Biochem. J. (1981) **199**, 557–564
Printed in Great Britain

N-Terminal sequence analysis of polypeptide at the picomole level

Jui-Yoa CHANG
Pharmaceuticals Research Laboratories, CIBA–GEIGY Ltd., 4002 Basle, Switzerland

(*Received 30 April 1981/Accepted 23 July 1981*)

This paper describes a manual method for *N*-terminal sequence analysis of polypeptides at subnanomole sensitivity. The polypeptide is degraded stepwise by using the dimethylaminoazobenzene isothiocyanate/phenyl isothiocyanate double-coupling method, and the released dimethylaminoazobenzenethiohydantoins of amino acids were identified by reversed-phase high-pressure liquid chromatography. The dimethylaminoazobenzenethiohydantoins are coloured compounds and can be detected in the visible region with the sensitivity limit of 1 pmol (signal-to-baseline noise ratio 5). A high-pressure liquid-chromatographic method was developed for complete analysis of all amino acid dimethylaminoazobenzenethiohydantoin derivatives, including the by-products of serine and threonine. Thus, without use of an automatic sequenator or radioactive materials, it is possible to determine the complete sequence of peptides and *N*-terminal sequence of proteins with less than 1 nmol of material.

The automatic Edman degradation procedure (Edman & Begg, 1967) has been adapted for sequencing both peptides and proteins at the subnanomole level (Hunkapiller & Hood, 1978). Among those refinements, the use of h.p.l.c. for analysis of amino acid PTH derivatives (Zimmerman *et al.*, 1977) and the introduction of Polybrene for minimizing extraction losses of polypeptide (Tarr *et al.*, 1978; Klapper *et al.*, 1978) have been shown to have a major impact on the development of micro sequencing techniques.

To perform subnanomole sequence analysis with current automatic techniques, one needs an extensively modified sequenator (Wittmann-Liebold, 1973) as well as a computer-assisted h.p.l.c. system (Hunkapiller & Hood, 1980). Alternatively, one can use automatic solid-phase Edman degradation (Laursen, 1971) with radioactive [^{35}S]PITC (Bridgen, 1976). All those techniques, despite their capabilities, require highly specialized instruments. Thus there is a real need for developing a comparable micro-sequencing method that can be performed with less expense and complexity.

We have introduced a coloured Edman reagent, DABITC (Chang, 1977, 1979*b*; Chang *et al.*, 1978), to replace radioactive PITC for high-sensitivity sequence analysis. The DABITC degradation produces coloured amino acid DABTH derivatives, which can be identified by t.l.c. at the 5–15 pmol level. The DABITC/PITC double-coupling method (Chang *et al.*, 1978; Chang, 1979*b*) has enabled us to sequence manually 2–5 nmol of polypeptide containing 20–30 residues and has been successfully applied to the sequence determination of ribosomal proteins (Allen & Wittmann-Liebold, 1978; Heiland & Wittmann-Liebold, 1979), human somatotropin (Lewis *et al.*, 1980), human lypho-blastoid interferon (Allen & Fantes, 1980), trypsin inhibitor (Richardson, 1979) etc.

The sensitivity of the DABITC/PITC double-coupling method, however, could be further increased by using h.p.l.c. for analysis of amino acid DABTH derivatives. The h.p.l.c. method has two specific advantages: (1) it is quantitative, and (2) it is about 5–10 times as sensitive as the t.l.c. method and as little as 1–2 pmol of amino acid DABTH derivative can be detected against a stable base-line. These points have allowed us to sequence 400–1000 pmol of polypeptide routinely, and results are presented in this paper.

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; DABITC, dimethylaminoazobenzene isothiocyanate; DABTC, dimethylaminoazobenzenethiocarbamoyl; DABTH, dimethylaminoazobenzenethiohydantoin; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin.

