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Amino acid analysis at the picomole level

Application to the *C*-terminal sequence analysis of polypeptides

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Amino acids labelled with dimethylaminoazobenzenesulphonyl chloride can be separated by reversed-phase high-pressure liquid chromatography and detected in the visible region (436 nm). All 19 naturally occurring amino acids can be separated on a Zorbax ODS column by employing two different gradient systems consisting of an acetonitrile/aqueous buffer mixture. As little as 2–5 pmol of an individual dimethylaminoazobenzenesulphonyl-amino acid can be quantitatively analysed with reliability, and only 10–30 ng of the dimethylaminoazobenzenesulphonylated protein hydrolysate is needed for each complete amino acid analysis. This new technique is as sensitive as any of the current amino acid analysis methods involving ion-exchange separation plus fluorescence detection, and is technically much simpler. By the combination of this sensitive amino acid-analysing technique with carboxypeptidase, we have been able to determine the *C*-terminal sequence of polypeptides at the picomole level.

The standard method for amino acid analysis is based on the technique developed by Spackman *et al.* (1958). Amino acid mixtures were separated by ion-exchange column chromatography (Moore & Stein, 1954; Hamilton, 1958) and detected by reaction with ninhydrin. This method has since been modified by adapting micro-bore column (Liao *et al.*, 1973; Hare, 1977) and fluorescent detection systems (Benson & Hare, 1975; Lee *et al.*, 1979; Böhlem & Mellet, 1979) in order to analyse amino acids at the picomole level.

A different approach for amino acid analysis is pre-column formation of derivatives of amino acids followed by separation with h.p.l.c. Among these newly emerging techniques, pre-column formation of *o*-phthalaldehyde derivatives is an especially attractive one (Hill *et al.*, 1979; Lindroth & Mopper, 1979). The *o*-phthalaldehyde method, despite the inherent limitation of the compound unable to react with imino acid (proline, hydroxyproline) is simple, sensitive and reproducible.

We now report a new method for amino acid analysis. Amino acid mixtures were subjected to pre-column formation of derivatives with DABS-Cl (Lin & Chang, 1975) and then separated on a Zorbax ODS column. Dabsylation of most amino acids was found to be quantitative and reproducible under selected conditions. Excess of DABS-Cl was quantitatively hydrolysed to its corresponding sulphonic acid (Methyl Orange) and can be well separated from all the DABS-amino acids.

Compared with the standard method, this new technique is as sensitive, more efficient and instrumentally simpler.

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; DABS-Cl, dimethylaminoazobenzenesulphonyl chloride; DABS or dabsyl, dimethylaminoazobenzenesulphonyl.

Materials and methods

Materials

DABS-Cl was purchased from Fluka and recrystallized by the following method. A 1 g portion of DABS-Cl was dissolved in 100 ml of boiling acetone (analytical grade), and the insoluble materials were removed by passing the solution through a sintered-glass filter funnel. The clear solution was left at −20°C overnight, and needle-shaped crystals were precipitated. Free amino acids and peptides were supplied by Sigma, Serva or Beckman. A CNBr-cleavage peptide derived from the heavy-chain variable region of anti-(streptococcal A) monoclonal antibody 2S1.1 was prepared by Mr. Hermann Herbst at our laboratory. Carboxypeptidase Y was provided by the Millipore Corporation. All other

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chemicals used in the present study were commercial analytical grade from Fluka and Merck and were used without any further purification. The acetate buffer used as solvent A in h.p.l.c. was prepared as follows: 5.44g of sodium acetate trihydrate was mixed with 7.7ml of acetic acid and diluted with water to 900ml.

Instrumentation

The h.p.l.c. system (Waters Associates) comprises two 6000A pumps, a 720 system controller, a U6K sample injector and a model 440 detector (fixed wavelength at 436nm). The sensitivity of the detector is routinely set at 0.01 AUFS (absorption unit, full scale) unless otherwise indicated. DABS-amino acids were analysed on a Zorbax ODS column (Dupont). Sample sizes of 5–25μl were usually injected, and peak area was integrated by an SP 4000 central processor (Spectraphysics).

