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ANALYSIS OF PHOSPHO-AMINO ACIDS AND AMINO ACID AMIDES AT THE PICOMOLE LEVEL USING 4'-DIMETHYLAMINOAZOBENZENE-4-SULPHONYL CHLORIDE

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SUMMARY

Two groups of biologically important unusual amino acids, (a) phospho-amino acids and (b) amino acid amides, can be quantitatively analysed at the pmol level using the dimethylaminoazobenzenesulphonyl chloride precolumn derivatization technique. Conditions for the complete high-performance liquid chromatographic separation of both phospho-amino acids and amino acid amides from common amino acids are described.

INTRODUCTION

4'-Dimethylaminoazobenzene-4-sulphonyl chloride (DABS-Cl) is a chromophoric reagent[1] employed for the labelling of amino acids, polypeptides and amines. DABS derivatives, having ε_{max} of 40,000 l mol^{-1} cm^{-1} at 420 nm[1], are highly stable, and are readily separated by both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) and detected at the pmol level. The DABS-Cl precolumn derivatization technique has been shown to be very useful for quantitative micro-analysis of amino acids[2–4], proteins[5], carcinogenic amines[6], amino acid neurotransmitters[7], sphingosines[8] and tyrosine sulphate[9].

Two groups of unusual amino acids are bbiologically important. (a) Phospho-amino acids, including phospho-serine, phospho-threonine and phospho-tyrosine, are products of protein phosphorylation which has been shown to regulate a large number of cellular process[10–18]. The analysis of phospho-amino acids thus aids in the elucidation of the molecular mechanism of cellular functions controlled by protein phosphorylation. (b) Amino acid amides frequently occur at the carboxyl terminal of polypeptide hormones. The analysis of amino acid amides following enzyme digestion[19,20] is a powerful chemical method for detecting the presence of polypeptide hormones[19].

Quantitative methods for analysis of amino acid amides[19,20] and phospho-amino acids[21–25] are available. However, sensitive and quantitative methods which allow simultaneous analysis of those unusual amino acids together with common amino acids are required. This paper describes the application of DABS-Cl for the quantitative micro-analysis of phospho-amino acids and amino acid amides.

MATERIALS AND METHODS

Materials

O-Phospho-L-serine, Ophospho-DL-threonine and O-phospho-L-tyrosine were purchased from Sigma (U.S.A.). Amino acid amides were obtained from Sigma, Vega (U.S.A.), Serva (F.R.G.) and Bachem (U.S.A.), DABS-Cl from Fluka (Switzerland). All other reagents and solvents used in both derivatization and HPLC analysis were commercial analytical grade from Merck, with minimum purity 99.5%.

Purity of DABS-Cl

As DABS-Cl degrades slowly in acetone during prolonged storafe, the following procedure is recommended for preparation of fresh DABS-Cl. Commercial DABS-Cl (Fluka) was first recrystallized[3]. A solution of DABS-Cl (4 nmol/μl, 1.31 mg/ml) in acetone was then prepared. Portions (100 μl) of this solution were pipetted into Eppendorf tubes (100 or more samples can be prepared each time) and dried under vacuum (first using a water vacuum pump, then an oil vacuum pump). The reagent samples were stored at -20°C, and redissolved in 100 μl of acetone shortly before derivatization.

Preparation of standard DABS derivatives of phospho-amino acids and amino acid amides

Standard mono-DABS-phospho-amino acids and amino acid amides were prepared as described previously[3]. Excesses of the amino acids were allowed to react with a defined quantity of DABS-Cl. In general, 80–90% of the DABS-Cl reacts with the amino acid, the remaining 10–20% being hydrolysed to DABS-ONa (methyl orange). By HPLC analysis of each standard, the precise concentration of each standard DABS-amino acid can be determined after correction of the percentage hydrolysed to DABS-ONa.

Bis-DABS derivatives of lysine amide, histidine amide and tyrosine amide were synthesized by reaction of defined quantities of amino acid amides with excesses ($\approx$ ten-fold) of DABS-Cl[3].

Derivatization of phospho-amino acids and common amino acids

Using the precolumn derivatization technique for amino acid analysis, one needs to know roughly the amount of protein being hydrolysed. The simple reason is that an excess of reagent has to be used. With the DABS-Cl method, a 5–10 fold molar excess of reagent added to the total amino acids is sufficient to achieve quantitative and reproducible derivatization.