Materials and methods

Materials

The commercial DABITC of Fluka (Switzerland) was recrystallized from boiling acetone. DABITC is very stable in acetone but slowly decomposed in

0306-3275/81/120557-08$01.50/1

pyridine. A stock solution of DABITC (1.41 mg/ml) in acetone was prepared. Portions (500 μl) of the stock solution were pipetted into Eppendorf tubes (1 ml size), dried *in vacuo*, stored at −20°C and redissolved in 250 μl volumes of pure pyridine freshly before used. Pyridine and trifluoroacetic acid (sequential grade) were prepared by the method described previously (Chang *et al.*, 1978) or purchased from Fluka and Pierce. PITC was supplied by Beckman. Acetic acid saturated with HCl was prepared by bubbling HCl gas through acetic acid (99.5%; Fluka) for 2 h. Distilled water was degassed for all uses (in both sequencing and h.p.l.c.). All other chemicals and solvents used were commercial analytical grade (Merck and Fluka) and were used without further purification. Polypeptides (angiotensin I, insulin B-chain, mellitin and lysozyme) were purchased from Sigma. Tryptic peptide L24-T12 was derived from ribosomal protein L24 (Wittmann-Liebold, 1979), and a CNBr-cleavage peptide from the constant region of monoclonal antibody 2S1.1 was prepared by Mr. Hermann Herbst at our laboratory.

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 436 nm). The sensitivity of the detector is set at 0.01 AUFS (absorption unit, full scale) unless otherwise indicated. Amino acid DABTH derivatives were analysed on a Zorbax ODS column (Dupont). Sample sizes of 5–25 μl were usually injected.

Preparation of standard amino acid DABTH derivatives

Amino acid (0.5 mg) was dissolved in 100 μl of triethylamine/acetic acid buffer (50 ml of water + 50 ml of acetone + 0.5 ml of triethylamine + 5 ml of 0.2 M-acetic acid, pH 10.65) and treated with 50 μl of DABITC solution (4 nmol/μl in acetone). The mixture was heated at 53°C for 1 h, dried *in vacuo* and then redissolved in 20 μl of water and 40 μl of acetic acid saturated with HCl. The acid solution was heated at 53°C for 50 min and then dried again *in vacuo*. The dried amino acid DABTH derivative was dissolved in 2 ml of 70% (v/v) ethanol and used as standard sample (approx. 1 nmol/10 μl).

The pH of lysine, histidine, aspartic acid and glutamic acid solutions usually falls below 8 and should be adjusted to 10 (by addition of 1 M-NaOH) before addition of DABITC solution.

Preparation of by-products of DABTH-Ser and DABTH-Thr

By-products of DABTH-Ser and DABTH-Thr were prepared and characterized as described previously (Chang, 1979*a*).

Preparation of α-DABTH-ε-DABTC-Lys (K_1)

L-Lys-L-Asp (20 nmol) was dissolved in 200 μl of aq. 50% (v/v) pyridine and treated with 100 μl of DABITC solution (10 nmol/μl in pyridine). The mixture was heated at 70°C for 1 h, and the excess of reagent was extracted by vortex mixing with 3 × 500 μl of heptane/ethyl acetate (2:1, v/v). The aqueous phase was dried *in vacuo* and redissolved in 40 μl of water and 80 μl of acetic acid saturated with HCl. The acid mixture was kept at 53°C for 50 min and then evaporated. The dried sample (containing α-DABTH-ε-DABTC-Lys and free aspartic acid) was dissolved in 200 μl of 70% (v/v) ethanol and used as standard.

Preparation of α-PTH-ε-DABTC-Lys (K_2) and α-DABTH-ε-PTC-Lys (K_3)

Lysine (800 nmol) was dissolved in 100 μl of triethylamine/acetic acid buffer, pH 10.65, and treated with 50 μl of DABITC solution (4 nmol/μl in acetone). After 30 min incubation at 53°C, 5 μl of pure PITC was added to the mixture and incubation was continued for another 30 min. The sample was dried *in vacuo* overnight and redissolved in 40 μl of water and 80 μl of acetic acid saturated with HCl. The acid mixture was heated at 53°C for 50 min, dried *in vacuo* and redissolved in 1 ml of 70% (v/v) ethanol. The final product shows one blue (α-PTH-ε-DABTC-Lys, K_2) and one red (α-DABTH-ε-PTC-Lys, K_3) spot on polyamide t.l.c. (Chang *et al.*, 1978) but gives only one peak on h.p.l.c. (Fig. 5).

