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Gas-chromatographic Analysis of Products of the Decomposition of Proteins and Nucleic Acids

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Various gas-chromatographic methods for analysing amino-acids, nucleic acid bases, nucleosides, and nucleotides, and also the use of pyrolytic gas chromatography to determine the composition of proteins and nucleic acids, are surveyed critically. A comparison is made of the best analytical results for mixtures of the above compounds by the use of gas-liquid chromatography and high-pressure liquid chromatography. A list of 243 references is included.

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I. INTRODUCTION

Molecular biology and kindred disciplines are unable to develop if the structures of proteins and nucleic acids, i.e. the sequences of monomeric units (amino-acids and nucleotides) in their molecules, are not established. Such a procedure is extremely laborious because of the high molecular weight of biopolymers. Therefore automation and acceleration of such determinations are highly significant.

Methods of liquid chromatography are widely used in separating mixtures of proteins and nucleic acids and in the qualitative and quantitative analysis of their decomposition products. However, the classic forms of such methods are laborious and insensitive, so that, immediately following the discovery of gas-liquid chromatography[1], research workers became interested in the possibility of using it to separate mixtures of amino-acids[2]. But the need to convert the latter into relatively unstable volatile derivatives presented considerable difficulties in the application of gas-liquid chromatography to their analysis.

In 1958 an automatic method was developed for analysing mixtures of α-amino-acids by ion-exchange chromatography[3-6] with a photocolorimeter to detect the amino-acid derivatives; the time required for a complete analysis was a few hours, when the sensitivity of a determination reached 10^{-7} mole. By the end of the 1960s the first models of high-pressure liquid chromatographs were manufactured with an ultraviolet-absorption detector, which shortened to 1 h the time required to analyse a mixture of α-amino-acids and improved the sensitivity to 10^{-9} mole. A limitation of these instruments is the need to obtain aromatic derivatives of amino-acids for detection by their ultraviolet spectra. It might appear that they would displace gas-liquid chromatography completely from laboratory research on biopolymers. However, this method possesses certain advantages over the high-pressure chromatographs—sensitivity enhanced by factors of 10^2–10^3, incomparably greater separating power, and cheaper apparatus (Table)—which explains the development of research on its application to the analysis of breakdown products of biopolymers and its widespread use together with high-pressure liquid chromatographs in practical biochemical investigations.

Comparison of optimum characteristics of gas-liquid chromatography and high-pressure liquid chromatographs in the analysis of degradation products from proteins and nucleic acids.

Characteristic	Gas-liquid chromatography	High-pressure liquid chromatograph
Separation of amino-acid mixtures:		
time of separation of 20 protein amino-acids	15 min	70 min
detection limit—average	10^{-8} g*	10^{-7} g
minimum	10^{-12} g**	10^{-8} g
stability of derivatives for analysis	moderate	high
resolution of optical isomers	possible	impossible
total duration of one determination	< 1 h	~1 h
Analysis of amino-acid phenylthiohydantoins:		
time of separation or identification	50 min	
limit of detection	10^{-10} g***	10^{-7} g
Analysis of amino-acid methylthiohydantoins:		
time of separation or identification	50 min	not used
Analysis of peptides:		
possible number of amino-acids in 1 mol.	4 (12****)	unlimited
establishment of structure	possible	impossible
Nucleic-acid bases:		
time of separation	24 min	
limit of detection	10^{-9} g	10^{-7} g
Cost of complete equipment, $1000	10	50

*Flame-ionisation detector.
**Electron-capture detector.
***Photometric flame detector.
****Theoretically possible number.

Aspects of the gas-chromatographic analysis of amino-acids and nucleic-acid bases (here and subsequently the Reviewer uses the term "nucleic-acid bases" for brevity to cover purine and pyrimidine bases, major and minor,

present in nucleic acids and their fragments—nucleotides and nucleosides) have been considered in > 500 papers. However, reviews on this topic either have become out of date[6-14] or deal with individual aspects[15-17]: relatively complete reviews of the gas–liquid chromatographic analysis of amino-acids have appeared only recently[18-20]. One of these[19] is concerned mainly with quantitative aspects of the analysis of amino-acids, and hardly mentions pyrolytic gas–liquid chromatography and the separation of hydantoin derivatives of the acids. Furthermore, several fundamentally new methods for the analysis of amino-acids and nucleic-acid bases have appeared during the past one or two years.

The present Review surveys critically methods for the gas-chromatographic analysis of amino-acids, nucleic-acid bases, nucleosides, nucleotides, peptides, proteins, and nucleic acids, and compares the best gas-chromatographic methods with results obtained by other chromatographic methods. Most of the above compounds cannot be determined directly by gas chromatography because they are non-volatile, so that attention is directed mainly to quantitative methods for obtaining volatile decomposition products of biopolymers†.

II. PREPARATION AND SEPARATION OF VOLATILE DERIVATIVES OF AMINO-ACIDS

Twenty α-amino-acids—termed "protein amino-acids"—may be present in proteins. Several other amino-acids are also met in Nature, and some derivatives are hormones. Amino-acids can be classified according to reactivity, which depends on the radical R attached to the amino-acid grouping $H_2N.CH.COOH$; the different groups are characterised by differences in the difficulty of obtaining volatile derivatives and of their gas-chromatographic separation. The first group—glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, and proline—exhibit minimal reactivity. A second group comprises threonine, serine, lysine, tyrosine, asparagine, and glutamine. The most active amino-acids—arginine, cysteine, tryptophan, and histidine—form the third group. In addition, several other amino-acids are encountered, e.g. norvaline, α-aminobutyric acid, norleucine, and cystine. Some amino-acids contain iodine (tyrosines); many contain sulphur.

When heated, amino-acids undergo decarboxylation to the corresponding amines. Over the temperature range 180–240°C members of the first group other than isoleucine are converted into amines containing one carbon atom fewer than in the original amino-acid; this applies also to threonine, serine, and norvaline[10]. Raising the temperature to 300°C forms complexes of ammonia with amines containing 1–5 carbon atoms[10]. Complete thermal decomposition of amino-acids results in the evolution of nitrogen, which was used earlier[10] for the qualitative identification of amino-acids. It is now more advisable to employ for this purpose thermionic detectors that are selective for nitrogen[21].

Deamination yields finally aldehydes, which are comparatively easy to determine by gas–liquid chromatography. The oxidation of amino-acids by sodium hypochlorite was examined[10] with α-aminobutyric acid, norvaline, valine, norleucine as examples, the resulting aldehydes being separated on a dinonyl phthalate column at 92°C. However, sodium hydrochlorite proved to be too strong an oxidant, causing degradation of the carbon skeleton, so that the yield of aldehyde from an amino-acid was not quantitative.

Ninhydrin was used to convert leucine and isoleucine into aldehydes, which were then separated on silicone oil at 69°C. Alanine, valine, isoleucine, leucine, norleucine, methionine, threonine, and α-aminobutyric acid were converted quantitatively into aldehydes after reaction for 15–30 min; glycine and phenylalanine were also converted into aldehydes. The amino-acids were oxidised by ninhydrin in a reaction vessel[10-22] comprising a column filled with a solid carrier impregnated with ninhydrin. Alanine, valine, norvaline, leucine, isoleucine, and norleucine were converted[10] into aldehydes with the reaction vessel at 125°C; the aldehydes were then separated on a glycerol–silicone oil column at 65°C.

Although the aldehyde method has not been successful with all protein amino-acids, it is extremely attractive in regard to the ease of separation of volatile derivatives. A recent paper[23] has reported the conversion of amino-acids into aldehydes by means of aqueous silver picolinate for analysis of a mixture of alanine, valine, leucine, isoleucine, proline, glycine, and norleucine, the aldehydes being separated on a polyethylene glycol 4000 column. A suggestion[10] that aldehydes obtained from amino-acids should be oxidised with permanganate to acids and then determined on a polypropylene glycol column at 150°C is hardly advisable, since acids are considerably more difficult than aldehydes to determine chromatographically. Attempts to use ninhydrin to obtain aldehydes from the second and third groups of amino-acids have proved unsuccessful.

Deamination occurs also on the addition of sodium nitrite in acetic acid; the resulting acid is esterified with diazomethane, and the methyl esters are separated on a silicone-oil column with temperature programming[18]. Examination of a mixture of glycine, alanine, valine, leucine, asparagine, and glutamine has shown[24] that the reaction is not quantitative, although the systematic error is constant within the range 2–10%. Esters of isoleucine, threonine, methionine, phenylalanine, histidine, and arginine have been obtained by the same method. The methyl esters from five amino-acids of the first group were separated on a capillary column with Apiezon L at 130°C.[18]

Decarboxylation of amino-acids by *N*-bromosuccinimide has been used to analyse derivatives of alanine, valine, leucine, phenylalanine, and norvaline[18]; the ratio of nitriles and aldehydes obtainable by this reaction varies within wide limits, but the former predominate. The products from alanine, valine, leucine, isoleucine, norleucine, norvaline, and α-aminobutyric acid were separated on a silicone-oil column. Nitriles of amino-acids are formed also by treating them with iodosobenzene; the reaction products were separated on polyethylene glycol and 1,2,3-tris-(2-cyanoethoxy)propane.[18]

The carboxy-group of an amino-acid is reduced to hydroxyl by lithium tetrahydroaluminate with formation of the corresponding aminoalcohol. Reduction products from the first group of amino-acids (other than proline) were separated on a column with 20% of SE-96 or SE-52 silicone oil over the range 155–200°C.[10] Under these conditions the aminoalcohol obtained from tryptophan does not emerge from the column, while the corresponding glycine derivative is masked by the solvent peak. A polar

†Because of limitations of space some of the references to original papers (mostly examples of applications) are quoted from published reviews.

stationary phase—polyethylene glycol 3000—has also been used to separate these products. This method has been employed to establish the composition of the hydrolysate from peptides [18].

Treatment of the first group of amino-acids with a mixture of hydrochloric and nitric acids yields α-chloro-derivatives, whose methyl esters are separated on a mixture of polyethylene glycol with silicone oil and stearic acid [18], a method used to detect norleucine and norvaline in natural products and to establish the structure of dipeptides [18]. Derivatives corresponding to phenylalanine and methionine are not recorded on the chromatogram when the amino-acids are converted into the methyl esters of α-chloro-acids, and treatment of serine gives two peaks. *N*-Thiocarbonyl derivatives have been obtained from the n-propyl esters of amino-acids; tryptophan does not undergo this reaction, while cystine and cysteine form only thiocarbamates [25].

Even the latter comparatively mild methods for obtaining volatile derivatives of amino-acids by removing active groups have not found application in practical analysis, owing to the difficulty of quantitative conversion of the second and third groups of amino-acids.

In the preparation of methyl esters of amino-acids in the presence of hydrogen chloride as catalyst the hydrochlorides of the esters are usually obtained. The free esters are formed by shaking the methanolic solution of the salt with an ion-exchange resin: the resulting solutions remained unchanged for 1–24 h. [10] Methyl esters containing a free amino-group were separated on a column of 2% of poly(neopentyl glycol succinate) on Fluoropak-80; diatomaceous carriers proved to be active for these systems. Great difficulties arose in the chromatographing of the methyl esters of tyrosine, histidine, and tryptophan. Attempts to convert the hydrochlorides into the free esters by thermal dissociation in the heated injector of the chromatograph have proved unsuccessful. Columns with the above sorbent have been used to separate derivatives of isomeric hydroxyprolines. Esters of amino-acids with C_{2-4} alcohols are relatively non-volatile and therefore less suitable for gas–liquid chromatography. Mass-spectrometric investigation of the structure of the ethyl esters of several amino-acids has shown [18] that all these derivatives are indeed ethyl esters, with the exception of ornithine, which cyclises into a lactam.

The most common method for obtaining esters is to treat the amino-acid with the anhydrous alcohol in the presence of hydrogen chloride; sometimes hydrogen bromide and sulphuric acid are used as catalyst. Alcohols can be replaced by derivatives of diazo-compounds. Moisture must be absent from reagents and from initial specimen for quantitative esterification. Small quantities of aqueous amino-acids can be dried by means of an electrically heated platinum spiral [26] in an atmosphere of an inert gas. Esterification is quantitative at 70°C after 30 min [23], although other régimes have been reported: 1.25 *N* methanolic hydrogen chloride at 20°C required 30 min [27]; the n-propyl ester of an amino-acid is formed by treatment with 8 *N* propanolic hydrogen chloride at 100°C for 20 min [28]. Acetyl chloride with methanol and an orthoformate in methanolic hydrogen chloride are also used as esterification catalysts. Another process for obtaining esters is to reflux an amino-acid and an alcohol in the presence of an ion-exchange resin [18]. Methylation by refluxing with dimethyl sulphite in methanolic hydrogen chloride is quantitative and rapid [18,29]; it can be effected also by thionyl chloride with methanol [30].

