Determination of Asparagine, Glutamine and Pyrrolidonecarboxylic Acid in Total Enzymic Hydrolysates of Peptides and Glycopeptides by Gas-Liquid Chromatography

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A new procedure for the qualitative and quantitative determination of asparagine, glutamine and pyrrolidonecarboxylic acid in total enzymic hydrolysates of peptides and glycopeptides based on g.l.c. has been developed. Under the conditions of esterification and trifluoroacetylation *N*-trifluoroacetylaspartic acid mono-*n*-butyl ester was formed from asparagine and *N*-trifluoroacetylglutamic acid mono-*n*-butyl ester from both glutamine and pyrrolidonecarboxylic acid. To distinguish between the latter two compounds, the esterification was carried out at room temperature yielding 30% of esterified pyrrolidonecarboxylic acid but less than 1% of esterified glutamine. In extending the g.l.c. of amino acids, the previously unknown positions in the g.l.c. elution pattern of the following amino acids could also be reproducibly determined: carboxymethylcysteine, homoserine, hydroxylysine and ϵ -methyl-lysine. Further, certain glycopeptides were investigated and the artifacts due to their carbohydrate moieties were determined.

Although several investigators have used g.l.c. for the determination of amino acids (Gehrke *et al.*, 1968; Gehrke & Stalling, 1970; Coulter & Hann, 1968; Darbre & Islam, 1968; Hagen & Black, 1965; McBride & Klingman, 1968), the identification of two of the common amino acids, asparagine and glutamine, has not been achieved. In the present study the g.l.c. technique has been extended to special applications for the elucidation of the amino acid sequences of peptides and glycopeptides, and a new method for the qualitative and quantitative determination of these two amides has been developed.

Materials and Methods

Chemicals

Tabsoarb (reagent no. TA-33; Regis Chemical Co., Chicago, Ill., U.S.A.) was used throughout this study as stationary phase. For the determination of histidine, arginine and cysteine an additional system employing Tabsoarb HAC (Regis Chemical Co., Chicago, Ill., U.S.A.) was necessary. The *N*-trifluoroacetyl *n*-butyl esters of the common amino acids (Regis no. TR-1, TAB kit; Regis Chemical Co.) were used as control compounds and references. L-Amino acids of the highest purity were purchased from Schwarz/Mann, Division of Becton, Dickinson and

Co., Orangeburg, N.Y., U.S.A. DL-Pyrrolidonecarboxylic acid (Schwarz/Mann), D-glucosamine, *N*-acetyl-D-glucosamine, D-galactose, D-mannose and *N*-acetylneuraminic acid (Sigma Chemical Co., St. Louis, Mo., U.S.A.), DL-allo- δ -hydroxylysine and L-hydroxyproline (Calbiochem, La Jolla, Calif., U.S.A.), DL-homoserine (Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.), carboxymethyl-L-cysteine (Schwarz/Mann Research Co.), L-asparagine (National Biochemicals Corp., Cleveland, Ohio, U.S.A.), Pronase (70000, p.u.k./g; Kaken Chemical Co., Tokyo, Japan), aminopeptidase M (Rohm and Haas, Darmstadt, Germany, obtained through Henley Co., New York, N.Y., U.S.A.) and certain peptides [9545 (Gln-Gln-Gln) and 8187 (His-Ser-Gln-Gly-Thr-Phe) from Schwarz/Mann, and A 0253 (Ala-Asp) from Sigma Chemical Corp.] were also used in this investigation. Additional peptides and desialized glycopeptides were isolated from enzymic hydrolysates of desialized α_1 -acid glycoprotein and exhibited a high degree of purity (over 95%) except when stated otherwise.

G.l.c. apparatus

An F & M model 402 gas chromatograph with a dual flame-ionization detector was used for this investigation. Dry O₂-free N₂ was used as carrier at a flow rate of 40 ml/min. This apparatus was provided with two glass columns (3 mm internal diam., 1.8 m long), two detectors and two recorders, permitting

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duplicate analyses to be carried out simultaneously. The columns were injected at an oven temperature of 75°C and then a temperature gradient of 4°C/min was applied until 220°C was reached. The flash heater was set to 100°C.

