 40 mins. at 194° on a column of 35% silicone oil. The lowest yield quoted by these workers is of the order of 97%. Heyns and Grutzmacher (1961) have reported some work on the mass spectrometry of these compounds.

(v) N-acetyl esters: Youngs (1959) carried out a study on N-acetyl n-butyl esters, since he found that the N-acetyl ethyl ester of glycine tended to crystallise out from mixtures so that homogeneous samples could not be taken. A synthetic mixture of N-acetyl butyl esters of glycine, alanine, valine, leucine, isoleucine and proline could be chromatographed though leucine and isoleucine were not resolved. These same components were tentatively identific in gelatin hydrolysates, and a number of unidentified peaks appeared after proline.

Johnson, Scott and Meister (1961) investigated the suitability of the N-acetylated methyl, n-butyl, isobutyl, n-amyl and isoamyl esters for GLC and concluded that the N-acetyl n-amyl esters gave better separations than the other compounds tried. Seventeen naturally occurring amino acids could be separated by chromatographing the N-acetyl n-amyl esters on columns packed with Carbowax 1540 at different temperatures. Table 3 shows the retention times obtained on an eight foot column which contained 1% of Carbowax 1540 on Chromosorb W. The column temperature was increased sharply, after the emergence of leucine, to 148°.

Less volatile esters could be separated on a 2 ft. column: the peaks representing glutamic acid, tyrosine and lysine emerged in 15 to 30 minutes. All but three of the important naturally occurring amino acids (arginine, histidine and tryptophan) could be chromatographed; arginine and histidine were esterified in poor yield and the acylation did not proceed smoothly. The method has not been adapted for quantitative analysis.

Subsequent to this work, Teuwissen, Lenain, Derlet and Leonis (1962) have published some of their results with N-acetyl amyl esters, but they appear to have little to add to the rather more detailed study by the former group.

It is interesting to note that Shlyapnikov, Karpeiski and Litvin (1963) who carried out a study of the separation of N-acetyl esters on columns coated with Apiezon grease, or polyethylene glycol adipate, found that the most complete and rapid separation of derivatives of glycine, alanine, amino butyric acid, valine, norvaline, leucine, isoleucine, norleucine, proline, serine, methionine, aspartic acid and phenylalanine was achieved with n-propyl esters on an Apiezon L column. They examined ethyl, n-propyl, isopropyl, n-butyl and amyl esters of N-acetyl amino acids.

TABLE 3

Retention times of N-Acetyl Amino Acid n-Amyl Esters

Conditions: 8 ft. column packed with Chromosorb W coated with 1% Carbowax 1540. Flow rate - 60ml/min. Starting temp. 125° - after 23 min. the temp. was abruptly increased to 148°C.

Amino Acid	Retention time mins.	Amino Acid	Retention time mins.
Alanine	14	Allothreonine	34
Valine	16	Threonine	35
α-Aminobutyric	17	Serine	39
Alloisoleucine	18	α-Aminoadipic	40
Isoleucine	19	Cysteine	44
Norvaline	20	S-Methylcysteine	47
Leucine	22	Methionine sulphone	49
β-Aminoisobutyric	23	Methioninesulphoxide	51
Norleucine	23	Methionine	51
Glycine	24	Ethionine	55
Pipecolic	26	Phenylalanine	60
β-Alanine	26	Hydroxyproline	69
β-Aminobutyric	28	Allohydroxyproline	75
Proline	29	Aspartic	84
γ-Aminobutyric	32	Glutamic	102
Ornithine	33	Tyrosine	132

Derivatives of arginine, histidine and tryptophan were not successfully chromatographed.

Graff, Wein and Winitz (1963) reported work on the quantitative estimation of amino acids by GLC as N-acetyl n-propyl esters. The derivatives were separated on columns coated with polyethylene glycol, and a high degree of quantitative precision was claimed for the preparation of derivatives. This work appeared as an abstract (April, 1963) and there has been no subsequent publication by this group. There is not sufficient evidence available to permit a critical assessment of this method and its potential.

(vi) N-trifluoroacetyl esters: (a) N-TFA methyl esters

The earliest studies involving N-TFA methyl esters were carried out on columns coated with silicone grease and polyesters (Bayer, 1958, Weygand, Kolb, Prox, Tilak and Tomida, 1960) Fluorine containing compounds have a relatively high vapour pressure (Weygand, Geiger and Swodenk, 1956) and so the retention times on the silicone column were unusually short, which resulted in crowding of peaks. By using two columns in series Weygand et al. (1960) obtained partial resolution of a mixture of ten amino acids. The method was also used to separate simple peptides. Weygand and Geiger (1959) had previously done some work on the gross distillation of protein hydrolysates as N-TFA amino acid methyl esters.

Saroff and Karmen (1960) obtained improved yields by using sulphonated polystyrene resin as the catalyst for formation of the methyl esters. They found that fourteen of the more common amino acids gave single peaks on columns of polyethylene glycol adipate at temperatures between 160 and 215°. Table 4 shows the retention times expressed as the ratio of derivative time to that of methyl stearate under identical conditions. Separations on other polyester columns were found to be similar although alanine/valine and glutamic acid/serine were not resolved.

TABLE 4

Retention times of N-Trifluoroacetyl Amino Acid Methyl Esters

Conditions: $\frac{1}{4}$"x6ft. column packed with 22% polyethylene glycol adipate on Chromosorb W. Linear temperature 160-215°. Argon carrier gas 80 ml/min. Ionisation detector - preheater block used.

Amino Acid	Temp. (approx)	Derivative time/ methyl stearate time
Alanine	160	0.059
Valine	160	0.059
Isoleucine	160	0.076
Leucine	160	0.100
Glycine	160	0.12
Proline	160	0.22
Aspartic Acid	190	0.34
Threonine	190	0.41
Methionine	190	0.71
Serine	190	0.79
Glutamic Acid	190	0.82
Phenylalanine	190	0.96
Hydroxyproline	215	1.4
Lysine	215	2.7

Tyrosine and arginine gave multiple peaks which may have been due to decomposition of the derivatives in the preheater block. Cystine, histidine and tryptophan gave negative results.

These derivatives were also investigated by Wagner and Winkler (1961) and by Ikekawa (1963). The latter separated the N-TFA methyl esters of 15 amino acids on columns of NPGS and SE-30 connected in series. The analysis took 50 minutes, and the retention times are shown in Table 5. Serine and methionine were not well resolved and arginine, cystine and histidine were not eluted. A linear relation between peak area and amount was shown for alanine (using a flame ionisation detector).

Cruickshank and Sheehan (1964) reported a complete separation of the N-TFA methyl esters on an NPGS column. They showed that different derivatives were obtained with hydroxyamino acids depending on whether they were first acylated and then methylated (monoacyl derivative) or vice versa (diacyl derivative). The optimum separation was achieved with a 2 ft. column and any lengthening of the column resulted in a poor separation of threonine and glycine. Figure 1 shows a typical chromatogram of the separation achieved by these workers and Table 6 gives the retention times. The method was applied quantitatively to a ribonuclease hydrolysate.

Hagen and Black (1964; 1965) separated the same derivatives of nineteen amino acids on two columns - one of Carbowax 1540 and one of Carbowax 20M. There appeared to be a constant relationship between the amount of each amino acid and the size of the recorded peak, indicating either that quantitative conversion to the derivative had been achieved or that a constant proportion of each amino acid was converted to the acyl ester.

TABLE 5

Retention Times of N-Trifluoroacetyl Amino Acid Methyl Esters

Conditions: 150cm. x 4mm. stainless steel column packed with Gas Chrom P coated with NPGS or SE-30. Detector temperature 230°, Flash heater 230°. a. N_2 - 60ml/min. b. 65ml/min. c. 75ml/min. d. 50ml/min.

	150cm 1% NGS				75cm. 1% NGS + 75cm. 1.5% SE-30			
	a Isothermal		Programmed at 4°/min.		d Isothermal		Programmed at 4°/min.	
Amino Acid	Time min.	Temp. °C	Time min.	Temp. °C	Time min.	Temp. °C	Time min.	Temp. °C
Alanine	2.0	110c	4.0		0.7	147	3.1	
Valine	2.3	110	4.5		0.8	147	3.7	
Glycine	4.2	110	7.0	116	1.0	147	5.2	112
Leucine	4.8	110	7.6		1.15	147	6.2	
Proline	2.0	147d	9.9	128	1.8	147	8.3	125
Aspartic Acid	3.4	147	13.7		2.8	147	11.6	
Threonine	4.9	147	15.3		3.7	147	13.4	
Serine	7.2	147	18.0	160	5.3	147	15.4	143
Methionine	7.2	147	18.0		5.6	147	15.8	
Glutamic Acid	8.6	147	18.9		6.5	147	16.6	
Phenylalanine	9.6	147	19.5	167	7.6	147	17.5	160
Hydroxyproline	1.4	220c	26.6		14.8	147	24.1	
Lysine	2.8	220	32.1	223			30.6	208
Tyrosine	6.7	220	38.8				36.3	
Tryptophan	15.8	220	50.6	225			44.0	225

FIGURE I

Separation of N-TFA amino acid methyl esters on a 2 ft. x 1.5 mm. stainless steel column packed with Gas Chrom P coated with 5% of NPGS. Flow rate - 18 ml./min. Temperature increased from 65° at 1.5^{o}/min. for 20 mins.; then at 2°/min. for 22.5 mins.; and then at 4°/min. to a maximum temperature of 210°. Sample size - 2 µl. Concentration of each amino acid adjusted to keep all peaks on scale without attenuation.

(Cruickshank and Sheehan, 1964).

FIGURE 1

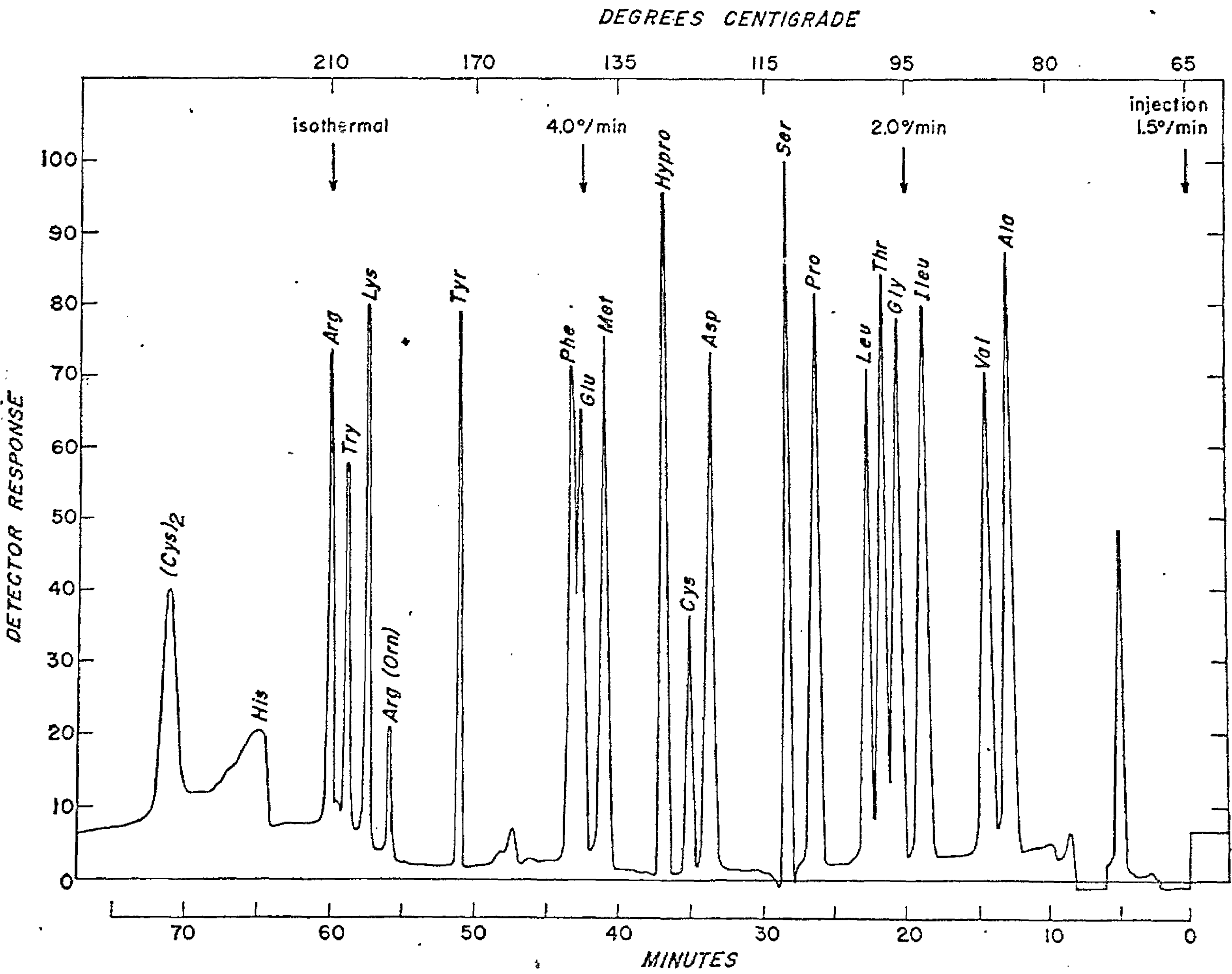


TABLE 6

Retention Times of N-trifluoroacetyl Amino Acid Methyl Esters

Conditions: 2 ft. x 1.5 mm stainless steel column packed with 80 - 100 Gas Chrom P coated with 5% NPGS. Argon flow rate - 18 ml/min. Sample injected at 65°, temperature increased by 1.5°/min for 20 mins; then by 2°/min for 22½ min; then at 4°/min. to final temperature of 210°.

NTFA methyl ester	Retention Time (mins.)
Alanine	12.5
Valine	14.0
Isoleucine	18.4
Glycine	20.1
Threonine	21.3
Leucine	22.4
Proline	26.3
Serine	28.3
Aspartic Acid	33.6
Cysteine	35.0
Hydroxyproline	36.7
Methionine	41.0
Glutamic Acid	42.6
Phenylalanine	43.4
Tyrosine	51.0
Ornithine	57.5
Lysine	55.9
Tryptophan	58.8
Arginine	60.0
Histidine	64.0-67.0
Cystine	70.8-71.3

More recently Makisumi and Saroff (1965) have synthesised and purified those derivatives for each of the twenty protein amino acids and characterised their gas chromatographic behaviour. The conditions for resolution of each of these using a three column system designed in the National Institutes of Health have been outlined. A complete description of this system for quantitative amino acid analysis is to be published (Saroff, 1966).

(vii) (b) N-TFA n-Butyl esters: The first complete GLC separation of all of the common protein amino acids was reported by Zomzely, Marco and Emery (1962). 1% NPGS was used, and the N-TFA n-butyl esters of these amino acids were separated in about forty minutes. Table 7 shows the retention times observed. The chromatogram shows considerable base line drift and aspartic acid and phenylalanine are poorly resolved. Preliminary studies have shown that the conversion of amino acids to n-butyl ester appears to be more than 90% complete, since infrared analysis of the crude free ester preparations showed the absence of carboxyl groups. However the yields in the trifluoroacetylation step do not appear to be good.

Marcucci, Mussini, Poy and Gagliardi (1965) have reported their studies on the separation of N-TFA n-butyl esters of eleven amino acids, using Dowex 50 resin as catalyst for the esterification. They showed by TLC that after conversion to n-butyl ester no unmodified amino acid could be detected.

The most complete study of these derivatives has been carried out by Gehrke and his collaborators at the University of Missouri. Lamkin and Gehrke (1965) reported that these derivatives had a number of advantages over other derivatives investigated, and developed a method for their preparation. Single peaks were obtained for all of the amino acids with the exception of tryptophan and arginine.

TABLE 7

Retention Times of N-trifluoroacetyl Amino Acid n-butyl Esters

Conditions: Column temperature increased from 75° to 220°; flow rate - 128ml/min; 2m x 6.3mm. stainless steel column packed with Gas Chrom A (60-120 mesh) coated with 1% NGS; N_2 as carrier gas; hydrogen flame detector; aspartic acid and phenylalanine poorly resolved.

Amino Acid	Retention Time mins.	Amino Acid	Retention Time mins.
Alanine	10.9	Aspartic Acid	24.3
Sarcosine	11.7	Phenylalanine	24.7
Valine	12.4	Cysteine	26.0
Isoleucine	14.5	Glutamic Acid	27.2
Glycine	15.7	Histidine	28.3
Leucine	16.4	Ornithine	30.8
Proline	18.4	Lysine	32.1
Serine	19.1	Arginine	32.7
Threonine	20.9	Tyrosine	35.3
Methionine	23.6	Tryptophan	38.8
Methionine sulphoxide	23.6	Cystine	41.9

Tryptophan could however be converted into a single derivative by longer acylation. Complete resolution of a mixture of 19 amino acids taken simultaneously through the analytical and chromatographic procedure was achieved using a mixed stationary phase of diethylene glycol succinate DEGS and EGSS-X (ethylene glycol succinate chemically combined with a methyl silicone). An acylated sample of arginine methyl ester, added to the mixture prior to chromatography, was also separated (Gehrke, Lamkin, Stalling and Shahrokhi, 1965) Table 8 details the retention behaviour observed, and Figure 2 shows a chromatogram of a separation.

A quantitative procedure for the acylation of arginine without decomposition to ornithine has been reported by Stalling and Gehrke (1966). Subsequent investigation (Stalling and Gehrke, 1966) showed that when the gas chromatograph was equipped with a metal flash heater, and a solution of arginine n-butyl ester hydrochloride dissolved in trifluoroacetic acid was introduced onto the column, two peaks were produced - the desired volatile derivative was being synthesised in the flash heater. A method was developed for acylation of the n-butyl ester of arginine in a sealed tube at 150°, and it was shown that this could be used for the other 19 protein amino acids without any loss of yield. Thus a method for quantitative analysis of amino acids as their N-TFA n-butyl esters has been developed.

(vi) (c) N-TFA n-amyl esters: Prior to their investigation of the N-TFA n-amyl esters Blau and Darbre (1963) examined esters of dimethyl amino acids prepared by the method of Bowman (1950) and the products obtained by esterification of the amino acids with most of the lower alcohols as well as cyclohexanol, and of acylation to form acetyl, propionyl, formyl and trifluoroacetyl derivatives.

TABLE 8

Gas Chromatographic Behaviour of N-trifluoroacetyl Amino Acid n-butyl esters

Conditions: 1m.x0.4mm. glass column, packed with 60/80 Chromosorb W and mixed substrate phases of 0.75/0.25% of DE65 and F65S-X. Column temperature programmed from 67° to 218° at 3.3°/min. Flow rate of nitrogen - 38 ml./min.

Amino Acid	Retention Temperature
Alanine	80°
Valine	87°
Isoleucine	94°
Glycine	96°
Leucine	101°
Threonine	107°
Proline	110°
Serine	117°
Cysteine	127°
Hydroxyproline	130°
Methionine	133°
Phenylalanine	137°
Aspartic Acid	140°
Glutamic Acid	150°
Tyrosine	160°
Lysine	175°
Tryptophan	180° (a) 203° (b)
Arginine	189°
Histidine	195°
Cystine	215°

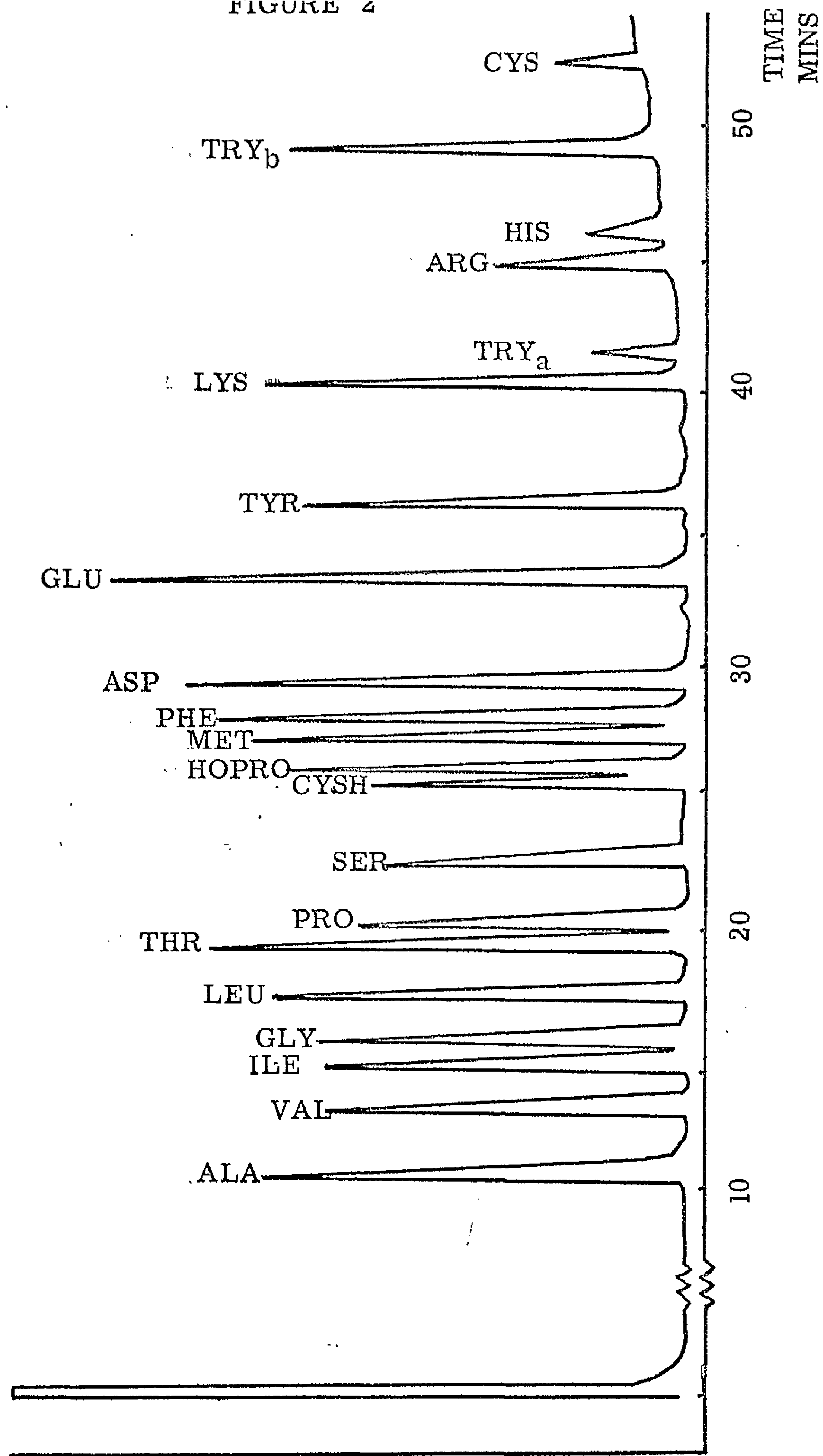
(a) - Diacyl derivative

(b) - Monoacyl derivative

FIGURE 2

Separation of N-TFA n-butyl esters of the amino acids on a 1 m. x 4 mm. glass column packed with Chromosorb W coated with DEGS/EGSS-X (0.75/0.25%). Flow rate - 38 ml./min. Temperature increased from 67° to 218° at 3.3°/min. Sample size - 5 μl. Each peak represents 2.5 μg. of amino acid. a. Diacyl derivative b. Monoacyl derivative - (Gehrke et. al., 1965).

FIGURE 2



DETECTOR RESPONSE

The dimethylation reaction was not found to be sufficiently general (French and Edsall 1945) and Blau and Darbre concluded that the N-TFA esters of the lower alcohols were so volatile as to make it impossible to obtain quantitative recoveries of the derivatives of alanine, valine, glycine and leucine; extensive losses occurred even on evaporating ethereal solutions at room temperatures.

Since the reaction of amino acid hydrochlorides with trifluoroacetic anhydride was rapid, convenient and gave excellent yields, a suitable esterification reaction was sought, and the choice fell on n-amyl alcohol. Esterification was carried out first, since the N-TFA group was found to be subject to hydrolysis during esterification. Optimum conditions for quantitative conversion of the aliphatic amino acids to those derivatives were determined.

A gas density balance was used as the detector in order that the detector response would be linear. This detector has the advantage that the area of any peak can be directly related to the weight of compound applied, provided its molecular weight is known (Phillips and Timms, 1961). Unfortunately with this detector the problem of separation has been tackled isothermally and it is aggravated by the fact that large amounts of sample must be applied, and so very broad peaks are obtained. Initially no single column was found that would separate the N-TFA n-amyl esters of alanine, valine, isoleucine, threonine, glycine, leucine and serine at temperatures of 140 - 150° (Darbre and Blau,1965) although recent work indicates that this separation can now be achieved on a single column (Blau, 1966). However, good resolution was achieved on a column of silicone MS710 of derivatives of leucine, cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid and glutamic acid (Blau and Darbre, 1965).