The amino acid mixture (0.2–1 μg of protein hydrolysate) was dissolved in 10 μl of 0.2 *M* $NaHCO_3$–NaOH buffer (pH 9.0) and added to 20 μl of DABS-Cl solution (4 nmol/μl in acetone). The tube was tightly stoppered and heated at 70°C for 10 min with occasional shaking. After derivatization, the sample was diluted to 200–1000 μl in 25 m*M* phosphate, pH 6.5–ethanol (1:1) and 20 μl of the diluted sample were injected for HPLC analysis. Although 70°C has been routinely used in this laboratory for derivatization, lower temperatures, such as 60 and 55°C, are equally effective. It is however important to employ the same temperature for derivatization of both unknown and standard samples.

Derivatization of amino acid amides

Amino acid amides exhibit relatively lower reactivities toward DABS-Cl than do amino acids. To achieve optimum derivatization of amino acid amides, the following conditions are recommended. Amino acid amides (containing 50–500 pmol of each amino acid amide) were dissolved in 10 μl of 0.2 *M* $NaHCO_3$–NaOH buffer (pH 9.0) and then added to 40 μl of DABS-Cl solution (2 nmol/μl in acetone). The mixture was allowed to stand at room temperature for 2 h with constant shaking and then heated at 60°C for 10 min in order to ensure complete hydrolysis of the excess of reagent. The derivatized sample was diluted to 200–1000 μl in 25 m*M* phosphate, pH 6.5–ethanol (1:1) and 20 μl of the diluted sample were injected for HPLC analysis.

Equipment

The HPLC system from Waters Associates was used[3]. The time constant of the Model 440 fixed-wavelength detector (at 436 nm) was modified from 0.5 to 1 sec in order to increase the baseline stability. Both DABS-phospho-amino acids and DABS-amino acid amides were analysed on a LiChrosorb RP-18 column (5 μm, Merck). Peak responses were measured by both peak height and peak area integration.

RESULTS AND DISCUSSION

Separation of DABS-phospho-amino acids

A chromatographic system for the complete separation of standard DABS-phospho-amino acids and common DABS-amino acids is shown in Fig. 1. For each new LiChrosorb RP-18 column, a slight modification of the conditions described in Fig. 1 may be required in order to achieve the optimum separation among the acidic DABS-amino acids. Crucial parameters are as follows: (a) increase of the pH of the phosphate buffer, *e.g.*, from 6.1 to 6.5, has slightly more effect in reducing the retention time of DABS-Asp and DABS-Glu than that of DABS-phospho-amino acids (Fig. 2); (b) increase of the salt concentration of the phosphate buffer, *e.g.*, from 12.5 to 25 m*M*, has slightly more effect in increasing the retention of DABS-phospho-amino acids than those of DABS-Asp and DABS-Glu (Fig. 2); (c) inclusion of dimethylformamide in the phosphate buffer can slightly improve the separation among the acidic DABS-amino acids.

Separation of DABS-amino acid amides

The separation pattern of DABS-amino acid amides (Fig. 3) is similar to that of common DABS-amino acids. The conditions described in Fig. 3 do not separate asparagine amide from glutamine amide and aspartic acid amide from glutamic acid amide. Amino acid amides differ from amino acids in lacking a free α-carboxyl group. DABS-amino acid amides are thus less hydrophilic than DABS-amino acids and require a higher percentage of organic solvent as the initial condition for elution (Fig. 3). This structural difference can also be exploited to achieve the complete separation of DABS-amino acid amides from common DABS-amino acids. Fig. 4 gives conditions which allow simultaneous separation of DABS-amino acid amides and common DABS-amino acids. The system employs a phosphate buffer with pH of 6.5 but with a much lower salt concentration (lower ionic strength) The low ionic strength en-