Preparation of the N-trifluoroacetyl n-butyl ester derivatives of asparagine, glutamine and pyrrolidone-carboxylic acid

For this purpose the procedure of Gehrke and co-workers (Gehrke *et al.*, 1968; Gehrke & Stalling, 1970) was modified as follows. An amount (50–100nm) of amino acids or a corresponding amount of a hydrolysate was placed in a 12ml conical test tube with a Teflon-lined screw cap and dissolved in 0.2ml of 1.25M-HCl in butan-1-ol. Esterification was

performed at 100°C for a critical period of 7min (4min for pyrrolidonecarboxylic acid). After esterification, the samples were cooled in ice-water (60s) and placed in a stream of N₂ (2min) for removal of the solvent. Trifluoroacetic anhydride (0.1ml) in methylene dichloride (25%) was added, and the reaction mixture was heated for 20min at 100°C. A portion (2–4μl) of the sample was then injected into the gas chromatograph.

The total enzymic hydrolysates of the peptides (0.2μmol in 0.2ml of 0.05M-NH₄HCO₃ buffer, pH8.0) were prepared by digestion at 37°C with 1% (w/w) of Pronase for 24h followed by further digestion with 4% (w/w) of aminopeptidase M for 17h. The NH₄HCO₃ buffer was used so that the digests could be desalted by freeze-drying. For comparison, in addition to total enzymic hydrolysis, these peptides

Table 1. *Elution temperatures and relative area factors of amino acids analysed as N-trifluoroacetyl n-butyl esters on a Tabsorb column*

Although the relative elution temperature of each peak is constant, the absolute values may vary $\pm 1^\circ\text{C}$ from run to run. The molar areas of the amino acids were related to that of glutamic acid, giving the relative molar area factors listed in this table. The esterification time for asparagine and glutamine was 7min and that for pyrrolidonecarboxylic acid 4min (see the text). The relative molar area factor of tyrosine was taken from Gehrke *et al.* (1968) and those of ornithine and ϵ -methyl-lysine were not determined (N.D.).

Amino acid	Elution temperature (°C)	Relative molar area factors
Alanine	96	0.50
Valine	107	0.68
Glycine	111	0.42
Isoleucine	115	0.77
Leucine	120	0.78
Proline	124	0.68
Threonine	128	0.67
Serine	136	0.54
Pyrrolidonecarboxylic acid	143	0.47
Glutamine	143	0.34
Cysteine	144	N.D.
Methionine	149	0.69
Homoserine	149	0.57
Hydroxyproline	151	0.82
Asparagine	152	0.30
Phenylalanine	155	1.16
Aspartic acid	159	0.92
Glutamic acid	173	0.100
Tyrosine	186	0.97
Stearate	190	
Ornithine	196	N.D.
Carboxymethylcysteine	197	0.91
ϵ -Methyl-lysine	197	N.D.
Lysine	203	0.82
Tryptophan	207	1.08
Hydroxylysine	{217 219}	0.70

were also hydrolysed with constant-boiling HCl in evacuated and sealed tubes at 110°C for 24h and their compositions were determined with a Technicon Autoanalyzer.

For the calculation of the amounts of each amino acid present in these hydrolysates the areas of the observed peaks (product of height $\times$ width at half-height of the peak) were divided by the relative molar response values of the corresponding amino acids (Table 1). The response values for asparagine and glutamine were calculated from the values derived from standard solutions of the free amino acids esterified for a period of 7min and that for pyrrolidonecarboxylic acid obtained after an esterification time of 4min. The reason for selecting these conditions is that, as shown below, the maximum molar responses of the latter three amino acids were obtained at these esterification times. The responses were then referred to the response of glutamic acid (full esterification with 3.0M-HCl in butan-1-ol at 100°C for 60min).

Preparation of the N-trifluoroacetyl n-butyl ester derivatives of the free amino acids and the hydrolysates mentioned

The procedure of Gehrke and co-workers (Gehrke *et al.*, 1968; Gehrke & Stalling, 1970) was used for this purpose. As standard, *n*-butyl stearate (Regis no. TA-20; 1mg/ml in butan-1-ol) was used except when stated otherwise. It is important that the standard be added to the amino acids or hydrolysates before esterification.