Teuwissen, Lenain, Derlet and Leonis (1962) also used N-TFA n-amyl esters and were able to identify the amino acids present in a hydrolysate of gramicidin and to determine their relative concentrations (since the peak areas were proportional to the concentration of the esters.) However, arginine, histidine, cysteine, cystine and tryptophan could not be determined by this method.

VII. Object of the Investigation

At the outset of this project in 1963, the situation was that although Zomzely et al (1962) had achieved a complete separation of the N-TFA n-butyl esters, and Graff et al (1963) had reported quantitative results, with the N-acetyl propyl esters, there was no method available for the quantitative estimation of amino acids by GLC.

In view of the potential advantages of gas chromatography over ion exchange chromatography in respect of speed of analysis, economy of operation, sensitivity of detection and resolving power, it seemed that the time was opportune for a thorough investigation of the pathways available for converting amino acids into volatile derivatives and their quantitative estimation by GLC. The investigation was limited principally to those amino acids which are commonly found in proteins: glycine, alanine, valine, leucine, isoleucine, serine, threonine, proline, hydroxyproline, cystine, cysteine, methionine, aspartic acid, glutamic acid, phenylalanine, tyrosine, tryptophan, lysine, histidine and arginine.

Initially the N-acetyl derivatives were studied using isothermal procedures. Subsequently, when temperature programming facilities became available, the N-TFA esters were investigated. The development of a method for quantitative analysis of amino acids by GLC and its application will be described and discussed.

MATERIALS

APPARATUS AND REAGENTS

1. Apparatus:

A Pye Argon Gas Chromatograph (W.G. Pye & Co. Ltd., Granta Works, Cambridge, England) with a -0.5 + 9.5 m.v. Honeywell Strip Chart Recorder (2 second full scale response) and a macro argon detector was used in the initial phase of this work. An argon line assembly for drying the carrier gas was used and is outlined in Figure 3. The instrument was modified by the construction of a preheater assembly which fitted on to the top of the chromatograph as shown in Figure 4, and was controlled by a Variac transformer. This necessitated the modification of columns for the instrument, which were altered as shown in Figure 5 by the addition of a T-shaped piece of tubing having two inlets, one of which was fitted with a silicone stopper having a through hole for use as a gas inlet and the other with a stopper of the same material, but with the hole only half way through, for use as a sample injection inlet. Argon was used as the carrier gas.

Subsequently a Perkin Elmer F-11 Gas Chromatograph (Perkin Elmer Ltd., Beaconsfield, Bucks., England) was obtained with a +2.5 to 0 m.v. Honeywell Strip Chart Recorder (1.2 second full scale response) and dual hydrogen flame ionisation detector. The instrument was used without any modification with nitrogen as carrier gas.

Samples were introduced for gas chromatography by means of a Hamilton 701N 10 microlitre (µl.) syringe (Hamilton Co., Inc., Whittier, Calif., U.S.A.) which could be read to 0.05 µl.

Other specialised equipment is described in those sections which deal with their use.

FIGURE 3

Argon Drier Assembly for Pye Argon Gas Chromatograph

The figure shows the arrangement of the argon line drier assembly used with the Pye Argon Gas Chromatograph.

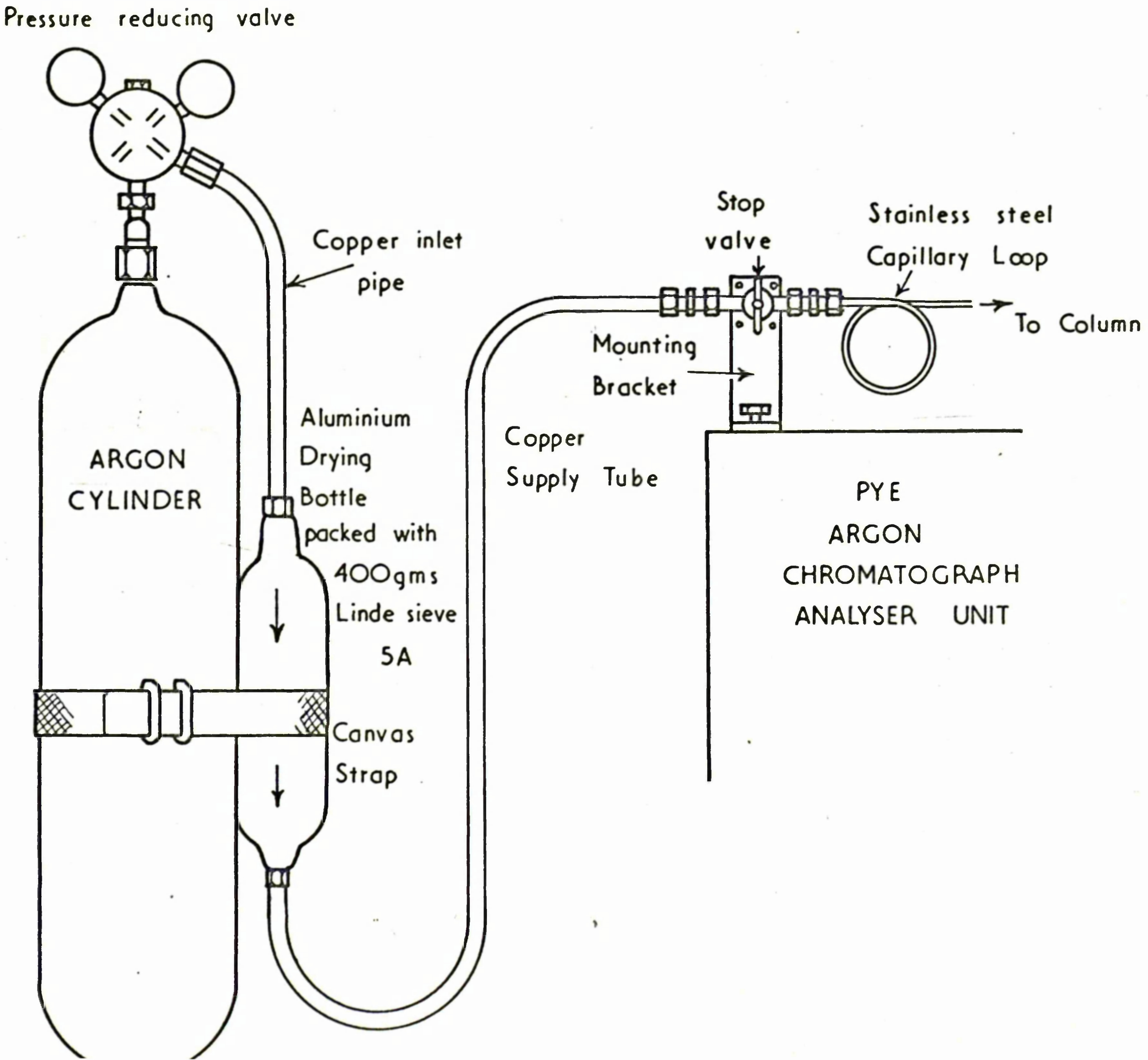


FIGURE 3

FIGURE 4

PREHEATER ASSEMBLY

The figure shows the design of the preheater assembly used with the Pye Argon Gas Chromatograph.

FIGURE 4

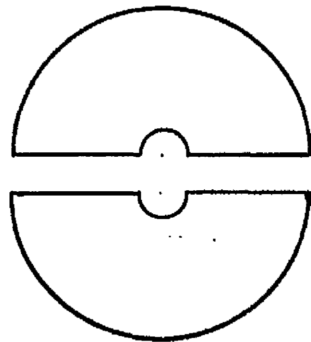
Top (Segmented)

Aluminium Block
(6" × 2" × 2")

1/2" diam. holes for columns

100 W. Cartridge heater

BLOCK

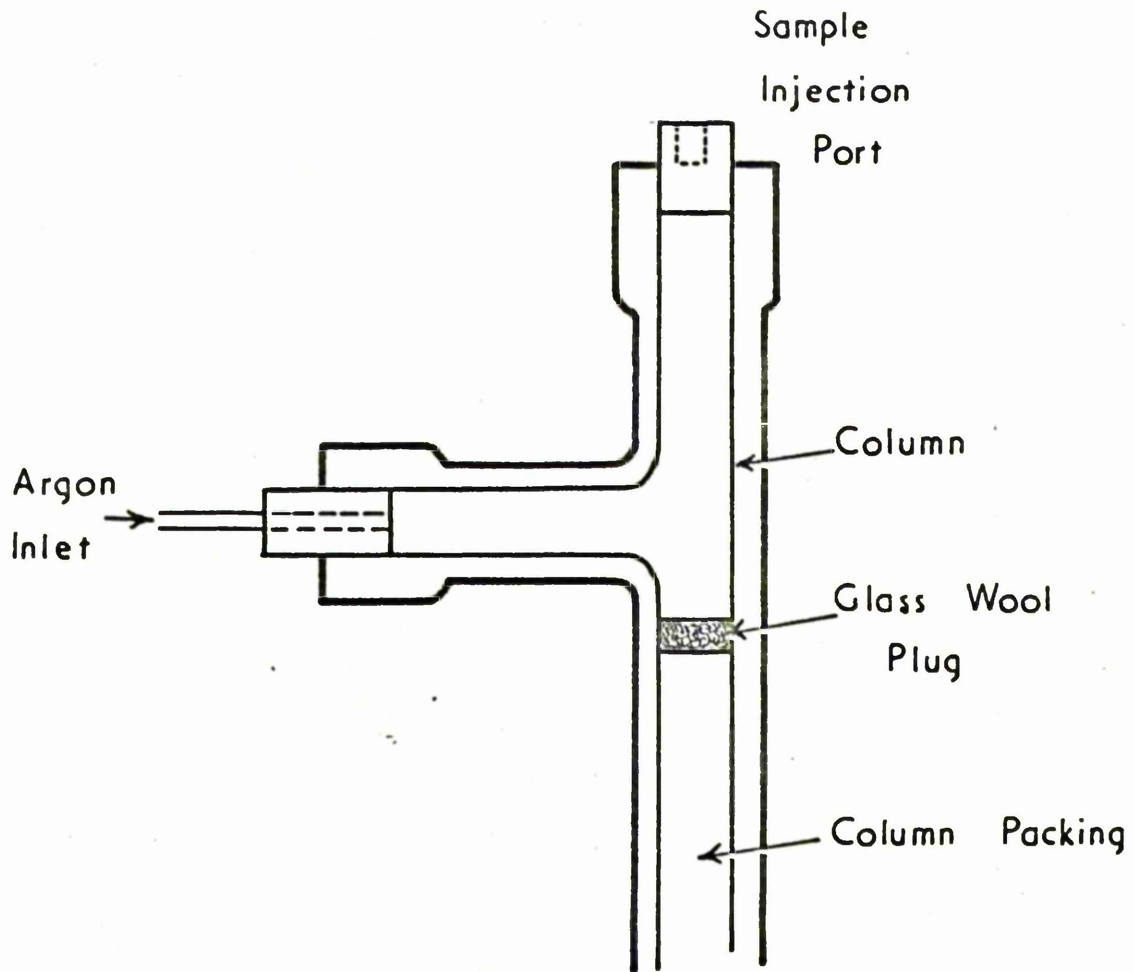


TOP

FIGURE 5

Modification of Columns for the Pye Argon Gas Chromatograph. The glass columns were modified by fitting a tube consisting of the T-junction shown to the straight column. The carrier gas entered via the silicone rubber stopper on the side limb, whilst the silicone rubber stopper on the top of the column had a hole halfway through the stopper, and served as a sample injection port.

FIGURE 5



II. Reagents:

All amino acids were purchased from the British Drug Houses Ltd., Poole, England with the following exceptions: L-lysine (L. Light & Co. Ltd., Colnbrook, England) and DL-threonine (Cambrian Chemicals Ltd., London, England). Amino acids used were either of the L- or DL- configuration, and no difference was observed in the chromatographic behaviour of derivatives prepared from L- or DL- amino acids.

Thionyl chloride, dimethyl sulphite, trifluoroacetic acid and trifluoroacetic anhydride were obtained from Eastman Kodak, Kirkby, Lancs., England. Nitrosan was obtained from Du Pont Co. (U.K.) Ltd., Fetter Lane, London, England and Diazald from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. All other reagents were Analar grade where possible.

To ensure that methanol was completely anhydrous, all methanol was dried by a modification of the method of Lund and Bjerrum (1931). 600 ml. of methanol was refluxed for two hours with 5 g. of magnesium turnings after allowing it to stand at room temperature until gas evolution had ceased, and it was then distilled in an all glass apparatus protected from atmospheric moisture by a calcium chloride drying tube.

Embacel (acid washed kieselguhr, 60 - 100 mesh) was obtained from May and Baker Ltd., Dagenham, England. Gas Chrom P, Z and Q were products of Applied Science Laboratories, State College, Penn., U.S.A. Stationary phases used in this investigation were obtained from Applied Science Laboratories and Table 9 enumerates these, and gives details of the supports.

Commercial preparations of cytochrome c (Seravac Labs., Colnbrook, Bucks.), insulin (British Drug Houses) and bovine serum albumen (Armour Pharmaceutical Co., Eastbourne, Sussex) were used.

TABLE - 9

(i) Stationary Phases used in the Investigation

PEGA	-	polyethylene glycol adipate.
NPGS	-	neopentyl glycol succinate polyester.
QF-1	-	trifluoropropyl methyl silicone polyester.
SE-30	-	methyl silicone polyester.
DEGS	-	diethylene glycol succinate polyester.
EGSS-X	-	ethylene glycol succinate polyester chemically combined with a methyl silicone.
EGSP-Z	-	ethylene glycol succinate polyester chemically combined with a phenyl silicone.
CHDMS	-	cyclohexane dimethanol succinate.

(ii) Supports used in the Investigation

Embacel	-	acid washed kieselguhr; 60 - 100 mesh.
Gas Chrom P	-	acid and base washed Celatom; 80 - 100 mesh.
Gas Chrom Z	-	acid washed, methyl dichlorosilane treated Celatom; 80 - 100 mesh.
Gas Chrom Q	-	acid and base washed, dimethyldichlorosilane treated Celatom; 80 - 100 or 100 - 120 mesh.

EXPERIMENTAL

EXPERIMENTAL

I. Preparation of Columns for GLC

(1) Preparation of Supports: In the initial stages of this investigation Celite 545 ('Embacel') was used; the mesh size was 60 - 100 and it had been acid washed to remove acid soluble material. The original column packings prepared with this material produced very unsymmetrical peaks when low concentrations of stationary phase were used, and since this was almost certainly due to adsorption of the solutes onto the support it was deemed advisable to pretreat the support in order to reduce the amount of adsorption and produce an inactive material.

Horning, Moscatelli and Sweeley (1959) recommend treatment of the support before addition of the stationary phase. They used dimethyldichlorosilane based on the procedure devised by Howard and Martin (1950). Bohemen, Langer, Perret and Purnell (1960) describe a method for treatment of columns *in situ* using hexamethyldichlorosilane, whilst the use of metallic silver is recommended by Ormerod and Scott (1959).

Embacel and Gas Chrom P were treated by the method of Horning, Moscatelli and Sweeley (1959) as follows: the dry support (25 g.) was placed in 100 ml. of a 5% solution of dimethyldichlorosilane in toluene in a Buchner flask. The pressure in the flask was reduced (by a water pump) for a period of a few minutes. The flask was shaken to dislodge bubbles from the surface of the support, and the pressure was allowed to return to atmospheric. The treated support was removed by filtration and washed with 100 ml. of toluene. It was then washed well with methanol, and after preliminary air drying, was dried at 80°.

In spite of such silanisation, skewness of peaks was sometimes observed, either because of inadequate treatment at the inactivation stage, or because of the development of active sites for adsorption during use. When this occurred, it was found that the column performance could be materially improved by treating the column packing with hexamethyldisilazane <u>in situ</u>. Horning, Vanden Heuvel and Creech (1963) recommended the injection of about 20 - 50 μl of hexamethyldisilazane into the column maintained at about 100° - 150° with a relatively low carrier gas pressure (5-10 psi.).

Recently the use of polyvinyl pyrrolidine has been proposed for the deactivation of supports for GLC work (Vanden Heuvel, Gardiner and Horning, 1963). However, the effect of coating the dry support with, for example, 2% of PVP, before coating with the stationary phase was generally to modify the behaviour of the stationary phase and so to alter the nature of the separation achieved with the column. This may be advantageous or disadvantageous. The support was coated with PVP as described in section (I (2) (ii)).

2. <u>Preparation of Column Packings</u>

(i) <u>The Procedure of Jones</u>: The precise amount of stationary phase required was dissolved in 30 - 40 ml. of a suitable solvent (acetone, chloroform, methylene chloride, toluene were used) placed in a 250 ml. round bottomed flask, and a weighed amount of support (Embacel or Gas Chrom P) was carefully added. The solvent was then removed on a warm water bath with application of moderate vacuum (water pump) and the flask very gently rotated periodically to ensure as even a distribution of the stationary phase as possible. It is essential that vigorous shaking be avoided since this results in excessive production of fine material, which adversely affects the flow of carrier gas through the column and also results

in low column efficiencies. After removal of the solvent, the coated support was heated for 30 - 60 minutes at 100° on a water bath under reduced pressure and was then ready for use.

(ii) <u>The Filtration Technique</u>: The evaporation of low boiling solvents on a rotary evaporator was not entirely satisfactory since it was difficult to obtain a uniform film of phase on the support and agitation of the mixture often led to the production of fine particles.

Horning, Moscatelli and Sweeley (1959) described a filtration procedure for preparing column packings, which necessitates the use of silanised support (Section I (i)). This was used in a modification proposed by Horning, Vanden Heuvel and Creech (1963).

The procedure involves addition of 50 ml. of a solution of liquid phase in an appropriate solvent to the support (9-12g) in a Buchner flask, (the concentrations used were usually 0.5 to 5% of phase in solution). The flask was maintained at reduced pressure (water pump) for a few minutes and gently shaken to dislodge bubbles from the surface of the support. The pressure was allowed to return to atmospheric and the mixture was allowed to stand for about five minutes. The slurry was then placed on a Buchner funnel with a rapid swirling motion of the flask, and the solution allowed to drain through the bed of support, suction being applied for about five minutes. At the end of this time the filtration process was complete, and the surface of the filter cake usually appeared to be damp but not wet. The coated support was spread

on a smooth surface for preliminary drying at room temperature. It was then dried in an oven at 80° - 100°. Column packings prepared in this way flowed freely and had the appearance of a powder.

When the filtration procedure is used for the preparation of thin-film column packings, the relationship between the concentration of phase in solution and the amount of phase on the support must be determined by experiment. For this purpose a 2 or 3 g. quantity of coated support was extracted exhaustively in a Soxhlet extractor, and the weight of phase determined after evaporation of the solvent. With Gas Chrom P, and with Gas Chrom Q it was found that the amount of phase on the support (w/w) is the same (to within 0.1%) as the concentration of phase in solution. If Celite 545 was used (Embacel) the amount of phase on the support was approximately twice the concentration of the phase in solution. These results are in agreement with the finding of Horning, Vanden-Heuvel and Creech (1963).

3. Packing of Columns:

(i) Columns for the Pye Argon Chromatograph were straight glass columns (4 ft. x 4mm.) and were packed by pushing a small plug of glass yarn down as far as the constriction near the bottom of the column. The column was held vertically with the bottom cone in contact with a vibrating glass rod (attached to a stirrer motor and fitted with a rubber tube to prevent breakage). Sufficient packing material to fill a 2.5 cm. length of column was added whilst the column was still vibrating and at the same time the outside of the column was tapped with a wooden ruler. This operation was repeated until a 15 cm. length of column had been packed. The column was then removed from the vibrator

and firmly tapped on the floor. After this the column was replaced in position and the above procedure repeated until the column was packed so that on insertion into the gas chromatograph the packing was just below the top of the heat reservoir bar. For the standard column (1383 mm. long) this meant that the packing was 8 cm. from the top of the column. Once packed, a 0.5 cm. plug of glass yarn was placed on top of the packing.

(ii) Columns for the Perkin Elmer F-11 were stainless steel 1/8th" outside diameter, 2mm. inside diameter coiled columns. These were packed by applying suction at one end of the column (water pump) and tapping the column firmly with a wooden ruler whilst slowly adding the support from a filter funnel at the open end of the column. Excessive vibration is to be avoided, since this may damage particles of the support producing new adsorption sites, but at the same time it is necessary to pack the column so that there are no discontinuities.

(iii) Conditioning of Columns: All columns were conditioned by allowing a slow flow of carrier gas (usually 10 - 20 ml. per minute) to pass through the column at the maximum temperature of operation of the column for 16 hours before the column was used.

4. Conditions for Gas-Liquid Chromatography:

Two instruments were used in this work with two types of detector.

(i) Macro Argon Detector (Pye Cat. No. 12216)

(ii) Flame Ionisation Detector (Perkin Elmer, Cat. No. 454-0061)

(i) Macro Argon Detector: Developed by Lovelock (1958) the Argon B ionisation detector utilised the B radiation from a ^{90}Sr source to produce a metastable state in the argon used as carrier gas. The presence of an organic

vapour in the column in amounts down to 2×10^{-12} mole results in a measurable ionisation current produced by the transfer of energy from the metastable argon atoms to the molecules of the organic compound. The detector is illustrated in figure 6. The detector is constructed of brass and contains a central cylindrical electrode that terminates in a small sphere. The electrode, which is a modified sparking plug, is insulated from the body of the detector by means of a ceramic sleeve. A radioactive source (strontium - 90) in the form of a foil is fitted concentric with the electrode. This detector is relatively insensitive to changes in temperature and flow rate.

(ii) Flame Ionisation Detector: McWilliam and Dewar (1958) introduced this detector which depends on the electrical conductivity of a flame burning a mixture of nitrogen and hydrogen in the presence of a combustible solute. A potential of up to 500V is applied between the flame and a negatively charged platinum electrode situated above it, and the resultant current recorded continuously. Figure 7 illustrates the basic design of the detector used. The detector is constructed of stainless steel and contains 2 flames, to enable maximum sensitivity and stability by its use as a differential detector. The electrode is insulated from the body of the detector by a teflon connection. The electrical conductivity of the flame burning hydrogen in air is low, but when an organic compound is fed into the flame a partial ionisation results and the conductivity increases. The system is relatively insensitive to flow rate changes, and is not affected by changes in the column temperature.

(iii) Operating Conditions: The column conditions are described in the appropriate part of the Results Section.

(iv) Calculation of Peak Areas: A triangulation method was used for determining peak areas. The area was estimated by determining the product of the

FIGURE 6

Macro Argon Detector used in Pye Argon Gas Chromatograph. The detector is constructed of brass and contains a central cylindrical electrode that terminates in a small sphere. The electrode, which is a modified sparking plug, is insulated from the body of the detector by means of a ceramic sleeve. A radioactive source (strontium-90) in the form of a foil is fitted concentric with the electrode.