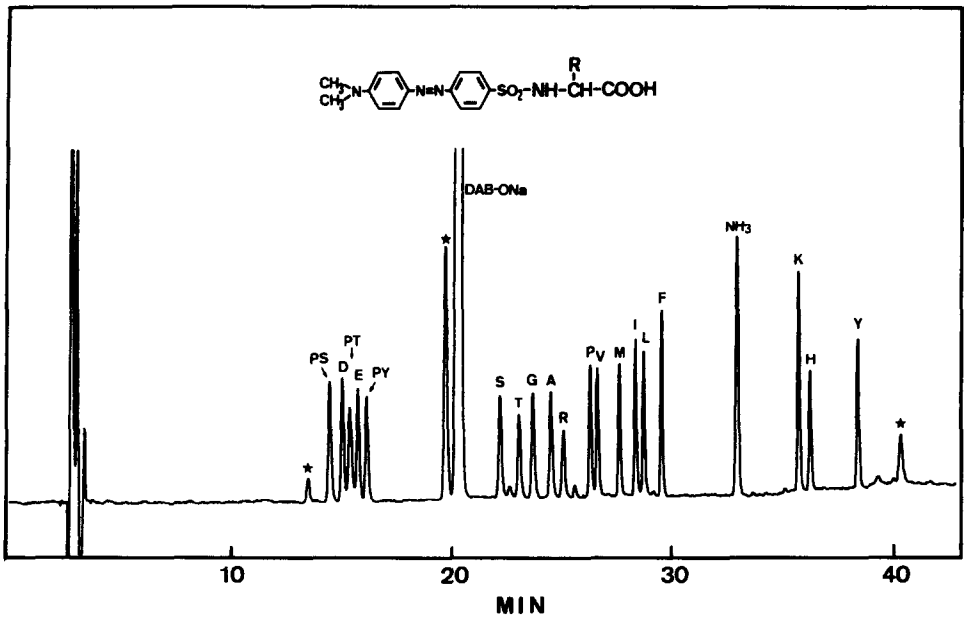

Fig. 1. Complete separation of standard DABS derivatives of phospho-amino acids and common amino acids (each *ca.* 7.5 pmol). Solvents: A, phosphate buffer (25 m*M*, pH 6.5) containing 4% (v/v) of dimethylformamide. B, acetonitrile. The gradient was 12–30% solvent B, in 0–20 min, 30–60% solvent B in 20–35 min, kept at 60% solvent B for 35–45 min, then 60–12% solvent B in 45–50 min. The flow-rate was 1 ml/min. The chart speed was 40 cm/h. Detector: 436 nm, 0.01 a.u.f.s. The column temperature was 50°C. Key: PS = DABS-phospho-serine; D = DABS-Asp; PT = DABS-phospho-threonine; E = DABS-Glu; PY = DABS-phospho-tyrosine; S = DABS-Ser; T = DABS-Thr; G = DABS-Gly; A = DABS-Ala; R = DABS-Arg; P = DABS-Pro; V = DABS-Val; M = DABS-Met; I = DABS-Ile; L = DABS-Leu; F = DABS-Phe; "NH_3" = DABS-NH_2; K = bis-DABS-Lys; H = bis-DABS-His; Y = bis-DABS-Tyr. By-products originating from the excess of reagent are marked by stars.

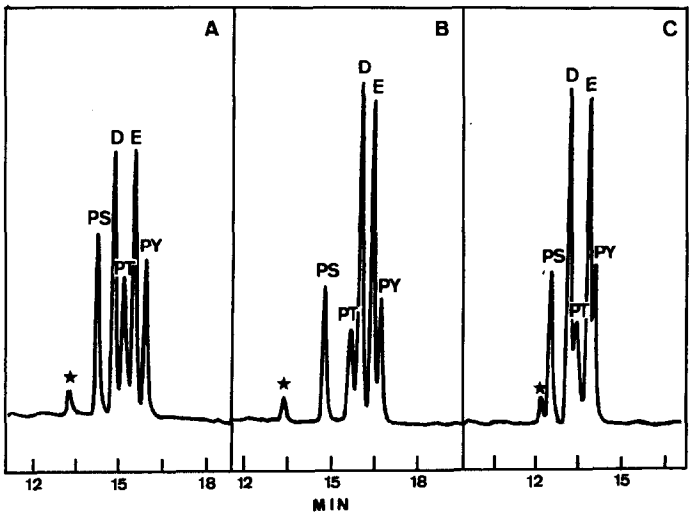

Fig. 2. Effect of the pH and salt concentration of the phosphate buffer (buffer A) on the separation of acidic DABS-amino acids. Phosphate buffers: A, 25 m*M*, pH 6.5; B, 25 m*M*, pH 6.1; C, 12.5 m*M*, pH 6.5. Other chromatographic conditions as in Fig. 1.

