MICROSEQUENCE ANALYSIS: III. AUTOMATIC SOLID-PHASE SEQUENCING USING DABITC

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1. Introduction

The automation of solid-phase sequencing, using the Edman-type degradation [1], was originally perfected by Laursen and co-workers [2,3]. Several books have been devoted to this subject [4,5]. The original idea was to provide a means for degrading insolubilized peptides and thus prevent the well-known 'washout' problem common to the liquid-phase instruments. Alternatively various groups have attempted to render the peptide or protein more polar (thus reducing its solubility in organic solvents) but only through the recent use of polybrene [6,7] has liquid-phase sequencing for most peptides at low concentrations (<50 nmol) become possible. In solid-phase technology on the other hand, emphasis has been directed toward improving coupling yields and providing the means to couple peptides arising from a variety of specific cleavages.

Prior to the introduction of DABITC [8] there had been little interest in developing substitutes for PITC which would simplify detection and increase sensitivity. In manual liquid- [9,10] and solid-phase [11,12] degradations with DABITC the identification of reaction products by TLC [8–12] is cumbersome. In [13] we have shown that HPLC can conveniently be used for DABTH detection in the visible (~440 nm) at low levels (<100 pmol). Here we report the adaption of DABITC to automatic solid-phase sequencing at the <10 nmol level, a quantity easily isolated by the peptide–HPLC system in [14]. In [15] we describe its application in a liquid-phase sequencer.

Abbreviations: AP, aminopropyl; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; DABITC, 4-*N*,*N*-dimethylazobenzene 4'-isothiocyanate; DABTH, 4-*N*,*N*-dimethylazobene 4'-thiohydantoin; DITC, phenylene diisothiocyanate; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MeOH, methanol; NBS, *N*-bromosuccinimide; PITC, phenylisothiocyanate; polybrene, hexadimethrinbromide; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TMS, trimethylsilane

2. Materials and methods

Two different instruments were adapted for the use of DABITC to automatic solid-phase sequencing. For both the final programs, the chemicals, the flowrates and the column specifications are given in fig.1. The first instrument, a Sequemat Mini-15 was equipped with the P-4 microsequencing unit which contained the DABITC solution. The door to this unit was left open to avoid temperature build up during operation. The syringe containing the DABITC solution was thus wrapped in foil to protect against photo-degradation. Conversions were performed automatically with a Sequemat P-6 programmed as follows: the TFA (540 μl) and MeOH (1 ml) washes were collected in the reaction vessel (maintained at 65°C) and dried for 10 min with N_2; 0.4 ml 30% TFA (in H_2O) were added, incubated for 15 min, then dried for 15 min with N_2; DABTHs were quantitatively transferred to the fraction collector of the sequencer with 4 repetitive washes of 0.3 ml

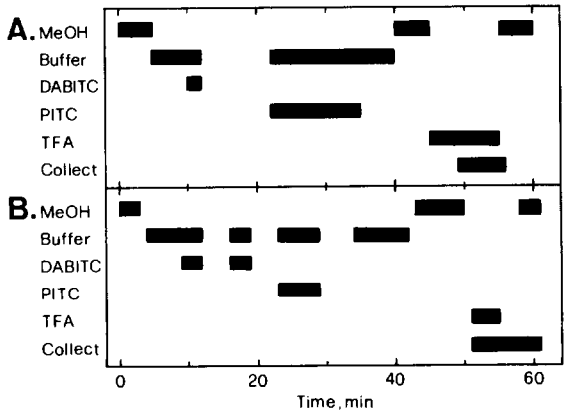

Fig.1. Sequencer programs for the use of DABITC in (A) the Sequemat Mini-15 or (B) the LKB 4020 solid-phase instruments. For (A) DABITC was 0.75% in DMF and PITC, 5% in DMF; (B) DABITC was 0.5% in DMF and PITC as in (A). The buffer was prepared as in [3] except that the final pH was 8.6. Column sizes and temperatures were (A) 3 × 100 mm and 55°C and (B) 2 × 98 mm and 50°C. The pumping rates for both instruments were 1.0 ml/min for MeOH, 0.1 ml/min for buffer and DABITC, and 50 μl/min for PITC. TFA pumping rates were 90 μl/min in (A) and 0.15 ml/min in (B).

ethylacetate. An extraction from 0.3 ml 0.4 M sodium phosphate (pH 6.8) was performed and the ethylacetate phases dried under N_2 prior to identification.