N-Terminal sequence analysis by using the DABITC/PITC double-coupling method

The protocol is mainly based on the procedures described previously (Chang *et al.*, 1978), but the quantities of reagents were scaled down and modified as follows. Polypeptide (0.4–1 nmol), freeze-dried in an acid-washed tube (0.6 cm internal diam. × 5 cm, with a V-shaped bottom) fitted with a glass stopper, was dissolved in 40 μl of aq. 50% (v/v) pyridine and treated with 20 μl of DABITC solution (10 nmol/μl in pyridine). The tube was flushed with N_2, sealed with a glass stopper and heated at 53°C for 50 min. After the first coupling, 5 μl of pure PITC was added and incubation was continued for 30 min at 53°C. The excess reagents were extracted by mixing the reaction mixture with three 250 μl portions of heptane/ethyl acetate (2:1, v/v) in a vortex mixer and centrifuging. The organic phase was thoroughly removed with a 5 ml syringe, and the aqueous phase was dried *in vacuo*. The dried residue was dissolved in 50 μl of anhydrous trifluoroacetic acid, flushed with N_2, and the tube was sealed with the glass stopper and heated at 53°C for 10 min. The trifluoroacetic acid mixture was dried *in vacuo*, 50 μl of water was added, and the cleaved *N*-terminal

amino acid was extracted with 150μl of butyl acetate. After removal of the butyl acetate extract, the peptide in the aqueous phase was dried *in vacuo* and subjected to the next degradation cycle.

For the conversion reaction, the butyl acetate extract was evaporated and the residue was dissolved in 15μl of water and 30μl of acetic acid saturated with HCl. Conversion of thiazolinone derivative into thiohydantoin derivative was performed at 53°C for 50min. The acid solution was dried and redissolved in 50–500μl of 70% (v/v) ethanol for h.p.l.c. identification of amino acid DABTH derivatives.

Results

Separation of amino acid DABTH derivatives

Separation of 20 amino acid DABTH derivatives on a Zorbax ODS column is presented in Fig. 1. All amino acid derivatives, except for those of leucine and isoleucine, can be separated by a linear gradient of acetate buffer/acetonitrile mixture. The DABTH-Leu/DABTH-Ile pair can be separated by a Zorbax CN column with a similar gradient system (Fig. 2). The base-line is stable regardless of the gradient change. The detection limit is less than 1pmol (signal-to-baseline noise ratio about 4–5).

With the DABITC/PITC double-coupling method, serine, threonine and lysine normally give multiple products (Chang *et al.*, 1978; Chang, 1979*a*,*b*). (1) DABTH-Ser is destroyed to give four by-products (Fig. 3) namely, DABTH-Ser$^{\triangle}$ (a dehydro product, retention time 15.3min), DABTH-Ser$^{\circ}$ (a presumably hydrated DABTH-Ser$^{\triangle}$ with a hydroxy group at the α-position, retention time 12.3min), DABTC-NH_2 (retention time 11.1min) and DABTH-Ser$^{\square}$ (polymerized DABTH-Ser$^{\triangle}$, retention time not established). (2) DABTH-Thr is accompanied by two additional by-products (Fig. 4), DABTH-Thr$^{\triangle}$ (a dehydro product, retention time 15.9min) and DABTH-Thr$^{\times}$ (uncharacterized, retention time 25.8min). (3) Lysine gives three

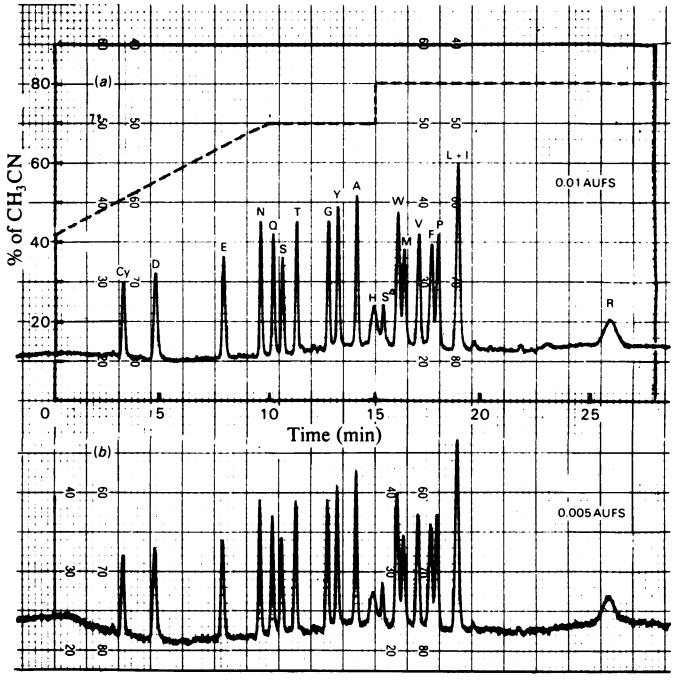

Fig. 1. *Separation of 10pmol (a) and 5pmol (b) each of a mixture of amino acid DABTH derivatives on a Zorbax ODS column*