FIGURE 6

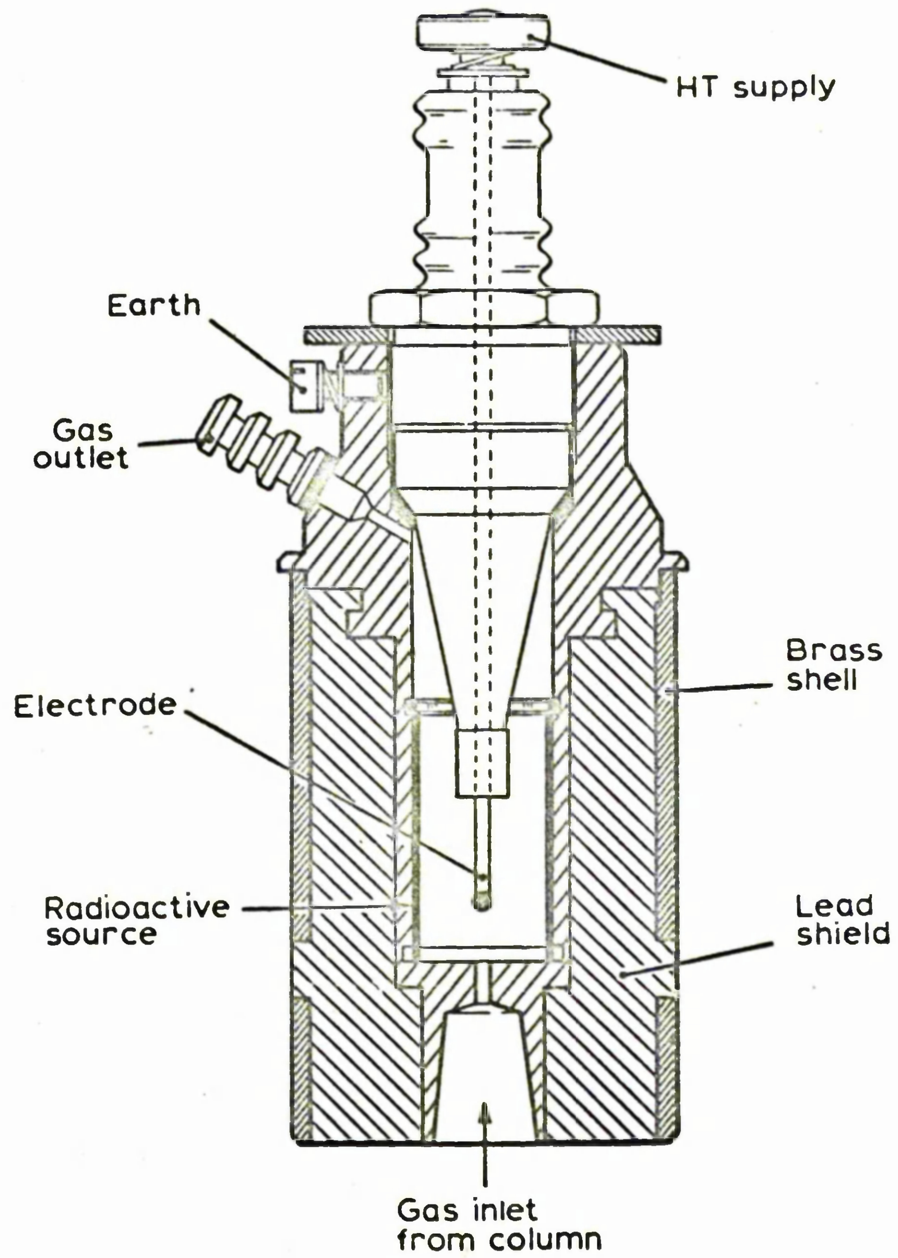


FIGURE 7a

Elevation of Flame Ionisation Detector used in the Perkin Elmer F-11 Gas Chromatograph. Carrier gas from the two columns, containing hydrogen which is introduced just beyond the end of the columns, passes to the detector jets where it is burned (in air, which enters at the side of the detector). The collecting electrode is held in position by an insulated support. The high tension supply to the detectors is via insulated connections on the side of the wall.

FIGURE 7a

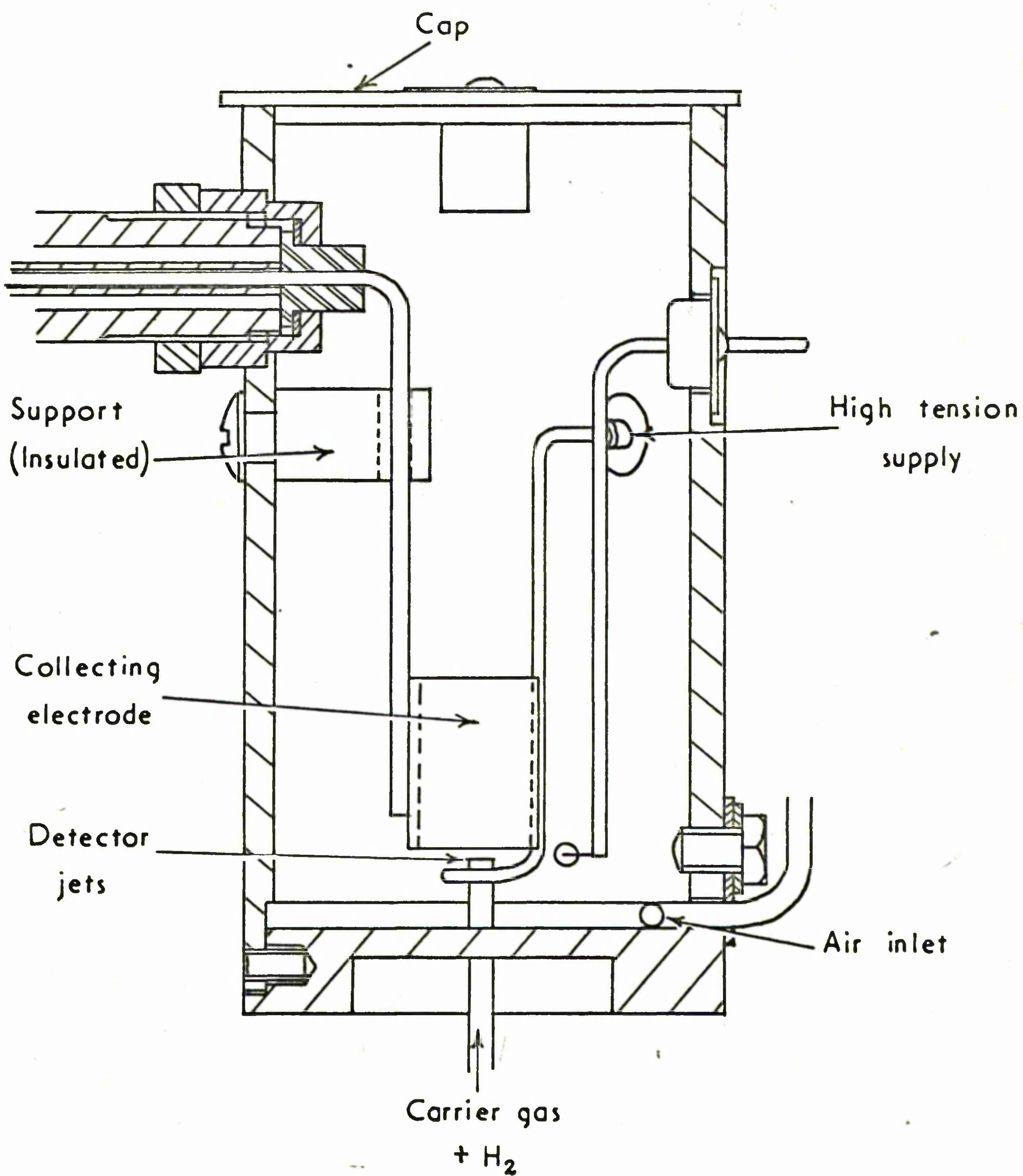
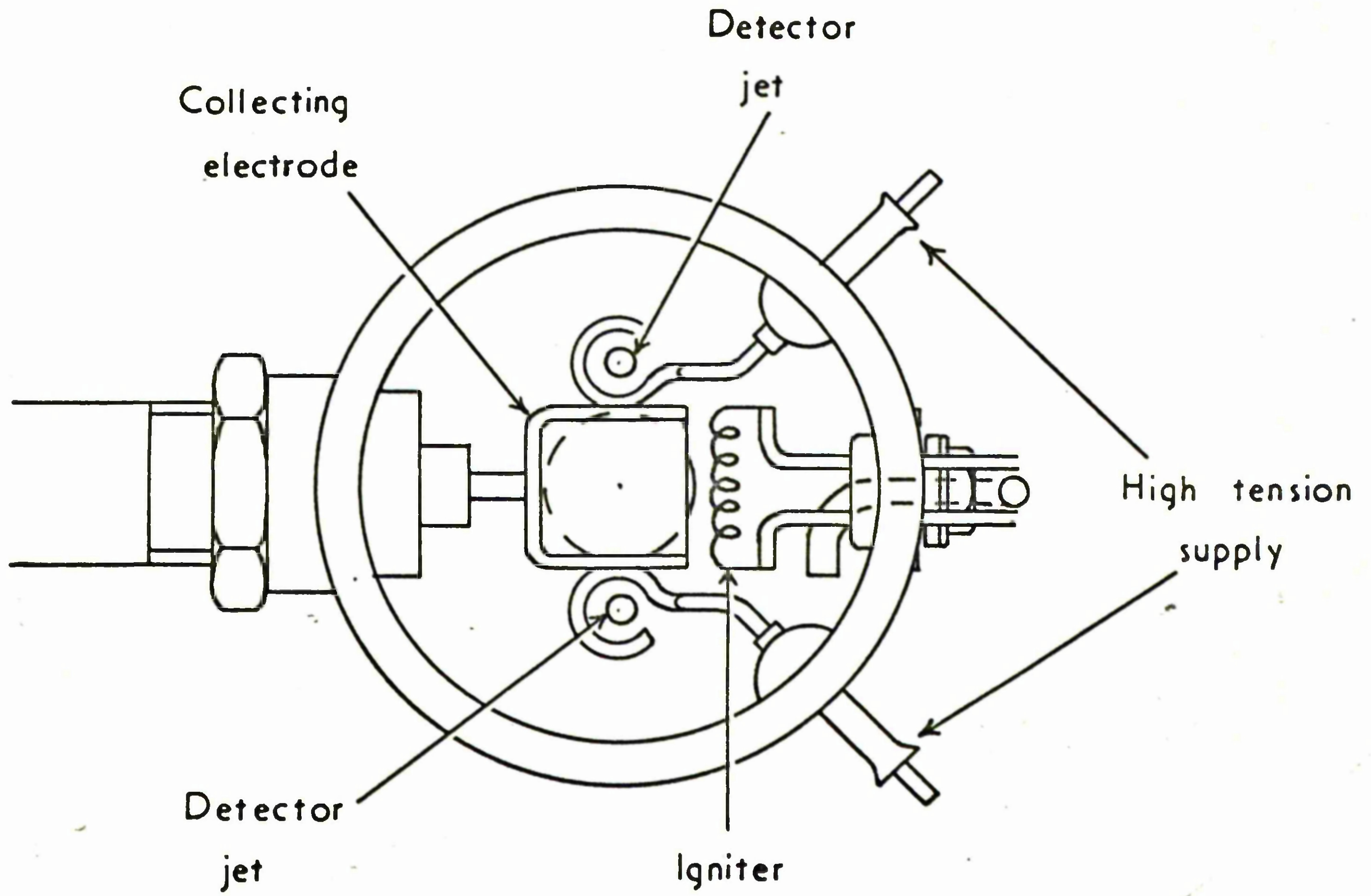


FIGURE 7b

Plan view of Flame Ionisation Detector used in the Perkin Elmer F-11 Gas Chromatograph. The figure shows the twin detector jets and the central collecting electrodes. The igniter for lighting the hydrogen flames is also visible.

FIGURE 7b



height of the peak by the width at half the height. Peaks which were not completely resolved were dealt with as outlined in Figure 8. In order to increase the area of the peaks, a recorder chart speed of 60 in./hr. was used for all peak area calculations.

II. Hydrolysis and Alcoholysis of Proteins and Peptides:

(i) Acid Hydrolysis: A weighed amount of the protein or peptide was sealed in a small diameter soda glass test tube with 1 or 2 ml. of 6 N HCl and hydrolysed for 16 hours in an oven at 115°. After hydrolysis the hydrochloric acid was removed either in a vacuum desiccator at room temperature, or on a rotary evaporator at 40° under vacuum.

(ii) Alcoholysis: A weighed amount of protein or peptide was sealed in a test tube (II, (i)) with 2 ml. of dry methanol or butanol containing 6 meq./ml. of dry HCl. The tube was placed in a metal bomb and incubated for various periods of time at various temperatures. The alcohol was removed on a rotary evaporator and the hydrochloric acid in a vacuum desiccator.

III. Preparation of N-acyl esters of the amino acids

(1) Esterification:

(i) Methylation with hydrogen chloride as catalyst: 1 mmole of amino acid was suspended in 10 ml. of dry methanol in a 50 ml. round bottomed flask, fitted with a ground glass stopper. The flask was attached to a condenser through which a tube from the hydrogen chloride generator was led (see Figure 9). The contents of the flask were refluxed with continuous slow addition of HCl using a micro bunsen, for 1 hour, and the flask was then removed to a rotary evaporator where the sample was taken to dryness at a temperature of 45° - 50° under reduced pressure. The mixture was suspended in 10 ml. of benzene/methanol (1/1 v:v.) and taken to dryness again. This procedure was repeated with 10 ml. portions of methanol/

FIGURE 8

Method used for estimation of Peak Areas of Unresolved Peaks. The unresolved parts of the peaks were extrapolated in such a way that the area a_1 was approximately equal to the area a_2. The widths of the peaks at half the height were then calculated using these extrapolated lines. Thus the area of the first peak was h_1w_1 and of the second h_2w_2.

FIGURE 8

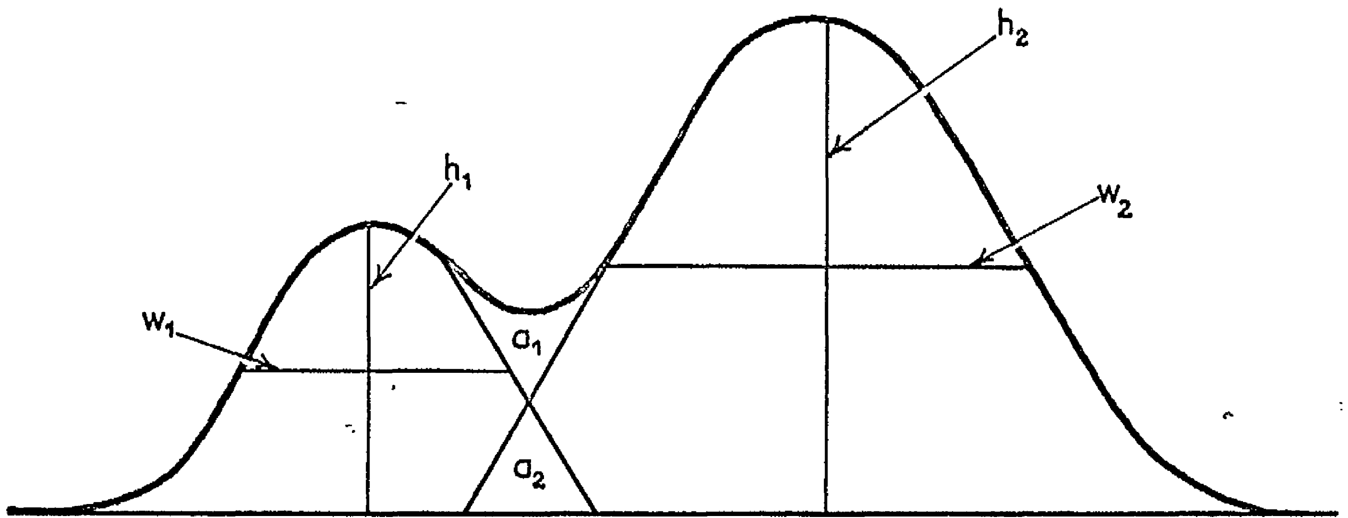
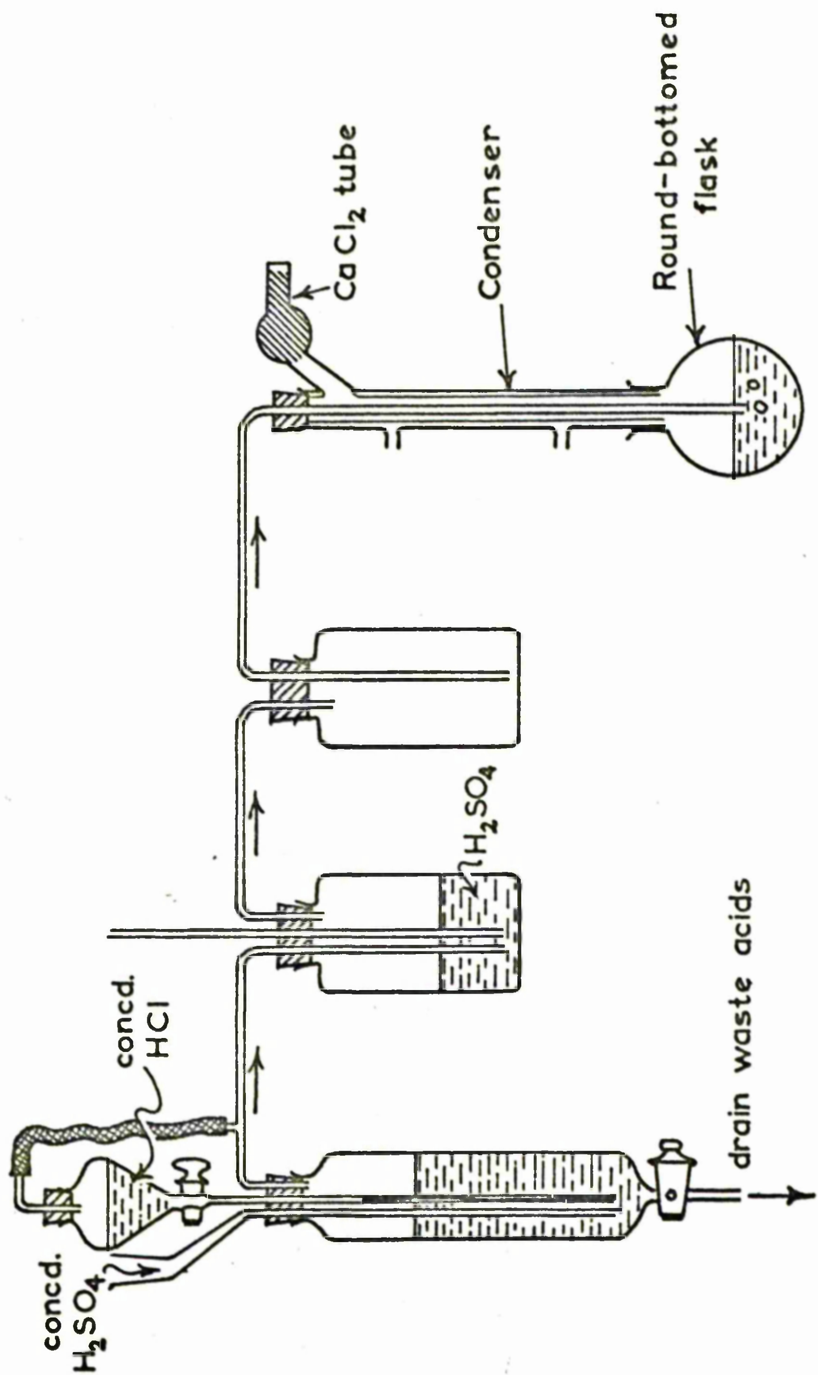


FIGURE 9

Schematic Diagram of HCl generator. The HCl gas was produced by running concentrated HCl into a separating funnel containing concentrated H_2SO_4. The HCl was dried by passage through concentrated H_2SO_4 and then passed through a second bottle (to prevent suck-back of the contents of the flask into the concentrated H_2SO_4) and into the reaction flask.

FIGURE 9



benzene and methanol/ethyl acetate, and the sample dried over sulphuric acid and sodium hydroxide in a desiccator under vacuum.

In some preliminary studies the amino acid(s) was (were) saturated with dry HCl gas and then refluxed without subsequent addition of the gas for 1 hour.

(ii) <u>Methylation with hydrogen chloride and dimethyl sulphite as catalyst</u>: For samples containing 100 μmoles or less of amino acids the sample was suspended in 5 ml. of methanol which was then saturated with anhydrous hydrogen chloride (the mixture was cooled in an ice bath during this procedure). 1 ml. of dimethyl sulphite was added and the solution heated under reflux (the moisture being excluded by a calcium chloride drying tube). After 30 minutes the methanol and excess dimethyl sulphite were removed under reduced pressure and the residue of amino acid methyl ester hydrochlorides was dried under high vacuum.

(iii) <u>Methylation with thionyl chloride as catalyst</u>: The method of Brenner and Pfister (1951) was used; this procedure apparently depends on the formation of methyl sulphite as intermediate which serves as the source of the methyl groups. Approximately 20 ml. of methanol was placed in a 100 ml. round-bottomed flask, the amino acid mixture added (100 μmoles) and the flask shaken well at room temperature. The flask was then placed in a cold bath (dry ice in methyl cellosolve) and 2 - 4 ml. of water clear thionyl chloride added drop by drop to the methanol, the flask being agitated throughout. After addition of the thionyl chloride, the flask was stoppered with a calcium chloride drying tube and kept at 40° for 2 hours. The contents were then reduced to a very small volume under reduced pressure on a rotary evaporator at room temperature, and dried in a desiccator under vacuum.

The method was modified and the reaction carried out under reflux for 30 or 60 minutes using the same amounts as above.

(iv) Direct esterification with Dowex 50 as catalyst: The use of ion exchange resins as catalysts for esterification of amino acids was described by Marcucci et al. (1965) following the procedures of Sussman (1946) and Mill and Crimmin (1957).

Dowex 50 is a strongly acidic cation exchange resin composed of sulphonated polystyrene crosslinked with divinyl benzene. It is insoluble in water and most organic liquids and can be easily removed from the reaction mixture by filtration. The amino acid hydrochlorides (total amount from 2.5 – 5 mg.) were esterified in 10 ml. of alcohol (either n-propanol or n-butanol) together with 100 – 200 mg. of dry Dowex 50 (50 – 100 mesh) in the hydrogen form as catalyst. After refluxing for 2-3 hours the alcohol was decanted and the resin washed twice with 10 ml. portions of alcohol. 10 ml. of citrate buffer, pH 6.9 and 20 ml. of methylene chloride were then added to the washed resin. After shaking for 5 minutes, the aqueous phase was discarded and the organic layer containing the esters of the amino acids was concentrated on a rotary evaporator and stored in a desiccator over concentrated sulphuric acid.

(v) Methylation with p-toluene sulphonic acid as catalyst: 100 μmoles of amino acid and 200 mg. of p-toluene sulphonic acid were added to a 100 ml. round bottomed flask and 30 ml. of an azeotropic mixture of alcohol and benzene added. The mixture was refluxed for 2-3 hours with a Soxhlet extractor attached (filled with anhydrous magnesium sulphate as drying agent). At the end of the reaction the mixture was filtered and evaporated to dryness on a rotary evaporator. The residue was stored in a desiccator over concentrated sulphuric acid *in vacuo*.

(vi) Methylation with diazomethane: The use of diazomethane for methylation of the amino acids was not often used. It was more usual for treatment with diazomethane (the preparation of which is detailed below) to be applied to the N-acyl amino acids. A solution (or, in the case of free amino acids, a suspension) of the amino acid derivatives in methanol was treated with an excess of diazomethane in ether and the excess colour discharged immediately with a drop of glacial acetic acid. The solution was concentrated where necessary by evaporation on a rotary evaporator at room temperature under reduced pressure, and dried in a vacuum desiccator.

Preparation of Diazomethane: The method of Moore and Reid (1961) was used in most of the earlier work. An efficient condenser (2 condensers each 45 cm. in length in series was used) was fitted with an adaptor to which a short length of teflon tubing had been attached extending to the bottom of a 50 ml. conical flask which served as a distillation receiver. The receiver was placed in a well mixed ice-salt mixture and sufficient anhydrous ether was added to cover the tip of the adaptor. Two methods were used to generate diazomethane.

Method 1: (Moore and Reid, 1961). In a 50 ml. round bottomed flask was placed 30 ml. of solvent grade ether (sodium-dried) 4.5 ml. of diethylene glycol monoethyl ether and 4.5 ml. of 40% aq. sodium hydroxide solution. The mixture was chilled in an ice bath at 0° and 1.8 g. (5 mmoles) of bis-(N-methyl N-nitroso) terephthalimide (Nitrosan) was added in one portion. The flask was immediately transferred to a water bath at 35° and connected by a goose neck to the condenser. The yellow colour of diazomethane appeared in the receiver almost immediately. About 20 ml. of ether was distilled in 30 minutes; at this point the distilling ether was practically colourless. The tip of the receiver adaptor was kept just below the surface of the distillate during the distillation.

The distillate contained 7.6-8.6 mmoles (76 - 86% yield) of diazomethane.

Method 2: (De Boer, 1954): Ethanol (95%, 25 ml.) was added to a solution of potassium hydroxide (5 g.) in water (1 ml.) in a 25 ml. round bottomed flask fitted with a dropping-funnel and an efficient condenser set downward for distillation. The condenser was connected to a receiving flask which contained 10 ml. of ether. The receiver was cooled to 0°.

The flask containing the alkaline solution was heated in a waterbath to 65° and a solution of 2.15 g. (0.01 mole) of Diazald (N-methyl-N-nitroso-p-toluenesulphonamide) in about 15 ml. of ether was added through the dropping-funnel in about 20 minutes. The rate of distillation was approximately equal to the rate of addition. When the dropping-funnel was empty, another 2 ml. of ether was added slowly, and the distillation continued until the distilling ether was colourless. The combined ethereal distillate contained about 0.3 g. of diazomethane.