The second instrument was an LKB 4020 solid-phase sequencer adapted for microsequencing. The only modification was to replace the vial normally used for the radioactive PITC with a 100 ml reagent (reservoir) bottle. A scintillation vial containing the appropriate volume of DABITC solution for the experiment was then introduced and pressurized as recommended in the LKB manual for microsequencing. The pump was covered with foil and the perspex panels were maintained in position in an attempt to protect the DABITC solution from light. Conversions were performed according to [1] in 1 N HCl and the ethylacetate extract dried under N_2. The DABTHs were identified by HPLC and HPTLC as described in [13].

Protein or peptide samples (usually 10–50 nmol) were dissolved in 1.5 ml 0.25 M Quadrol buffer (Fluka, sequencer grade) (pH 9.0) and 150 mg DITC–glass added. Following incubation at 50°C with shaking (30 min), 200 μl ethanolamine (Fluka) were added and incubation continued (10 min). The beads were thoroughly washed with 50% pyridine, MeOH and ethylacetate (~20 ml each) and vacuum dried. The AP–glass was prepared from CPG/100 glass (Pierce) and 3-aminopropyltriethoxy-silane (Fluka) as in [16]. The reaction of 80 mg DITC (Pierce) and 150 mg AP–glass was carried out in 1.5 ml DMF at 50°C for 30 min followed by extensive washing with DMF and MeOH (~20 ml each) and ~3 ml Quadrol buffer. The DITC–glass was not dried prior to coupling as described above. CNBr peptides were attached via the lactone on AP–glass as in [17], diluted 10–100-fold with TMS-silanised glass microbeads (Sequemat), depending on the amount of coupled protein, thoroughly mixed and packed firmly into the column by light tapping. The initial cycle of each run was done using only PITC, and not the double-couple program, since this was found to drastically reduce background. Tryptic digestions of peptides attached to glass were performed in 0.2 M *N*-ethylmorpholine acetate (pH 8.0) at 37°C for 18 h using a 1:10 mole ratio of trypsin: peptide.

All chemicals used in the sequencers were sequencer and/or analytical grades from either Fluka or Pierce. DABITC, purchased from either of the firms, gave identical results. Amino acid analyses were carried out on either a Durrum D-500 or a Liquimat III analyzer following 6 N HCl hydrolysis.

3. Results and discussion

Both commercially available solid-phase instruments, from Sequemat and LKB, can readily be operated with DABITC by simply using the adaptions supplied by the manufacturers for radioactive ([^{35}S]-PITC) sequencing. Initial experiments were performed with the reagent dissolved in pyridine and the PITC, i.e., for the double-couple methodology [10], in acetonitrile. Degradation of the DABITC solution upon standing, however, severely limited the use of this solvent for extended sequencer runs. The solubility and stability was tested and DMF was found to provide, optimally, both properties (see [15]). However, both commercially available DABITC preparations have small amounts of DMF-insoluble material which should be removed prior to use. Currently, a fresh solution of the reagent is made at the beginning

of each experiment and the reagent syringe (Sequemat), or reservoir (LKB), wrapped in foil to prolong the usefulness of the DABITC. Chemical quality, of the reagents, sequencer or analytical grade, does not appear to significantly influence either the repetitive yields or lengths (no. residues) that can be sequenced (see fig.2; all chemicals used, except for the TFA in the sequencer and the pyridine in the buffer (distilled from KOH), were of analytical grade).

