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GAS–LIQUID CHROMATOGRAPHY OF THE N(O)-HEPTAFLUOROBUTYRATES OF THE ISOAMYL ESTERS OF AMINO ACIDS*

I. SEPARATION AND QUANTITATIVE DETERMINATION OF THE CONSTITUENT AMINO ACIDS OF PROTEINS

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SUMMARY

A quantitative gas–liquid chromatographic method has been developed for the analysis of amino acids. The method involves the conversion of free amino acids into their isoamyl N(O)-heptafluorobutyryl esters and chromatography on 3% SE-30–Gas-Chrom Q. The method can be applied to acid-hydrolysed proteins or glycoproteins. It is extremely sensitive (0.1 ng) and all common protein amino acids can be accurately determined in a single chromatographic run with pipecolic acid as internal standard.

INTRODUCTION

GEHRKE and co-workers[1–7] have developed methods for the determination of amino acids by gas–liquid chromatography (GLC) after the formation of volatile amino acid derivatives by esterification and acylation of the polar groups. This method is much more sensitive and rapid than the more classical techniques[8–15] of ion-exchange chromatography. We have used the method of GEHRKE *et al.*[7] in routine determinations. In general, this method is satisfactory, but it does have two disadvantages, both of which are related to the nature of the derivatives.

Firstly, we have not been able to find a stable stationary phase on which all the protein amino acids could be separated. MCBRIDE AND KLINGMAN[16] were able to resolve all these amino acids, but the stationary phase (PDEAS) was too unstable for routine analysis.

The second problem is that the N(O)-trifluoroacetyl derivatives used by GEHRKE and co-workers are volatile, and excess of the acylation reaction mixture cannot be eliminated before injection without substantial loss of the more volatile derivatives of glycine, alanine, valine, serine, threonine, isoleucine, leucine, proline and cysteine. A concentration step is necessary when analysing low amounts of

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material, and the volatility of the derivatives is therefore a major disadvantage. To overcome this difficulty, ZUMWALT *et al.*[17] have developed a method for the injection of large volumes of sample into the gas–liquid chromatograph, but major modifications to the conventional apparatus are required, however.

We have used a different pair of blocking agents, which give less volatile derivatives that can be separated on a single column. Silyl derivatives were not tested as previous work[18–20] did not suggest that they would be applicable. Heptafluorobutyric anhydride was chosen as the acylation reagent as it gives less volatile derivatives than trifluoroacetic anhydride. Three esterifying reagents were tested. Although the *n*-propyl esters could be separated on a single column[21], they were too volatile. The *n*-butyryl derivatives were less volatile but could not be resolved on a single column. Isoamyl alcohol (3-methyl butan-1-ol) derivatives were found to be satisfactory.

EXPERIMENTAL

Reagents

Standard amino acids were obtained from Fluka A.G. (Buchs, Switzerland) and standard amino acid mixtures from Hamilton Co. (Whittier, Calif., U.S.A.). Bovine pancreatic ribonuclease, pig pancreatic amylase and subtilisin were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Beef chymotrypsin was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), bovine serum albumin and heptafluorobutyric anhydride from Fluka A. G. (Buchs, Switzerland) and acetonitrile was of UVASOL grade from Merck A. G. (Darmstadt, G.F.R.).

Methanol and isoamyl alcohol were re-distilled in an all-glass apparatus after refluxing them for 2 h over magnesium turnings[7], then stored under dry condition at low temperature. Methanol–1.25 *M* HCl and isoamyl alcohol–1.25 *M* HCl were prepared at 0° by dissolving HCl vapour, produced by the action of concentrated sulphuric acid on sodium chloride and dried in concentrated sulphuric acid, in the alcohols. These reagents were stored under dry conditions at −20° in order to avoid a decrease in the acid concentration with the corresponding production of water[22].

Chromatographic materials and apparatus

The stationary phase (3% SE-30-Gas-Chrom Q) was obtained from Applied Science Inc. (State College, Pa., U.S.A.). All analyses were carried out on a Varian Aerograph gas chromatograph, Model 2100, equipped with a temperature programmer, a four-column oven, four flame ionization detectors and two differential electrometers. The chromatograms were recorded with two dual-pen Varian Aerograph Model 20 recorders.