Preparation of standard mono-DABS-amino acids

Amino acids (1mg) were dissolved in 100μl of 0.1M-sodium bicarbonate buffer, pH9.0, and treated with 100μl of DABS-Cl (2nmol/μl). The pH of the aqueous buffer should be checked before addition of DABS-Cl. In cases where the pH falls (e.g. with serine and threonine), it should be adjusted to 9.0 by addition of 0.1M-NaOH. The mixture was heated at 70°C for 10min, dried *in vacuo* and redissolved in 2ml at 70% (v/v) ethanol. The final concentration of the standard DABS-amino acid is approx. 1nmol/10μl. The exact concentration has to be calculated by analysing each DABS-amino acid sample on h.p.l.c., since 3–10% of the DABS-Cl added was usually hydrolysed to DABS-ONa.

Preparation of standard bis-DABS-amino acids (those of lysine, tyrosine and histidine)

Amino acid (50nmol) was dissolved in 100μl of 0.1M-sodium bicarbonate buffer, pH9.0, and treated with 200μl of DABS-Cl solution (4nmol/μl in acetone, 1.3mg/ml). The mixture was heated at 70°C for 10min, dried *in vacuo* and redissolved in 500μl of 70% (v/v) ethanol. The final concentration of the standard bis-DABS-amino acid is 1nmol/10μl.

Amino acid composition analysis of polypeptide

Polypeptide (0.1–1μg) was placed at the bottom of a silicone-treated glass tube (3mm internal diam. × 10cm) and freeze-dried. (The 0.1–1μg of polypeptide should result in 1–10nmol of total amino acids after hydrolysis. We emphasize this range, since excess of polypeptide lowers the reagent/amino acid molar ratio and may possibly cause incomplete dabsylation under the conditions described.) Then 20μl of 6M-HCl was added and the tube was sealed (2cm from the top) under reduced pressure. After 24h incubation at 110°C, the tube was gently centrifuged and opened at the point of 5cm from the tube bottom. The sample was evaporated *in vacuo* and subjected to dabsylation.

Dabsylation of peptide hydrolysate was performed as follows. To the dried hydrolysate was added 10μl of 0.1M-sodium bicarbonate buffer, pH9.0, followed by 20μl of DABS-Cl solution (4nmol/μl in acetone). The tube was sealed with a rubber stopper and heated at 70°C for 10–15min, with occasional shaking. After derivative formation, the sample was diluted with 70% (v/v) ethanol to 100–500μl, and 10μl of the diluted sample was subjected to h.p.l.c.

A Hamilton standard (100pmol each of amino acid) was also subjected to acid hydrolysis, under conditions identical with those for the unknown sample, followed by dabsylation.

C-Terminal sequence analysis of polypeptide

Polypeptide (500pmol) was pipetted into an Eppendorf tube and freeze-dried. The sample was dissolved in 30μl of 50mM-sodium acetate buffer, pH5.4, 5μl of carboxypeptidase Y solution (0.1mg/ml) was added and the mixture was incubated at room temperature. At time intervals, 7μl samples were removed into Eppendorf tubes and freeze-dried immediately. Dabsylation and amino acid analysis of the removed sample were performed exactly as described for the amino acid composition analysis.

Results

Purity of DABS-Cl

DABS-Cl from Fluka is about 98.8% pure as analysed by h.p.l.c. The product contains approx. 1% of the sulphonic acid derivative (Methyl Orange) as well as approx. 0.2% of unknown impurities that can be partially reduced after recrystallization. Those uncharacterized impurities were fortunately all eluted at well-separated positions under the chromatographic conditions described (Fig. 5).

Separation of standard DABS-amino acids

Two solvent and gradient systems were selected for separation of DABS-amino acids. In the first system, with acetate buffer (pH4.13)/acetonitrile (Fig. 1), DABS-amino acids (except for the aspartic acid and serine derivatives), including the cysteic acid, carboxymethylcysteine, asparagine and glutamine derivatives, were all separated and eluted as sharp peaks. This system also separated the homoserine (Fig. 7), tryptophan (Fig. 6), taurine, β-alanine and γ-aminobutyric acid (results not shown) derivatives. In the second system, with phosphate buffer (pH7.2)/acetonitrile (Fig. 2), DABS-Asp can be separated from DABS-Ser, but other DABS-amino acids were not separated as well as those in first system.

