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A Gas-Liquid Solid Phase Peptide and Protein Sequenator*

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A new miniaturized protein and peptide sequenator has been constructed which uses gas phase reagents at the coupling and cleavage steps of the Edman degradation. The sample is embedded in a matrix of Polybrene dried onto a porous glass fiber disc located in a small cartridge-style reaction cell. The protein or peptide, though not covalently attached to the support, is essentially immobile throughout the degradative cycle, since only relatively apolar, liquid phase solvents pass through the cell. This instrument can give useful sequence data on as little as 5 pmol or protein, can perform extended sequence runs (>30 residues) on subnanomole quantities of proteins purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and can sequence hydrophobic peptides to completion. The sequenator is characterized by a high repetitive yield during the degradation, low reagent consumption, low maintenance requirements, and a degradative cycle time of only 50 min using a complete double cleavage program.

Since its introduction in 1967, the use of automated Edman degradation in the spinning cup sequenator has been the most widely used method for determining the primary structure of polypeptides (1). For many years, the major limitations of the technique were the large amounts of sample required (10 to several hundred nmol) and difficulty in sequencing short (less than 50 amino acids) peptides. These problems were mainly due to sample loss during the repeated extractions with organic solvents used to remove nonvolatile reagents and byproducts from the sample film. Early attempts to minimize sample loss included the use of more volatile coupling buffers (2, 3), reduction in the volume of extraction solvents (4, 5), and the addition of various nonprotein carriers to the cup to add mass and stability to the sample film (3). However, the major breakthrough came with the introduction of a polymeric quaternary ammonium salt, Polybrene, to spinning cup sequencing technology (6, 7). This substance effectively anchors small quantities of both proteins and peptides in the cup and allows sequencing of even short hydrophobic peptides to completion. By combining the use of Polybrene with more extensive purification of reagents and solvents, improvements in spinning cup sequenator design, and analysis of >PhNCS[1]-derivatives by reverse phase HPLC, two of us (M. W. H. and L. E. H.) have previously obtained extended NH_2-terminal sequence information on subnanomole quantities of a variety of peptides and proteins (8, 9).

The solid phase sequencing system of Laursen (10), wherein the protein or peptide is covalently attached to a derivatized glass or polystyrene support, was developed in parallel with the spinning cup system and provides another answer to the problem of extractive sample loss. However, the major limitations of this technique are that it is rarely possible to achieve quantitative attachment of sample to the support phase, another set of reagents and solvents is required for covalent sample attachment, and gaps appear in the amino acid sequence where attachment has occurred. The identity of these attached residues cannot be determined directly.

In the present report, we describe a new type of sequenator which, based partly on previous work (11-14), employs gas phase reagents instead of liquid phase reagents at critical points in the Edman degradation. This modification means a miniaturized cartridge-type reaction cell can replace the spinning cup assembly and the elaborate hardware associated with it. The advantages of this new miniaturized gas-liquid solid phase system are as follows: high sensitivity sequencing, very low reagent and solvent consumption, a much shorter degradative cycle, and low maintenance and running costs. Unlike the Laursen solid phase sequencing system, which also employs a cartridge-style reaction cell, the present system does not require covalent attachment of sample to the support phase.

SEQUENATOR DESIGN

The gas-liquid solid phase sequenator has certain features in common with the liquid phase spinning cup sequenator described by Hunkapiller and Hood (8, 9, 15)[2] and some critical differences, as shown in the schematic (Fig. 1).

The similarities include the following: the use of argon, delivered through a series of filters, to pressurize the reagent and solvent reservoirs; similar argon pressure-regulation manifold, reservoir pressurizing valves, and reservoir-venting manifold; similar reagent/solvent reservoirs, some of which are modified to deliver vapor as opposed to liquid; the use of zero-holdup, pneumatically actuated diaphragm valves for delivery of reagents, solvents, and argon; and control of valve function by means of a solid state programmer.

The major differences are summarized as follows: the replacement of the cup and drive unit by a cartridge assembly which houses a miniature glass reaction chamber (internal volume, ~0.050 ml) and the presentation of the polypeptide sample in this chamber on a porous glass fiber disc. The cartridge is mounted in an insulated oven equipped with heating and cooling elements for programmed temperature control.

The pneumatically actuated diaphragm valves have been miniaturized to reduce their internal volume to one-tenth of that in the spinning cup system. These valves also control the vacuum to the cartridge and the >PhNCS conversion flask. The large electromagnetic vacuum valves that are used in the spinning cup instrument are not required. The reservoirs and various delivery valve blocks are mounted in the vertical plane directly above and below the reaction cartridge as appropriate to minimize the length of connecting tubing.

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[1] The abbreviations used are: >PhNCS, phenylthiohydantoin; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

[2] M. W. Hunkapiller and L. E. Hood, submitted for publication.