Preparation of volatile derivatives

Samples containing 1–50 μg of amino acid were freeze-dried, then dissolved in 200 μl of methanol–1.25 *M* HCl and placed in a 2-ml conical Pyrex tube stoppered with a PTFE-lined screw-cap (Sovirel, 92-Levallois, France). After 30 min at room temperature, the reagent was evaporated under a stream of nitrogen at 50°. The residue was dissolved in 200 μl of isoamyl alcohol–1.25 *M* HCl and heated

in an oven at 110° for 150 min. After cooling, the reagent was evaporated under a stream of nitrogen at 80°. The isoamyl esters were then dissolved in 100 μl of acetonitrile* and 20 μl of heptafluorobutyric anhydride was added. The tube was tightly capped and carefully placed in a sand-bath at 150°. After 10 min, the tubes were cooled and the reagent evaporated under a stream of nitrogen at room temperature until just dry. The residue was then dissolved in an appropriate volume of ethyl acetate for injection. If it was necessary to store the samples, they were kept in the acylation mixture and heated to 150° less than 24 h before chromatography.

Chromatography

Pyrex columns (3.50 m × 2 mm I.D.) were filled with 3 % SE-30–Gas-Chrom Q by gentle tapping under suction. They were conditioned overnight at 250° with carrier gas (nitrogen or argon) at 30 ml/min. If necessary, more stationary phase was added and the conditioning process repeated. Chromatographic conditions are given in Table I. The area of each peak was determined by planimetry.

TABLE I

CONDITIONS FOR GAS–LIQUID CHROMATOGRAPHY

Condition	*Value*
Column temperature:	
Initial	70°
Final	240°
Temperature programming rate	4°/min
Detector cell temperature	280°
Injector temperature	265°
Gas flow-rates:	
Hydrogen	30 ml/min
Air	300 ml/min
Carrier gas (N_2)	20 ml/min
Sample volume	1–10 μl

RESULTS

All the constituent amino acids of the proteins were resolved under the conditions used in this work (Table II and Fig. 1), in contrast to the situation with the trifluoroacetate derivatives of the *n*-butyl esters[1–7]. The separation is better than that of the *n*-propyl heptafluorobutyryl esters on OV-1 (ref. 21), or on 3 % SE-30–Gas-Chrom Q, which is a more satisfactory stationary phase. As the isoamyl heptafluorobutyryl esters are less volatile, they are more easily separated from the peak of heptafluorobutyric acid, which is not completely eliminated under the stream of nitrogen. With a temperature programme of 6°/min, the tyrosine and glutamic acid derivatives are only partially separated, but they can be satisfactorily determined by manual integration. The separation of most of the amino acids is

* As the solvent for the acylation step, acetonitrile has the advantage of ensuring solubility of the amino acid esters. Ethyl acetate, used by other workers[21], does not completely solubilize the amino acid esters, and also gives rise to numerous interfering peaks. However, acetonitrile does cause tailing of the solvent peak and should be thoroughly eliminated before injection.

TABLE II

RETENTION TEMPERATURES (R_T) OF THE ISOAMYL N(O)-HEPTAFLUOROBUTYRYL ESTERS OF AMINO ACIDS

Kyn = kynurenine; Pipe = pipecolic acid; Orn = ornithine.

Amino acid derivative	R_T (°C)
Ala	111.5
Arg	190.0
Asp	170.0
Cys	143.0
Cys2	230.0
Glu	182.5
Gly	114.0
His (diacyl derivative)	162.0
His (monoacyl derivative)	194.5
Hypro	150.0
Ile	133.0
Kyn	218.0
Leu	131.0
Lys	178.0
Met	155.0
Orn	168 0
Phe	165 0
Pipe	147.0
Pro	141.0
Ser	128.0
Thr	126.0
Trp	209.0
Tyr	181.0
Val	124.0