Chromatographic conditions were as follows. Solvent A was 35mM-acetate buffer, pH5.0, and solvent B was acetonitrile. The gradient was as indicated by the broken line. The flow rate was 1ml/min. The column temperature was 22°C. The chart speed was 40cm/h. Other experimental details are given in the text. Key: Cy, DABTH-Cya (where Cya represents cysteic acid); D, DABTH-Asp; E, DABTH-Glu; N, DABTH-Asn; Q, DABTH-Gln; S, DABTH-Ser; T, DABTH-Thr; G, DABTH-Gly; Y, DABTH-Tyr; A, DABTH-Ala; H, DABTH-His; W, DABTH-Trp; M, DABTH-Met; V, DABTH-Val; F, DABTH-Phe; P, DABTH-Pro; L, DABTH-Leu; I, DABTH-Ile; S$^{\triangle}$, dehydro product of DABTH-Ser.

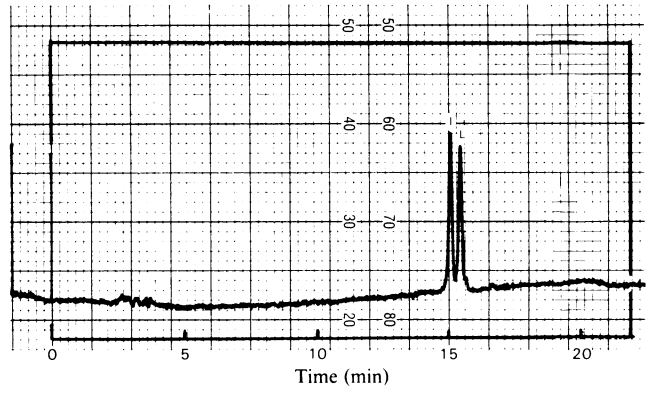

Fig. 2. *Separation of DABTH-Leu and DABTH-Ile on a Zorbax CN column*
Chromatographic conditions were as follows. Solvent A was 35 mM-acetate buffer, pH 5.0, and solvent B was acetonitrile. The gradient was 42% solvent B to 70% solvent B in 15 min (linear). The flow rate was 1 ml/min. The column temperature was 22°C. The chart speed was 40 cm/h. The sample size was 10 pmol. DABTH-Ile overlapped with DABTH-Pro in this system. Other experimental details are given in the text. For key to symbols see the legend to Fig. 1.

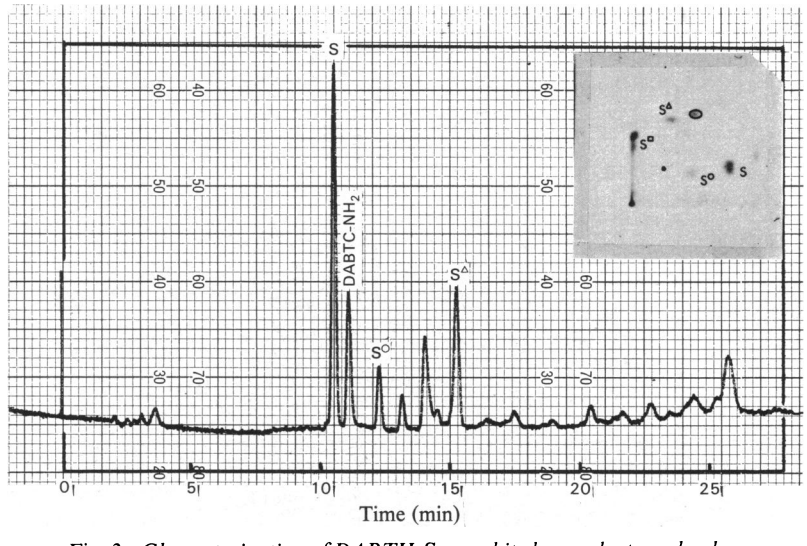

Fig. 3. *Characterization of DABTH-Ser and its by-products on h.p.l.c.*
The products were obtained from the sequence analysis of Ser-Ala by the DABITC/PITC method described in the text. Four major by-products ($S^{\triangle}$, a dehydro product of DATH-Ser; S°, a presumably hydrated product of $S^{\triangle}$ with a hydroxy group at the α-position of the thiohydantoin ring; $S^{\square}$, a presumably polymerized product of $S^{\triangle}$; and DABTC-NH_2) were usually observed on polyamide t.l.c. (for their structures see Chang, 1979*a*), but only the elution times of $S^{\triangle}$, S° and DABTC-NH_2 are established on h.p.l.c. By-products $S^{\triangle}$ and S° are eluted at discrete positions, and DABTC-NH_2 overlaps with DABTH-Thr. Conditions for column chromatography were identical with those described in the legend to Fig. 1.