(vii) Interesterification with hydrogen chloride as catalyst: The amino acid or mixture (60 mg.) was placed in a 50 ml. round bottomed flask and 10 ml. of anhydrous methanol was added. The suspension was then treated with anhydrous hydrogen chloride to dissolve the amino acid(s) and the solution was then refluxed on a rotary evaporator at 60° under vacuum. 10 ml. of n-propanol, or n-butanol, was added and the solution was refluxed for 2-3 hours with continuous slow addition of anhydrous HCl. The alcohol was removed on the rotary evaporator at 60° and the sample stored in a desiccator over concentrated sulphuric acid and sodium hydroxide.

(2) Acylation:

(i) Acetylation: N-acetylation was carried out, either on free amino acids, or on amino acid ester hydrochlorides. The amino acid or mixture (100 μmoles) was

suspended in 10 ml. of acetic anhydride and stirred vigorously at room temperature for 20 minutes on a magnetic stirrer. In the case of ester hydrochlorides these were dissolved in 10 ml. of acetic anhydride and allowed to stand at room temperature for 30 minutes. The solutions in both cases were taken to dryness on a rotary evaporator at 60° and stored *in vacuo* over sulphuric acid.

(ii) Trifluoroacetylation: Initially this was carried out prior to ester formation. 100 μmoles of amino acid was dissolved in 1 ml. of anhydrous trifluoroacetic acid. Trifluoroacetic anhydride (1 ml.) was added and the solution was heated under reflux for 5 minutes. The solvent and excess reagent were removed in a jet of dry nitrogen gas.

However, subsequently the trifluoroacetylation was carried out after the ester preparation in a number of modifications.

(a) Method of Cruickshank and Sheehan (1964) Trifluoroacetic anhydride (1 ml.) was added to the flask containing the amino acid ester hydrochlorides, and the resulting solution was heated under reflux for 10 minutes with the apparatus protected from moisture by a calcium chloride tube. The excess trifluoroacetic anhydride and trifluoroacetic acid were evaporated in a stream of dry nitrogen and the residue re-dissolved in trifluoroacetic anhydride for chromatography.

(b) Method of Hagen and Black (1964) The amino acid ester hydrochlorides were treated with 1-2 ml. of trifluoroacetic anhydride and allowed to stand at room temperature for 1 to 2 hours. The excess anhydride and acid were removed on a rotary evaporator at room temperature.

(c) Method of Makisumi and Saroff (1965) A total of approximately 0.8 mmole of amino acid ester hydrochlorides was treated with either 2 ml.(14.5mmole) o

trifluoroacetic anhydride, or with 0.4 ml. (2.9 mmole) of trifluoroacetic anhydride in 1.6 ml. of ethyl acetate at room temperature for 30 minutes with stirring. A sample of the resulting solution was used directly for gas chromatography.

(d) Method of Lamkin and Gehrke (1965). Ester hydrochlorides (60 mg.) were trifluoroacetylated by adding 5 ml. of methylene chloride and 0.5 ml. of trifluoroacetic anhydride and stirring for 120 minutes at room temperature on a magnetic stirrer. The trifluoroacetic anhydride and solvent were removed on a rotary evaporator at room temperature and the N-trifluoroacetyl esters dissolved in anhydrous chloroform prior to gas chromatography.

(e) Method of Stalling and Gehrke (1966). These workers developed a method for acylation of arginine which they found to be applicable to the other protein amino acids. Their method was modified by dissolving the amino acid mixture (50 μmoles) in 2 ml. of trifluoroacetic anhydride and carrying out the reaction for 5 minutes at 150° in a sealed soda glass tube ($\frac{3}{8}$" diameter). The sealed tube was placed in a metal can fitted with a screw top cap and lined with cotton wool. Samples were used for chromatography directly after opening the tube once it was cool.

IV. Synthesis of Individual Acyl Esters of Amino Acids

(1) N-acetyl methyl esters:

(i) Acetylation: 50 mmoles of amino acid was placed in a 200 ml. Erlenmeyer flask fitted with a calcium chloride drying tube. The amino acid was mixed with 45 ml. of glacial acetic acid and brought to the boil with gentle agitation on an electric hot plate. The mixture was removed from the hot plate to cool for a minute or two, and 7.5 ml. (75 mmoles) of acetic anhydride was carefully added in portions so as to avoid boiling or superheating. The resulting solution was returned to the hot plate, brought to the boil, and held

at this point for 2 minutes and then allowed to cool to room temperature. The solution was then taken to dryness *in vacuo* on a rotary evaporator at 40° and the residue treated several times with water followed each time by evaporation *in vacuo*. After a final evaporation with benzene to remove the last traces of water, the syrupy residue was taken up in the minimum volume of hot, dry ethyl acetate and the solution chilled in an ice bath. On scratching or seeding the N-acetyl amino acid usually crystallised out. After standing overnight at 4° the crystals were filtered rapidly with suction, washed with dry ether and dried *in vacuo* at room temperature.

If the N-acetyl amino acid did not crystallise out, the ethyl acetate solution was filtered and taken to dryness *in vacuo*; the crude acyl derivative was then used for the subsequent esterification.

In the case of lysine, the mono-hydrochloride was first converted to the monoacetate using ion exchange resin and then treated as above.

2. Preparation of N-acetyl amino acid methyl esters: 1 mmole of N-acetyl amino acid in a 50 ml. round-bottomed flask was dissolved in the minimum volume of dry methanol. An ethereal solution of diazomethane, prepared as described in Section III 1. (vi) was added to the flask placed in a salt/ice mixture until the yellow colour of the diazomethane was no longer discharged. Excess diazomethane was removed by addition of a drop of acetic acid and the reaction mixture was taken to dryness on a rotary evaporator at room temperature. The derivatives were recrystallised from a suitable solvent and the melting points determined. Table 10 gives the melting point of the derivatives prepared, and lists the solvents used for recrystallisation.

TABLE 10

N-Acetyl Amino Acid Methyl Esters

Amino Acid	Melting Point ° Observed Value	Melting Point ° Literature Value	Solvent for recrystallisation
DL-Alanine	43	45.2-46.4[1]	methanol
L-Aspartic Acid	63	60-61[2]	chloroform
L-Glutamic Acid	56-58	60.1-61.0[1]	ethyl acetate
Glycine	55	57.4-58.0[1]	ethyl acetate
L-Isoleucine	51-52	53.3-54.1[1]	ethyl acetate/methanol
DL-Leucine	72	75.9-76.5[1]	ethyl acetate
L-Lysine	oil	-	-
DL-Methionine	80-81	82.6-83.2[1]	ethyl acetate
DL-Phenylalanine	62-63	66.1-66.8[1]	ethyl acetate
DL-Proline	oil	36.3-37.0[1]	-
DL-Serine	101	100.9-101.6[1]	ethyl acetate/methanol
DL-Threonine	102	103.8-104.6[1]	ethyl acetate/methanol
DL-Tryptophan	150-151	153.5[3]	ethyl acetate
DL-Tyrosine	128-130	136-137[4]	ethyl acetate
DL-Valine	54	57.4-58.1[1]	ethyl acetate

1. Andersson, Ryhage and Stenhagen (1962).
2. Hardegger and Braunschweiger (1961).
3. Huang and Niemann (1951).
4. Jackson (1952).

(2) N-trifluoroacetyl methyl esters

(i) Preparation of methyl esters: The amino acid ester hydrochlorides were prepared using methanol and dry HCl gas (Greenstein and Winitz, 1961). 2g. of amino acid was placed in a 50 ml. round-bottomed flask and 30 ml. of anhydrous methanol added. The flask was gently refluxed for one to three hours with slow addition of a stream of dry HCl gas via a gas inlet tube, moisture being excluded by a calcium chloride tube. The flask was then allowed to stand for one hour at room temperature. If at the end of this time an insoluble product was formed, the flask was allowed to stand overnight in the cold, then filtered and the precipitate washed with ice cold methanol, then with ether and the product dried in vacuo over caustic soda pellets. Re-crystallisation was effected from methanol. If the product was soluble, the methyl ester hydrochloride was concentrated to dryness in vacuo at 50° on a rotary evaporator. 10 ml. of methanol was added, and the ester hydrochloride again taken to dryness. The dry residue was rubbed with anhydrous ether and the resulting crystalline suspension filtered and washed with dry ether. The product was dried in vacuo over NaOH, and re-crystallised from dry methanol by addition of anhydrous ether. The melting points of the ester hydrochlorides are given in Table 12.

In the case of threonine the ester hydrochloride was not obtained in the pure form as crystals. The crude syrup was therefore used in the acylation procedure. The behaviour of these compounds on thin layer chromatograms is presented in Table 11.

TABLE - 11

Thin Layer Chromatography Results

Amino Acid	TLC Solvent	Rf Ester	TLC Solvent	Rf N-TFA Ester
Alanine	1	0.49	3	0.95
Arginine	2	0.37	4	0.31
Aspartic Acid	1	0.70	3	0.91
Cysteine	1	0.48	3	0.75
Cystine	1	0.40	3	0.84
Glutamic Acid	1	0.60	3	0.80
Glycine	1	0.40	3	0.75
Histidine	2	0.65	4	0.51
Isoleucine	1	0.75	3	0.95
Leucine	1	0.75	3	0.95
Lysine	2	0.30	4	0.94
Threonine	1	0.68	3	0.55
Tryptophan	1	0.83	3	0.80
Tyrosine	1	0.81	3	0.63

TABLE 12

Amino Acid Methyl Ester Hydrochlorides

Methyl Ester Hydrochloride	Melting Point °	
	Observed Value	Literature Value[1]
DL-Alanine	158-159	158-158.5
DL-Valine	123-125	120-122
L-Isoleucine	97-99	100.5-101
DL-Threonine	Syrup	125 (decomposes)
Glycine	170-173	175
DL-Leucine	114	113-114
L-Aspartic Acid	112-115	116-117
DL-Glutamic Acid	146-148	149
L-Tyrosine	184-187	190
L-Lysine	210-211	212
L-Tryptophan	206-210	214 (decomposes)
L-Histidine	193-197	200-201
L-Arginine	181-183	195 (decomposes)
L-Cystine	170-172	173

1. Greenstein and Winitz (1961).

(ii) Preparation of N-trifluoroacetyl methyl esters:

The monoacylated derivatives of alanine, aspartic acid, glycine, iso-leucine, leucine and valine were prepared by adding to a suspension of 10 mmoles of amino acid methyl ester hydrochloride in 10 ml. of ethyl acetate 2 ml. (15 mmoles) of trifluoroacetic anhydride. The mixture was stirred at room temperature for 45 minutes. The ester hydrochloride dissolved gradually. The solution was evaporated under reduced pressure at room temperature on a rotary evaporator and the evaporation was repeated twice with addition of about 10 ml. of ethyl acetate. After drying *in vacuo* over sodium hydroxide and conc. sulphuric acid, the residual oil was distilled under pressure of approximately 1 - 2 mm, of mercury.

The N-, O-, (or S-) di-trifluoroacetyl methyl esters of cysteine, threonine and tyrosine were prepared by the same method as above, using 4 equivalents of trifluoroacetic anhydride to amino acid ester hydrochloride. The crude derivatives of tyrosine and cysteine solidified before distillation. They were crystallised from ethyl acetate containing a small amount of trifluoroacetic anhydride with addition of petroleum ether. Trifluoroacetic anhydride was used to prevent cleavage of the O- (or S-) trifluoroacetyl linkage during the crystallisation procedure.

N-trifluoroacetyl histidine methyl ester was prepared by treating 1.2 g. (5 mmoles) of histidine methyl ester hydrochloride suspended in 40 ml. of ethyl acetate with 5 ml. (37 mmoles) of trifluoroacetic anhydride with stirring at room temperature. After 20 minutes, an additional 5 ml. of trifluoroacetic anhydride was added to the solution. After an hour the clear solution obtained was evaporated on a rotary evaporator under reduced pressure at room temperature, and the evaporation was twice repeated with the addition of 10 ml. of ethyl acetate.

To the residue was added 10 ml. of ethyl ether and the resulting mixture was allowed to stand overnight at room temperature. The crystals thus obtained were recrystallised from warm ethyl acetate with the addition of ethyl ether.

Trifluoroacetamide was obtained from ammonium hydrochloride by the same method as for alanine using a 2.5 times molar ratio of trifluoroacetic anhydride to ammonium chloride.

Table 13 presents the melting points of these derivatives and Table 11 details their behaviour on TLC.

V. Thin Layer Chromatography

1. Principles: Chromatography on thin layers of adsorbent combines the advantages of greater speed and sensitivity as compared to chromatography on paper. This is because on thin layers of such adsorbents as Silica Gel G less broadening of the spots occurs and so separations can be achieved by a shorter length of run and smaller amounts of substances separated chromatographically can be detected. Thus 0.01 µg. of many amino acids can be detected on layers of Silica Gel G after TLC, whereas 0.1 µg. is the lower limit of detection of the same amino acids on paper chromatograms (Brenner et al., 1964).

It will be convenient to detail the stages of the process of TLC, which may be summarised as follows. A glass plate is covered with an aqueous slurry of an adsorbent powder containing a binder, so that a uniform, coherent film adheres to the glass. After the plate has been allowed to dry a solution to be analysed is spotted onto the film, and the charged plate is developed in the usual way in a closed tank containing a suitable solvent, and the components of the mixture identified by a suitable method.

TABLE 13

N-TFA Amino Acid Methyl Esters

N-TFA Amino Acid Methyl Ester	Melting Point °		Boiling Point °	
	Observed Value	Literature Value[1]	Observed Value	Literature Value[1]
DL-Alanine			86/15mm.Hg	58/0.8mm.Hg
DL-Valine			-	62/1.10mm.Hg
L-Isoleucine			-	72/1.35mm.Hg
DL-Threonine	oil	52		
Glycine			-	66.5/.9mm.Hg
DL-Leucine			98/15mm.Hg	72/.78mm.Hg
L-Aspartic Acid	42-44	45-46		
L-Histidine	Syrup	111		
L-Arginine	Syrup	Syrup		
L-Cystine	151-153	153		

1. Makisumi and Saroff (1965).

Since the rate of flow of solvent by capillary action is fastest at the beginning of the run (the length covered by the solvent front is proportional to the square root of the time of development), more time is saved than would be expected on the basis of the distance moved by the solvent. Thus a two dimensional thin layer chromatogram of amino acids requires only 4 - 5 hours, whereas a paper chromatogram may require 2 days to develop.

2. Preparation of Thin Layers: Thin layers were prepared using Desaga thin layer equipment (supplied by Camlab, Glass Ltd., Cambridge, England) on 20 x 20 cm. glass plates and on microscope slides using equipment supplied by Quickfit and Quartz Ltd., Stone, Staffs., England. The method of preparing the thin layers was as described by Brenner, Niederwiesser and Pataki (1964). Before use they were allowed to dry overnight in air. The thickness of the layers was approximately 0.25 mm.

3. Application of Samples: Samples for TLC were applied with 5 or 10 μl. capillaries as discrete spots 1.5 - 2 cms. from the edge of the plate; the amount spotted was usually 1 to 3 μl. The size of the spot was kept as small as possible and care was taken not to disturb the layer during sample applications.

4. Development of Chromatograms: 20 x 20 cm. plates were developed in tanks fitted with a ground glass lid, which had been lightly greased. Layers on microscope slides were developed in Coplin jars with the lids on. The solvent systems used are detailed below.

(i) Amino Acids:
(a) chloroform/methanol/17% ammonia (2:2:1 by volume)
(b) n-butanol/acetic acid/water (4:1:1 by volume)
(c) phenol/water (3:1 by volume) containing 20 mg. of KCN per 100 g. of mixture.
(Brenner and Niederwiesser, 1960)

(ii) Amino Acid Methyl Ester Hydrochlorides:

(a) ethanol/water (3:2 by volume)

(b) phenol/water (3:1 by weight)

(Cruickshank and Sheehan, 1964)

(c) benzene/n-butanol (3:1 by volume)

(Marcucci et al., 1965).

(iii) N-acyl amino acid esters:

(a) chloroform/acetone (9:1 by volume)

(b) chloroform/methanol (9:1 by volume)

(Cruickshank and Sheehan, 1964).

The thin layer plates were developed until the solvent had travelled halfway up the plate, and were then removed from the tank, dried at room temperature and then in an oven at 120°.

5. Detection of compounds on TLC plates: Free amino acids and amino acid methyl ester hydrochlorides were detected on TLC plates by spraying the plate with a ninhydrin solution and developing the colour at 120° for 5 minutes. Two such spray solutions were used.

(i) ninhydrin/collidine: 100 mg. of ninhydrin was dissolved in 50 ml. of ethanol and 15 ml. of glacial acetic acid and 2 ml. of collidine (2,4,6 - trimethylpyridine) added.

(ii) ninhydrin/cadmium acetate: 100 mg. of cadmium acetate, 10 ml. of water, 5 ml. of glacial acetic acid, 100 ml. of acetone and 1 g. of ninhydrin were mixed in the above order.

N-acylated amino acids and derivatives were detected by spraying the TLC plate with a 0.5% solution of Morin in methanol, and viewing under an ultra-violet light. The N-substituted amino acids appeared as dark spots on a light background.

RESULTS

I. Retention Data

(1) Introduction:

It was clear that until conditions could be found whereby the twenty amino acids which commonly occur in proteins could be separated in the form of a suitable volatile derivative by GLC, there was little point in pursuing quantitative aspects of this problem. Accordingly, before undertaking any study of the quantitative estimation of the amino acids, it was first of all necessary to establish what was the most suitable derivative in respect of ease of preparation and of resolution, and what were the optimum conditions for the separation of these derivatives of the twenty protein amino acids.

At the outset of the investigation there was very little information available on gas chromatography of amino acid derivatives. It was clear from the literature that the degradative type of procedure outlined in the Introduction (IV, (iii), A.) was of limited applicability, and it seemed from the observations of Saroff and Karmen (1960) and Johnson Scott and Meister (1961) that the most promising derivatives were the N-acyl esters of the amino acids. On the basis of availability of reagents and ease of preparation it seemed that the N-acetyl amino acid methyl esters would be the most convenient starting point, since there was at that time a considerable body of information available on the N-acetylation of amino acids and preparative procedures had been described for the N-acetyl methyl esters of a number of amino acids (Andersson, Ryhage and Stenhagen, 1962). However, the separation of these derivatives was not very satisfactory.

Subsequent to this, when Hagen and Black (1964) and Cruickshank and Sheehan (1964) published their accounts of the separation and estimation of

N-trifluoroacetyl methyl esters of the amino acids it was decided to turn attention to those derivatives. This was advantageous in that facilities for chromatography using gradual increase of the column temperature (temperature programming), became available shortly after this, and it had become apparent that this was necessary for separation of these derivatives.

The retention data are presented in the subsequent parts of this section. Chromatographic results obtained isothermally on the Pye Argon are presented in terms of retention times. For data obtained on the Perkin Elmer F-11, with which temperature programming was possible, retentions are given relative to leucine as unity.

(2) N-acetyl amino acid methyl esters:

(i) Preparation of derivatives: These derivatives were in general prepared as outlined in Experimental Section III, 1., (vi) and III, 2., (i), or in the case of pure derivatives Section IV, 1. The acetylation preceded the methylation with diazomethane. In a few cases the methyl esters were prepared first using p-toluene sulphonic acid as catalyst; the acetylation then followed the methylation.

(ii) Conditions of Chromatography: All of the N-acetyl amino acid methyl esters which were successfully chromatographed were analysed using glass columns packed with silanised Embacel, of the dimensions 4 ft. x 4 mm., in the Pye Argon Gas Chromatograph. The flow rate of argon in all cases was 100 ml./min., and the preheater was operated at 240°. In all cases the sample size was in the range 1 to 5 µl., and contained 0.02 to 0.1 µmole of amino acid derivative. The detector voltage was 1500 volts in all cases except for the results of Table 16

where the voltage was 1250 volts. In all except the last table (No.18), the data was obtained isothermally.

(iii) <u>Retention data:</u> Tables 14 - 18 present the retention times for the N-acetyl amino acid methyl esters. Table 14 gives some preliminary results with columns coated with 2% of PEGA and 1% of QF-1. The peaks were very asymmetrical due to tailing. This is a result of adsorption onto the support and could be reduced either by applying a higher percentage of stationary phase, or treating the support again with dimethyl-dichlorosilane.

The retention times on a column coated with 2% of NPGS are presented in Table 15. At the lower temperatures the peaks were very skew and showed considerable tailing. Even at 200° this was still apparent, though less pronounced. The resolution of alanine from valine was incomplete both at 115° and at 140°. The need for some means of gradually increasing the column temperature is illustrated by the fact that at 140°, when alanine, valine and leucine are rapidly eluted from the column, phenylalanine is not eluted at all.

PEGA is a stationary phase frequently used for separation of fatty acid esters and Table 16 gives the retention times of some N-acetyl amino acid methyl esters on a column containing 31% of PEGA (the column contained 25 parts of PEGA to 80 parts of the support). With the exception of isoleucine and leucine the peaks were all completely resolved, and were all perfectly symmetrical. One obvious disadvantage of this column is that the retention times of glutamic acid and aspartic acid are very long. Since PEGA columns cannot normally be used above 200°, it seems unlikely that this column would be of any use for routine analysis of all the amino acids.

TABLE 14

Retention Times of N-Acetyl Amino Acid Methyl Esters on 2% PEGA and 1% QF-1 Column

Amino Acid	Temp. °C	Retention Time mins.
1. 2% PEGA column		
Alanine	125°	5.3
Glycine	125°	10.3
2. 1% QF-1 column		
Glycine	115°	2.5
Leucine	115°	3.8
Leucine	125°	2.4
Leucine	135°	1.3
Phenylalanine	135°	8.7

TABLE 15

Retention Times of N-Acetly Amino Acid Methyl Esters on

2% NPGS Column

Amino Acid	Temp oC	Retention Time mins.
Alanine	115	7.5
Valine	115	10.3
Leucine	115	18.0
Alanine	140	2.7
Valine	140	3.8
Leucine	140	6.3
Aspartic Acid	175	3.7
Glutamic Acid	175	9.0
Leucine	200	3.0
Phenylalanine	200	5.3

TABLE 16

Retention Times of N-Acetyl Amino Acid Methyl Esters on 3% EGA Column

Amino Acid	Temp. °C	Retention Time mins.
Alanine	165	18.7
Glycine	165	29.7
Leucine	165	31.0
Serine	180	9.7
Alanine	180	10.3
Valine	180	12.6
Glycine	180	16.5
Leucine	180	18.0
Isoleucine	180	16.9
Proline	180	37.3
Aspartic Acid	180	67.6
Glutamic Acid	180	146.7

In an attempt to reduce the asymmetry obtained with the 2% NPGS column, a 5% NPGS column was prepared and Table 17 gives the retention times observed. Valine and glycine were not resolved well at 150°, but the peaks were very symmetrical, and lysine gave a reasonable retention time at 200°. At this temperature however, alanine, glycine, leucine and valine were all eluted as a single peak immediately after the solvent, whereas at lower temperatures these derivatives were resolved. This again emphasises the requirement for the capacity to vary the temperature in the course of the analysis. It seems probable from these results that reasonable retention times for these amino acid derivatives would be obtained if the temperature was gradually increased from 125° to 200°.

An attempt was made to achieve a gradual increase in the temperature of the column in the Pye Argon Chromatograph using a 2% NPGS column (which gave much less asymmetry than the column used in Table 15). The method used was to set the temperature at the required starting level, and once the sample had been injected, to set the temperature to the final value required and switch on the boost heater. This gave a temperature increment of about 2°/min. The disadvantage of this method is that the heater of the chromatograph consists of an aluminium block with holes bored in it for the columns to pass through. Although the temperature of this can be increased at 2°/min., it is not possible to achieve a comparable rate of cooling. Thus, even with a blast of cold air being blown through it, the block took 1½ to 2 hours to cool and stabilise to the starting temperature. This placed a severe restriction on the number of analyses which could be performed in a day.

TABLE 17

Retention Times of N-Acetyl Amino Acid Methyl Esters on 5% NPGS Column.

Amino Acid.	Temp. °C	Retention Time mins.
Alanine	150	5.3
Valine	150	7.7
Glycine	150	9.0
Leucine	150	12.0
Aspartic Acid	150	13.7
Glutamic Acid	150	22.7
Leucine	175	4.3
Proline	175	8.0
Phenylalanine	175	23.7
Proline	200	3.3
Aspartic Acid	200	5.7
Glutamic Acid	200	10.5
Lysine	200	25.0

Table 18 gives the retention times of 15 amino acids on this column, with the temperature increased from 140° to 190° at about 2°/min. When a mixture was chromatographed, the resolution of alanine/valine and glycine/leucine was poor, and the pairs threonine/methionine, phenylalanine/glutamic acid and histidine/lysine were not separated. No peak was obtained for tryptophan or cystine using derivatives which were prepared from pure N-acetyl amino acids, obtained from commercial sources.