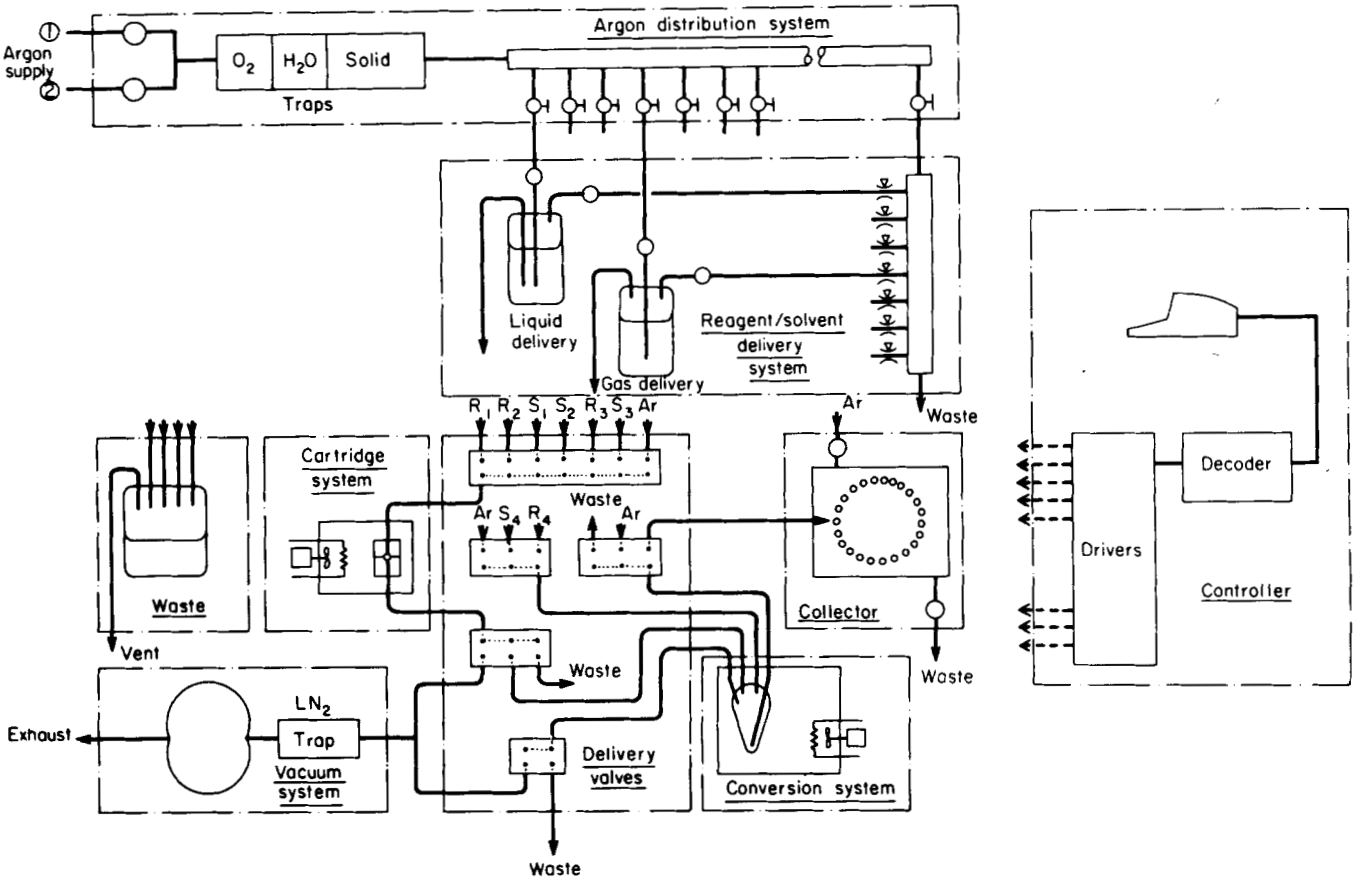

FIG. 1. **Schematic diagram of gas-liquid solid phase sequenator.**

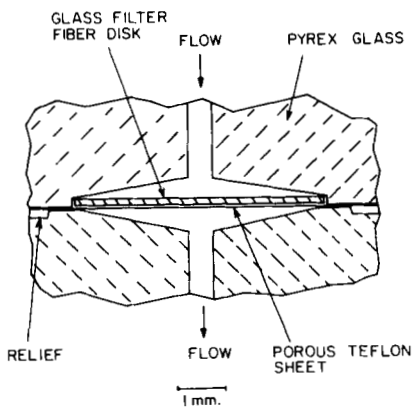

FIG. 2. **Enlarged detail of reaction chamber.**

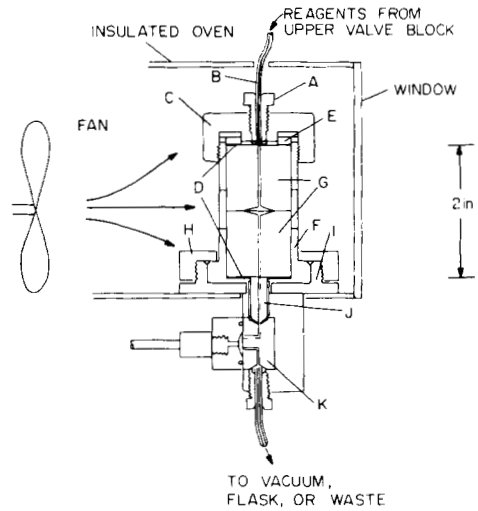

FIG. 3. **Cartridge assembly.** *A*, aluminum top fitting; *B*, Teflon tubing; *C*, aluminum cap; *D*, Teflon washer; *E*, keyed aluminum washer; *F*, 304 stainless steel cartridge body; *G*, Pyrex glass rod; *H*, aluminum locking ring; *I*, aluminum mounting base; *J*, Kel-F bottom fitting; *K*, Kel-F valve block.

The automatic conversion flask and fraction collector are miniaturized, and the vacuum pump and liquid nitrogen trap also are correspondingly smaller.

Reaction Cartridge—The cartridge is constructed from two pieces of Pyrex glass rod (1 inch diameter × 1 inch long) with finely ground vacuum-flat surfaces at both ends (Wilmad Glass Company, Buena, NJ). Each piece of glass is ultrasonically machined (L. C. Miller Co., Monterey Park, CA) so that it has a 0.020-inch diameter central capillary flared out to give a conical recess at one end. An important feature is that the hole and recess in each piece of glass are mutually concentric to within 0.001 inch. The recessed ends of the two pieces of glass are clamped together in a metal cylinder to form a central chamber. The conical cavity in the upper piece of glass has a small recess sufficient to hold the glass fiber disc (GF/C Glass Microfibre Filter, Whatman Ltd., England) on which the protein or peptide is supported. Clamped between the two pieces of glass is a fibrous porous Teflon disc (Zitex filter membrane, extra-coarse grade, Chemplast Inc., Wayne, NJ). This Teflon disc physically supports the glass fiber disc and is crushed between the abutting glass surfaces to give a vacuum seal. Enlarged detail of this arrangement is shown in Fig. 2.