Figure 2 illustrates the HPLC chromatograms from a 'typical' series of degradations using DABITC in the Sequemat instrument and converting automatically. Here a CNBr fragment from the α-chain of human globin (residues 33–76; pool D of fig.4 in [14] was coupled via the homoserine lactone to AP–glass (77.6% yield) and 8.9 nmol were sequenced with an average repetitive yield of 90.4% (calculated on Phe and Val residues). As with most sequences thus far determined 'carry-over' or 'over-lap' is minimal through the first 20 or so cycles. However, the gradual accumulation of an unidentified peak which co-elutes with DABTH-Ala often constitutes a problem, e.g., in cycle 31, 33, 37 and 39. The appearance of this peak seems to be due to a side-reaction of the reagent with the glass which, after a number of cycles, begins to elute. This is suggested by:

(i) The peak's presence even when a fresh DABITC solution was used after 20 cycles;
(ii) The peak's absence, for the first 20 or so cycles, even when using an 'aged' (24–48 h) DABITC solution from the beginning of an experiment.

Another point illustrated in this series of chromatograms is the ability to identify DABTH-His directly (cycle 13, as a single peak, and as doublets at positions 18, 26 and 40) and the absence of a DABTH-Lys peak (cycles 8, 24, 28 and 29). Since DABTH-Arg elutes at the position of His, this is the only amino acid derivative which is not readily identified by one or the other of the HPLC systems [14]. DABTH-Pro usually exhibits a characteristic double peak, the second of which elutes at the position of Ala and is probably DABTH-Pro$^{\Delta}$ (residues 5 and 12). Although no tryptophanyl residue was encountered in these series, it is partially destroyed under the conversion conditions used here. The necessity of using automatic conversion, however, is supported by the acceptable recoveries of Ser-derivatives (at 3, 17 and 20), of Thr-derivatives (at 6, 7, 9 and 35) and the limited, if any, deamidation of either Gln- (at 22) or Asn- (at 36) derivatives.

Since the carboxyl-terminal sequence is not as illustrative as desired, a tryptic digestion was performed on 7.1 nmol of the material and the resulting bound peptide(s) sequenced (fig.2B). As the chromatograms show, the tryptic digestion resulted in an approximately equal cleavage at lysine residues 28 and 29, thereby yielding two peptides which degrade simultaneously. Although the DABTH-residues from the first cycle (Val, Ala) were not identified (present as their PTH derivatives) the subsequent amino acid sequence is readily recognized and the homoserine residue in the final cycle is obvious. Numerous other experiments [18] have indicated that limited fragmentations, either chemical or enzymatic and performed on either the soluble or insolubilized peptide (protein), are useful in providing overlap peptides. Additionally, new fragments are often generated for sequencing which would not be obtained by extended cleavage.

The results shown here (fig.2A,B) are representative of the 'cleaner' chromatograms which we observe when peptides have been coupled via the carboxy-terminal lactone. Conversely, background accumulation with peptides coupled through lysine residues on DITC–glass would prohibit an injection of 100% of the sample (cycles 33–44, fig.2A). Unfortunately the lactone peptides are slowly hydrolyzed from the solid support and thus their repetitive yields are lower (90.4% for fig.2A) than for those coupled via lysines (>93–95%, results not shown). Although there is a slight difference due to a buffer change in the elution times between fig.2A and 2B (~9 s), it is the gradient

Fig.2. Gradient HPLC analyses from the solid-phase degradations of CNBr fragments of human α-globin. (A) 8.9 nmol of the fragment, isolated in pool D as in [14], and attached to AP–glass via the lactone was degraded and conversions performed automatically (see section 2). (B) 7.1 nmol of the attached CNBr fragment was digested with trypsin (see section 2) and then subjected to degradation. Gradient chromatography was carried out as in [13].