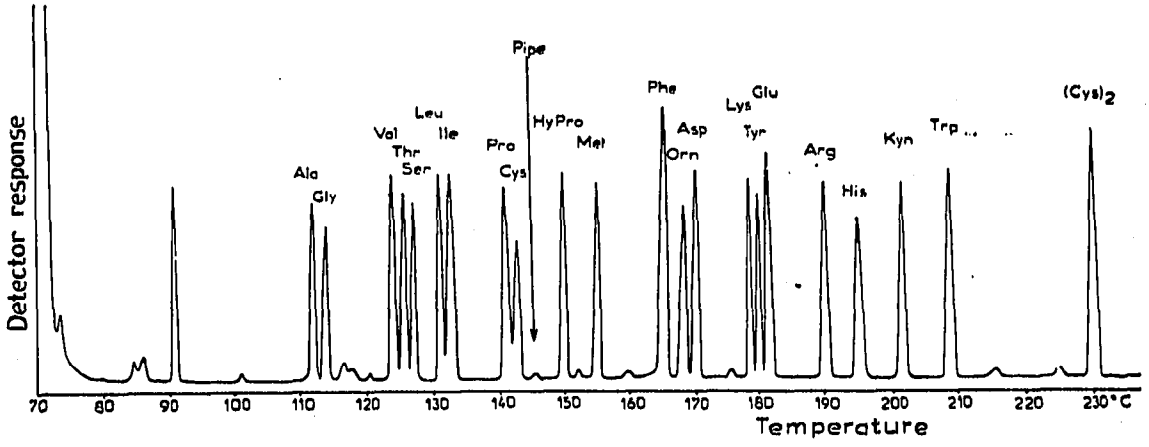

Fig. 1. Resolution of the isoamyl N(O)-heptafluorobutyryl esters of amino acids. Pipe = pipecolic acid; Orn = ornithine; Kyn = kynurenine. GLC conditions are given in Table I.

extremely reproducible, but the flow-rate of the carrier gas is critical for the resolution of these two amino acids.

The molar responses of the various amino acids relative to glutamic acid (RMR_{glu}) have been determined for ten different synthetic amino acid mixtures.

Derivatives were formed from 0.1–100 nmole of each of the amino acids, and parallel analyses were performed before and after concentration of the sample. The means of at least thirty determinations are given in Table III. The RMR_{Glu}

TABLE III

RELATIVE MOLAR RESPONSES (*RMR*) OF THE ISOAMYL N(O)-HEPTAFLUOROBURYRYL ESTERS OF AMINO ACIDS

Amino acid	RMR_{Glu}	
	Before concentration	*After concentration*
Ala	540	540
Arg	860	860
Asn[a]	910	910
Asp	910	910
Cys[b]	750	750
Cys2	1450	1450
Gln[a]	1000	1000
Glu	1000	1000
Gly	510	510
His	750[c]	450[d]
Hypro	810	810
Ile	810	810
Leu	810	810
Lys	930	930
Met	660	660
Orn	860	860
Phe	1010	1010
Pro	750	750
Ser	690	690
Thr	750	750
Trp	1005	1005
Tyr	980	980
Val	650	650
Pipe	810	810

[a] Analyzed as the corresponding acid.
[b] Partially oxidized to cystine.
[c] Diacyl derivative.
[d] Monoacyl derivative.

values were extremely reproducible, and in no instance was the variability greater than ± 2 %, which is the variability to be expected from errors in measuring the surface areas of the peaks. The *RMR* values were not changed after concentration, in contrast to the lowered *RMR* values of the *n*-butyl trifluoroacetate[1-7] and *n*-propyl heptafluorobutyryl[21] derivatives, caused by loss of the more volatile components. Only with histidine was the *RMR* value lowered, due to conversion of the diacyl to the monoacyl derivative[23].

The conditions for the esterification and transesterification reactions adopted by GEHRKE *et al.*[7] were found to be satisfactory for the formation of the isoamyl N(O)-heptafluorobytyryl esters. However, if the esters were formed directly in isoamyl alcohol–1.25 *M* HCl, cystine could not be determined, and the recoveries of some other amino acids were decreased. This effect seems to be due to their relative insolubilities in *n*-butanol or isoamyl alcohol[7]. When each amino acid

derivative was formed separately, all were stable during storage, elimination of the acylation reagent and injection. However, threonine was slowly degraded over long periods (more than a week) in the reaction medium, and a peak that had the same retention temperature (119°) as α-aminobutyric acid appeared. A similar peak occurred if the injection was prolonged for more than 10 sec, as with the *n*-butyl trifluoroacetate esters[7] or when a metallic injector was used. Cysteine is partially oxidized to cystine during the formation of derivatives (probably during the transesterification), but this oxidation does not affect the analysis. Histidine forms a diacyl derivative (R_T = 162°) in the presence of excess of reagent at high temperature, but during the elimination of the reagent it is quantitatively converted into the monoacyl derivative (R_T = 194.5°), as previously observed[21,23]. Glutamine and asparagine are completely converted into the corresponding acids during transesterification, and therefore cannot be determined separately. All other amino acids could be determined without problems by using the present method.

