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A GAS CHROMATOGRAPHIC METHOD FOR THE SEPARATION
AND
ESTIMATION OF THE N-TRIFLUOROACETYL METHYL
DERIVATIVES OF AMINO ACIDS

TO
MY PARENTS

19257

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Abbreviations

The following abbreviations are used in the thesis:

AMINO ACIDS

ala	:	alanine
arg	:	arginine
asp	:	aspartic acid
cys	:	cysteine
glut	:	glutamic acid
gly	:	glycine
hist	:	histidine
hyp	:	hydroxyproline
ileu	:	isoleucine
leu	:	leucine
lys	:	lysine
met	:	methionine
phe	:	phenylalanine
prol	:	proline
ser	:	serine
thre	:	threonine
tryp	:	tryptophan
tyr	:	tyrosine
val	:	valine
att'n	:	attenuation
conc. rec'd	:	concentration recovered
HETP	:	height equivalent to one theoretical plate
I. U.'s	:	integrator units (excursions of integrator pen)
NPG	:	neopentyl glycol
Rel. ret'n	:	relative retention
Res'n	:	resolution
TFA	:	trifluoroacetic
DNP	:	dinitrophenol

ABSTRACT

A description is given of a method of forming amino acid methyl esters with thionyl chloride in methanol. The esters are treated with trifluoroacetic anhydride to form the N-trifluoroacetyl methyl esters. These are taken up in methanol and separated by gas chromatography on three types of columns. With a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column it is possible to cleanly separate the derivatives of cysteine, valine, leucine, glycine, proline, aspartic acid, threonine, methionine, glutamic acid, phenylalanine, serine, hydroxproline, arginine and lysine in that order. Alanine and isoleucine are separated on a 10' x $\frac{1}{8}$ " 1% Carbowax 1540 column. Tyrosine, histidine and tryptophan are separated on a 2' x $\frac{1}{8}$ " 1% Carbowax 20M column after the other amino acids have come off at lower temperatures. An F & M hydrogen flame ionization detector was used. The carrier gas was nitrogen. When mixtures containing 1 mg, 2.5 mg, 5 mg, 10 mg, or 20 mg of each of the above amino acids were carried through the entire chemical and chromatographic procedure, there was for each amino acid a linear relation between the amount of amino acid originally present and the area of the peak.

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SECTION I

INTRODUCTION

The aim of the work reported in this thesis was to develop a gas chromatographic procedure for the separation and estimation of the amino acid composition of a protein hydrolysate.

Since in studies of protein chemistry it is important to have information on the amino acid composition of the proteins being studied a number of methods for the separation and estimation of the amino acid composition of protein hydrolysates have been devised. The most useful procedures have been developed over the past fifteen years and involve electrophoresis or chromatography on paper or on ion exchange resins. The most successful of these procedures is an ion exchange separation of amino acids followed by a photometric measurement of the amount of each amino acid present (Moore & Stein 1954). The whole procedure has been automated in several ways and equipment for this purpose is available commercially. Despite their sensitivity and accuracy such automated procedures have the disadvantages of the considerable expense of the apparatus and of the need of a virtually full-time person to maintain and operate it.

A number of attempts to separate derivatives of amino acids by gas chromatography have been recorded in the literature over the past ten years. None of these studies reports a complete quantitative analysis of all 19 amino acids commonly found in proteins.

Following a review of the pertinent literature a description is given of the development of procedures for the preparation and estimation by gas chromatography of N-trifluoroacetyl methyl esters of the 19 amino acids commonly found in protein.

Review of the Literature

Even though the ability to separate various derivatives of amino acids by fractional distillation (Fischer 1901) (Weygand 1959) has encouraged the view that it should be possible to convert amino acids to volatile derivatives which could be separated and estimated by gas chromatography, reports of such studies are few in relation to other fields of gas chromatography.

The first actual reported attempt to separate amino acids by gas chromatography was that of Hunter, Dimick and Corse (1956) who oxidized the amino acids with ninhydrin and separated the aldehydes of valine, leucine, isoleucine and alanine by gas-liquid chromatography. In the years of 1957 and 1958 Bayer, Reuther and Born (1957) separated amino acids by two different methods. In the first method they oxidized amino acids to their corresponding aldehydes with alkaline hypochlorite. However, the acidic and sulphur-containing amino acids formed complex mixtures and some amino acids produced the same compound on oxidation. Using a dionyl phthalate column at 92°C they were able to separate the aldehydes of alanine, α -aminobutyric acid, norvaline, valine, norleucine and leucine.

The second approach to the problem employed by Bayer et al (1957) was to separate the methyl esters of valine, norvaline, leucine, norleucine, glutamic acid and phenylalanine. Bayer et al (1957)

also separated the methyl esters from an hydrolysate of albumin, obtaining the methyl esters of alanine, proline, valine, leucine, isoleucine, aspartic and glutamic acid, thus being the first workers to apply gas chromatography to the analysis of a protein.

Bier and Teitelbaum (1959) developed a microprocedure for the oxidation of α -amino butyric acid, valine and leucine prior to separation by gas-liquid chromatography. The amino acids were treated with ninhydrin in a sealed ampoule and the products extracted into a chlorinated hydrocarbon solvent. The aldehydes were analyzed on a silicone column at 78°C.

Applying the techniques of Hunter et al (1956) and of Bier and Teitelbaum (1959), Zlatkis, Oro and Kimball (1960) developed an automatic gas chromatographic method for the analysis of volatile amino acid aldehydes. Employing a continuous flowing system, the amino acid was treated with ninhydrin and injected into the system. The sample passed through a column containing 30% ninhydrin on C-22 firebrick. The separated aldehydes were then carried to a reactor filled with a nickel kieselguhr catalyst at temperatures of 425°C. The aldehydes were cracked on the catalytic column to methane and water. The methane was dried on a molecular sieve drying tube and detected by a thermal conductivity cell. Zlatkis et al separated the aldehydes of alanine, α -aminobutyric acid, valine, norvaline, leucine, isoleucine and norleucine. The analysis time was 50 minutes and they were able to detect 1 μ gm amounts of the amino acids. Zlatkis et al analyzed a hydrolysate of casein and obtained alanine, valine, leucine and isoleucine.

Baraud (1960) also tried to separate the oxidation products of amino acids. The amino acids were oxidized with alkaline permanganate and the

aldehydes were esterified forming the methyl esters. The esters were separated on a polypropylene glycol column at 150°C. Only alanine, valine, norvaline, leucine, isoleucine and phenylalanine were separated.

Youngs (1959) was the first to substitute both functional groups of the amino acid prior to gas chromatography. Youngs separated the N-acetyl butyl esters of alanine, glycine, valine, leucine / isoleucine (not resolved) and proline on a 6 foot safflower oil column at 220°C. The analysis time was 1 hour. Youngs also chromatographed the esters of a gelatin hydrolysate obtaining the above mentioned amino acids. The results were quantitative for only the above amino acids.

Weygand, Kolb, Prox, Tilak and Tomido (1960) separated the N-trifluoroacetyl methyl esters on silicone grease and polyester columns. They esterified and completely separated the N-trifluoroacetyl methyl esters of valine, proline, aspartic acid, threonine, methionine and phenylalanine. However the pairs leucine-isoleucine and glycine-alanine were not resolved and proline-aspartic were only partly resolved. Tyrosine and arginine gave four and two peaks respectively. The multiple peaks were attributed either to a breakdown of the derivatives on the column or to impurities.

Weygand et al (1960) partially hydrolysed the synthetic peptide H.ala.glyc.glyc.leuc.glyc.OH and separated a number of N-TFA dipeptide methyl esters on a silicone oil column. Another interesting result of the work of Weygand was the separation of the esters of the stereoisomeric dipeptide pair of L-alanine L-phenylalanine and L-alanine D-phenylalanine on a silicone oil column. Others workers have not so far continued the work of Weygand on dipeptides. These results hold great

promises for future work on the separation of dipeptides from protein hydrolysates, giving possible clues to the amino acid sequence of many proteins.

Continuing the work of Baraud (1960), Melamed and Renard (1960) converted the L-monoamino monocarboxylic acids to the L-chloro acids with a mixture of concentrated HCl and HNO_2 . The L-chloro acids were then converted to the corresponding methyl esters with diazomethane. Using polyethylene glycol and silicone oil columns, Melamed and Renard were able to separate the esters of alanine, glycine, α -aminobutyric acid, valine, norvaline, leucine, isoleucine and norleucine.

Saroff and Karmen (1960) reported the separation of the N-TFA methyl esters of 14 amino acids on a polyethylene glycol adipate column. In their separation alanine-valine and glutamic acid-serine were not resolved. The conversion of threonine, serine and lysine to this derivative was difficult, giving multiple peaks on chromatography. Tyrosine and arginine derivatives decomposed at high temperatures. Saroff and Karmen could not succeed in forming the N-TFA methyl esters of cystine, histidine or tryptophan.

Johnson, Scott and Meister have reported the most systematic study to date. They made a study of N-acetylated methyl, butyl, isobutyl, amyl and isoamyl amino acid esters and found the N-acetyl amyl amino acid esters most suitable for chromatography. Using columns of 1% Carbowax 1540 columns they resolved 16 amino acids but could not form the derivative of tryptophan, histidine or arginine. Johnson et al produced no data for the reproducibility of their procedure nor did they mention whether the conversion to the derivative was quantitative.

Wagner and Winkler (1961) published a paper describing the separation

of N-TFA methyl esters a year after the papers of Weygand et al and of Saroff and Karmen. Wagner separated the derivatives of the amino acids on an Apiezon L column containing sodium caproate. Wagner only succeeded in converting and separating the esters of valine, leucine, threonine, methionine and phenylalanine.

Rulmshtn and Giesecke (1961) separated the N-Trimethylsilyl amino acids of glycine, alanine, leucine, isoleucine, valine, glutamic and phenylalanine on a silicone oil column at 165°C.

Zomzely, Marco and Emery (1962) separated the N-TFA butyl derivatives of amino acids on a neopentyl glycol succinate column. Although they resolved 19 amino acids in 45 minutes many of the peaks were wide and the resolution of glycine-leucine, proline-serine, aspartic-phenylalanine was not good enough for the estimation of peak areas for quantitative purposes. The esterification and acylation procedures of Zomely et al are rather cumbersome and they presented no evidence for the quantitative or reproducible conversion of the amino acids.

Blau and Darbre (1963) reported the quantitative formation and separation of the N-TFA amyl esters of 10 amino acids. The esters were separated on different columns of polyethylene glycol adipate, fluoro-alkylsilicone QF-1 or silicone oil. Only the esters of glycine, alanine, valine, leucine, isoleucine, threonine, serine, methionine, proline and phenylalanine were quantitatively formed.

Landowne and Lipsky (1963) briefly reported the separation of several DNP methyl esters of amino acids on an XE-60 column using an electron capture detector. However details of this separation are scanty.

Lahli and Witkop (1963), in studying the structure of Gramicidin A

tried several methods to analyze the amino acids by gas chromatography. Using the systems employed by Waygand (1960), Saroff (1960) and Zomely (1962) (alkyl esters of TFA) and of Johnson (1961) (amyl esters of acetyl derivatives) they succeeded in establishing the presence of alanine, glycine, valine, leucine, isoleucine and the hydroxy-amino acids serine and threonine in the structure of Gramicidin A. They also separated the DNP amino acids esters of the above named amino acids. With the aid of gas chromatography they were able to show that Gramicidin A was a heterogeneous cyclopeptide antibiotic containing a mixture of 60-80% valine-gramicidin and 40-20% isoleucine-gramicidin.

Within the past year several reports of the gas chromatographic separation of amino acid derivatives have been published.

Ikekawa (1963) reported the separation of the N-TFA methyl esters of 15 amino acids. However the separations do not appear to be satisfactory for quantitative estimation, particularly the resolution of serine and methionine. Ikekawa did not detect the presence of cysteine, arginine or histidine. Ikekawa showed that for alanine there was a linear relationship of the concentration to the peak area. However he failed to show quantitative results for the other amino acids.

Weinstein and Fenselau (1964) have reported the separation of the amino alcohols of glycine, alanine, valine, leucine, serine, isoleucine, methionine and phenylalanine on columns of methyl silicone fluid and methyl phenyl silicone gum. The amino acids were converted to their alcohols with LiAlH_4 . This procedure is limited in its application to the amino acids that can be converted to alcohols.

Iwamoto and Morimoto (1964) have recently reported the chromatography

of some N-Cyanoethyl amino acids, esterifying the amino acids with either butanol or ethanol. They reported the separation of 11 amino acids. However this method, particularly the use of cyanoethyl acid appears to offer no improvement over the Trifluoroacetyl methods.

Recently, while the work presented in this thesis was in progress, Cruickshank and Sheehan (1964) reported the separation of the 19 common protein amino acids by conversion to their N-TFA methyl esters. The separation was carried out on a short 5% neopentyl glycol succinate column in an analysis time of 75 minutes. However, the illustrations do not show satisfactory separations necessary for the exact estimation of peak areas for quantitative results. The resolution of threonine-leucine, glutamic-phenylalanine and histidine-cysteine pairs were very poor. The histidine-cysteine peaks were very wide and could not be extrapolated to the base line. Changing the column dimensions altered the resolution of these above mentioned poorly resolved amino acids. The separation of threonine, glycine and leucine required special care in temperature, program rate and carrier gas flow for a good separation. Arginine showed two different peaks on the chromatograph. Although the majority of the relative peak areas of the amino acids were constant with six repetitions of the procedure, the quantitative work was carried out at only one amino acid level instead of at several different levels as described in this thesis. The methods of esterification and acylation were cumbersome.

Cruickshank et al (1964) analyzed the amino acid content of a ribonuclease hydrolysate. All of the amino acids known to occur in ribonuclease were obtained by gas chromatography but the separation was poor and there were marked losses in the peak areas of threonine and serine.

Brief Description of the Subject of the Thesis.

The following sections of this thesis describe work leading to the development of a reproducible gas chromatographic method for the separation and estimation of the N-trifluoroacetyl methyl esters of the 19 amino acids commonly obtained by hydrolysis of proteins. This method is quantitative and reproducible. It involves the rapid conversion of the amino acids to their N-trifluoroacetyl methyl esters and the separation of these derivatives by chromatography with programmed rising temperatures on two types of columns. A 20' x $\frac{1}{8}$ " Carbowax 20M column is used for the separation and quantitation of the derivatives of valine, leucine, glycine, proline, aspartic acids, threonine, methionine, glutamic acid, phenylalanine, serine, arginine, hydroxyproline and lysine. It is possible also to separate tyrosine, histidine and tryptophan on this column but separation is very slow and the peaks quite broad. One other drawback of this system is that alanine and isoleucine can only be separated at low temperatures and low gas flows. Under these conditions many of the other amino acids are retained on the column for a long time and the separation takes several hours. However, alanine and isoleucine can be rapidly separated at low temperature on a Carbowax 1540 column. Likewise tyrosine, histidine and tryptophan can be much more rapidly separated at high temperatures on a short column of Carbowax 20M.

SECTION II

MATERIALS AND GENERAL METHODS

A MATERIALS

REAGENTS

1. Amino Acids:

The L isomer of the 19 amino acids commonly found in proteins were used in this experiment: cysteine, valine, alanine, isoleucine, leucine, glycine, proline, aspartic acid, threonine, methionine, glutamic acid, phenylalanine, serine, arginine, hydroxyproline, lysine, tyrosine, histidine and tryptophan.

A-grade L-amino acids were purchased from Calbiochem. Corp., Los Angeles, Calif. These amino acids were of the free base form with the exception of cysteine and lysine which were purchased as the hydrochloride.

2. Methanol:

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont., in 4 litre quantities. This methanol has a distillation range of not less than 95% between 64.5-65.5°C and a 0.1% water content. Methanol was dried with magnesium turnings (see method on page 14).

3. Thionyl Chloride:

Purchased from Fluka S.G. Buchs SG Schweiz, Switzerland. This water-clear reagent was stored in sealed ampoules in 25 ml lots.

Thionyl chloride was also purchased from Fisher Scientific Co., Fairlawn, N.J. Both the "Laboratory Chemical" and "Purified" grades (#T-105) were tried. However, Fisher thionyl chloride had a yellow

11.

color and had to be purified (see method on page 16) before use in the esterification process.

4. Trifluoroacetic Anhydride:

Purchased from Matheson, Coleman and Bell, Norwood, Ohio. The trifluoroacetic anhydride was stored in 25 ml lots in sealed glass ampoules.

5. Acetic Anhydride:

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont.

6. Ammonium Carbonate:

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont.

7. Amyl Alcohol:

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont.

8. Anhydrous Hydrogen Chloride and Hydrogen Bromide:

Purchased from the Matheson Co., E. Rutherford, N.J.

9. Benzene:

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont.

10. Boiled Linseed oil:

Commercial grade purchased from a local hardware store.

11. Diethyl Ether:

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ontario.

12. Dimethoxypropane (Acetone dimethyl acetal):

Purchased from Matheson, Coleman and Bell, Norwood, Ohio.

13. Dimethylformamide:

"Spectroquality Reagent" grade purchased from Matheson, Coleman and Bell, Norwood, Ohio.

14. Dimethyl Sulfite:

Purchased from Eastman Organic Chemicals, Rochester 3, N.Y.

15. Magnesium Turnings:

Laboratory reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont.

16. Methylene Chloride:

"Spectroquality Reagent" grade purchased from Matheson, Coleman and Bell, Norwood, Ohio.

17. Sodium Carbonate (anhydrous):

"Analar" reagent grade purchased from British Drug Houses (Canada) Ltd., Toronto, Ont.

MATERIALS

GAS CHROMATOGRAPHY

1. Apparatus

An F & M model 720 gas chromatograph equipped with a hydrogen flame detector attachment Model 1609 was purchased from F & M Scientific Corp., Avondale, Pa.

The recorder was an Electronik 15°C Model #153 manufactured by Minneapolis-Honeywell Regulator Co., Brown Instruments Div. Philadelphia 44, Pa.

A Disc-chart integrator Model 201-B, attached to the Brown recorder, manufactured by Disc Instruments Inc., Santa Ana, Calif., was purchased from F & M Scientific Co., Avondale, Pa.

2. Carrier Gases

(a) Nitrogen--- purchased from the Linde Corp., Welder's Supply Ltd., Winnipeg, Canada.

(b) Helium--- purchased from Matheson of Canada Ltd., Whitby, Ont.

(c) Hydrogen (repurified)--- purchased from Matheson of Canada Ltd. Whitby, Ont.

3. Glass Wool:

Purchased from Canadian Laboratory Supplies, Toronto, Ont.

4. Metal Tubing

(a) Copper Tubing--- Anaconda American Brass Ltd., New Toronto, Ont. purchased from Alloy Metal Sales, Winnipeg, Canada.

(b) Stainless Steel--- purchased from Alloy Metal Sales, Winnipeg, Canada.

5. Microliter Syringes:

purchased from the Hamilton Co. Inc., Whittier, Calif. in 1pl, 5pl, 10pl and 20pl sizes.

6. Column Packings

The following column packings were purchased ready for use from F & M Scientific Corp., Avondale, Pa. in 100 gm. lots.

- 1% Carbowax 20M on 80-100 mesh Diatoport S.
- 2% Carbowax 20M on 80-100 mesh Diatoport S.
- 10% Carbowax 20M on 80-100 mesh Diatoport S.
- 1% Neopentyl glycol succinate on Diatoport S (80-100 mesh).
- 10% Neopentyl glycol succinate on Diatoport S (80-100 mesh).
- 10% Silicone Grease DC-11 on Diatoport S (80-100 mesh).
- 10% Apiezon on Diatoport S (80-100 mesh).

The following column packings were purchased from Research Specialties Co., Richmond, Calif.

- 1% Carbowax 1540 on 80-100 mesh Diatoport S.
- 0.5% Carbowax 1540 on 80-100 mesh Diatoport S.
- 1% Neopentyl glycol succinate on Chromosorb PAW 60-80 mesh.

7. Silica Gel

Laboratory reagent grade (6-16 mesh) purchased from Fisher Scientific Co., Fairlawn, N. J.

B METHODS

(a) PREPARATION OF MATERIALS

(1) Drying of Methanol

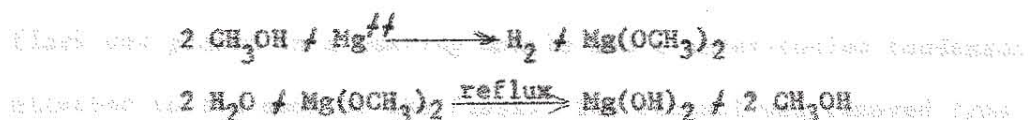
Approximately 200 ml of methanol was placed in a 500 ml round bottom distillation flask. About two dozen magnesium turnings (amount not critical) were added to the methanol and the contents shaken.

The flask was placed in a heating mantle and a 2 foot fractionation column was placed upright in the neck of the flask. A thermometer was placed in the upper end of the fractionation column. A water-cooled condenser was attached to the side arm of the fractionation column. At the lower end of the condenser was attached a collecting flask. A calcium chloride tube was attached to a side arm in the neck of the collection flask.

The reaction between magnesium and methanol was allowed to go to completion at room temperature. The heating mantle was then set to 65°C and the methanol collected by distillation. The first 20-25 ml distilling over were discarded and the distillation stopped after enough methanol had been collected for experimentation.

The methanol was stored in the distillation flask completely sealed from the atmosphere. As more methanol was required more magnesium turnings were added to the distillation flask and the procedure repeated. However, after two or three distillations the residue ($Mg(OH)_2$) remaining in the flask was washed out with methanol.

The chemical reactions for the drying of methanol by this method are as follows:



The chemical reactions for the drying of methanol by this method are as follows: The flask was placed in a heating mantle and a 2 foot fractionation column was placed upright in the neck of the flask. A thermometer was placed in the upper end of the fractionation column. A water-cooled condenser was attached to the side arm of the fractionation column. At the lower end of the condenser was attached a collecting flask. A calcium chloride tube was attached to a side arm in the neck of the collection flask.

(ii) Purification of Thionyl Chloride

Thionyl chloride and quinoline were added to a 500 ml flask in a 2:1 ratio and thoroughly mixed. The flask was placed on a heating mantle and attached to a 1 foot Vigreux column and condenser and heated. Thionyl chloride was collected at 78-80°C

The distillation of thionyl chloride was carried out in a fume hood with adequate ventilation.

The thionyl chloride, distilled from the quinoline, was mixed with the washed, boiled, linseed oil (see below). The flask was placed on a heating mantle and a water-cooled condenser attached. The mixture was heated to 75 °C in a fume hood. Colorless thionyl chloride was collected.

The water-clear thionyl chloride was placed in brown-colored glass bottles which were tightly capped and stored in a refrigerator. Whenever a yellow appearance was observed the thionyl chloride was redistilled from linseed oil. The linseed oil, since it solidifies after distillation, was discarded immediately.

The linseed oil used above was purified as follows: approximately 50 ml of boiled linseed oil was shaken with 200 ml of ethanol by agitation for several minutes in a 500 ml distillation flask. The flask was placed in a heating mantle and a water-cooled condenser attached to the neck of the flask. The ethanol was removed from the linseed oil by distillation at 78-80°C. This step is necessary since the boiled linseed oil is not a chemically defined compound and may contain components which would distill over at the same temperature as thionyl chloride.

(b) METHODS FOR GAS CHROMATOGRAPHY**(i) Cleaning of Metal Tubing**

Since copper or stainless steel tubing may contain impurities inside the tubing that will cause bleeding from the resin, it was advisable to wash the tubing before use. One end of the tubing was attached to a water-suction pump and the following solutions drawn through the metal tubing in the following order: 100 ml each of laboratory detergent solution, distilled water, methanol, acetone and benzene. The metal tubing was then thoroughly dried with a gas flame before packing with resin.

(ii) Packing of Metal Columns for Gas Chromatography

The appropriate length of tubing was cut with a tube cutter and the ends of the tube smoothed with a metal file. One end of the metal tube was plugged with glass wool. The plugged end was placed onto an electric vibrator (a Vortex Jr. mixer was used). A funnel was attached to the open end of the metal tube and the resin poured into the funnel while the column was continuously vibrating. The pouring of the resin was stopped at intervals to allow the resin to settle and air to escape. To insure complete packing of the resin the tubing was frequently knocked with a heavy metal object. The column was considered packed when no more resin could be added to the

tubing. The open end was then plugged with glass wool.

(iii) Coiling of Columns

The metal columns had to be coiled into a spiral in order to fit into the oven of the flame ionization attachment. The internal diameter of this spiral was 15 cm. A steel cylinder of these dimensions was attached to a power lathe. With the cylinder turning slowly, the metal tubing was then carefully wound around the cylinder. About 10 cm on each end of the column was left straight and bent to a 90° angle for attachment to the block by Swagelok fittings. The connections were tested for gas leaks under a high gas pressure with a SNOOP detergent solution.

(iv) Measurement of Gas Flow

The carrier gas flow was measured with a bubble flow meter attached to the end of the column nearest the injection port. A soap-film meter as suggested by James and Martin (1952) consisting of a graduated burette with a rubber bulb attached to the end and filled with detergent solution was used.

The gas sample from the column enters the burette. The rubber connection is pinched and the soap solution ascends into the gas inlet tube. A bubble is formed, rises in the graduated tube and its rate of ascent in the calibrated portion of the burette is timed with a stopwatch. The flow rate is calculated in ml of gas per minute.

(v) Drying of Gases

It was found necessary, particularly under

conditions of high humidity, to prevent water from entering the gas lines. Since traces of water had a drastic effect on the stability of the flame detector, metal tubes containing silica gel and closed at both ends with glass wool were inserted into the gas lines between the gas cylinders and the detector unit.

(vi) Seasoning of the Columns

In order to clean (or season) the columns and their resins of impurities and to stabilize them to gas flow and temperature changes, newly prepared columns were heated to their maximum allowable temperatures i.e. 275°C for Carbowax 20 M columns and 150°C for Carbowax 1540 columns, from 16 to 24 hours before use. A high carrier gas flow i.e. 150 ml/min. was used to sweep off the impurities. Once a column had been properly seasoned it was only necessary to repeat the process for a few hours when the column was to be reused.