products (Fig. 5), namely α-DABTH-ε-DABTC-Lys (K_1, retention time 21.9 min), α-PTH-ε-DABTC-Lys (K_2, retention time 16.2 min) and α-DABTH-ε-PTC-Lys (K_3, retention time 16.2 min). Most of those by-products were eluted at discrete positions and can be easily identified.

The mechanism of the separation of amino acid DABTH derivatives on a reversed-phase column is

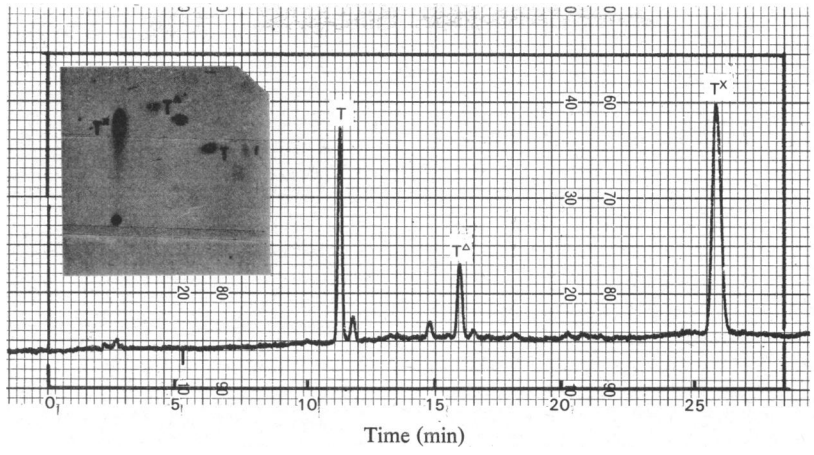

Fig. 4. *Characterization of DABTH-Thr and its by-product on h.p.l.c.*
The products were obtained from the sequence analysis of Thr-Phe by the DABITC/PITC method described in the text. T^{Δ} is a dehydro product of DABTH-Thr, and $T^{\times}$ is an uncharacterized product. Their migration positions on polyamide t.l.c. are shown on the left. Conditions for column chromatography were as described in the legend to Fig. 1.

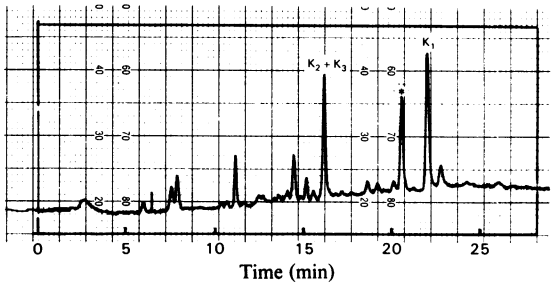

Fig. 5. *Identification of lysine as multiple products released from the first degradation of Lys-Glu-Thr-Tyr-Ser-Lys (Serva) by the DABITC/PITC double-coupling method*
K_1 is α-DABTH-ε-DABTC-Lys, K_2 is α-PTH-ε-DABTC-Lys and K_3 is α-DABTH-ε-PTC-Lys. Derivatives K_2 and K_3 overlap with DABTH-Trp. The peak marked with an asterisk originated from an impurity of the peptide. Conditions for column chromatography were as described in the legend to Fig. 1.

governed by (1) hydrophobic interaction between the solute and the hydrocarbon ligand of the stationary phase (Horvath *et al.*, 1976) and (2) ionic interaction between the charged solute with the negatively charged silica surface of the stationary phase (Schmidt *et al.*, 1980). The ionic interaction is important only for charged solute and accounts for the exceptionally long retention time of DABTH-Arg (pK_a of side chain is 12.48). However, this ionic interaction can be diminished, and thus the retention time of DABTH-Arg can be decreased, by increasing the salt concentration of the acetate buffer.

The gradient system described in Fig. 1 has given reproducible elution profiles of amino acid DABTH derivatives (except for DABTH-Arg and DABTH-His) on at least four commercial Zorbax ODS columns. For each new column, the elution positions of DABTH-Arg and DABTH-His can be optimized by simply adjusting the salt concentration of the acetate buffer.