Determination of the amino acid composition of proteins

The results obtained by using the present method to determine the amino acid compositions of the acid hydrolysates (6 *M* HCl, 115°, 24 h) of proteins are in good agreement with the results obtained by the more classical techniques[8-15] (Table IV).

During acidic hydrolysis in the presence of oxygen, cysteine and cystine are oxidized to cysteic acid, which is not volatile and cannot be determined. Similarly, methionine is partially converted into methionine sulphone, which is poorly resolved from glutamic acid. Also, the methionine sulphone peak is asymmetrical, and is therefore difficult to integrate. Hydrolyses should be carried out under nitrogen in order to prevent low recoveries of cysteine and methionine (Fig. 2).

Some difficulties were observed during the determination of isoleucine owing to the well known stability of peptide bonds involving this amino acid. The rapidity

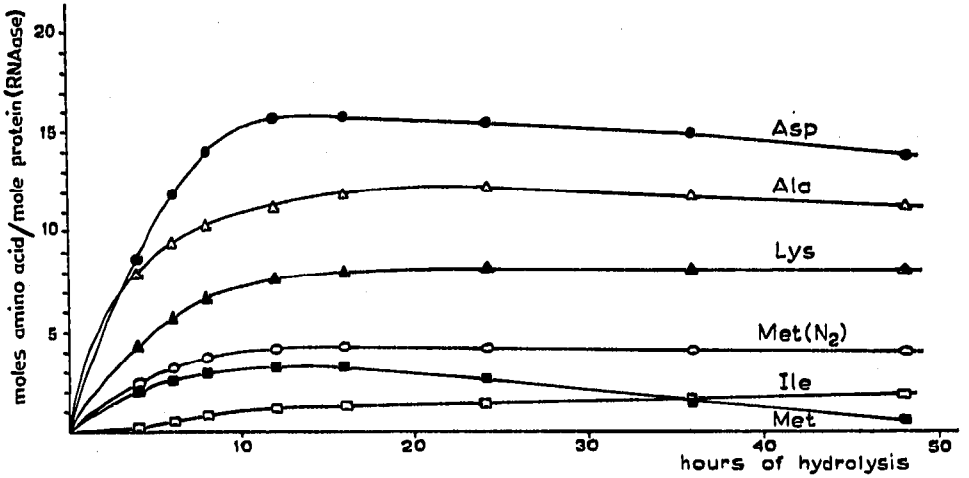

Fig. 2. Kinetics of liberation of some amino acids from ribonuclease by hydrolysis in 6 *M* HCl at 115°. Amino acids were analyzed by GLC of the isoamyl N(O)-heptafluorobutyryl derivatives. Met(N_2): hydrolysis was performed under nitrogen. Met: hydrolysis was performed under air.

and sensitivity of the GLC determination facilitates systematic kinetic studies, which are the only means of establishing amino acid compositions accurately. These kinetic studies should be carried out with the use of an internal standard. Of the various possible standards, we have used pipecolic acid (added after the hydrolysis). Ornithine can be used as the internal standard, but as arginine can be partially degraded to ornithine, the internal standardization is not as satisfactory as that obtained by using pipecolic acid. The standard can be added after hydrolysis because both hydrolysis and the formation of derivatives are carried out in the same tube, thus eliminating losses.

Certain common contaminants of protein preparations can interfere in the determinations. Salts, in high concentrations, prevent the formation of derivatives and should be eliminated by dialysis or gel filtration. Lipids give rise to fatty acid peaks and should be eliminated by exhaustive extraction with chloroform–methanol. Nucleic acids give rise to a number of interfering peaks, and the resulting chromatograms often cannot be interpreted. The nucleic acids should therefore be completely eliminated. In contrast, if glycoproteins are analysed, the carbohydrates are almost completely degraded and the low-molecular-weight products are eluted with the solvent peak. Only the hexosamines give a weak peak (R_T = 163°) that is well separated from the amino acid peaks. The method is therefore applicable to glycoproteins without modification.