SECTION III

PRELIMINARY EXPERIMENTS ON THE ESTERIFICATION OF AMINO ACIDS

1. Preparation of N-Acetyl Amyl Amino Acid Esters

Since the method described by Johnson, Scott and Meister (1961) was developed in the most systematic manner so far reported attempts were made to use it for quantitative conversion. The procedure was followed as described in the original paper. A volume of 20 ml amyl alcohol was added to a mixture containing the 19 amino acids (listed on page 10) in a 100 ml round bottom flask. The methanol was saturated with anhydrous hydrogen bromide gas and the amino acids dissolved. This mixture was refluxed at 165°C for 30 minutes and then cooled to room temperature. The contents of the flask was then evaporated on a rotatory evaporator at 50°C under reduced pressure.

A volume of 8 ml acetic anhydride was added to the residue and the mixture was left standing at room temperature for 5-10 minutes. The excess anhydride was then evaporated as before and the residue dissolved in benzene or amyl alcohol to a volume of 5 ml.

This method met with limited success in our hands. It was difficult to obtain reproducible peak areas with cysteine or the basic amino acids. Some modifications of the method were tried, e.g. lower refluxing temperatures and a longer acetylation time, but without improvement.

When the amino acid esters were chromatographed on a 10' x 1/4" 1% Carbowax 1540 column the esters of methionine, phenylalanine, hydroxyproline and aspartic acid were very wide and flat, and the yields were not very great.

A 10' x 1/4" 1% neopentyl glycol succinate column was tried but the peaks were again broad and flat, particularly towards the end of the separation. It was difficult to resolve alanine / valine, isoleucine / leucine and glycine due to tailing of the esters. It was difficult to record peak areas from the integration of the peaks.

Since water is an end-product in the esterification reaction, benzene was used to form an azeotropic mixture with the water in order to further complete the esterification. This met with limited success, improving the peak heights of a few amino acids but still not removing the lack of reproducibility with the basic amino acids.

2. Preparation of N-Trifluoroacetyl Methyl Amino Acid Esters

Several methods were tried:

(a) Anhydrous HCl or HBr method

Approximately 20 ml dry methanol was acidified with anhydrous HCl or HBr and the amino acid mixture added to the solution. A few drops of dimethoxypropane -- a water scavenger -- was sometimes added at this point. The mixture was then refluxed at temperatures from 60-80°C for 30-60 minutes. The contents were then evaporated at either room temperature or 50°C on a rotatory evaporator.

A 3 ml volume of trifluoroacetic anhydride was added and left standing at room temperature for one hour. The excess anhydride was then evaporated and the residue dissolved in methanol to 5 ml.

This method, though more successful than the N-acetyl amyl amino acid method, was still not reproducible. Azeotroping

of the ester with benzene and further methylation improved the peak heights of the amino acids but there was still difficulty with the basic amino acids, particularly histidine.

The peaks for cysteine, proline, methionine, glutamic acid and the basic amino acids arginine, lysine and histidine were very small. This suggested that only a small percentage of these amino acids were converted. Methionine was not very stable and decomposed within a few hours.

When injected alone in high concentrations histidine gave three peaks, one where histidine normally occurs, one peak overlapping the glycine peak and another peak overlapping the lysine peak. It is not known whether the multiple peaks are due to contaminants in the original histidine sample or else due to the formation of several histidine derivatives.

Since it is possible that the presence of different amounts of water as an end product may be one reason for the difficulty in achieving quantitative esterification other procedures were attempted.

(b) Dimethylformamide Method

The anhydrous HCl or HBr method was modified by a procedure similar to that described by Zomely et al (1962).

A 15 ml volume of methanol was acidified with a small amount of anhydrous HBr. A few drops of dimethoxypropane -- a water scavenger -- were added at this point. The acidified methanol was added to the amino acid mixture and the amino acids dissolved. A 10% volume of dimethylformamide (1.5 ml) was added

to the methanol and the solution saturated with anhydrous HBr. The contents were then evaporated on a rotatory evaporator under negative pressure at 50-60°C.

At this stage the residue was neutralized with a 1 N solution of ammonium carbonate in methanol or a solution of 1 N sodium carbonate. The esters were then removed from the solution by repeated extractions with methylene chloride in a separatory funnel. The extracts were evaporated on a rotatory evaporator under negative pressure at 30°C.

Methylene chloride (5 ml), 0.1 ml of dimethylformamide and 2 ml of trifluoroacetic anhydride were added to the residue. The contents were allowed to stand at room temperature for 30-60 minutes and then evaporated and the residue dissolved in methanol and dimethylformamide.

This method was not successful. When the derivatives were analyzed on the gas chromatograph multiple peaks for many amino acids were obtained. Any dimethylformamide present also took a long time to come off the column. The column was also damaged and severe bleeding resulted.

(c) First Method for Making N-Trifluoroacetyl Methyl Esters with Thionyl Chloride

To 20 ml of dry methanol were added 4 ml of thionyl chloride drop by drop, the flask being placed in a cold bath (dry ice and methyl cellosolve). The solution was then added to the amino acid mixture and the amino acids dissolved. The solution was then refluxed at 60-65°C for 1 - 2 hours and the contents evaporated on a rotatory evaporator under negative pressure at 30°C.

A volume of 5 ml of trifluoroacetic anhydride was added and left at room temperature for one hour. The excess anhydride was then evaporated and the residue dissolved in methanol to a volume of 5 ml.

This method gave better results than any of the previously used procedures. However, it was observed that the esterification could proceed just as well if the mixture were left standing overnight at room temperature. Also, it was thought that refluxing might cause loss of some of the volatile esters.

The method finally adopted is given in detail in the following section.

SECTION IV
FINAL METHOD FOR THE
PREPARATION AND SEPARATION OF THE AMINO ACID
N-TRIFLUOROACETYL METHYL ESTERS

a) Procedure

Approximately 20 ml of dry methanol was added to the amino acid mixture in a 100 ml round bottom flask, and the amino acids dissolved as well as possible by shaking. 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 with constant mixing. The flask was then stoppered with a calcium chloride drying tube and kept at 40°C for two hours in a silicone oil bath. The heated mixture was then cooled to room temperature. The flask was again placed in a cold bath for a few minutes and one to two milliliters of Trifluoroacetic anhydride added. The flask was left at room temperature for one to two hours and the excess Trifluoroacetic anhydride (or acid) removed on a rotatory evaporator at room temperature.

The residue was then dissolved in dry methanol and the volume adjusted to 5 ml. The esters were left standing at room temperature for 3 - 4 hours. Up to 20 µl of the sample was injected onto the gas Chromatography column with a Hamilton microliter syringe.

b) Gas Chromatography

The separations were carried out on an F & M Model 720 chromatograph with a hydrogen flame detector attachment Model 1609. The hydrogen flow rate was 65 ml/minute and the air flow 450 ml/minute. The injection port temperature was 305°C and the block temperature 280°C. Either stainless steel or copper tubing was used. The separations were made on three

types of columns: a 20' x $\frac{1}{8}$ " 1% Carbowax 20M on 80-100 mesh diatop-
port S, a 2' x $\frac{1}{8}$ " 1% Carbowax 20M on 80-100 mesh diatop-
port S and a 10' x $\frac{1}{8}$ " 1% Carbowax 1540 on diatop-
port S.

The carrier gas was nitrogen at a flow rate of 80 ml/minute with
the Carbowax 20 M columns and 50 ml/minute with the Carbowax 1540
column.

A different temperature program was used for each column. For
chromatography on the 20' Carbowax column the temperature was 80°C at
the time of injection. The temperature was programmed to rise at
5°/minute to 185°C. The temperature was then held at 185°C for
30 minutes, then the temperature was again programmed to rise
2°/minute to 230°C. The column temperature was then held at 230°C for
the rest of the run, approximately a further forty minutes. Cysteine,
valine, alanine / isoleucine (not separated), leucine, glycine, proline,
aspartic acid, threonine, methionine, glutamic acid, phenylalanine,
serine, arginine, hydroxyproline and lysine were all quantitatively
separated.

For chromatography on the 2' Carbowax 20 M column the injection
was made with the column at room temperature and the temperature raised
to 270°C within two to three minutes. The tyrosine peak appeared with-
in 18 minutes and was followed immediately by the histidine and tryp-
tophan peaks.

For chromatography on the 1% Carbowax 1540 column the injection was
made at room temperature. After five minutes the temperature was set to
120°C and then raised 5°/minute to 170°C. Cysteine, valine, alanine,
isoleucine, leucine, glycine and proline were quantitatively separated

on the 1540 column, with threonine-methionine, glutamic acid and phenylalanine being the final amino acids to appear. (A detailed study of the gas chromatography of the N-Trifluoroacetyl amino acid methyl esters, including the diagrams of the separations on each of the three columns is given in the section on Gas Chromatography page 44).

The above procedure gave reproducible, quantitative results. Every step in the procedure was carefully worked out to insure maximum peak areas for all amino acids. The peak areas were expressed in "integrator units", each excursion of the integrator being described as a unit. The following sections describe in detail the variations tried in the esterification and trifluoroacetylation of the amino acids.

SECTION VI

CONDITIONS FOR THE REPRODUCIBLE FORMATION OF N-TRIFLUOROACETYL AMINO ACID METHYL ESTERS

Each step in the formation of N-trifluoroacetyl methyl amino acid esters given in the preceding section had to be studied, e.g. the necessity for purification of thionyl chloride, the optimum temperature and time for methylation and the concentration of thionyl chloride required for maximum methylation. Likewise the volume of trifluoroacetic anhydride needed and the optimum time for trifluoroacetylation were also studied. Throughout the one criterion of conversion was the obtaining of large, reproducible peak areas on the chromatograms for given amounts of amino acids.

For some amino acids esterification did not seem to be complete after standing overnight. For these amino acids maximum peak heights seemed to require a further reaction in the final methanol after the removal of trifluoroacetic anhydride.

1. METHYLATION OF AMINO ACIDS

(a) Importance of Water-Clear Thionyl Chloride

During the first part of the work industrial thionyl chloride was used in all experiments. While some batches were satisfactory, others did not give reproducible results. Fluka water-clear thionyl chloride was therefore tried and since it was found to be satisfactory was used throughout all the later work. The Fisher (industrial) thionyl chloride was then purified by the method described on page 16.

(b) Unpurified vs Water-Clear Thionyl Chloride

Under constant experimental conditions only tyrosine, histidine and tryptophan failed to show reproducible peaks when unpurified thionyl chloride was used. For this reason a study of the conversion of these amino acids alone was made on a short 2' x $\frac{1}{4}$ " 1% Carbowax 20M column.

In this experiment the use of unpurified Fisher thionyl chloride was compared with Fisher thionyl chloride redistilled from quinoline and washed linseed oil. A sample containing 5 mg of each amino acid was esterified in a 100 ml round bottom flask in a cold bath ^(-4°C) with 4 ml of either the redistilled or unpurified thionyl chloride in 20 ml of dry methanol. The flasks were then heated to 40°C for 2 hours in a silicone oil bath. After evaporation on a rotatory evaporator under negative pressure at room temperature, 3 ml of trifluoroacetic anhydride was added and the flasks allowed to stand at room temperature for 2 hours. After removal of the excess anhydride by evaporation the final deriv-

TABLE 1

UNPURIFIED VS WATER-CLEAR THIONYL CHLORIDE

Injected

10 µg of each amino acid

INTEGRATOR UNITS

AMINO ACID	Cys	Val	Leu	Asp	Ser	Tyr	Hist	Tryp
ATTENUATION x	160	640	640	640	160	160	80	160
UNPURIFIED SOCl ₂	4	8	9	7	---	30	5	0
UNPURIFIED SOCl ₂	8	9	11	7	4	30	8	0
Mean:	6	8.5	10	7	2	30	6.5	0
Water-Clear SOCl ₂	5	9	11	7	4	37	16	18
Water-Clear SOCl ₂	7	11	12	7	4	38	16	9
Mean:	6	10	11.5	7	4	37.5	16	13.5

atives were dissolved in 5 ml of dry methanol. A volume of 10 μ l (containing the derivative obtained from 10 μ g of each amino acid) was injected onto a 2' x $\frac{1}{8}$ " 1% Carbowax 20M column. The experiments were run in duplicate.

The results are given in Table I.

The peaks due to serine, tyrosine, histidine and tryptophan showed an increase in size when water-clear thionyl chloride was used.

(c) Optimum Temperature for the Methylation of Amino Acids

Several experiments were carried out, allowing the samples to stand at room temperature overnight (i.e. 26°C-16 hours).

Procedure

Approximately 20 ml dry methanol was added to 100 ml round bottom flasks containing 5 mg of each amino acid. The flasks were then placed in a cold bath and 4 ml of "Fluka" water-clear thionyl chloride added drop by drop to the methanol. The flasks were stoppered with calcium chloride drying tubes and allowed to stand at room temperature for 16 hours. The contents of the flasks were then evaporated under negative pressure at room temperature. Tri-fluoroacetic anhydride (3 ml) was added and the flasks allowed to stand at room temperature for two hours and then evaporated as before. The esters were then dissolved in 5 ml dry methanol and 10 μ l samples (equivalent to 10 μ g of each amino acid) injected onto the chromatography columns.

This procedure, with variations, was used in succeeding experiments.

TABLE 2
CONVERSION AT ROOM TEMPERATURE - 16 HOURS

Injected: 10 µg of each amino acid

	INTEGRATOR UNITS																					
	Ileu																					
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp				
ATTENUATION	160	640	-----						160	320	-----						160	-----			80	160
26°C--16 hours	8	10	17	10	5	10	6	8	13	12	21	4	4	16	3	35	6	7				
26°C--16 hours	6	11	16	10	4	8	5	6	12	13	22	3	4	15	5	29	1	7				
26°C--16 hours	8	10	16	11	5	9	6	7	12	12	21	4	4	15	4	30	5	4				
Mean:	7	10	16	10	4.5	9	5.5	7	12	12	21	4	4	15	4	31	4	6				

TABLE 3
METHYLATION AT VARIOUS TEMPERATURES

Injected: 10 µg each amino acid

	INTEGRATOR UNITS																		
	Ileu																		
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp	
ATTENUATION x	160	640						160	320			160					80	160	
26°C--16 hours	8	10	16	11	5	10	6	7	12	12	21	4	4	15	4	29	8	8	
40°C--2 hours	8	9	12	11	4.5	9	6	4	13	11	22	3	3	14	10	33	12	14	
40°C--16 hours	9	8	14	10	5	4.5	6	6	12	13	23	4	4	17	4	34	6	8	
60°C--1 hour	0	0		2												3	0	0	

TABLE 4
REPRODUCIBILITY OF ESTER FORMATION AT 40°C

Injected: 10 µg of each amino acid

	INTEGRATOR UNITS																			
	Ileu																			
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp		
ATTENUATION x	160	640	-----							160	320	-----							80	160
40°C---2 hours	5	10	9.5	10	4	8	5	3	12	11	20	3	2.5	15	8	30	14	12		
40°C---2 hours	5	9	14.5	11	4.5	10	4	4	10	11	20	3	4	16	10	33	11	11		
40°C---2 hours	3	10	13.5	11	5.5	9	6	3.5	14	12	23	3	3	17	15	31	10	18		
Mean:	4.3	9.6	12.5	10.6	5	9	5	4	12	11.3	21	3	3	16	11	31	12	14		

The derivatives were separated on a 20' x 1/4" 1% Carbowax 20 M column and tyrosine, histidine and tryptophan on a 20' x 1/4" 1% Carbowax 20 M column.

The results are listed in Table 2.

There is a wide variation in the results obtained for tyrosine, histidine and tryptophan in the three experiments. A series of experiments were tried in which the boiling action was carried out by heating the flasks. Since the boiling point of methanol is 65°C and since some of the esters are volatile in this range, care was taken in selecting the right temperature.

By the procedure described on page 25, four lots of amino acids were methylated at temperatures of 260°C for 16 hours, 400°C for 2 hours, 400°C for 16 hours and 600°C for 1 hour. Exactly 10 ml (equivalent to 10 mg of each amino acid) of each sample was injected onto a 20' x 1/4" 1% Carbowax 20 M column. The amino acids from cysteine to lysine were separated while tyrosine, histidine and tryptophan were separated on a 20' x 1/4" 1% Carbowax 20 M column.

The results are listed in Table 3.

These results indicate that 400°C for 2 hours is the best temperature for all of the amino acids in this series of experiments. There is a marked increase in the peak area due to lysine, histidine and tryptophan. A temperature of 400°C for 16 hours is too long a period of time at 400°C since the peaks for proline, lysine, histidine and tryptophan all show a decrease.

ESTERIFICATION IN THE COLD (3°C)

Injected:

10 µg each amino acid

INTEGRATOR UNITS

AMINO ACID	Cys	Val	Leu	Asp	Ser	Tyr	His	Tryp
ATTENUATION x	160	640-----	160	160	160	160	80	160
cold 16 hours	3	4	8	4	2	29	9	4.5
cold 36 hours	9	8	10	4	5	24	9	4

TABLE 5

A temperature of 60°C is too drastic for the purpose of this work.

Several experiments were run at 40°C to see if reproducibility could be obtained at this temperature. Three samples containing 5 mg of each amino acid were subjected to the procedure described on page 25, at 40°C for 2 hours.

Exactly 10 µl of each sample was injected onto a 20' x 1/4" 1% Carbowax 20 M column and a 2' x 1/4" 1% Carbowax 20 M column as before. The results are listed in Table 4.

With a few exceptions e.g. lysine, a greater reproducibility was obtained at 40°C than in previous experiments.

Esterification of amino acids was also tried in the cold (3°C), to minimize any hydrolysis of the methyl esters that may occur.

Two samples each containing 5 mg of each amino acid were treated with thionyl chloride and methanol in the cold (3°C) for 16 hours and for 36 hours respectively before adding

the trifluoroacetic anhydride. Otherwise the procedure was as described on page 25. Samples of 10 µl were injected onto a 20' x 1/4" 1% Carbowax 20 M column. The results are listed in Table 5.

Tyrosine, histidine and tryptophan were lower than previously (40°C for 2 hours) when esterification was carried out in the cold.

TABLE 6

OPTIMUM TIME FOR METHYLATION AT 40°C

Injected:

10 µg of each amino acid

		INTEGRATOR UNITS																																																		
		Ileu																																																		
AMINO ACID		Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp																																	
ATTENUATION		160	640															160	320															160															80	160		
40°C	30 min.	0	4	10	9															4															2															30	14	2
40°C	1 hour	6	7	10	10	4	8	5	4	11	11	21	3	2.5	14	2.5	30	13	14																																	
40°C	2 hours	8	11	15	11	5.5	12	6	6	16	16	31	4	9	20	7	34	13	15																																	
40°C	4 hours	5	9	13	11	4	3.5	6	5	13	14	24	4	5	16	8	33	12	15																																	
40°C	8 hours	6	11	15	10	5	2	7	4	12	14	28	4	4	15	6	29	8	10																																	
40°C	16 hours	6	8	14	10	5	4.5	6	6	12	12	23	5	4	17	4	34	6	7																																	
40°C	19 hours	5	9	15	9	4.5	.5	5	5	11	12	23	3	3	11	2	30	7	8																																	

(d) Optimum Time for the Methylation of Amino Acids
at 40°C and room temperature

Having verified 40°C as being the optimum temperature for the methylation of amino acids, it was now necessary to find the optimum time for methylation at 40°C.

A large number of experiments were carried out in which the esterification step proceeded at 40°C for various time intervals. The data given represent the mean values of these experiments. Twenty ml of dry methanol was added to 100 ml round bottom flasks containing 5 mg of each amino acid. The flasks were placed in a cold bath (dry ice in methyl cellosolve) and 4 ml of Fluka water-clear thionyl chloride added drop by drop to the methanol. The flasks were stoppered with calcium chloride drying tubes and placed in a silicone oil bath at 40°C.

The time intervals for methylation at 40°C were: 30 mins., 1 hour, 2 hours, 4 hours, 8 hours, and 16 hours (i.e. 6 different samples). Another sample was also methylated at 40°C for 19 hours.

The contents of the flasks were then evaporated on a rotatory evaporator under negative pressure at room temperature. Approximately 3 ml of trifluoroacetic anhydride was added and the flasks left standing at room temperature for 2 hours and then evaporated as before. The derivatives were dissolved in dry methanol to a volume of 5 ml. 10 µl samples (10 µgm of each amino acid) were injected onto a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column to separate the amino acids from cysteine to lysine and onto a 2' x $\frac{1}{8}$ " 1% Carbowax 20 M column to separate tyrosine, histidine and tryptophan. The 40°C-- 30 min. sample was injected only onto

a 2' x $\frac{1}{8}$ " 1% Carbowax 20 M column.

The mean values in Table 6 were obtained for a number of experiments at the time intervals given.

All of the amino acids reach their maximum peak area at 40°C--2 hours. With prolonged heating at 40°C there is a decrease in the peak areas of several amino acids. Proline, lysine, histidine and tryptophan show a marked decrease in peak size, whereas arginine and hydroxyproline show a slight decrease in peak size on prolonged heating.

Methylation at 40°C for 2 hours was therefore used in all subsequent experiments.

Since it was found convenient to esterify amino acids at room temperature overnight. A time study was therefore conducted on methylation at room temperature.

The procedure on page 25 was followed with the exception that esterification was carried out at room temperature (26°C) for the following time intervals: 1 hour, 2 hours, 4 hours, and 16 hours. Samples of 10 μ l (containing 10 μ gm of each amino acid) were injected onto 20' x $\frac{1}{8}$ " 1% Carbowax 20 M columns and onto 2' x $\frac{1}{8}$ " 1% Carbowax 20 M columns. A 10' x $\frac{1}{8}$ " 1% Carbowax 1540 column was also used to separate the alanine and isoleucine.

The amino acids show the largest peak areas at 26°C - 16 hours. However there is a gap between 4 hours and 16 hours. During this interval complete esterification could have taken place. For convenience it was found most suitable to let the esterification take place overnight. The peak areas of all the

TABLE 7

OPTIMUM TIME FOR METHYLATION AT ROOM TEMPERATURE

The results are tabulated in TABLE 7

Injected:

10 µg of each amino acid

INTEGRATOR UNITS

AMINO ACID	Cys	Val	Ala	Ileu	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp							
ATTENUATION	160	640	-----										160	-----										160	80	160
26°C 1 hour	5	2	2	1	4	3	1	2	1	6	5	10	0	0	8	1	1	0	0							
26°C 2 hours	4	6	7	4	11	5	9	6	3	10	8	15	1	0	10	2	2	0	0							
26°C 4 hours	6	8	8	5.5	11	5	7	6	3	11	12	21	2	2	11	2	0	0	0							
26°C 16 hours	8	10	7	9	11	5	10	6	7	12	12	21	4	4	15	4	30	9	8							

amino acids esterified at 26°C for 16 hours are with the exception of tryptophan, approximately equal to the peak areas obtained at 40°C; the results obtained with either of the two methods were reproducible.

(e) The amount of Thionyl Chloride required to react with Methanol and Amino Acids for the Maximum Methylation of the Amino Acids

Having obtained the optimum temperature and time for the methylation of amino acids it was now necessary to find the optimum concentration of thionyl chloride required for reaction with dry methanol for the maximum methylation of amino acids. A constant volume of dry methanol (20 ml) was used in all of the experiments. Again the criterion of maximum methylation was maximum peak size.

Approximately 20 ml of dry methanol was added to 100 ml round bottom flasks containing 5 mg of each amino acid. The flasks were placed in a cold bath (dry ice in methyl cellosolve) and the following volumes of water-clear "Fluka" thionyl chloride added dropwise to their respective flasks: 0.5 ml, 1 ml, 2 ml, 4 ml, and 8 ml. The flasks were then stoppered with calcium chloride drying tubes and placed in a silicone oil bath at 40°C for 2 hours. The contents of the flasks were then evaporated on a rotatory evaporator under negative pressure at room temperature. Approximately 3 ml of trifluoroacetic anhydride was added and the flasks left standing at room temperature for 2 hours and then evaporated as before. Samples of 10 µl (equivalent of 10 µg of each amino acid) were injected onto 20' x 1/4" 1% Carbowax 20 M columns and 2' x 1/4" 1% Carbowax 20 M columns. By mistake the 8 ml thionyl chloride sample was injected onto a 10' x 1/4" 1%

TABLE 8

OPTIMUM VOLUME OF THIONYL CHLORIDE FOR METHYLATION

Injected:

10 µg of each amino acid

		INTEGRATOR UNITS																				
		Ileu																				
AMINO ACIDS		Cyst	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp			
ATTENUATION x		160	640	-----							160	320	-----			160	-----				80	160
0.5	ml SOCl ₂	0	1	5	4	2.5	3	1	0	0	4	6	0	1	7	0.5	14	9	12			
1	ml SOCl ₂	2	4	8	9	4	5	4	4	12	10	23	4	4	9	3	28	8	11			
2	ml SOCl ₂	2	4	7	8	4	7	4	4	12	10	23	4	4	9	3	30	11	9			
4	ml SOCl ₂	5	10	16	11	5	10	5	4.5	13	12	24	5	4	16	13	31	10	12			
8	ml SOCl ₂	4	8	12	12	6	12	7	---	---	---	---	---	---	---	---	20	2.5	4			

TABLE 9

10 µg of each amino acid

		INTEGRATOR UNITS																		
		Ileu																		
AMINO ACID		Cyst	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp	
ATTENUATION x		160	640							160	320				160			80	160	
Method I		4	10	14	11	5.5	10	5	4	14	12	23	4.5	3	17	15	36	10	12	
Method II		6	9	18	10	4.5	9.5	5.5	3	12	12	24	6	5	16	11	38	10	15	

Carbowax 1540 column instead of the 20' Carbowax 20 M column.

Since a number of experiments were carried out with varying thionyl chloride concentrations, the results listed in Table 8 represent the mean values of these experiments.

There is a large increase in peak size for all amino acids between 0.5 ml and 1 ml of thionyl chloride. It is interesting to note that histidine and tryptophan have reached maximum peak areas at 0.5 ml of thionyl chloride. All of the amino acids reach their optimum peak areas at 4 ml of thionyl chloride. There is a sharp decrease in the size of the peaks due to tyrosine, histidine and tryptophan at 8 ml of thionyl chloride.

In early experiments the thionyl chloride was added first to the dry methanol in the cold bath and then 20-25 ml of this mixture was added to the amino acid mixture. By this method, some of the amino acids were difficult to dissolve and mechanical means had to be used to get them into solution. In order to dissolve the dry amino acids it was found advantageous to add the dry methanol directly to the amino acid mixture and then add the thionyl chloride drop by drop to the methanol. The amino acids went into solution when only a few drops of thionyl chloride had been added. The results obtained from both methods, named Methods I and II respectively, were very similar. These results are listed in Table 9.