The sparing solubility of amino-acids in high alcohols complicates their esterification, and this applies especially to cysteine, lysine, and histidine. It has been proposed [18] to overcome such difficulties by initially obtaining the methyl esters and then transesterifying (e.g. with 1.25 *N* n-butanolic hydrogen chloride at 100°C for 150 min [23]). In practical analysis a more common method for dispersing amino-acids in C_{3-5} alcohols is the use of ultrasound: a suspension of the amino-acid in n-butanol was subjected to ultrasonic treatment for 15 s. [31] Recently, however, it has been found [32] that only 50% of the total quantity of amino-acid is esterified under these conditions, and therefore treatment of the suspension for 5 min is recommended, but more complete dissolution of the amino-acid can be effected by adding dichloromethane (10% on the total quantity). Esterification with n-butanol is accomplished in 3.5 *N* hydrogen chloride at 150°C for 15 min [32]; isoleucine is the most difficult to esterify (by 85%), but tryptophan is 15% decomposed; when esterification is prolonged to 2.5 h, isoleucine is completely esterified, but then tryptophan is also completely decomposed [31-36]. The dissolution of amino-acids in alcohols can be improved by adding trifluoroacetic acid; lysine hydrochloride is then completely esterified in a stream of dry hydrogen chloride at 108°C. [37] Amino-acids can be esterified also by means of isodobutane in the presence of methanolic phenyltrimethyl- or tetramethyl-ammonium hydroxide; this reaction takes place in dimethylformamide at room temperature within 10 min. [38]

Thus almost all derivatives of the protein amino-acids can be obtained quantitatively by esterifying the carboxy-group. However, such esters are either hydrochlorides or free esters of the amino-acids with free amino-groups: i.e. they contain at least one active group, which lowers the volatility of the derivative and causes appreciable adsorption on diatomaceous carriers of the compounds to be separated.

Formic acid in acetic anhydride has been used to acylate the second active group [10]. The resulting derivatives were separated on a column with 25% of a high-vacuum oil at 194°C, when conversion into the *N*-formylated methyl esters was at least 95%. The latter compounds contain no hydrogen atoms capable of forming a hydrogen bond, but such esters are highly polar, which prevents their effective chromatographic separation. Indeed, the molecule of an α-amino-acid comprises a radical and an amino-acid group, and such acids differ only in the nature of the radical, so that their successful separation by gas–liquid chromatography requires that the effect of the amino-acid group (free or esterified and acylated) on the retention of the compound should be minimal. This requirement is satisfied only when the amino-acid group is attached to the stationary phase merely by dispersion forces, i.e. with complete shielding of polar bonds by alkyl radicals. A methyl group usually shields polar bonds only to a slight extent. These considerations were confirmed by experiment: comparison of the gas-chromatographic behaviour of *N*-formyl and *N*-acetyl esters revealed [39] comparable retention times, but separation of the latter esters was incomparably better than that of the former, so that use of the *N*-formyl esters to analyse mixtures of amino-acids had to be rejected. The logarithm of the retention time of an ester of a C_{2-5} alcohol with an *N*-acetylated amino-acid is proportional to the molecular weight of the alcohol, whether the stationary phase used is polar or non polar [40]; this indicates fairly complete shielding of polar groups by the ethyl radical.

Acetic anhydride must be used to obtain acetyl derivatives, but its acylating power is not strong enough to acylate quantitatively the hydrochloride of an arginine ester even at 150°C. It is therefore proposed[28] to pass this ester through an ion-exchange resin or to neutralise the salt with an alkali-metal carbonate; it is also possible to convert arginine enzymatically into ornithine, and histidine by ozonolysis into asparagine[18], but such methods are too laborious to be recommended. It is more advisable to acylate amino-acid esters with a mixed reagent comprising acetone, triethylamine, and acetic anhydride in the proportions 5 : 2 : 1 [41] at 60°C for 30 s; arginine then undergoes 78% acylation reproducibly.

The gas-chromatographic behaviour has been studied of methyl and ethyl esters of *N*-acetylated amino-acids[10,39], propyl and isopropyl esters[10,28,41-44], butyl and isobutyl esters[10,44], and pentyl and isopentyl esters[10,45].

The best results for the separation of a mixture of protein amino-acids have been obtained[42] by esterifying the acids in 8 *N* n-propanolic solution at 110°C for 20 min, and acylating with a 5 : 2 : 1 mixture of acetone, triethylamine, and acetic anhydride at 60°C for 30 s. At room temperature the resulting esters do not decompose for several days, and at 4°C they can be kept for several months. The injector unit and the detector were respectively at 250 and 300°C, and the temperature of the column was programmed to rise from 125 to 180°C at 8 deg min^{-1}, and from 180 to 250°C at 32 deg min^{-1}. A column 90 cm in length and 3 mm in diameter was filled with a sorbent comprising a mixed stationary phase (0.31% Carbowax 20 M + 0.28% Silar 5CP + 0.06% Lexan) deposited on Chromosorb W. Before application of the stationary phase the carrier was heated at 400°C for 1 h. The efficiency of the column was 2250–2400 theoretical plates with respect to a tryptophan derivative, with norleucine as standard. The sample was introduced by means of a Perkin–Elmer automatic capsule injector. Under these conditions derivatives of all three groups of protein amino-acids were separated, as well as ornithine and hydroxyproline, with a detection threshold of 10^{-12} mole; the reproducibility of a quantitative analysis was 2–5%. Certain difficulties arose in the analysis of cystine, which have been overcome[46] by first converting cystine and cysteine into their *S*-methoxycarbonyl derivatives, when the sum of these amino-acids is determined. Similar mixtures had been separated in earlier work[43,44], but with poorer results. Separation of amino-acids as *N*-acetylated n-propyl esters has only recently been made possible by the appearance of new equipment and the development of new forms of high-temperature chromatography; in the early stages of the development of gas–liquid chromatography efforts were directed mainly at preparing more volatile derivatives of amino-acids, such as esters of the perfluorinated acids. The emergence of these derivatives from the column is more rapid than that of their non-fluorinated analogues by factors of 3–6, which is why amino-acids were analysed for a long time as such perfluorinated derivatives. The preparation and the gas-chromatographic properties of methyl esters of *N*-trifluoroacetylated amino-acids have been reported[18,35,47-56]; although derivatives of all 20 proteins amino-acids could not be separated in a single analysis, these investigations enriched significantly the experience of working with derivatives of perfluorinated esters. It was observed in particular that *O*- and *S*-trifluoroacetylated amino-acids decompose on polar stationary phases such as polyethers and cyano-derivatives.

Acylated amino-acid esters can in principle be obtained in two ways: (*a*) the ester is obtained initially, and then acylated; or (*b*) initial acylation is followed by esterification. In the latter case esters of hydroxy-acids are not formed, and arginine and histidine do not give volatile products; hence the first method is used for quantitative analysis. Hydrochloride salts of methyl esters of amino-acids are acylated quantitatively with trifluoroacetic anhydride at 120°C in 20 min[10]. It has been recommended[33,34,36] that the dry ester be acylated with a 50-fold excess of the anhydride at 180°C for 5 min; the most difficult to acylate are the arginine and histidine esters, while derivatives of cysteine and of a hydroxylated amino-acid are most liable to hydrolysis. It must be borne in mind that trifluoroacetylated esters are extremely sensitive to moisture, so that it is advisable to introduce the reaction mixture into the chromatograph injector without preliminary vaporisation of the reagents and the solvent. Evaporation of the solvent may involve an indeterminate loss of the most volatile esters of alanine, glycine, and valine, so that the temperature of the mixture should not exceed 0°C during removal of the solvent. Since the histidine derivative is the least stable, it is recommended[57] that it be converted into the *N*-ethoxycarbonyl-*N*-trifluoroacetylated n-butyl ester, which greatly improves its detection. Several authors[58-60] advise that, to prevent hydrolysis of the histidine derivative, excess of the acylating agent should be added before the sample is introduced, since its partial vaporisation from the reaction medium is responsible for the appearance on the chromatogram of a peak due to the mono-acylated histidine derivative[61]. At room temperature all protein amino-acids other than arginine and histidine are acylated within a short time.

In the temperature-programmed analysis of *N*-trifluoroacetylated methyl esters of the 20 protein amino-acids on a column with neopentyl glycol succinate, glycine and leucine remained unresolved, as well as glutamine and phenylalanine; furthermore, the arginine derivative gave three peaks, and considerable difficulties were experienced in the analysis of cysteine and histidine[18]. The best separation of *N*-trifluoroacetylated methyl esters of amino-acids is obtained with a column 3.25 m long full of Diatoport S with 1.5% of a mixed stationary phase (silicone oils XE-60, QF-1, and MS-200 in the proportions 46 : 27 : 27), the temperature being programmed from 90 to 231°C; under these conditions the histidine derivative does not emerge from the column. The time of separation is 85 min.

Widespread use of methyl esters is typical of the first stages in the application of perfluorinated derivatives of amino-acids, but their high polarity impairs separation, while the great volatility diminishes the accuracy of quantitative analysis, especially for the alanine, valine, glycine, and leucine derivatives. As a consequence of these disadvantages attention was directed to esters of alcohols having higher boiling points—ethyl[10], isopropyl[10,62,63], n-propyl[18,44], s-butyl[18,19,64,65], n-butyl[18,31,33,34,58,66-71], n-pentyl and isopentyl[18,37,72-74], and benzyl—as well as to vinyl esters[18].

The n-butanol derivatives have been most widely used for gas-chromatographic analysis, largely because of the work of the Gehrke school. The quite large alkyl radical shields the polar ester group well, and the derivatives possess moderate volatility. Such amino-acid derivatives have been completely separated[33,34] on two columns both 1.5 m long, one filled with Supelcoport impregnated with 2% of silicone oil OV-17 and 1% of silicone oil OV-210,

and the other filled with Chromosorb W impregnated with 0.65% of polyethylene glycol adipate, with temperature programming of the columns from 60 to 225°C. Histidine, arginine, and cysteine do not emerge from the latter column: the former column serves for their determination. Furthermore, hydroxyproline and ornithine are also separated, as well as all the protein amino-acids. With chromatograms from the two columns recorded in parallel the total time for separation does not exceed 30 min. The life of the columns is 2–6 months. Other workers have somewhat varied the quantities of stationary phases on these carriers [75], with 1% of polyethylene glycol adipate and 4.5% of silicone oil OV-17.

In the chromatographic analysis special attention is paid to absence of contact between vapours of the amino-acid derivatives and heated metal surfaces: the injector, the column, and the inlet to the detector should be made of glass. The carrier gas is dried with molecular sieves (the glutamine and asparagine derivatives are the first to be hydrolysed). All glass surfaces in contact with vapours of amino-acid derivatives, including the glass wool used to seal the columns, should be silylated with a 10% solution of dimethyldichlorosilane in toluene at room temperature for 10–12 h, then washed with methanol and toluene, and subsequently dried at 200°C. When not in use, the apparatus should be purged with the carrier gas at 200°C.

The above methods involve the use of small quantities of the stationary phase on a carrier. Under such conditions, however, the form of the retention volume is especially strongly influenced by the various adsorption effects due to the presence of active interfaces in the chromatographic column. The influence of interfacial adsorption on the retention volume of amino-acid derivatives is so great that variation in the quantity of stationary phase on the carrier over the range 0.5–2% changes the sequence in which the methionine, hydroxyproline, and phenylalanine derivatives emerge, as well as the leucine–isoleucine pair [76]. Such effects are extremely undesirable in establishing reliable reproducible methods for commercial analyses. The chief active interface in the separation of amino-acid derivatives is that between the solid carrier and the stationary phase, so that special care is required in the choice of carrier. When polyethylene glycol is used as stationary phase, the inertness of the carrier towards the derivatives increases in the sequence—Chromosorb W AW, Chromosorb W AW DMCS, Gasochrome Q, Chromosorb W HP or G HP [28,77]—but sometimes by no means the most inert carriers, e.g. Chromosorb W AW, [78] have proved quite unexpectedly to be the best. The arginine derivative emerges from a column with OV-17 deposited on Chromosorb W HP, but does not separate when a sorbent based on Chromosorb G HP is used.