(3) <u>N-trifluoroacetyl amino acid methyl esters - Isothermal Operation</u>

(i) <u>Preparation of Derivatives</u>: Amino acids were trifluoroacetylated as described in the Experimental Section III, (2), (ii), (b) and then methylated with diazomethane (Experimental Section III, (1), (vi)).

(ii) <u>Conditions of Chromatography</u>: The N-TFA methyl esters were chromatographed on the Pye Argon Gas Chromatograph with the preheater set at 240° and the detector voltage at 1250 volts. The sample size was in the range 3 to 6 μl, and contained 0.1 to 0.2 μmole of amino acid derivative. Details of the chromatographic conditions are presented in the tables.

(iii) <u>Retention Data</u>: Tables 19 to 23 give the retention data for certain N-TFA amino acid methyl esters obtained isothermally using the Pye Argon Gas Chromatograph.

Table 19 gives the retention times of the N-TFA methyl esters of alanine, valine, isoleucine, threonine, glycine, leucine and proline on a column packed with Embacel coated with 3.5% of NPGS. The resolution of alanine and valine at 75° was not complete, and isoleucine and threonine were hardly resolved at all at 90° on this column. Glycine and leucine were not completely resolved at 90°.

Table 20 shows the effect of increasing the amount of stationary phase. At a higher temperature (110°) the five aliphatic amino acids were all well resolved.

TABLE 18

Retention Times of N-Acetyl Amino Acid Methyl Esters on 2% NPGS Column - Temperature Programmed

Amino Acid	Temp. °C At 2°/min.	Retention Time Mins.
Alanine	140-190	11.3
Valine	140-190	12.5
Glycine	140-190	14.2
Leucine	140-190	14.0
Isoleucine	140-190	15.3
Proline	140-190	17.0
Aspartic Acid	140-190	22.5
Methionine	140-190	26.0
Threonine	140-190	26.6
Phenylalanine	140-190	28.3
Glutamic Acid	140-190	28.3
Histidine	140-190	37.9
Lysine	140-190	37.7
Arginine	140-190	40.5
Tyrosine	140-190	42.9

TABLE - 19

N-TFA Amino Acid Methyl Esters

Conditions: 4 ft. x 4 mm. glass column packed with Embacel (silanised) coated with 3.5% of NPGS. Argon flow rate - 50 ml./min.

Amino Acid	Retention Time (min.) Column Temperature		
	75°	83°	90°
Alanine	10.05	6.00	-
Valine	12.15	-	-
Isoleucine	21.00	11.90	7.10
Threonine	-	-	7.35
Glycine	25.35	14.55	8.95
Leucine	34.00	18.80	10.35
Proline	-	-	18.05

TABLE - 20

N-TFA Amino Acid Methyl Esters

Conditions: 4 ft. x 4 mm. glass column of Embacel (silanised) coated with 5% of NPGS. Argon flow rate 50 ml./min.

Amino Acid	Retention Time (min.) Column Temperature 110°	150°	160°
Alanine	5.65		
Valine	7.00		
Isoleucine	10.00		
Glycine	12.60		
Leucine	14.90		
Proline		5.5	3.6
Aspartic Acid		10.0	5.9
Methionine		22.9	13.1
Glutamic Acid		26.9	16.3
Phenylalanine		30.1	18.8

At 150° the resolution of glutamic acid from phenylalanine was quite good, but at 160° they were less well resolved.

The effect of treating the support with PVP is illustrated by comparison of the results in Tables 21 and 22. The retention times of two groups of amino acids at two temperatures on a column of silanised Gas Chrom P coated with 1% of CHDMS without use of PVP are presented in Table 21. Alanine and valine were just resolved at 64° and the resolution of isoleucine and glycine at this temperature was incomplete. Methionine, glutamic acid and phenylalanine were poorly resolved at 125° on this column, but the other two amino acids in this group were well resolved. On a similar column which had been coated with 2% of PVP prior to coating with 1% of CHDMS, the peaks for the five monoamino monocarboxylic aliphatic amino acids were so asymmetric that no satisfactory retention values could be quoted. The order of elution was unchanged, but alanine/valine and isoleucine/glycine/leucine were not resolved. The behaviour of the other five amino acid derivatives was better in that the peaks were less skew. The retention times for aspartic acid and phenylalanine at 125° were about two and a half times their values on the untreated column. The order of elution was unchanged and the resolution of methionine, glutamic acid and phenylalanine was improved in that glutamic acid and phenylalanine were now completely resolved. The effect of PVP in increasing the retention times is indicated by the fact that at 135° these last three amino acids had longer retention times on the PVP treated column than on the untreated column at 125°.

A column of silanised Gas Chrom P coated with 5% of QF-1 gave the results presented in Table 23. At 110° the first three amino acids were resolved, but isoleucine and leucine were not resolved at all. On this column at 150°,

TABLE - 21

N-TFA Amino Acid Methyl Esters

Conditions: 4 ft. x 4 mm. glass column packed with Gas Chrom P (silanised) coated with 1% CHDMS. Argon flow rate - 50 ml./min.

	Retention Time (min.)	
	Column Temperature	
Amino Acid	84°	125°
Alanine	3.3	
Valine	3.9	
Isoleucine	6.75	
Glycine	7.7	
Leucine	10.2	
Proline		2.15
Aspartic Acid		4.5
Methionine		11.85
Glutamic Acid		12.5
Phenylalanine		13.6

TABLE - 22

N-TFA Amino Acid Methyl Esters

Conditions: 4 ft. x 4 mm. glass column packed with Gas Chrom P, treated with 2% PVP and then coated with 1% CHDMS. Argon flow rate - 50 ml./min.

Amino Acid	Retention Time (min.) Column Temperature		
	115°	125°	135°
Proline	4.5		
Aspartic Acid	16.35		
		11.0	
Methionine	51.25		
			14.2
Phenylalanine		36.0	
			19.2
Glutamic Acid			15.4

TABLE - 23

N-TFA Amino Acid Methyl Esters

Conditions: 4 ft. x 4 mm. glass column packed with Gas Chrom P (silanised) coated with 5% of QF-1. Argon flow rate - 50 ml./min.

Amino Acid	Retention Time (min.) Column Temperature	
	110°	150°
Alanine	5.1	
Glycine	6.5	
Valine	8.5	
Leucine	13.0	
Isoleucine	13.0	
Aspartic Acid		8.0
Proline		8.6
Methionine		13.7
Glutamic Acid		17.5
Phenylalanine		17.5

aspartic acid and proline were not well resolved, and glutamic acid and phenylalanine were not resolved at all.

(4) N-TFA Amino Acid Methyl Esters - Temperature Programmed Operation

(i) Introduction: It had become apparent from the results with both the N-acetyl and N-trifluoroacetyl methyl esters that there was a requirement for an instrument which would enable the temperature to be gradually raised in the course of an analysis, and rapidly lowered at the end. The Perkin Elmer F-11 Gas Chromatograph fulfilled this requirement in that it is equipped for parallel operation of 2 columns, which allowed use of temperatures near the top of the operating range for the packing, since the blank signal produced by the bleeding of stationary phase at high temperatures from the two columns was balanced out. The trifluoroacetyl methyl esters had the advantage of greater volatility over the N-acetyl esters and so were used in the temperature programmed analysis. Preliminary observations with isothermal operation had suggested that NPGS was the most suitable phase to use for the separation of these derivatives, and so it was used extensively in the investigation, particularly since a number of workers had reported separations of N-TFA methyl esters using this phase.

(ii) Preparation of Derivatives: Cruickshank and Sheehan (1964) observed that when trifluoroacetylation preceded methylation, the monoacylated derivatives of serine, threonine, hydroxyproline and tyrosine were formed, whereas when trifluoroacetylation followed methylation the diacylated derivatives (N-, O-trifluoroacetyl methyl esters) of these amino acids were formed. For this reason it was decided that the order of preparation should be methylation followed by trifluoroacetylation. Usually the methylation was carried out as described in Experimental, Section III, (1), (i), using anhydrous methanol saturated with dry

HCl. The trifluoroacetylation was carried out either using the method described in Experimental, Section III, (2), (ii), b, or that in Section III, (2), (ii), c.

(iii) <u>Conditions of Chromatography</u>: The F-11 was set up as outlined in the manufacturer's handbook. The compressed air was set at a pressure of 30 p.s.i. and the hydrogen at 18 p.s.i. The pressure of the nitrogen used as carrier gas varied from 2 to 20 p.s.i. depending on the length of the column and the flow rate required. Usually the reference column was identical to the column used for the analysis with an identical flow rate of carrier gas. The preheaters were operated at 240-260°. The sensitivity was usually in the range 10^{-8} - 10^{-9}A. full scale deflection. The retentions of the amino acid derivatives are given relative to leucine as unity.

(iv) <u>Retention Data on NPGS columns</u>: Table 24 gives the relative retentions of the N-TFA amino acid methyl esters on a 5% NPGS column, 2 ft. long. The conditions are given on the table, and represent an attempt to repeat the published separation of Cruickshank and Sheehan (1964), who used a 2 ft. x 1.5 mm. column. The flow rate was adjusted to 32 ml./min. in order to achieve the same relative flow rate within the wider 2 mm. tube. The separation achieved with this column is illustrated in Figure 10. n-Nonadecane was included in the mixture. The resolution of alanine/valine/isoleucine and aspartic acid/cysteine is poor, and glutamic acid and phenylalanine are not resolved at all. Cystine is eluted as a broad peak which overlaps the histidine peak.

The effect of lengthening the column is shown in the results of Table 25, which presents the relative retentions of the derivatives on a 2 m. column using various conditions. It was found that the best separation was achieved using a rate of temperature increase of 3° per minute from 60° to 215° at a flow rate of 32 ml./min. Figure 11 presents a typical separation. The resolution of glycine

TABLE 24

N-TFA Amino Acid Methyl Esters

Conditions: 2 ft. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate - 32 ml./min. Temperature increased from 65° at 1.5°/min. for 20 min.; then at 2°/min. for a further $22\frac{1}{2}$ min.; and then at 4°/min. to a final temperature of 210°.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Alanine	0.58	Hydroxyproline	1.57
Valine	0.66	Methionine	1.73
Isoleucine	0.72	Glutamic Acid	1.79
Threonine	0.85	Phenylalanine	1.81
Glycine	0.92	Tyrosine	2.04
Leucine	1.00 (16.4 min.)	Lysine	2.17
Serine	1.16	Tryptophan	2.23
Proline	1.23	Arginine	2.31
Aspartic Acid	1.46	Histidine	2.56
Cysteine	1.52	Cystine	2.61

FIGURE 10

Separation of N-TFA amino acid methyl esters on a 2 ft. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate - 32 ml./min. Temperature increased from 65° at 1.5°/min. for 20 mins.; then at 2°/min. for 22.5 mins.; then at 4°/min. to a final temperature of 210°. 5 µl. of sample applied, containing about 10^{-8} moles of each amino acid.

FIGURE 10

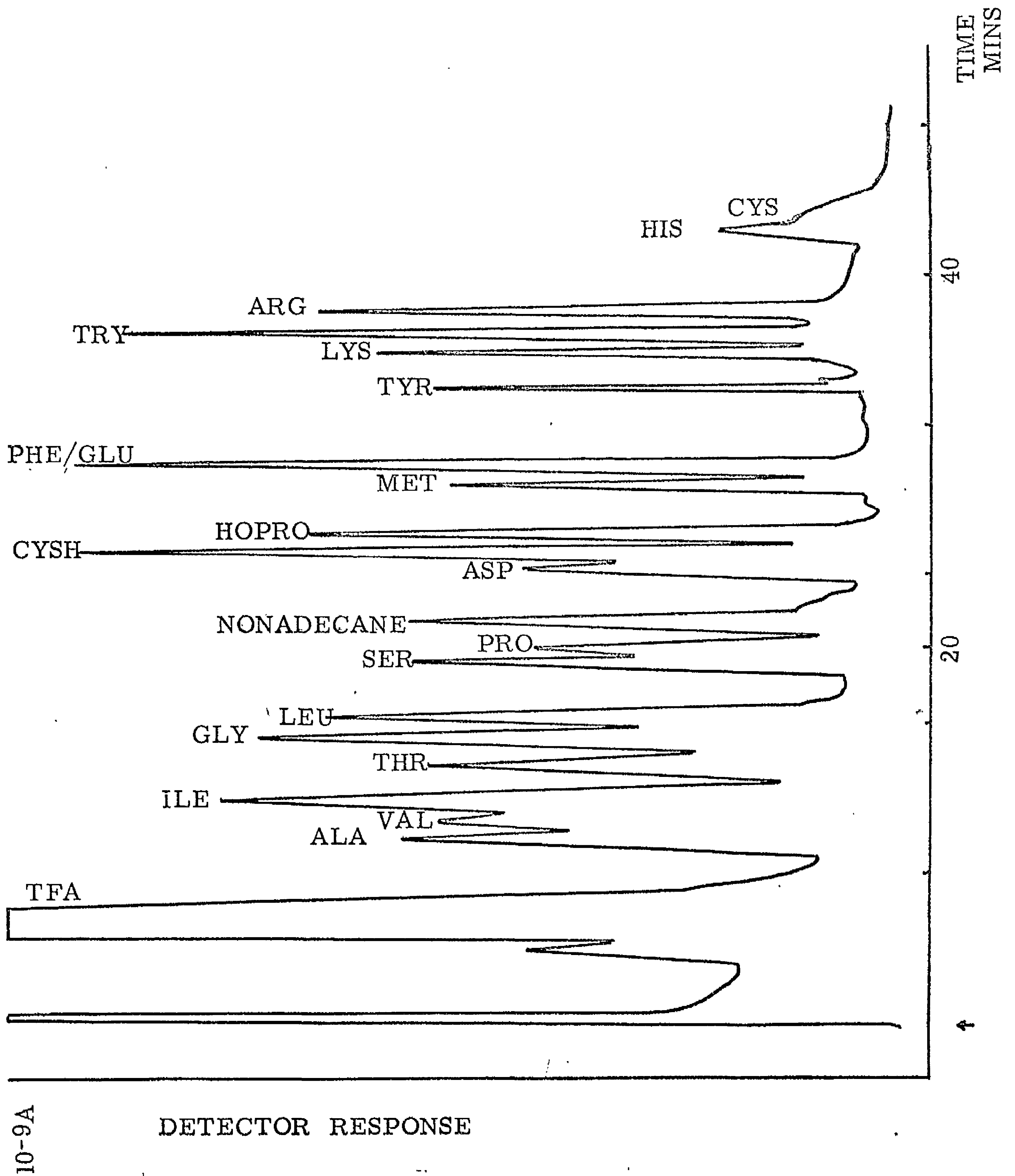


TABLE 25

N-TFA Amino Acid Methyl Esters

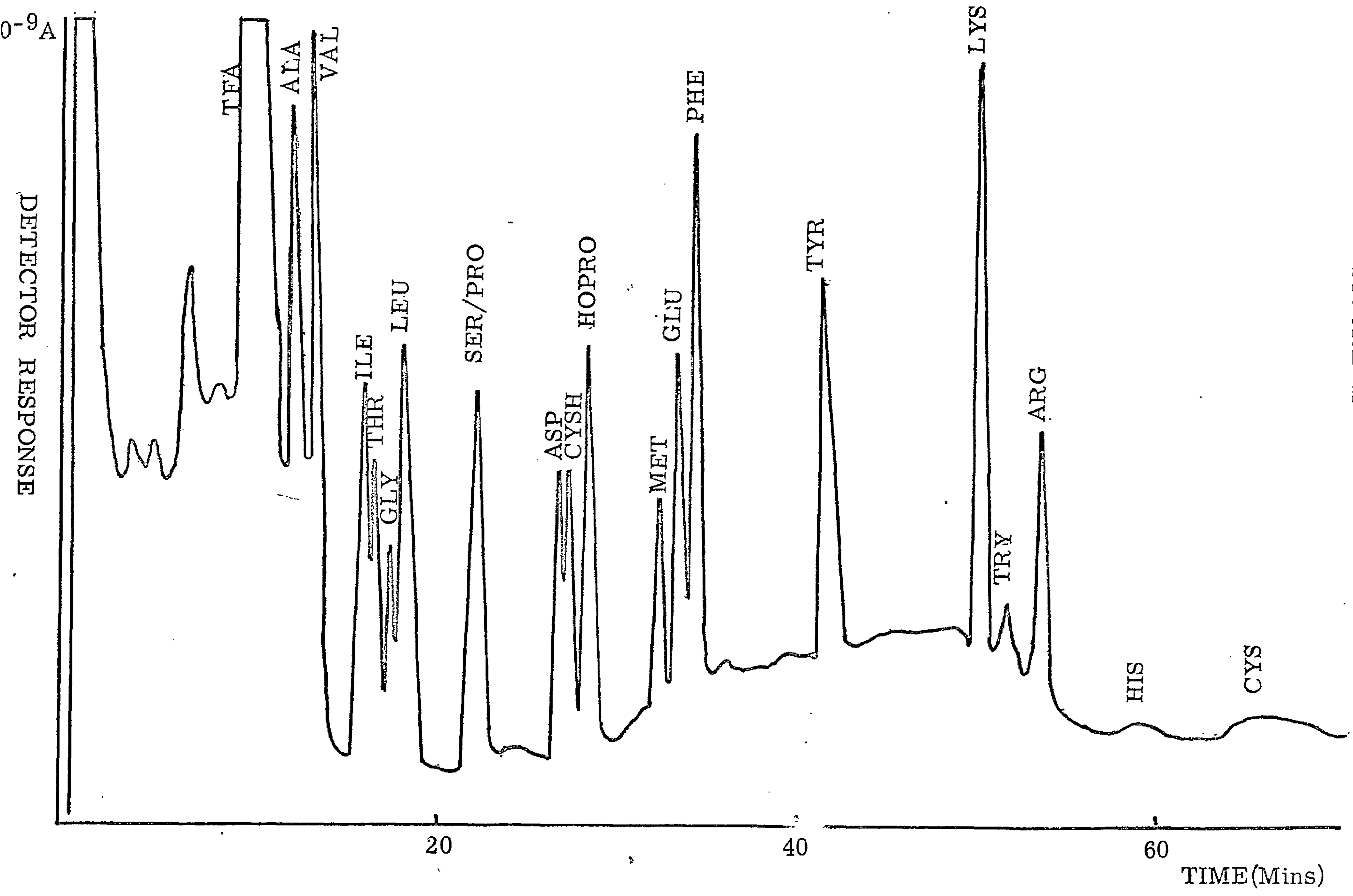
Conditions: 2 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rates - (a) 20 ml./min.; (b) 32 ml./min.; (c) 40 ml./min. Temperature increased at 4°/min. from 60° to 200° in (a) and (c) and at 3°/min. from 60° to 210° in (b).

	Relative Retention				Relative Retention		
Amino Acid	(a)	(b)	(c)	Amino Acid	(a)	(b)	(c)
Alanine	0.75	0.65	0.69	Hydroxyproline	1.41	1.55	1.40
Valine	0.79	0.72	0.73	Methionine	1.55	1.77	1.53
Isoleucine	0.90	0.89	0.87	Glutamic Acid	1.60	1.83	1.58
Threonine	0.94	0.91	0.93	Phenylalanine	1.63	1.87	1.62
Glycine	0.97	0.96	0.94	Tyrosine	1.91	2.26	1.92
Leucine	1.00 (25.2 min.)	1.00 (18.4 min.)	1.00 (17.2 min.)	Lysine	2.12	2.73	2.17
Serine	1.14	1.22	1.16	Tryptophan	-	2.84	-
Proline	1.16	1.22	1.16	Arginine	-	2.92	-
Aspartic Acid	1.35	1.47	1.34	Histidine	-	3.18	-
Cysteine	1.37	1.50	1.36	Cystine	-	3.58	-

FIGURE 11

Separation of N-TFA amino acid methyl esters on a 2 ft. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% NPGS. Flow rate - 32 ml./min. Temperature raised from 60° at 3°/min. to 215°. 5 μl. of sample applied containing about 10^{-8} moles of each amino acid.

FIGURE 11



from threonine and leucine, and of aspartic acid and cysteine are poor. Serine and proline are not resolved at all. On this column histidine and cystine were eluted as low, broad peaks.

The effect of increasing the amount of stationary phase is given in Table 26. A column packed with Gas Chrom Q coated with 6% of NPGS gave poorer results than the 5% column in that threonine/glycine, serine/proline, aspartic acid/cysteine and glutamic acid/phenylalanine were not resolved at all.

The effects of lengthening the 5% column to 4 m. are given in Table 27. The resolution of aspartic acid and cysteine was greatly improved, but threonine/glycine and serine/proline were not resolved.

(v) Retention Data on other columns: Attention was turned to a number of other stationary phases and combinations of these. A column packing coated with 4% of EGSS-X gave the retentions detailed in Table 28. Alanine and valine were very poorly resolved and isoleucine/threonine and glutamic acid/phenylalanine were not resolved at all. All of the other amino acid derivatives were well resolved on this column. It is of interest to note that on this column (and on all of the columns containing EGSS-X or EGSP-Z) leucine is eluted before glycine.

Table 29 gives the relative retentions on 1 and 2 m. columns packed with Gas Chrom Q coated with 0.75% of DEGS and 0.25% of EGSS-X. This particular phase has been used by Gehrke and his collaborators for the N-TFA n-butyl esters. Alanine/valine, threonine/isoleucine, glutamic acid/phenylalanine were not resolved at all on this column.

The effect of increasing the percentage of phase is to resolve isoleucine and threonine as shown in Table 30. However, the resolution of aspartic acid and hydroxyproline is now very poor, and alanine/valine and glutamic acid/

TABLE - 26

N-TFA Amino Acid Methyl Esters

Conditions: 2 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 6% of NPGS. Flow rate - 60 ml./min. Temperature raised by 3°/min. from 60° to 200°.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Alanine	0.48	Aspartic Acid	1.76
Valine	0.56	Cysteine	2.00
Isoleucine	0.78	Hydroxyproline	2.00
Threonine	0.89	Methionine	2.20
Glycine	0.89	Glutamic Acid	2.26
Leucine	1.00 (18.8 min.)	Phenylalanine	2.27
Serine	1.26	Tyrosine	2.83
Proline	1.26	Lysine	3.44

TABLE 27

N-TFA Amino Acid Methyl Esters

Conditions: 4 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate - 20 ml./min. Temperature raised from 60° to 210° at 4°/min.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Alanine	0.73	Aspartic Acid	1.27
Valine	0.78	Cysteine	1.34
Isoleucine	0.88	Hydroxyproline	1.44
Threonine	0.92	Methionine	1.54
Glycine	0.94	Glutamic Acid	1.59
Leucine	1.00 (29.1 min.)	Phenylalanine	1.66
Serine	1.14	Tyrosine	1.84
Proline	1.12	Lysine	1.94

TABLE - 28

N-TFA Amino Acid Methyl Esters

Conditions: 2m. x 2mm. stainless steel column packed with Gas Chrom Q coated with 4% EGSS-X. Flow rate - 25 ml./min. Temperature increased from 60° to 210° at 3°/min.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Valine	0.71	Hydroxyproline	1.72
Alanine	0.74	Methionine	2.06
Isoleucine	0.82	Glutamic Acid	2.23
Threonine	0.80	Phenylalanine	2.23
Leucine	1.00 (15.1 min)	Tyrosine	2.56
Glycine	1.11	Lysine	2.96
Serine	1.29	Arginine	3.06
Proline	1.43	Histidine	3.20
Cysteine	1.57	Cystine	3.32
Aspartic Acid	1.66		

TABLE - 29

N - TFA Amino Acid Methyl Esters

Conditions: 1 and 2 m x 0.2 mm. stainless steel columns packed with Gas Chrom Q coated with DEGS/EGSS-X (0.75/0.25%) 1 m column - flow rate 20 ml/min. 2m column - flow rate 30 ml/min. Temperature increased by 4°/min. from 60° to 200°.