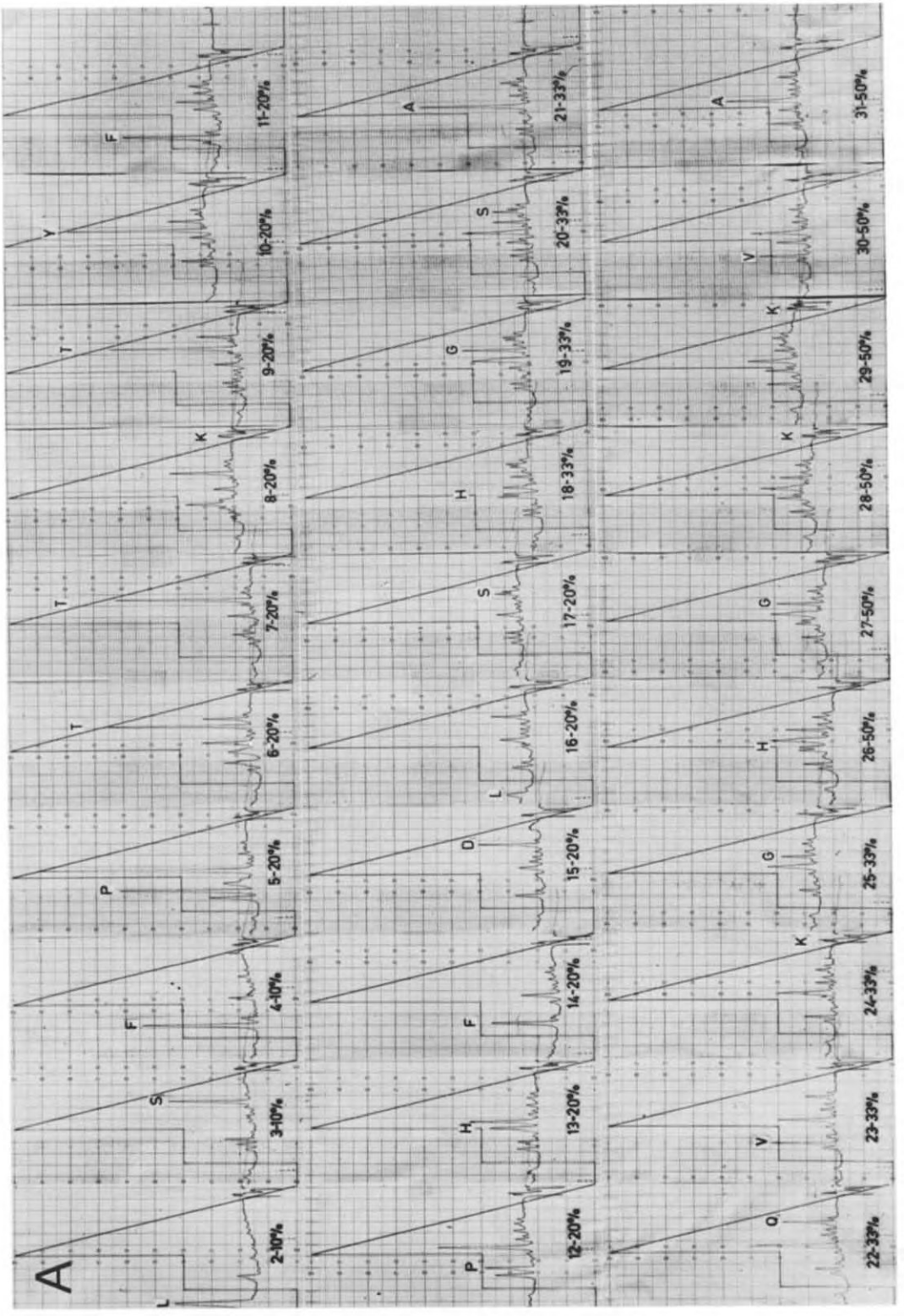
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Fig.2A