A general diagram of the cartridge assembly and its relationship to the upper valve block (argon, reagent, and solvent delivery) and the lower valve block (vacuum, delivery to flask, and delivery to waste) is shown in Fig. 3. Critical features of the cartridge design are that alignment of the 0.020-inch diameter hole running from the upper valve block to the lower valve block is reproducibly maintained and that the whole system is vacuum tight. These points are particularly important, since the top fitting (*A*) is disconnected and the cartridge is removed from the mounting base (*I*) each time a sample is loaded.

TABLE I

Comparison of the reagent and solvent consumption per cycle of Edman degradation in a spinning cup (see Footnote 2) and a gas-liquid solid phase sequenator

Reagent/solvent (spinning cup)	Volume	Reagent/solvent (gas-liquid)	Volume
	ml		*ml*
R1: 5% phenylisothiocyanate in *n*-heptane	0.4	15% phenylisothiocyanate in *n*-heptane	0.05
R2: Quadrol/trifluoroacetic acid, pH 9.0, in water/propanol (4:3)	0.7	25% trimethylamine in water	5 cc/min argon flow
R3: Heptafluorobutyric acid, 0.01% dithiothreitol	0.5	Trifluoroacetic acid, 0.01% dithiothreitol	5 cc/min argon flow
R4: 25% trifluoroacetic acid in water, 0.01% dithiothreitol	0.3	25% trifluoroacetic acid in water, 0.01% dithiothreitol	0.05
S1: Benzene	7	Benzene	1.1
S2: Ethylacetate, 0.05% acetic acid, 0.002% dithiothreitol	16	Ethylacetate, 0.05% acetic acid, 0.002% dithiothreitol	2.7
S3: 1-Chlorobutane, 0.001% dithiothreitol	7	1-Chlorobutane, 0.001% dithiothreitol	1.2
S4: Acetonitrile, 0.001% dithiothreitol	4	Acetonitrile, 0.001% dithiothreitol	0.3[a]

[a] Excluding 1-ml flask rinse.

The upper aluminum washer (*E*) is keyed into the cartridge wall so that when the cap (*C*) is tightened, the two pieces of glass (*G*) do not rotate. The washer also serves to precisely align the upper fitting (*A*) relative to the central capillary. The upper and lower Teflon washers (*D*) protect the top and bottom glass surfaces. A vacuum seal is achieved at the top fitting (*A*) by a direct flanged Teflon tubing fit on the glass surface. A vacuum seal at the bottom of the cartridge is achieved by pressurized contact between the glass surface and a Kel-F fitting (*J*), and between this Kel-F fitting and the Kel-F valve block (*K*).

Delivery Valves—The delivery valves are of the basic pneumatically actuated diaphragm type described by Whittmann-Liebold (16) and Hunkapiller and Hood (15).[2] However, in this case, significant design modifications have been made. The most notable change is that the valves have been substantially miniaturized. The internal valve capillaries are 0.020 inch in diameter as opposed to 0.060 inch, and the length, depth, and width of the Kel-F valve blocks are reduced. The net effect is a reduction of over 10-fold in the internal volume accessible to liquid. The reduction in size has resulted in closer hole-spacing in the valve block. This has necessitated a thinner and smoother diaphragm to ensure good sealing properties. Appropriately, for these valves, the original 0.010-inch thick Teflon membrane has been replaced by a 0.005-inch thick FEP-Teflon membrane.

Reagent and Solvent Reservoirs—The reservoirs are smaller than those used in the spinning cup sequenator in order to be compatible with the much lower consumption and the need to maintain a fairly rapid turnover of reagents and solvents. Since reagent 2 is trimethylamine/water vapor instead of Quadrol buffer, and reagent 3 is trifluoracetic acid vapor instead of liquid heptafluorabutyric acid, delivery lines in the reservoirs for reagent 2 and reagent 3 are appropriately modified (see Fig. 1) to allow vapor rather than liquid delivery. An adjustable flow meter (Gilmont Instruments, Great Neck, NY) fitted with relatively inert perfluoroelastomer O-rings (Kalrez, E. I. du Pont de Nemours & Co., Wilmington, DE) is connected between the reservoir and the delivery valve block and is used to control and monitor the gas flow rates for reagent 2 and reagent 3.

Fraction Collector and Conversion Flask—The fraction collector is a vacuum tight unit with a carousel designed to house one hundred 300-μl tubes. The conversion flask, similar in design to that described by Hunkapiller and Hood (15)[2] and Wittmann-Liebold *et al.* (17), has been miniaturized to an internal volume of about 2.0 ml. This reduction ensures efficient transfer of sample and washing of the flask walls with small volumes of solvent.

Reagents and Solvents—The reagents and solvents are listed in Table I along with their approximate per cycle consumption. Great emphasis has been placed on the purity of reagents and solvents used for the Edman degradation. Details of appropriate distillation procedures for all reagents and solvents except trimethylamine will be given elsewhere.[2] Trimethylamine, which replaces Quadrol buffer as reagent 2, was obtained from Eastman Organic Chemicals (Rochester, NY) and is purified by refluxing with phthallic anhydride (Matheson, Coleman, and Bell, Norwood, OH) at 5 °C for 1–2 h. This procedure is followed by distillation through a glass column filled with phthallic anhydride into a receiving flask cooled in a dry ice/acetone bath. The trimethylamine is stored under argon as a 25% (v/v) solution in water at −20 °C.