A two-column scheme of analysis is not the optimum arrangement, for in particular two pen-recorders are required. The amino-acid derivatives could have been analysed successively on each column, and recorded on a single instrument, but this would lengthen to 50 min the time required for an analysis. The use of switches to transfer the recording from one column to the other and some staggering of the time of introduction of the sample into each column enables this time to be shortened to 35 min.[79]

The chief obstacle to a single-column analysis of protein amino-acids is the poor selectivity of stationary phases having little or no polarity; polyether stationary phases possess good selectivity for the separation of these compounds, but they react with *O*- and *S*-trifluoroacetylated amino-acids. A mixture could be separated only by increasing the quantity of stationary phase (Apiezon M) in the column to 10% on the weight of Chromosorb W.[80] Such quantities of stationary phase lower adsorption effects, which makes the method more reproducible. Eighteen derivatives of protein amino-acids, including cystine and hydroxyproline, have been separated in 35 min on a 2.5 m column with such a sorbent and the temperature programmed to rise from 90 to 260°C. A proportion of the derivatives (14% of methionine, 37% of histidine, and 54% of cysteine) does not emerge from the column, but these quantities are reproducible and do not diminish the value of the quantitative method. The relative error of a determination is 5%.

The use of simple devices to remove excess of reagent and solvent [62] enables down to 10^{-9} g of amino-acid to be determined by means of a flame-ionisation detector, and down to 10^{-12} g with an electron-capture detector. A thermal ionisation detector with rubidium salts, which detects nitrogen-containing compounds, is intermediate in sensitivity [81]. This last detector is extremely convenient for numerical calculations, since its signal is proportional to the number of nitrogen atoms in the molecule of the test compound, so that preliminary calibration is unnecessary; the sensitivity of such a detector exceeds that of a flame-ionisation detector by factors of 3–20. The sensitivity of the determination of amino-acids can be enhanced by acylation with a chlorophosphate in diethyl ether or in acetonitrile in the presence of triethylamine; the reaction takes 30 min at room temperature, although not all amino-acids give a quantitative yield of products. These derivatives are detected by means of a thermal ionisation detector having a threshold of 10^{-12} g.[82] Such derivatives of the first group of amino-acids, asparagine, glycine, and β-alanine are separated on a 2-m column containing 6% of silicone oil OV-17 on Chromosorb G HP with the temperature programmed from 210 to 250°C. This method has been used to establish the amino-acid sequence in the proteins of gramicidin S.

The extremely high sensitivity of gas chromatography has enabled amino-acids to be determined in specimens of the moon's surface brought back by the crews of Apollo 11 and 12.[83]

The time consumed in preparing volatile amino-acid derivatives is comparable with that required to separate these compounds: a series of papers therefore appeared on accelerating the preparation of samples. For example, three separately heated aluminium blocks can be used having depressions for ampoules, in which all operations to prepare derivatives are conducted [84]. The most radical solution to the problem was suggested to obtain methyl esters of *N*-heptafluorobutyrylated amino-acids [85] using an MS-41 (Perkin–Elmer) automatic capsule injector. The specimen and the methylating reagents were placed in a gold capsule, which was then closed; the process was conducted at 80°C for 10 min. The capsule was then opened, and moisture was removed by azeotropic distillation with dichloromethane. Hydrochlorides of methyl esters of amino-acids can be kept in a desiccator for a long time. Before an analysis an acylating agent was added to the capsule, and reaction occurred in the closed capsule at an elevated temperature; the cooled capsule was placed in the carrier of the automatic injector. Subsequent analytical operations and calculation of the results were effected automatically.

The search for amino-acid derivatives suitable for gas–liquid chromatography was not confined to the trifluoroacetyl compounds: acylation with pentafluoropropionic[59,86-89] and with heptafluorobutyric[59,61,85,86,90,91] acids was tried. The use of derivatives containing a large number of fluorine atoms enhances the sensitivity of an electron-capture detector, and enlarging the non-polar radical has a favourable influence on the selectivity of columns. The diminished polarity of the heptafluorobutyryl derivatives tends to shorten the retention time by 35% in comparison with the trifluoroacetyl compounds. The former are not so sensitive to moisture as the latter, and can be washed with organic solvents without danger of hydrolysis, while removal of the excess of reagent and solvent from the reaction mixture presents no difficulties[91]. For example, solutions of n-propyl esters of heptafluorobutyrylated amino-acids can be evaporated without risk of a change in quantitative composition of the amino-acid derivatives; at the same time the tendency of the solvent peak to mask the most volatile components of the amino-acid mixture becomes less marked. Among the disadvantages of heptafluorobutyric anhydride are its high cost, its toxicity, and its persistent unpleasant odour.

n-Propyl esters of heptafluorobutyryl derivatives of 19 protein amino-acids have been separated in 35 min on a glass capillary column with chemically bound polydimethylsiloxane and the temperature programmed from 70 to 230°C[159]; isoleucine forms two peaks, one due to isoleucine itself, and the other to alloisoleucine. Such esters from 18 amino-acids, including hydroxyproline and cystine, have been separated in 43 min on a 3.6-m glass column with 3% of silicone oil OV-1 and the temperature programmed from 100 to 250°C.[185] Determination of histidine is improved by introducing into the column, after addition of the sample, a small quantity of acetic anhydride; methionine and asparagine are not completely separated.

Eighteen protein amino-acids, pipecolic acid, ornithine, kynurenine, and cystine have been separated in 35 min as the isopentyl esters of the heptafluorobutyryl derivatives on a 3.5-m glass column with 3% of silicone rubber SE-30 and the temperature programmed from 70 to 240°C [18]; the sensitivity of the determination by means of a flame-ionisation detector is 10^{-10} g of the amino-acid. Large quantities of glutamine in the mixture impair its separation with lysine and tyrosine. Somewhat poorer results in separating the same amino-acid derivatives have been obtained[91] on a glass column 2.4 m long full of a carrier with 15% of Dexsil-300 and the temperature programmed from 140 to 270°C; serine and valine are not separated, but hydroxyproline gives two peaks, one of which corresponds to its allo-isomer. Isopentyl esters of heptafluorobutyryl derivatives have been regarded[61] as most suitable for the analysis of amino-acids, because preparation of the samples involves no loss of ester by vaporisation.

Perfluoroacyl derivatives esterified with various alcohols are widely used in the analysis of mixtures of non-protein amino-acids—those of peat[92], metabolites of tyrosine and tryptophan[93], iodinated[94] and cyanoethylated[18] amino-acids, mixtures of hydroxyproline and proline[18], and mixtures containing β-alanine[95]—and several publications[96-99] have dealt with the separation of acylated esters in the determination of amino-acids in various biological materials and individual aspects of such analyses. Thus esterification and acylation enable volatile derivatives to be obtained quantitatively from all protein and many non-protein amino-acids. Although a large proportion of the investigations have been concerned with separating volatile esters of perfluorinated acids, the most rapid determinations have been made by separating n-propyl esters of *N*-acetyl derivatives.

Because the use of acylated esters has the important disadvantage of involving two stages, efforts have been made to develop a single-stage process for obtaining volatile compounds, primarily trimethylsilyl derivatives of amino-acids. Silylating agents react both with the carbonyl and with amino- and hydroxy-groups, which makes it possible to conduct the whole treatment in a single stage. The amino-group is most difficult to silylate, which limits the overall rate. The first report on trimethylsilyl derivatives of amino-acids appeared in 1959,[100] almost simultaneously with the start of investigations on the separation of acylated esters. Trimethylchlorosilane was used as silylating agent for the first group of amino-acids, when the process was quantitative. All these derivatives, other than the methionine and proline esters, were separated on a column with silicone oil at 165°C; the cysteine, lysine, and histidine derivatives underwent partial decomposition in the column.

All silyl derivatives of amino-acids are extremely sensitive to moisture and to oxygen[15,101]: even a fresh rubber washer for the injector must be conditioned two or three times before an analysis to remove moisture. The great lability of trimethylsilyl derivatives of amino-acids and the difficulties of quantitative silanisation of all protein amino-acids led to a prolonged interruption of research on their application in gas–liquid chromatography. To increase the stability of the trimethylsilyl derivatives the starting materials must be carefully purified and the diethylamine that is formed must be removed from the reaction zone. Because of the inadequate silylating power of trimethylchlorosilane a search was begun for optimal silanising agents. A mixture of trimethylchlorosilane with hexamethyldisilazane has been used[15]. Leucine, serine, and asparagine are then the most difficult to silanise; their silanisation is improved by the use of trimethylsilyldiethylamine in the presence of aluminium and silicon oxides as catalysts. Detailed investigation showed that derivatives containing different numbers of trimethylsilyl groups are formed, those with more groups being less volatile, so that it is advisable to arrest the process in the early stages[102]. Various compounds—trimethylsilyldiethylamine, trimethylsilylamine, bistrimethylsilylacetamide, bistrimethylsilyltrifluoroacetamide, and *N*-methyl-*N*-trimethylsilylacetamide—have been proposed as silanising agents.

Amines proved the best agents when non-polar solvents were used[103]. *N*-Trimethylsilylimidazole and trifluoro-*N*-methyl-*N*-trimethylsilylacetamide have also been tried[15]. When polar solvents have been used, the best results have been obtained with amides[104], especially bistrimethylsilylacetamide. In separation on silicone stationary phases, however, the peaks of the glycine and alanine derivatives are masked by the solvent peak, so that the use of bistrimethylsilyltrifluoroacetamide is preferable[105,106]. A mixture of such derivatives of all protein amino-acids has been separated in 42 min on a 2-m column full of Supelcoport with 10% of silicone oil OV-11 and the temperature programmed from 80 to 210°C.[107-109]; ornithine and arginine are not separated. Lengthening the column to 6 m enables good separation to be obtained for all the mixture components and ornithine. Polyether stationary phases are not used in the separation of trimethylsilyl derivatives of amino-acids, since they

cause decomposition[110]. The separation of such derivatives of 18 protein amino-acids on a 3.7-m glass column with 3% of silicone oil DC-550 and the temperature programmed from 60 to 280°C required 24 min [111-113]; proline and glutamine were not separated.

Careful choice of esterification parameters is necessary in obtaining trimethylsilyl derivatives of amino-acids. The principal difficulties in quantitative esterification lie in the need for control at the stages of mono- and di-substitution in the amino-group; this is especially important with glycine, tryptophan, and lysine. If a non-polar solvent is used for silylation, glycine gives only the mono-substituted product, but arginine does not react; addition of 1% of trimethylchlorosilane to bistrimethylsilyltrifluoroacetamide enables arginine to be silanised, but disubstituted products of other amino-acids appear. In a polar solvent (acetonitrile) addition of trimethylchlorosilane can be avoided, but the time required for complete silanisation at 150°C is 2.5 h. With a polar solvent under optimum conditions the reaction is conducted for 1.5 h, which sharply diminishes the content of disubstituted products, with some incompleteness of reaction.

Several non-protein amino-acids undergo structural changes during silanisation. Thus cyclic products are formed from glutamic and $\alpha\gamma$-diaminobutyric acids[114]; derivatives of ω- and $\alpha\omega$-amino-acids emerge from the column as two peaks with different numbers of trimethylsilyl groups in the molecule.

Like the acylated ester derivatives, the trimethylsilyl compounds can be prepared in hermetically sealed gold capsules, which are then introduced into the chromatograph by means of an automatic injector.

The time required for the separation of protein amino-acids as their trimethylsilyl derivatives is double that required for the acylated esters. An undoubted advantage of the former is that they can be obtained in a single stage, but the duration of silanisation is far longer than for esterification and acylation. Furthermore, trimethylsilyl derivatives are rather less stable than the acylated esters; arginine is difficult to silanise, but histidine and tryptophan do not readily yield acylated esters; and the composition of a mixture of amino-acids can be determined more reliably by combining these two methods. Flame-ionisation and thermoionic detectors with a threshold of $\sim 10^{-9}$ g are usually employed for trimethylsilyl derivatives of amino-acids.