TABLE 10

VOLUME OF TRIFLUOROACETIC ANHYDRIDE

FOR

MAXIMUM PEAK SIZE

Injected:10 μ g of each amino acid

AMINO ACID	Cys	Val	Leu	Asp	Ser	Tyr	Hist	Tryp
ATTENUATION $\times$	160	640	640	640	160	160	80	160
2 ml TFA anhydride	5	11	12	7	3.5	30	14	12
3 ml TFA anhydride	9	9	11	8	4	34	13	12
4 ml TFA anhydride	8	10	11	6	4	30	15	16
5 ml TFA anhydride	5	9	11	7	4	37	16	9
6 ml TFA anhydride	4	11	12	7	3.5	33	8	10
8 ml TFA anhydride	5.5	11	10.5	5.5	3	33	6	10

2. Trifluoroacetylation of Methyl Amino Acid Esters

(a) Volume of Trifluoroacetic Anhydride for Maximum Peak Size

Experiments were carried out in which various volumes of trifluoroacetic anhydride were used for the trifluoroacetylation of methyl amino acid esters in an attempt to find the optimum amount for trifluoroacetylation.

Approximately 20 ml of dry methanol was added to 100 ml round bottom flasks containing 5 mg of each amino acid. A volume of 4 ml of thionyl chloride was added drop by drop to the methanol in a cold bath. The flasks were then stoppered with calcium chloride drying tubes and then placed in a silicone oil bath at 40°C for 2 hours. The contents of the flasks were then evaporated on a rotatory evaporator under negative pressure at room temperature.

The following volumes of trifluoroacetic anhydride were added to their respective flasks: 2 ml, 3 ml, 4 ml, 5 ml, 6 ml and 8 ml. The flasks were then left at room temperature for 2 hours, then evaporated as before. Dry methanol was added to the esters to a volume of 5 ml. A 10 μ l volume of each sample was injected onto a 2' x $\frac{1}{8}$ " 1% Carbowax 20 M column.

The results shown in Table 10 represent the mean value of several experiments carried out with varying volumes of trifluoroacetic anhydride.

The volume of trifluoroacetic anhydride had little effect on the peak areas of the amino acids. However high volumes of trifluoroacetic anhydride appeared to decrease the peak areas of histidine and tryptophan.

TABLE 11

OPTIMUM TIME FOR TRIFLUOROACETYLATION
OF
METHYL AMINO ACID ESTERS

Injected:

10 µg of each amino acid

INTEGRATOR UNITS

AMINO ACID	Cys	Val	Ala	Ileu	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp		
ATTENUATION x	160	640	-----								160	320	-----			160	-----			80	160
15 mins.	0.5	7	5	7	7	3.5	2	5	4	12	11	13	3	3	11	2	30	8	12		
30 mins.	5	10	7	10	11	5	5.5	6	6	12	12	18	3	5	15	4	33	10	14		
1 hour	5	10	7	10	11	5	7	6	6	12	12	24	4	5	17	8	35	9	16		
2 hours	6	10	8	10	12	5	8	6	5	11	11	24	3	4.5	18	10	36	9	16		
3 hours	6	9.5	7	9	11	5.5	10	6	4	12	13	28	4	5	18	12	34	10	15		
4 hours	7	11	7	8	11	5	10	6	6	9	12	24	3	8	20	13	35	8	14		
16 hours	8	9	7	9	11	4.5	10	7	6	11	12	24	4	4	16	10	32	11	14		

(b) Optimum Time for Trifluoroacetylation of Methyl
Amino Acid Esters

The procedure described on page 25 was used for the esterification of amino acids. Approximately 3 ml trifluoroacetic anhydride was added to the samples and they were left at room temperature for the following time intervals: 15 min., 30 min., 1 hour, 2 hours, 3 hours, 4 hours and 16 hours. The samples were evaporated and then dissolved in dry methanol to a volume of 5 ml. Exactly 10 μ l samples were injected onto three columns: a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M, a 2' x $\frac{1}{8}$ " 1% Carbowax 20 M and a 10' x $\frac{1}{8}$ " 1% Carbowax 1540 column.

The results in Table II, represent the mean values of several experiments.

Trifluoroacetylation reaches a maximum after one hour at room temperature after which there is little change in peak areas over a period of 16 hours.

Therefore a volume of 2-3 mls of trifluoroacetic anhydride at room temperature for 1-2 hours is sufficient for maximum peak size.

3. The Effect of Time on Standing in the Final Solvent
(Methanol) upon Peak Size

When the optimum conditions for formation of the amino acid derivatives, given in detail in the preceding sections, were adhered to it was frequently observed that the peak areas of some of the amino acids were not always at their maximum. This was only the case when the amino acids were injected shortly after dissolving in the final solvent. If the samples were left standing overnight at room temperature and then injected onto the

EFFECTS OF STANDING UPON PEAK SIZE

TABLE 12

Injected: 10 µg of each amino acid

INTEGRATOR UNITS

	Ileu																	
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp
ATTENUATION x	160	640	-----	-----	-----	-----	-----	160	320	-----	160	-----	-----	-----	-----	-----	80	160
zero time	3	9	15	9	4.5	9	5	4	11	12	23	3	0	7	2	29	4	16
zero time	0	10	9.5	7.5	4	8	4	5	12	11	27	0	2	8	3.5	36	3	15
Mean:	1.5	9.5	12	8	4.5	8.5	4.5	4.5	11.5	11.5	25	1.5	1	7.5	3	32	3.5	15.5
16 hours	4	8	13	8	4	9	5.5	5.5	11	12	21	3	5	11	5	37	8	18
16 hours	6	9	14	11	5	10	5.5	6.5	14	13	26	5	4	17	5	36	11	15
Mean:	5	8.5	13.5	9.5	4.5	9.5	5.5	6	12	12.5	24	4	4.5	14	5	36.5	9.5	16.5

TABLE 13

Injected: 10 µg of each amino acid

INTEGRATOR UNITS

AMINO ACID	Ileu														
	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys
ATTENUATION	160	640	-----	-----	-----	-----	-----	160	320	-----	160	-----	-----	-----	-----
zero time	3	10	17	10	4.5	10	6.5	7	9	12	26	0	---	14	6
1 hour	4	9.5	13	9	5.5	9	5	3	6	10	21	0	---	16	9
4 hours	5	9.5	15	11	5.5	10.5	6	3	12	12	25	3	6	19	9
16 hours	6	10	14	10	4	8	5	4	11	11	22	4	5	16	10

chromatography columns, the peak areas of all the amino acids would reach a maximum. The amino acids that were most affected by standing-- serine, arginine, hydroxyproline, lysine and histidine-- all have either secondary amine groups, imino groups, indole nitrogen or hydroxyl groups which might undergo further methylation or trifluoroacetylation on standing at room temperature in the final solvent.

The following procedure was used: approximately 20 ml of dry methanol was placed in a 100 ml round bottom flask containing 5 mg of each amino acid. The flasks were placed in a cold bath and 4 ml of water-clear "Fluka" thionyl chloride added drop by drop to the methanol. The flasks were stoppered with calcium chloride drying tubes and placed in a silicone oil bath at 40°C for 2 hours. The contents of the flasks were then evaporated on a rotatory evaporator at negative pressure at room temperature. Approximately 3 ml of trifluoroacetic anhydride was added to the flasks which were left at room temperature for 2 hours. The excess anhydride was then evaporated. Dry methanol was added to the esters and the volume adjusted to 5 ml.

Duplicate samples were carried through the above procedure and exactly 10 µl injected onto a 20' x 1/4" 1% Carbowax 20 M column and a 2' x 1/4" 1% Carbowax 20 M column. The samples were injected at zero time i.e. immediately after dissolving the N-trifluoroacetyl amino acid methyl ester in the dry methanol and again 16 hours later.

The results and the mean values for the duplicate samples at zero time and 16 hours are listed in Table 12.

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	Item	Cut	Vol	Air	Low	Qty	Xo	App	Time	Net	Gist	The	Ser	Are	Hyp	Lvs	Tyr	Wht	Type
ATTENTION x		160	640						160	320				160				80	160
4 hours		3	9.5	15	11	5.5	10.5	6	3	12	12	23	3	6	19	9			
6 days		6	13	21	13	6	13	7	7	10	16	33	4	5	19	9			
14 days		5	10	18	10	4.5	9.5	5.5	5	12	12	24	3	5	16	11	30	14	15

aspartic acid -- are not affected by standing in the final methanol. By the time chromatography on the column is complete all the amino acids should be completely converted to the derivative and can therefore be chromatographed on the remaining two columns.

4. Stability of N-Trifluoroacetyl Amino Acid Methyl Esters

The samples used in the experiment above were left standing at room temperature for several days. The stability of the N-Trifluoroacetyl methyl derivatives was then tested by chromatographing them after standing for 8 days and 14 days. Exactly 10 μ l samples were injected onto a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column. The sample injected after 14 days standing was also injected onto a 2' x $\frac{1}{8}$ " 1% Carbowax 20 M column. The results are listed in Table 14.

The N-Trifluoroacetyl methyl amino acid esters were shown to be very stable after standing at room temperature for 8 and 16 days. The higher results obtained after 8 days are probably due to a pipetting error.

5. Attempts to Accelerate the Conversion of Amino Acids to their N-Trifluoroacetyl Methyl Esters

Since it requires up to 4 hours standing at room temperature in the final solvent for complete esterification of all the amino acids, attempts were made to accelerate the conversion of these amino acid derivatives.

The amino acid derivatives were heated at 40°C for 1 hour, 2 hours, 3 hours, 4 hours, and 6 hours in the final methanol solvent. Exactly 10 μ l samples (containing 10 μ g of

TABLE 15

40°C in final methanol

Injected:

10 µg of each amino acid

INTEGRATOR UNITS

	Ileu																	
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp
ATTENUATION x	160	640						160	320			160					80	160
zero time	4	9	13	10	4.5	9	4	5	13	12	24	2	3.5	13	5	29	0	13
1 hour	6	9	14	10	4.5	9	5.5	2	11	11	23	1.5	5	13	5	30	5	13
2 hours	5	9	13	10	5	10	5	1.5	13	12	23	2.5	4	14	6	33	10	17
3 hours	5	10	15	10	4.5	10	5.5	2	10	11	22	3	4	14	10	34	12	18
4 hours	9	10	16	12	5.5	12	7	3	16	16	31	4	9	20	7	36	13	19
6 hours	5	10	15	11	5	10	5	2	11	12	23	2	5	15	7.5	35	14	16

TABLE 16

Injected:

10 µg of each amino acid

INTEGRATOR UNITS

	Ileu																	
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp
ATTENUATION x	160	640						160	320			160					80	160
azeotroped																		
methanol	3	9	15	11	4.5	10	6	3	11	12	25	3	4	12	12	34	1	16
usual procedure	5	10	14	10	4	8	5	4	11	11	22	4	5	16	10	36	11	15

TABLE 17

Injected:

10 µg of each amino acid

INTEGRATOR UNITS

	Ileu																	
AMINO ACID	Cys	Val	Ala	Leu	Gly	Pro	Asp	Thre	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	Hist	Tryp
ATTENUATION x	160	640	-----	-----	-----	-----	-----	160	320	-----	-----	160	-----	-----	-----	-----	80	160
2nd methanol																		
40°C--2 hours	2	7.5	12	9	4	7	5	2.5	11	13	23	3.5	3	9	3.5	30	2	10
normal method	5	10	14	10	4	8	5	4	11	11	22	4	5	16	10	36	11	15

each amino acid) were injected onto a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column after heating at 40°C for the time intervals given above as well as at zero time. The results are listed in Table 15.

There was an increase in the size of the peaks due to serine, arginine, hydroxyproline, lysine, tyrosine, histidine and tryptophan after heating the final ester at 40°C which reached maximum peak areas after 4 hours. These results were the same as those obtained at room temperature for 4 hours. Therefore, heating the final ester at 40°C has little effect on accelerating the complete esterification.

The incomplete conversion of the amino acids to the final derivative was most noticeable at low amino acid concentrations. It was noticed that with small amounts of amino acids, after methylation and evaporation, there was always a liquid residue remaining which could only be removed by heating at a high temperature. Since thionyl chloride was probably added in excess for esterification of the smallest amounts of amino acids, the residue was likely either sulphurous acid or HCl. Water was also a possibility. This residue could possibly be interfering with the further esterification of these amino acids. Attempts were made to remove this residue without harming the methyl amino acid esters.

The first procedure used was to add more dry methanol to the residue after methylation had occurred (procedure on page 25) in an attempt to remove the residue by azeotroping it. The residue decreased in volume after re-evaporating it and

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MINICOL

Cys Val Ala Leu Gly Pro Asp Thr Ser Glu Phe Ser Asn Hyp Tyr Tyr Met Tyr

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and in some cases it was completely removed. However, as the results in Table 16 will show, this procedure was not successful.

There is a decrease in peak size due to cysteine and a large decrease in peaks due to histidine when treated with dry methanol.

Since only cysteine and histidine were affected, an attempt was made at heating the methyl esters in the second lot of dry methanol at 40°C for 2 hours. The results, together with that of the normal procedure, are listed in Table 17.

The experiment was not successful. There was a large decrease in the peak areas of cysteine, threonine, hydroxyproline, lysine and histidine.

Attempts were made to azeotrope with benzene after the methylation and evaporation procedures or to dry the residue with ether.

The results of these two experiments are listed in Table 18.

There is a decrease in the size of the peaks due to cysteine, phenylalanine, serine, arginine, hydroxyproline, lysine, histidine and tryptophan when benzene is used to azeotrope the residue. Drying with ether was also not successful since the peak areas of histidine and tryptophan were decreased.

Other experiments were carried out in an attempt to accelerate the esterification of amino acids.

A few drops of dimethoxypropane-- a water scavenger-- was added to the final solvent, in the belief that water

TABLE 19

Injected:10 μ gm of each amino acid

INTEGRATOR UNITS

AMINO ACID	Tyr	Hist	Tryp
ATTENUATION $\times$	160	80	160
Usual procedure	34	12	16
dimethoxypropane	21	4	3
2 ml dimethyl-sulfite	16	1	3
3 ml dimethyl-sulfite	18	2	0
4 ml dimethyl-sulfite	17	1	4
1. 4ml TFA- 4 hours	1	0.5	11
2 4ml $\text{SOCl}_2/\text{CH}_3\text{OH}$ 40°C --2 hours			

may be inhibiting complete esterification.

Dimethylsulfoxide, believed to be a possible intermediate in the methylation reaction, was used in the methylation of amino acids. Following the procedure described on page 25, varying amounts of dimethylsulfoxide was added to the thionyl chloride--methanol--amino acid mixture.

Finally, the esterification procedure described on page 25 was carried out in the reverse order. 4 ml of Trifluoroacetic anhydride was added to the amino acid mixture, which was then dissolved by gently heating at 40°C for a few minutes and then standing at room temperature for 2 hours. The contents were then evaporated as described on page 25 and 20 ml of dry methanol plus 4 ml of Fluka water-clear thionyl chloride added to the residue. The flask was then heated at 40°C in a silicone oil bath for 2 hours.

The results of the above experiments are listed in Table 19. The amino acid derivatives were chromatographed on a 2' x 1/8" 1% Carbowax 20 M column. Only tyrosine, histidine and tryptophan derivatives are recorded and compared to the usual amino acid esterification procedure.

None of the above experiments was successful. There was a sharp decrease in peak heights due to tyrosine, histidine and tryptophan, with the largest decrease occurring when trifluoroacetylation was carried out first.

SECTION VI

THE SEPARATION OF N-TRIFLUOROACETYL AMINO ACID METHYL
DERIVATIVES BY GAS-LIQUID CHROMATOGRAPHY

In the preceding sections, the procedure for the conversion of the amino acids into volatile derivatives suitable for gas chromatography was discussed. It is now appropriate to describe experiments to determine optimum conditions for the separation of these derivatives by gas chromatography. The selection of the liquid phases for efficient separation, the best temperature program to resolve amino acids on a particular column, the optimum flow rate of carrier gas through the column and the dimensions of a column have all been studied.

A GENERAL PROCEDURE FOR GAS CHROMATOGRAPHIC SEPARATION OF N-TFA METHYL
DERIVATIVES AMINO ACIDS

N-TFA methyl amino acid esters prepared by the method described on page 25 were separated under the following chromatographic conditions:

1. Chromatograph

An F & M model 720 dual column programmed temperature gas chromatograph equipped with a temperature programmer with a temperature range from 0.5°C to 300°C per minute.

2. Detector

An F & M model 1609 hydrogen flame ionization detector attachment operated at 110 volts with a platinum ring collector electrode. The hydrogen flow rate was 65 ml/min. and the air flow rate 450 ml/min.

3. Temperatures

The injection port temperature was 305°C and the block temperature (detector) was 280°C.

4. Recorder

A Minneapolis-Honeywell, Brown instruments div. Electronik model 153 with a continuous balance potentiometer. It has a millivolt range of -0.20 to 1 millivolt, a full scale balance speed of 1 second with a chart speed of 1 inch/4 minutes or approximately 6.25 cm/minute.

5. Columns

Three columns were used in the final analysis, a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M on diatopost S (80-100 mesh), a 2' x $\frac{1}{8}$ " 1% Carbowax 20 M on diatopost S (80-100 mesh) and a 10' x $\frac{1}{8}$ " 1% Carbowax 1540 column on diatopost S (80-100 mesh) Copper or stainless steel tubing I. D. $\frac{1}{8}$ " was used.

6. Carrier Gas

The carrier gas was nitrogen with a flow rate of 80 ml/minute for the Carbowax 20 M columns and 50 ml/minute for the Carbowax 1540 columns. The pressures at the inlet and outlet were 60 psig and atmospheric respectively.

7. Temperature programs

(a) 20' x $\frac{1}{8}$ " 1% Carbowax 20 M

The sample was injected at a column temperature of 80°C and the column held at this temperature for 24 minutes. The temperature was then programmed to rise 50°/minute to 185°C. The temperature was held at 185°C for 30 minutes, after which time the temperature was again programmed to

made at room temperature and after 6 minutes the temperature is identified. In this alternate programming the injection is sample since all of the amino acids present can be readily scanning is an aid in the rapid identification of an unknown. An alternate programming can also be used. This program is about 24 minutes.

is followed by histidine and tryptophan. The analysis time 5 minutes. The tyrosine peak appears within 18 minutes and temperature and the column temperature raised to 270°C within. The sample is injected with the column at room temperature.

(b) 2' x 1/4" 1% Carbowax 20 M

actions, 19 amino acids, a necessity for proper quantitative estimation. 2 hours. This time is necessary to resolve cleanly 14 of the. The time for separation on this column is about separation is very slow and the peaks are quite broad. With this length of column tyrosine, histidine and tryptophan is necessary to separate arginine, hydroxyproline and lysine. are separated above 190°C and a temperature of 230°C-240°C online, methionine and glutamic acid. Phenylalanine and serine to hold the temperature at or below 185°C to separate three temperatures between 120°C and 185°C whereas it is necessary leucine, glycine, proline and aspartic acid are separated at obtain cysteine. Valine, alanine / isoleucine (not resolved), The low temperature (80°C-90°C) is necessary to approximately another 40 minutes. rise at 2°/minute to 230°C-240°C for the rest of the run.

is raised to 120°C and held for 16 minutes; the temperature is then programmed to rise 10°C/minute to 260°C-270°C. This program is ideal for a rapid semi-quantitative identification of all amino acids within less than 1 hour. However, the tyrosine, histidine and tryptophan peaks are sharper when the temperature is raised rapidly to 270°C. When working with a sample containing very small amounts of amino acids the sharpness of these peaks is essential for a good estimation.

(c) 10' x 1/4" 1% Carbowax 1540 Column

The injection is made with the column at room temperature. After 14 minutes the temperature is set to 120°C and held there for about 15 minutes. The temperature is then set at 170°C for the remainder of the run.

This column is necessary for the separation of alanine and isoleucine. Cysteine, valine, leucine, glycine, proline, aspartic acid, threonine / methionine, glutamic acid and phenylalanine can also be separated and estimated on this column. Analysis time is about 1 hour and 30 minutes.

The total analysis time is about 4½ hours if all three columns are used.

8. Sample Size

A volume of 1 to 20 µl of a sample containing between 1 to 20 mg of each amino acid in 5 ml of methanol. This gave a sensitivity of between 13×10^{-6} moles for glycine and 5×10^{-6} moles for tryptophan.

9. Operation of the Electrometer

The flame ionization attachment is equipped with an electrometer which delivers 120 volts to the ion collector electrode and a range of 4×10^{-12} amps full scale. The electrometer determines the sensitivity

of the detection of the amino acids by the hydrogen flame detector.

The range selector attenuates the input to the electrometer with a scale range of 10,000 down to 1 (by degrees of $\times 10$).

The attenuator divides the output, in scale readings of 256, 128, 64, 32, 16, 4, 2 and 1, from the electrometer. The sensitivity expressed in amperes is determined by the range $\times$ attenuation $\times 4 \times 10^{-12}$.

For amino acid analysis a range of 10 and attenuator settings of 256 to 2 were found to be adequate for analysis of up to 10^{-8} moles of amino acid. It could therefore be possible with a range settings of 1 and with the attenuator set between 16 and 1 to detect samples as small as 10^{-15} or 10^{-16} moles of amino acid.

B CRITERIA FOR THE DETERMINATION OF OPTIMUM CONDITIONS FOR THE SEPARATION OF VOLATILE DERIVATIVES OF AMINO ACIDS

The conditions for separation outlined above were only achieved after all of the physical conditions necessary for an optimum gas chromatographic separation had been carefully worked out.

A review of these physical criteria and their relationship to the success of the final analysis is given. Once these 'laws' have been obeyed, future analysis should proceed with relative ease.

1. Choice of Chromatography Columns

Gas-liquid chromatography columns were packed in our laboratory (see page 17). The choice of packings was made on the basis of the following criteria.

(a) The Stationary Phase

The studies of Johnson et al (1961) on the efficiency of different liquid phases and on the percentage of liquid phase loaded on the solid support initially served as a guide to the type of liquid phase to be used. Since a stationary phase stable at high temperatures was necessary for the separation of the basic amino acids the choice of resins was restricted.

Some masking of the polar groups of amino acids was achieved by forming the N-TFA methyl derivatives. This also renders the amino acids volatile within the limiting temperatures of the resins. Thus alanine with an original melting temperature of 297°C has a N-trifluoroacetyl methyl derivative which melts at about 130°C . The temperature at which the N-TFA methyl esters of tyrosine, histidine and tryptophan will melt is in the range of 210°C - 230°C . Therefore a column packing had to be chosen which was stable at temperatures above 230°C , yet capable of resolving the other amino acids over a wide temperature range.

Carbowax 20 M (polyethylene glycol) stable up to a temperature of 250°C to 275°C was satisfactory in this respect.

The stationary phase should also be liquid at the operating temperature range of the column and have a low viscosity.

Since the amino acids would be expected to separate on a basis of differences in polarity, a polar liquid should be used. Polyethylene glycol (Carbowax) is ideal.

(b) Packing Ratio

(V/W)

A ratio of liquid phase to solid phase of 1% was found to be satisfactory. A ratio of 2% or more did not give satisfactory separations of the amino acid derivatives. A ratio of 0.5% was less satisfactory than a ratio of 1%.

(c) Column Dimensions

A 20' column was found to be necessary to separate the group of threonine, methionine and glutamic acid since the derivatives of these amino acids have low separation factors. Presumably their melting points and vapor pressures are close together.

No difference was found when either copper or stainless steel columns were used. Copper tubing was used in most columns because of ease of handling and bending, the tubing being bent into a spiral. Care was taken that the diameter of the coils was more than 15 cm.

2. Detectors

Since a thermal conductivity detector was not sensitive enough for this work, a hydrogen flame detector was used in order to achieve as great a sensitivity as possible.

3. Selection of Temperature(a) Injection Block and Detector

Since the injection block temperature should be just above the boiling point of the least volatile amino acid derivative and since tryptophan was the least volatile and regulated temperatures between 250°C-275°C for elution, the injection block temperature was set at 305°C.

Since tyrosine, histidine and tryptophan were eluted at temperatures between 250°C-275°C, the detector temperature was set at 260°C.

(b) Temperature Programming

Temperature programming was found to be a most important factor in obtaining a good separation of the amino acid derivatives. If the temperature was too low the peaks were very broad and often not resolved, however when the temperature was too high the peaks came off the column too rapidly without separation.

In order to obtain a good separation of the most volatile amino acid derivatives it was necessary to inject the sample at low temperatures and the partition of these amino acids continued at the low temperature for a sufficient length of time. The temperature was then increased as rapidly as possible to a height which permitted the elution of less volatile derivatives.

4. Flow Rate of Carrier Gas

An optimum flow rate of carrier gas (nitrogen) had to be found which was suitable both at high and low column temperatures. Gas flow rates were measured by a soap film meter as described on page 18.

5. Purity of Gas

Nitrogen was found to be pure enough for our work. However it was often necessary to remove water from nitrogen, hydrogen or air since water had an adverse effect on both the column

and detector causing fluctuations in the recordings. A column of silica gel (6-16 mesh) was placed between the gas cylinder reducing valve and the chromatograph inlet valve.

6. Injection of Sample

The sample to be injected was usually first thoroughly mixed on a vortex mixer because it sometimes appeared to be partially separated into more than one phase.

The samples were injected with Hamilton microinjector

syringes with cemented-on needles in 1 ml, 5 ml or 10 ml sizes.

The syringe and needle was cleaned by flushing

methanol through it with a water-suction pump and dried by suction.

7. Seasoning of Columns: (see page 19)

The identification of the chromatographic peaks due to the N-Tri-fluoro-acetyl methyl ketone of amino acids

The sequential order of amino acid derivatives appearing

on a chromatographic tracing is determined by the retention times of the individual derivatives on a particular column, the nature of the packing, the flow rate and volume of carrier gas required to elute the derivative, the temperature of the column and the dimensions of the column. Therefore, in order to describe the

identification and separation of amino acid derivatives on a particular column or to make comparisons between different columns, it is necessary to use a standard procedure of evaluation which can be

interpreted for use in experiments with other apparatus and under

different conditions.

Relative retention times and HETP are given in the following discussion. A description of the significance of these terms is given in the appendix.

1. Procedure for the Identification of Amino Acid

Mixtures of known concentrations of all or a portion of the 19 amino acids were converted to their N-trifluoroacetyl methyl esters and chromatographed. The relative retentions of the amino acids in the mixture were then calculated.