SEQUENATOR OPERATION

Sample Loading—The procedure for loading protein or peptide into the cartridge is as follows. The top fitting (Fig. 3, *A*) is disconnected and the cartridge is removed from the mounting base (*I*) by unscrewing the locking ring (*H*). The cap (*C*) is removed and the two glass blocks (*G*) are withdrawn from the cartridge body (*F*) for cleaning. The used porous Teflon disc is replaced with a new one. The used glass fiber disc is discarded and replaced by a new disc (12-mm diameter) cut from Whatman GF/C sheet. An aqueous solution (0.025 ml) of Polybrene (60 mg/ml) and glycylglycine (1 μmol/ml) is spotted onto the disc and dried under vacuum. After reassembly of the cartridge, the sequenator program is run for four cycles. Following this, the cartridge is once again disassembled and the upper glass block holding the GF/C disc is removed. Without removing the disc from its recess in the glass block, the solution of protein is spotted onto the disc and dried under vacuum. If the sample volume is greater than 0.025 ml, it is spotted and dried in aliquots. The cartridge is then reassembled and the sequence run is commenced.

Program—The sequenator program is listed in Table II[3] and is generally similar to that described by Hunkapiller and Hood (8, 15)[2] for their modified spinning cup sequenator. However, since the present instrument uses a miniaturized cartridge system and gas phase reagents, there are several features worth noting. 1) Only sufficient phenylisothiocyanate solution to completely wet the glass fiber disc is delivered (~20 μl). The heptane is removed by briefly flushing the cartridge with argon. 2) The coupling stage is effected by slowly bleeding trimethylamine/water vapor through the cartridge to waste. Likewise, cleavage is effected by slowly bleeding trifluoroacetic acid vapor through the cartridge to waste. 3) Line flush is used to pressurize the cartridge with argon after a vacuum step, or, with the cartridge waste valve open, to flush the delivery valve block, lines, and cartridge with argon. 4) The miniaturized conversion flask has two argon supplies: line flush, which flushes the flask delivery valve block and

[3] A portion of this paper (Table II) is presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20814. Request Document No. 81M970, cite author(s), and include a check for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

lines and pressurizes the flask; and flask argon, which delivers argon into the bottom of the flask to aid drying and agitation of fluids. 5) The conversion flask, in addition to the vapor-vent valve, has a liquid waste outlet (see Fig. 1), so that the flask can be thoroughly rinsed with solvent during each cycle (steps 65 and 66). 6) To confine the sample to the lower tip of the miniaturized flask and also perform efficient extraction, the amino acid anilinothiazolinone is extracted from the glass fiber filter with several small volumes of chlorobutane (steps 77–83). Continuous drying of the flask contents is necessary

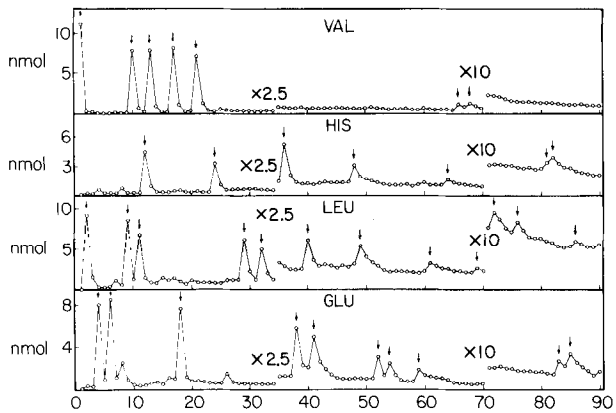

FIG. 4. **Yields of >PhNCS valine, histidine, leucine, and glutamic acid derivatives from an NH_2-terminal amino acid sequence analysis of 10 nmol of sperm whale apomyoglobin.** Aliquots (40%) from each cycle were analyzed by HPLC as described under "Results." HPLC peak heights were converted to nanomoles for each derivative using values from a standard mixture of >PhNCS-derivatives, and the yields were normalized to 100% injection.

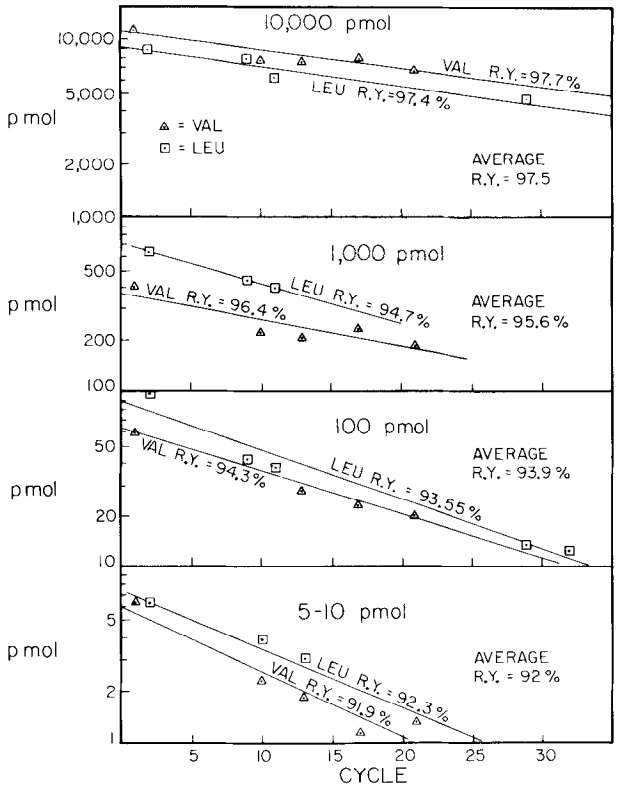

FIG. 5. **Sequenator repetitive cycle yield with various amounts of sperm whale apomyoglobin.** Semilogarithmic plots of >PhNCS valine (△) yields (cycles 1, 10, 13, 17, and 21) and >PhNCS leucine (□) yields (cycles 2, 9, 11, 29, and 32) *versus* sequenator cycle are shown for analysis of 10 nmol, 1 nmol, 100 pmol, and 10 pmol of protein. The repetitive yield (*R.Y.*) at each cycle was calculated from the slopes of the linear-least-squares-fitted straight lines for the plots of >PhNCS yields.