Amino Acid	Relative Retention 1m col.	Relative Retention 2m.col.	Amino Acid	Relative Retention 1m col.	Relative Retention 2m col.
Alanine	0.78	0.68	Cysteine	1.38	1.60
Valine	0.78	0.66	Aspartic Acid	1.49	1.62
Threonine	0.87	0.83	Hydroxyproline	1.52	1.73
Isoleucine	0.89	0.84	Methionine	1.65	1.85
Leucine	1.00 (17.5) (min.)	1.00 (15.2) (min.)	Glutamic Acid	1.93	2.10
Glycine	1.07	1.03	Phenylalanine	1.96	2.08
Serine	1.14	1.25	Tyrosine	2.03	2.34
Proline	1.33	1.30	Lysine	2.16	2.64

TABLE - 30

N-TFA Amino Acid Methyl Esters

Conditions: 1m. x 2mm. stainless steel column packed with Gas Chrom Q coated with DEGS/EGSS-X (1.5/0.5%). Flow rate - 25 ml./min. Temperature increased by 3°/min. from 60° to 200°.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Alanine	0.60	Cysteine	1.95
Valine	0.56	Aspartic Acid	2.23
Isoleucine	0.74	Hydroxyproline	2.21
Threonine	0.85	Methionine	2.54
Leucine	1.00 (13.1 min)	Glutamic Acid	2.72
Glycine	1.22	Phenylalanine	2.70
Serine	1.50	Tyrosine	3.48
Proline	1.54	Lysine	4.22

phenylalanine are still not resolved.

Table 31 gives the retentions on 1 and 2 m. columns coated with DEGS/EGSS-X in the ratio 1 to 2% respectively. Surprisingly on the 2 m. column isoleucine and threonine are not resolved, whereas they are quite well resolved on the 1 m. column at both rates of temperature increase. The effect of altering the rate of temperature increase is shown in that the resolution of aspartic acid and glutamic acid is better at 3°/min. than at 4°/min. on the 1 m. column. On the 2 m. column they are not resolved. Alanine/valine and glutamic acid/phenylalanine remain unresolved with this column packing.

The effect of increasing the percentage of phase to 4% is given in Table 32. With a 2 m. column, alanine and valine were very poorly resolved, but isoleucine and threonine were well resolved, whereas with a 4 m. column neither pair were resolved to any appreciable extent. On the 4 m. column there was an incipient separation of glutamic acid and phenylalanine. On both columns histidine was eluted before arginine; this is the reverse of the order for the NPGS columns. Aspartic acid and hydroxyproline, which were not resolved on the 2 m. column were well resolved on the longer column. The resolution of glycine and serine on the 4 m. column was just satisfactory, whereas on the 2 m. column they were resolved by a considerable amount.

EGSP-Z is similar to EGSS-X except that a phenyl silicone replaces the methyl silicone of the EGSS-X. The results of Table 33 show that with a 4 m. column of DEGS/EGSP-Z gives similar results to the DEGS/EGSS-X, although the relative retentions differ. The introduction of an isothermal 'stop' at the start of the analysis improved the separation of threonine and isoleucine; however under these

TABLE - 31

N-TFA Amino Acid Methyl Esters

Conditions: 1m. or 2m. x 2mm. stainless steel column packed with Gas Chrom Q coated with DEGS/EGSS-X (1/2%). Flow rate - 20ml./min. Temperature increased from 60° to 200° at rate shown.

	Relative Retention		Relative Retention
	1m column		2m column
Amino Acid	3°/min.	4°/min.	3°/min.
Alanine	0.71	0.72	0.74
Valine	0.71	0.70	0.74
Isoleucine	0.80	0.81	0.88
Threonine	0.87	0.88	0.90
Leucine	1.00 (14.4 min)	1.00 (12.5 min)	1.00 (20.0 min)
Glycine	1.15	1.13	1.08
Serine	1.29	1.26	1.20
Proline	1.41	1.39	1.28
Cysteine	1.62	1.56	1.45
Aspartic Acid	1.78	1.70	1.54
Hydroxyproline	1.82	1.73	1.54
Methionine	2.06	2.00	1.76
Glutamic Acid	2.23	2.11	1.86
Phenylalanine	2.23	2.11	1.86
Tyrosine	2.62	2.42	2.14
Lysine	3.27	3.02	2.80

TABLE - 32

N-TFA Amino Acid Methyl Esters

Conditions: 2 and 4 m. x 2mm. stainless steel columns packed with Gas Chrom Q coated with DEGS/EGSS-X (3/1%). Flow rate 20 ml./min. Temperature increased from 60° to 200°.

Amino Acid	Relative Retention 2m column 3°/min.	Relative Retention 4m column 4°/min.	Amino Acid	Relative Retention 2m column 3°/min.	Relative Retention 4m column 4°/min.
Alanine	0.68	0.85	Aspartic Acid	2.05	1.60
Valine	0.64	0.88	Hydroxyproline	2.07	1.68
Isoleucine	0.78	0.93	Methionine	2.42	1.80
Threonine	0.91	0.96	Glutamic Acid	2.58	1.94
Leucine	1.00 (13.2 min)	1.00 (16.0 min)	Phenylalanine	2.61	1.96
Glycine	1.25	1.22	Tyrosine	3.12	2.40
Serine	1.42	1.26	Lysine	3.82	2.92
Proline	1.55	1.38	Histidine	3.65	3.23
Cysteine	1.80	1.46	Arginine	4.05	3.50

TABLE - 33

N-TFA Amino Acid Methyl Esters

Conditions: 4m. x 2mm. stainless steel column packed with Gas Chrom Q coated with DEGS/EGSP-Z (3/1%). Flow rate - 50ml./min. Temperature increased from 60° to 200°; A - 6 min. at 60° then at 4°/min. B - 3°/min. from 60° with no initial isothermal step.

Amino Acid	Relative Retention A.	Relative Retention B.	Amino Acid	Relative Retention A.	Relative Retention B.
Alanine	0.68	0.62	Cysteine	1.42	1.62
Valine	0.71	0.66	Aspartic Acid	1.53	1.78
Isoleucine	0.84	0.80	Hydroxyproline	1.51	1.78
Threonine	0.92	0.86	Methionine	1.71	2.22
Leucine	1.00 (17.1 min)	1.00 (12.6 min)	Glutamic Acid	1.79	2.33
Glycine	1.08	1.16	Phenylalanine	1.79	2.31
Serine	1.20	1.31	Tyrosine	2.03	2.75
Proline	1.32	1.42	Lysine	2.46	3.48

conditions alanine and valine were still not resolved. Nor were these amino acids resolved by the other method of raising the temperature, despite the apparently different relative retentions - since the peaks were very broad at this temperature, they overlapped to such an extent that no resolution occurred.

A composite column was prepared consisting of 1 m. of the 5% NPGS packing and 1 m. of the DEGS/EGSS-X (1.5/0.5%) packing and the retention values are given in Table 34. As might have been expected, alanine and valine were not very well resolved, whilst leucine and glycine were eluted together - this is not entirely surprising since glycine is eluted before leucine on the NPGS packing, and after leucine on the DEGS/EGSS-X columns. Serine and proline were just adequately resolved, cysteine and aspartic acid were not separated at all, and the resolution of glutamic acid from phenylalanine was poor.

Table 35 gives the results obtained when a ternary mixture of DEGS, EGSS-X and EGSP-Z was used in the ratio 1.5 to 1.5 to 3%. Although alanine and valine were not resolved at all, the resolution of isoleucine from threonine was quite good, and all of the other amino acids chromatographed were well resolved. Figure 12 shows a typical separation on this column.

By using this column in conjunction with the 2 m., 5% NPGS column, it was possible to achieve a complete separation of all of the N-TFA amino acid methyl esters. Only serine and proline were unresolved on the NPGS column and these were well resolved on the column coated with the ternary mixture. However, it was decided that the n-propyl and n-butyl esters of the N-TFA amino acids should be investigated to see if a complete separation of either of these derivatives on a single column was possible.

TABLE - 34

N-TFA Amino Acid Methyl Esters

Conditions: 2m. x 2mm. stainless steel column of Gas Chrom Q coated with NPGS (5%) for detector half of column and DEGS/EGSS-X (1.5/0.5%) for injection half of column. Flow rate 25 ml/min. Temperature increased from 60° to 200° at 3°/min.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Alanine	0.74	Cysteine	1.43
Valine	0.76	Aspartic Acid	1.43
Isoleucine	0.88	Hydroxyproline	1.49
Threonine	0.94	Methionine	1.67
Leucine	1.00 (22.0 min)	Glutamic Acid	1.73
Glycine	1.00	Phenylalanine	1.76
Serine	1.16	Tyrosine	2.06
Proline	1.20	Lysine	2.65

TABLE - 35

N-TFA Amino Acid Methyl Esters

Conditions: 4m. x 2mm. stainless steel column packed with Gas Chrom Q coated with DEGS/EGSS-X/EGSP-Z (1.5/1.5/3%). Flow rate - 26ml./min. Temperature increased from 60° to 200° at 3°/min.

Amino Acid	Relative Retention	Amino Acid	Relative Retention
Alanine	0.80	Proline	1.33
Valine	0.80	Cysteine	1.37
Isoleucine	0.88	Aspartic Acid	1.45
Threonine	0.91	Hydroxyproline	1.49
Leucine	1.00 (24.8 min)	Methionine	1.93
Glycine	1.06	Glutamic Acid	2.01
Serine	1.12	Phenylalanine	2.04

FIGURE 12

Separation of N-TFA amino acid methyl esters on a 4 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with DEGS/EGSS-X/EGSP-ZSP-Z (1.5/1.5/3%). Flow rate - 26 ml./min. Temperature increased from 60° to 200° at 3°/min. 5 µl of sample applied, containing about 10^{-8} moles of each amino acid.

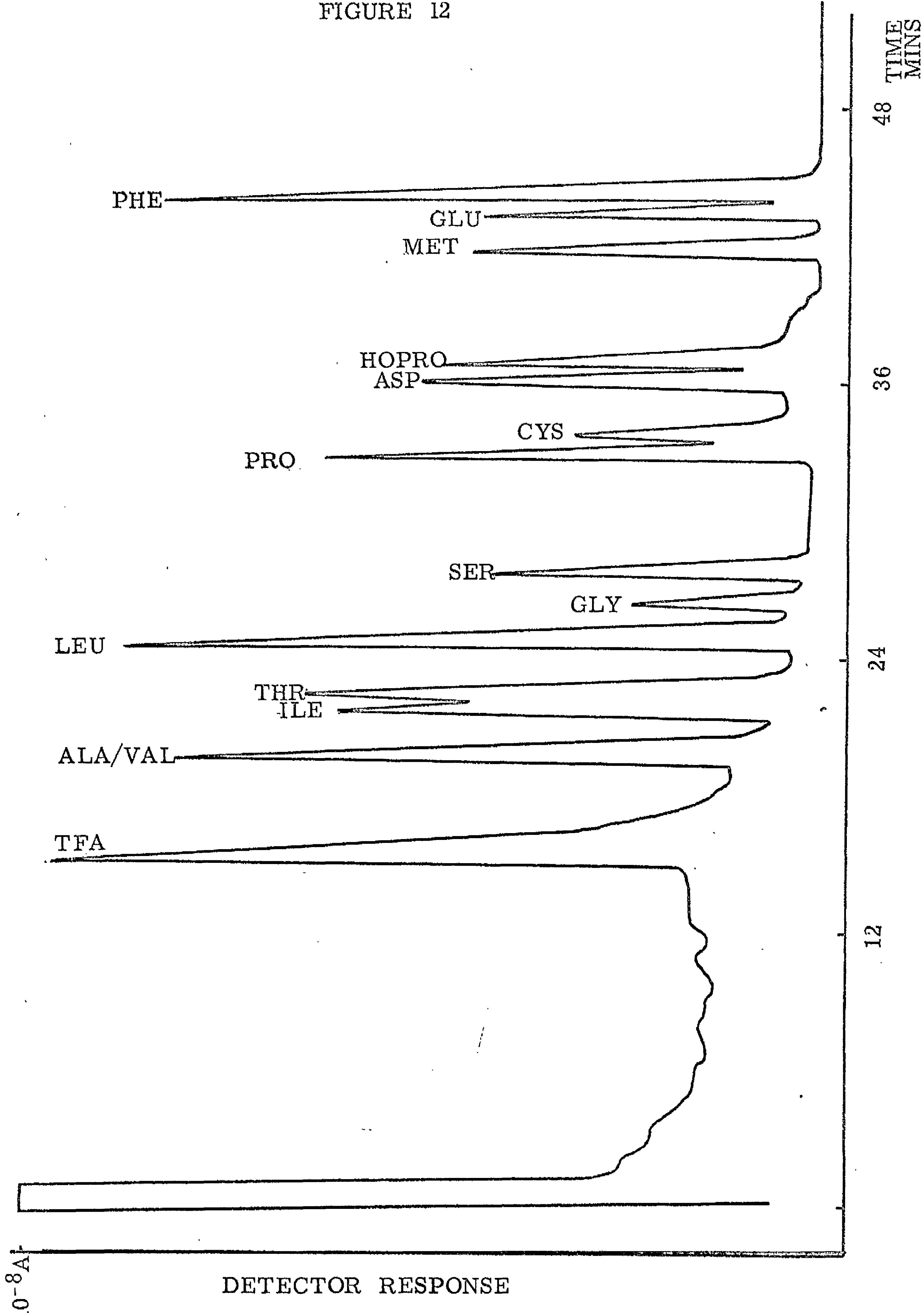


FIGURE 12

5. N-TFA amino acid n-propyl esters:

(i) Preparation of derivatives: The n-propyl esters of the amino acids were prepared either by esterification with Dowex 50 as catalyst (Experimental Section III (1) (iv) or by interesterification with HCl as catalyst (Experimental Section III (1) (vii). Trifluoroacetylation was carried out using the method described in Experimental Sections III (2) (ii), (c) or (e).

(ii) Conditions of Chromatography: The conditions were as described in the Results Section I (4) (iii).

(iii) Retention Data: Table 36 gives the relative retention of N-TFA amino acid n-propyl esters on 2 m. columns coated with either 5% of NPGS or 4% of EGSS-X. Surprisingly on the NPGS column, valine preceded alanine, threonine preceded isoleucine and leucine preceded glycine. Alanine and threonine were not resolved when a mixture was chromatographed. Aspartic acid and glutamic acid were retarded compared to the behaviour of the methyl esters, aspartic acid being eluted after cysteine, and just being resolved from hydroxyproline (though not very well) and glutamic acid being eluted after phenylalanine. All the other amino acid derivatives were well resolved.

On the EGSS-X column valine/threonine and glycine/serine were not resolved. Again threonine preceded isoleucine. Aspartic acid was eluted after hydroxyproline which was not well resolved from cysteine. Glutamic acid was also retarded and emerged after methionine which was eluted after phenylalanine. These three were not completely resolved from one another.

6. N-TFA amino acid n-butyl esters

(i) Preparation of derivatives: The amino acids were interesterified with HCl as catalyst (Experimental Section III (1) (vii).) The trifluoroacetylation

TABLE - 36

N-TFA Amino Acid n-Propyl Esters

Conditions: 2m. x 2mm. stainless steel columns packed with Gas Chrom Q coated with either 5% of NPGS or 4% of EGSS-X. Flow rates 32.5 ml./min. and 25 ml./min. respectively. Temperature increased from 60° to 230° at 3°/min.

Amino Acid	NPGS	EGSS-X	Amino Acid	NPGS	EGSS-X
Alanine	0.88	0.65	Aspartic Acid	1.45	1.67
Valine	0.82	0.72	Hydroxyproline	1.46	1.53
Isoleucine	0.94	0.77	Methionine	1.51	1.82
Threonine	0.90	0.73	Phenylalanine	1.55	1.79
Leucine	1.00 (26.5) min.	1.00 (17.6) min.	Glutamic Acid	1.67	1.99
Glycine	1.09	1.07	Tyrosine	1.89	2.19
Serine	1.09	1.06	Lysine	2.14	2.67
Proline	1.28	1.25	Tryptophan	2.23	2.72
Cysteine	1.33	1.47			

was carried out by the method of Stalling and Gehrke (1966) (Experimental Section III, (2) (ii), (e).)

(ii) Conditions of chromatography: The conditions were as detailed in the Results Section I (4) (iii).

(iii) Retention Data: The relative retentions of the N-TFA amino acid n-butyl esters on columns coated with 4% EGSS-X and DEGS/EGSS-X (0.75/0.25%) are given in Table 37. On the former column, alanine/valine and methionine/phenylalanine were not resolved. The resolution of glutamic acid and tyrosine was incomplete. It is interesting that on this column serine is eluted before glycine. Once again as with the n-propyl esters, the dicarboxylic acids are retained for much longer times than the methyl esters. Thus aspartic is eluted well after hydroxyproline and glutamic is eluted after tyrosine.

On the DEGS/EGSS-X column the separation was very poor. Serine and threonine gave peaks which tailed very badly on this column, and as a result, although alanine, valine and isoleucine were eluted sharply, glycine, leucine and threonine emerged as a single peak, as did serine and proline; aspartic acid and methionine were not resolved either. Again on this column the dicarboxylic acids were considerably retarded, even more so than the n-propyl esters (Table 35).

II. Quantitation Data

(1) Introduction:

Since no single column was found with which the N-TFA amino acid methyl, n-propyl or n-butyl esters could be completely separated, it was decided that the quantitative aspects of the problem would be best investigated using the two column separation of the N-TFA methyl esters, described in the previous section (Results, Section I, 4, iv and v). Initially the pure derivatives of

TABLE 37

N-TFA amino acid n-butyl esters

Conditions: 2 m. x 2 mm. stainless steel columns packed with Gas Chrom Q coated with either 4% EGSS-X or DEGS/EGSS-X (0.75/0.25%). Flow rates - 25 ml./min. and 15 ml./min. respectively. Temperature raised from 60° to 200° at 3°/min.

Amino Acid	Relative Retention EGSS-X	Relative Retention DEGS/EGSS-X	Amino Acid	Relative Retention DEGS	Relative Retention DEGS/EGSS-X
Alanine	0.78	0.74	Cysteine	1.41	1.34
Valine	0.78	0.81	Hydroxyproline	1.50	1.40
Isoleucine	0.87	0.88	Aspartic Acid	1.69	1.59
Threonine	0.82	0.96	Methionine	1.76	1.58
Leucine	1.00 (20.3 min.)	1.00 (20.5 min.)	Phenylalanine	1.75	1.67
Glycine	1.12	1.00	Tyrosine	1.92	1.79
Serine	1.07	1.14	Glutamic Acid	1.97	1.86
Proline	1.31	1.18	Lysine	2.53	2.39

a number of amino acids were prepared. However, in view of the fact that they proved to be relatively unstable on storage, it was decided to concentrate attention on the reproducibility of derivative formation. The available methods for preparation of derivatives were compared, and the method which gave the best results was used in all subsequent work.

(2) Comparison of Preparative Methods:

(i) Calculation of Molar Response Factors: Owing to the variation of the detector response with the conditions under which the detector is operated, it is impossible to determine an absolute value for the molar response of any compound and therefore measurements must be made relative to some chosen standard. Initially an attempt was made to use as standards the N-TFA methyl esters of the amino acids, prepared on a relatively large scale in the pure form, as described in the Experimental Section IV, 2. This was frustrated by two difficulties: (i) the difficulty of accurately placing a precisely known amount of sample onto the column; this could be overcome by the use of a suitable internal standard. (ii) the difficulty of instability of the derivatives; it was found that the response varied with conditions of chromatography and with the age of the sample.

It was decided that, since the reproducibility of preparation of the derivatives of the simple aliphatic amino acids was good, these would be suitable for use as a standard. Of these, leucine was chosen as the standard. Thus to calculate the relative molar response, the area of the peak for a particular amino acid derivative was divided by the area of the leucine peak, and also by the molar ratio of the two amino acids in the original mixture. The composition of this mixture is given in Table 38.

TABLE - 38

Composition of Standard Amino Acid Mixture

	mmole/250 ml.
Alanine	2.011
Valine	1.000
Isoleucine	0.991
Threonine	1.025
Glycine	2.003
Leucine	0.967
Serine	1.004
Proline	0.994
Aspartic Acid	0.971
Cysteine	1.008
Hydroxyproline	0.997
Methionine	0.992
Glutamic Acid	0.994
Phenylalanine	0.994
Tyrosine	1.005
Lysine	2.012
Tryptophan	0.998
Arginine	2.003
Histidine	1.998
Cystine	2.000

(ii) Comparison of methylation procedures: Three methods of methylation were compared. These were:-

(1) methylation with methanol saturated with dry HCl (Experimental, Section III, (1), (i)).

(2) methylation with dimethyl sulphite and HCl as catalyst (Experimental, Section III, (1), (ii)).

(3) methylation with thionyl chloride as catalyst (Experimental, Section III, (1), (iii)).

5 ml. samples of the standard amino acid mixture containing 20 - 40 μmoles of each amino acid were taken to dryness on the rotary evaporator. The samples were then methylated by one of the three methods given above, three samples being used for each method. Trifluoroacetylation was carried out by the method described in the Experimental, Section III, (2), (ii), (e). The samples were then chromatographed on the 2 m. 5% NPGS column, so that the only amino acids not resolved were serine and proline. For each analysis, the peak areas were determined and the relative molar response calculated as described in the Results, Section II, (2), (i). The results are given in Table 39. A combined relative molar response factor is given for serine and proline. Arginine, histidine, tryptophan and cystine were not included in this analysis.

Consistently higher values for alanine, valine, isoleucine, threonine, cysteine, methionine and tyrosine were obtained with method 3 than with the other two methods. Cruickshank and Sheehan (1964) had observed that four amino acids (valine, isoleucine, threonine and lysine) were not completely esterified in 30 minutes with dimethyl sulphite as catalyst. However, lysine does not appear to be esterified in poor yield by method 1. The agreement of the values within each of the methods is quite good in view of the accuracy of peak area estimation.

TABLE - 39

N-TFA Amino Acid Methyl Esters

Comparison of Methylation Procedures

Amino Acid	Relative Molar Response								
	Method 1			Method 2			Method 3		
	1.	2.	3.	1.	2.	3.	1.	2.	3.
Alanine	.487	.481	.481	.481	.490	.490	.498	.506	.514
Valine	.917	.916	.927	.932	.967	.922	.955	1.005	1.000
Isoleucine	.853	.843	.902	.887	.877	.893	.958	.939	.976
Threonine	.688	.668	.712	.697	.726	.706	.705	.733	.752
Glycine	.280	.281	.282	.282	.266	.253	.266	.273	.276
Leucine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Serine/Proline	1.230	1.197	1.225	1.196	1.196	1.184	1.196	1.171	1.138
Aspartic Acid	.580	.544	.518	.518	.536	.540	.516	.532	.518
Cysteine	.461	.438	.394	.173	.175	.138	.474	.481	.469
Hydroxyproline	.753	.732	.727	.741	.741	.753	.714	.734	.713
Methionine	.493	.459	.481	.496	.480	.474	.532	.557	.459
Glutamic Acid	.528	.494	.509	.471	.469	.458	.501	.494	.435
Phenylalanine	1.063	1.056	1.068	1.117	1.062	1.060	1.093	1.088	1.063
Tyrosine	.624	.619	.663	.661	.673	.641	.712	.684	.728
Lysine	.618	.516	.542	.546	.594	.581	.552	.598	.531

The values for glutamic acid and methionine in one of the samples by method 3 are rather low. The factor for cysteine with method 2 is very low, possibly due to interaction of the sulphydryl group with the thionyl chloride. In conclusion, it seems that method 3 gives the best yield of amino acid derivatives, and the most consistent factors.

(iii) Comparison of trifluoroacetylation methods: The amino acid methyl ester hydrochlorides were prepared by the method which gave the best results in the previous section (method (1)). In order to eliminate inconsistencies due to the methylation procedure, 45 ml. of amino acid standard mixture was taken to dryness and methylated with methanol saturated with dry HCl (Experimental, Section III, (1), (i)) and the sample then divided between nine flasks. The trifluoroacetylation was carried out by three methods, using three samples for each method:

(1) at room temperature for 30 minutes (Experimental, Section III, (2), (ii), (c)).

(2) refluxing for 10 minutes (Experimental, Section III, (2), (ii), (a)).

(3) at 150° for 5 minutes (Experimental, Section III, (2), (ii), (e)).