A known amount of each amino acid was converted to the derivative independent of other amino acids. The amino acid derivative whose identity was to be determined was then mixed with the mixture of the derivatives of all the amino acids and chromatographed again. If the peak due to the particular amino acid had increased in size in comparison to the other peaks then the identity of that amino acid had been established. However there was a danger that two amino acid derivatives might have the same retention times on the column. A mixture of the 19 amino acids lacking only the one particular amino acid was converted to the N-trifluoroacetyl methyl ester derivative. The absence of a peak on the chromatograph then indicated that the peak represented only one amino acid.

When amino acids were identified by 'spiking' with a large concentration of a specific amino acid sometimes multiple peaks would result. On the deletion of this amino acid these multiple peaks would disappear, or if they originally had the same retention times as other amino acids in the mixture the peak heights would be diminished. These multiple peaks were therefore

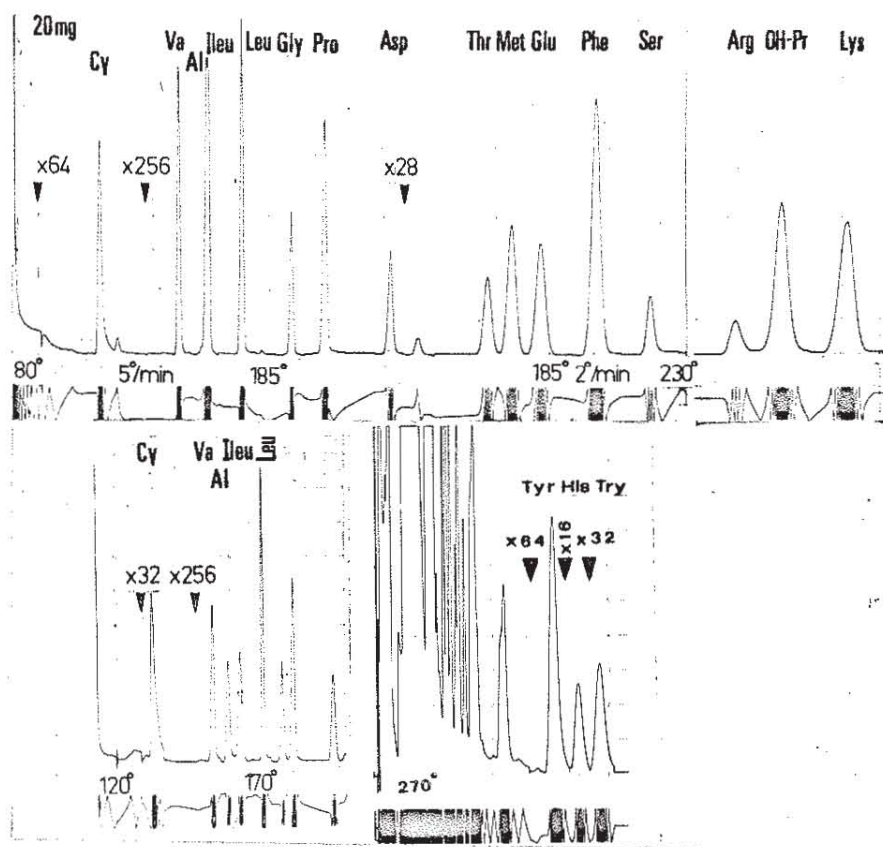


Figure 1.

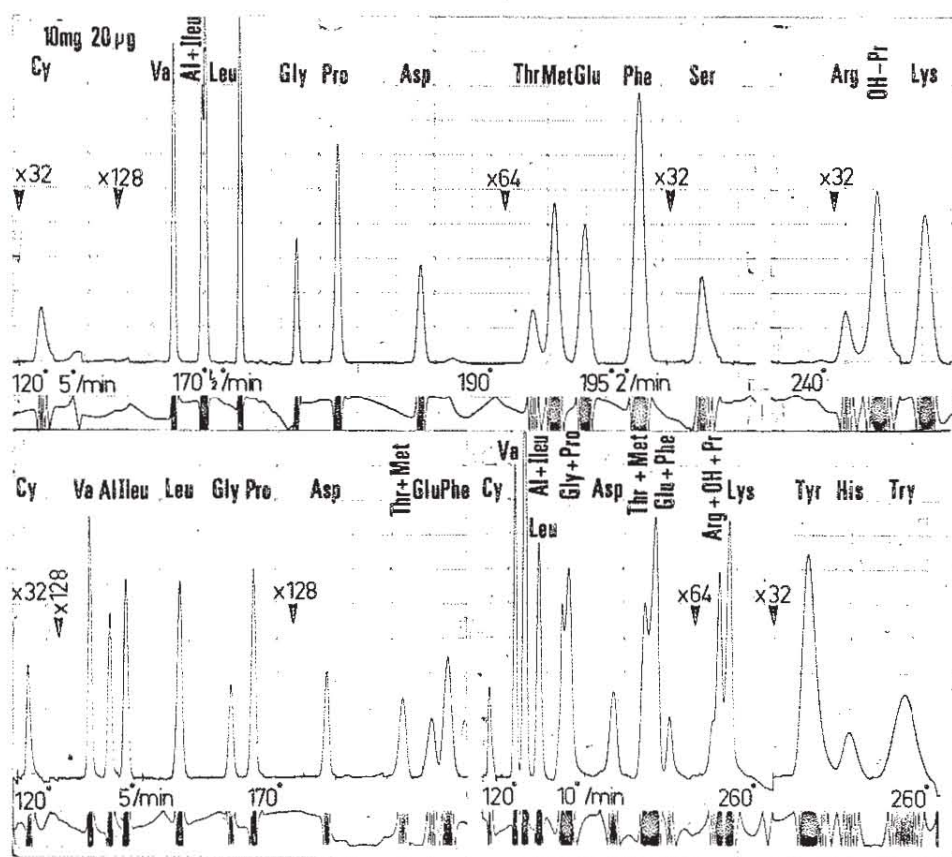


Figure 2.

LEGENDS TO FIGURES

Figure 1. Chromatographs of a mixture containing 20 mg each of 19 amino acids of which 40 μ g were injected into each column. The top tracing shows the separation on Carbowax 20 M 20' x 1/4". The alanine and isoleucine peaks overlap and the tyrosine, histidine and tryptophan peaks are not present. The gap in the tracing indicates a time interval of 20 minutes. The lower left chromatograph with Carbowax 1540 10' x 1/4" shows the separation of alanine and isoleucine. The lower right chromatograph with Carbowax 20 M 2' x 1/4" shows the separation of tyrosine, histidine and tryptophan.

Figure 2. The same as Figure 1 except that the amino acid mixture contained 10 mg of each amino acid of which 20 μ g were injected into each column. In addition the temperature has been raised more rapidly for the chromatographs on Carbowax 1540 (lower left) and on Carbowax 20 M.

attributed to contaminants, mostly other amino acids, in the standard amino acid rather than multiple peaks of a single amino acid. The derivative in question was also chromatographed alone and the contaminants identified by 'spiking' with the suspected amino acid contaminants. Pure standard amino acids were obtained which were free of contamination from other amino acids. When the derivatives of these pure amino acids were chromatographed only single peaks occurred on the recording. Thus the derivative of each of the 19 amino acids emerged as a single peak on the chromatogram.

2. The Relative Retentions of Amino Acids on the Three Chromatography Columns

The separation of the 19 amino acids is shown in Figures 1 and 2. The relative retention values of the amino acids on each column relative to aspartic acid, is now described.

(a) 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column

In figure 1, the top tracing shows the separation on the 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column. The injection was made with the column at 80°C. After 24 minutes the temperature was programmed to rise 5°/minute to 185°C and held here for 30 minutes, after which the temperature was again programmed to rise 2°/minute to 230°C. The temperature was then held at 230°C for the remainder of the run.

Cysteine is eluted at a low temperature. Valine and alanine plus isoleucine (not resolved) are eluted at temperatures between 130°C and 140°C. Leucine is eluted at 170°C and glycine, proline, aspartic acid, threonine,

TABLE 20(a)

RELATIVE RETENTIONS OF N-TFA METHYL AMINO ACIDS ON A 20' x 1/8" 1% CARBOWAX 20 M COLUMN

Gas Flow = 80 ml/minute

Reference = Aspartic Acid
Relative Retention
of 1 @ 53 mins.

Temperature = 80°C (24 min.); 5°/min. to 185°C (30 min.); 2°/min. to 230°C.

AMINO ACID	Relative Retentions	Temperature (°C)	Resolution
Cysteine	.42	90	
Valine	.57	130	
Alanine	.63	140	) 0.38
Isoleucine	.64	140	
Leucine	.70	170	
Glycine	.79	185	
Proline	.87	185	
Aspartic Acid	1.00	185	
Threonine	1.19	185	) 1.97
Methionine	1.25	185	
Glutamic Acid	1.30	185	
Phenylalanine	1.40	190	
Serine	1.51	220	
Arginine	2.05	230	) 2.50
Hydroxyproline	2.15	230	
Lysine	2.26	230	

methionine and glutamic acid at 185°C. Phenylalanine is eluted at 190°C and serine at 220°C. Arginine, hydroxyproline and lysine are eluted at 230°C. 185°C is necessary for the resolution of threonine and methionine whereas the slow rise to 230°C is necessary for the partitioning of arginine and hydroxyproline.

The retention times and relative retentions of the M-TFA amino acid methyl esters are listed in Table 20 (a).

The top tracing in Figure 2 represents an alternate programming sometimes used on the 20' x $\frac{1}{8}$ " 12 Carbowax 20 M column. This programming is slower than the separation above and was used only when the column had aged for some time. For some reason the faster programming was not always reproducible on an old column. This could be due to a drop in liquid: solid ratio in the packing with repeated use.

The sample was injected with the column at 80°C. After 24 minutes the temperature was programmed to rise 5°/minute to 170°C and then programmed to rise $\frac{1}{2}$ °/minute to 195°C. After the elution of glutamic acid the temperature was programmed to rise 2°/minute to 240°C and held there for the remainder of the run.

The slow linear programming after 170°C was necessary for the resolution of threonine and methionine.

TABLE 20(b)

RELATIVE RETENTIONS OF N-TFA METHYL AMINO ACIDS ON A 20' x 1/8" 1% CARBOWAX 20 M COLUMN

Gas Flow = 80 ml/minute

Reference = Aspartic Acid
Relative Retention
of 1 @ 67 mins.

Temperature = 80°C (24 min.); 5°/min. to 170°C; 1/2°/min. to 195°C; 2°/min. to 240°C.

<u>AMINO ACID</u>	<u>Relative Retentions</u>	<u>Temperature (°C)</u>	<u>Resolution</u>
Cysteine	.40	120	
Valine	.61	168	
Alanine	.66	170	)
Isoleucine	.66	170	
Leucine	.72	172	
Glycine	.81	175	
Proline	.87	177	
Aspartic Acid	1.00	187	
Threonine	1.16	193	)
Methionine	1.21	194	
Glutamic Acid	1.26	195	
Phenylalanine	1.34	200	
Serine	1.44	210	
Arginine	1.91	240	)
Hydroxyproline	1.96	240	
Lysine	2.03	240	

it was programmed to rise 10°/minute to 260°C and held raised to 120°C, then held for 16 minutes, after which room temperature and after 6 minutes the temperature was acid mixture. The injection is made with the column at recommended for the rapid analysis of an unknown amino with a slower, temperature program. This method is The lower right hand tracing in Figure 2 is that separated. The retention values are listed in Table 21(a).

there until tyrosine, histidine and tryptophan were 5 minutes the temperature was raised to 270°C and held acids were injected at room temperature and after Figure 1, lower right hand corner, the amino

(b) 2' x 1/4" 12 Carbowax 20 M column

the faster programming than it is with the slow program. proline i.e. 1.34 as compared to 2.50 is better with 1.97 as compared to 1.76 and of arginine and hydroxy- The resolution of threonine and methionine i.e. acid and aspartic acid is decreased. are decreased i.e. the separation time between the amino 0.05. At higher temperatures the relative retentions from cysteine to serine are similar within values of values relative to aspartic acid for each amino acid and 190°C shows that with either program the retention Carbowax 20 M column, at temperatures between 90°C comparison of the two programs on the 20' x 1/4" 12 Alanine and isoleucine are not resolved. The retention data are listed in Table 20 (b).

RELATIVE RETENTIONS OF N-TFA METHYL AMINO ACIDS ON A 2' x 1/4" 1% CARBOWAX 20 M COLUMN

TABLE 21(a)

Gas Flow = 80 ml/minute

Reference = Aspartic Acid
Relative Retention
of 1 @ 9 min.

Temperature = room temperature (5 min.); 270°C

AMINO ACID	Relative Retentions	Temperature (°C)	Resolution
Aspartic Acid	1.00	270	
Tyrosine	2.00	270	1.88 1.20
Histidine	2.33	270	
Tryptophan	2.55	270	

TABLE 21(b)

Gas Flow = 80 ml/minute

Reference = Aspartic Acid
Relative Retention
of 1 @ 23 min.

Temperature = room temperature (6 min.); 120°C (16 min.); 10°/min. to 260°C

AMINO ACID	Relative Retentions	Temperature (°C)	Resolution
Cysteine	.43	120	
Valine	.57	120	
Alanine /	.61	120	
isoleucine			
Leucine	.65	120	
Glycine /	.78	120	
proline			
Aspartic Acid	1.00	150	
Threonine /	1.13	180	
Methionine			
Glutamic Acid /	1.18	190	
Phenylalanine			
Serine	1.26	220	
Arginine /	1.48	240	
Hydroxyproline			
Lysine	1.52	250	
Tyrosine	1.92	260	2.0 3.4
Histidine	2.10	260	
Tryptophan	2.44	260	

for the remainder of the run. The retention values are listed in Table 21 (b).

Although the resolution for tyrosine, histidine and tryptophan is better with the slower programming, the peaks are too broad and flat thus it is difficult to obtain a good quantitation of histidine. The rapid programming is recommended for quantitation of these three amino acids.

Note that in Table 21 (b) the relative retention values for cysteine to serine are within the range of those obtained on the 20' Carbowax 20 M column.

(c) 10' x 1/8" 1% Carbowax 1540 column

Figure 1, lower left hand corner is a tracing of a separation on this type of column. This column is used to resolve alanine and isoleucine. Cysteine, valine, leucine, glycine, proline, aspartic, threonine / methionine (not resolved), glutamic acid and phenylalanine are also separated. The injection is made with the column at room temperature and after 14 minutes the temperature is raised to 120°C and held there till isoleucine is eluted; this takes another 14 minutes. The temperature is then raised to 170°C and held there for the remainder of the run. The retention values are listed in Table 22 (a).

An alternate programming is shown in the left hand corner of Figure 2. The injection is made with

the column at room temperature and after 5 minutes the temperature is raised to 120°C. After the elution of isoleucine the temperature is programmed to rise 5°/minute to 170°C. The retention values are listed in Table 22 (b).

Alanine and isoleucine are resolved. The faster program gave lower retention times and increased the relative retentions i.e. compressed the separation to values closer to the reference aspartic acid. However the resolution of glycine and proline is slightly better with the slow program.

D Estimation of N-TFA Amino Acid Methyl Esters by Gas Chromatography

The quantitative estimation of N-TFA methyl amino acids is discussed on page 100. The peak areas of the curves obtained in Figures 1 and 2 were calculated. The peak areas of the curves were measured by means of mechanical integration.

A Disc integrator attachment was used for this purpose. The integrator operates a pen that moves in continuous back and forth motions at a speed proportional to the displacement of the pen from the base line. The integrated area is proportional to the number of strokes made by the pen. The tracing made by the integrator pen can be seen on the bottom of the recordings in Figures 1 and 2.

determination of minute quantities of amino acids since many proteins

The analysis had to be sufficiently sensitive for the

impossible if quantitative measurements were to be made.

resolved on a single column. However, by our methods this was

one in which the derivatives of all the amino acids could be

To simplify analysis, the ideal separation would be

acids was necessary.

meant that complete resolution of the derivatives of all the amino

Therefore accurate estimation of amino acids was essential, which

same time the method had to be quantitative as well as qualitative.

determination, speed of analysis was a prime requisite. At the

method was to compare favorably with other systems of amino acid

amino acid derivatives many factors had to be considered. If the

In arriving at the proposed method of separating the

2. Requirements for the separation of amino acid derivatives by Gas Chromatography

at separating amino acids in a chronological order.

The following sections will attempt to describe the attempts made

achieving these conditions consisted mainly of trial and error.

ditions found after such experimental work. The methods used in

esters (page 54 and Figures 1 and 2) represents the optimum con-

The technique proposed here to separate the amino

1. Introduction

METHYL ESTERS BY GAS-LIQUID CHROMATOGRAPHY

A DETAILED STUDY OF THE SEPARATION OF N-TVA AMINO ACID

TABLE 23

SEPARATION OF N-TFA METHYL AMINO ACIDS ON 1% NEOPENTYL GLYCOL SUCCINATE COLUMNS

5' x 1/8" 1% NPG Succinate

Gas Flow = 50 ml/minute
 Reference = Aspartic Acid
 Relative Retention
 of 1 @ 34 min.

10' x 1/8" 1% NPG Succinate

Gas Flow = 50 ml/minute
 Reference = Aspartic Acid
 Relative Retention
 of 1 @ 59 min.

AMINO ACID	Relative Retentions	Resolution	HETP	Relative Retentions	Resolution	HETP
Cysteine	.24			.37		
Alanine	.59		0.19	.54		.142
Valine	.71			.61		
Isoleucine	.79	)	0.36	.61	)	
Leucine	.82	)		.71	)	0.11
Glycine	.91	)	0	.79		
Proline	.91	)		.85		
Aspartic Acid	1.00			1.00		
Methionine	1.11		0.003	1.14	)	.042
Threonine	1.18			1.14	)	0
Serine	1.26			1.17	)	
Glutamic Acid	1.32	)	0	1.18	)	0.28
Phenylalanine	1.32	)		2.00	)	
Hydroxyproline	1.42	)	0.50	2.05	)	.082
Lysine	1.43	)			)	

are often in limited supply or may contain low concentrations of certain amino acids.

Certain of the amino acid derivatives appear to have similar melting points and vapor pressures. A suitable temperature programming had to be found that would separate these derivatives but at the same time would not hinder the resolution of less volatile amino acids. The amino acids that were difficult to separate appeared to fall into four main groups. Alanine and isoleucine had to be resolved at 120°C, threonine, methionine and glutamic acid are resolved only at 185°C-190°C, arginine, hydroxyproline and lysine are resolved at 230°C-270°C. The resolution temperatures of the amino acid groups are dependent upon the type of liquid phase used and the flow rate of the carrier gas.

3. Approach to the Problem

The first consideration was given to the choice of liquid phase. This had to fulfill the requirements listed above and also to be stable at the temperatures required for resolution of the least volatile amino acids. Since amino acids vary in their degree of polarity, a liquid phase should be selected that would selectively separate the amino acid derivatives on this basis without unduly retaining any of them. Indeed, it was possible on the basis of the studies of Johnson et al (1961) on the suitability of several polyethylene glycol derivatives to considerably narrow the choice of liquid phase.

Once a suitable column packing was obtained, empirical studies were made on the effect of column length and diameter, of carrier gas flow rate and of temperature programming on the resolution of the amino acid derivatives.

The following sections give an account of the approach used in separating the amino acid derivatives from the earliest attempts at separation to the method of separation finally adopted for use.

4. Column Packings

(a) Separation of Amino Acids on a 1% Neopentyl Glycol Succinate Column

A 1% neopentyl glycol succinate (NPG) on Chromosorb PAW (60-80 mesh) was the first resin tried. Zomlefer et al (1962) had first reported the separation of butyl N-TFA amino acid esters on a 9' x 1/8" 1% NPG succinate on 60-80 mesh gas Chromosorb P column and about the time our studies were almost complete Ikeshawa (1963) reported the separation of 13 of 19 N-TFA amino acid methyl derivatives on a 150 cm 1% NPG succinate column by temperature programming.

Many experiments were performed with the 1% NPG succinate on columns varying in length from 2 feet and 5 feet to 10 feet and 20 feet. Various carrier gas flows and temperature programs were tried. Table 23 represents the retention data from a 5' x 1/4" and a 10' x 1/4" 1% NPG columns. The separation on the 5' x 1/4" 1% NPG column was carried out with the column temperature at 60°C for the injection of the esters. After 12 minutes the temperature was programmed to rise 2°/minute to 250°C.

The amino acids on the 10' x $\frac{1}{4}$ " 1% NPG column were injected with the column temperature at 30°C and the temperature programmed to rise 3°/minute to 260°C.

Under none of the conditions employed could a satisfactory separation be obtained. On the 5' column isoleucine and leucine, glycine and proline, glutamic acid and phenylalanine were not resolved. An increase in column length did not improve the resolution of these amino acids. In fact in some cases with the temperature program adopted the relative positions of some of the amino acids changed. On the 10' column isoleucine came off with valine instead of with leucine.

Altering the conditions of gas flow, column length or temperature were found to change the relative order of elution of amino acid derivatives even on the same column. Depending on the conditions adopted alanine and valine, valine and isoleucine, glycine and proline, isoleucine and leucine changed their retention times and could not be resolved. This made it difficult to identify the peaks due to the amino acids and to obtain optimum conditions for separation.

The peaks were frequently wide and considerable tailing occurred. The tailing became more marked as the column length was increased. The column efficiency, expressed as HETP is given for alanine, methionine and hydroxyproline-lysine. The lower the HETP the more efficient the column. But as the column length was

TABLE 24

SEPARATION OF N-TFA METHYL AMINO ACIDS ON 8' x 1/4" 1% CARBOWAX 1540 COLUMNS

Column 1*

Gas Flow = 30 ml/minute
 Reference = Aspartic Acid
 Relative Retention
 of 1 @ 74 min.

Column 2*

Reference = Aspartic Acid
 Relative Retention
 of 1 @ 76 min.

<u>AMINO ACID</u>	<u>Relative Retention</u>	<u>Temp. (°C)</u>	<u>Res'n</u>	<u>HETP</u>	<u>Relative Retention</u>	<u>Temp. (°C)</u>	<u>Res'n</u>	<u>HETP</u>
Cysteine	.13	80			.06	80		
Valine	.32	100		0.04	.32	95		.10
Alanine	.38	110	)		.38	100	)	
Isoleucine	.42	110	)	2.50	.42	105	)	2.50
Leucine	.55	115			.55	115		
Glycine	.68	120			.67	120		
Proline	.73	120			.73	125		
Aspartic Acid	1.00	145			1.00	142		.009
Threonine	1.20	160	)		1.20	155	)	
Methionine	1.20	160	)	0	1.20	155	)	0
Glutamic Acid	1.24	160			1.25	160		
Phenylalanine	1.28	160			1.29	165		
Serine	----				1.41	175		
Hydroxyproline	----				1.97	200	)	1.88
Arginine	----				2.13	200	)	

* For Temperature Programs see Pages 64.

increased the HETP increased, which is contrary to the expected on increasing the length. Changes in gas flow or temperature programming had little effect on the resolution, the HETP or on the tailing.

(b) Separation of Amino Acid Esters on a 1% Carbowax 1540 Column

After having had little success with the 1% NPG succinate columns, 1% Carbowax 1540 on diatoport S (80-100 Mesh) was tried. Johnson et al (1961) had reported the separation of N-Acetyl amyl esters of 15 common amino acids (except tryptophan, histidine and arginine) with the combined use of an 8' 1% Carbowax 1540 and a 2' 0.5% Carbowax 1540 column.

Since N-TFA methyl esters should be even more volatile than N-acetyl amyl esters attempts were made at separating the esters with 1% Carbowax 1540 resin.

Numerous gas flow rates and temperature programs were tried on the Carbowax 1540 columns. This section describes the steps in the development of separation in terms of column length, gas flow and temperature programming. Table 24 gives the retention data for 8' x $\frac{1}{8}$ " 1% Carbowax columns.

The first columns tried were 8' x $\frac{1}{8}$ " 1% Carbowax 1540 on diatoport S (80-100 mesh), similar to the column employed by Johnson. After a few attempts at separation it was apparent that threonine and methionine would be difficult to separate. Slow gas flows (30 ml/min.) and a slow rate of programming were used. There was unfort-

unately a limitation on the maximum temperature at which the amino acid derivatives could be separated since Carbowax 1540 is only stable up to a temperature of 150°C-170°C. The separation was therefore limited to the derivatives of cysteine, valine, alanine, isoleucine, leucine, glycine, proline, aspartic, threonine / methionine, glutamic and phenylalanine in that order. All of the amino acids were resolved with the exception of threonine and methionine. A representative 8' 1% Carbowax 1540 separation can be seen in Table 24.

The derivatives were injected with the column temperature at 70°C and then programmed 1°/minute to a maximum of 160°C and held at this temperature for the remainder of the run.

Phenylalanine was the last amino acid to be eluted at 160°C after 95 minutes. Cysteine came off quickly and was often caught up in the solvent front. In an attempt to elute more amino acids from the column the programming was extended to 200°C (column 2 of Table 24) and at this temperature hydroxyproline and arginine were recovered. There was however severe bleeding of the column and the peaks above 175°C were very wide. The resolution for alanine and isoleucine was very good, the two amino acids that gave difficulty on the Carbowax 20 M resin.

In an attempt to separate threonine and methionine and to elute cysteine at higher temperatures the column length was increased to 10' x $\frac{1}{8}$ ". This column improved the

TABLE 25

SEPARATION OF N-TFA METHYL AMINO ACIDS ON 10' x 1/8" 1% CARBOWAX 1540 COLUMNS

Column 1*

Gas Flow = 30 ml/min.

Reference = Aspartic Acid

Rel. Ret'n 1 @ 76 min.

AMINO ACID	Relative Retention	Temp. (°C)	Res'n	HETP
Cysteine	.16	90		
Valine	.36	105		
Alanine	.39	110	) 1.66	
Isoleucine	.42	120		
Leucine	.55	130		
Glycine	.66	133		
Proline	.70	150		
Aspartic Acid	1.00	160		
Threonine	1.18	160	) 0	
Methionine	1.18	160		
Glutamic Acid	1.25	160	) 1.24	
Phenylalanine	1.30	160		

Column 2*

Gas Flow = 50 ml/min.

Reference = Aspartic Acid

Rel. Ret'n 1 @ 81 min.