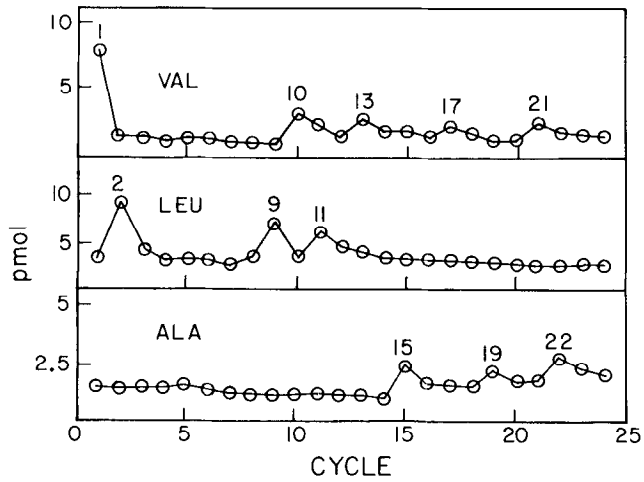

FIG. 6. **Yields of >PhNCS valine, leucine, and alanine derivatives from an NH_2-terminal amino acid sequence analysis of 10 pmol of sperm whale apomyoglobin.** Yields were calculated as described in the legend to Fig. 4.

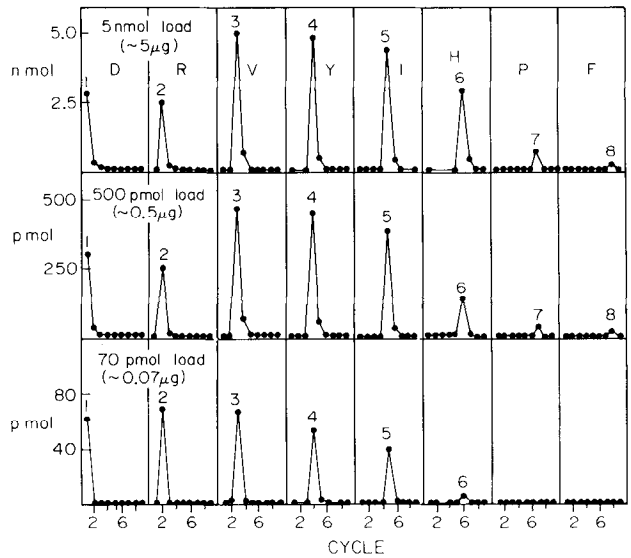

FIG. 7. **Yields of >PhNCS-derivatives from NH_2-terminal amino acid sequence analyses of 5 nmol, 500 pmol, and 70 pmol angiotensin II (sequence: H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH).** Yields were calculated as described in the legend to Fig. 4.

and is effected by flushing the flask with argon during these steps in order to minimize the time between extraction from the disc and addition of reagent 4 to the flask.

In the present program, the cartridge is maintained at 42 °C. The basic program incorporates a single coupling stage with two deliveries of phenylisothiocyanate and two complete cleavage stages. The amino acid anilinothiazolinone released by the shorter first cleavage stage (step 71) is extracted and directed to the conversion flask, and the derivative extracted after the second cleavage is directed to waste. This procedure reduces exposure of the more labile amino acid derivatives to strong anhydrous acid, since most of the derivative is released during the first cleavage, yet maintains an adequate overall cleavage time. In most cases, a sequence run is initiated with a double coupling. This is performed by completing the first degradative cycle with the reagent 3 valve switched off.

Protein and Peptide Preparation—Human angiotensin II was obtained from Pierce Chemical Co. (Rockford, IL). Carboxymethylated insulin B chain was obtained from Mann Research Laboratories (New York, NY). [$Ala^{3,14}$]Somatostatin was synthesized by J. Rivier and R. Guillemin (Salk Institute, San Diego, CA). Neuropeptide B from *Aplysia californica* (18) was a gift from J. Yeakley and F. Strumwasser (Division of Biology, California Institute of Technol-

ogy). Dynorphin was purified and supplied by A. Goldstein (Addiction Research Foundation, Palo Alto, CA). Sperm whale myoglobin (Sigma) was purified and converted to the apoprotein according to the method of Edman and Begg (1) or obtained from Beckman Instruments (Palo Alto, CA). Larval cuticle proteins of *Drosophila melanogaster* purified by isoelectric focusing on polyacrylamide gels containing urea were supplied by M. Snyder and N. Davidson (Division of Chemistry and Chemical Engineering, California Institute of Technology) and D. Silvert and J. Fristrom (Department of Genetics, University of California, Berkeley, CA). The 22,000-dalton membrane phosphoprotein from *A. californica* was purified by SDS-polyacrylamide gel electrophoresis and supplied by L. Kaczmarek, K. Jennings, and F. Strumwasser (Division of Biology, California Institute of Technology). The α and β chains of human histocompatibility antigen HLA-DR were purified and supplied by L. Walker and R. Reisfeld (Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, CA). Human erythropoietin was purified and supplied by E. Goldwasser (Department of Biochemistry, The University of Chicago, Chicago, IL). Human melanoma cell-surface antigen supplied by J. Brown (Division of Tumor Immunology, Fred Hutchison Cancer Research Center, Seattle, WA) was purified by SDS-polyacrylamide gel electrophoresis.