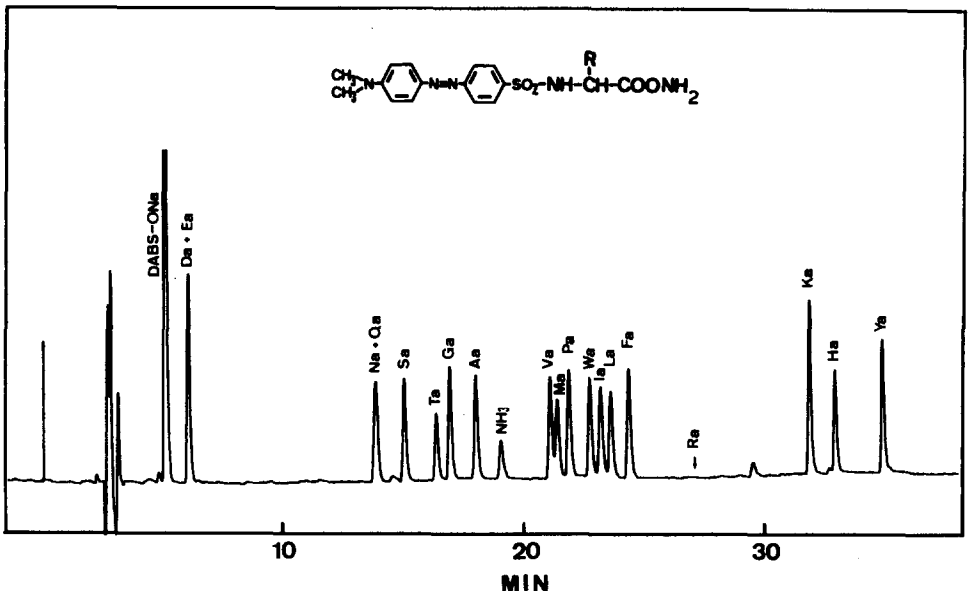

Fig. 3. Separation of standard DABS-amino acid amides (each *ca.* 7.5 pmol). Solvents: A, phosphate buffer (4 m*M*, pH 6.5) containing 4% of dimethylformamide. B, acetonitrile. The gradient was 25–60% solvent B in 0–25 min, 60–75% solvent B in 25–30 min, held at 75% solvent B for 30–40 min, then 75–25% solvent B in 40–45 min. Other conditions as in Fig. 1. Key: Da = DABS-Asp amide; Ea = DABS-Glu amide; Na = DABS-Asn amide; Qa = DABS-Gln amide; Sa = DABS-Ser amide; Ta = DABS-Thr amide; Ga = DABS-Gly amide; Aa = DABS-Ala amide; "NH_3" = DABS-NH_2; Va = DABS-Val amide; Ma = DABS-Met amide; Pa = DABS-Pro amide; Wa = DABS-Trp amide; Ia = DABS-Ile amide; La = DABS-Leu amide; Fa = DABS-Phe amide; Ra = DABS-Arg amide; Ka = bis-DABS-Lys amide; Ha = bis-DABS-His amide; Ya = bis-DABS-Tyr amide.

hances the charge–charge interaction between the negatively charged column support and the negatively charged carboxyl groups of DABS-amino acids and thus drastically reduces the retention time of all DABS-amino acids. Although mono-DABS-amino acids are easily separated from all DABS-amino acid amides (except for aspartic acid amide and glutamic acid amide), the hydrophobic bis-DABS derivatives of lysine, histidine and tyrosine are not. The following steps can be taken to achieve optimum separation: (a) decrease of the molar concentration of phosphate buffer, *e.g.*, from 4.5 to 4 m*M* (see Fig. 4), will slightly reduce the retention time of all DABS-amino acids (except for DABS-Arg), but have essentially no effect on the retention time of all DABS-amino acid amides and DABS-NH_2; (b) increase of the salt concentration of the phosphate buffer, *e.g.*, from 4.5 to 5 m*M*, will have the opposite effect to those in (a). This system is rather insensitive to change of pH (between 6.2 and 7) when the salt concentration is fixed.

Peak response and reproducibility

Using the derivatization conditions described, most amino acids and amino acid amides with uncharged side groups gave comparable peak responses (Table I). Bis-DABS derivatives of lysine, histidine, tyrosine, lysine amide, histidine amide and tyrosine amide gave *ca.* 1.5–2 times higher peak responses than amino acids having