Results and Discussion

Study of asparagine and glutamine

Asparagine and glutamine, when subjected to the esterification procedure generally used for total amino acid analysis (3M-HCl in butan-1-ol, 100°C, 60min), gave very small characteristic responses. Under these conditions the amide groups were almost completely hydrolysed (Gehrke *et al.*, 1968), leading to their essentially quantitative recoveries as aspartic acid and glutamic acid. The maximum characteristic response of the two amides was observed when the esterification was limited to 2–3min. Since it was difficult to maintain an exact esterification time of between 2 and 3min, and to establish the maximum response values of asparagine and glutamine, the following additional series of experiments was carried out. The amides were esterified in 1.25M-HCl in butan-1-ol for increasing periods of time with the bis-*n*-butyl ester of aspartic acid or glutamic acid as standard. Both asparagine (Fig. 1) and glutamine (Fig. 2) showed maximum responses at an esterification time of 7min (in these and all subsequent experi-

Fig. 1. Effect of esterification on asparagine as a function of time in butan-1-ol containing 1.25M-HCl at 100°C

Results are expressed in molar yields of asparagine (○) and of the concomitantly formed aspartic acid (■). For comparison the corresponding analyses of authentic aspartic acid (●) are also included. Note that at short esterification times the sum of the areas of asparagine plus aspartic acid does not equal that of the authentic aspartic acid. Each point of the curves represents the average of two measurements, except that of the maxima, which represent the average of six determinations.

Fig. 2. Effect of esterification on glutamine as a function of time in butan-1-ol containing 1.25M-HCl at 100°C

Results are expressed in molar yields of glutamine (○) and the concomitantly formed glutamic acid (■). For comparison the corresponding analyses of authentic glutamic acid (●) are also included. Note that at short esterification times the sum of glutamine plus glutamic acid does not equal that of authentic glutamic acid. Each point of the curves represents the average of two measurements, except those of the maxima, which represent the average of six determinations.

Fig. 3. Effect of hydrolysis on asparagine and glutamine during esterification in butan-1-ol containing 1.25M-HCl at 100°C for 7, 15 and 150 min

The area ratios of asparagine to aspartic acid and of glutamine to glutamic acid clearly indicate the progressive hydrolysis of these two amides. Note that trace amounts only of asparagine and a small percentage of glutamine remain after 150 min of esterification.

ments *N*-trifluoroacetylation was carried out after esterification). The amount of the two amides decreased with greater esterification times. This effect is further illustrated in the patterns of Fig. 3. The quantity of formed aspartic acid and glutamic acid increased with esterification time (Figs. 1 and 2). In addition, the control experiments demonstrated that aspartic acid and glutamic acid were completely esterified after 40 min. It also should be noted that, because of the steep slopes of the curves observed at short esterification times (Figs. 1 and 2), a small error in the time of esterification would lead to a large error in the yields of the amides and hence in the ratio of the amides to their acids (see below).

From the results described above and additional duplicate measurements the maximum relative molar responses of asparagine and glutamine (observed after an esterification time of 7 min) were 0.30 and 0.34 respectively (Table 1).

For the calculation of the amide contents it is

Table 2. Area ratios of asparagine to aspartic acid and of glutamine to glutamic acid

The mixtures of asparagine and aspartic acid and of glutamine and glutamic acid in the molar ratios indicated were esterified in 1.25M-HCl in butan-1-ol at 100°C for 7 min, followed by trifluoroacetylation. The area ratios were calculated from the two peaks observed.

Molar ratio		Area ratio	
Asn	Asp	Asn	Asp
1	: 0	0.84	: 1.00
3	: 1	0.66	: 1.00
2	: 1	0.50	: 1.00
1	: 1	0.37	: 1.00
1	: 2	0.28	: 1.00
1	: 3	0.20	: 1.00
0	: 1	0.07	: 1.00

Molar ratio		Area ratio	
Gln	Glu	Gln	Glu
1	: 0	1.84	: 1.00
3	: 1	1.02	: 1.00
2	: 1	0.60	: 1.00
1	: 1	0.40	: 1.00
1	: 2	0.25	: 1.00
1	: 3	0.13	: 1.00
0	: 1	0.00	: 1.00

important to take into account that when aspartic acid was esterified for 7 min, an additional peak at the asparagine position was observed, the area of which accounted for approx. 7% of that of aspartic acid. Glutamic acid, however, did not lead to a peak at the position of glutamine. By comparison, asparagine, when esterified for the same period of time, yielded peaks of asparagine and aspartic acid whose areas had a ratio of 0.8:1.0. The corresponding ratio of the two peaks derived from glutamine was 1.8:1.0. The area ratios of asparagine to aspartic acid and glutamine to glutamic acid, determined for various molar ratios of the amides to their free acids, are given in Table 2.