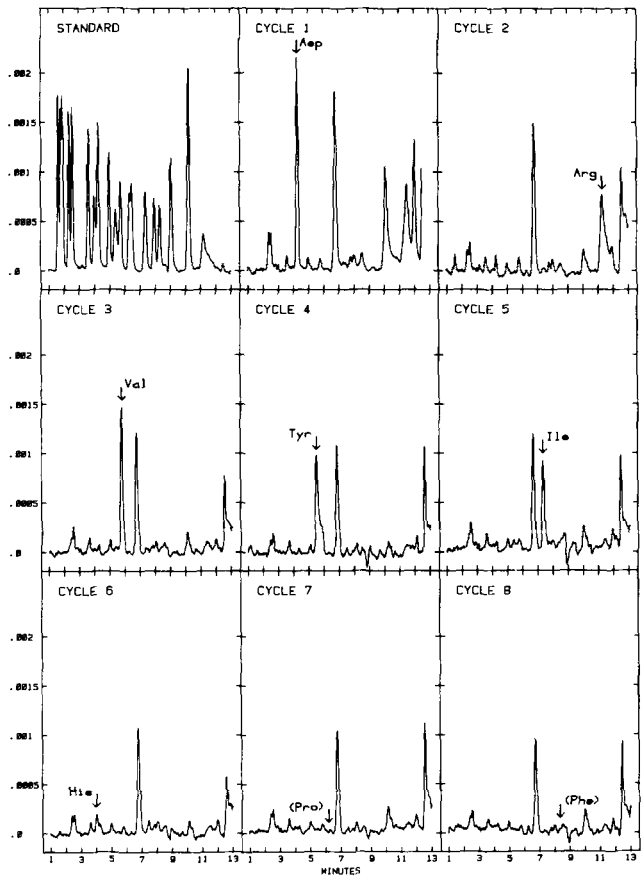

FIG. 8. **HPLC traces from an NH_2-terminal amino acid sequence analysis of 50 pmol of human angiotensin II.** The absorbance meter of the HPLC detector was set at 0.005 absorbance units full scale. The chromatograms were recorded on a Hewlett-Packard series 3354 laboratory automation computer and the chromatographic solvent background from a blank injection (10 μl of acetonitrile) was subtracted by the computer to give the *traces* shown in the figure. The order of elution of >PhNCS-derivatives (12.5 pmol of each) in the standard mixture (*upper left corner*) is Asn, Ser, Thr, Gln, Gly, Ala, His, AspOMe, GluOMe, Tyr, Val, Pro, Met, Ile, Leu, Phe, Trp, Lys, and Arg. Ten-μl aliquots, representing 40% of each sample, were injected. The positions of the >PhNCS-derivatives assigned in the traces for cycles 1 through 6 are indicated by the *arrows* and the *three-letter amino acid designations*. The positions of the expected >PhNCS-derivatives for cycles 7 and 8 are indicated in a similar manner, although they are not assignable in this experiment.

>PhNCS-derivative Analysis—Sequenator fractions were analyzed by reverse phase HPLC using Altex Ultrasphere Cyano columns (Beckman Instruments). Details of the procedure have been described previously (19).

RESULTS

Sperm whale apomyoglobin was sequenced using various programs and at several levels of sample loading in order to test the efficiency of the gas-liquid solid phase sequenator in performing the Edman degradation. The first 90 amino acid residues were identified from a single sequenator run with a 10-nmol load of protein (Fig. 4). The yields of individual >PhNCS-derivatives are similar to those reported previously (8); those of authentic >PhNCS serine and >PhNCS threonine, two of the most troublesome, are 15 and 30%, respectively. The reproducibility of the background of >PhNCS-derivatives (Fig. 4) afforded by the automatic conversion system allows accurate residue assignment even late in the run when the signal-to-background ratio is low. Based upon quantitation of the amount of protein loaded on the cartridge disc by amino acid analysis of a companion aliquot, the yields of >PhNCS valine and >PhNCS leucine at cycles 1 and 2, respectively, were in excess of 90%.

The repetitive cycle yield for runs with myoglobin loads ranging from 10 nmol to 5 pmol varied from 98% (10 nmol) to 96% (500 pmol) to 94% (50 pmol) to 92% (5 pmol) (Fig. 5). The drop in repetitive yield as the sample load is decreased most likely reflects the effect of sample washout during the solvent extractions and/or trace levels of oxidants in the sequenator system. Trace levels of oxidants are suspected, because only in the lowest sample load (5 pmol) was identification of serine and tryptophan residues, both of which are very sensitive to oxidative destruction, impossible. Even with these effects, partial sequence data to residue 22 was obtained on the 5-pmol myoglobin run (Fig. 6).

Human antiogensin II was used to test the ability of the new sequenator to handle short, relatively hydrophobic peptides. The yields of >PhNCS-derivatives for runs with angio-

TABLE III

Polypeptides analyzed with the gas-liquid solid phase sequenator

Sample	Residues identified/ total residues	Amount	
		pmol	*μg*
Angiotensin	8/8	500	0.5
Angiotensin	6/8	50	0.05
Somatostatin	14/14	1,400	2.0
Insulin, B chain	30/30	300	1.0
Neuropeptide B from *Aplysia*	31/34	500	2.0
Dynorphin	14/17	20	0.04
Myoglobin	90/153	10,000	165
Myoglobin	22/153	5	0.08
Larval cuticle protein 1 from *Drosophila*[a]	55/166	850	15
Larval cuticle protein 3 from *Drosophila*[a]	36/96	900	9
22,000-dalton membrane phosphoprotein from *Aplysia*[b]	23/200	15	0.3
Human histocompatibility antigen HLA-DR, α chain	49/300	700	23
Human histocompatibility antigen HLA-DR, β chain	39/240	500	13
Human erythropoietin[c]	28/150	100	1.6
Human melanoma cell surface antigen[b]	13/850	60	5.5

[a] Purified by isoelectric focusing in polyacrylamide gels containing urea.

[b] Electrophoretically eluted from Coomassie blue-stained, SDS-polyacrylamide gel.

[c] 60% carbohydrate by weight.

tensin loads ranging from 5 nmol to 50 pmol are shown in Fig. 7. The complete sequence of the octapeptide could be determined using 5 nmol and 500 pmol of sample, and all but the last 2 residues (proline and phenylalanine) could be determined using 50 pmol. The HPLC traces from a 50-pmol angiotensin run are shown in Fig. 8. Very little extraneous 254 nm absorbing material appears in the chromatograms. Those artifacts that are present, mainly a small peak eluting just after >PhNCS methionine derivative, do not seriously interfere with identification. Interference with identification of low levels of >PhNCS aspartic acid and >PhNCS glutamic acid derivative, which can be obscured by the reduced and oxidized forms of dithiothreitol, is avoided by methylating the acidic side chains with a 1 N methanolic HCl solution prior to HPLC analysis (20).