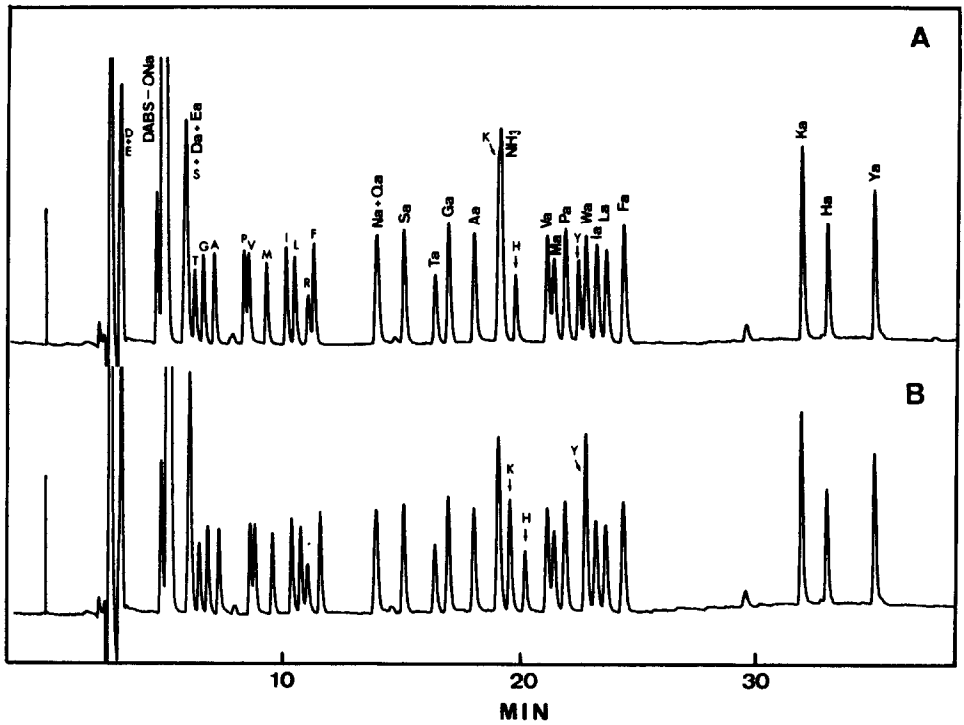

Fig. 4. Complete separation of DABS-amino acid amides from common DABS-amino acids. Chromatographic conditions: A, as in Fig. 3, the concentration of the phosphate buffer being 4 m*M*; B, as in A, but with 4.5 m*M* phosphate buffer. Adjustment of the phosphate buffer from 4.5 m*M* (B) to 4 m*M* (A) slightly reduces the retention time of common DABS-amino acids and can be used to effect the separation of bis-DABS-amino acids (Lys, His and Tyr) fromm DABS-amino acid amides. Symbols as in Figs. 1 and 3.

neutral side groups, while amino acids having charged side chains and amino acid amides typically gave 40–60% of the peak response of neutral amino acids. Despite these variations, linear relationships between peak responses and amounts of amino acids (or amino acid amides) subjected to derivatization are found to be highly reproducible for all amino acids and amino acid amides studied so far (Table I). Phospho-amino acids are known to be unstable to acid. The release of phospho-amino acids from protein usually requires partial acid hydrolysis[27,27] or total enzymatic hydrolysis. However, phospho-amino acids appear to be completely stable during derivatization with DABS-Cl, as evidenced by the observations that: (a) phospho-Ser and phospho-Thr gave comparable peak responses to other acidic amino acids, like Asp and Glu; (b) derivatization of phospho-Ser, phospho-Thr and phospho-Tyr produces no side products (except for excess of reagent) which could have arisen from decomposition of phospho-amino acids.

Sensitivity

The detection limit of DABS-amino acid is about 1 pmol, and could be reduced to the fmol level provided an optimized detector is available (optimized time constant,

TABLE I

PEAK HEIGHT RESPONSES (cm) OF 10 pmol OF DABS-PHOSPHO-AMINO ACIDS AND AMINO ACID AMIDES UNDER VARIOUS CONCENTRATIONS OF AMINO ACIDS AND A FIXED CONCENTRATION OF DABS-Cl

Derivatization conditions as described in the text. Quantities of standards are prepared by weight from individual phospho-amino acids and -amino acid amides (sources indicated in the text).

Amino acid	*A**	*B***	*C****	*D*§
Phospho-Ser	1.7	1.6	1.6	1.5
Phospho-Thr	1.5	1.4	1.5	1.4
Phospho-Tyr	2.5	2.6	2.6	2.4
Ser amide	3.0	3.2	3.2	3.1
Thr amide	2.8	2.8	2.9	2.7
Gly amide	4.2	4.5	4.2	4.3
Ala amide	4.7	5.1	5.0	5.0
Val amide	4.9	5.3	5.3	5.2
Met amide§§	2.5	2.5	2.4	2.4
Pro amide	5.5	5.8	5.8	5.7
Trp amide	3.8	4.1	4.1	4.0
Ile amide	5.0	5.3	5.2	5.2
Leu amide	4.6	4.9	4.9	4.7
Phe amide	3.7	4.0	4.1	4.0
Lys amide	7.1	7.5	7.3	7.3
His amide	4.9	5.3	5.2	5.1
Tyr amide	6.6	6.8	6.9	6.6

* 250 pmol od amino acids were treated with 80 nmol of DABS-Cl and 4% of the derivatized sample were analysed.