Sequence analysis of polypeptide

The method has been applied to sequence determination of polypeptides with both known and unknown structures. The repetitive yield is somehow dependent on the amount subjected to sequence analysis. For example, with 1.2 nmol of angiotensin I (a decapeptide) (Fig. 6), the first nine residues can be easily identified (with repetitive yield of 88%), but only the first eight residues were confirmed when starting with 400 pmol of peptide (with repetitive yield of 82%). The sequences of insulin B-chain (26/30, 0.8 nmol), mellitin (20/26, 1 nmol), a tryptic peptide L24-T12 from ribosomal protein (15/18, 1.5 nmol), a CNBr-cleavage peptide from the constant region (residues 117–216) of monoclonal antibody 2S1.1 light chain (15/39, 0.75 nmol) and oxidized lysozyme (15/129, 0.75 nmol) have also been successfully determined with repetitive yields ranging from 80 to 92%.

The cysteic acid (residues 7 and 19 of insulin B-chain, and residue 6 of lysozyme) cannot be detected owing to its insolubility in butyl acetate. Lysine (residue 13 of lysozyme) can also generate a

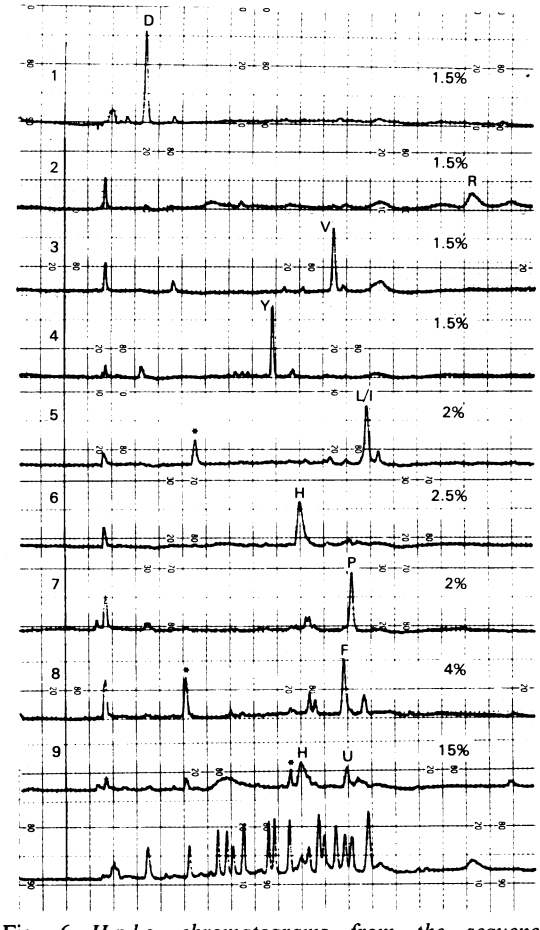

Fig. 6. *H.p.l.c. chromatograms from the sequence analysis of angiotensin I (1.2 nmol, based on the weight claimed by Sigma) by the DABITC/PITC method described in the text*

Percentages of extracts of amino acid DABTH derivatives used for h.p.l.c. analysis at each step are indicated. The average yield during the first eight degradations was 88%, but fell drastically at the ninth degradation and the last amino acid (Leu) was not identified. Chromatogram of a standard mixture of amino acid DABTH derivatives (5 pmol each) is shown at the bottom panel. Conditions for column chromatography were as described in the legend to Fig. 1 except for the use of 45 mM-acetate buffer instead of 35 mM-acetate buffer because of aging of the column. U is a by-product. For key to symbols see the legend to Fig. 1.

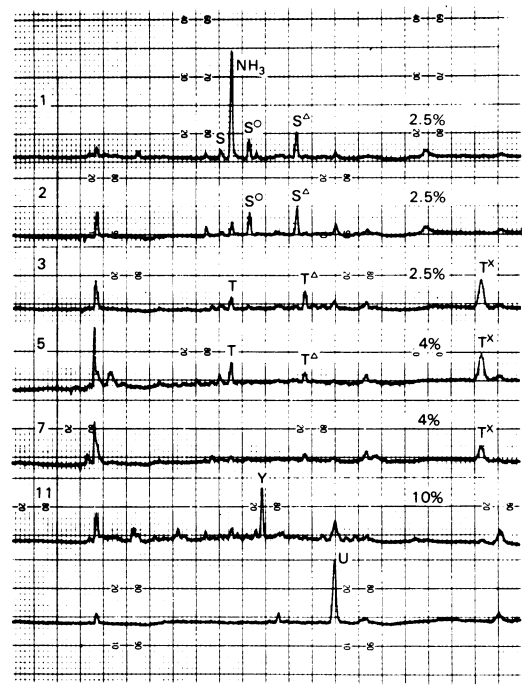

Fig. 7. *Chromatograms from the selected steps of sequence analysis of an immunoglobulin peptide (750 pmol) by the DABITC/PITC method*