Selected proteins and peptides that have been analyzed in the new sequenator are listed in Table III. Several of these polypeptides presented potential sequencing problems that, in fact, did not interfere seriously with sequenator performance. These problems (see "Discussion") included the presence of numerous proline residues in the sequence and the contamination of the samples by residue from the techniques used in their preparation, which included SDS-polyacrylamide gel electrophoresis and isoelectric focusing.

DISCUSSION

Sample Immobilization—The chief feature that distinguishes the new gas-liquid solid phase sequenator described in this report from previously described sequenators is the method by which the sample is immobilized during the coupling and cleavage stages of the Edman chemistry. In the classic Edman spinning cup sequenator, the sample is held in solution in a thin film of liquid on the interior wall of the cup (1). In the Laursen solid phase sequenator, the sample, covalently attached to a solid support, is immersed in a stream of liquid reagent (10). In the new sequenator, the sample is embedded in a matrix of Polybrene into which the combination of liquid and gaseous reagents diffuse to carry out the Edman chemistry.

Since, in the Edman spinning cup sequenator, the sample is freely soluble in the reagents and would be removed from the reaction cup by a continuous stream of reagents, an elaborate system for metering in precise aliquots of reagents every degradation cycle is required. The aliquots must reproducibly and precisely cover the sample film every cycle or the reactions may not go to completion. In practice, the required reproducibility is not easily maintained. Moreover, the requirements for an open reaction cup lead to an inefficient solvent extraction system for removal of nonvolatile reagent components, reaction byproducts, and amino acid anilinothiazolinones cleaved from the polypeptide. A thin liquid film must be maintained by mechanically rotating the cup at relatively high speeds, and this process is subject to interference by imperfections in the interior of the cup and corrosion of the drive bearings on the spinning cup assembly.

Many of these problems are absent in the Laursen solid phase sequenator. However, a new difficulty that in practice is often more difficult to overcome than those discussed above is introduced. Since the sample is exposed to continuous streams of liquid reagents that would tend to quickly remove it from the reaction cell, it must be held in place by covalent attachment to a solid support such as derivatized polystyrene or glass beads contained in the cell. Covalent attachment requires the introduction of another set(s) of chemical reagents and steps, is often quite inefficient (less than 20% attachment is common) even under seemingly ideal circumstances, and is particularly difficult with samples that contain very little polypeptide and relatively large amounts of extraneous material remaining from the peptide purification procedures. In any case, the peptide is lost once tbe sequencing progresses inward from its NH_2 terminus to the last attachment site. Also, the identity of those amino acid residues directly involved in the covalent linkage cannot be determined directly.

In the new gas-liquid solid phase sequenator, the sample is never exposed to liquids in which it is appreciably soluble. Therefore, neither an open, spinning cup type reaction chamber nor covalent attachment to a solid support is necessary to retain the sample in the reaction vessel. The primary requirement for preventing sample loss is a means of protecting the polypeptide from being dislodged by mechanical shearing forces as the extraction solvents (in which the sample is generally quite insoluble) flow past it. This requirement is met by embedding the sample in a thin film of Polybrene dispersed on a porous glass disc. The disc, comprised of a mesh of overlapping fibers, is held transversely across the reaction chamber. This structure possesses a relatively high total surface area (hence allowing a very thin film) with a minimum dimension in the direction of fluid flow. The Polybrene film is readily permeable to the reagent vapors and to the extraction solvents, so that flowing gases or liquids can diffuse into and out of the film to carry out chemical reactions or extractions without mechanically disturbing the sample. The Polybrene forms a cohesive film that adsorbs strongly to the porous glass disc and is insoluble in the extracting solvents.

Component Miniaturization—An important characteristic of the reaction cartridge built around the sample support disc is the ease with which the sample containment area can be miniaturized. This, along with the simple flow-through nature of the cartridge assembly, allows the reagent and solvent consumption to be reduced to one-tenth or less of that used in commercial instruments. This has several benefits worth noting. The first is a significant reduction in operating costs. A second is the increased practicality of providing the required amounts of ultrapure reagents and solvents, an important consideration since many of the commercially available chemicals require additional purification to provide the desired level of purity. Yet a third advantage is the increase in speed with which samples can be sequenced. This results from the decreased time required for mass transfer in the miniaturized system and from the very rapid changeover from one sample to another. Cycle time is only 45–55 min, and sample reloading (including cartridge cleanup and Polybrene precycling) is only 3–4 h. A final benefit and the one that is perhaps most important, is that lower reagent and solvent usage per cycle results in a reduced accumulation of impurities accompanying the >PhNCS-derivative samples that are analyzed by HPLC. The low HPLC backgrounds shown in Fig. 8 attest to the low background level of the sequenator, and this miniaturization of artifacts is essential to sequencing at ultramicro levels.

Efficiency of Edman Chemistry—The efficiency with which the new sequenator performs the Edman chemistry, as judged by its repetitive cycle yield, is at least as good as the best available spinning cup sequenators and better than the Laursen-type solid phase instruments. Average repetitive yields of 98% are obtainable with as little as 10 nmol of protein, and 92% cycle yields can be obtained with only 5 pmol of protein (Fig. 5). Sequencing efficiency also is high for peptide analysis, with complete sequencing of small to medium peptides possible with subnanomole quantities.

Several factors contribute to this high efficiency. One is the thoroughness with which the sample cartridge can be cleansed with the extracting solvents. Another is the protection of the sample from removal from the reaction chamber. Other fac-

tors include the thoroughness with which the entire reaction system can be protected from leaks and cross-contamination, purged with argon, and evacuated. Also, the coupling base used in this system, trimethylamine, is very easily purified by distillation, whereas Quadrol and other buffers used in classical sequenators are difficult to purify and are prone to contain impurities, such as aldehydes, which block the Edman chemistry. Further, since the Edman reactions can proceed to completion rapidly at 42 °C (rather than the usual 55 °C), the acid-catalyzed splitting of the polypeptide chain that generates background >PhNCS-derivative signals and reduces repetitive yield is lessened (1, 21). The miniaturization even increases the efficiency of the analysis of the >PhNCS-derivative fractions. The >PhNCS-derivatives generated in the automatic conversion flask are transferred directly into the 300-μl conical tubes used in the autosampler for the HPLC system. This saves time, avoids sample loss, reduces chances of sample contamination, and improves quantitation.