Trimethylsilyl derivatives of the amino-acids containing sulphur and selenium that are necessary for the efficient nutrition of animals can be separated by means of columns with silicone elastomer SE-30: at 117°C derivatives of cysteine, methionine, and their sulphur and selenium analogues are separated[115]. Selenium-containing amino-acids decompose when heated with bistrimethylsilylacetamide, and therefore the reagent "trisil" is used for their silanisation. With cysteine, cystine, homocystine, cysteic acid, and *L*-2-thiohistidine dark-coloured oxidation products appear on silanisation.

Trimethylsilyl derivatives of amino-acid esters have also been investigated[15]: silanised n-butyl esters of the 20 protein amino-acids have been separated in 32 min on a glass column containing glass beads with 0.2% of OV-17 at from 90 to 225°C; cysteine emerges together with hydroxyproline. However, the involvement of yet another stage, esterification, cannot be regarded as desirable for analytical work.

The difficulty of obtaining trimethylsilyl derivatives and their instability have resulted in continuous search for mild new methods of obtaining amino-acid derivatives. It has been proposed[116] that treatment of the hydrochlorides of the methyl esters with sodium carbonate, isobutyraldehyde, and a hydrogen sulphite should be followed by reduction to give stable esters resistant to hydrolysis. Another suggestion[117,118] is to introduce trifluoroacetylated amino-acids directly into the heated zone of the chromatograph injector, when volatile oxazolin-5-ones are formed. Eleven oxazolines of the first and second groups of amino-acids have been separated on a column with polyethylene glycol adipate deposited on Chromosorb W HP with the temperature programmed from 40 to 140°C. Cyanoethyl and cyanobutyl esters of amino-acids have been examined[119]. A mixture of dimethylformamide with a dialkyl acetal and 1,3-dichlorotetrafluoroacetone has been used to obtain volatile derivatives of amino-acids in a single stage[120]. This reagent alkylates the carboxyl and condenses with the amino-group; however, hydroxy-groups do not react, and additionally require acylation. The total time of the reaction is ~1 h, and the greatest difficulties are encountered in obtaining the asparagine derivative.

Halogenated acetones provide an extremely promising method for obtaining volatile derivatives of amino-acids. A rapid procedure developed recently[121] involves condensation with dichlorotetrafluoroacetone in weakly alkaline medium at room temperature to give a cyclic structure within a few minutes; both hydroxy-groups and also ω-amino-groups must be acylated. These derivatives are stable for several months, but the acylated derivatives for only a week (when kept at 4°C). A column 1.8 m long with 5% of silicone elastomer SE-30 and the temperature programmed from 150 to 230°C has been used; satisfactory separation is achieved also by means of silicone oil OV-17.[122] Amino-acids containing iodine have been determined by these methods.

It has been proposed[123] to determine tryptophan as the trimethylsilyl derivative of 2,3,4,5-tetrahydro-*S*-carboline-4-carboxylic acid on silicone elastomer SE-30. Methyl esters of *N*-isobutoxycarbonylated amino-acids (protein amino-acids other than arginine) have been separated on a column containing 0.7% of a mixed stationary phase (Poly-A-101A, Poly-A-103, and FFAP in the proportions 3 : 3 : 1).[124]

The above survey of various methods for obtaining volatile derivatives of amino-acids has shown that a convenient single-stage method is not yet available. However, the development of several methods, e.g. cyclisation, will probably enable stable volatile derivatives to be obtained in the near future.

III. SEPARATION OF OPTICAL ISOMERS OF AMINO-ACIDS

Optical isomers are resolved mainly by two methods: (1) derivatives of amino-acids with optically active reagents are obtained, and are resolved on the usual stationary phases; (2) volatile derivatives obtained without the use of optically active reagents are separated on an optically active stationary phase. A necessary condition for resolution by gas–liquid chromatography was the presence of at least two asymmetric carbon atoms either in the sorbate molecule or in a molecule of the stationary phase. This rule enables derivatives of hydroxy-amino-acids to be separated on the usually stationary phases[18], since the molecules of such amino-acids contain two optically active carbon atoms.

1. Separation of an Optically Inactive Stationary Phase

Any optically active alcohol, e.g. butan-2-ol or a homologue, can be used as reagent. Esterification is accomplished in a 4–8 *N* solution of hydrogen chloride in the alcohol at 100°C for 1 h. Esters of secondary alcohols and amino-acids of the first group, including asparagine, lysine, glutamine, and hydroxyproline, acylated with trifluoroacetic anhydride, were separated on a 50-m capillary column with polypropylene glycol or fluorosilicone oil FS-1265.[125,126] Optical isomers of derivatives of octan-2-ol are separated better than are those of butan-2-ol, but the former are less volatile, and amino-acids are sparingly soluble in the latter alcohol. Optical isomers of s-butyl esters of *N*-trifluoroacetylated protein amino-acids, other than histidine, arginine, and cysteine, were separated on a capillary column with polyethylene glycol or Yukon LB-550X.[127,128] Partial resolution of optical isomers of a mixture of alanine, valine, leucine, threonine, serine, cysteine, phenylalanine, and tyrosine as the s-butyl esters of the *N*-trifluoroacetylated derivatives has been effected on a 1.8-m column full of the carrier with 0.65% of polyethylene glycol adipate and the temperature programmed from 80 to 215°C. All these compounds, together with isoleucine, proline, and methionine, have been separated[129] under the same temperature conditions but with a lower content (0.32%) of polyethylene glycol adipate on the carrier. Splitting of peaks into optically active components was not observed for lysine and ornithine. The total time required to separate isomers of 17 amino-acids was 80 min. The use of sorbents with a small quantity of stationary phase is open to the same objections as in the separation of acylated esters.

Gas-chromatographic separation of optical isomers of amino-acids as s-pentyl and s-butyl esters has been regarded[129–133] as more advisable. The influence of the structure of the alcohols on the resolution of optically isomeric amino-acids has been examined with phenylalanine as example[134]: increasing the length ratio of the radicals attached to the asymmetric atom in the ester improves the resolution; elimination of branching of the carbon chain from the optically active carbon has a similar effect. The best separation has been obtained with the 1,2,2-trimethylpropyl esters, which forms the basis[135] of a method for separating optical isomers of 16 amino-acids (arginine, histidine, and cysteine were not studied). Volatile derivatives of these amino-acids were separated on a 3-m column full of Chromosorb W with 10% of silicone oil OV-17. Esters of other alcohols have also been used—those of pentan-2-ol to resolve valine isomers, methyl esters[2], and t-butoxycarbonyl derivatives treated with (*D*)-1,3-dimethylbutylamides.[18]

Esters of C_{5-8} secondary alcohols and acyl derivatives from trifluoroacetic, pentafluoropropionic, and heptafluorobutyric anhydrides have been examined[130] to determine optimum conditions for the gas-chromatographic separation of isomeric amino-acids. Optical isomers of asparagine (*N*-trifluoroacetylated 1,2,2-trimethylpropyl and *N*-perfluorobenzoylated s-butyl esters) have been resolved on a capillary column 50 m long with Yukon LB-550X [136–141] and with polyethylene glycol.[128]

An alternative method for obtaining optically active volatile derivatives of amino-acids is acylation with optically active agents. The carbonyl chlorides—α-chloropropionyl,[136,142,143] α-bromopropionyl,[144] α-chloroacetyl,[142,143] *N*-trifluoroacetylthiazolin-4-ylcarbonyl,[145] and *N*-trifluoroacetylprolyl [146–152]—have been used for this purpose. The applicability of the chlorides of various α-chloro-carboxylic acids for acylating amino-acids has been studied[143–153], and use of a polar stationary phase shown to give the best separation of isomers. α-Bromopropionic derivatives gave the best resolution of optical isomers of phenylalanine on a capillary column 46 m long with polypropylene glycol or silicone oil XE-60 [154]; however, difficulties were noted in preparing the optically pure reagent. Acylation of methyl esters of amino-acids with *N*-trifluoroacetyl-*L*(or *D*)-prolyl chloride is most widely used in practical analysis; this reagent is prepared by treating the *L*-proline with thionyl chloride[146]. Although the preparation is made under very mild conditions, racemisation of the product is not excluded[147]. Measures have been suggested[147] to control individual stages in the preparation with the aim of preventing racemisation.

Hence either esterification with optically active alcohols or acylation with optically active agents can be used to obtain optically active volatile derivatives of amino-acids for their subsequent separation by gas–liquid chromatography. Only capillary columns are normally employed in the former method, because of the small differences in the retention of optical isomers. Differences in the retention times of derivatives are larger in the latter case, and their separation is usually accomplished on packed columns.

2. Separation on Optically Active Stationary Phases

Optically active stationary phases can be divided into three groups—(*a*) ureides, (*b*) ethers, and (*c*) dipeptides—among which the last group find the greatest analytical use.

The separation of optically active isomers of amino-acids was first accomplished as their *N*-trifluoroacetyl-*L*-prolyl derivatives on an optically active stationary phase[146,154,155]; it was later shown that the best stationary phase is the cyclohexyl ester of *N*-trifluoroacetyl-*L*-valyl-*L*-valine.[156–158] The upper temperature limit at which this stationary phase can be used is 110°C, which rises by 10 deg when the same ester of *N*-trifluoroacetyl-*L*-phenylalanyl-*L*-leucine is employed[159,160]. Some results[161–165] indicate that the latter stationary phase retains its effectiveness up to 140°C, when the use of perfluoropropionylated isopropyl derivatives of amino-acids enables retention times to be shortened considerably. Mixtures of optical isomers of 17 amino-acids have been separated on short and long capillary columns (30 and 120 m) under isothermal conditions at temperatures of 110–125°C.[156–160]

The separation of derivatives of optical isomers of amino-acids on an optically active stationary phase has been attributed to the formation of triple hydrogen bonds between the interacting molecules, steric hindrance to hydrogen bonding being minimal if sorbate and sorbent belong to the same optical class. The selectivity of stationary phases is increased by using esters of alcohols having branched chains. Comparison of the separating properties of cyclohexyl esters of *N*-trifluoroacetyl-*L*-valyl-*L*-valine and *N*-trifluoroacetyl-*L*-valyl-*DL*-valine showed that better results are obtained when the latter stationary phase is used[168].

An appreciable disadvantage of optically active stationary phases is their low thermal stability: the maximum temperature at which tripeptides can be used does not exceed 120°C, while solid polypeptides, though stable up to 200°C, are incapable of resolving optically active isomers[18]. If the formula of an amino-acid derivative is

written $CF_3.CO.NH.CHR.COOR'$, the achievement of maximum isomeric selectivity requires that R > COOR'.[169,170]

Since differences in the retention of optical isomers are small, it is preferable to use capillary columns. For example, isobutyl esters of *N*-trifluoroacetamido-acids have been separated on a dipeptide optically active stationary phase in a capillary column 120 m long; a time of 70 min did not result in complete separation of the isomers of alanine, valine, threonine, isoleucine, serine, and leucine; and tyrosine and arginine isomers were resolved on a short (12 m) column[159]. Isoproyl esters of *N*-trifluoroacetamido-acids were separated on capillary columns 30–150 m long with the cyclohexyl ester of *N*-trifluoroacetyl-*L*-valyl-*L*-valine [108]; on the cyclohexyl ester of *N*-trifluoroacetyl-*L*-valyl-*L*-leucine at 110°C mixtures of isomers of valine, alanine, threonine, isoleucine, leucine, and proline were separated with a column length of 30 m in 130 min[167]. A mixture of isomeric isopropyl esters of *N*-trifluoroacetylated alanine, valine, threonine, isoleucine, leucine, serine, proline, and glycine has been separated on a column 120 m long with cyclohexyl *N*-trifluoroacetyl-*L*-α-aminobutyrate at 100°C.[136] Optical isomers of such ester derivatives of amino-acids have been resolved also on a glass column with the cyclohexyl ester of *N*-trifluoroacetyl-*L*-valyl-valine at 110°C,[171] a procedure that has been used for several applied investigations[18].

Each of the above methods for determining optically active isomers of amino-acids has its advantages. However, the first method requires reagents of high optical purity, and a highly efficient column must be used to separate esters of branched alcohols and amino-acids. The second method does not demand high optical purity of the stationary phase, but its possibilities are restricted by the relatively low limiting temperature at which such stationary phases can be used.