Relative Retention	Temp. (°C)	Res'n	HETP
.38	80		
.58	110		.011
.63	120	) 1.66	
.66	125		
.74	135		
.82	145		
.85	150		
1.00	170		.0075
1.12	170	) 0	
1.12	170		
1.18	170	) 1.24	
1.20	170		

Column 3*

Gas Flow = 50 ml/min.

Reference = Aspartic Acid

Rel. Ret'n 1 @ 65 min.

AMINO ACID	Relative Retention	Temp. (°C)	Res'n	HETP
Cysteine	.43	100		
Valine	.66	120		.006
Alanine	.71	125	) 1.24	
Isoleucine	.72	130		
Leucine	.78	150		
Glycine	.85	160		
Proline	.89	165		
Aspartic Acid	1.00	170		
Threonine	1.12	170	) 0	.006
Methionine	1.12	170		
Glutamic Acid	1.17	170	) 1.24	
Phenylalanine	1.20	170		

Column 4*

Gas Flow = 50 ml/min.

Reference = Aspartic Acid

Rel. Ret'n 1 @ 50 min.

Relative Retention	Temp. (°C)	Res'n	HETP
.32	120		
.48	120		.020
.52	120	) 1.24	
.54	120		
.64	130		
.76	155		
.80	160		
1.00	170		.005
1.08	170	) 0	
1.08	170		
1.12	170	) 1.24	
1.16	170		

* For Temperature Programs See Pages 65-67.

cysteine resolution but there was no separation of threonine and methionine. Since the resolution of these amino acids is equal to zero, further attempts at separation were abandoned. Work with the column was then limited to improving the separation of alanine and isoleucine, in the shortest possible time. As can later be seen, alanine and isoleucine are very difficult to resolve on a Carbowax 20 M column and it is therefore more convenient to separate them on a 1% Carbowax 1540 column.

The retention data from experiments aimed at improving the separation are listed in Table 25. Programming similar to that used on an 8' column was tried. The amino acid derivatives were injected at 75°C and the temperature programmed to rise 1°/minute to 160°C, where it was held for the remainder of the run. The carrier gas flow was 30 ml/minute. Little improvement was made in the resolution of cysteine which still had a very low relative retention. The resolution of alanine and isoleucine was lower than on the 8' column (1.66 as compared to 2.50).

The amino acid derivatives were then injected with the column at room temperature and the temperature held for 3 minutes to allow partitioning of the esters. The temperature was programmed to rise 2°/minute to 170°C where it was held for the remainder of the run. The carrier gas flow was increased to 50 ml/minute. The relative

retention of cysteine had doubled from 0.16 to 0.38 and was completely separated from the initial solvent peak. The total analysis time was 97 minutes.

A similar program was used (column 3) but the temperature was raised to 170°C at $3^{\circ}/\text{minute}$. The relative retention of cysteine had increased to 0.43 and the analysis time was only 78 minutes. The resolution of alanine and isoleucine had decreased from 1.66 to 1.24 but was not critical since the column efficiency had increased from a HETP of .025 to .006 cm.

It was observed that the best resolution of alanine and isoleucine was at a temperature of 120°C . Therefore a method of programming was devised in which the derivatives were injected with the column at room temperature and after 8 minutes the temperature was increased to 120°C and held there until isoleucine came off, then the temperature was either programmed at $5^{\circ}/\text{minute}$ to 170°C or increased isothermally to 170°C . The relative retention of cysteine was not affected and the resolution of alanine and isoleucine was still 1.24 and the column efficiency (HETP) was decreased slightly to 0.020 cm. The most favorable factor was the analysis time which was only 58 minutes. Since this appeared to be the shortest analysis time for good separation on this column, this program was the one that was finally adopted.

A 20' x $\frac{1}{4}$ " 1% Carbowax 1540 column was also tried but did not separate threonine and methionine. Although the resolution of alanine and isoleucine was improved the analysis time was over 100 minutes. An increase in the rate of rise of temperature or in the rate of gas flow were not shown to improve the separation on the 10' x $\frac{1}{4}$ " column.

(c) Separation of the Amino Acid Derivatives
on a 1% Carbowax 20 M Column

Carbowax (polyethylene glycol) was shown to be an effective liquid phase in the separation of amino acids. The amino acids were cleanly separated with no tailing and the main advantage was that the relative retentions for each amino acid remained constant under all experimental conditions.

Since Carbowax 1540 has only a maximum temperature stability of 150°C-170°C it is limited in use. It was therefore decided to obtain a Carbowax phase stable at higher temperatures. Carbowax 20 M, stable up to^a temperature of 275°C was found suitable.

1% Carbowax 20 M on diatoport S (80-100 mesh), from the very beginning was shown to be an effective resin. A concerted effort was made with Carbowax 20 M to find conditions for the best possible separation of the amino acid derivatives. The next section will discuss experiments with 1% Carbowax 20 M columns under the sub-headings of column length, column width, temperature programming, carrier gas flow rate and ratio of liquid phase to solid support. The experimental

TABLE 26

8' x 1/8" 12 Carbowax 20 M Column (Gas Flow = 50 ml/min.)

COLUMN 1* COLUMN 2* COLUMN 3* COLUMN 4* COLUMN 5*

Aspartic= 1.00 @ 50 min. Asp= 1.00 @ 42 min. Asp= 1.00 @ 46 min. Asp= 1.00 @ 43 min. Asp= 1.00 @ 23 min.

AMINO ACID	Rel. Ret'n	(°C) Temp	Res'n	Rel. Ret'n	(°C) Temp	Res'n	Rel. Ret'n	(°C) Temp	Res'n	Rel. Ret'n	(°C) Temp	Res'n	Rel. Ret'n	(°C) Temp	Res'n
Cyst	.58	140		.24	90		.15	90		.18	90		.00	120	
Val	.66	140		.48	90		.35	90		.44	120		.35	120	
ala	.68	140)	0	.62	120)	0	.52	120)	0	.60	150)	0	.48	130)	0
Ileu	.68	140)		.62	120)		.52	120)		.60	150)		.48	130)	
Leuc	.72	140		.74	140		.61	144		.74	170		.61	140	
Gly	.82	140		.86	165		.78	150		.84	180		.78	160	
Pro	.88	140		.91	170		.83	180		.88	180		.83	170	
Asp	1.00	190		1.00	200		1.00	180		1.00	180		1.00	185	
Thre	1.08	220)	0.66	1.07	200)	1.22	1.10	190)	1.00	1.12	180)	1.20	1.25	190)	0.40
Meth	1.12	225)		1.10	200)	2.00	1.13	190)		1.14	180)	1.80	1.25	195)	
Glut	1.13	230)	0.50	1.12	200)		1.16	190		1.19	180)		1.28	210)	0.84
Phe	1.13	240		1.12	200		1.20	190		1.23	180		1.30	220	
Ser	1.16	250		1.19	200		1.26	195		1.32	195		1.39	240	
Arg	1.28	270)		1.47	260)	0.74	1.54	260)	1.66	1.65	240)	1.50	1.92	250)	2.00
Hyp	1.29	270)	0.50	1.48	265)		1.56	265)		1.68	240)		2.00	260)	
Lys	1.30	270)	0.66	1.52	270		1.58	270		1.74	240		2.18	270	
Tyr	1.56	270		1.86	270		1.83	270		2.05	270		2.75	270	
Hist	----	270		2.10	270		1.96	270		2.25	270		----	270	
Trypt	1.94	270		2.34			2.13	270		2.42	270		3.30	270	

* For Temperature Programs See Pages 68 - 71.

evidence presented includes only the highlights of many experiments which are described in chronological order.

1. Separation of Amino Acid Derivatives on a
8' x $\frac{1}{4}$ " 1 Carbowax 20 M Column

An 8' x $\frac{1}{4}$ " 1% Carbowax 20 M column was chosen arbitrarily because an 8' x $\frac{1}{4}$ " 1% Carbowax 1540 column had been successful.

From the first few experiments it became obvious that the resolution of alanine-isoleucine, arginine-hydroxyproline and threonine-methionine would be difficult. The proper temperature had to be found for resolving these amino acids and at the same time an attempt was made to separate all 19 amino acids on the one column. Temperature programming became a critical factor in reaching these objectives.

Initially the carrier gas flow was kept constant at 50 ml/minute in all the programs. These experiments are described below and the retention data listed in Table 26.

In the first run the amino acids were injected with the column at room temperature. After 24 minutes the temperature was raised to 140°C isothermally until proline was eluted and the temperature then programmed to rise 7.5°/min to 270°C. The long delay at room temperature was necessary to elute the solvent from the column. Alanine and isoleucine were not resolved at 140°C, threonine-methionine-glutamic acid

were poorly resolved at 220°C and the resolution of arginine-hydroxyproline was poor at 270°C. Tyrosine and tryptophan were eluted but histidine did not come off the column. The analysis time was 100 minutes.

In the second run the rate of rise of temperature was reduced. After the esters were injected at room temperature the oven temperature was immediately raised to 90°C and held there for 20 minutes until the solvent was eluted. The temperature was programmed to rise 5°/minute to 200°C and held there until phenylalanine was eluted then programmed to rise at 5°/minute to 270°C. The retention times were shorter and cysteine appeared after 10 minutes as part of the solvent peak. Alanine and isoleucine were not resolved at 120°C (the same temperature at which they were resolved on the Carbowax 1540 column). The separation of threonine and methionine was improved at 200°C (resolution of 1.22) while methionine and glutamic showed better separation (resolution of 2.00). Tyrosine, histidine and tryptophan were eluted at 270°C, but due to the long retention times permitting considerable longitudinal diffusion their peaks were wide. Histidine appeared to be partly retained on the column since its peak was much lower than before, attenuating at only $\times 40$ instead of the expected $\times 160$ for that particular concentration.

In the next run a slower temperature program was adopted. The amino acid derivatives were injected with the column at 90°C and after 20 minutes the temperature was programmed to rise 3°/minute to 190°C and held there until phenylalanine was eluted, after which the temperature was programmed to rise 5°/minute to 270°C. Cysteine was eluted too quickly (7 minutes), alanine and isoleucine were not resolved at 120°C. Threonine and methionine gave resolution of 1.00 at 200°C and the separation of arginine-hydroxyproline was improved at 260°C. The tyrosine, histidine and tryptophan peaks were too wide because of their long retention times.

Injection of the sample at 90°C gave sharper peaks, but cysteine was eluted too quickly on an 8' column. The temperatures were lowered to 180°C for threonine and methionine and to 240°C for arginine and hydroxyproline. The esters were injected at 90°C and after 20 minutes the temperature was again programmed to rise 5°/minute to 240°C. After lysine was eluted the temperature was raised isothermally to 270°C. (column 4).

Alanine and isoleucine were not resolved. Threonine were partially resolved at 180°C while 240°C improved the resolution of arginine-hydroxyproline. All amino acids were eluted with an analysis time of 104 minutes.

An attempt was made to sharpen up the peaks giving better resolutions by raising the temperature more rapidly. (column 5 of Table 26).

The amino acid derivatives were injected at 120°C and after 6 minutes the temperature was programmed to rise 5°/minute to 220°C then raised isothermally to 270°C. This program was too fast. Threonine and methionine, although resolved previously at 185°C, were not resolved in this run. Arginine, and hydroxyproline were resolved at 240°C. Histidine was not eluted.

From these experiments it was deduced that an initial temperature of 80 - 90°C with a delay to allow for elution of the solvent and for partitioning of the amino acids at a low temperature was apparently necessary for maximum resolution. Alanine and isoleucine could only be resolved by increasing the efficiency of the column by increased column length.

Maximum resolutions of 1.20 for threonine-methionine at 180-190°C and 2.00 for arginine-hydroxyproline at 240°C were obtained and could not be improved with this column length. In order to improve the resolutions the column length was increased.

Similar results were obtained on a 10' x 1/4" 1% Carbowax 20 M column.

TABLE 27

12' x $\frac{1}{8}$ " 1% Carbowax 20 M Column

(Gas Flow = 50 ml/min.)

Aspartic Acid = 1.00 @ 44 mins.
 Temperature = room temp; 90°C; 5°/min to 200°C;
 5°/min to 250°C

Aspartic Acid = 1.00 @ 28 mins.
 Temperature = 90°C; 7.5°/min.
 to 220°C; 270°C

AMINO ACID	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
Cysteine	.50	90		.36	100	
Valine	.68	135		.57	160	
Alanine	.73	150	) 0.66	.69	170	) 0.50
Isoleucine	.74	170	)	.71	170	)
Leucine	.82	180		.79	180	
Glycine	.89	190		.86	190	
Proline	.93	200		.93	200	
Aspartic	1.00	200		1.00	220	
Threonine	1.11	200	) 0.50	1.08	220	) 0.66
Methionine	1.12	200	)	1.10	220	) 0.66
Glutamic Acid	1.14	200	) 1.10	1.12	220	)
Phenylalanine	1.18	200		1.16	220	
Serine	1.23	210		1.22	220	
Arginine	1.55	250	) 1.10	1.78	220	
Hydroxyproline	1.57	250	) 1.34	1.86	220	
Lysine	1.62	250		2.07	220	
Tyrosine	1.86	250		2.54	270	
Histidine	2.00	250		2.68	270	) Peak areas are
Tryptophan	2.18	250		3.35	270	) very low

2. Separation of Amino Acid Derivatives on a 12' x $\frac{1}{8}$ " 12 Carbowax 20 M Column

By increasing the column length, attempts were still made to separate all the amino acids on a single column.

The amino acids were injected at room temperature and after 8 minutes the temperature was raised to 90°C isothermally and held for another 12 minutes. The temperature was then programmed to rise 5°/minute to 250°C. (see Table 27)

For the first time alanine and isoleucine showed signs of becoming separated. At 150°C there was a split between alanine and isoleucine with a resolution of 0.66. This was promising. However the separation of threonine and methionine was very poor at 200°C and arginine-hydroxyproline resolution was poor at 250°C. Peaks for all the amino acids were obtained. Since the threonine and methionine peaks were wider than on the 8' column it was thought that higher temperatures might improve the resolution by sharpening the peaks.

The amino acids were injected at 90°C and after 8 minutes the temperature programmed to rise 7.5°/minute to 220°C. After the elution of lysine the temperature was raised isothermally to 270°C. (see Table 27).

Alanine and isoleucine were still split at 170°C

but not resolved. Threonine and methionine were not resolved at 220°C even with the sharper peaks. However separation of arginine and hydroxyproline was improved at 220°C. This indicated that threonine and methionine were only resolved at 180-190°C and this resolution was not dependent on the sharpness of the peaks. The derivative of all of the amino acids were eluted but the peaks due to histidine was still low indicating retention on the column.

Summary

From chromatography on the 8 and 12 foot 1% Carbowax 20 M columns several important observations were made. For resolution of alanine-isoleucine a very long column would be required to give an increase in the number of theoretical plates for the partitioning of these amino acids. The temperature of elution of these amino acids is between 140-160°C. Resolution of threonine and methionine requires temperatures between 180-190°C. On the 8' columns only resolutions of 1.20 were achieved. Therefore in order to obtain ideal resolutions of 2.0 or more an increased column length would be required. At temperatures of 230-240°C arginine and hydroxyproline gave resolutions of 1.50-2.00. Increase in column length should also improve the separation of these amino acids.

If ideal conditions for the above amino acids were adhered to it became apparent that separation of all 19 amino acids on a single column would be impossible since these conditions would require increased retention times resulting in diffusion of the less volatile tyrosine, histidine and tryptophan derivatives. Therefore attempts to separate these amino acids on a single column were abandoned and work was concentrated on obtaining a good separation of the first 16 amino acids on one column.

3. Separation of Amino Acid Derivatives on a
20' x $\frac{1}{8}$ " 1% Carbowax 20 M Column

Once a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column was shown to give a separation of most of the amino acids the majority of our chromatographic work was done on this column.

When the length of the column was doubled, it was necessary with the same temperature program to increase considerably the carrier gas flow (from 40 ml/minute to 80 ml/minute) so that the acids would pass through the column within the same temperature range. Moreover since the number of theoretical plates had doubled, twice the gas flow rate is needed to obtain the required efficiency with sharp peaks and very little diffusion. All of the experiments with this column were carried out with a gas flow of 80 ml/minute.

In the succeeding experiments much time was spent in finding the best temperature program to obtain complete resolution of alanine and isoleucine, threonine and methionine and arginine and hydroxyproline. It was assumed that the separation of tyrosine, histidine and tryptophan would be best achieved with shorter columns.

From the experiments discussed previously it seemed clear that in order to obtain complete resolution of the foregoing pair of amino acid derivatives the temperature programming would have to be arranged to elute alanine and isoleucine at 140-150°C, threonine and methionine at 180-190°C and arginine and hydroxyproline at 230-240°C.

(1) Continuous Temperature Programming

In these experiments a continuous rise in temperature was used from the injection temperature to the final column temperature. It was found that the amino acid derivatives had to be injected with the column temperature above ambient.

A column temperature for injection was found which would permit partition of the amino acid derivatives without eluting those of low volatility.

These experiments established the necessity for partitioning the amino acids at a low tem-

TABLE 28

20' x 1/4" 1% Carbowax 20 H Column

(Gas flow = 80 ml/minute)

Continuous Temperature Programming

AMINO ACID	Column 1.*			Column 2.*			Column 3.*			Column 4.*		
	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
Cysteine	.37	100		.60	180		.36	115		.41	125	
Valine	.54	125		.66	180		.54	150		.54	140	
Alanine	.60	135	) 0	.70	180	) 0	.60	150	) 0	.61	148	) 0
Isovaline	.60	135		.70	180		.60	155		.61	148	
Isoleucine	.69	150		.74	180		.68	160		.68	160	
Glycine	.82	165		.82	180		.80	170		.81	178	
Proline	.88	180		.88	180		.88	180		.88	186	
Aspartic	1.00	190		1.00	180		1.00	195		1.00	195	
Threonine	1.12	210	) 1.50	1.20	180	) 2.14	1.13	205	) 1.50	1.12	210	) 1.66
Methionine	1.14	212		1.26	180		1.16	208		1.16	215	
Glutamic	1.16	220	) 1.50	1.32	180	) 2.30	1.19	210		1.17	218	) 1.00
Phenylalanine	1.21	240		1.44	205		1.24	215		1.22	225	
Serine	1.25	250		1.52	230		1.30	220		1.27	230	
Arginine	1.66	250	) 1.50	2.02	240	) 1.88	1.80	235	) 1.00	1.68	240	) 1.88
Hydroxyproline	1.69	250		2.08	240		1.84	235		1.73	240	
Lysine	1.71	250		2.14	240		1.92	235		1.80	240	

* For Temperature Programs See Pages 76 - 77.

perature for a determined period of time.

In column 1 of Table 28 are shown the results of an experiment in which the amino acid derivatives were injected with the column at 60°C and the temperature immediately programmed to rise 2°/minute to 250°C. With this programming threonine and methionine were eluted at 210°C with a resolution of 1.50 and arginine-hydroxyproline were eluted at 250°C but were poorly resolved. These temperatures were in fact too high for resolution. Alanine and isoleucine were not resolved at 135°C.

In the experiment shown in column 2 of Table 28 the temperature was held at 60°C for 24 minutes after injecting to see if the amino acids were partitioned at the initial temperature. After 24 minutes the temperature was programmed to rise 5°/minute to 240°C.

Threonine and methionine were separated at 180°C with a resolution of 2.14 while arginine and hydroxyproline were resolved at 250°C with a resolution of 1.88. Alanine and isoleucine were not resolved at 180°C. The increased resolutions were due to either the lower elution temperatures or the long delay at the initial temperature for partitioning of the amino acids.

partitioning of the derivatives at the initial column temperature.

The effects of partitioning at a higher initial temperature (70°C) were then tried. In the experiment shown in column 3 the derivatives were injected at 70°C and the temperature immediately programmed to rise 3°/minute to 150°C until isoleucine was eluted and it was then programmed at 2°/minute to 240°C. A fair separation of threonine and methionine was obtained at 195°C with a resolution of 1.50. However arginine and hydroxyproline were poorly separated at 240°C, a temperature that usually gave a very good resolution. This poor resolution could be due to the fact that the esters were not partitioned at the initial column temperature.

In the experiment shown in column 4 the amino acid derivatives were again injected at 70°C and the temperature maintained for 16 minutes. The temperature was then increased isothermally to 120°C and then programmed to rise 2°/minute to 240°C. The separation of threonine and methionine was improved at 210°C but the temperature was still too high for their complete resolution. A very good resolution was obtained for arginine and hydroxyproline at 240°C. This resolution therefore must have been due to the partitioning of the derivatives at the initial column temperature.

TABLE 29(a)

20' x $\frac{1}{8}$ " 1% Carbowax 20 M Column

(Gas Flow = 80 ml/minute)

Discontinuous Temperature Programming

AMINO ACID	Column 1.*			Column 2.*			Column 3.*		
	Asp = 1.00 @ 51 min.			Asp = 1.00 @ 48 min.			Asp = 1.00 @ 67 min.		
	Ret'n	Temp (°C)	Res'n	Ret'n	Temp (°C)	Res'n	Ret'n	Temp (°C)	Res'n
Cysteine	.41	120		.44	100		.33	100	
Valine	.57	150		.64	150		.54	155	
Alanine	.61	170	) 0	.71	160	) 0	.58	160	) 1.00
Isoleucine	.61	170		.71	180		.59	160	
Leucine	.71	180		.77	195		.66	165	
Glycine	.81	185		.86	195		.76	170	
Proline	.86	186		.92	195		.84	173	
Aspartic	1.00	185		1.00	195		1.00	180	
Threonine	1.20	185	) 1.68	1.13	195	) 2.50	1.20	188	) 1.66
Methionine	1.24	183		1.16	195		1.24	190	
Glutamic	1.30	185	) 1.68	1.21	195	) 2.50	1.28	195	
Phenylalanine	1.38	200		1.27	195		1.38	210	
Serine	1.43	215		1.38	205		1.46	215	
Arginine	1.96	235	) 2.50	1.90	245	) 2.20	1.94	240	) 0.80
Hydroxyproline	2.08	235		1.94	245		1.97	240	
Lysine	2.12	235		2.00	245		2.08	240	

* For temperature program see pages 79 - 84.

Table 29 gives the retention values for the chromatographic separations described below for discontinuous temperature programming.

All chromatography was performed with a carrier gas flow of 80 ml/min.

The chromatographic separation shown in Column 1 of Table 29 was obtained by injecting at 80°C. After a partition period of 10 minutes the temperature was programmed to rise 40°/minute to 185°C and held at this temperature until glutamic acid was eluted. The temperature was then programmed to rise 30°/minute to 235°C.

A fair separation was obtained for threonine and methionine at 185°C while the separation of arginine and hydroxyproline was very good at 235°C. Alanine and isoleucine were not separated at 165°C.

The main problem with the above program was that the peaks were wide. A similar program was tried with a longer partition period and a faster temperature rise to 195°C.

(Column 2 of Table 29) The amino acids derivatives were injected at 80°C and after 16 minutes the temperature was programmed to rise at 5°/minute to 195°C, held there until

phenylalanine was eluted and then programmed at 3° /minute to 245°C .

The retention times were shorter. Threonine and methionine were separated at 195°C with a resolution of 2.50. Arginine and hydroxyproline were also well separated at 245°C . The analysis time was 96 minutes. However there was no sign of even a split between alanine and isoleucine at 160°C . Since even a split would be helpful for the qualitative identification of these amino acids in a protein of unknown composition it was decided to attempt to obtain this by further modifying temperature programming.

In the first attempt at splitting alanine and isoleucine (column 3 of Table 29 (a)) it was decided to delay temperature programming at either 150°C or 160°C to prevent the overlapping of alanine and isoleucine on elution. The amino acids were injected at 80°C . After 18 minutes the temperature was programmed to rise 5° /minute to 160°C and the programming set for a rise of $\frac{1}{2}^{\circ}$ /minute to 190°C or until methionine was eluted. The temperature was then programmed to rise 2° /minute to 240°C .

Alanine and isoleucine were now split at 160°C with a resolution of only 1.00. This split seemed to be due to either the delay in temperature rise or the slow rise in temper-

TABLE 29 (b)

20' x 1/4" 1% Carbowax 20 M Column

(Gas Flow = 80 ml/min.)

Discontinuous Temperature Programming

AMINO ACID	Column 4.* Asp = 1.00 @ 62 min.			Column 5.* Asp = 1.00 @ 67 min.			Column 6.* Asp = 1.00 @ 53 min.		
	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
Cysteine	.39	100		.40	120		.42	90	
Valine	.58	170		.61	168		.57	130	
Alanine	.61	172)	1.30	.66	170)	0.38	.63	140)	0.38
Isoleucine	.62	172)		.66	170)		.64	140)	
Leucine	.68	175		.72	172		.70	170	
Glycine	.77	178		.81	175		.74	185	
Proline	.84	180		.87	177		.87	185	
Aspartic	1.00	185		1.00	187		1.00	185	
Threonine	1.24	188)	1.30	1.16	193)	1.76	1.19	185)	1.97
Methionine	1.29	190)		1.21	194)		1.25	185)	
Glutamic	1.36	200		1.26	195		1.30	185	
Phenylalanine	1.44	210		1.34	200		1.40	190	
Serine	1.55	220		1.44	210		1.51	220	
Arginine	2.05	240)	1.30	1.91	240)	1.34	2.05	230)	2.50
Hydroxyproline	2.10	240)		1.96	240)		2.15	230)	
Lysine	2.20	240		2.03	240		2.26	230	

* For Temperature programs see pages 79 - 84.

ature; the threonine and methionine peaks were wider giving a lower resolution of only 1.66. The separation of arginine and hydroxyproline was poor at 240°C due to some diffusion on the column.

It was observed that good separations of arginine and hydroxyproline were obtained if the programming after reaching 180-195°C was delayed until phenylalanine was eluted. This indicated that another partitioning occurred at these temperatures.

The next program (column 4) was similar except that the initial temperature and partition time were increased to see if this would improve the separation of threonine-methionine and arginine-hydroxyproline while at the same time trying to obtain a better split of alanine and isoleucine at 170°C.

The amino acid derivatives were injected with the column temperature at 90°C. Partitioning was allowed to proceed for 22 minutes and then the temperature programmed to rise 7.5°/minute to 170°C and then programmed ½°/minute to 185°C and immediately programmed at 2°/minute to 240°C.