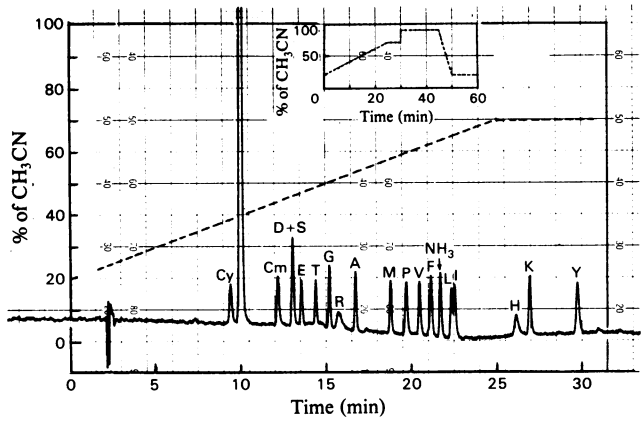

Fig. 1. *Separation of 18 DABS-amino acids on a Zorbax ODS column with the 50 mM-acetate buffer (pH 4.13) (solvent A)/acetonitrile (solvent B) system*

The gradient was 20% solvent B to 70% solvent B in 25 min (linear). The flow rate was 1.2 ml/min. The column temperature was 22°C. The chart speed was 40 cm/h. The sample size was 3 pmol (histidine, lysine and tyrosine) or 5 pmol (other amino acids). Key: Cy, DABS-Cya (where Cya represents cysteic acid); Cm, DABS-CmCys (where CmCys represents carboxymethylcysteine); D, DABS-Asp; S, DABS-Ser; E, DABS-Glu; T, DABS-Thr; G, DABS-Gly; R, DABS-Arg; A DABS-Ala; M, DABS-Met; P, DABS-Pro; V, DABS-Val; F, DABS-Phe; NH_3, DABS-NH_2; L, DABS-Leu; I, DABS-Ile; H, DABS-His; K, DABS-Lys; Y, DABS-Tyr.

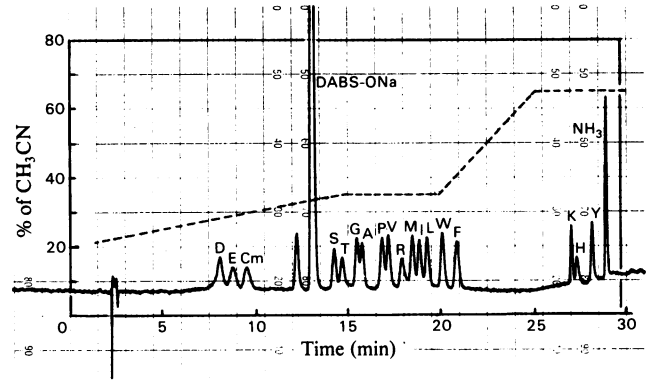

Fig. 2. *Separation of 18 DABS-amino acids on a Zorbax ODS column with the 28 mM-phosphate buffer (pH 7.2) (solvent A)/acetonitrile (solvent B) system*

The gradient was indicated by the broken line. The flow rate was 1.2 ml/min. The column temperature was 22°C. The chart speed was 40 cm/h. The sample size was 3 pmol (histidine, lysine and tyrosine) or 5 pmol (other amino acids). Other experimental details are given in the text. For key to symbols see the legend to Fig. 1.

Dabsylation of amino acid

Dabsylation is performed in the acetone/sodium bicarbonate buffer mixture (2:1, v/v). The reagent (dissolved in acetone) was precipitated on addition to the aqueous buffer and redissolved after heating at 70°C. Quantitative dabsylation of most amino acids requires a minimum reagent concentration (2.66 mM; Fig. 3) and an optimum pH (Fig. 4). Under these selected conditions, there is a linear relationship (for all amino acids) between the peak response and quantity of amino acid subjected to dabsylation within the reagent/amino acid molar ratio range 4:1–80:1 (Table 1).

Amino acid composition analysis with DABS-Cl

Amino acid analysis was performed with polypeptides of known structure, and the results were compared with those obtained from a conventional

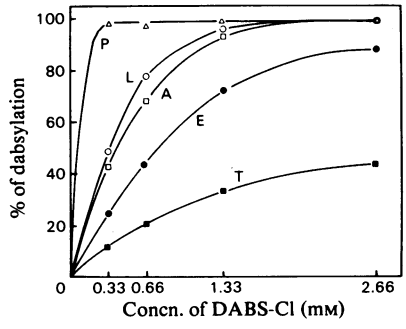

Fig. 3. *Dependence of amino acid dabsylation on the reagent concentration*

Dabsylation was performed at 70°C for 10min in the sodium bicarbonate buffer (pH9.0)/acetone (1:2, v/v) solution with fixed amino acid concentration of 0.066mM. Other experimental details are given in the text. For key to symbols see the legend to Fig. 1.