IV. SEPARATION OF PEPTIDES

The same methods as for amino-acids are used to protect active groups in peptides, while the thermal stability of the derivatives is limited by the temperature of rupture of peptide bonds (200–250°C). The possibility of the gas-chromatographic separation of peptides as volatile derivatives is interesting from the two aspects of (*a*) the extent to which large polymeric molecules possess the required volatility and (*b*) the separating power needed by a chromatographic column to separate all dipeptides (400 are possible for protein amino-acids). The reduction of peptides with lithium tetrahydroaluminate gives polyaminoalcohols, which are separated on a column with 8% of Apiezon L. This procedure has been used to separate mixtures of di-, tri-, and tetra-peptides.[18,160] Methyl esters of *N*-trifluoroacetylated penta- and hexa-peptides have been obtained, and finally volatile derivatives of peptides containing up to 12 amino-acids have been reported[172]. The thermal stability of peptides is largely determined by the nature of the monomer units, so that not all tri- and poly-peptides can be analysed by gas–liquid chromatography. Volatile derivatives of these compounds have been described[173–178].

Methyl esters of *N*-trifluoroacetylated dipeptides have been separated at 180–220°C, and the corresponding derivatives of tripeptides at 250°C.[18] The gas-chromatographic separation of esters of acetylated, trimethoxyacetylated, trifluoroacetylated, and pentafluoropropionylated derivatives of 37 dipeptides has been examined on various stationary phases[179]. Separation of dipeptides as their *N*-trifluoroacetylated esters is usually accomplished by high-temperature gas–liquid chromatography with a small quantity of stationary phase on the carrier and with temperature programming. Peptides containing arginine form volatile derivatives with difficulty. In several cases the inertness of the solid carrier surface plays a decisive part in separating volatile derivatives of dipeptides: the first few samples are chemisorbed, and preliminary introduction of three or four samples into the column is necessary to obtain quantitative information.

A highly promising approach is to combine mass spectrometry with gas chromatography to determine the composition of di- and poly-peptides. In contrast to liquid chromatography, the great efficiency of gas–liquid chromatography enables many isomeric compounds to be separated, and then their structure to be established mass-spectrometrically; moreover, by withdrawing separate portions of the chromatographic zones emerging from the column, it is possible by means of a mass spectrometer to establish the presence of unseparated compounds.

The above results indicate the great possibilities of the gas-chromatographic method for separating peptides containing up to 12 monomer units in the molecule. An especially promising approach to the analysis of peptides is to combine high-pressure liquid chromatographs and gas–liquid chromatography, which in many respects are complementary.

V. APPLICATION OF PYROLYTIC GAS CHROMATOGRAPHY

The principle of pyrolytic gas chromatography consists in almost instantaneous decomposition of a microsample (0.1–1 mg) at a controlled temperature. Only products of the initial degradation reactions are then observed, so that the composition of the test substance can be determined unambiguously. It is a qualitative method, suitable mainly for identifying a single compound or for establishing its structure.

Studies in which volatile derivatives of amino-acids were obtained by heating to 300°C were mentioned at the beginning of the Review. The use of higher temperatures in pyrolytic gas chromatography enables characteristic spectra of decomposition products (pyrograms) to be obtained[180–182]. With pyrolysis on a platinum spiral heated to redness and use of a squalane separating column, for example, several characteristic peaks were observed[183] for phenylalanine and alanine. In the absence of temperature control of the spiral, however, this method of pyrolysis is not generally applicable: histidine, tryptophan, and tyrosine do not form volatile products, and about half the protein amino-acids do not give characteristic pyrograms. Only the use of pyrolysers with strict temperature control (e.g. with ferromagnetic heaters having a definite Curie point or with electronic temperature control of the spiral) has enabled characteristic pyrograms to be obtained for amino-acids. Pyrolysis of protein amino-acids at 700°C with mass-spectrometric identification of the products will yield characteristic pyrograms, which show that the chief decomposition products from amino-acids are nitriles[184–187].

Lowering the pyrolysis temperature and using a column 2.4 m long with 5% of 1,2,3-tris-(2-cyanoethoxy)propane and temperature programming from −180 to 125°C gave different products[188,189]. The same study showed that the

pyrolysis spectra of ten different dipeptides were combinations of the characteristic pyrograms of their constituent amino-acids.

Investigation of the pyrolysis of equimolecular mixtures of amino-acids in an atmosphere of nitrogen at 850°C indicates [190-192] that the addition of non-aromatic amino-acids to those containing aromatic rings lowers appreciably the quantity of aromatic hydrocarbons among the pyrolysis products. However, this does not prevent qualitative determinations, since only the proportions of the decomposition products change, but the characteristic peaks on the pyrograms do not disappear. Hence the pyrolysis of peptides will provide information on their amino-acid composition; the temperature of pyrolysis may be either low (300°C) or high (about 800°C), and this temperature influences the composition of the products [193-196].

The determination of pyrolysis products is of interest also for study of the mechanism of the thermal breakdown of biopolymers. In several investigations [197-199] the products of pyrolysis at 500°C were identified by means of a mass spectrometer, and separation was effected on the polymeric sorbent Tenax with the temperature programmed from 25 to 285°C. At these temperatures aliphatic amino-acids undergo decarboxylation with the formation of amines and several other compounds; this is accompanied by condensation of the amino-acids with their conversion into dipeptides and subsequent formation of the corresponding dioxopiperazine. While losing ammonia, β-amino-acids are converted into unsaturated acids, whereas γ- and ω-amino-acids form on pyrolysis 2-oxopyrrolidines and 2-oxopiperidines respectively. The presence of large quantities of hydrogen cyanide among the pyrolysis products as a consequence of cyclisation reactions provides evidence of such a mechanism [197]. Since dioxopiperazines are quite volatile, it is of interest to use them as derivatives of the dipeptides for gas-chromatographic analysis. Thus products of the pyrolysis of dipeptides at 400°C have been separated on a column with 5% of silicone liquid OV-17 at 240°C. [198] The dioxopiperazine method is restricted by the need for condensation of the amino-acid (in the determination of individual amino-acids). This can be effected e.g. by heating the methyl esters of the amino-acids in molten phenol at 150°C. [192] Silanisation of the pyrolysis products with bistrimethylsilylacetamide at 80°C for 10 min permits the time required for the analysis to be greatly shortened and the symmetry of the peaks to be improved, which removes the difficulties encountered [19] in determining dioxopiperazines.

Pyrolytic gas chromatography has been used to determine the sequence of amino-acids in peptide molecules: pyrolysis of the initial peptide is followed by detachment of the terminal amino-acid and then pyrolysis of the product, when comparison of the two pyrograms enables the terminal amino-acid to be identified [200]. This method has been used to establish the primary structure of the proteins in four actinomycins [198]. Several amino-acid phenylthiohydrantoins have been studied by pyrolytic gas chromatography [201].

Pyrograms of proteins and nucleic acids have been obtained with pyrolysis temperatures of 800°C; although they contain the same numbers of characteristic peaks, the proportions of the latter can be used to identify the macromolecules. Such determination of proteins and nucleic acids is considerably simplified by use of a thermal ionisation detector sensitive to nitrogen; the pyrograms contain a total of six characteristic peaks, the relative heights of which enable the molecule to be identified. Furthermore, pyrolytic gas chromatography has proved an extremely effective procedure for classifying proteins, nucleic acids, individual cells, viruses, etc.; combination with mass spectrometry is widely used to study the nature of biopolymers [18].

VI. DETERMINATION OF THE SEQUENCE OF AMINO-ACIDS IN PROTEINS

Several methods of successively detaching terminal amino-acids have been developed to establish the primary structure of protein molecules. Pyrolytic gas chromatography can be used to determine the amino-acid composition of proteins, but the amino-acid sequence can be studied only by the application of mild methods. From two to five successively combined amino-acids can be determined in a single procedure: since the molecular weight of proteins may reach several millions, determination of the primary structure of a protein is obviously a most laborious operation.

Among several methods of detachment the most widely used is Edman's [18], in which the starting material is treated with phenyl isothiocyanate, after which the terminal group is split off in hydrochloric-acid medium; the final product is a phenylthiohydantoin, whose bicyclic molecule contains an imino-group, a carbonyl group, and the radical of the original amino-acid. The absence of a carboxy-group enhances the volatility of the derivative, while the presence of the benzene ring is extremely useful for application of a high-pressure liquid chromatograph, with which an ultraviolet detector is used. The phenylthiohydantoin derivative of only one amino-acid is obtained after each unit operation, so that the task of gas–liquid chromatography is to establish the nature of the derivatives from retention data. Demands on the analytical technique are increased when it is necessary to determine in one sample the products of 2–5 detachment stages without elution.

Amino-acids can be divided into three groups according to the volatility of the phenylthiohydantoin derivatives: (*a*) asparagine, glutamine, and cysteine give low volatility, and silanisation is necessary for their gas-chromatographic determination; (*b*) serine, threonine, and histidine give moderate volatility, and silanisation is desirable; and (*c*) silanisation of the remaining amino-acid phenylthiohydantoin derivatives makes no appreciable difference to their volatility. Silanisation is effected in a mixture of bistrimethylsilyltrifluoroacetamide with acetonitrile (1 : 1) and added pyridine [202]. The first separation of phenylthiohydantoin derivatives of amino-acids revealed [176,203] considerable difficulties in analysing the derivatives of serine, threonine, tyrosine, and hydroxylated amino-acids. The separation of 18 such derivatives has been reported [204]; silanisation has been used to increase their volatility [205]. These techniques have been applied to determine the sequence of amino-acids in proteins [18].

The analytical procedure for phenylthiohydantoin derivatives of amino-acids has been described in some detail [206]. As with the esters, contact with heated metal surfaces must be avoided. Silanisation of the derivatives directly in the column by successively injecting the silanising agent is recommended, but under these conditions not all such derivatives react: in particular, it is advisable to silanise those of asparagine, cysteine, and glutamine before introduction of the sample, while those of lysine and *S*-carboxymethylcysteine are extremely

difficult to silanise. The trimethylsilylated phenylthiohydantoin derivatives of asparagine, glutamine, and cysteine are adsorbed appreciably on the carrier, while the phenylthiohydantoin derivatives of tyrosine and tryptophan are not very volatile. A mixture of derivatives can be separated on a mixed stationary phase (11% SP-400 + 8% silicone liquid OV-210 + 1% OV-225) in a 1.2-m column with temperature programming from 165 to 270°C; complete separation is found with 13 derivatives. The sample is analysed twice—the initial mixture and the silanised products—to ensure reliable identification. Although the phenylthiohydantoin derivatives of serine, threonine, histidine, glutamine, asparagine, and lysine are partly decomposed in the column, this is not significant for qualitative analysis. The total time of separation is 40–50 min.

Trimethylsilylated phenylthiohydantoin derivatives of protein amino-acids can be partly separated on a column 3.25 m long containing 5% of silicone liquid OV-17 and 1% of Dexsil-300.[207] Nineteen of the twenty trimethylsilylated amino-acid phenylthiohydantoins have been separated on a capillary column of length 4.5 m and diameter 0.13 mm with a mixture of silicone liquids OV-101 and OV-225; the phenylthiohydantoin of histidine is evolved only at high temperatures, cysteine was converted into cystine, and arginine into ornithine[208]. Special difficulties arise in analysing the trimethylsilylated phenylthiohydantoin of arginine: on no stationary phase could escape of this derivative be achieved. The peak of the arginine phenylthiohydantoin emerges only after the derivative has been acylated with a mixture (4 : 1) of acetic anhydride and pyridine at 100°C for 5 min [209]; acylation can be effected also by introducing the phenylthiohydantoin sample with acetic anhydride directly into the heated injector of the chromatograph. Such derivatives have been used to establish the molecular structure of several proteins[18].

Volatile derivatives of amino-acid phenylthiohydantoins have been obtained also by treating proteins with perfluorophenyl isocyanate[210]. These derivatives of protein amino-acids other than arginine and histidine have been separated on a 1.2-m column full of Chromosorb W with 10% of silicone liquid DC-500 and 2% of OV-25 with temperature programming from 160 to 240°C.

The sensitivity and the selectivity of the gas-chromatographic determination of amino-acid phenylthiohydantoins are considerably enhanced by use of a flame-photometric detector, which records only compounds containing sulphur. This enables the aggregate fractions from removal of terminal amino-acids to be analysed successfully without the need to wash away the products after each stage. The sensitivity of a determination is 10^{-9} g of amino-acid phenylthiohydantoin.