Alanine and isoleucine were split at 170°C with a resolution of 1.50. Threonine-

TABLE 30

20' x 1/4" 1% Carbowax 20 M Column

Effects of Carrier Gas Flow Rate

<u>Column 1.*</u>	<u>Column 2.*</u>	<u>Column 3.*</u>
Gas Flow = 10 ml/min.	Gas Flow = 40 ml/min.	Gas Flow = 40 ml/min.
Asp = 1.00 @ 80 min.	Asp = 1.00 @ 62 min.	Asp = 1.00 @ 66 min.

AMINO ACID	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
Cysteine	.50	180		.45	120		.44	110	
Valine	.58	182		.68	160		.62	165	
Alanine	.59	185)	0.75	.73	180)	0.50	.66	170)	0.50
Isoleucine	.60	185)		.74	180)		.67	170)	
Leucine	.65	187		.79	190		.73	173	
Glycine	.75	192		.85	195		.80	176	
Proline	.85	195		.90	195		.88	181	
Aspartic	1.00	200		1.00	195		1.00	185	
Threonine	1.20	206)	1.66	1.14	195)	1.24	1.26	189)	1.12
Methionine	1.25	208)		1.18	195)		1.29	191)	
Glutamic	1.30	210		1.21	195		1.38	193	
Phenylalanine	1.42	215		1.29	205		1.44	195	
Serine	1.52	220		1.38	220		1.55	210	
Arginine	2.28	245)	1.50	1.86	240)	1.24	2.05	240)	1.50
Hydroxyproline	2.36	248)		1.90	240)		2.10	240)	
Lysine	2.48	253		1.98	240		2.18	240	

* For temperature programs see pages 85-86.

methionine resolution had dropped to 1.50.

It became apparent that although the slow temperature rise was satisfactory for these amino acids it was necessary to maintain a steady temperature at 180-190°C resulting in a steadier base line and less overlapping of the peaks. Arginine and hydroxyproline were better separated at 240°C but they still required a temperature delay at 185°C for partitioning.

In the experiments shown in column 5 the amino acid derivatives were chromatographed with a long partition period, the temperature was held at 195°C to separate threonine-methionine cleanly and to partition arginine and hydroxyproline.

The injection temperature was 80°C and after a 16 minute delay the temperature was programmed to rise 5°/minute to 170°C and then programmed at ½°/minute to 195°C where it was held until glutamic acid was eluted. The temperature was then programmed to rise 2°/minute to 240°C.

Alanine and isoleucine were split at 170°C and threonine and methionine were separated with a resolution of 1.76 at 195°C. Arginine and hydroxyproline were separated at 240°C although not as well as was expected.

In the experiment shown in column 6, after a partition period, continuous temperature programming to 185°C with a longer delay at this temperature was tried. The amino acid derivatives were injected with the column temperature of 80°C and after a 20 minute delay the temperature was programmed to rise 5°/min. to 185°C and held there until glutamic acid was eluted. The temperature was then programmed at 2°/minute to 230°C.

Alanine and isoleucine were split at 140°C. The delay at 185°C improved the separation of threonine and methionine and greatly improved the separation of arginine and hydroxyproline.

Chromatography with other types of programs failed to improve the separations. Therefore the procedure of the latter two experiments (columns 5 and 6 of Table 29 (b) Figures 1 and 2 page 54) served as the basis for the procedure finally adopted.

The average analysis time on this column was 2 hours.

Conclusion

All of the amino acid N-trifluoroacetyl methyl esters require a partition period after injection onto the column at temperatures below that of the most volatile amino acids. Arginine, hydroxyproline and lysine require a

further partitioning at 180°-190°C for maximum resolution.

Alanine and isoleucine can be split at temperatures between 140°C-170°C if the temperature is allowed to rise very slowly over this temperature range. Threonine and methionine are best resolved between 180-190°C, with a steady column temperature.

With Carbowax 20 M as the liquid phase under constant conditions of column length and gas flow and when the elution temperatures for each amino acid derivatives is within the required range for elution, the relative retention values for the amino acids are consistent over all program conditions deviating only by ± 0.05 from the mean relative retention value. The relative retention values are therefore a specific characteristic of each amino acid derivative on the column.

5. The Effects of Carrier Gas Flow on the Separation of Amino Acid
N-Trifluoroacetyl Methyl Esters on a 20' x $\frac{1}{8}$ " 1% Carbowax
20 M Column

With a temperature program similar to that employed on the 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column with a carrier gas flow of 80 ml/minute, a study was made of the effects of changes in the rate of carrier gas flow on the separation of amino acid derivatives.

Chromatography was carried out at a very low gas flow of 10 ml/minute. The amino acid derivatives were injected with the column temperature at 90°C and after a 28 minute delay the temperature was increased to 180°C and programmed to rise $\frac{1}{2}$ °/minute to 250°C. The retention values are listed in column 1 of Table 30.

The 28 minute delay after injection was necessary for complete elution of the solvent with this low gas flow. Alanine and isoleucine were split at 180°C. Threonine and methionine were separated at 200°C and arginine and hydroxyproline at 245°C.

The low gas flow did not affect the efficiency of the column. The peaks were still sharp with no diffusion being noticeable. The analysis time was 3½ hours instead of 2 hours for a flow rate of 80 ml/minute.

Chromatography was then carried out at a gas flow rate of 40 ml/minute. The amino acid derivatives were injected at 90°C and after a 26 minute delay the temperature was programmed to rise 5°/minute to 195°C where it was held until glutamic

acid was eluted and then programmed to rise 2° /minute to 240°C . (Column 2.)

The solvent was slow in eluting off of the column at this gas flow. Alanine and isoleucine were split at 180°C but there was no improvement in their resolution. At 195°C the resolution of threonine and methionine was poor (1.24).

Arginine and hydroxyproline were poorly resolved at 240°C .

The decreased carrier gas flow rate did not affect the analysis time, which was still 2 hours. The poor resolutions were due to diffusion resulting in broader peaks.

The same carrier gas flow (40 ml/minute) was tried again, this time programming the temperature at $\frac{1}{2}^{\circ}$ /minute from 170°C . The injection temperature was 90°C and after 24 minutes the temperature was programmed to rise 5° /minute to 170°C and then programmed at $\frac{1}{2}^{\circ}$ /minute to 195°C until phenylalanine was eluted. The temperature was then programmed at 2° /minute to 240°C . (Column 3).

Alanine and isoleucine were split at 170°C but still were not as well separated as would be expected. The resolution of threonine and methionine was poor at 190°C . Arginine and hydroxyproline were better resolved at 240°C but their peaks were very broad.

Conclusion

The low carrier gas flow rates gave unduly broad amino acid peaks. The better resolutions and sharper peaks at

a flow rate of 10 ml/minute is attributed to the increased elution temperatures. Thus to attain the same resolution at a low gas flow rate higher elution temperatures are needed. Since no gain in column efficiency was achieved by varying the gas flow rate, attempts were made to increase the efficiency of the column by increasing its column length.

6. The Effects of Increasing the Length of 1% Carbowax 20 M Columns on the Separation of Amino Acid Tri-fluoro-acetyl Methyl Esters

Having obtained good separations of threonine-methionine and arginine-hydroxyproline on a 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column attempts were made to resolve alanine and isoleucine by increasing the column length, i.e. to increase the column efficiency by increasing the number of theoretical plates.

(a) Separation of Amino Acid Derivatives on a 30' x $\frac{1}{8}$ " 1% Carbowax 20 M Column

The amino acids were injected with the column temperature of 90°C and after 28 minutes the temperature was programmed to rise 5°/minute to 130°C, then 1°/minute to 200°C. After the elution of phenylalanine the temperature was programmed to rise 2°/minute to 230°C.

Alanine and isoleucine were better resolved at 150°C than they were on the 20' column (a resolution of 1.00 as compared to 0.38). The resolution of threonine and methionine was improved, a resolution of 2.00. Arginine and hydroxyproline were poorly resolved. This was due to the prolonged retention time causing longi-

TABLE 31

EFFECTS OF INCREASED COLUMN LENGTH

AMINO ACID	Column 1.* 30' x 1/4" 1% Carbowax 20 M Asp= 1.00 @ 100 min.			Column 2.* Asp= 1.00 @ 94 min.			Column 3.* 40' x 1/4" 1% Carbowax 20 M Column Asp= 1.00 @ 68 min.			Column 4.* Asp= 1.00 @ 68 min.		
	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
Cysteine	.35	130		.36	130		.32	140		.37	140	
Valine	.51	145		.52	150		.44	140		.50	160	
Alanine	.57	150)	1.00	.59	156)	1.50	.50	140)	1.20	.58	160)	1.00
Isoleucine	.58	150)		.60	156)		.52	140)		.60	160)	
Leucine	.66	160		.67	164		.65	170		.69	200	
Glycine	.78	172		.79	172		.80	200		.82	200	
Proline	.86	180		.89	180		.88	200		.91	200	
Aspartic	1.00	194		1.00	192		1.00	200		1.00	200	
Threonine	1.14	200)	2.00	1.14	200)	1.88	1.13	200)	2.20	1.12	200)	3.00
Methionine	1.18	200)		1.17	200)		1.18	200)		1.16	200)	
Glutamic	1.21	200		1.20	200		1.21	200		1.19	200	
Phenylalanine	1.28	200		1.28	200		1.31	200		1.28	240	
Serine	1.36	210		1.34	210		1.43	220		1.34	240	
Arginine	1.94	230)	1.14	1.81	240)	1.00	2.05	250)	0.84			
Hydroxyproline	1.98	230)		1.84	240)		2.08	250)				
Lysine	2.11	230		1.90	240							

* For temperature programs see pages 87 - 89.

tudinal diffusion of their derivatives on the column.

(See Table 31, column 1).

(b) Separation of Amino Acid Derivatives on a 40' x $\frac{1}{8}$ "
1% Carbowax 20 M Column

The column length was again increased to improve the resolution of alanine and isoleucine.

The amino acid esters were injected at 80°C and after a 28 minute delay, for elution of the solvent front, the temperature was programmed to rise 5°/minute to 140°C, then 1°/minute to 200°C. After the elution of phenylalanine the temperature was programmed to rise 2°/minute to 240°C. A good resolution (1.50) for alanine and isoleucine was obtained at 155°C. Threonine and methionine were separated with a resolution of 1.88 at 200°C. The arginine and hydroxyproline peaks were still broad and had a resolution of only 1.00. (See Table 31, column 2).

A lower temperature was then tried for the separation of alanine and isoleucine. The amino acids were injected with the column at room temperature. The column temperature was immediately raised to 140°C and held for 36 minutes until the isoleucine peak had appeared. The temperature was then programmed to rise 5°/minute to 200°C and held there until phenylalanine was eluted and then programmed again at 5°/minute to 250°C.

The separation of alanine and isoleucine was not improved at 140°C. Threonine and methionine peaks were sharper with the increased rate of temperature^{rise} and their

resolution was very good (2.20). Arginine and hydroxyproline were very diffuse giving wide, flat peaks. Their resolution was only 0.84.

A higher column temperature for injecting the amino acids was then tried. The injection temperature was 30°C and the column was held at this temperature for 22 minutes, after which the temperature was raised to 160°C where it was held until isoleucine was eluted. The temperature was then raised isothermally to 200°C and held until serine was eluted. The separation of arginine, hydroxyproline and lysine was not attempted on this column.

The separation of alanine and isoleucine was not as good at 160°C (resolution of 1.00) without partitioning at a low temperature. The separation of threonine and methionine was very good giving the highest resolution value yet obtained --- a resolution of 3.00.

Conclusion

Increasing the column length resulted in better resolution of the low boiling point amino acid derivatives. However, the amino acid derivatives with high boiling points diffused on the column to give wide peaks. Alanine and isoleucine, as well as threonine and methionine were completely separated. Arginine and hydroxyproline peaks were wide and diffuse.

TABLE 32

Effects of Column Width

AMINO ACID	Column 1*			Column 2*			Column 3*			Column 4*		
	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
	20' x 1/8" 1% Carbowax 20 M			20' x 3/16" 1% Carbowax 20 M			10' x 1/8" 1% Carbowax 20 M			12' x 3/16" 1% Carbowax 20 M		
	Asp= 1.00 @ 84 min.			Asp= 1.00 @ 47 min.			Asp= 1.00 @ 22 min.			Asp= 1.00 @ 38 min.		
Cysteine	.52	120		.45	120		.27	125		.32	90	
Valine	.65	140		.62	160		.41	135		.55	170	
Alanine	.67	143)	0.50	.68	170		.50	140)	0	.61	175	
Isoleucine	.67	143)		.69	170		.50	140)		.66	175	
Leucine	.73	148		.75	180		.59	150		.71	180	
Glycine	.81	132		.83	180		.73	160		.76	185	
Proline	.85	158		.87	180		.82	165		.87	185	
Aspartic	1.00	170		1.00	180		1.00	180		1.00	185	
Threonine	1.13	180)	1.00	1.15	180		1.14	190)	0.66	1.02	185	
Methionine	1.16	182)		1.19	180		1.16	190)		1.08	185	
Glutamic	1.19	185		1.26	180		1.23	190)	1.20	1.18	185	
Phenylalanine	1.24	188		1.34	200		1.28	195)		1.26	185	
Serine	1.31	195		1.40	210		1.32	205		1.82	185	
Arginine	1.84	215)	1.00	1.74	260		2.05	220)	0.50	1.85	240	
Hydroxyproline	1.88	218)		1.77	260		2.10	220)		1.90	240	
Lysine	1.94	220		1.79	260		2.23	225		2.20	240	
Tyrosine							3.20	250		2.42	270	
Histidine										2.63	270	
Tryptophan										2.80	270	

*

For temperature programs see pages 90-92.

7. The Effects of Column Width on the Separation of Amino Acid Derivatives

Since narrower columns have been reported to be successful in other types of chromatographic work, it was decided to try them for the separation of amino acid N-trifluoroacetyl methyl esters.

(a) Separation of Amino Acid N-TFA Methyl Esters on a 20' x 1/8" 1% Carbowax 20 M Column

The amino acid derivatives were injected with the column at 30°C and after a 12 minute delay the temperature was programmed to rise 3°/minute to 140°C then 1°/minute to 250°C. (Column 1, Table 32).

The carrier gas flow rate was 40 ml/minute at 40 psig at the beginning of the run. At 140°C the gas flow dropped to 10 ml/minute. In order to maintain a constant gas flow

the gas pressure had to be adjusted to 60 psig with the rotameter on the flow regulator wide open. The gas flow for the remainder of the run was impossible to control.

This difficulty was attributed to the high resistance of the resin on the 1/8" column to gas flow. As a result of the resistance of the column and the fluctuating gas flow by periodic adjustments throughout the chromatography, all of the amino acid peaks were wide and the resolution

of alanine-isoleucine, threonine-methionine and arginine-hydroxyproline was poor.

(b) Separation of Amino Acid N-TFA Methyl Esters on a 20' x 3/16" 1% Carbowax 20 M Column

The column width was increased slightly by using copper tubing with 3/16 inch inside diameter.

TABLE 33

Effects of Varying Ratio of Liquid Phase to Solid Support

AMINO ACID	Column 1.* 20' x 3/16" 2% Carbowax 20 M Asp= 1.00 @ 75 min.			Column 2.* 10' x 3/16" 2% Carbowax 20 M Asp= 1.00 @ 48 min.			Column 3.* 5' x 1/8" 10% Carbowax 20 M Asp= 1.00 @ 28 min.		
	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n	Rel. Ret'n	Temp (°C)	Res'n
Cysteine	.39	180		.42	100		.72	160	
Valine	.51	190		.63	150		.79	190	
Alanine	.54	190)	0.50	.65	153		.82	200	
Isoleucine	.55	190)		.66	153		.82	200	
Leucine	.61	190)		.71	158		.86	210	
Glycine	.72	190		.79	163		.93	230	
Proline	.83	190		.85	165		.97	250	
Aspartic	1.00	190		1.00	170		1.00	250	
Threonine	----	---		1.16	180		1.06	250	
Methionine	1.27	190)	1.16	1.21	185		1.07	250	
Glutamic	1.31	190)		1.23	190		1.07	250	
Phenylalanine	1.42	190)		1.29	195		1.13	250	
Serine	----	---		1.36	200		1.14	250	
Arginine	2.06	240)	0.54	1.67	240		----	---	
Hydroxyproline	2.10	240)		1.68	240		1.64	250	
Lysine	2.20	240)		1.73	240		----	---	

*For temperature programs see pages 93 - 94.

The amino acid derivatives were injected at 90°C and after 14 minutes the temperature was programmed to rise 5°/minute to 180°C and held until glutamic acid was eluted. The temperature was then programmed at 5°/minute to 260°C. (Column 2, Table 32).

The gas flow of 50 ml/minute was maintained throughout the run and no resistance to gas flow was encountered. The resolution of alanine-isoleucine was poor as well as the resolution of arginine and hydroxyproline. Threonine and methionine were separated but not resolved completely.

There seemed little to be gained by using this column rather than a 1/8" column.

(c) Separation of Amino Acid N-TFA Methyl Esters on a
10' x 1/8" 1% Carbowax 20 M Column

The length of the 1/8" column was decreased to 10 feet to see if the decreased length would lower the resistance to gas flow. The gas flow, however, was still difficult to control at a steady rate although a lower pressure head could be used. It was found advantageous to use a low carrier gas flow rate of 10 ml/minute (30 psig) and to maintain this flow by periodic adjustments throughout the chromatography.

The amino acid derivatives were injected at 30°C and after 6 minutes the temperature was raised to 120°C isothermally and then programmed at 3°/minute to 200°C then 2°/minute to 250°C. (Column 3, Table 32).

Although the retention times of the amino acids were lower the separation of all of the amino acids was poor.

Separation on a 10' x 3/16" column was just as poor.

(d) Separation of the Amino Acid Derivatives on a
12' x 3/16" 1% Carbowax 20 M Column

The amino acids were injected onto the column at 90°C and after 12 minutes the temperature was raised to 130°C then programmed at 5°/minute to 170°C. The temperature was then programmed at 1°/minute to 185°C and held there until serine was eluted. The temperature was then raised to 240°C, then programmed 5°/minute to 270°C after the elution of lysine. The gas flow rate was 80 ml/minute. (Column 4, Table 32).

On this column alanine and isoleucine were split at 170°C, however threonine-methionine and arginine-hydroxyproline were poorly separated. All of the amino acids including tyrosine, histidine and tryptophan were separated in a time of 100 minutes. The amino acid peaks were sharp.

This column clearly has possibilities for obtaining a separation of all of the amino acid esters but attempts at doing this were not pursued since it was not clear that it would have any advantage over the 1/4" column.

Conclusion

Although narrow columns may be more efficient for gas Chromatography it was impossible by our techniques to obtain good separations probably largely due to the resistance of the column to the carrier gas flow. The high resistance was due mainly to the solid support. The separations obtained on the 12' x 3/16" 1% Carbowax 20 M column indicate that narrow columns may be suitable.

8. The Effects of Varying the Ratio of Liquid Phase to Solid Support upon Separation of N-TFA Amino Acid Methyl Esters

As well as 1% Carbowax 20 M on diatoport 8, 2% and 10% Carbowax 20 M was tried.

(a) Separation of Amino Acid Derivatives on a 20' x 3/16" 2% Carbowax 20 M Column

The esters were injected at 110°C and after 24 minutes the temperature was programmed to rise 7.5°/minute to 140°C and held until serine was eluted. The temperature was then raised to 240°C. (Column 1, Table 33).

Alanine and isoleucine were split at 190°C. There was no elution of threonine or serine. The amino acids were poorly separated and the peaks were wide due to diffusion on the column. This indicated that 2% Carbowax gave poor partitioning of the amino acids between the liquid and mobile phase. Similar results were obtained on a 20' x 1/8" 2% Carbowax 20 M column.

(b) Separation of Amino Acid Derivatives on a 10' x 3/16" 2% Carbowax 20 M Column

A shorter 2% Carbowax 20 M Column was tried to reduce the diffusion of amino acids on the column.

The amino acids were injected at 90°C and after 10 minutes the temperature was programmed to rise 5°/minute to 150°C, then 1°/minute to 195°C until serine was eluted. The temperature was then raised to 240°C. (Column 2, Table 33).

Alanine and isoleucine had a slight split between their peaks. Although all the amino acids were eluted, they were poorly separated.

(c) Separation of Amino Acid Derivatives on a
5' x $\frac{1}{8}$ " 10% Carbowax 20 M Column

A short column with a large percentage of liquid phase was used for gas chromatography.

The amino acids were injected at 30°C and after 6 minutes the temperature was programmed to rise 10°/minute to 250°C. (Column 3, Table 33).

No arginine or lysine was eluted from the column. The peak areas were very small, indicating either that the esters were lost in the solvent front or they were not completely eluted from the column.

9. Separation of N-TFA Amino Acid Methyl Esters of Tyrosine, Histidine and Tryptophan on short 1% Carbowax 20 M Columns

Columns varying in length from 1 foot to 4 feet, were used to obtain separations for tyrosine, histidine and tryptophan--- the least volatile of all the amino acids derivatives. The most difficult elution problem was encountered with histidine. As previously mentioned it was difficult to elute histidine from 8' or 15' columns when its retention time was long. The same problem existed on the short columns.

Early in these experiments it was discovered that if the amino acid derivatives were injected with the column at room temperature and the temperature immediately raised to 260°C-270°C the recovery of histidine from the column would be at its maximum.

(a) Separation of Amino Acid Derivatives on a
18" x $\frac{1}{8}$ " 1% Carbowax 20 M Column

A carrier gas flow of 40 ml/minute was used in all of these experiments.

TABLE 34

Separation on a 18" x 1/4" 1% Carbowax 20 M Column (Gas Flow = 40 ml/minute)

Asp = 1.00 @ 30 min.

Asp = 1.00 @ 22 min.

Asp = 1.00 @ 21 min.

AMINO ACID	Rel. Temp		Res'n I.U.'s	Rel. Temp		Res'n I.U.'s	Rel. Temp		Res'n I.U.'s
	Ret'n	(°C)		Ret'n	(°C)		Ret'n	(°C)	
Cysteine	.37	80		.41	140		.48	140	
Valine	.57	110		.55	140		.57	140	
Leucine	.73	130		.64	140		.67	140	
Aspartic	1.00	170		1.00	170		1.00	160	
Tyrosine	1.90	270	1.40	1.96	260	1.32	1.96	260	30
Histidine	2.04	270	(x640)	2.18	260	(x640)	2.15	260	(x640)
Tryptophan	2.20	270	(x320)	2.45	260	(x320)	2.38	260	(x320)
			(x640)			(x640)			(x640)

TABLE 35

Separation on a 2' x 1/4" 1% Carbowax 20 M Column

Column 1.*

Column 2.*

Column 3.*

Column 4.*

Asp=1.00 @17min.

Asp= 1.00 @ 12 min.

Asp= 1.00 @ 8 min.

Asp= 1.00 @ 9 min.

AMINO ACID	Rel. Temp		Res'n I.U.'s	Rel. Temp		Res'n I.U.'s	Rel. Temp		Res'n I.U.'s	Rel. Temp		Res'n I.U.'s
	Ret'n	(°C)		Ret'n	(°C)		Ret'n	(°C)		Ret'n	(°C)	
Cysteine	.41	140										
Valine	.53	140										
Leucine	.65	140										
Aspartic	1.00	170		1.00	260		1.00	270		1.00	270	
Tyrosine	1.88	255	0.67	1.75	260	2.86	2.25	270	3.34	2.00	270	1.88
Histidine	2.12	255	(x640)	2.08	260		2.75	270	(x640)	2.33	270	(x640)
Tryptophan	2.47	255	(x320)	2.42	260		3.25	270	(x320)	2.55	70	1.20
			(x640)						(x640)			

* For temperature programs see pages 95 - 97.

A continuous temperature program was tried. The amino

acids were injected at room temperature and after a 6 minute delay the temperature was programmed to rise 50/minute to 270°C. The analysis time was 60 minutes. (Column 1, Table 34). The peaks were still broad and poorly resolved.

The peaks were wide and low due to histidine and tryptophan. However by this programming all of the amino acids could be identified. The resolution of tyrosine and histidine was only 1.40. There was a long delay after 270°C was reached before tyrosine appeared. (Column 2, Table 34).

The programming was arranged to elute tyrosine as soon as the temperature reached 260°C-270°C in a hope that this would sharpen the peaks. The derivatives were injected onto the column at room temperature and after 6 minutes the temperature was raised to 140°C and held there until proline was eluted. The temperature was then programmed to rise 10°/minute to 260°C. The peaks were still wide, however the peak areas for histidine and tryptophan had increased (see Table 34). The resolution of tyrosine and histidine was still poor (1.32).

A similar temperature programming was used with a longer partition period. The amino acids were injected and the column kept at room temperature for 8 minutes. The temperature was then raised to 140°C and held until aspartic acid was eluted, after which the temperature was programmed to rise 10°/min. to 270°C. (Column 3, Table 34).

The resolution of tyrosine and histidine was still poor (1.32).

The peaks were sharper with the longer partition period. The peak area for histidine had increased and the resolution of tyrosine and histidine had increased to 1.70.

(b) Separation of Amino Acid Derivatives on a
2'x $\frac{1}{8}$ " 1% Carbowax 20 M Column

A larger column was employed to obtain a better resolution of tyrosine and histidine.

In Column 1 of Table 35 the derivatives were injected with the column at room temperature and after a 4 minute delay the temperature was raised to 140°C and held until proline was eluted, then raised to 255°C abruptly.

Histidine and tryptophan peak areas had decreased with either the lower temperature (255°C) or the increased retention time.

The temperature program was then speeded up to obtain sharper peaks with lower retention times. The amino acids were injected at room temperature and after 6 minutes the temperature was raised abruptly to 260°C.

Histidine and tryptophan peaks were considerably longer and the resolution of tyrosine and histidine was 2.86, the best resolution thus far obtained. (Column 2, Table 35).

A similar program was then employed but with the maximum temperature at 270°C. The amino acid derivatives were injected at room temperature and after 2 minutes the temperature was raised abruptly to 270°C. (Column 3, Table 35).

This program gave good recoveries of tyrosine and histidine and the resolution of tyrosine and histidine (3.34) was the highest resolution that had been obtained.

The separation pictured in Figure 1, page 54 was similar to the one described above, except the partition time was 5 minutes. The resolution of tyrosine and histidine was less (1.88), although the peak areas were the same. (column 4).

A slower programming was also used on the 2' 1% Carbowax 20 M column. The amino acid derivatives were injected onto the column at room temperature and after 6 minutes the temperature was raised abruptly to 120°C and held until proline was eluted. The temperature was then programmed to rise 10°/minute to 260°C. This separation is pictured in Figure 2.