From the results described above, the content of each of the two amides was calculated by the following procedure. First analysis: from the analysis for the two amides for which the total enzymic hydrolysate of the peptides was used (with 1.25M-HCl in butan-1-ol, 100°C, 7 min, followed by trifluoroacetylation), the area ratios of the two amides to their acids were calculated. From Table 2 the corresponding molar ratios of the amides to their acids were obtained. The content of each amide was then derived from these molar ratios and from the total number of residues of each of the two pairs of amino

Table 3. *Determination of asparagine and glutamine in ten peptides by g.l.c.*

For the determination of the amides, these hydrolysates were esterified at 100°C in butan-1-ol containing 1.25M-HCl for a period of 7 min. For the determination of their amino acid compositions, the enzymic hydrolysates of these peptides were esterified at 100°C in butan-1-ol containing 3M-HCl for a period of 60 min. These amino acid compositions were identical with those obtained by the Technicon Autoanalyzer (see also Table 4). Peptides 1-4 and 8 and 9 were derived from enzymic hydrolysates of human plasma α_1 -acid glycoprotein (Schmid *et al.*, 1971). The A chain of bovine insulin was a gift of Dr. R. A. Laursen. Values in parentheses are the nearest integers corresponding to the values determined.

Amino acid	Peptide ...	Amino acid composition (residues/mol of peptide)									A chain of bovine insulin*
		1	2	3	4	9545	A0253	8187	8	9	
Ala							1.13 (1)		0.90 (1)		
Val		1.32 (1)	2.02 (2)	1.32 (1)							
Gly			0.99 (1)	2.17 (2)	1.00 (1)			1.00 (1)		0.89 (1)	
Ile									1.34 (1)		
Leu			1.17 (1)								
Thr								0.79 (1)	0.72 (1)		
Ser			1.09 (1)		0.96 (1)			0.98 (1)			
Phe				0.86 (1)				1.20 (1)	1.10 (1)	1.22 (1)	
Asx		3.68 (4)	4.21 (4)				0.95 (1)				2.0
Glx		1.00 (1)	0.97 (1)	1.65 (2)	3.92 (4)	1.00					4.0
Lys		0.97 (1)	1.18 (1)		0.92 (1)			1.08 (1)	0.77 (1)	0.90 (1)	
His											
Trp			0.30 (1)					1.04 (1)			

Content of aspartic acid and glutamic acid and their amides determined by the 'area ratio' techniques	
Asn/Asp	
Area ratio	
Found	0.39:1.00 0.39:1.00
Theoretical†	0.37:1.00 0.37:1.00
Molar ratio	2:2 2:2
(of peptide)‡	
Gln/Glu	
Area ratio	
Found	0.00:1.00 0.00:1.00 0.41:1.00 0.11:1.00 1.71:1.00
Theoretical†	0.00:1.00 0.00:1.00 0.40:1.00 0.13:1.00 1.84:1.00
Molar ratio	0:1 0:1 1:1 1:3 1:0
(of peptide)‡	
Content of aspartic acid and glutamic acid and their amides determined by 'relative response factors'	
Asn/Asp	
Gln/Glu	
Independent analysis	
§	2.0:2.2 0.0:1.0
§	0.0:1.0 1.0:0.0
§	§
§	§,
§	2.3:0.0
§	2.3:1.7

* Taken from Dayhoff (1969).

† These ratios are taken from Table 2 and represent the values that are closest to the found ratios.

‡ The molar content of free acids and their amides of the peptides were calculated from Asx and Glx and the molar ratios of Asn/Asp and Gln/Glu respectively, which were taken from Table 2 and corresponded to the area ratios.

§ The same results were obtained by high voltage electrophoresis at pH6.4

|| The same results were obtained with the direct Edman degradation procedure.

acids (Asx and Glx respectively) derived from the subsequent analysis. Second analysis: the total content of asparagine plus aspartic acid and that of glutamine plus glutamic acid were determined from the analysis of the total acid or enzymic hydrolysates of the peptides as aspartic acid and glutamic acid respectively (with 3.0M-HCl in butan-1-ol, 100°C, 60min, followed by trifluoroacetylation). The area and molar ratios of asparagine to aspartic acid and of glutamine to glutamic acid in ten peptides are listed in Table 3.

In an alternative procedure, the contents of asparagine and glutamine were determined from the areas of their peaks and their relative molar area factors (Table 1). A 7% correction, owing to the presence of the mentioned artifact of aspartic acid, was used to calculate the asparagine content. The results obtained (Table 3) are in good agreement with those derived by the area ratio technique.