The peptide (40 amino acid residues) is derived from the light-chain constant region (*C*-terminal peptide) of an anti-(streptococcal A) monoclonal antibody 2S1.1. DABTC-NH_2 appeared in step 1, most possibly as a result of the ammonia contamination rather than destruction product of DABTH-Ser (Fig. 3), since the peptide was eluted from gel filtration by 50 mM-ammonium bicarbonate. A chromatogram (bottom panel) resulting from the analysis of 5 μl of third washing extract (see the Materials and methods section) shows a major by-product U, which is a coupling product of PITC and hydrolysed DABITC (Chang *et al.*, 1978). U is eluted just between DABTH-Phe and DABTH-Pro. Conditions for column chromatography were as described in the legend to Fig. 1, but 45 mM-acetate buffer was used instead of 35 mM-acetate buffer. The retention time of T^X is slightly different from that in Fig. 4. For key to symbols see the legends to Figs. 1, 3 and 4.

gap, for unknown reasons. There are no difficulties in identifying serine and threonine from their distinct patterns of multiple peaks (Fig. 7), but it is practically impossible to measure those residues quantitatively. The parent DABTH-Ser and DABTH-Thr are mostly destroyed and the yields of their by-products are never reproducible (Fig. 7).

For most peptides, the interference from the unremoved excess of reagent is minimal under the washing scheme described in the Materials and methods section. However, the complete removal of excess of reagent is sometimes difficult with proteins (see the Discussion section). A major component of

the by-products is U (a coupling product of PITC and hydrolysed DABITC), which is eluted between DABTH-Phe and DABTH-Pro. Another uncharacterized minor component overlaps with DABTH-Ala (Fig. 7).

Discussion

The sensitivity limit of Edman degradation is primarily dependent upon the detection limit of amino acid PTH derivatives. Amino acid PTH derivatives are colourless compounds. Their detection by either t.l.c. or h.p.l.c. relies on the absorption of the thiohydantoin ring in the u.v. region. One of the major concerns of detecting the amino acid PTH derivatives at the low picomole level is the interference from the u.v.-absorbing impurities that reside in both sequencing buffer and chromatographic solvent. The identification of amino acid DABTH derivatives by the DABITC/PITC double-coupling method offers an alternative to circumvent this problem as well as additional advantages: (1) the molar absorption coefficient of amino acid DABTH derivatives at 420nm (34000) is double that of amino acid PTH derivatives at 269nm (16000); (2) detection in the visible region avoids interference from the u.v.-absorption contaminants and the base-line rise due to gradient change; (3) detection of amino acid DABTH derivatives is based on the chromophore dimethylaminoazobenzene instead of the thiohydantoin ring, and therefore it is possible to identify all the by-products of DABTH-Ser and DABTH-Thr at the same wavelength with the same sensitivity; (4) amino acid DABTH derivatives can also be analysed by micro t.l.c. at the sensitivity level of 5–15pmol. The t.l.c. method, although not quantitative, has the specific advantage of being able to distinguish the by-product from the amino acid DABTH derivative by their colour difference (Chang *et al.*, 1978), and thus can be used to complement the h.p.l.c. method for analysis of amino acid DABTH derivatives.