Methylthiohydantoins have also been used to determine the amino-acid sequence in proteins; the process of sequencing is then simplified, and the greater volatility of these derivatives compared with the phenylthiohydantoins favours their gas-chromatographic separation. Silanisation is not essential at least for 16 amino-acid methylthiohydantoins, which can be separated on two columns, with silicone elastomer SE-30 and silicone liquid OV-17.[211] Five other methylthiohydantoins—those of asparagine, serine, arginine, and cystine—are determined only after silanisation, which is conducted in the same way as for the phenylthiohydantoins[207,212]. It is recommended[213] that a solution of bistrimethylsilylacetamide in acetonitrile be used at 20°C for 5 min; the yield of the histidine ester is doubled by introducing the sample with excess of the silylating agent. To complete the reaction the temperature is raised to 100°C and the exposure is extended to 10 min.[214,215] Nineteen amino-acid methylthiohydantoins have been separated[208] in 55 min on a capillary glass column 4.5 m long full of a mixed stationary phase (95% of silicone liquid OV-101 and 5% of OV-225) with the temperature programmed from 120 to 280°C. The initial compounds were treated with a mixture of bistrimethylsilyltrifluoroacetamide and 2% of trimethylchlorosilane, when several of the methylthiohydantoins formed disubstituted derivatives; that of arginine could not be determined. Optimum conditions for the separation of methylthiohydantoins of amino-acids (other than arginine) involve[214,215] a column 1.65 m long full of Gasochrome Q with 2% of silicone liquid OV-17 and the temperature programmed from 145 to 230°C, when the time of the determination is 45 min. Further separation of the pair of methylthiohydantoins of asparagine and phenylalanine requires 4.5 min.

The greatest difficulties arise in the silylation of the methylthiohydantoins of arginine and histidine. It is therefore recommended that these derivatives should be extracted with ethyl acetate, and then silanised and separated independently[204].

Another method used to determine the primary structure of proteins is 2,4-dinitrophenylation of the amino-acids[18]. The methyl esters of such derivatives of 13 amino-acids were separated on a 4-m column with 1.5% of silicone elastomer SE-30 and temperature programming from 170 to 230°C [216]; the threonine and serine derivatives were not separated. This method has been used to determine the amino-acid sequences in the proteins of malformin A and gramicidin A.[18]

VII. SOME APPLICATIONS OF GAS–LIQUID CHROMATOGRAPHY TO THE DETERMINATION OF AMINO-ACIDS IN BIOLOGICAL MATERIALS AND IN GEOLOGY

Difficulties arise in the analysis of biological media in connection with preparation of the sample and removal of interfering impurities. The most convenient procedure[35] for the gas-chromatographic analysis of a protein hydrolysate involves initial ultrasonic removal from the sample of air, which would impede the determination of methionine and cysteine. The protein is hydrolysed in 6 *N* hydrochloric acid at 145°C for 4 h (whereas in the usual process this operation lasts 18–24 h, with tryptophan, theonine, serine, tyrosine, methionine, and cysteine partly decomposing, while the links with valine and leucine are not completely hydrolysed). Inorganic acids are removed from the hydrolysate by means of an ion-exchange resin, and the residue is dried, esterified, and acylated. The amino-acid composition of ribonuclease and the protein of grains and fish has been determined in this way, in good agreement with analytical results obtained by other methods.

Protein hydrolysates have been analysed[217–219] by means of the isopropyl esters of the *N*-acetylated amino-acids, with 2,2-dimethoxypropane added to the initial mixture to diminish hydrolysis. The threshold of detection of amino-acids is 5×10^{-8} g. Gas–liquid chromatography has been used to analyse the amino-acid composition of diverse proteins, in particular of fossil animals, soil, and antibiotics.[18]

In addition, free amino-acids in various media can be determined by gas–liquid chromatography. It is recommended[33,34] that 0.1–0.4 ml of plasma be denatured with

4 volumes of saturated aqueous picric acid, the solution centrifuged, and the filtrate passed through a column with an ion-exchange resin (the column is washed first with water and then with aqueous ammonia). The view has been expressed[220,221] that this method is unsuitable for arginine and lysine, and it is therefore proposed that 0.1 ml of plasma be diluted with 10 volumes of 1 *N* acetic acid and passed through the ion-exchange column, whence amino-acids are washed out with water and aqueous ammonia; this involves loss of part of the amino-acids.

Histidine, histamine, *N*-methylhistamine, and *N*-dimethylhistamines have been separated as their trimethylsilyl derivatives and their trifluoroacetylated and heptafluorobutyrylated esters by means of silicone liquid OV-17.[222] The methyl esters of *N*-nitroso-derivatives of proline, sarcosine, and pinacolinic acid have been separated on a diethylene glycol succinate column at 180°C.[223]

Trimethylsilyl derivatives are widely used for analysing hormones and products of vegetable origin[18].

The extraordinarily high sensitivity of the gas-chromatographic method permits use of the component determination of amino-acids in microquantities on various materials, as well as in objects of extraterrestrial origin[224].

VIII. SEPARATION OF BASES OF NUCLEIC ACIDS, NUCLEOSIDES, AND NUCLEOTIDES

Heterocyclic bases—purines and pyrimidines—are present in nucleic acids. The carbonyl group is readily enolised, which is the reason for strong hydrogen bonding and correspondingly diminished volatility. Volatile derivatives of nucleic-acid bases are obtained mainly by silanisation.

The first studies on the separation of methylated derivatives of adenosine and uridine by gas–liquid chromatography appeared in 1964.[225,226] Better results were obtained with silanised derivatives[227]. Noteworthy attempts were made to separate nucleic-acid bases as triacetates[228]. Separation of trimethylsilyl derivatives of adenosine, cytidine, adenosine 5′-sulphate, 5′-monopropionate, and 2′-, 3′-, and 5′-monophosphates, and nicotinamide–adenine dinucleotide (NAD) has been accomplished[15] on a column with silicone elastomer SE-30 at temperatures from 228 to 249°C; silanisation may yield[229,230] several derivatives with different numbers of trimethylsilyl groups attached to the amino-nitrogen atom. Treatment of five nucleic-acid bases with bistrimethylsilylacetamide at 150°C for 45 min gives quantitative yields of trimethylsilyl derivatives; bistrimethylsilyltrifluoroacetamide reacts under the same conditions, and the reagent peak is more sharply distinguished from the product peaks. Trimethylsilyl derivatives of nucleic acids were separated on a 1-m column with 4% of silicone elastomer SE-30 and temperature programming from 95 to 230°C in 24 min; xanthin, 5-methylinosin, and hypoxanthin were also determined[212]. The minimum detectable quantity of nucleic-acid bases is $3–5 \times 10^{-9}$ g. A mixture of uridin, inosin, adenosine, xanthosin, and cytidine was separated under the same conditions, with an error of 3% in the determination; cytidine gave two peaks, and silylation of adenosine produced small quantities of a tetrakistrimethylsilyl derivative[229].

In the silanisation of thymin it is proposed[231] to deactivate excess of the silylating agent with methanol and remove in a vacuum substances that tend to promote hydrolysis. In the gas-chromatographic determination of trimethylsilyl derivatives of guanosine and adenosine it was recommended[175] first to obtain the methoxy-derivatives, and then to silanise them. Bistrimethylsilyltrifluoroacetamide derivatives of several minor nucleosides were obtained, and separated by gas–liquid chromatography[230]. A comprehensive investigation of the gas-chromatographic behaviour of trimethylsilyl derivatives of 32 nucleosides has used[232] two silicone stationary phases—OV-17 and SE-30.

Gas-chromatographic methods of analysing bases of nucleic acids and nucleosides are widely used in practical analysis[19,233–236].

In a technique[56] for the gas-chromatographic investigation of hydrolysates from ribonucleic and deoxyribonucleic acids at three levels—micro, semimicro, and semiquantitative analysis, with samples respectively of 10^{-3}, 10^{-4}, and 5×10^{-7} g—the hydrolysate is freed from perchlorates, phosphates, and other inorganic ions on an ion-exchange column, where ribose, which interferes with the determination, is also removed. Results of gas-chromatographic analysis agree well with those obtained by other methods.

Nucleic-acid bases are usually analysed by combining thin-layer chromatography with spectrophotometry, when semiquantitative results are obtained with a detection limit of 10^{-4} g, the time taken being considerably longer than in gas-liquid chromatography. Electrophoretic methods enable down to 5×10^{-5} g of nucleic-acid bases to be detected[18]. Gas-chromatographic determination of these bases enables the sensitivity of detection to be improved by factors of 10^2 and separation to be effected in a short time (24 min). An appreciable disadvantage of gas–liquid chromatography is the need to silylate the initial substances and the attendant difficulties.

Phosphorus was determined in nucleotides and nucleic acids[237–242] by silylating salts of phosphates of hexadimethyldisilazane with trimethylchlorosilane in pyridine at high temperatures. Derivatives are obtained also by treating the initial substance with a 10–20-fold excess of bistrimethylsilylacetamide or bistrimethylsilyltrifluoroacetamide, and are determined on a 1.8-m column containing silanised Chromosorb W impregnated with 5% of silicone liquid OV-101 at 155°C.[243]

Recent achievements of gas–liquid chromatography and high-pressure liquid chromatographs in the determination of proteins, nucleic acids, and their breakdown products enable conclusions to be reached on the limits of the application of these methods. With the time required for preparation of the sample taken into account, analysis of the 20 protein amino-acids is quicker by the former method, but derivatives for the latter are obtained more easily and are more stable. However, the separating power of gas–liquid chromatography is incomparably greater, so that this procedure is more advisable for analysing mixtures of protein and non-protein amino-acids; its greater separating power also enables optical isomers of amino-acids to be resolved. Sequencing is appreciably less laborious than with high-pressure liquid chromatographs. The extraordinarily high sensitivity makes gas–liquid chromatography unique in analysing traces of amino-acids, and combination with mass spectroscopy enables the structure of unknown amino-acids and peptides to be established.

The possibilities of gas–liquid and high-pressure liquid chromatography in separating nucleic-acid bases are comparable, but again the former is more sensitive. The Table lists comparative characteristics of the two methods

in analysing degradation products of proteins and nucleic acids. It is evident that these methods are largely complementary, but not mutually exclusive. The choice of method depends on the problems involved, the skill of the investigator, and the availability of the necessary equipment.