** 500 pmol of amino acids were treated with 80 nmol of DABS-Cl and 2% of the derivatized sample were analysed.

*** One nmol of amino acids was treated with 80 nmol of DABS-Cl and 1% of the derivatized sample was analysed.

§ Two nmol of amino acids were treated with 80 nmol of DABS-Cl and 0.5% of the derivatized sample were analysed.

§§ The exceptional low response may be due to an impurity in commercial Met amide.

optimized light energy and absorbance units full scale as low as 0.002). However, in practice, amounts as much as 10–25 times the detection limit are generally used for derivatization with DABS-Cl in order to dilute the contamination. For instance, to detect quantitatively a single tyrosine phosphate residue in a polypeptide with a molecular weight of 2000, the minimum amount of phosphorylated protein required for hydrolysis would be 20–50 ng.

REFERENCES

1 J.-K. Lin and J.-Y. Chang, *Anal. Chem.*, 47 (1975) 1634–1638.
2 J.-Y. Chang, R. Knecht and D. G. Braun, *Biochem. J.*, 203 (1982) 803–806.
3 J.-Y. Chang, R. Knecht and D. G. Braun, *Methods Enzymol.*, 91 (1983) 41–48.
4 J.-K. Lin and C.-H. Wang, *Clin. Chem.*, 26 (1980) 579–583.
5 M.-C. Tzeng, *Anal. Biochem.*, 128 (1983) 412–414.
6 J.-K. Lin and C. C. Lai, *Anal. Chem.*, 52 (1980) 630–635.
7 J.-Y. Chang, P. Martin, R. Bernasconi and D. G. Braun, *FEBS Lett.*, 132 (1981) 117–120.

8 G. Rosenfelder, J.-Y. Chang and D. G. Braun, *J. Chromatogr.*, 272 (1983) 21–27.
9 J.-Y. Chang, *FEBS Lett.*, 164 (1983) 307–313.
10 P. Cohen, *Nature (London)*, 296 (1982) 613–620.
11 E. G. Krebs and J. A. Beavo, *Annu. Rev. Biochem.*, 48 (1979) 923.
12 C. S. Rubin and O. M. Rosen, *Annu. Rev. Biochem.*, 44 (1975) 831–887.
13 C. Klee and T. H. Crouch, *Annu. Rev. Biochem.*, 49 (1981) 489–515.
14 T. Hunter and B. M. Sefton, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1311–1315.
15 H. Ushiro and S. Cohen, *J. Biol. Chem.*, 255 (1980) 8363–8365.
16 O. N. Witte, A. Dasgupta and D. Baltimore, *Nature (London)*, 283 (1980) 826–831.
17 Y.-C. Tse, K. Kirkegaard and J. C. Wang, *J. Biol. Chem.*, 255 (1980) 5560–5565.
18 P. G. Rothberg, T. J. R. Harris, A. Nomoto and E. Wimmar, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 4868–4872.
19 K. Tatemoto and V. Mutt, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 4115–4119.
20 W. H. Simmons and G. Meisenberg, *J. Chromatogr.*, 266 (1983) 483–489.
21 J. C. Yang, J. M. Fujitaki and R. A. Smith, *Anal. Biochem.*, 122 (1982) 360–363.
22 J.-P. Capony and J. G. Demaille, *Anal. Biochem.*, 128 (1983) 206–212.
23 G. Swarup, S. Cohen and D. L. Garbers, *J. Biol Chem.*, 256 (1981) 8197–8201.
24 W.-C. Chang, M.-L. Lee, C.-K. Chou and S.-C. Lee, *Anal. Biochem.*, 132 (1983) 342–344.
25 A. W. Steiner, E. R. Helander, J. M. Fujitaki, L. S. Smith and R. A. Smith, *J. Chromatogr.*, 202 (1980) 263–269.
26 D. B. Bylund and T. S. Huang, *Anal. Biochem.*, 73 (1976) 477–485.
27 B. M. Seftonn, T. Hunter, E. H. Ball and S. J. Singer, *Cell*, 24 (1981) 165–174.