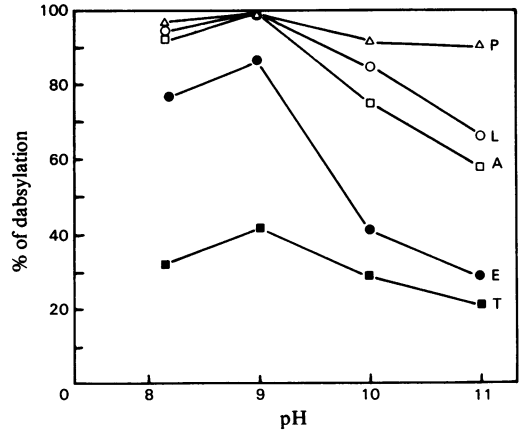

Fig. 4. *Dependence of amino acid dabsylation on the pH of aqueous buffer*

Dabsylation was performed at 70°C for 10min in the aqueous buffer/acetone (1:2, v/v) solution. Aqueous buffers were 0.1M-sodium bicarbonate (pH8.15) adjusted to pH9, 10 and 11 by addition of 0.1M-NaOH. Reagent concentration was 2.66mM. Amino acid concentration was 0.066mM. Other experimental details are given in the text. For key to symbols see the legend to Fig. 1.

amino acid analyser (Biotronik) (Table 2). DABS-amino acids were determined by measuring either peak height or peak area. Quantitative analysis based on peak height is critically dependent on the reproducibility of sharpness of the peaks. With the h.p.l.c. system and column working at optimum conditions, the results were usually reliable and

Table 1. *Peak-height response of 10pmol of DABS-amino acids under dabsylation conditions with varied concentration of amino acids and fixed concentration of DABS-Cl*

Condition A: 50pmol of Hamilton standard was dabsylated with 80nmol of DABS-Cl (see the Materials and methods section), and 20% of the dabsylated sample was injected. Condition B: 200pmol of Hamilton standard was dabsylated with 80nmol of DABS-Cl, and 5% of the dabsylated sample was injected. Condition C: 500pmol of Hamilton standard was dabsylated with 80nmol of DABS-Cl and 2% of the dabsylated sample was injected. Condition D: 1nmol of Hamilton standard was dabsylated with 80nmol of DABS-Cl, and 1% of the dabsylated sample was injected.

Amino acid	Peak-height response (cm) Condition ... A	B	C	D
Asp + Ser	5.2	5.5	5.6	5.2
Glu	2.9	3.3	3.3	3.1
Thr	1.5	1.6	1.6	1.5
Gly	4.5	4.8	4.9	4.7
Arg	1.0	1.0	1.0	0.9
Ala	3.9	4.1	4.2	4.2
Met	4.0	3.6	3.7	3.8
Pro	3.8	3.8	3.8	3.6
Val	3.5	3.6	3.5	3.5
Phe	4.0	4.0	3.9	3.7
Leu*	4.8	4.8	4.7	4.6
Ile	3.7	3.6	3.5	3.5
His	2.3	2.4	2.3	2.2
Lys	6.5	6.4	6.0	6.1
Tyr	5.3	5.2	5.0	5.0

* The peak-response of leucine is 25% higher than that of a home-made standard.

precise. Fig. 5 gives the original chromatograms of the amino acid analysis of a Hamilton standard (10pmol), 7ng (2pmol) of melittin and 30ng (2pmol) of ribonuclease A. It should be pointed out that the standard gives an exceptionally high response of leucine (approx. 25% higher than the home-made standard), and we have no idea about the origin of this leucine contamination.

C-Terminal sequence analysis

C-Terminal analysis of polypeptide at the picomole level was achieved by combining the use of carboxypeptidase Y and DABS-Cl method. The eight amino acid residues at the *C*-terminal end of glucagon were quantitatively released by carboxypeptidase Y within 15min (Fig. 6). Time-course release of *C*-terminal amino acids is shown in Table 3. It is difficult to establish the *C*-terminal sequence of glucagon from the kinetic curve except for identifying threonine as the *C*-terminus. However, it

Table 2. *Amino acid compositions of polypeptides determined by the DABS-Cl method*
Results given for the DABS-Cl method are averaged from four separate analyses (10–30ng each) of the same dabsylated sample. Analyses by the standard method were performed with a Biotronik analyser, and results given are averaged from two separate analyses (5–10μg each). N.D., Not determined. Numbers given in the parentheses are expected values.