REFERENCES

1. A.T. James and A.J.P. Martin, Biochem. J., 50, 679 (1952).
2. J.R. Hunter, K.P. Dimick, and J.W. Corse, Chem. and Ind., 294 (1956).
3. S. Moore, D.H. Spackman, and W.H. Stein, Anal. Chem., 30, 1185 (1958).
4. D.H. Spackman, W.H. Stein, and S. Moore, Anal. Chem., 30, 1190 (1958).
5. P.B. Hamilton, Anal. Chem., 30, 914 (1958); 35, 2055 (1963).
6. P.B. Hamilton, D.C. Bouge, and R.A. Anderson, Anal. Chem., 32, 1782 (1960).
7. T. Hashidzume and U. Sasaki, Proteins, Nucleic Acids, Enzymes, 13, 735 (1968).
8. E.C. Horning and W. Vanden Heuvel, Ann. Rev. Biochem., 32, 709 (1963).
9. S.R. Lipsky and R.A. Landown, Ann. Rev. Biochem., 29, 649 (1960).
10. B. Potteau, Bull. Soc. chim. France, 3747 (1965).
11. P. Neri and P. Tarli, Quand. sclavo diagn. diu. e lab., 5, 1 (1969).
12. Z.E. Sikorski, Przem. spozywczy, 19, No. 12, 19 (1965).
13. B. Weinstein, in "Methods of Biochemical Analysis", edited by D. Glick, Wiley, New York, 1966, Vol. 14, p. 203.
14. J.R. Coulter and C.S. Hahn, in "New Techniques in Amino-acid, Peptide, and Protein Analysis" (Translated into Russian), Mir, Moscow, 1974, p. 85.
15. V. Marek, Chem. listy, 68, 250 (1974).
16. J. Rosmus and Z. Deyl, J. Chromatog., 70, 221 (1972).
17. V. Miller and V. Pacakova, Chem. listy, 67, 1121 (1973).
18. P. Husek and K. Maček, J. Chromatog., 113, 139 (1975).
19. S.V. Vitt, M.B. Saporovskaya, G.V. Avvakumov, and V.M. Belikov, Uspekhi Khim., 45, 548 (1976) [Russ. Chem. Rev., No. 3 (1976)].
20. R. Kaiser and A. Prox, in "Amino-acids, Peptides, and Proteins", edited by T. Deveny and J. György (Translated into Russian), Mir, Moscow, 1976, p. 115.
21. R.F. Coecard and P. Smith, J. Chromatog., 61, 329 (1971).
22. T.G. Clarke, N.A. Hampson, J.B. Lee, J.R. Morley, and B. Scanton, J. Chem. Soc., 815 (1970).
23. A.J. Cliffe, N.J. Berridge, and D.R. Westgarth, J. Chromatog., 78, 333 (1973).
24. D.B. Lakings and C.W. Gehrke, J. Chromatog., 62, 347 (1971).
25. B. Halpern, V.A. Close, A. Wegmann, and J.W. Westley, Tetrahedron Letters, 3119 (1968).
26. B. Teuwissen and A. Darbre, J. Chromatog., 49, 298 (1970).
27. D.L. Stalling and C.W. Gehrke, Anal. Biochem., 18, 118 (1967).
28. J.R. Coulter and C.S. Hahn, in "New Techniques in Amino-acid, Peptide, and Protein Analysis" edited by A. Niederwisser and G. Pataki, Ann. Arbor Sci. Publ., Ann Arbor, Mich., 1971, p. 75.
29. S.R. Tannenbaum, W.G. Tilly, and P. Issenberg, Analyt. Chem., 40, 1723 (1968).
30. R.E. Summons, W.E. Pereira, W.E. Reynolds, T.C. Rindfleish, and A.M. Duffield, Anal. Chem., 46, 582 (1974).
31. D. Roach and C.W. Gehrke, J. Chromatog., 43, 303 (1969).
32. P. Carcalon and J.D. Klingman, J. Chromatog. Sci., 12, 349 (1974).
33. C.W. Gehrke, K. Kuo, and R.W. Zumwalt, J. Chromatog., 57, 209 (1971).
34. C.W. Gehrke, R.W. Zumwalt, and K. Kuo, J. Agric. Food Chem., 19, 605 (1971).
35. F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt, and K.C. Kuo, J. Chromatog., 94, 113 (1974).
36. D. Roach and C.W. Gehrke, J. Chromatog., 44, 269 (1969).
37. K. Blau and A. Darbre, J. Chromatog., 26, 35 (1967).
38. R.H. Greeley, J. Chromatog., 88, 228 (1974).
39. S.V. Shlyapnikov, M.Ya. Karpeiskii, and A.I. Yakushina, Biokhimiya, 30, 457 (1965).
40. S.C. Fu and D.S.H. Mak, J. Chromatog., 78, 211 (1973).
41. J.R. Coulter and C.S. Hahn, J. Chromatog., 36, 42 (1968).
42. R.F. Adams, J. Chromatog., 95, 189 (1974).
43. L.J. Everett and J. Graff, Amer. Lab., 3, 51 (1971).
44. R.E. McGregor, G.M. Britten, and M.S. Sharon, Clinica Chim. Acta, 48, 65 (1973).
45. J. Metz, W. Ebert, and H. Weicner, Chromatographia, 2, 259 (1971).
46. J. Moodie and R. George, J. Chromatog., 124, 315 (1976).
47. J. Monjardino, Anal. Biochem., 21, 308 (1967).
48. M. Butler and A. Darbre, Biochem. Soc. Trans., 1, 610 (1973).
49. M. Greer and C.M. Williams, Anal. Biochem., 19, 40 (1967).
50. A. Islam and A. Darbre, J. Chromatog., 43, 11 (1969).
51. E. Bayer and W.A. Koenig, J. Chromatog. Sci., 7, 95 (1969).
52. A.J. Cliff, N.J. Berridge, and D.E. Westgarth, J. Chromatog., 78, 333 (1973).
53. A. Islam and A. Darbre, J. Chromatog., 71, 223 (1972).
54. M. Butter and A. Darbre, J. Chromatog., 101, 51 (1974).
55. C. Hedcoth and M. Jocoban, Anal. Biochem., 25, 55 (1968).
56. D.L. Stalling, C.W. Gehrke, and R.W. Zumwalt, Biochim. Biophys. Acta, 31, 616 (1968).
57. J. McArthur, J. Chromatog., 99, 445 (1974).
58. C.W. Gehrke, D. Roach, R.W. Zumwalt, D.L. Stalling, and L.L. Wall, "Quantitative GLC of Amino-acids in Protein and Biological Substances", Anal. Biochem. Lab., Columbia, 1968.
59. J. Jönsson, J. Eyem, and J. Sjöquist, Anal. Biochem., 51, 204 (1973).
60. C.W. Moss, M.A. Lambert, and F.J. Diaz, Anal. Biochem., 44, 458 (1971).
61. J.P. Zannetta and G. Vingendon, J. Chromatog., 76, 91 (1973).

62. R. P. Collins and K. Kalnins, Phyton, 29, 89 (1972).
63. W. A. Koenig, W. Parr, H. C. Lichtenstein, E. Bayer, and J. Oro, J. Chromatog., [no details given].
64. G. E. Pollock and L. H. Frommhagen, Anal. Biochem., 24, 18 (1968).
65. H. Bober, Beckman Rept., No. 2, 10 (1973).
66. C. W. Gehrke and D. L. Stalling, Separation Sci., 2, 101 (1967).
67. C. W. Gehrke, R. W. Zumwalt, and L. L. Wall, J. Chromatog., 37, 398 (1968).
68. C. W. Gehrke and K. Leimer, J. Chromatog., 53, 195 (1970).
69. H. H. Hediger, R. L. Stevens, H. Brandenberger, and K. Schmid, Biochem. J., 133, 551 (1973).
70. N. Lachovitzki and B. Björklud, Anal. Chem., 38, 446 (1970).
71. F. Marucci and F. Mussini, J. Chromatog., 25, 11 (1966).
72. T. Bhatti and J. R. Clamp, Biochim. Biophys. Acta, 229, 293 (1971).
73. A. Darbre and K. Blau, J. Chromatog., 29, 49 (1967).
74. D. L. Stalling and C. W. Gehrke, Biochem. Biophys. Res. Comm., 22, 329 (1966).
75. P. Morard, M. Garcia, and J. P. Cabassy, Analysis, 3, 451 (1975).
76. M. Stefanovic and B. L. Walker, Anal. Chem., 39, 710 (1967).
77. E. D. Smith, F. M. Oathout, and G. T. Cooh, J. Chromatog. Sci., 8, 291 (1970).
78. B. Thom and J. W. Parsons, J. Chromatog., 90, 370 (1974).
79. A. Applequist and B. M. Nair, J. Chromatog., 124, 239 (1976).
80. C. W. Gehrke and H. Takeo, J. Chromatog., 76, 66 (1973).
81. W. A. Aue, C. W. Gehrke, R. C. Tindle, D. L. Stalling, and C. D. Ruyle, J. Gas Chromatog., 5, 381 (1967).
82. G. Erlingshausen, C. W. Gehrke, and W. A. Aue, Separation Sci., 2, 681 (1967).
83. R. W. Zumwalt, K. Kuo, and C. W. Gehrke, J. Chromatog., 57, 193 (1971).
84. J. M. L. Mee and C. C. Brooks, J. Chromatog., 62, 138 (1971).
85. B. Kolb and W. Hoser, Chromatographia, 6, 28 (1973).
86. C. W. Moss, M. A. Lambert, and F. J. Diaz, J. Chromatog., 60, 134 (1971).
87. D. D. Clarke, S. Wilk, and S. E. Gittlow, J. Gas Chromatog., 4, 310 (1967).
88. G. E. Pollock, Anal. Chem., 39, 1194 (1967).
89. E. Anggard and G. Sedvall, Anal. Chem., 41, 1250 (1969).
90. S. L. McKenzie and T. Tenaschuk, J. Chromatog., 97, 19 (1974).
91. C. W. Moss and M. A. Lambert, Anal. Biochem., 59, 259 (1974).
92. F. D. Ganchel, K. Beyermann, and R. K. Zahn, Z. Analyt. Chem., 259, 177 (1972).
93. P. W. Albro and L. Fishbein, J. Chromatog., 55, 297 (1971).
94. F. Sharokhi and C. W. Gehrke, J. Chromatog., 36, 31 (1968).
95. P. Tarli, S. Benocci, and P. Neri, Farmaco Ed. prat., 25, 504 (1970).
96. L. Campos, M. Renard, and M. Severin, Amer. Inst. Super. Agron., 31, 151 (1968–1970).
97. A. Daussin and R. Bourdo, Ann. Biol. clin., 30, 417 (1972).
98. J. M. Gee, J. Chromatog., 87, 155 (1973).
99. C. W. Gehrke and H. Takeo, J. Chromatog., 76, 77 (1973).
100. K. Rühlmann, J. prat. Chem., 4, 86, 515 (1959).
101. E. D. Smith, K. E. Sorrells, and R. Q. Swinea, J. Chromatog. Sci., 12, 101 (1974).
102. A. E. Pierce, "Silylation of Organic Compounds", Pierce Chem. Co., Ill., 1968, p. 221.
103. E. D. Smith and K. L. Shewbart, J. Chromatog. Sci., 7, 704 (1969).
104. L. Bikofer and M. Donike, [J. Chromatog.,], 26, 270 (1967).
105. D. L. Stalling, C. W. Gehrke, and R. M. Zumwalt, Biochem. Biophys. Res. Comm., 31, 616 (1968).
106. E. D. Smith, J. M. Oathout, and G. T. Cook, J. Chromatog. Sci., 8, 291 (1970).
107. C. W. Gehrke and K. Leimer, J. Chromatog., 90, 370 (1974).
108. C. W. Gehrke, H. Nakamoto, and R. W. Zumwalt, J. Chromatog., 45, 24 (1969).
109. C. W. Gehrke and K. Leimer, J. Chromatog., 53, 201 (1970).
110. S. N. Alan and R. H. Hall, Anal. Biochem., 40, 424 (1971).
111. K. Bergström and J. Gütler, J. Chromatog., 26, 481 (1967).
112. K. Bergström, J. Gütler, and R. Blomstrand, Anal. Biochem., 34, 74 (1970).
113. R. L. Stern, B. L. Karger, W. J. Keane, and H. C. Rose, J. Chromatog., 39, 17 (1969).
114. K. Bergström and J. Gütler, Acta Chem. Scand., 25, 175 (1971).
115. K. A. Caldwell and A. L. Tappel, J. Chromatog., 32, 635 (1968).
116. J. M. Davis and A. Furst, Anal. Chem., 40, 1910 (1968).
117. O. Grahl-Nelsen and E. Solheim, J. Chem. Soc. Chem. Comm., 1092 (1972).
118. O. Grahl-Nelsen and E. Solheim, J. Chromatog., 69, 366 (1972).
119. E. Iwamoto and S. Morimoto, Nippon Kagaku Zasshi, 85, 341 (1964).
120. J. P. Thenot and E. C. Horning, Anal. Letters, 5, 519 (1972).
121. P. Husek, J. Chromatog., 91, 475 (1974).
122. O. Grahl-Nelsen and E. Solheim, Anal. Chem., 47, 333 (1975).
123. B. S. Middeditch, Anal. Letters, 8, 397 (1975).
124. M. Makita, S. Jamamoto, M. Kono, K. Sakai, and M. Shiraishi, Chem. and Ind., 355 (1975).
125. E. Gil-Av, R. Charles, and G. Fischer, J. Chromatog., 17, 408 (1965).
126. B. Halpern and J. W. Westley, Austral. J. Chem., 19, 1533 (1966).
127. G. E. Pollock, A. K. Miyamoto, and V. J. Oyama, "Life Sciences and Space Research", North-Holland, Amsterdam, 1970, Vol. 8, p. 99.
128. G. E. Pollock and A. H. Kawauchi, Anal. Chem., 40, 1356 (1968).
129. F. Raulin and B. N. Khare, J. Chromatog., 75, 13 (1973).
130. G. E. Pollock, Anal. Chem., 44, 2368 (1972).
131. K. A. Kvenvolden, E. Peterson, and G. E. Pollock, Nature, 221, 141 (1969).
132. K. A. Kvenvolden, J. C. Lawless, and C. Ponnomperuna, Proc. Nat. Acad. Sci. U.S.A., 68, 486 (1971).