Study on pyrrolidonecarboxylic acid

The maximum response of pyrrolidonecarboxylic acid (Fig. 4) was obtained after 4min of esterification with 1.25M-HCl in butan-1-ol at 100°C (followed by trifluoroacetylation). The molar response was 0.47 relative to glutamic acid. A gradual decrease in the area of the characteristic pyrrolidonecarboxylic acid peak, which was eluted at 143°C, was observed with increasing esterification times. After 2.5h essentially all of this acid had been converted into a compound that was eluted at 173°C and represented glutamic acid. For the identification of the pyrrolidonecarboxylic acid peak, a series of experiments with authentic pyrrolidonecarboxylic acid *n*-butyl ester and esterification of pyrrolidonecarboxylic acid at room temperature was carried out and demonstrated that trifluoroacetyl mono-*n*-butyl ester of glutamic acid is the compound formed.

To distinguish between pyrrolidonecarboxylic acid and glutamine it was found that when a solution of these two amino acids was esterified at room temperature with 1.25M-HCl in butan-1-ol for 15min followed by trifluoroacetylation and analysis by the present g.l.c. technique, approx. 30% of pyrrolidonecarboxylic acid, but less than 1% of glutamine, were recovered.

It is therefore clear that for the determination of pyrrolidonecarboxylic acid of certain natural peptides and proteins (Ikenaka *et al.*, 1966; O'Donnell *et al.*, 1970; Iwanaga *et al.*, 1967; Dayhoff, 1969) that are known to have *N*-terminal pyrrolidonecarboxylic acid, they should first be subjected to digestion by the highly specific enzyme, pyrrolidonecarboxyl peptidase (Doolittle, 1972). Subsequently, the liberated pyrrolidonecarboxylic acid can be measured by the present g.l.c. procedure. Doolittle (1972) described

Fig. 4. Effect of esterification on pyrrolidonecarboxylic acid as a function of the esterification time in butan-1-ol containing 1.25M-HCl at 100°C

The results are expressed in molar yields of pyrrolidonecarboxylic acid (○) and of the concomitantly formed glutamic acid (■). For comparison the corresponding analyses of authentic glutamic acid (●) are also included. Note that at short esterification times the sum of pyrrolidonecarboxylic acid plus glutamic acid does not equal that of authentic glutamic acid. Each point of the curves represents the average of two measurements except those of the maxima which represent the average of seven determinations.

an alternative procedure for the determination of pyrrolidonecarboxylic acid.

Elution temperatures and relative area factors of the common and certain other amino acids

These values are listed in Table 1. The separation of the common amino acids, including asparagine and glutamine, from each other was excellent and highly reproducible. Asparagine was eluted just ahead of phenylalanine, and glutamine was located between serine and methionine. The relative area factors, expressed as values relative to glutamic acid, are very close to those reported by Gehrke *et al.* (1968).

The following observations were especially notable.

- (1) Carboxymethylcysteine was eluted close to the position of ornithine, but this is not considered to be a disadvantage, as ornithine has not been found to be a constituent of proteins.
- (2) Homoserine appeared at the position of methionine. Again this is not a disadvantage, since homoserine is formed only on reaction of methionine with CNBr followed by acid hydrolysis and transformation of homoserine lactone to homoserine.
- (3) The hydroxylysines and hydroxyprolines separated well from each other and the other

Table 4. Comparison of the amino acid composition of five peptides and two glycopeptides as determined by g.l.c. on a Tabsoarb column and by ion-exchange chromatography on a Technicon Autoanalyzer

For details see the text. These peptides and glycopeptides were derived from enzymic digests of human plasma α_1 -acid glycoprotein (Schmid *et al.*, 1971).