	Melittin		Insulin B-chain		Myoglobin		Ribonuclease
	DABS-Cl method	Standard method	DABS-Cl method	Standard method	DABS-Cl method	Standard method	DABS-Cl method
Cya	—	—	2.07 ± 0.08	2.40 (2)	—	—	7.7 ± 0.2 (8)
Asp	0.65 ± 0.04*	1.09 (1)	1.09 ± 0.17*	1.06 (1)	8.0 ± 0.2*	8.7 (8)	15.6 ± 0.3* (30)
Ser		—		1.07 (1)		5.7 (6)	
Glu	2.07 ± 0.02	2.18 (2)	2.92 ± 0.12	3.36 (3)	17.6 ± 0.5	19.1 (19)	12.4 ± 0.2 (12)
Thr	2.00 ± 0.10	1.89 (2)	1.01 ± 0.04	0.96 (1)	5.0 ± 0.1	5.0 (5)	8.9 ± 0.1 (10)
Gly	2.98 ± 0.20	3.09 (3)	2.91 ± 0.13	2.97 (3)	10.4 ± 0.2	11.3 (11)	3.3 ± 0.1 (3)
Arg	1.85 ± 0.04	1.89 (2)	0.96 ± 0.10	0.90 (1)	4.1 ± 0.1	4.1 (4)	3.9 ± 0.2 (4)
Ala	2.09 ± 0.07	1.96 (2)	2.10 ± 0.15	1.80 (2)	16.8 ± 0.1	16.5 (17)	11.0 ± 0.2 (12)
Met	—	—	—	—	1.9 ± 0	2.2 (2)	N.D. (4)
Pro	1.11 ± 0.09	N.D. (1)	0.91 ± 0.07	N.D. (1)	4.4 ± 0.1	N.D. (4)	4.1 ± 0.1 (4)
Val	2.22 ± 0.05	1.98 (2)	3.02 ± 0.04	2.77 (3)	8.0 ± 0.2	7.7 (8)	9.0 ± 0.1 (9)
Phe	—	—	2.96 ± 0.11	3.33 (3)	6.4 ± 0.2	6.7 (6)	3.1 ± 0.1 (3)
Leu†	3.23 ± 0.15	3.05 (4)	3.28 ± 0.20	2.97 (4)	14.4 ± 0.4	14.0 (18)	1.7 ± 0.1 (2)
Ile	2.93 ± 0.07	2.84 (3)	—	—	8.9 ± 0.1	8.8 (9)	2.5 ± 0.2 (3)
His	—	—	1.94 ± 0.15	1.74 (2)	12.2 ± 0.2	11.5 (12)	4.6 ± 0.2 (4)
Lys	3.05 ± 0.05	2.87 (3)	1.10 ± 0.07	0.98 (1)	18.9 ± 0.4	18.7 (19)	10.0 ± 0.3 (10)
Tyr	—	—	2.20 ± 0.12	1.65 (2)	3.4 ± 0.1	3.0 (3)	5.2 ± 0.2 (6)

* Aspartic acid and serine were determined together. The numbers given in the table are the average values for aspartic acid and serine.
† The leucine content of the Hamilton standard is 25% higher than a home-made standard.

may be noticed that the reaction drastically slowed down at the aspartic acid residue, and by selecting an optimum hydrolysis time (12–15 min) one may prepare a desoctapeptide-(22–29)-glucagon with a purity of 96–98%.

Fig. 7 shows the original chromatograms from the *C*-terminal analysis of a 50-residue CNBr-cleavage peptide (with homoserine as the *C*-terminus). This peptide is derived from the heavy-chain variable region (residues 33–82) of anti-(streptococcal A) monoclonal antibody 2S1.1. The peptide was eluted from a Sephadex G-50 gel column and was contaminated by approx. 25% of a 17-residue peptide with the known *C*-terminal sequence -Pro-Gly-Gly-Ser-Hse (where Hse represents homoserine). *N*-Terminal sequence analysis with the automatic sequenator provided sequences of only the first 47 residues and gave an incomplete *C*-terminal sequence of -Val-Tyr-(...)-Hse. Analysis of major amino acids released by carboxypeptidase Y digestion gave the quantitative results (serine, glycine and homoserine were not calculated) shown in Table 4, and clearly establish the *C*-terminal sequence as -Val-Tyr-Leu-Gln-Hse.