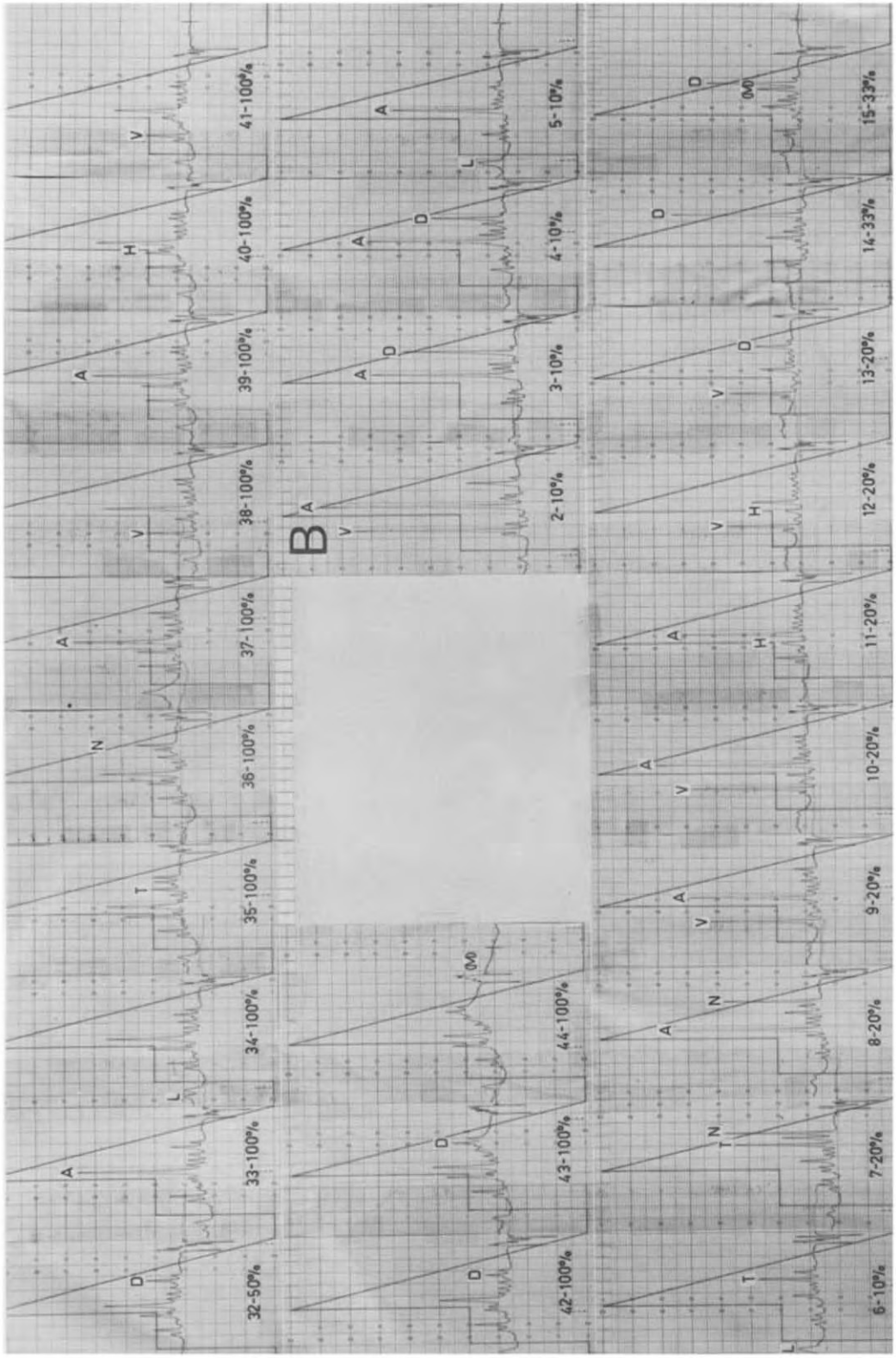
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Fig. 2B

reproducibility by the microprocessor-controlled pumps which makes this detection system so efficient and accurate.

The combination of solid-phase degradation using DABITC, autoconversion and HPLC identification represents a considerable improvement in automatic sequencing. Routinely, sequences of >30 residues have been obtained at the <10 nmol level from such diverse sources as myoglobin, insulin growth factor, α- and β-globin chains from human as well as goldfish hemoglobins, and fragments thereof. The main disadvantage is that a peptide lacking either a lactone, generated by CNBr, BNPS-skatole or NBS [18], or a lysine residue cannot be coupled in high yields. However the use of the DABITC methodology presented in the following article [15], for automatic liquid-phase sequencing will solve the problem when such instances arise.

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