133. K. A. Kvenvolden, E. Peterson, and F. S. Brown, Science, 169, 1079 (1970).
134. J. W. Westley, B. Halpern, and B. L. Karger, Anal. Chem., 40, 2046 (1968).
135. G. S. Ayers, R. E. Monroe, and J. H. Mossholder, J. Chromatog., 63, 256 (1971).
136. P. Y. Howard and W. Parr, Chromatographia, 7, 283 (1974).
137. S. Lande and R. A. Landown, Tetrahedron, 22, 3085 (1966).
138. S. Nakaparskin, E. Gil-Av, and J. Oro, Anal. Biochem., 33, 374 (1970).
139. H. Okamo and T. Okuyama, J. Chem. Soc. Japan, 90, A46, 834 (1969).
140. W. Parr and P. Howard, J. Chromatog., 66, 141 (1972).
141. G. E. Pollock, V. J. Oyama, and R. D. Johnson, J. Gas Chromatog., 3, 174 (1965).
142. B. Halpern, J. Richs, and J. W. Westley, Austral. J. Chem., 20, 389 (1967).
143. B. Halpern, J. W. Westley, and B. Weinstein, Nature, 210, 259 (1966).
144. R. A. Landowne, Chim. analyt., 47, 589 (1965).
145. B. Halpern, J. W. Westley, J. Wredenhagen, and J. Lederberg, Biochem. Biophys. Res. Comm., 20, 710 (1965).
146. B. Halpern and J. W. Westley, Biochem. Biophys. Res. Comm., 19, 361 (1965).
147. W. A. Bonner, J. Chromatog. Sci., 10, 159 (1972); 11, 101 (1973).
148. J. C. Dabrowiak and D. W. Cooke, Anal. Chem., 43, 791 (1971).
149. E. Bayer, E. Gil-Av, W. A. König, S. Nakaparksi, J. Oro, and W. Parr, J. Amer. Chem. Soc., 92, 1978 (1970).
150. H. Iwase and A. Murai, Chem. and Pharm. Bull. (Japan), 22, 8 (1974).
151. H. Iwase, J. Chromatog., 93, 233 (1974).
152. E. Solem, E. Jellum, and L. Eldjarn, Clinica Chim. Acta, 50, 393 (1974).
153. T. Nambara, J. Goto, K. Taguolu, and T. Iwata, J. Chromatog., 100, 184 (1974).
154. M. Halpern and J. W. Westley, Chem. Comm., 246 (1965).
155. B. Halpern and J. W. Westley, Tetrahedron Letters, 21, 2282 (1966).
156. E. Gil-Av, B. Feibush, and R. Charles-Sigler, in "Gas Chromatography", edited by A. B. Littlewood, Institute of Petroleum, London, 1967, p. 227.
157. E. Gil-Av and B. Feibush, Tetrahedron Letters, 35, 3345 (1967).
158. S. Nakaparskin, P. Birrel, E. Gil-Av, and J. Oro, J. Chromatog. Sci., 8, 177 (1970).
159. W. A. Koenig, W. Parr, H. O. Lichtenstein, E. Bayer, and J. Oro, J. Chromatog. Sci., 8, 183 (1970).
160. K. Biemann and W. Vetter, Biochem. Biophys. Res. Comm., 3, 578 (1960).
161. W. Parr, J. Pleterski, C. Young, and E. Bayer, in "Advances in Chromatography", edited by Z. Zlatkis, University of Houston, Houston, Texas, 1970, p. 277.
162. W. Parr, J. Pleterski, C. Young, and E. Bayer, J. Chromatog. Sci., 9, 141 (1971).
163. W. Parr, J. Pleterski, C. Young, and E. Bayer, J. Chromatog., 50, 510 (1970).
164. W. Parr, J. Pleterski, C. Young, and E. Bayer, J. Chromatog. Sci., 8, 591 (1970).
165. W. Parr, C. Young, E. Bayer, and E. Gil-Av, see Ref. 161, p. 287.
166. W. Parr, C. Young, E. Bayer, and E. Gil-Av, J. Chromatog. Sci., 8, 591 (1970).
167. W. Parr and P. Howard, Chromatographia, 4, 162 (1971).
168. J. A. Corbin, J. E. Rhoad, and L. B. Rogers, Anal. Chem., 43, 327 (1971).
169. B. Feibush, E. Gil-Av, and T. Tamari, Israel J. Chem., 8, 50 (1970).
170. R. L. Stein, B. L. Karger, W. J. Keane, and H. C. Rose, J. Chromatog., 39, 17 (1969).
171. W. A. Koenig and G. J. Nicholson, Anal. Chem., 47, 951 (1975).
172. B. C. Pettitt and J. E. Stouffer, J. Chromatog. Sci., 8, 735 (1970).
173. H. R. Morris, FEBS Letters, 22, 257 (1972).
174. H. R. Morris, R. J. Dickinson, and D. H. Williams, Biochem. Biophys. Res. Comm., 51, 247 (1973).
175. M. Jacobson, J. F. O'Brien, and C. Hedicoth, Anal., Biochem., 25, 363 (1968).
176. F. Weygand, A. Prox, W. König, and H. H. Fessel, Angew. Chem., 75, 724 (1963).
177. E. Fahr, R. Pastille, and M. Mageri, Z. Naturforsch., 28b, 454 (1973).
178. Yu. Ovchinnikov and A. A. Kiryushkin, FEBS Letters, 21, 300 (1972).
179. H. Lindley and P. C. Davis, J. Chromatog., 100, 117 (1974).
180. E. Kirret, E. Kyullik, and M. Lewis, Izv. Akad. Nauk SSSR, Ser. Khim. Geol., 19, 183 (1970).
181. J. Ulehla, Sb. českosl. akad. zemed. ved. zyvoc. vyroba, 5, 567 (1960).
182. L. N. Winter and P. W. Albro, J. Gas Chromatog., 2, 1 (1964).
183. J. Janak, Coll. Czech. Chem. Comm., 25, 1780 (1960).
184. J. Volimin, P. Kreimler, J. Omura, J. Seible, and W. Simon, Microchem. J., 11, 73 (1966).
185. M. A. Ratcliff, E. E. Madley, and P. G. Simmonds, J. Org. Chem., 39, 1481 (1974).
186. P. G. Simmonds, E. E. Madley, M. A. Ratcliff, and G. P. Shulman, Anal. Chem., 44, 2060 (1972).
187. P. G. Simmonds, G. P. Shulman, and C. H. Stembridge, J. Chromatog. Sci., 7, 36 (1969).
188. C. Merritt and D. H. Robertson, J. Gas Chromatog., 5, 96 (1967).
189. L. N. Winter and P. W. Albro, J. Gas Chromatog., 2, 1 (1964).
190. J. M. Patterson, M. L. Baedecher, R. Musik, and W. T. Smith, Tobacco Sci., 13, 26 (1969).
191. J. M. Patterson, W. Chen, and W. T. Smith, Tobacco Sci., 15, 98 (1971).
192. J. M. Patterson, N. F. Naidar, E. P. Papadopulos, and W. T. Smith, J. Org. Chem., 38, 663 (1973).
193. W. T. Smith, T. B. Harris, and J. M. Patterson, J. Agric. Food Chem., 22, 480 (1974).
194. E. B. Higman, J. Sehmeltz, and W. S. Schlofzhaner, J. Agric. Food Chem., 18, 636 (1970).
195. M. C. Stack, Biochem. J., 96, 56P (1965).
196. M. V. Stack, J. Gas Chromatog., 5, 22 (1967).
197. W. R. Johnson and J. C. Kang, J. Org. Chem., 36, 189 (1971).
198. B. Manger, Chem. Comm., 39 (1971).
199. A. B. Manger, J. Chromatog., 37, 315 (1968).
200. R. A. W. Johnstone, T. J. Povall, and J. D. Baty, Chem. Comm., 392 (1973).
201. C. Merritt, C. Dipietro, and D. H. Robertson, J. Chromatog. Sci., 12, 668 (1974).
202. J. J. Pisano and T. J. Bronzert, Anal. Biochem., 45, 43 (1972).

203. J. J. Pisano, W. J. A. Vanden Heuvel, and E. C. Horning, Biochem. Biophys. Res. Comm., 7, 82 (1962).
204. P. E. Harmon, J. L. Paterson, and W. J. A. Vanden Heuvel, [?Anal. Biochem.,], 25, 452 (1968).
205. M. A. Hermondson, L. H. Ericson, K. Titoni, H. Neurdth, and K. A. Welsh, Biochemistry, 11, 449 (1972).
206. J. J. Pisano, "Methods in Enzymology", New York, 1972, Vol. 25, p. 27.
207. M. Rangersgan, R. A. Ardrey, and A. Darbre, J. Chromatog., 87, 499 (1973).
208. J. Eyem and J. Sjöquist, Anal. Biochem., 52, 255 (1973).
209. A. S. Jnglis and P. W. Nicholls, J. Chromatog., 86, 117 (1973).
210. R. M. Legnin and H. D. Niall, Biochim. Acta, 257, 76 (1972).
211. J. E. Attrill, W. C. Butts, W. T. Reiner, and J. W. Holleman, Anal. Letters, 3, 59 (1970).
212. C. W. Gehrke and C. D. Ruyle, J. Chromatog., 38, 473 (1968).
213. D. E. Vance and D. S. Feingold, Anal. Biochem., 36, 30 (1970).
214. W. M. Lamkin, N. S. Jones, T. Pan, and D. N. Ward, Anal. Biochem., 58, 549 (1974).
215. W. M. Lamkin, J. W. Weatherford, N. S. Jones, T. Pan, and D. N. Ward, Anal. Biochem., 58, 422 (1974).
216. N. Thekawa, O. Hoshino, and R. Watanuki, Anal. Biochem., 17, 16 (1966).
217. J. R. Coulter and C. S. Han, J. Chromatog., 36, 42 (1968).
218. C. W. Moss, M. A. Lambert, and F. J. Diaz, J. Chromatog., 60, 134 (1971).
219. J. R. Coulter and C. S. Han, in "New Techniques in Amino-acid, Peptide, and Protein Analysis", Ann Arbor Sci. Publ., Ann Arbor, 1971, Chapter 2, p. 75.
220. F. P. Zscheite and B. L. Brannaman, Anal. Biochem., 49, 442 (1972).
221. E. D. Pellizzari, J. H. Brown, P. Talbot, and L. F. Fabre, J. Chromatog., 55, 281 (1971).
222. H. Narert, J. Chromatog., 106, 218 (1975).
223. T. Ishibashi, M. Matsui, and T. Kawabata, Bunseki Kagaku, 24, 102 (1975).
224. J. J. Räsh, C. W. Gehrke, R. W. Zumwalt, K. C. Kuo, K. A. Kvenvolden, and D. L. Stalling, J. Chromatog., Sci., 10, 444 (1972).
225. J. MacGee, Fed. Proc., 23, 531 (1964); Anal. Biochem., 14, 305 (1966).
226. R. L. Hancock, J. Gas Chromatog., 2, 363 (1966).
227. R. L. Hancock and D. L. Coleman, Anal. Biochem., 10, 365 (1966).
228. H. Miles and H. Fales, Anal. Chem., 34, 860 (1962).
229. R. L. Hancock, J. Gas Chromatog., 6, 431 (1968).
230. R. L. Hancock, J. Gas Chromatog., 7, 366 (1969).
231. M. A. Quilliam, K. K. Ogilvie, and T. B. Westmore, J. Chromatog., 105, 297 (1975).
232. S. E. Hattox and J. A. McCloskey, Anal. Chem., 46, 1378 (1974).
233. F. D. Ganchel, K. Beyermann, and R. K. Zahn, FEBS Letters, 6, 141 (1970).
234. C. W. Gehrke and D. B. Lakings, J. Chromatog. Sci., 61, 45 (1971).
235. V. Pacakova, V. Miller, and J. J. Cernohorsky, Anal. Biochem., 42, 549 (1971).
236. D. B. Lakings and C. W. Gehrke, Clinical Chem., 18, 810 (1972).
237. W. C. Butts, J. Chromatog. Sci., 8, 474 (1970).
238. T. Hashizuma and Y. Sasaki, Anal. Biochem., **2** 15, 199 (1966).
239. T. Hashizuma and Y. Sasaki, Anal. Biochem., 21, 316 (1967); 24, 232 (1968).
240. Y. Sasaki and T. Hashizuma, Anal. Biochem., 16, 1 (1966).
241. W. C. Butts, Anal. Letters, 3, 29 (1970).
242. W. C. Butts and W. T. Rainey, Anal. Chem., 43, 538 (1971).
243. V. Pacakova and J. Nekvasil, J. Chromatog., 91, 459 (1974).

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