Discussion

Separation of DABS-amino acids

The gradient system described in Fig. 1 has given very reproducible separations of DABS-amino acids on at least three Zorbax ODS columns. There are, however, slight differences with retention times of DABS-Arg and DABS-NH_2 among those columns. Therefore each new column should be optimized by selecting a suitable aqueous buffer. Here we outline some of our experiences. (1) At fixed salt concentration, decreasing the pH (between 4 and 5.5) of aqueous buffer (ionizing carboxy groups and thus decreasing the polarity of the DABS-amino acid), increases the retention time of most DABS-amino acids, but has little or no effect on the retention time of DABS-NH_2, DABS-ONa or DABS-Cya. Thus, if DABS-NH_2 is found to be too close to, or overlapping with DABS-Leu, decreasing the pH of the aqueous buffer (e.g. from 4.2 to 4.1) would solve the problem. (2) At fixed pH, increasing the salt concentration (increasing the ionic strength and thus suppressing the negative charge on the silica surface

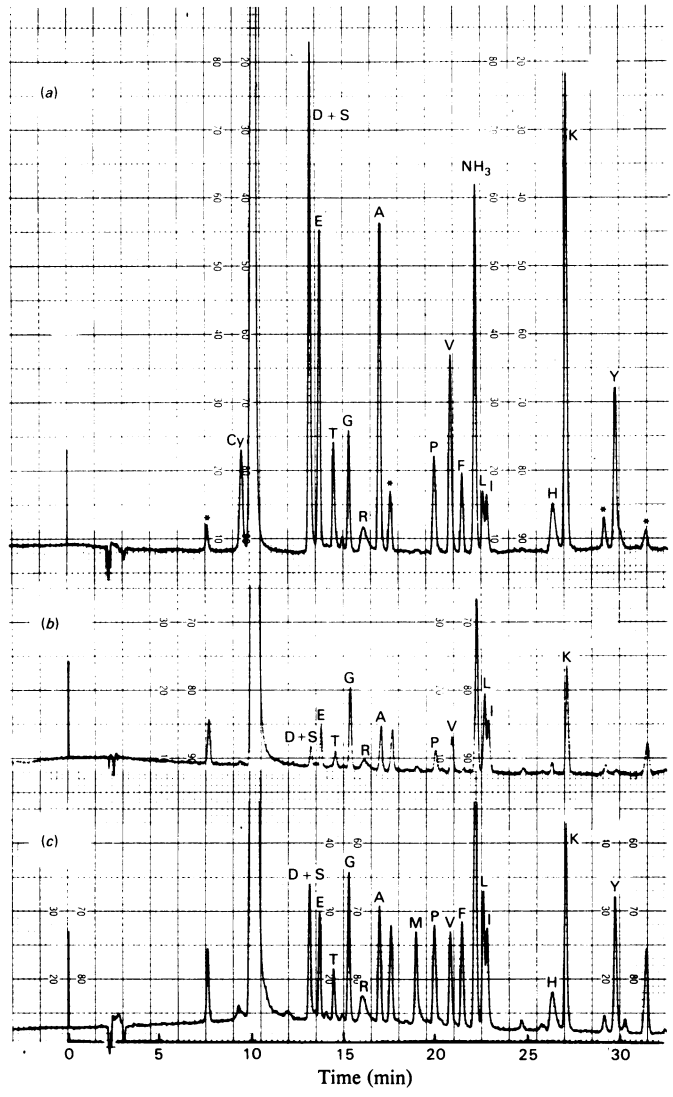

Fig. 5. *Original chromatograms of amino acid analysis by the DABS-Cl method*
(*a*) Oxidized ribonuclease A (1 μg) was hydrolysed and dabsylated, and one-thirtieth of the dabsylated sample (33 ng) was injected. (*b*) Melittin (0.3 μg) was hydrolysed and dabsylated, and one-fortieth of the dabsylated sample (7.5 ng) was injected. (*c*) Hamilton standard (100 pmol) was subjected to acid hydrolysis and dabsylated, and one-tenth of the total sample was injected. For full experimental details see the text. Amino acid compositions calculated from these chromatograms are presented in Table 2. Peaks marked by asterisks are by-products originating from the reagent. Conditions for column chromatography are identical with those described in Fig. 1, except for the use of second Zorbax ODS column. Slight differences in retention time as compared with the first Zorbax ODS column (Fig. 1) were observed. For key to symbols see the legend to Fig. 1.

of the stationary phase; Schmidt *et al.*, 1980) decreases the interaction between the basic DABS-Arg and the silica surface, and as a consequence decreases the retention time of DABS-Arg.