DISCUSSION

The method described for the analysis of amino acids by the GLC of their isoamyl N(O)-heptafluorobutyryl esters has various advantages over other methods. All the amino acids that commonly occur in proteins can be separated on single columns with a stable stationary phase, which enables routine assays to be made over a long period of time. The method is more sensitive than others (0.1 ng of each amino acid can be detected), owing to the ease with which samples can be concentrated without loss. The derivatives used by GEHRKE and co-workers[1–7] and by MOSS AND LAMBERT[21] cannot be concentrated without loss of material. Moreover, the stability of the stationary phase provides a very stable base-line, and the gas–liquid chromatograph can therefore be run at high sensitivity.

Provided that appropriate precautions are taken during the acidic hydrolysis, this method is very convenient for the determination of the amino acid compositions of proteins. Routine analyses can be carried out on 1–25 μg of protein in a single chromatographic run of either 30 or 45 min. The sensitivity and rapidity of the method facilitates kinetic studies. The method is directly applicable to glycoproteins, and can be applied on the same sample after determination of the carbohydrate composition by the previously described method[30].

Sodium dodecyl sulphate, which is commonly used for the solubilization as commercially supplied, is often a mixture of long-chain alkyl sulphates that give rise to a number of interfering peaks. It can be eliminated before the hydrolysis, however, by acidification and ether extraction of an aqueous suspension of the protein. The protein can then be freeze-dried or precipitated with methanol.

Two further advantages of the method can be envisaged. The derivative peaks are narrow and well separated, and it seems likely that much more complex

TABLE IV

AMINO ACID COMPOSITIONS OF KNOWN PROTEINS

Amino acid	*RNAase*		*Serum albumin*		*Ovalbumin*		*Subtilisin*		*α-Chymotrypsin*		*α-Amylase*	
	Found	*Literature value*[24]	*Found*	*Literature value*[25]	*Found*	*Literature value*[26]	*Found*	*Literature value*[27]	*Found*	*Literature value*[28]	*Found*	*Literature value*[29]
Ala	10.25	9.68	8.70	8.20	9.80	9.50	15.00	13.43	10.70	8.98	9.80	9.05
Arg	3.41	3.23	4.12	3.95	3.85	4.13	0.79	0.73	1.64	1.63	4.14	3.90
Asp	12.75	12.10	10.05	9.50	9.43	8.80	11.15	10.20	9.50	9.39	13.75	12.72
Cys	7.65	6.45	6.83	6.30	2.06	1.95	0	0	3.68	4.08	2.37	2.22
Glu	9.70	9.68	13.00	13.10	14.20	14.10	6.00	5.46	6.55	6.12	8.70	8.35
Gly	2.63	2.42	2.37	2.84	5.22	5.10	12.65	12.00	11.20	9.39	10.72	10.44
His	3.31	3.23	2.91	3.00	1.78	1.91	2.54	2.18	0.80	0.82	2.87	2.93
Ile	1.07	2.42	1.90	2.35	4.21	6.72	2.93	4.73	2.82	4.08	3.29	10.25
Leu	1.93	1.61	10.32	10.90	8.22	8.83	5.30	5.46	8.05	7.75	7.30	
Lys	8.12	8.06	10.10	10.70	5.47	5.44	3.86	4.00	5.60	5.72	4.14	3.92
Met	2.72	3.23	0.58	0.63	4.35	4.40	1.27	1.83	0.80	0.82	1.90	1.91
Phe	2.32	2.42	4.75	4.55	4.67	5.83	1.32	1.09	2.34	2.45	6.80	7.16
Pro	3.09	3.23	4.75	4.80	3.54	3.93	4.95	5.09	3.76	3.67	2.70	2.67
Ser	11.72	12.10	4.95	4.70	8.23	4.26	12.85	13.45	11.70	11.85	4.50	4.57
Thr	8.00	8.06	5.65	5.67	5.04	4.27	4.82	4.73	9.50	9.39	4.00	3.83
Trp	n.d.	0	n.d.	n.d.	n.d.	0.63	n.d.	1.09	n.d.	3.27	n.d.	3.83
Tyr	4.67	4.83	3.42	3.25	2.64	2.55	3.98	3.64	1.64	1.63	3.84	3.42
Val	7.33	7.26	5.47	5.80	7.40	7.57	11.00	10.90	9.50	9.39	8.20	7.77

n.d. = not determined.

separations of amino and phenolic acids or amines could be performed on the same column. Also, by coupling the heptafluorobutyrate derivatives with electron capture detection, the sensitivity of the method should be markedly increased.