Amino acid	Peptide 1		Peptide 2		Peptide 3		Peptide 4*		Peptide 5		Glycopeptide 1		Glycopeptide 2	
	G.l.c.	Ion-exchange	G.l.c.	Ion-exchange	G.l.c.	Ion-exchange	G.l.c.	Ion-exchange	G.l.c.	Ion-exchange	G.l.c.	Ion-exchange	G.l.c.	Ion-exchange
Ala					1.01	1.03	1.00	1.00						
Val	0.43	0.40			1.11	1.16	1.16	1.01						
Gly	1.88	2.00			1.86	2.01	1.87	2.00	1.07	0.99				
Ile			1.00	1.00	2.00	2.00			1.00	1.00				
Leu							1.00	1.04						
Pro											1.21	1.00		
Thr							0.44	0.46	0.79	1.00	1.82	1.85		
Phe	1.00	1.00									1.00	0.99		
Asp	1.88	2.02					0.24	0.37	1.00	1.12	2.20	1.96	2.08	1.82
Glu			0.91	0.94	1.65	2.10	0.79	1.07	0.80	1.02	1.00	1.01	2.00	1.93
Tyr			0.74	0.95							0.70	0.91	0.35†	0.82
Lys					1.85	2.04	0.27	0.35	0.70	0.90	0.97	0.87	0.98	1.00
Trp									0.25‡	0.27‡				

* This peptide is a mixture of two peptides.

† Certain impurities of this preparation led to partial destruction of tyrosine in the column.

‡ On total acid hydrolysis of carbohydrate-free peptides a considerable fraction of this amino acid may be recovered (Bradshaw *et al.*, 1969).

common amino acids. The former appeared as a double peak well after tryptophan and the latter as a single peak between methionine and phenylalanine. (4) ϵ -Methyl-L-lysine, a constituent of certain proteins (DeLange *et al.*, 1969; Huszar & Elzinga, 1969; Hardy *et al.*, 1970), was eluted at the position of carboxymethylcysteine, i.e., between stearate and lysine. (5) The *N*-1- and *N*-3-methylhistidines were not eluted.

Amino acid composition of peptides

Acid hydrolysates of twenty peptides and glycopeptides isolated from enzymic hydrolysates of α_1 -acid glycoprotein (Schmid *et al.*, 1971) were analysed by the g.l.c. technique (esterification with 3M-HCl in butan-1-ol at 100°C for 60min) and by ion-exchange chromatography. Seven pairs of such analyses are presented in Table 4. As control, Pronase and aminopeptidase M, after autolysis for 36h, were analysed by the present g.l.c. technique (3M-HCl). The amount of free amino acids found accounted for approx. 2% of those formed from the enzymic hydrolysis of the peptides. These results, together with the analyses of the other peptides (Tables 3 and 5), indicated that both procedures give essentially the same results. Hence, g.l.c. proved to be an effective and very economical technique for analysing peptides for their amino acid composition. Conveniently, one g.l.c. analysis is completed within approx. 35min.

Application of g.l.c. of amino acids as an adjunct to the subtractive Edman degradation procedure

A peptide with the sequence Asp-Val-Asn-Asp-Glu-Lys-Asn-Trp-Gly-Leu-Val-Ser was subjected to subtractive Edman degradation (Elzinga *et al.*, 1968). After each step a sample of the degraded peptide was completely hydrolysed and analysed for its amino acid composition (Table 5). The g.l.c. patterns obtained (Fig. 5) were also in excellent agreement with the amino acid sequence. It is noteworthy that ϵ -phenylthiocarbamoyl-lysine as the *N*-trifluoroacetyl *n*-butyl ester was not observed on the g.l.c. elution diagram but that phenylisothiocyanate and *N*-allyl-*NN*-dimethylamine, which, if not removed completely in the Edman degradation procedure and rendered volatile by the present g.l.c. technique, were eluted at the position of aspartic acid and as a broad peak at the positions of tyrosine and serine respectively.

Studies on glycoproteins

Because many fundamentally important biological functions are associated with glycoproteins and in view of the wide distribution of these conjugated proteins in Nature (Schmid, 1972; Jamieson & Greenwalt, 1971), the monosaccharides and artifacts produced during acid hydrolysis of glycopeptides were investigated by this g.l.c. procedure. When a total acid hydrolysate of a glycopeptide derived from human plasma α_1 -acid glycoprotein (Schmid *et al.*, 1971) was subjected to the procedure de-

Table 5. G.l.c. analysis of the peptides remaining after each subtractive Edman degradation step of a dodecapeptide

The amides and the position of tryptophan were established by additional, independent measurements. The amino acid removed at each step is underlined. The content of serine was not corrected for destruction due to hydrolysis.