The major and perhaps the only shortcoming of the DABS-Cl method, as compared with the standard method, is the incomplete separation of all DABS-amino acids with any one single solvent system. However, with the dramatic improvement of column qualities in recent years (Horvath, 1980) and with the types of reversed-phase column that are commercially available, we believe that it is only a matter of time before optimum conditions are found that would overcome this problem.

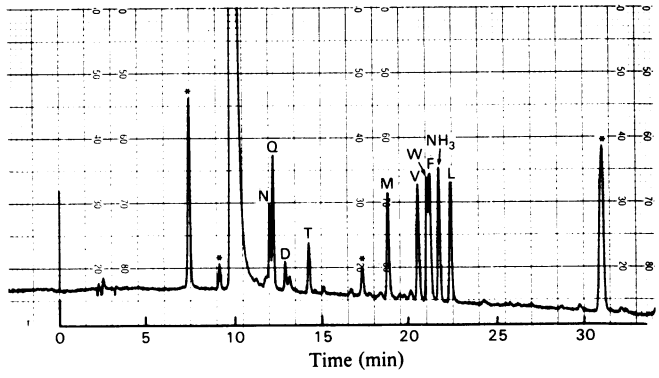

Fig. 6. *Analysis of amino acid released from glucagon after 70 min digestion with carboxypeptidase Y*
For experimental details of the digestion see the text. The chromatogram shows good separation of DABS-Asn (N) and DABS-Gln (Q), and partial separation of DABS-Trp (W) from other amino acids. Peaks marked by asterisk are by-products originating from the reagent. Conditions for column chromatography are identical with those described in Fig. 1. For key to symbols see the legend to Fig. 1.

Table 3. *Time-course release of C-terminal amino acids from glucagon by carboxypeptidase Y digestion*
A 500 pmol sample of glucagon was used for digestion, and the released amino acids were determined by the DABS-Cl method. Other experimental details are given in the text and the legend to Fig. 6.

Period of digestion	C-Terminal sequence* and amino acids released ...	DABS-amino acid released (units of 10 pmol)									
		- Gln	- Asp	- Phe	- Val	- Gln	- Trp	- Leu	- Met	- Asn	- Thr
3 min		—	0	0.27	0.41	0.40	0.41	0.40	0.47	0.47	0.53
15 min		—	0.05	1.02	1.03	0.97	1.00	0.89	0.95	0.90	1.07
70 min		—	0.28	1.08	1.06	1.30	1.03	0.91	0.92	1.02	1.09

* Bromer *et al.* (1971).

Amino acid analysis with the DABS-Cl method

From Table 2 it is apparent that amino acid analysis obtained with the DABS-Cl method at the picomole level is just as reliable as that obtained with a standard analyser at the nanomole level. In contrast with the fluorescent method (Hare, 1977; Böhlem & Mellet, 1979; Lee *et al.*, 1979), the DABS-Cl method can analyse the imino acids together with the amino acids at the same degree of sensitivity.

Only 10–30 ng of the dabsylated hydrolysate is required for each analysis. This sensitivity is particularly noteworthy, since it is approx. 10-fold higher than that of the most sensitive analyses reported (Lee *et al.*, 1979; Kang & Drescher, 1979). However, it is necessary to point out that we have routinely hydrolysed 0.1–1 μg of polypeptide and applied only one-tenth to one-fortieth of the total dabsylated sample for each analysis run. It is feasible to start the hydrolysis with less than 50 ng of sample, although this would produce relatively larger amounts of by-products (mainly DABS-ONa) and result in a higher risk of amino acid contaminations.

It is also relevant to mention that in the present study we have not put any effort into purifying the chemicals and solvents (except for recrystallizing DABS-Cl) and have found that background produced by a blank run contained usually less than 0.5 pmol of individual DABS-amino acids. This is not surprising, since the possible source of contamination resides only in the dabsylation buffer, whose volume is relatively small (10–15 μl).