The resolution of tyrosine and histidine had increased from 1.88 to 2.00 but the peak areas of histidine and tryptophan were lower.

The final two programs were adopted as the most useful for further work.

(c) Separation of Amino Acid Derivatives on a
4' x 1/4" 1% Carbowax 20 M Column

A 4' column was tried to see if the increased length would improve the efficiency of the separation of tyrosine and histidine.

The amino acids were injected at 90°C and after 8 minutes the temperature was programmed to rise 10°/minute to 270°C. The analysis time was 44 minutes.

The analysis time was too long resulting in very low histidine peaks. With the increased amount of liquid phase

TABLE 36

Separation on a 4' x 1/4" 1% Carbowax 20 M Column

AMINO ACID	Column 1.* Asp = 1.00 @ 18 min.				Column 2.* Asp = 1.00 @ 7 min.				Column 3.* Asp = 1.00 @ 6 min.				Column 4.*			
	Rel. Ret'n	Temp (°C)	Res'n	I.U.	Rel. Ret'n	Temp (°C)	Res'n	I.U.	Rel. Ret'n	Temp (°C)	Res'n	I.U.	Rel.** Ret'n	Temp (°C)	Res'n	I.U.
Cysteine	.17	90														
Valine	.33	90														
Leucine	.72	130														
Aspartic	1.00	200			1.00	250			1.00	270						
Tyrosine	1.89	270)	36		1.90	250)	24		2.50	270)	36		.12	250)		
		)	2.80 (x640)			)	1.50 (x640)			)	2.50 (x640)			)	2.60	
Histidine	2.12	270)	2.5		3.15	250)	9		3.00	270)	12		.20	250)		
			(x320)				(x320)				(x320)					
Tryptophan	2.33	270	16		3.85	250	20		3.67	270	24		.30	250		
			(x640)				(x640)				(x640)					

*For temperature programs see pages 97 - 98

** refers to retention time in minutes

there was considerable bleeding of the resin and the shifting baseline made it difficult to obtain a good resolution of tyrosine and histidine. (Column 1, Table 36).

A faster program with a lower maximum temperature (250°C) was tried in order to prevent the resin from bleeding. The amino acids were injected at room temperature and after 2 minutes the temperature was raised abruptly to 250°C . (Column 2, Table 36).

The width of the peaks indicated that this temperature was too low. There was a poor resolution of tyrosine and histidine and the base line still shifted due to bleeding from the resin.

The amino acid derivatives were then injected at room temperature and after 2 minutes the temperature was raised isothermally to 270°C .

A good resolution of tyrosine and histidine was obtained (2.50) and the peaks due to histidine and tryptophan were very good. However the base line still shifted. (Column 3, Table 36).

Finally the amino acid derivatives were injected onto the column at 250°C . The analysis time was 34 minutes. (Column 4, Table 36).

Although there was a good resolution of tyrosine and histidine, the peaks were very broad and the base line shifted considerably. Although the 4' column it gave good separations the instability of the baseline made it of doubtful value.

TABLE 37

THE QUANTITATIVE ESTIMATION OF N-TFA METHYL AMINO ACIDS BY GAS LIQUID CHROMATOGRAPHY

AMINO ACID		Cys	Val	Ala	Ileu	Leu	Gly	Pro	Asp	Thr	Met	Glut	Phe	Ser	Arg	Hyp	Lys	Tyr	His	Tryp
AMOUNT	ATT'N	320	2560							1280					640				160	320
INJECTED	I.U.'S	10	7.5	3.5	4	8	3	7	4.5	4	8.5	8.5	19	3	4	18	23	15	8	13.5
20 µgms	I.U.'S	160	970	448	512	1024	384	896	575	256	545	545	1215	192	128	575	735	480	64	216
	@ 20																			
10 µgms	ATT'N	160	1280							640					320				80	160
	I.U.'S	8	6.5	4.5	5.5	7	3	6.5	4.5	5	9.5	9	20	3	5	17.5	25.5	14	7.5	12
	I.U.'S	64	416	288	352	449	193	416	288	160	304	288	640	96	80	280	407	224	30	96
	@ 20																			
5 µgms	ATT'N	80	640							320					160				40	80
	I.U.'S	10.5	26.5	3.5	4	7	3	6.5	5.5	5	9.5	9	20	3	4	17.5	24	13	7	13
	I.U.'S	41	208	112	128	224	96	208	160	80	152	144	320	48	32	140	192	96	14	52
	@ 20																			
2.5 µgms	ATT'N	40	320							160					80				20	40
	I.U.'S	8.5	6.5	3.5	4	7	3.5	7	4.5	4	8.5	8.5	18	3.5	5	13	19	11	7	13
	I.U.'S	17	104	56	64	112	56	112	72	32	68	68	144	28	20	52	76	44	7	26
	@ 20																			
1 µgm	ATT'N	16	128							64					32				8	16
	I.U.'S	10.5	7.5	3.5	4	8.5	3	6.5	4.5	4	8.5	8	19	3.5	4.5	16	20	12	6.5	12
	I.U.'S	8.4	48	23	26	54	19	42	29	13	27	26	61	11.2	7.5	26	32	19	3	10
	@ 20																			

Summary

From a consideration of the results of experiments described in the previous sections, it was concluded that the best conditions for reproducible separations of amino acid N-TFA methyl esters were those described in detail on pages 54-8. That is, amino acids are best separated by employing three separate columns: a 20' x 1/4" 1% Carbowax 20 M column for separation of 14 of the 19 amino acids; a 2' x 1/4" 1% Carbowax 20 M column for the separation of tyrosine, histidine and tryptophan and a 10' x 1/4" 1% Carbowax 1540 column for the separation of alanine and isoleucine. These columns were used successfully in qualitative work and in the estimation of the amino acid composition of proteins (page 102). The total analysis time on the three columns is just under 4 hours.

There is still considerable room for improvement. All of the amino acid derivatives could be completely separated in the shortest possible analysis time if the right liquid phase could be found and used with the best program conditions. Since the two types of Carbowax used in the present study----Carbowax 1540 and Carbowax 20 M----are at opposite extremes in terms of molecular weight, there may be an efficient Carbowax resin lying somewhere between these two Carbowax resins with the right molecular weight permitting the separation of all of the amino acids on a single column. The possibility of using long Golay capillary columns coated with only the liquid phase of Carbowax

should also be considered in future studies.

F The Quantitative Estimation of N-Trifluoroacetyl Amino Acid Methyl
Derivatives by Gas Chromatography

If gas chromatography is to be used as a reliable tool for amino acid analysis the method of forming the derivatives should be reproducible. The preceding work has been primarily concerned with the preparation and estimation of N-trifluoroacetyl amino acid methyl esters. The use of the procedure for quantitative purposes will now be discussed.

Mixtures of the 19 common amino acids in differing amounts were weighed out and converted to the volatile derivatives by the method described on page 25 . Five samples containing 1 mg, 2.5 mg, 10 mg and 20 mg of each amino acid were carried through the procedure and the final N-Trifluoroacetyl methyl derivatives were taken up in dry methanol and made to a volume of 5 ml. Each sample was then chromatographed on the three columns: 20' x $\frac{1}{8}$ " 1% Carbowax 20 M, 2' x $\frac{1}{8}$ " 1% Carbowax 20 M and 10' x $\frac{1}{8}$ " 1% Carbowax 1540.

If 5 μ l samples are injected, then the actual amounts of N-trifluoroacetyl amino acid methyl derivatives injected onto the column were equivalent to 1 μ g, 2.5 μ g, 10 μ g and 20 μ g of amino acid respectively.

The area under the peak for each amino acid was calculated from the number of strokes made by the Disc-Chart Integrator attached to the recorder. The areas were expressed as Integrator Units (excursions) and plotted against the amount of each amino acid injected. A linear relationship was found between the amount of each amino acid injected and

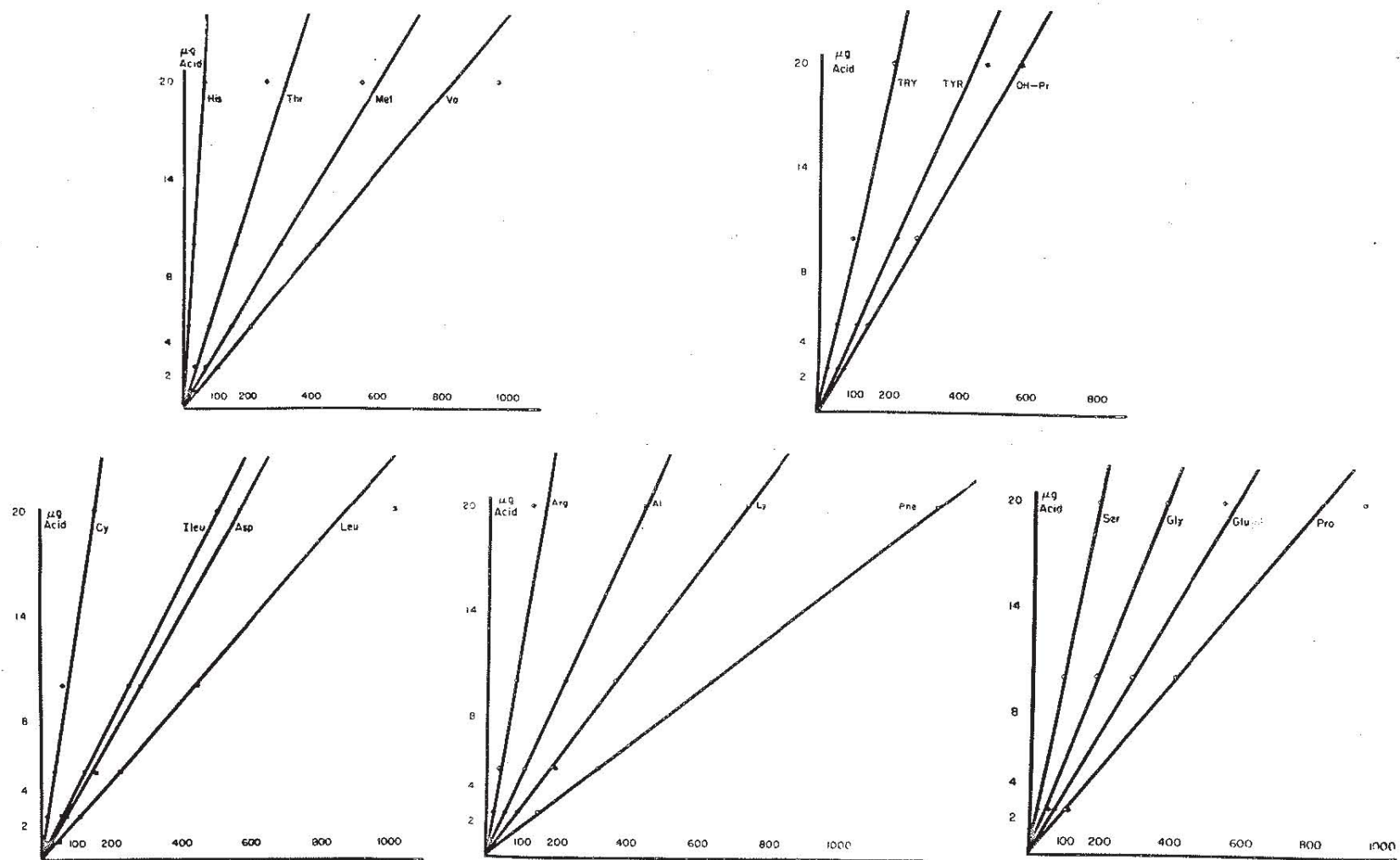


Figure 3. Graphs showing the relationship between the amount of amino acid in the mixture and the area under the peak. Units: Ordinate μg amino acid, Abscissa number of disc integrator units.

the peak area. (See Figure 3 and Table 37). If the occasional deviation from linearity is attributed to weighing or pipetting errors, then either the conversion of the amino acid to its volatile derivative is complete or a constant proportion of each amino acid is converted to the volatile derivative.

The deviations from linearity are mostly at the 20 μ g amino acid concentration. This is attributed to weighing errors, since the 20 mg amino acid samples were weighed on a different and less sensitive balance than that used to weigh the lower amounts of amino acid. Although the amino acid derivatives were estimated at different attenuations the areas were corrected to a common attenuation ($\times 20$) for linear evaluation.

The calibration curves were reproducible from day to day for all of the amino acids.

SECTION VII

THE DETERMINATION OF THE AMINO ACID COMPOSITION OF SOME
PROTEIN HYDROLYSATES BY GAS-LIQUID CHROMATOGRAPHY

1. Introduction

The amino acid composition of three proteins: casein, insulin and ovalbumin were determined. The proteins were subjected to acid hydrolysis and the free amino acids were esterified forming the N-TFA methyl derivatives. The derivatives were then subjected to gas chromatography according to the three systems previously described.

Although the values obtained for the majority of amino acids were within 80-100% of the published values for these proteins, the method of analysis may not yet be sufficiently foolproof to obtain the precision necessary for accurate protein work.

2. Experimental(1) Preparation of Constant Boiling Hydrochloric Acid

A mixture containing equal volumes of concentrated hydrochloric acid (BDH 'analar' reagent) and distilled water was prepared giving a specific gravity of approximately 1.10.

The mixture was placed in a round bottom flask and placed on a heating mantle. The flask was connected to a water cooled distillation apparatus. The azeotropic mixture was distilled and the distillate collected. The distillate collected up to 108°C was discarded, saving only the distillate collected between 108-110°C. This fraction is the constant boiling point hydrochloric acid. The constant boiling Hydrochloric acid was stored in a glass

stoppered bottle and used for the hydrolysis of proteins.

(11) Preparation of Proteins for Amino Acid Analysis

Protein samples were obtained commercially in the dry form or in the case of insulin, precipitated by a common protein precipitant and freeze-dried. The dry protein was then weighed into a 10 ml glass ampoule. A hot solution of constant boiling hydrochloric acid was added to the ampoule, filling it to the constriction in the neck. Nitrogen gas may be introduced to drive out oxygen. The hot 6N HCl will drive out oxygen, lessening the chance of any humin forming in the hydrolysate.

The glass ampoule was sealed at the open tip, heating the ampoule while sealing it, to drive out any residual oxygen that may remain. The protein was then hydrolysed with the 6N HCl in a steam autoclave at 110°C for 12-16 hours.

The hydrolysate was then placed into a small evaporation flask and the HCl removed by evaporation on a rotatory evaporator under negative pressure at 40°C. The residue was then washed three times with 50 mls of dry methanol and evaporated as before.

The residue was then either dried overnight in a dessicator with phosphorus pentoxide or immediately esterified.

Twenty ml dry methanol was added to the hydrolysate and the flask was placed in an ice bath. Four ml water-clear thionyl chloride was added dropwise to the methanol, mixing constantly. The mixture was heated at 40°C for 2 hours in

an oil bath. The contents of the flask were then evaporated on a rotatory evaporator under negative pressure at room temperature.

To the residue was added 3 ml of Tri-fluoroacetic anhydride and the flask left at room temperature for 2 hours. The excess Tri-fluoroacetic anhydride was then removed by evaporation as before.

The residue was dissolved in dry methanol to a volume of 5 ml. 5 to 20 μ l samples were injected onto each of the three chromatography columns: 20' x $\frac{1}{8}$ " 1% Carbowax 20 M, 2' x $\frac{1}{8}$ " 1% Carbowax 20 M and a 10' x $\frac{1}{8}$ " 1% Carbowax 1540, employing the temperature programs described on pages 54 - 58.

For a rapid rough qualitative analysis, it was advantageous to first identify the amino acids present on the 2' x $\frac{1}{8}$ " 1% Carbowax 20 M column using the slow program described on page 54.

3. The Amino Acid Composition of Casein as Determined by Gas-Liquid Chromatography

(1) Experimental

Thirty mgm of dry casein powder, purchased from Merck and Co., was weighed out into a 10 ml glass ampoule. The casein was then hydrolysed with constant boiling point HCl (page 102) in an autoclave at 110°C for 16 hours.

The HCl was then evaporated and the residue washed (see page 103). The dry residue was then esterified by the procedure described on page 103 and the esters dissolved in dry methanol to a volume of 5 ml.

TABLE 38

THE AMINO ACID COMPOSITION OF CASEIN

Concentration: 30 µg/5 ml methanol

Injected: 60 µg in 10 µls

AMINO ACID	Rel.	Stand.	I.U.'s @ Att'n	I.U.'s	Conc.	Gm a.a./ 100 gm protein	Published Values	
	Ret'n	Ret'n		x 20	Rec'd		Tristram*	Haurowitz**
Cysteine/2	.40	.40	6 (x40)	12	0.4µgm	0.67	0.34	0.43
Valine	.58	.61	11 (x320)	176	4.2 "	7.00	7.20	6.30
alanine	.63	.66	3.5 (x320)	56	2.6 "	4.33	3.20	3.70
Isoleucine			6 (x320)	96	3.8 "	6.33	6.10	6.40
Leucine	.69	.72	17 (x320)	272	6.0 "	10.00	9.20	7.90
Glycine	.78	.81	2 (x320)	32	2.7 "	2.00	2.00	2.80
Proline	.85	.87	10 (x640)	320	7.8 "	13.00	10.60	8.20
Aspartic	1.00	1.00	6 (x320)	96	3.3 "	5.50	7.10	8.40
Threonine***	1.16	1.16	2 (x320)	32	2.50"	4.16	4.90	4.90
Methionine	1.20	1.21	6.5 (x160)	52	1.8 "	3.00	2.80	2.50
Glutamic	1.24	1.24	6.5 (x640)	416	14.2 "	23.70	22.40	22.50
Phenylalanine	1.30	1.28	6 (x640)	192	3.0 "	5.00	5.00	4.60
Serine	1.36	1.44	2.5 (x256)	32	3.5 "	5.83	6.30	6.30
Arginine	1.76	1.91	4 (x160)	32	3.6 "	6.00	4.10	4.30
Hydroxyproline	0	1.96	0	0	0	0	0	0
Lysine	1.89	2.03	11 (x256)	141	6.70"	6.70	8.20	8.90
Tyrosine			10 (x128)	64	5.00"	5.00	6.30	8.10
Histidine			0	0	0	0	3.10	2.90
Tryptophan			0	0	0	0	1.20	1.60

* G. R. Tristram The Proteins vol. 1, Part A pg. 216; Neurath and Bailey. (1953).

** F. Haurowitz; The Chem. and Function of Proteins, pg. 198, (1963).

*** 30 µls (180 µgms) Injected.

Ten or 30 μ l samples were injected onto the three chromatography columns previously described (p.54). This represents a concentration of 60 and 180 μ g total protein respectively.

The esters were injected onto the 20' x $\frac{1}{4}$ " 1% Carbowax 20 M column at 80°C and after 24 minutes the temperature was programmed to rise 50°/minute to 170°C then $\frac{1}{2}$ °/minute to 190°C. After glutamic was eluted the temperature was programmed to rise 20°/minute to 240°C.

The peak areas were obtained at the particular attenuation and the peak areas at an attenuation of $\times 20$ (range 10 and attenuation of 2) were calculated. The amino acid concentration for each peak area at $\times 20$ was then obtained from the graphs of the standard amino acids (see graphs page 101).

(11) Results and Discussion

Table 38 gives the concentration of amino acids recovered from casein in μ m/100 μ m of protein. These values are compared to data previously obtained by other authors. The relative retention values and the peak areas (integrator strokes) are also listed.

No hydroxyproline, histidine, or tryptophan were found in casein. No hydroxyproline has been reported in casein by other authors, but histidine (3 μ m/100 μ m protein) and tryptophan (1.9 μ m/100 μ m protein) have been reported by every author.

The lack of these amino acids is attributed to the

hydrolysis procedure, possibly being too acidic for histidine and tryptophan may have formed humin. (See page 117).

The values received for cysteine, valine, isoleucine, leucine, glycine, aspartic, methionine, glutamic, phenyl-alanine and serine agree with the published values within a 0-15% range. Considering the errors that may occur during the weighing, hydrolysis, esterification and chromatography processes these results are within the bounds of experimental error.

Proline and arginine are 20-30% too high. Threonine, lysine and aspartic are 20-30% too low.

The majority of the amino acids are therefore in very close agreement with the published values.

The relative retention values are very close to those obtained for a standard amino acid mixture under the same temperature programming conditions. The relative retention values are a reliable aid in the positive identification of amino acids.

4. The Amino Acid Composition of Insulin as Determined by Gas-Liquid

Chromatography

(1)

Experimental

Forty mg of commercial insulin was obtained (Connaught Medical Research Labs., Toronto, Ont.). After precipitation dialysis to remove the zinc and drying, the insulin was hydrolysed in a 10 ml glass ampoule with 6N constant boiling HCl in an autoclave at 110°C for 16 hours. The HCl was then evaporated and the residue washed (page 103). The dry

TABLE 39

THE AMINO ACID COMPOSITION OF INSULIN

Concentration: 40 mg/5 ml methanol

Injected: 120 µg in 15 µl

AMINO ACID	Rel. Ret'n	Stand. Ret'n	I.U.'s @ Act'n	I.U.'s x 20	Conc. Rec'd	On a.a./ 100 gm protein	Published Values Formageot*	Tristram**
Cysteine	.42	.42	10 (x160)	80	9.6 µgm	8.00	12.50	12.50
Valine	.60	.57	7.5 (x1280)	480	11.5 "	9.60	7.41	7.75
Alanine***	.64	.63	4.5 (x1280)	288	13.0 "	3.25	4.58	4.50
Isoleucine***	.64	.63	3.8 (x1280)	243	9.5 "	2.38	1.58	2.77
Leucine	.76	.70	6 (x2560)	770	17 "	14.20	14.63	13.38
Glycine	.80	.79	8 (x320)	128	6.7 "	5.60	4.51	4.30
Proline	.88	.87	6 (x640)	192	4.7 "	1.96	2.02	2.53
Aspartic	1.00	1.00	7.5 (x640)	240	8.3 "	6.90	5.51	6.80
Threonine	1.20	1.19	7 (x80)	28	1.8 "	1.50	2.34	2.08
Methionine	0	1.25	0	0	0 "	0	0	0
Glutamic	1.32	1.30	18 (x640)	576	20 "	16.70	19.30	18.60
Phenylalanine	1.44	1.40	18 (x640)	576	9.0 "	7.50	8.30	8.14
Serine	1.52	1.51	18 (x160)	32	6.3 "	5.25	5.16	5.23
Arginine***	1.89	2.05	12 (x320)	192	28 "	3.50	2.86	3.07
Hydroxyproline	0	2.15	0	0	0 "	0	0	0
Lysine	2.12	2.26	10 (x80)	40	2.4 "	2.00	2.72	2.51
Tyrosine***			25 (x640)	800	36 "	9.00	12.10	13.00
Histidine			9 (x80)	36	8.5 "	2.13	4.98	4.91
Tryptophan			0		0	0	0	0

* C. Formageot, Cold Spring Harbor Symposia Quart. Biol. 14, 49 (1950).* G. R. Tristram, Advances in Protein Chem. 5, 83 (1949).

*** 50 µls (400 µgms) injected.

residue was esterified by the procedure described on page 103 and the esters dissolved in dry methanol to a volume of 5 ml.

Fifteen μ l was injected onto the three chromatography columns described previously (page 54), the concentration injected was 120 μ g of total protein.

The esters were injected onto the 20' x $\frac{1}{8}$ " 1% Carbowax 20 M column at 80°C and after 14 minutes the temperature was programmed to rise 5°/minute to 180°C and held until glutamic was eluted, then programmed to rise 3°/minute to 240°C.

The other two columns were used with the standard programs described on pages 54-8.

The peak areas were obtained at the particular attenuation and the peak areas at an attenuation of x20 were calculated. The amino acid concentration for the peak areas at x20 were obtained from the graphs of the standard amino acid solutions (page 101).

(11) Results and Discussion

Table 39 gives the amounts of the amino acids found in the Insulin. These values are compared to data published by various workers using other methods. The relative retention values and the peak areas (integrator--strokes) are also listed.

No methionine, hydroxyproline or tryptophan were found in insulin. This result agrees with the results obtained by other workers.

The values obtained for valine, alanine, isoleucine, leucine, glycine, proline, aspartic, glutamic, phenylalanine, serine, arginine, lysine and tyrosine are in the range of $\frac{1}{2}$ 0-20% of the published values.

Cysteine, threonine and histidine are low, the values ranging from 30-50% of published values. This is possibly a fault of the chromatography, but since this was a sample of commercial insulin it is more likely due to contamination of the insulin with another protein.

5. The Amino Acid Composition of Ovalbumin as Determined by Gas-Liquid Chromatography

(1) Experimental

Twenty-six mg of crystallized hen egg albumin, purchased from Sigma Chemical Company, St. Louis, Mo., was placed in a 10 ml glass ampoule. The ovalbumin was hydrolysed at 110°C for 16 hours with constant boiling 6N HCl (page 102) in an autoclave. The HCl was then evaporated and converted to the N-trifluoroacetyl methyl ester by the procedure described on page 103 and the derivatives dissolved in dry methanol to a volume of 5 ml.

Fifty μ l samples were injected onto the three chromatography columns described previously. The amount of hydrolysed ovalbumin injected represented 260 μ g of the original protein. The derivatives were injected onto the 20' x $\frac{1}{8}$ " 1% Carbowax column at 80°C. After 14 minutes the temperature was programmed to rise 5°/minute to 180°C, held there until the glutamic was eluted and then programmed to rise 3°/minute to 240°C.