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REFERENCES

1 W. M. LAMKIN AND C. W. GEHRKE, *Anal. Chem.*, 37 (1965) 383.
2 C. W. GEHRKE, W. M. LAMKIN, D. L. STALLING AND F. SHAHROKHI, *Biochem. Biophys. Res. Commun.*, 19 (1965) 328.
3 D. L. STALLING AND C. W. GEHRKE, *Biochem. Biophys. Res. Commun.*, 22 (1966) 329.
4 C. W. GEHRKE AND F. SHAHROKHI, *Anal. Biochem.*, 15 (1966) 97.
5 D. L. STALLING, G. GILLE AND C. W. GEHRKE, *Anal. Biochem.*, 18 (1967) 1.
6 C. W. GEHRKE AND D. L. STALLING, *Separ. Sci.*, 2 (1967) 101.
7 C. W. GEHRKE, D. ROACH, R. W. ZUMWALT, D. L. STALLING AND L. L. WALL, *Quantitative Gas–Liquid Chromatography of Amino-Acids in Proteins and Biological Fluids*, Analytical Biochemistry Laboratories Inc., Columbia, Mo., 1968.
8 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 192 (1951) 663.
9 D. H. SPACKMANN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
10 S. MOORE, D. H. SPACKMANN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
11 R. WOODS AND R. L. ENGEL, JR., *Ann. N.Y. Acad. Sci.*, 87 (1960) 764.
12 K. A. PIEZ AND L. MORRIS, *Anal. Biochem.*, 1 (1960) 187.
13 P. B. HAMILTON, *Anal. Chem.*, 30 (1958) 914.
14 P. B. HAMILTON, D. C. BOGUE AND R. A. ANDERSON, *Anal. Chem.*, 32 (1960) 1782.
15 P. B. HAMILTON, *Anal. Chem.*, 35 (1963) 2055.
16 W. J. MCBRIDE, JR., AND J. D. KLINGMAN, *Anal. Biochem.*, 25 (1968) 109.
17 R. W. ZUMWALT, K. KUO AND C. W. GEHRKE, *J. Chromatogr.*, 55 (1971) 267.
18 D. L. STALLING, C. W. GEHRKE AND R. W. ZUMWALT, *Biochem. Biophys. Res. Commun.*, 31 (1968) 616.
19 C. W. GEHRKE, H. NAKAMOTO AND R. W. ZUMWALT, *J. Chromatogr.*, 45 (1969) 24.
20 C. W. GEHRKE AND K. LEIMER, *J. Chromatogr.*, 57 (1971) 219.
21 C. W. MOSS AND M. A. LAMBERT, *J. Chromatogr.*, 60 (1971) 134.
22 C. C. SWEELEY AND D. E. VANCE, in G. V. MARINETTI (Editor), *Lipid Chromatographic Analysis*, Vol. I, Marcel Dekker, New York, 1967, p. 465.
23 D. ROACH, C. W. GEHRKE AND R. W. ZUMWALT, *J. Chromatogr.*, 43 (1969) 311.
24 D. G. SMITH, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 238 (1963) 227.
25 G. WEBER AND L. B. YOUNG, *J. Biol. Chem.*, 239 (1964) 1424.
26 H. D. SPRINGALL, *The Structural Chemistry of Proteins*, Butterworths, London, 1954.
27 E. L. SMITH, F. S. MARKLAND, C. B. KASPER, R. J. DELANGE, M. LANDON AND W. H. EVANS *J. Biol. Chem.*, 241 (1966) 5974.
28 B. S. HARTLEY, *Nature (London)*, 201 (1964) 1284.
29 M. L. CADWELL, E. S. DICKEY, U. M. HANRAHAN, H. C. KUNG, J. T. KUNG AND M. MISKO *J. Amer. Chem. Soc.*, 76 (1954) 143.
30 J. P. ZANETTA, W. C. BRECKENRIDGE AND G. VINCENDON, *J. Chromatogr.*, 69 (1972) 291.