C-Terminal sequence analysis of polypeptide

There has been a desperate need for a high-sensitivity *C*-terminal sequencing method to match the well-established *N*-terminal sequencing techniques (Edman & Begg, 1967; Hunkapiller & Hood,

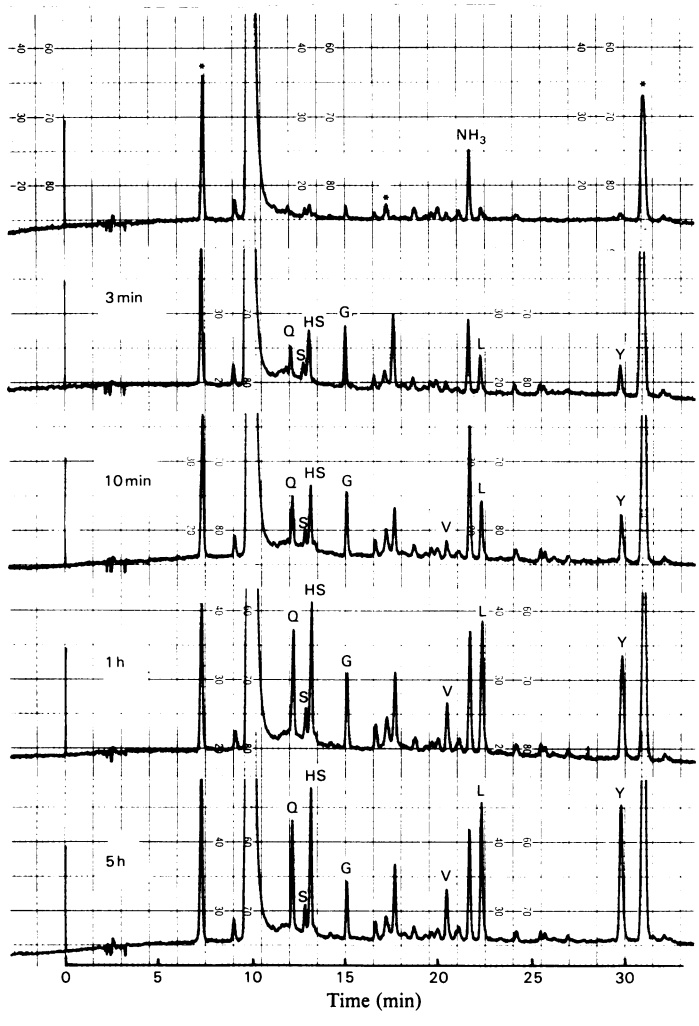

Fig. 7. *Time-course release of amino acids from carboxypeptidase Y digestion of a mixture of two CNBr-cleaved peptides from the heavy-chain variable region of anti-(streptococcal A) monoclonal antibody 2S1.1*

The major CNBr-cleaved peptide (approx. 80%, 50 amino acid residues) has the incomplete *C*-terminal sequence of -Val-Tyr-(. . .)-Hse. The minor CNBr-cleaved peptide (approx. 20%, 17 amino acid residues) has the known *C*-terminal sequence -Pro-Gly-Gly-Ser-Hse. About 500 pmol of polypeptide was subjected to carboxypeptidase Y digestion. Then 100 pmol each was removed at time intervals (after 3 min, 10 min, 1 h and 5 h) and dabsylated, and only one-tenth of each dabsylated sample was subjected to h.p.l.c. Key: HS, DABS-Hse (where Hse represents homoserine); for other symbols see the legend to Fig. 1. A blank run (sample constituting only carboxypeptidase Y and buffer) is shown on the top panel. The major by-products derived from reagent are marked with asterisk (top panel). Another uncharacterized by-product (possibly DABS-peptide) was eluted at 17.7 min, which does not correspond to the value for any known DABS-amino acid. Conditions for analysing DABS-amino acids were identical with those described in the legend to Fig. 1. For the quantitative results see Table 4.

1978). The availability of various carboxypeptidases (Ambler, 1972; Hayashi, 1977) with distinctive specificities should further enhance the versatility of this *C*-terminal sequencing method. This new technique should provide a useful tool in peptide structure analysis, not only because of its high sensitivity but also because of its speed (only 3–4 h with the use of carboxypeptidase Y).

Table 4. *Time-course release of C-terminal amino acids from a CNBr-cleaved peptide by carboxypeptidase digestion*
A 500pmol sample of the peptide was used for digestion, and the released amino acids were determined by the DABS-Cl method. The source of the CNBr-cleaved peptide and other experimental details are given in the text and the legend to Fig. 7. N.D., Not determined.

Time of digestion	*C*-Terminal sequence and amino acids released ...	DABS-amino acid released (units of 10pmol)				
		- Val -	- Tyr -	- Leu -	- Gln -	- Hse -
3min		0.1	0.19	0.27	0.30	N.D.
10min		0.14	0.33	0.43	0.50	N.D.
1h		0.44	0.72	0.98	1.11	N.D.
5h		0.50	0.96	1.04	1.11	N.D.

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