TABLE 40

THE AMINO ACID COMPOSITION OF OVALBUMIN

Concentration: 26 mg/ 3 ml methanol

Injected: 260 µg in 50 µl

AMINO ACID	Rel. Ret'n	Stand. Ret'n	I.U.'s @ Att'n	I.U.'s x 20	Conc. Rec'd	Gm a.s./ 100 gm protein	Published Values
Cysteine	.42	.42	8 (x80)	32	3.8 µgm	1.46	
Valine	.58	.57	10.5 (x1280)	672	16.2 "	6.22	1.15
Alanine	.64	.63	6.5 (x640)	328	14.5 "	5.58	5.96
Isoleucine	.64	.63	8 (x1280)	512	14.9 "	6.80	5.36
Leucine	.69	.70	6 (x2560)	768	17.2 "	6.60	6.04
Glycine	.81	.79	3.0 (x640)	96	5 "	1.92	7.94
Proline	.86	.87	4.5 (x1280)	280	6.8 "	2.62	2.32
Aspartic	1.00	1.00	3.5 (x1280)	448	17.5 "	6.72	3.04
Threonine	1.21	1.19	4.5 (x320)	72	5.8 "	2.23	8.04
Methionine	1.27	1.25	11 (x320)	176	5.8 "	2.23	3.42
Glutamic	1.33	1.30	6 (x2560)	768	28.5 "	11.00	1.57
Phenylalanine	1.46	1.40	11 (x1280)	705	11.2 "	4.32	14.48
Serine	1.58	1.51	4.5 (x640)	144	15 "	5.78	6.68
Arginine	2.12	2.05	32 (x80)	128	19 "	7.30	6.75
Hydroxyproline	0	2.15	0	0	0 "	0	5.13
Lysine	2.25	2.26	10 (x640)	320	8.8 "	3.40	0
Tyrosine			10 (x160)	160	7.7 "	2.80	5.52
Histidine			10.5 (x40)	21	5.5 "	2.12	3.33
Tryptophan			6 (x40)	12	1.0 "	0.85	2.08
							1.09

The peak areas recorded at each particular attenuation, were corrected to values at a 20 x attenuation.

(11) Results and Discussion

Table 40 gives the amounts of amino acids recovered from ovalbumin. These values are compared to data published by various authors using other methods of determination. The relative retention values and the peak areas (integrator strokes) are also listed.

All of the amino acids except hydroxyproline were found in ovalbumin. This result agrees with the amino acid composition reported by other authors.

Cysteine, valine, alanine, isoleucine, leucine, glycine, proline, aspartic, threonine, phenylalanine, serine, arginine, tyrosine, histidine and tryptophan are within the range of $\pm$ 0-20% of the published values.

Methionine, glutamic and lysine are within the range of $\pm$ 20-40% of the published values. However, since different preparations of ovalbumin from various sources have slight differences in amino acid composition it is probable that the values reported here represent very closely the actual composition of ovalbumin used in these studies.

SECTION VIII

DISCUSSION

1. The Esterification of Amino Acids

For the gas chromatographic separation of amino acids a principal difficulty has been the reproducible conversion of non-volatile amino acids of high melting points to volatile derivatives suitable for separation within the limits of a gas chromatograph.

Many workers have used various methods to catalyse the esterification of the carboxyl group. The most popular catalyst is anhydrous hydrochloric acid. However this method does not facilitate adequate esterification of either the basic amino acids or tryptophan and cysteine.

One difficulty encountered by previous workers has been the esterification of the basic amino acids. Difficulty has also been encountered with cysteine, tryptophan and the hydroxyamino acids threonine and serine.

(1) The Use of Thionyl Chloride to Catalyse Ester Formation

In the typical esterification reaction-

$$\text{R.CH(NH}_2\text{).COOH} + \text{CH}_3\text{OH} \xrightarrow{\text{HCl}} \text{R.CH(NH}_2\text{).COOCH}_3 + \text{H}_2\text{O}$$

water is an end product.

There is a definite possibility that water formed in this reaction will prevent the esterification going to completion. The ideal reagent for the esterification of amino acids would therefore be one which could act as a water scavenger and at the same time catalyse the esterification. Thionyl chloride is a good catalyst for the formation of acid anhydrides and has been found to be useful in this respect.

Since Brenner and Pfister (1951) first used thionyl chloride for the methylation of L-methionine, which was to be used in

peptide synthesis, no one has reported a study of thionyl chloride as a catalyst for esterifying amino acids. Although it was used by Iwamoto and Morimoto (1964) to form the N-cyanoethyl butyl esters of some amino acids they did not find it particularly useful in the preparation of the butyl esters.

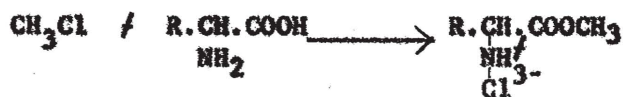
Thionyl chloride is well known to be an efficient water scavenger.



Thionyl chloride has been shown to play an active role in the methylation of organic acids. The reaction proposed by Gould in which methyl chloride can act as a source of methyl groups for esterification of carboxyl groups might apply to the methylation of amino acids.



The methanol could react with the thionyl chloride forming methyl chlorosulfite, an intermediate which on heating releases sulfur dioxide forming methyl chloride. The methyl chloride might then react with the amino acid to form the methyl amino acid ester hydrochloride.



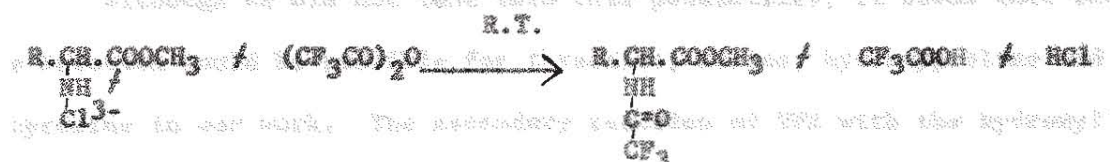
Experimental evidence presented in section V indicates that thionyl chloride is a good catalyst for the formation of amino acid methyl esters.

It might be postulated that the thionyl chloride reacts first with the methanol forming methyl chloride and releasing

sulphur dioxide and at the same time removing water produced in the esterification reaction. This reaction requires heating for completion. The methyl chloride then supplies the methyl groups for the methylation of amino acids. Both reactions require heat for a rapid conversion, the optimum temperature being 40°C for a time interval of two hours.

2. The Acylation of the Methyl Amino Acid Esters

The acylation of amino acids did not present comparable difficulties to the esterification. Trifluoroacetic anhydride reacts quickly with the amino group at room temperature, the reaction being



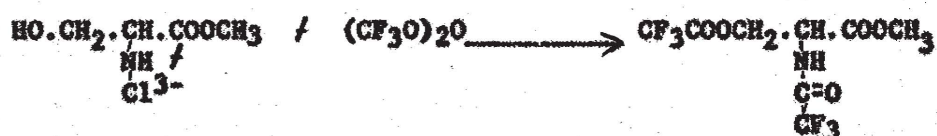
The concentration of trifluoroacetic anhydride was in excess to the amino group. On standing in the methanol solution and adding the amount needed for reaction, in fact it served as the solvent for this reaction.

3. Secondary Reaction of Amino Acids.

When the amino acid esters were injected on the column immediately after their preparation the derivatives of threonine, serine, arginine, hydroxyproline, lysine, histidine and tryptophan often gave low peak areas. On standing overnight the peak areas increased to a maximum value. This suggested that a secondary reaction might be taking place. Since the final derivatives were dissolved in dry methanol and since trifluoroacetic acid and methyl trifluoroacetate would be present in trace amounts further methylation or acylation would be possible. The amino acids all had one factor in common, they all possessed either secondary hydroxyl groups or secondary amino or imino groups which could

engage in secondary reactions.

By the use of infrared spectroscopy Cruickshank and Sheehan (1964) showed that the hydroxyamino acids threonine, serine, hydroxyproline and tyrosine could form N,O-bistrifluoroacetyl derivatives. This reaction only took place if the α carboxyl group was first esterified with a methyl group. Thus a typical reaction would be, using serine as an example:



Although we did not look into this possibility, it seems that such a reaction would be possible for threonine, serine, hydroxyproline and tyrosine in our work. The secondary reaction of TFA with the hydroxyl group would be expected to be slow, the TFA showing a preference for the α amino group. On standing/overnight in the methanol threonine and serine peak heights decrease with time, but the decrease reaches a minimum after which there is no further decrease. This would indicate that the trifluoroacetyl bond of the hydroxyl group of these amino acids is unstable.

The amino acids with secondary amino groups i.e. lysine and the guanido group of arginine could also undergo trifluoroacetylation. In these reactions it is assumed that the trifluoroacetylation of the

α -amino group would take place immediately while the ϵ -amino group of lysine or the guanido group of arginine would react much slower.

4. The Separation of N-Trifluoroacetyl Methyl Amino Acids by Gas-Liquid Chromatography

(1) Factors Affecting the Separation of Amino Acids

The prime requisite for the separation of amino acids by gas chromatography is that their esters should be volatile at the operating temperature of the chromatography column. The free amino acids are non-volatile, having very high melting points in relation to their low molecular weights. Masking their functional polar groups with volatile substituents could lead to an increase in their volatility. The problem is to prepare esters which will give high vapor pressures of the derivatives at temperatures within the tolerance of the liquid phase of the column. Since Carbowax 20 M maximum temperature is 275°C, it would be desirable to obtain derivatives with boiling points well below this temperature.

Weygand (1959) has determined the boiling points of 10 N-TFA methyl amino acids by fractional distillation. These are listed below together with the melting points or the decomposition temperatures of the unesterified amino acids.

Amino Acids	M.wt.	Melting point of amino acid	Boiling point of N-TFA methyl ester
Glycine	75	290°C	106°C
alanine	89	297	87
valine	117	315	98
leucine	131	337	114
isoleucine	131	284	109
proline	115	222	94
phenylalanine	165	284	115
tyrosine	181	222	148
aspartic	133	284	107
glutamic	147	334	121
lysine	146	270	154

Comparing the melting points of the amino acids with the boiling points of the N-TFA methyl esters obtained by Weygand (1959),

TABLE 41

THE RELATIVE RESPONSE OF THE IONIZATION DETECTOR TO CARBON NUMBER OF N-TFA

METHYL AMINO ACIDS

Injected: 10 µg of each amino acid
onto a 1% Carbowax 20 M
Column

AMINO ACID	Molecular weight	Molarity	Relative peak areas	Relative Molar Response	Carbon Number	% Linear Deviation
Cysteine	121	.083	*.22 **.26	2.70	3	-63%
Valine	117	.086	1.45 1.07	16.80	5	0
Alanine	89	.112	1.00 1.00	8.90	3	-10%
Isoleucine	131	.076	1.26 1.14	16.60	6	-18%
Leucine	131	.076	1.56 2.12	20.50	6	0
Glycine	75	.134	.67 .96	5.00	2	-23%
Proline	115	.087	1.45 1.85	16.70	5	0
Aspartic	133	.075	1.00 1.57	13.40	4	0
Threonine	119	.084	.56 .63	6.70	4	-47%
Methionine	149	.067	1.06 3.69	15.80	5	-7%
Glutamic	147	.068	1.00 1.45	14.70	5	-14%
Phenylalanine	165	.061	2.23 7.39	36.50	9	+20%
Serine	105	.095	.33 .49	3.50	3	-71%
Arginine	174	.058	.28 .55	4.80	6	-57%
Hydroxyproline	131	.076	.98 1.32	12.90	5	-23%
Lysine	146	.069	1.42 3.70	20.60	6	0
Tyrosine	181	.055	.78 3.38	14.20	9	-54%
Histidine	155	.065	.09 .48	1.40	6	-95%
Tryptophan	204	.049	.33 3.74	6.80	11	-82%

* Determined with a hydrogen flame ionization detector.

** Determined by Cruickshank with an Argon ionization detector.

an increase in
it can readily be seen that volatility has been achieved.

5. The Response of the Hydrogen Flame Ionization Detector to N-TFA Methyl
Amino Acid Esters

Dewar (1961) who along with McWilliams (1958) developed the flame ionization detector, made a study of the detector response to several hydrocarbon series of varying chain lengths. Dewar observed a linear relationship between the carbon number and the detector output. He also noted the hydrogen flame responds primarily to the CH-bond and that any functional groups present on the molecule that are attached to the CH-bond will result in a loss in the detector response. Zomzely et al (1962) and Saroff and Karman (1960) have reported an unequal varying response for equal amounts of amino acids. Zomzely states that the varying response is due to the presence of functional groups on the amino acid molecules. Saroff states that this is due only to either an unequal response caused by the detector or else to unequal yields. Since up to this time no one has obtained quantitative results for the amino acids the unequal yield theory could not be discounted.

In our experiment, our quantitative yields for the amino acids enabled us to look at this problem from both viewpoints. Using the values for the peak areas of the 10 µg amino acid samples used in the quantitative results described on page 100 the relative peak areas for the amino acids were calculated relative to alanine. The relative molar response was then obtained, correcting for the molecular weight of the amino acids at 10 µg. These values were then plotted against the carbon number of the amino acids, excluding the carbons of the TFA and methyl groups, and the best possible line drawn between the points. See Table 41 and Figure 4. Cruickshank et al (1964) have also reported the relative peak areas of

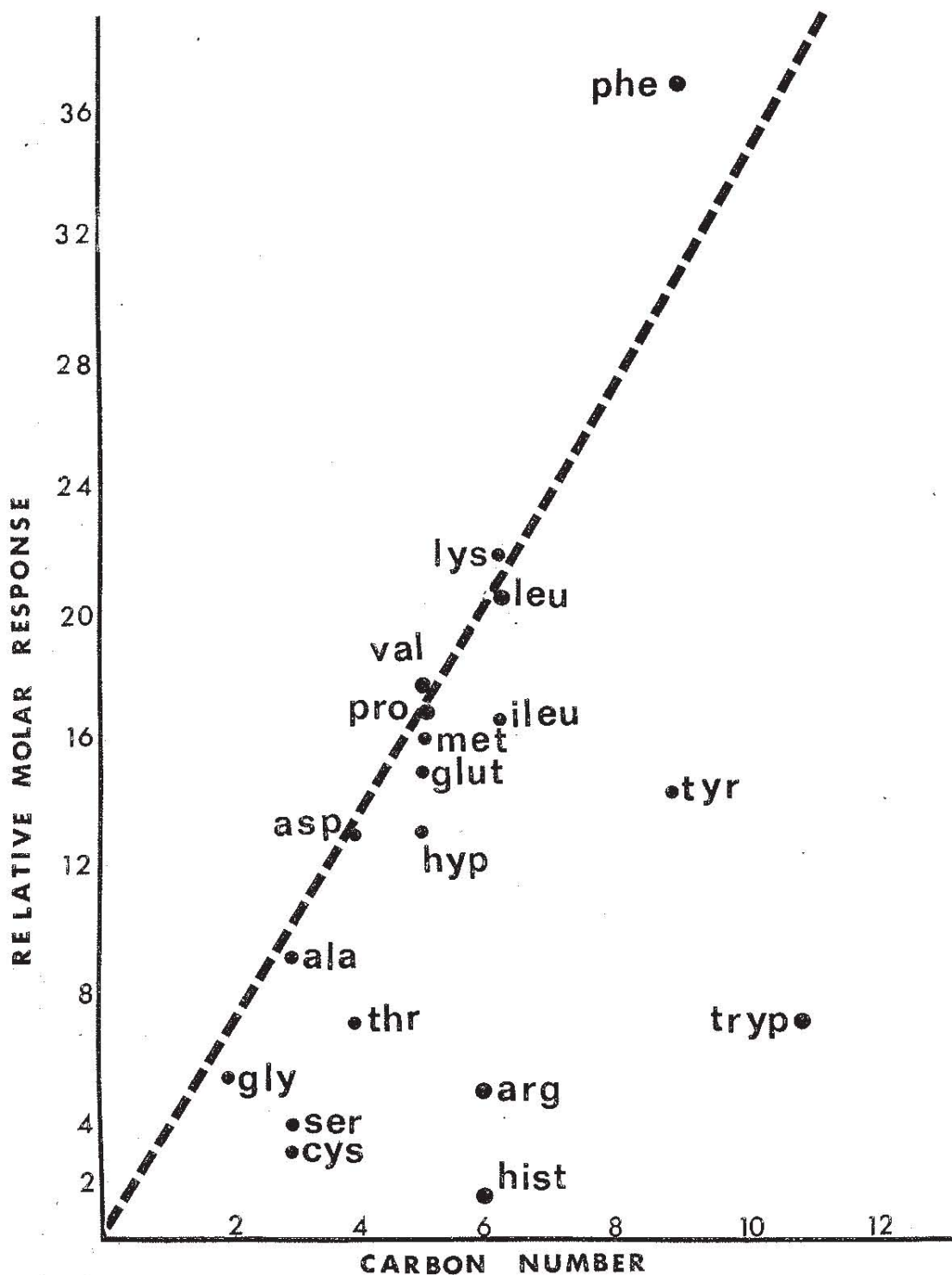


Figure 4. The relative molar response of the Flame-Ionization Detector to the carbon number of the N-TFA methyl amino acid esters.

the N-TFA methyl ester using an argon ionization detector and a 5% neopentyl glycol succinate column. These values are also listed in Table 41 for purposes of comparison between the two types of detectors.

The amino acids valine, alanine, isoleucine, leucine, glycine, proline, aspartic, methionine, glutamic, hydroxyproline, and lysine all show a linear relationship between the carbon number and the detector response. The percent deviation of these amino acids from linearity is only 0-20%, a range that is not too variable considering experimental errors. These amino acids are all aliphatic monosamine monocarboxylic or dicarboxylic and have no functional side groups, with the exception of lysine. The ϵ -amino of lysine does not appear to alter the detector response. However the decrease from linearity is marked when functional substituent groups are present. The decrease from the expected value is first noticeable with the hydroxyl and thiol amino acids as well as the guanido group of arginine. These groups show a linear deviation of 50-70% in the detector response. The largest deviation is with the heterocyclic histidine and tryptophan, deviations of 95 and 82% respectively from the expected molar response.

These findings agree with those of Dewar (1961) i.e. that there is a linear relationship between the carbon number and the detector response and that this response is altered by the presence of functional groups.

It is interesting to note that the relative peak areas obtained with the hydrogen flame detector, with the exception of methionine, phenylalanine, lysine, tyrosine and tryptophan, have approximately the same relative values as those obtained by Cruickshank with the argon ionization detector.

6. The Application of Gas Chromatography for the Determination of Protein Composition

The amino acid composition of acid hydrolysates of casein, ovalbumin and insulin were determined by gas chromatography. The amino acid composition of these was found to be similar to the composition reported by other workers.

Histidine and tryptophan gave the most difficulty in these proteins, the results being lower than the published values. Cysteine and/or cystine also posed some difficulties. Perhaps these difficulties could be overcome by using some other method of hydrolysis protecting the basic acids and tryptophan. Alkaline hydrolysis might be preferable in this respect.

It might be advisable to reduce cystine to cysteine with a reducing agent. Such a method was used successfully by Cruickshank (1964).

From the crude estimations of amino acid composition performed on protein hydrolysates and the agreement achieved in most cases with published values, gas chromatography may hold considerable promise for the future in the determination of protein composition.

Perhaps if the separation of dipeptides in partial hydrolysates described by Weygand (1960) is further explored gas chromatography could become an invaluable tool for the determination of amino acid composition and sequence.

When the conditions of chromatography are selected, the relative

1. H. G. Weygand, *Gas Chromatography*, 2nd ed., Vol. 1, (1960).

2. H. G. Weygand and H. G. Weygand, *Gas Chromatography*, 2nd ed., Vol. 1, (1960).

SECTION IX

APPENDIX*

1. Calculation of Retention Values

In calculating the retention values of amino acid derivatives there are many variables, which should be kept constant. The flow rate of carrier gas can be eliminated as a variable by using retention volumes which represent the volume of gas required to elute the amino acid.

This is expressed by the formula $V_R = t_R F_c$, where V_R = retention volume, t_R = retention time and F_c = volumetric flow rate of the carrier gas. This calculation is only necessary when the gas flow changes during a separation or when comparing columns with different flow rates.

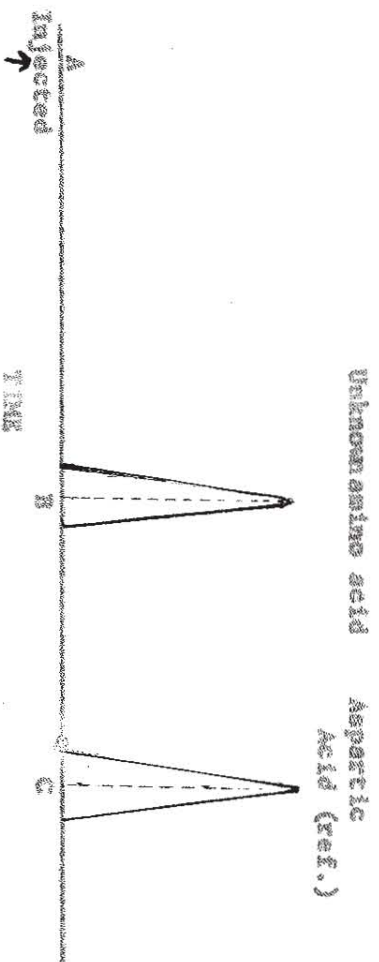
To describe the sequential order of amino acids on a column, relative retentions are used. These are retention volumes expressed relative to the retention volume of a standard component or 'marker'. In this experiment the aspartic acid derivative was chosen since it appears nearly halfway along all chromatographs, and is easily separated and identified with no interference from other amino acid derivatives and is constantly consistent from column to column.

The relative retentions of amino acids relative to aspartic acid is expressed as $r_1 r_2 = \frac{V_{R1}}{V_{R2}} = \frac{t_{R1} F_c}{t_{R2} F_c}$ where V_{R1} = retention volume of the amino acid and V_{R2} = retention volume of aspartic acid. Since the carrier gas flow remains constant throughout a normal separation it will cancel out of the above equation. Therefore only the relative retention times are required to describe a separation.

When the conditions of chromatography are constant, i.e. column

*E. Bayer, Gas Chromatography, Elsevier Publ. Co., Amsterdam (1961).
S. Dal Nogare and R.S. Juvet, Gas-Liquid Chromatography, Interscience Publ., New York (1962).

length and gas flow, the relative retention values remain constant varying slightly only with temperature changes. Thus, an amino acid can readily be identified by its relative retention value in relation to the other amino acids.



In the diagram AB = retention time of the unknown amino acid derivatives, AC = aspartic acid retention time and AB = relative retention time of unknown amino acid. Therefore aspartic acid would have a relative retention value of 1 and amino acids separating below aspartic acid would have values less than 1 while those appearing after aspartic acid would have values greater than 1. The temperature at which a particular amino acid appears must be known when recording the data.

2. Calculation of Efficiency of Columns.

When comparing columns it is necessary to appraise the efficiency or separating power of different columns. The separation power or factor is the ratio of the retention times of two components to be separated i.e. $\alpha = \frac{t_{R1}}{t_{R2}}$. A value of unity is obtained for two compounds which can not be separated. However frequently amino acid peaks are broad and overlap even if the separation factor is large. Therefore a better measurement would be one involving peak widths. When the peak widths and retention times are considered, the resolution of two amino acid derivatives can be calculated. Thus resolution

$$= 2 \frac{(\text{Difference between retention times})}{\text{sum of peak widths.}}$$

The peak widths are calculated from the recording chart in minutes. If the resolution approaches zero it is difficult to separate a pair of amino acids. The higher the value for resolution the better the separation of two particular amino acids. The resolution can often be improved by increasing the column efficiency.

Column efficiency is measured by the number of theoretical plates. A theoretical plate is a step at which perfect equilibration of the solute between the mobile and liquid phases is established. The greater the number of theoretical plates (n) the better the separation. Thus $n = 16 \frac{(\text{retention time})^2}{\text{peak width}}$ gives the number of theoretical plates required for the separation of a particular amino acid derivative.

In gas chromatography perfect equilibrium between the gas and liquid phase cannot be achieved. Only the height of a column that will give a separation equivalent to one theoretical plate can be calculated. This quantity is termed the height equivalent to one theoretical plate (i.e. HETP) and is calculated thus; $\text{HETP} = L/n$ where L is the effective length of the chromatographic column. The smaller HETP the better is the separation. The number of theoretical plates can be increased by increasing the length of a column.

Calculations of the number of theoretical plates and HETP are used in comparing the efficiency of different columns, while resolution is used in comparing the separation of two amino acids on different columns.

BIBLIOGRAPHY

- J. Baraud, Bull. Soc. France, 785 (1960)
- E. Bayer, K. A. Reuther and F. Born, Angew. Chem., 69, 640 (1957)
- M. Bier and P. Teitelbaum, Ann. N. Y. Acad. Sci., 72, 641 (1959)
- K. Blau and A. Darbre, Biochem. J., 88, 8P (1963)
- M. Brenner and R. W. Pfister, Helv. Chim. Acta, 34, 2085 (1951)
- P. A. Cruickshank and J. C. Sheehan, Anal. Chem., 36, 1191 (1964)
- R. A. Dewar, J. Chrom., 6, 312 (1961)
- E. Fischer, Ber. Deut. Chem. Ges., 34, 433 (1901)
- E. S. Gould, Mechanisms and Structure in Organic Chemistry, Holt, Rinehart and Winston, 295 (1959)
- I. R. Hunter, K. P. Dimick and J. W. Corse, Chem. & Ind. (London), 294 (1956)
- N. Ikekawa, J. Biochem. (Tokyo), 54, 279 (1963)
- S. Ishii and B. Witkop, J. Am. Chem. Soc., 85, 1832 (1963)
- H. Wamoto and S. Morimoto, Nippon Kagaku Zasshi, 85, 341 (1964)
- A. T. James and A. J. P. Martin, Biochem. J., 50, 679 (1952)
- D. E. Johnson, S. T. Scott and A. Meister, Anal. Chem., 33, 669 (1961)
- D. A. Landowne and S. R. Lipsky, Fed. Proc., 22, Part 1, 235 (1963)
- I. G. McWilliams and R. A. Dewar in D. H. Desty, Gas Chromatography, Butterworths Sci. Publ., London, p. 142 (1958)
- N. Melamed and M. Renard, J. Chrom., 4, 339 (1960)
- S. Moore and W. H. Stein, J. Biol. Chem., 211, 893 (1954)
- K. Ruhlman and W. Giesecke, Angew. Chem., 73, 113 (1961)
- H. A. Saroff and A. Karmen, Anal. Biochem., 1, 344 (1960)
- J. Wagner and G. Winkler, Z. Anal. Chem., 183, 1 (1961)

B. Weinstein and A. H. Fenselau, J. Chrom., 15, 149 (1964)

F. Weygand, E. Kolb, A. Prox, H. A. Tilak and I. Tomido, Z. Physiol. Chem., Hoppe-Seyler's, 322, 38 (1960)

F. Weygand and A. Ropsch, Chem. Ber., 92, 2039 (1959)

G. C. Youngs, Anal. Chem., 31, 1019 (1959)

A. Zlatkis, J. F. Oro and A. P. Kimball, Anal. Chem., 32, 162 (1960)

C. Zomzely, G. Marco and E. Emery, Anal. Chem., 34, 1414 (1962)