There are some technical features concerning the DABITC/PITC method requiring further refinement, and, if improved, they may enable this manual method to be used for routine sequencing of less than 100pmol of polypeptide. (1) There is always a drastic decrease of yield when sequencing is performed near the *C*-terminal end of a peptide. The decrease is mainly attributed to the extraction loss after coupling reaction. This problem may be overcome by selecting a more suitable extraction solvent or simply adjusting the heptane/ethyl acetate ratio from 2:1 to 3:1 or 4:1. An alternative for preventing extraction loss is to use the solid-phase DABITC/PITC method (Chang, 1979*b*). The solid-phase Edman degradation (Laursen, 1971) should become a very powerful micro sequencing technique provided that a general and reliable immobilizing method is available. (2) Most proteins tend to be precipitated and embody trace amounts of excess of reagent during the heptane/ethyl acetate washing. This excess of reagent (mainly U; see Fig. 7) will become prominent and may interfere with the identification of DABTH-Phe and DABTH-Pro when a higher percentage of extract of amino acid DABTH derivatives has to be applied. (3) Serine and threonine usually give multiple DABTH derivatives. The yield of those by-products seems to be dependent on the amount of the peptide subjected to sequence analysis, and was not reproducible even with the same peptide under the same degradation protocol. The destruction of DABTH-Ser and DABTH-Thr was found to be initiated by side-chain dehydration (Ilse & Edman, 1963), which mainly occurred in anhydrous acid solution (Chang, 1979*a*). However, in the single *N*-terminal determination (Chang, 1980), the yield of DABTH-Ser and DABTH-Thr can be as high as 80–98% when aqueous acid is used directly for cleavage and conversion reaction. The parent DABTH-Ser could also be largely saved by using automatic solid-phase DABITC/PITC degradation (Hughes *et al.*, 1979), as the cleaved thiazolinone derivative eluted by trifluoroacetic acid is immediately collected in aqueous solution, thus avoiding long exposure to anhydrous acid.

With the availability of an *N*-terminal sequencing method at the picomole level, it is equally important to have a peptide separation method, an amino acid analysis method as well as a *C*-terminal sequencing method that could all operate at the same sensitivity level. We have developed and described those techniques in the preceding papers (Chang, 1981; Chang *et al.*, 1981), and it is relevant to point out that all the techniques were performed with the same instrument. By taking advantage of those techniques, one may expect, in the future, to determine the sequence of a protein with only 10–20nmol of material.

I gratefully acknowledge the generous support of Dr. Dietmar G. Braun and Dr. Peter Dukor with this work.

References

Allen, G. & Fantes, K. H. (1980) *Nature (London)* **287**, 408–411

Allen, G. & Wittmann-Liebold, B. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1509–1525

Bridgen, J. (1976) *Biochemistry* **15**, 3600–3604

Chang, J. Y. (1977) *Biochem. J.* **163**, 517–520

Chang, J. Y. (1979*a*) *Biochim. Biophys. Acta* **578**, 175–187

Chang, J. Y. (1979*b*) *Biochim. Biophys. Acta* **578**, 188–195

Chang, J. Y. (1980) *Anal. Biochem.* **102**, 384–392

Chang, J. Y. (1981) *Biochem. J.* **199**, 537–545
Chang, J. Y., Brauer, D. & Wittmann-Liebold, B. (1978) *FEBS Lett.* **93**, 205–214
Chang, J. Y., Knecht, R. & Braun, D. G. (1981) *Biochem. J.* **199**, 547–555
Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80–91
Heiland, I. & Wittmann-Liebold, B. (1979) *Biochemistry* **17**, 4605–4611
Horvath, C., Melander, W. & Molnar, I. (1976) *J. Chromatogr.* **125**, 129–156
Hughes, G. J., Winterhalter, K. H., Lutz, H. & Wilson, K. J. (1979) *FEBS Lett.* **108**, 92–97
Hunkapiller, M. W. & Hood, L. E. (1978) *Biochemistry* **17**, 2124–2133
Hunkapiller, M. W. & Hood, L. E. (1980) *Science* **207**, 523–525
Ilse, D. & Edman, P. (1963) *Aust. J. Chem.* **16**, 411–416
Klapper, D. A., Wilde, C. E. & Capra, J. D. (1978) *Anal. Biochem.* **85**, 126–131
Laursen, R. A. (1971) *Eur. J. Biochem.* **20**, 89–102
Lewis, U. J., Bonwald, L. F. & Lewis, L. J. (1980) *Biochem. Biophys. Res. Commun.* **92**, 511–516
Richardson, M. (1979) *FEBS Lett.* **104**, 322–326
Schmidt, D. E., Jr., Giese, R. W., Conron, D. & Karger, B. L. (1980) *Anal. Chem.* **52**, 177–182
Tarr, G. E., Beecher, J. F., Bell, M. & Mckean, D. J. (1978) *Anal. Biochem.* **84**, 622–627
Wittmann-Liebold, B. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1415–1431
Wittmann-Liebold, B. (1979) *FEBS Lett.* **108**, 75–80
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem.* **77**, 569–573