The samples were chromatographed on a 2 m. 5% NPGS column, and Table 40 gives the results obtained. Once again there is some difference between the methods, and for example, the value for cysteine is higher by method 1 than by the other two methods. However, the only really significant difference between the methods is that the value obtained for arginine by method 3 is very much higher than by the other two methods. It was for this reason that Stalling and Gehrke (1966) suggested the use of this method for acylation. The reason given by these workers

TABLE 40

N-TFA Amino Acid Methyl Esters

Comparison of Trifluoroacetylation Procedures

Amino Acid	Relative Molar Response								
	Method 1			Method 2			Method 3		
	1.	2.	3.	1.	2.	3.	1.	2.	3.
Alanine	.452	.463	.457	.494	.461	.478	.491	.487	.472
Valine	.816	.830	.851	.727	.809	.823	.843	.850	.864
Isoleucine	.670	.639	.647	.697	.712	.708	.823	.855	.871
Threonine	.690	.704	.693	.636	.688	.642	.698	.707	.671
Glycine	.231	.249	.251	.248	.261	.249	.252	.260	.247
Leucine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Serine/Proline	1.206	1.195	1.173	1.174	1.205	1.183	1.138	1.160	1.195
Aspartic Acid	.568	.514	.559	.520	.543	.572	.533	.560	.577
Cysteine	.793	.578	.556	.524	.548	.561	.535	.518	.523
Hydroxyproline	.823	.815	.822	.780	.769	.793	.818	.855	.794
Methionine	.624	.639	.636	.652	.668	.671	.625	.607	.637
Glutamic Acid	.658	.616	.624	.580	.621	.632	.659	.619	.658
Phenylalanine	1.332	1.365	1.317	1.269	1.348	1.360	1.262	1.280	1.332
Tyrosine	.714	.726	.703	.652	.720	.659	.647	.621	.686
Lysine	.702	.692	.680	.724	.695	.705	.630	.621	.607
Arginine	.147	.236	.152	.043	.215	.147	.581	.620	.593

for the values reported earlier for arginine derivatives by, for example, Cruickshank and Sheehan (1964), was that the arginine derivative was not being formed during incubation with trifluoroacetic anhydride at room temperature, but in the metal preheater of the gas chromatograph. These workers also showed that when the preheater temperature was greater or less than 250°, the value obtained for the relative molar response of N-TFA arginine n-butyl ester fell. Thus at preheater temperature of 300°, the relative peak area of the arginine derivative to n-butyl stearate was 0.3 whereas at a preheater temperature of 250°, the relative peak area was 2.0.

It was found in the present work that a preheater temperature of 260° gave good and reproducible values for the arginine derivative. However, if the temperature of the preheater varied, the area of the arginine peak altered, in agreement with the observations of Stalling and Gehrke. This may explain why the values for the arginine derivative obtained by method 1 vary so much, since this sample was chromatographed at the beginning of the day, when the preheater temperature was less than 250° and at that time the importance of the preheater temperature was not realised. The samples prepared by method 3 were chromatographed with the preheater at 250 - 260°. In view of these results it was decided to use method 3 for the subsequent studies as the routine method for trifluoroacetylation.

(3) Evaluation of relative molar response factors

(i) Comparison with pure reference standard: Although the results quoted above indicate that reproducible conversion of the amino acids to N-TFA amino acid methyl esters was being achieved, it was desirable to determine the absolute degree of conversion. To do this a comparison of the factor obtained starting with leucine, with that obtained for pure, freshly distilled N-TFA leucine methyl

ester, was carried out.

It was found that when a known amount of freshly prepared N-TFA leucine methyl ester (Experimental, Section IV, (2)) was chromatographed with an appropriate amount of n-nonadecane (as internal standard), a value was obtained which corresponded closely with the value obtained when N-TFA leucine methyl ester was prepared by the routine procedure used for mixtures of amino acids (I, (2), (iv) of the Results Section) and chromatographed along with the same amount of internal standard. Table 41 gives the results obtained in this experiment. The agreement between the three replicates is good and the yield of the N-TFA leucine methyl ester in all three cases, although not 100% is 96% or better.

(ii) Relative Molar Response Factor on NPGS packing: Table 42 gives the results of a series of analyses performed on the mixture of amino acids (Table 37) prepared by the method which gave the most consistent results (Section 1, (2), (iv) of this section). The final column of the Table gives the mean value and standard error. Difficulty had been encountered in the preparation of derivatives of arginine, histidine, cystine and tryptophan using methods which involved trifluoroacetylation at room temperature. Some improvement was noted when the samples were refluxed for 10 minutes, but the most consistent results were obtained for these four amino acids using incubation at 150° for five minutes in a sealed tube. The values given in Table 42 with a 2 m. 5% NPGS column show a good level of consistency in the relative molar response. The greatest variation is shown by these amino acids which have reactive side chains, and form di- and tri-trifluoroacetyl derivatives. The cystine and histidine peaks proved difficult to estimate, since on this column these amino acid derivatives gave very low broad peaks. The cystine derivative frequently crystallised from the trifluoroacetic

TABLE 41

Molar Response Factor for N-TFA Leucine Methyl Ester

Conditions: 2m. x 2mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate -32ml./mm. Temperature - 125°.

1. Pure sample of N-TFA leucine methyl ester

	Area A sq.cm.	Concentration C um./ml.	A/C	Molar Response Factor
Leucine	23.48	21.43	1.094	0.513
n-Nonadecane	7.21	3.36	2.142	1.00

2. Samples of N-TFA leucine methyl ester prepared by method described in Experimental section III, (2).

	Area A sq.cm.	Concentration C um./ml.	A/C	Molar Response Factor
Leucine	14.80	20	0.740	0.497
n-Nonadecane	11.15	7.46	1.491	1.00
Leucine	15.70	20	0.785	0.493
n-Nonadecane	11.90	7.46	1.593	1.00
Leucine	13.95	20	0.698	0.508
n-Nonadecane	10.20	7.46	1.367	1.00

TABLE A2

N-TFA Amino Acid Methyl Esters

Relative Molar Response Factors on NPGS Column

Amino Acid	Relative Molar Response								Mean Value ± Standard Error
Alanine	.498	.506	.514	.491	.487	.472	.490	.475	.492 ± .006
Valine	.955	.930	.942	.956	.955	1.005	1.000	.967	.964 ± .009
Isoleucine	.917	.945	.958	.939	.976	.950	.926	.962	.947 ± .008
Threonine	.705	.733	.752	.698	.707	.671	.705	.710	.710 ± .008
Glycine	.252	.260	.266	.273	.276	.247	.270	.260	.263 ± .004
Leucine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Serine/Proline	1.138	1.360	1.190	1.196	1.171	1.138	1.150	1.190	1.165 ± .009
Aspartic Acid	.533	.560	.577	.516	.532	.518	.510	.530	.535 ± .008
Cysteine	.535	.518	.523	.474	.481	.469	.468	.442	.489 ± .010
Hydroxyproline	.714	.735	.713	.682	.695	.680	.732	.227	.710 ± .008
Methionine	.532	.557	.540	.502	.510	.543	.513	.536	.529 ± .007
Glutamic Acid	.504	.501	.494	.509	.494	.528	.504	.510	.506 ± .004
Phenylalanine	1.093	1.088	1.063	1.060	1.062	1.080	1.110	1.115	1.084 ± .004
Tyrosine	.686	.712	.684	.728	.673	.695	.705	.703	.698 ± .007
Lysine	.598	.630	.621	.607	.618	.594	.598	.581	.606 ± .007
Tryptophan	.207	.215	.203	.241	.182				.210 ± .010
Arginine	.581	.618	.593	.610	.586				.600 ± .006
Histidine	.093	.082	.087	.103	.125				.096 ± .006
Cystine	.378	.514	.406	.294	.362				.391 ± .032

anhydride solution, and this may explain some of the inconsistency in the results for this amino acid.

The value for the tryptophan derivative varied widely, and results for this amino acid would be correspondingly unreliable. This was probably due to some form of decomposition, for samples containing N-TFA tryptophan methyl ester rapidly turned green on standing. For the analysis of acid hydrolysates of proteins, this would be of little moment, since tryptophan is destroyed by acid hydrolysis. However, it means that a different procedure would be required for estimation of this amino acid.

(iii) Relative Molar Response Factors on Ternary Phase Column: The results of chromatographing a series of samples of N-TFA amino acid methyl esters prepared from the standard mixture on the 4 m. column packed with Gas Chrom Q coated with DEGS/EGSS-X/EGSP-Z (1.5/1.5/3%) are presented in Table 43. The value of 1.338 for alanine and valine is somewhat lower than sum of the relative molar responses of these amino acids on the NPGS column (1.456). The sum of the mean values for serine and proline on the ternary phase column is 1.167, which compares very well with the value of 1.165 obtained for the two amino acid derivatives on the NPGS packing. The agreement of the other values for this column with those for the NPGS column are quite good, with the exception of hydroxyproline (0.530 and 0.710, respectively) and tyrosine (0.584 and 0.698). The results obtained on the ternary phase column are quite consistent. The column was not operated above 200° because of the 'bleed' of DEGS; thus, the less volatile amino acid derivatives were not eluted.

III. Alcoholysis

(1) Methanolysis:

Leucylglycylglycine was treated with methanol containing 6 meq. of dry

TABLE 43

N-TFA Amino Acid Methyl Esters

Relative Molar Response Factors on Ternary Phase Column

Amino Acid	Relative Molar Response 1.	2.	3.	4.	5.	Mean Value ± Standard Error
Alanine/Valine	1.334	1.307	1.328	1.352	1.367	1.338 ± .011
Isoleucine	0.942	0.943	0.897	0.938	0.917	0.927 ± .009
Threonine	0.740	0.716	0.724	0.761	0.728	0.734 ± .009
Leucine	1.00	1.00	1.00	1.00	1.00	1.00
Glycine	0.273	0.259	0.248	0.246	0.265	0.258 ± .006
Serine	0.431	0.426	0.434	0.399	0.421	0.422 ± .006
Proline	0.731	0.746	0.735	0.762	0.750	0.745 ± .007
Cysteine	0.512	0.483	0.533	0.496	0.502	0.505 ± .005
Aspartic Acid	0.531	0.537	0.502	0.524	0.541	0.527 ± .011
Hydroxyproline	0.530	0.527	0.524	0.520	0.551	0.530 ± .009
Methionine	0.552	0.571	0.556	0.563	0.559	0.560 ± .004
Glutamic Acid	0.510	0.534	0.531	0.526	0.542	0.529 ± .006
Phenylalanine	1.120	1.084	1.079	1.092	1.110	1.097 ± .008
Tyrosine	0.560	0.572	0.614	0.620	0.565	0.584 ± .011

HCl per ml. in a sealed tube at 125° and 155° for varying lengths of time. The samples were then taken to dryness and acylated (Experimental, Section III, (2), (ii), (e)). The results are given in Table 44. Areas are expressed relative to N-TFA leucine methyl ester. From the results it appears that the methanolysis of leucylglycine is slower than of glycylglycine. At the higher temperature the reaction is not complete in 90 minutes, whilst at 125° after 4 hours there is still some of the original tripeptide left. After 12 hours at 125° there was no trace of the dipeptides and only a very small amount of the tripeptide (or some contaminent which gave a peak at the same place as the tripeptide). Since leucine-containing peptides were shown by Synge (1945) to be sterically hindered with respect to acid hydrolysis, it seems likely that if leucylglycylglycine can be completely methanolysed in 12 hours at 125°, most other peptide bonds will be cleaved in this period of time.

(2) Butanolysis:

The results of cleavage of leucylglycylglycine with butanol/HCl are given in Table 45. Clearly, the rate of butanolysis is more rapid than the rate of methanolysis, since only leucine and glycine appeared on chromatograms after 3 hours at 125°. The complete disappearance of the tripeptide and dipeptide derivatives in this time confirm this.

IV. Amino Acid Analysis of Peptides by GLC

(1) Hydrolysis followed by analysis:

(i) Pure Peptides: A number of synthetic peptides (obtained from Nutritional Biochemicals Corpn., Cleveland, Ohio, U.S.A.) were hydrolysed and N-TFA amino acid methyl esters prepared as described in the Experimental Section (III, (2)), Table 46 gives the results of these analyses. The amino

TABLE - 44

Methanolysis of Leucyglycylglycine

Conditions: 2 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate - 32 ml./min. Temperature raised from 60° to 210° at 3°/min.

N-TFA Methyl Ester	Relative Peak Area						
	125°				155°		
	Methanolysis Time				Methanolysis Time		
	1 hr.	2 hr.	4 hr.	12 hr.	30 min.	60 min.	90 min.
Glycine	1.19	1.07	0.63	0.56	1.34	1.07	0.71
Leucine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycylglycine	0.14	0.15	0.08	0.00	0.12	0.10	0.05
Leucylglycine	0.78	0.71	0.32	0.00	0.76	0.50	0.20
Leucylglycylglycine	1.28	0.45	0.15	0.01	1.37	0.60	0.19

TABLE 45

Butanolysis of Leucylglycylglycine

Conditions: 2 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 4% of EGSS-X. Flow rate — 25 ml./min. Temperature raised by 3°/min. from 60° to 200°.

N-TFA n-butyl ester	Relative Peak Area			
	Time of Butanolysis at 125°			
	1 hr.	2 hr.	3 hr.	22 hr.
Leucine	1.00	1.00	1.00	1.00
Glycine	0.84	0.69	0.52	0.54
Glycylglycine	0.23	0.04	0.01	0.00
Leucylglycine	0.26	0.02	0.00	0.00
Leucylglycylglycine	0.17	0.04	0.00	0.00

TABLE 46

Amino Acid Analysis of Peptides

N-TFA Amino Acid Methyl Esters

Conditions: 2 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate 32 ml./min. Programmed from 60° to 210° at 3°/min.

(a) observed value (hydrolysis) (b) observed value (methanolysis) (c) actual value

Amino Acid	Peptide Relative Molar Ratio											
	1			2			3			4		
	a.	b.	c.	a.	b.	c.	a.	b.	c.	a.	b.	c.
Alanine	1.00	1.00	1				1.00	1.00	1			
Valine	1.05	1.01	1									
Glycine				1.98	1.99	2						
Leucine				1.00	1.00	1				1.00	1.00	1
Phenylalanine							1.12	0.97	1			
Tyrosine										0.97	0.93	1

acid composition of the peptides is in good agreement with the expected value; the amount of material applied to the column for analysis corresponded to 50 µg. of peptide.

(ii) Peptides eluted from fingerprints: Several peptides which had been eluted from fingerprints were kindly supplied by Dr. C. Milstein. The results of the analysis of these peptides after hydrolysis and derivative preparation as for the peptides of IV, (1), (i), are given in Table 47. The values obtained by ion-exchange chromatography are given in the same table. There appears to be no obvious correlation between the observed values and the actual values. However, when a 2 cm.^{2} piece of Whatman 3MM filter paper which had been used for fingerprinting was eluted with dilute HCl and the residue, after taking to dryness, was taken through the process of hydrolysis and derivative formation, the chromatogram shown in Figure 13 was obtained. Clearly the reason for the poor correlation of the results is that a number of components are being eluted from the paper which give peaks after being taken through the methylation and trifluoroacetylation procedures. It is not certain how consistent this background would be, but it would interfere to a much greater extent with a small amount of peptide than with a large amount.

V. Amino Acid Analysis of Proteins by GLC

(i) Cytochrome c: The results of amino acid analysis of horse heart cytochrome c (commercial sample, 94% pure) are given in Table 48. Method 1 refers to amino acid analysis carried out using an amino acid analyser which was designed and built by Dr. G. Leaf. The design was based on the model of Spackman et. al. (1958) and had been modified to incorporate the improvements of Piez and Morris (1960). A full account of this apparatus has been published (Black, 1964). The results are presented as µmoles of amino acid per mg. of

TABLE 47

Amino Acid Analysis of Peptides, N-TFA Amino Acid Methyl Esters

Conditions: 2m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate - 32ml./min. Temperature raised from 60° to 230° at 3°/min. (a) value obtained by GLC (b) value obtained by ion-exchange chromatography.

Amino Acid	Peptide Molar Ratio 2de2 (a)	2de2 (b)	27ab (a)	27ab (b)	2gl2 (a)	2gl2 (b)	2de4b (a)	2de4b (b)
Alanine	1.8		1.6	0.9	1.3		0.6	0.85
Valine	1.2		1.0	0.9	0.4		0.2	0.9
Isoleucine	1.5		1.8		1.0		0.2	
Threonine	-		0.4	1.5	-		2.2	2.8
Glycine	1.1	0.99	0.8		0.6		2.5	0.86
Leucine	1.0		0.6		0.6		1.0	
Serine/Proline	1.1	2.81	1.2	2.2	0.7	1.10	1.1	2.07
Aspartic Acid	1.3		1.7		1.5	1.87	0.3	
Cysteine	0.7		0.5	1.0	-		-	
Methionine	0.3		-		-		-	
Glutamic Acid	1.0	1.07	1.7	1.0	1.1	1.15	0.3	1.00
Phenylalanine	-		-		0.1		1.9	
Tyrosine	-		-		-		1.2	
Lysine	0.9		1.0		0.7	0.9	0.4	0.9
Arginine	2.7	0.97	2.1		0.9		3.2	
Histidine	1.5		1.9		0.5		0.2	

FIGURE 13

Chromatogram obtained when 5 µl. of a sample extracted from a fingerprint, hydrolysed, methylated and trifluoroacetylated was applied to a 2 m. x 2 mm. stainless steel column packed with Gas Chrom Q coated with 5% of NPGS. Flow rate - 32 ml./min. Temperature increased from 60° to 215° at 3°/min.

FIGURE 13

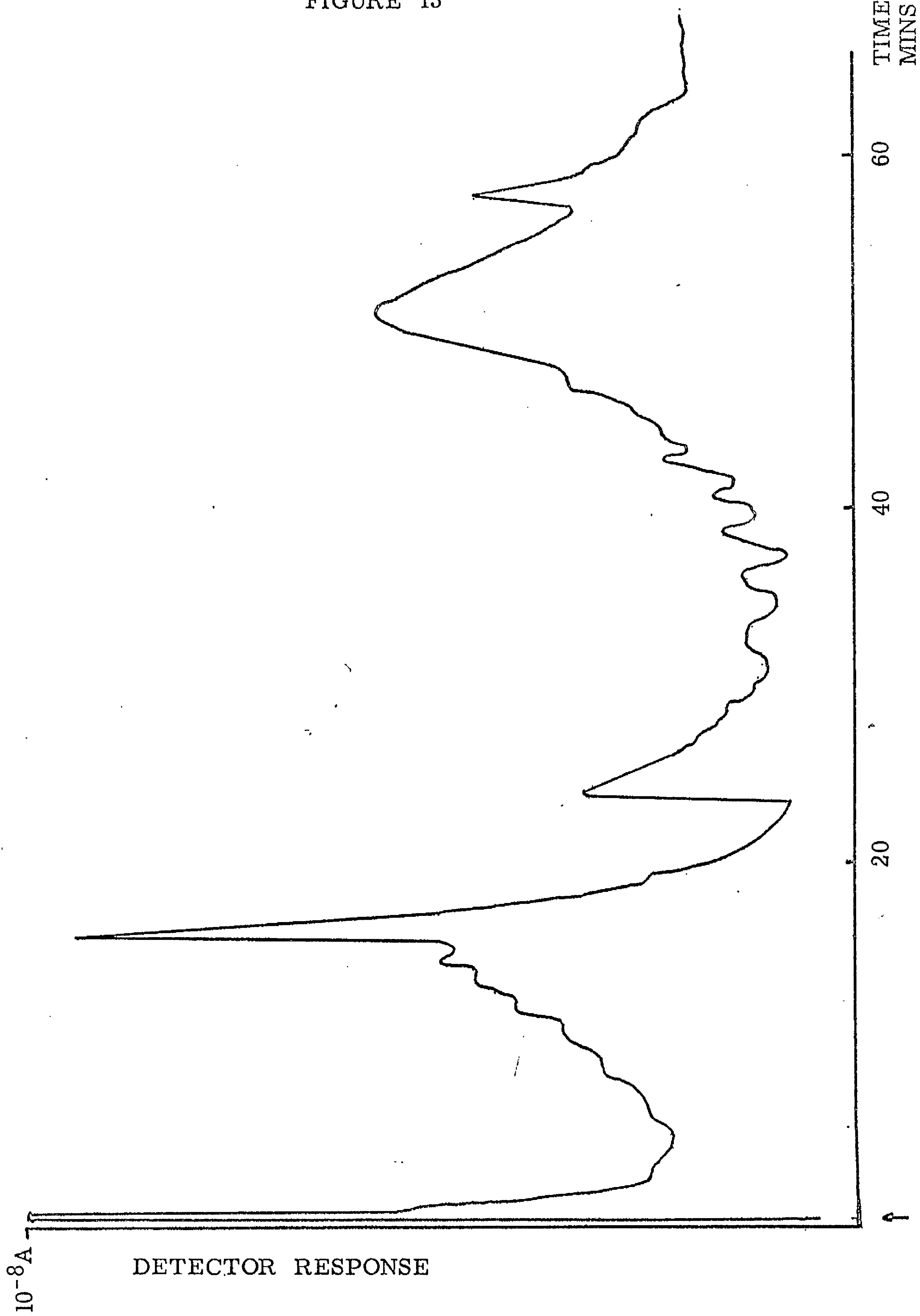


Table 48

Amino Acid Composition of Horse Heart Cytochrome c

Amino Acid	Method 1		Method 2		Method 3		Residues/ mole of protein
	umoles/mg. protein	Ratio	Relative molar area	Ratio	Relative molar area	Ratio	
Alanine	.627	7.56	6.87	5.18	9.05	6.38	6
Valine	.268	3.21	3.21	2.42	0.37	0.26	3
Isoleucine	.468	5.61	6.70	5.02	3.41	2.41	6
Threonine	.782	9.38	11.08	8.38	8.02	5.67	10
Glycine	.969	11.65	10.45	7.87	24.0	16.9	12
Leucine	.479	5.74	8.00	6.00	6.70	4.73	6
Serine	.083	0.996	0.77	0.58	0.47	0.33	-
Proline	.369	4.44	4.71	3.54	6.32	4.64	4
Aspartic Acid	.744	8.90	10.05	7.58	11.50	8.10	8
Cysteine	-	-	1.73	1.31	3.73	2.63	2
Methionine	-	-	-	-	-	-	2
Glutamic Acid	.994	11.91	16.9	12.7	17.3	12.18	12
Phenylalanine	.340	4.08	5.58	4.20	6.44	4.54	4
Tyrosine	.256	3.01	4.00	3.00	4.27	3.00	4
Lysine	1.410	16.96	20.5	15.5	23.3	16.50	19
Arginine	.206	2.48	3.71	2.79	3.69	2.58	3
Histidine	.251	3.0	3.02	2.27	2.85	2.01	3

protein and ratio of each of the amino acids present is given in the adjacent column. No values are given for cysteine (which appeared as cysteic acid) or methionine, which did not appear on the chromatogram. The sample used was only six months old, but it clearly had undergone some changes; it also contained serine, which is not normally present in cytochrome c.

Method 2 refers to analysis of N-TFA amino acid methyl esters prepared from a hydrolysate of the protein (Experimental Section II, (i) and Section III, (1); (i), and III, (2), (ii), (e)) and method 3 to analysis of N-TFA amino acid methyl esters prepared from a methanolysate of the protein (Experimental, Section II, (ii) and Section III, (2), (ii), (e)). The amino acid derivatives were chromatographed on the 2 m. 5% NPGS column, and the values for serine and proline were obtained by analysing the preparation on the 4 m. ternary phase column. The results are expressed as relative molar areas - that is to say the value given for leucine is the area observed in sq. cm., and the other values represent the area of the other amino acids multiplied by the relative molar response factor (Tables 42 and 43). The ratio of the amino acids to tyrosine are given in the adjacent columns. The number of residues of each amino acid per mole of protein is given in the final column of the table.

The agreement between the values obtained by methods 1 and 2 is not very good. However, when the results of method 2 are compared with the number of residues in the protein, the agreement is better. The values for glycine and lysine are very low, but for most of the other amino acids the values are in fairly good agreement, though many of them are on the low side. On the other

hand, the values obtained by method 3 are very poor in the case of those amino acids where steric hindrance is thought to cause a slow rate of peptide bond cleavage. Thus for example, the value for valine is one tenth of its previous value. The picture is further complicated by the appearance of a number of additional peaks on the chromatogram, presumably due to di- and tripeptides. From this evidence, it seems that the methanolysis has been incomplete, in the case of peptide bonds involving certain amino acids such as valine, isoleucine, threonine and leucine. This time the value for glycine is rather higher than expected, as is the value for cysteine. No peak was obtained for methionine by either method of preparation of derivatives.

(ii) Bovine Insulin: Table 49 gives the results of amino acid analysis obtained by ion-exchange chromatography, and by GLC for bovine insulin. The two sets of values are in quite good agreement although the values for valine, leucine, glutamic acid and phenylalanine are rather high. The final column of the table gives the number of residues per mole of protein. The values for glycine, isoleucine, serine, proline and cysteine are rather low.

(iii) Bovine Serum Albumen: Table 50 presents the results of amino acid analysis by the two methods. The agreement here is quite good with the exception of glutamic acid, phenylalanine and lysine which are much higher in the GLC analysis. The cysteine value is almost certainly not an accurate reflection of the amount of cysteine in the protein. The results of methanolysis of both insulin and bovine serum albumen have not been included because the chromatograms obtained contained very few peaks which could be conclusively identified as amino acids.