Edman cycle no.	Amino acid composition (mol/mol of peptide)						
	Asp	Val	Glu	Lys	Gly	Leu	Ser
0	4.14 (4)	2.11 (2)	0.95 (1)	0.83 (1)	1.02 (1)	1.00 (1)	0.66 (1)
1	<u>2.85</u> (3)	1.92 (2)	1.02 (1)	0.61 (1)	1.04 (1)	1.00 (1)	0.54 (1)
2	<u>2.87</u> (3)	<u>1.21</u> (1)	0.78 (1)	0.52 (1)	0.82 (1)	1.00 (1)	0.56 (1)
3	<u>1.60</u> (2)	<u>1.19</u> (1)	0.66 (1)	0.22 (1)	0.86 (1)	1.00 (1)	0.50 (1)
4	<u>1.20</u> (1)	1.58 (1)	0.87 (1)	0.25 (1)	1.08 (1)	1.00 (1)	0.58 (1)
5	<u>0.92</u> (1)	1.00 (1)	<u>0.18</u> (0)	0.36 (1)	1.11 (1)	1.00 (1)	0.81 (1)
6	0.84 (1)	1.13 (1)	—	<u>0.00</u> (0)	0.97 (1)	1.00 (1)	0.67 (1)
7	<u>0.20</u> (0)	1.13 (1)	—	—	1.06 (1)	1.00 (1)	0.60 (1)
8*	—	1.00 (1)	—	—	1.18 (1)	1.00 (1)	0.51 (1)
9	—	1.26 (1)	—	—	<u>0.05</u> (0)	1.00 (1)	0.68 (1)
10	—	1.00 (1)	—	—	—	<u>0.10</u> (0)	0.50 (1)

Sequence: Asp-Val-Asn-Asp-Glu-Lys-Asn-Trp-Gly-Leu-Val-Ser

* Tryptophan was not determined in the analyses.

Fig. 5. G.l.c. of a dodecapeptide with known amino acid sequence and the peptides obtained after each degradation by the subtractive Edman procedure

The amino acid analysis pattern of the original peptide is termed (0), and the peak of the amino acid removed by the first Edman step is indicated by the thicker line in pattern (0). The amino acid analysis pattern of the peptide obtained after the first Edman step is termed (1). The amino acid which was removed from this peptide at the second Edman step is indicated by the thicker line in pattern 1, etc. At the eighth step tryptophan was removed. Since this amino acid was not determined in this series of analyses, the pattern of the eighth step was omitted. Because of the partial reaction of the ϵ -amino group with phenylisothiocyanate and the resistance of its ϵ -carbamoyl derivative towards acid hydrolysis, the lysine peak gradually decreased.

scribed, three major (A, B and C) and occasionally two minor (a and b) artifacts were observed (Fig. 6). These peaks were well separated from the common amino acids, except for the minor peak b which was

found in the position of phenylalanine. Under the conventional conditions of hydrolysis of proteins and peptides, sialic acid and fucose are completely destroyed, but considerable amounts of galactose and

Fig. 6. G.l.c. of a total acid hydrolysate of a desialized glycopeptide

The esterification was carried out in butan-1-ol containing 3M-HCl at 100°C for 60min. The artifacts due to the carbohydrate moiety of the glycopeptide that were always observed are indicated by the solid peaks, and those that were seen occasionally are indicated by cross-hatched peaks.

Table 6. Elution temperatures of certain monosaccharides as *n*-butyl glycosides on a Tabsorb column

These monosaccharides were esterified and trifluoroacetylated as described for amino acids. Under the conditions of esterification, the *N*-acetyl group of *N*-acetylglucosamine is cleaved. Hence, both *N*-acetylglucosamine and glucosamine lead to the same two peaks.

Monosaccharides	Elution temperature (°C)
D-Galactose	176*
D-Mannose	185†
D-Glucosamine	197, 219
<i>N</i> -Acetyl-D-glucosamine	197, 219
<i>N</i> -Acetylneuraminic acid	217

* Double peak.

† Asymmetrical peak.

mannose are not. However, a relatively large percentage of glucosamine remains unchanged. The peaks eluted at approximately 200°C and 220°C were probably due to this amino sugar, as demonstrated by independent experiments with authentic glucosamine (Table 6). One of these peaks was located close to that of carboxymethylcysteine, as shown by the analysis of a hydrolysate of reduced and carboxymethylated α_1 -acid glycoprotein. It is noteworthy that if a desialized glycopeptide (Schmid *et al.*, 1971) was subjected to esterification (3M-HCl

in butan-1-ol, 60min) and trifluoroacetylation (20min) without previous acid hydrolysis, peaks were observed with elution temperatures that corresponded to those of mannose, galactose and *N*-acetylglucosamine (Table 6).

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