TABLE 49

Amino Acid Composition of Bovine Insulin

	Amino Acid Analyser		GLC Method		
Amino Acid	um./mg. protein	Ratio	Relative Molar Area	Ratio	Residues/mole of protein
Alanine	.329	3.3	7.13	3.85	3
Valine	.484	4.9	10.8	5.77	5
Isoleucine	.050	0.5	0.93	0.5	1
Threonine	.093	1.0	1.87	1.0	1
Glycine	.376	3.8	5.31	2.85	4
Leucine	.516	5.2	12.28	6.5	6
Serine	.279	2.75	3.40	1.82	3
Proline	.104	1.05	1.13	0.6	1
Aspartic Acid	.310	3.05	5.17	2.75	3
Cysteine	.424[1]	4.3	6.74	3.6	6
Glutamic Acid	.776	7.85	18.3	9.8	7
Phenylalanine	.308	3.1	8.05	4.3	3
Tyrosine	.340	3.45	4.87	2.6	4
Lysine	.135	1.35	1.32	0.7	1
Arginine	.155	1.55	1.97	1.05	1
Histidine	.198	2.0	3.02	1.6	2

TABLE 50

Amino Acid Composition of Bovine Serum Albumen

Amino Acid	Amino Acid Analyser		GLC Method		
	um./mg. of protein	Ratio	Rel. molar area	Ratio	Residues/mole of protein
Alanine	.402	3.60	3.85	3.74	42.3
Valine	.280	2.51	2.49	2.42	37.4
Isoleucine	.112	1.00	1.03	1.00	18.6
Threonine	.267	2.40	2.66	2.57	27.3
Glycine	.136	1.22	0.77	0.75	15.8
Leucine	.475	4.26	5.40	5.25	66.5
Serine	.201	1.80	2.61	2.53	17.3
Proline	.298	2.67	3.32	3.23	28.3
Aspartic Acid	.410	3.68	4.73	4.61	57.7
Cysteine	.258	2.23	0.78	0.75	23.9
Methionine	.003	0.03	1.19	1.15	4.9
Glutamic Acid	.703	6.30	10.64	10.30	74.6
Phenylalanine	.252	2.26	4.83	4.68	26.3
Tyrosine	.152	1.36	2.36	2.29	20.2
Lysine	.628	5.63	9.25	8.98	50.4
Arginine	.215	1.92	2.18	2.11	23.7
Histidine	.181	1.62	2.10	2.04	15.9

DISCUSSION

DISCUSSION

I. Separation of Amino Acid Derivatives by GLC

The adaptation of gas chromatographic techniques for the quantitative analysis of amino acids presents two distinct, but overlapping problems. First of all a suitable derivative must be found, and column conditions established for completely separating these derivatives of the twenty protein amino acids. Secondly, there is the problem of quantitative estimation of these derivatives.

Although a number of groups have published reports of the separation of all of the protein amino acids, as the N-TFA methyl esters (Hagen and Black, 1964; Cruickshank and Sheehan, 1964; Makisumi and Saroff, 1965), and as the N-TFA n-butyl esters (Zomzely, Marco and Emery, 1962; Gehrke, Lamkin, Stalling and Shahroki, 1965), it has not been possible to repeat the separations claimed by these workers, though many attempts have been made. Thus, Figure 10 shows the results obtained when the conditions used by Cruickshank and Sheehan were reproduced, in every regard except the column diameter, and Table 37 gives the retentions of N-TFA n-butyl esters on a column similar to that of Gehrke et. al. except that Gas Chrom Q was used instead of Chromosorb W, that the column was of stainless steel instead of glass, and was of 2 mm. internal diameter instead of 4 mm. Indeed in this latter case, no better results were obtained using a commercially prepared column packing supplied by Applied Science Labs. coated with DEGS/EGSS-X (0.75/0.25%).

The reasons for this failure to reproduce the separations of other workers are not evident. It may be that column conditions must be absolutely reproduced - and the number of variables involved here include the nature of the column itself, the type of support used, the stationary phase used (which varies from batch to

batch), the column dimensions (both length and diameter), the carrier gas flow rate, the rate of temperature increase and the method of coating the support and packing the column. Lack of technical skill may not be the only factor involved here, for the fact that so many methods are described in the literature suggests that others have experienced similar difficulties. In any event, if the method is to be satisfactory for routine use it cannot rely on chance alone for producing good columns, or on the possession of highly specialised technical skill on the part of the operator.

Though the search for a single column which would separate derivatives of all of the protein amino acids was unsuccessful partial separations were achieved which, on combination, allowed of complete analysis using two columns. By using the 2 m. 5% NPGS column and the 4 m. ternary phase columns described in the Results Section I, 4, a, complete analysis of the N-TFA methyl esters of all of the amino acids could be carried out in just over two hours, thus enabling four complete analyses to be completed per day.

The retention data for the amino acid derivatives studied are presented in Tables 14 to 37. The reasons for choosing the N-TFA methyl esters are underlined by the results shown on Table 51 (Makisumi, Nicholls and Saroff, 1963). Of the N-acyl esters, the N-TFA methyl esters are the most volatile. This does not mean that they will be the most easily resolved, but it does mean that relatively high concentrations of stationary phase can be used without the amino acid derivatives having extremely long retention times.

When one examines the order of elution of the N-TFA amino acids esters it is clear that van der Waals forces are of primary importance. However, certain anomalous behaviour is apparent. Thus, glycine is eluted after alanine, valine

TABLE 51

Retention times of N-acylated esters of Leucine

Conditions: 6 ft. x 3/16 in. glass column packed with 2% of NPGS on Fluoropak 80 or Chromosorb. Sample size 0.2 umole.

Ester	Trifluoroacetylated Leucine		N-Acetylated Leucine	
	142° (min.)	181° (min.)	142° (min.)	181° (min.)
Methyl	1.9	0.5	11.0	1.6
Ethyl	2.1			
Propyl	3.0			
Butyl	4.4	1.0	25.7	3.1
Amyl	6.5			

and isoleucine on most columns, and threonine appears before serine. These effects may be due to polarity, or to steric factors which for instance enable the carboxymethyl and the trifluoroacetyl groups of N-TFA glycine methyl ester to interact. The effect of increasing the molecular weight of the N-TFA aspartic acid and glutamic acid esters is to alter the order of elution by retarding these two amino acids. There is however, for the amino acid derivatives studied, no obvious correlation between structure and chromatographic behaviour, such as is found in the case of the fatty acid esters.

So far as ease of separation was concerned, the N-TFA n-propyl and n-butyl esters did not appear to give a better separation than the N-TFA methyl esters under the conditions used.

The effect of increasing the length of the column did not always produce a better separation, nor did increasing the percentage of stationary phase used, as Tables 29 to 32 show. This accords with the observations of Cruickshank and Sheehan (1964) who found that on any column longer than 2 ft. the resolution of threonine and glycine worsened.

II. <u>Quantitative Estimation of Amino Acids by GLC</u>

So far as the quantitative estimation of amino acids by GLC is concerned there are two aspects to be considered;

(i) the preparation of derivatives for GLC in quantitative yields

(ii) the estimation of this derivative by gas chromatography

(i) <u>Preparation of Derivatives</u>:

While it seems likely that N-TFA methyl esters of such amino acids as leucine, alanine and glycine can be prepared in good yields by the preparative methods used, it is questionable whether the yields of derivatives of, for instance, histidine or arginine would be as good. In fact, the use of TLC indicated that

there was virtually no free amino acid left after methylation with methanol/HCl for 1 hour, whereas in the case of the trifluoroacetylation, there was clear evidence of a small amount of unconverted amino acid ester in certain cases (arginine, tryptophan, cystine, histidine) after 30 minutes at room temperature. However, when the trifluoroacetylation was carried out at 150° for 5 minutes, there was a considerable reduction in the amount of unconverted ester on the thin layers. Only in the case of histidine and arginine was there a very small amount of ninhydrin-positive material near the origin of the thin layer plate. The fact that the relative molar response factors are fairly constant also confirms that for most of the amino acids the derivative preparation is carried out in reproducible yield, even if this is not 100%.

(2) Quantitative Estimation of N-TFA Amino Acid Methyl Esters by GLC:

In any gas chromatographic analysis, there are five main sources of error, which can affect the accuracy of the result (Evans and Scott, 1963).

(i) The sample obtained for analysis must be representative and must be stored in such a way that it remains unaltered throughout a series of duplicate or replicate tests. This criterion was not initially met. This was due to the fact that the N-TFA methyl ester of cystine tended to crystallise out of the trifluoroacetic anhydride solution, and the N-TFA derivatives of many other amino acids decomposed on storage. In order to completely fulfil this criterion it was routine practice to put the sample on to the column as soon as it had been trifluoroacetylated. Samples which had been methylated were stored in an evacuated desiccator over concentrated sulphuric acid (since it was assumed that the methyl ester hydrochlorides would be quite stable) and were only removed for trifluoroacetylation immediately prior to chromatography.

(ii) The injection system must be capable of placing a representative sample

on the column and the injection device must not permit the loss of low boiling materials. Its absolute precision must be such as to permit a charge to be placed on the column to produce peaks of the required size. Although an amount could be applied to the column which would produce peaks of the required size, it was not possible to directly estimate the precise amount of amino acid derivative applied to the column due to the inaccuracy of syringing, and the volatility of the solvent, trifluoroacetic anhydride. Some difficulty was encountered due to loss of certain amino acid derivatives in the preheater block of the gas chromatograph. The instability of the arginine derivative has already been mentioned (Results, Section II, 2, iii). It was found that if a low preheater temperature (230°) was used, spurious peaks were obtained on chromatograms. This was presumed to be due to the fact that the preheater temperature is related to the oven temperature, so that when the oven is at room temperature the temperature of the preheater block is lower than when the oven temperature is high. Thus, in the course of the day, with the oven temperature being increased and decreased every hour or so, the preheater was observed to increase in temperature. At this stage material which was not volatile enough to come off at lower temperatures may bleed out of the preheater, so producing spurious peaks. With these low preheater temperatures, however, much more satisfactory results were obtained for the less volatile amino acid derivatives. This was due to the fact that at preheater temperatures above about 275°, the N-TFA esters of histidine, arginine, cystine and tryptophan decomposed in the injection block and produced either no peaks, or else very small peaks. To overcome this, the preheater was normally maintained as closely as possible in the range 250 - 260°.

(iii) The column system must be capable of giving adequate separation of the substances chromatographed, and the adsorption effects of the support or column wall must be reduced to the limit where the accuracy of the quantitative

analysis is not affected. This requirement was more adequately fulfilled by the use of presilanised supports than with untreated support. However, although very little evidence of peak assymmetry was observed, some difficulties arose due to poor resolution. Thus, when trifluoroacetic anhydride was used which contained trifluoroacetic acid, formed as a result of hydrolysis of the anhydride during storage, very large trifluoroacetic acid peaks were obtained, and since this compound tailed badly on all of the columns used, the alanine and valine peaks, which were eluted shortly after the trifluoroacetic acid, were sometimes difficult to estimate because of the trifluoroacetic acid tail. To improve this, the trifluoroacetic acid was removed by distillation of the anhydride from phosphorus pentoxide. Also, if the column was not properly stabilised at 60° before the start of an analysis, there was sometimes a disparity between the rate of temperature increase set, and the temperature of the oven. This sometimes led to less sharp resolution of the isoleucine/threonine, and glycine/leucine peaks on the NPGS column, and of isoleucine/threonine on the ternary phase columns. The method used for estimation of the area of peaks which were not completely resolved (Figure 8) was found to be quite adequate when the size of the two peaks was about the same. It might not, however, give satisfactory results when the two peaks were of greatly disparate size. In the case of high concentration ratios, of the order of say 20 to 1, it would be necessary to run the sample at a higher sensitivity in order to estimate the components present in small amounts.

(iv) The detecting system must have a linear or known response. In fact the hydrogen flame ionisation detector has a linear response over a concentration range of $1 - 10^4$ under normal conditions (Fowlis, Maggs and Scott, 1964).

(v) The chromatogram obtained must be carefully interpreted. Since the

separation of the N-TFA amino acid methyl esters required the use of temperature programming, it was essential to use peak area measurements for the estimation of peak size.

(3) Reproducibility of Relative Molar Response Factors:

The values given in Tables 42 and 43 were obtained using the optimum preparative and chromatographic conditions. It was found that if proper precautions were taken, results of this level of consistency were obtained. However, if the methanol was not absolutely anhydrous, or the HCl was not dry the reproducibility of the method fell. Likewise, if the trifluoroacetic anhydride contained more than 3% of trifluoroacetic acid, the factors for arginine, histidine and cystine altered.

III. Application of the Method

(1) Amino Acid Analysis of Peptides:

The amino acid analyses of hydrolysates of pure peptides give very good results and the use of methanolysis to eliminate one stage of the derivative preparation does not materially alter the results obtained. The method is clearly of use for this task, but the results obtained with peptides isolated in the normal way from fingerprints show the danger of assuming the general applicability of the method. Since the paper obviously contains a number of components which interfere, it would be necessary to try to remove these prior to chromatography, or else to ascertain whether the 'background' chromatogram was reproducible in order to use the method for this type of analysis. Removal of the interfering components might be effected by use of an ion-exchange resin. This would introduce an extra step, which is unnecessary in other methods of amino acid analysis, and mitigates against the use of GLC in this context. Set against this, however, is the potentially high sensitivity of GLC, which may in some

circumstances make it worthwhile to develop methods of overcoming this difficulty.

(2) Amino Acid Analysis of Proteins:

The use of GLC for amino acid analysis of proteins has been discussed in the Results, Section V. Unfortunately, very few analyses were carried out with the method. The results for certain of the amino acids such as glycine and lysine seem to be consistently low, and on two occasions the values for glutamic acid and phenylalanine are rather high. Another difficulty which presents itself is that the values for cysteine are unlikely to be accurate, since some breakdown to cysteic acid and cystine in the course of hydrolysis is likely. So far no peak has been obtained for cysteic acid after taking it through the methylation and acylation procedures. One possible solution to this problem would be to reduce and alkylate the protein with iodoacetate prior to hydrolysis, and to chromatograph the N-TFA methyl ester of S-carboxymethyl cysteine. Whether this would be possible or not depends on whether carboxymethyl cysteine forms a volatile derivative which can be readily estimated.

Another problem which arises when dealing with protein hydrolysates is the estimation of ammonia. When ammonia is present, it would presumably be converted to trifluoroacetamide by the process of derivative formation. Trifluoroacetamide was observed to chromatograph between the solvent peak, and the trifluoroacetic acid peak, but very inconsistent results were obtained for this compound and no quantitative results have been presented. The response factor for trifluoroacetamide is very low and variable. The problem is further complicated by the fact that peaks were sometimes observed to appear in the position of trifluoroacetamide even when mixtures of amino acids which contained no ammonia were chromatographed, suggesting that ammonia in the atmosphere of

the laboratory might interfere with the estimation of ammonia in the samples analysed.

Unfortunately, the results of methanolysis of cytochrome c, given in Table 48, indicate that under the conditions used complete splitting of the protein to amino acid methyl ester hydrochlorides does not occur. Better results might be obtained by altering the concentration of HCl, or lengthening the period of incubation, or by using a higher temperature.

Stack and his colleagues at Bristol (Stack, 1966) have found that butanolysis at 110° for 16 hours gives complete cleavage of proteins to n-butyl ester hydrochlorides and this accords with the observation (Table 45) that butanolysis of leucylglycylglycine is more rapid than methanolysis. This may be a good reason for preferring n-butyl esters to methyl esters for GLC studies on amino acids.

IV. Perspectives

The most widely used method for amino acid analysis in routine use at present is ion-exchange chromatography. If gas chromatography is to find application for amino acid analysis, it must measure up to the requirements fulfilled at present by the amino acid analyser. The potential advantages of GLC, in respect of economy of operation, resolving power, speed and sensitivity have already been mentioned.

With regard to economy of operation, there is no doubt that GLC is much cheaper than ion-exchange chromatography so far as capital costs and running costs are concerned. However, so far as speed and resolving power, the situation is less clear cut. If alcoholysis of proteins could be carried out completely overnight, it would be possible to obtain four complete analyses per day by GLC, which would compare very favourably with the amino acid analyser, particularly

since the technical operations involved in the GLC method are much less involved than with ion-exchange chromatography. However if additional time had to be spent in the esterification of each hydrolysate, this would reduce the number of samples which could be analysed in a day to three.

One of the potential advantages of GLC is that the resolving power of a column can be simply increased - an increase in column length increases the number of theoretical plates available for partition, and so a longer column should give a more efficient separation. This is however only true if a purely partition phenomenon is involved in the separation, and so far as the amino acid derivatives are concerned, it seems that effects due to polarity and steric factors play an important part in the separation, and so increasing the column length does not produce a better separation. It may produce a different order of elution, but one cannot predict that increasing the column length will necessarily separate two amino acids which are not well resolved on a particular column. The effect of increasing the amount of stationary phase is also not something that produces a predictable change in relative retentions.

One way to increase the resolving power by a considerable amount is to use capillary columns, in which the stationary phase is coated on the walls of a narrow bore capillary. Such capillary columns have been used to resolve diastereoisomeric amino acids by Gil-Av, Charles and Fischer (1965) and by Pollock, Oyama and Johnson (1965) using 150 ft. and 300 ft. columns respectively. This approach may enable a complete separation of all of the N-TFA amino acid methyl esters to be achieved and it has the advantage that capillary columns can be operated at very high flow rates, so that in this way the time required for analysis would also be considerably reduced.

In respect of sensitivity, the potential of GLC is as yet incompletely

utilised. Thus, at present 10^{-9} moles of an amino acid can be estimated by the method developed in this investigation, although the sensitivity of detection of the instrument could be increased by a factor of 10^{3}. Although 10^{-9} moles of amino acid are routinely estimated, the sample used must contain about 100 times this amount of amino acid. This is because at least 1 ml. of trifluoroacetic anhydride is required to dissolve the amino acid methyl ester hydrochlorides from the walls of the flask in which the methylation has been carried out, and only 5 or 10 ul. of this solution is used for chromatography.

Saroff (1966) in a private communication has described a method for carrying out the entire sequence of reactions on a plug of glass wool placed in a small capillary tube which is fitted with teflon caps at both ends. At the end of the reaction sequence, the capillary can be placed in a specially constructed valve which introduces the whole capillary into the gas stream and blows the sample directly onto the column for analysis. This enables the whole sample to be applied, eliminates losses due to transfer, and would certainly permit the estimation of amino acids in the range 10^{-10} - 10^{-12} moles by GLC.

The considerable increase in sensitivity which can be obtained with GLC detectors is offset by the lack of specificity. This is apparent in the results of analysis of amino acids from hydrolysates of peptides isolated from fingerprints. Here, the lack of specificity of detection, which is in many cases an advantage of GLC, becomes a considerable disadvantage. Nevertheless, in view of the high sensitivity of the method it may well be worth introducing a purification step to remove the contaminating material eluted from the paper. One other alternative possibility would be to introduce an element of specificity into the detection of the amino acid derivatives, such as Landowne and Lipsky

(1963) did by their use of an electron capture detector for DNP and PTH derivatives of amino acids.

In the present investigation, the potential of GLC for amino acid analysis has not been fully exploited. However, apart from the economic attractiveness of GLC for amino acid analysis, it seems likely that the method could be developed to a point where it would be much more rapid than the amino acid analyser, and be capable of handling much smaller amounts of amino acids for analysis. The advantages that would accrue from this increase in sensitivity, particularly in the estimation of amino acids in biological fluids, where the concentrations are very low, and where the amounts which can be made available are very small, are considerable, and clearly justify the investigation which has been the subject of this discourse.

SUMMARY

SUMMARY

Ion Exchange chromatography is the most widely used method for analysis of amino acids. However, in respect of economy, speed, sensitivity and resolving power, gas-liquid chromatography is potentially better than the conventional amino acid analyser. The technique requires that the amino acids be converted into some suitable volatile derivative prior to their separation by GLC.

1. N-acetyl amino acid methyl esters were examined with regard to their suitability for gas chromatography, and were found to be not sufficiently volatile.

2. When the N-trifluoroacetyl amino acid methyl esters were investigated, no single column was found on which all of the protein amino acids could be resolved. However, it was found possible to separate all of the twenty amino acids which occur in proteins by using two columns - one coated with NPGS (neopentyl glycol succinate polyester) and the other coated with a mixture of DEGS (diethylene glycol succinate polyester), EGSS-X (ethylene glycol succinate polyester chemically combined with a methyl silicone) and EGSP-Z (ethylene glycol succinate polyester chemically combined with a phenyl silicone).

3. No better resolution of the N-TFA n-propyl or n-butyl esters of the amino acids was obtained when these derivatives were chromatographed on a number of columns than had been achieved with the N-TFA methyl esters.

4. Taking the response of the flame ionisation detector to N-TFA leucine methyl ester as unity, the response of the detector to the corresponding other amino acids was determined. Good reproducibility in the response factors was observed on both of the columns used for all of the amino acid derivatives with

the exception of histidine, tryptophan and cystine.

5. In order to assess the potential of the method, it was applied to hydrolysates of a number of pure peptides, and also to methanolysates of these peptides, prepared with methanol/HCl. Good agreement was obtained by both procedures. However, when the method was applied to peptides which had been eluted from fingerprints, the results were poor. This was found to be due to the fact that a considerable amount of material was eluted from the chromatography paper which produced spurious peaks on the chromatograms.

6. The method was applied to the analysis of hydrolysates of cytochrome c, insulin and bovine serum albumen. The results were in poor agreement with the results of amino acid analysis carried out on the amino acid analyser, though the agreement with the number of residues per mole of protein was somewhat better. The chromatogram obtained for a methanolysate of cytochrome c gave considerably reduced values for a number of amino acids such as valine, isoleucine and threonine. It appears that under the conditions of methanolysis used the cleavage of peptide bonds involving these amino acids does not proceed to completion.

7. Butanolysis of leucylglycylglycine was found to proceed to completion in three hours at 125°, whereas methanolysis of this peptide required twelve hours at this temperature. This result, taken in conjunction with the result for methanolysis of cytochrome c suggests that the N-TFA n-butyl esters of the amino acids may be more suitable derivatives for GLC studies.

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SUMMARY

Ion exchange chromatography is the most widely used method for the analysis of amino acids. However, in respect of economy, speed, sensitivity and resolving power, gas-liquid chromatography is potentially better than the conventional amino acid analyser. The technique requires that the amino acids be converted into some suitable volatile derivative prior to their separation by GLC.

N-acetyl amino acid methyl esters were examined with regard to their suitability for gas chromatographic analysis, but were found to be not sufficiently volatile.

When the N-trifluoroacetyl methyl esters were investigated no single column was found on which all of the amino acids could be resolved. However, it was found possible to separate all of the N-trifluoroacetyl methyl esters of the twenty amino acids which occur in proteins by using two columns - one coated with NPGS (neopentyl glycol succinate polyester) and the other coated with a mixture of DEGS (diethylene glycol succinate) EGSS-X (ethylene glycol succinate polyester chemically combined with a methyl silicone) and EGSP-Z (ethylene glycol succinate polyester chemically combined with a phenyl silicone).

No better resolution of the N-trifluoroacetyl n-propyl or n-butyl esters of the amino acids was obtained when these derivatives were chromatographed on a number of columns than had been achieved with the N-trifluoroacetyl methyl esters.

Taking the response of the flame ionisation detector to

N-trifluoroacetyl leucine methyl ester as unity, the response of the detector to the corresponding derivatives of the other amino acids was determined. Good reproducibility in the response factors was observed on both of the columns used for all of the amino acid derivatives with the exception of histidine, tryptophan and cystine.

In order to assess the potential of the method, it was applied to hydrolysates of a number of pure peptides and also to methanolysates of these peptides, prepared with methanol/HCl. Good agreement was obtained by both procedures. However, when the method wasapplied to peptides which had been eluted from peptide maps, the results were poor. This was found to be due to the fact that a considerable amount of material was eluted from the chromatography paper which produced spurious peaks on the chromatograms.

When the method was applied to the analysis of hydrolysates of cytochrome c, insulin and bovine serum albumen, the results obtained were in poor agreement with the results of amino acid analysis carried out on the amino acid analyser. The chromatogram obtained from a methanolysate of cytochrome c gave reduced values for a number of amino acids such as valine and isoleucine. It appears that under the conditions of methanolysis used, cleavage of bonds involving these amino acids does not proceed to completion. Butanolysis of leucylglycylglycine was found to be much more rapid than methanolysis of this peptide, and so the N-trifluoroacetyl n-butyl esters of the amino acids may be more suitable derivatives for GLC studies.