Versatility in Sequencing a Variety of Samples—In order for the new gas-liquid solid phase sequenator to be a general purpose instrument, it was designed to handle as wide a variety of polypeptides as possible. A representative listing of the samples that have been analyzed with this instrument illustrates its versatility (Table III). Proteins can be analyzed using less than 10 pmol (0.2 μg) of sample; peptides can be sequenced with less than 100 pmol (0.1 μg) of sample. Both hydrophobic peptides and proteins, including integral membrane proteins, can be sequenced. Proteins purified by SDS-polyacrylamide gel electrophoresis and by electrofocusing in polyacrylamide gels can be analyzed after the proteins are electrophoretically eluted from the gels. Polypeptides, such as *Aplysia* neuropeptide B (18), that contain several proline residues followed by amino acid residues with bulky side chains (arginine, phenylalanine, tyrosine) also pose no serious problems if the cleavage time is doubled to minimize overlap due to incomplete reaction (22).

Future Developments in Protein Sequencing—The sensitivity of the new sequenator is such that further increases in sensitivity of amino acid sequencing by this technique will require parallel improvements in analysis of the >PhNCS (or similar) derivatives and in purification techniques for submicrogram quantities of proteins. If the sequencing sensitivity can be increased to the 1 pmol level, which seems within the range of the present sequenator, then proteins isolated from analytical two-dimensional polyacrylamide gel electrophoresis (23) can be screened by NH_2-terminal sequence analysis for identification. Because of the enormous resolving power of this separation method, partial characterization of even very rarely expressed proteins would be possible. This partial sequence analysis can then be used to synthesize nucleic acid primers for cDNA cloning of the mRNA from which the proteins are translated (24). Such techniques have already been used to clone such rare message genes as those coding for the interferons (25) and human histocompatibility antigens (26). Once the genes are cloned, then structural analysis of the proteins can be completed by rapid DNA sequence analysis. In this way, partial protein microsequencing, gene cloning, and DNA sequence analysis could be used to characterize a wide variety of biologically and biomedically important proteins even when they are present in trace amounts.

This combination of methods for protein and gene structural analysis is particularly important when studying multigene families such as immunoglobulins, histocompatibility antigens, and interferons. The task of correlating which gene corresponds to which gene product will require structural analyses of both the proteins and the genes in multigene families. Thus in studying multigene families, the phenotypic or protein sequence analyses will be as important as the DNA sequence analyses.

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Supplementary Material to "A Gas-Liquid Solid Phase Peptide and Protein Sequenator" by Rodney M. Hewick, Michael W. Hunkapiller, Leroy E. Hood and William J. Dreyer

TABLE II

Sequenator program

Step	Cartridge functions	Flask functions	Step time (s)	Vol (ml)
1	Line flush (pressurize)	Line flush (pressure)	2	
2	Vacuum	R4 vent and pressurize; line flush, vent flask	4	
3	Vacuum	R4 deliver, vent flask	8	0.050
4	Vacuum	Line flush, vent flask	10	
5	Vacuum	Conversion	40	
6	R3 vent and pressurize; line flush (pressurize)	Conversion	4	
7	R3 deliver, waste open (cleavage)	Conversion	400	5 cc/min (gas)
8	Line flush, waste open	Conversion	20	
9	Vacuum	Conversion	80	
10	Line flush (pressurize)	Conversion	2	
11	S3 vent and pressurize	Conversion	10	
12	S3 deliver, waste open	Conversion	7	0.025
13	Precipitation	Conversion	45	
14	S3 deliver, waste open	Conversion	30	1.000
15	Line flush, waste open	Conversion	20	
16	Vacuum, line flush	Conversion	60	
17	Vacuum	Conversion	2	
18	Line flush (pressurize)	Conversion	2	
19	Vacuum	Conversion	90	
20	Line flush (pressurize)	Conversion	2	
21	R1 vent and pressurize	Conversion	4	
22	R1 deliver, waste open	Conversion	4	0.030
23	Line flush, waste open	Conversion	60	
24	R2 vent and pressurize; line flush, waste open	Conversion	4	
25	R2 deliver, waste open (coupling)	Conversion	450	5 cc/min (gas)
26	Line flush, waste open	Conversion	20	
27	Vacuum, line flush	Conversion	30	
28	Vacuum	Conversion	30	
29	Line flush (pressurize)	Conversion	2	
30	R1 vent and pressurize	Conversion	4	
31	R1 deliver, waste open	Conversion	2	0.020
32	Line flush, waste open	Conversion	60	
33	R2 vent and pressurize	Conversion	4	
34	R2 deliver, waste open (coupling)	Conversion	410	5 cc/min
35	R2 deliver, waste open (coupling)	Vacuum, line flush	40	5 cc/min
36	Line flush, waste open	Vacuum, line flush	40	
37	Vacuum, line flush	Line flush (pressurize)	4	
38	Vacuum, line flush	Line flush, vent, Argon (flask dry)	56	
39	Line flush (pressurize)		2	
40	Vacuum		60	
41	Line flush (pressurize)		2	
42	Vacuum		90	
43	Line flush (pressurize)		2	
44	S1 vent and pressurize	Vacuum, line flush	10	
45	S1 deliver, waste open	Vacuum, line flush	3	0.025
46	Precipitation	Vacuum, line flush	40	
47	S1 deliver, waste open	Vacuum, line flush	60	1.075
48	S2 vent and pressurize; line flush, waste open	Vacuum, line flush	10	
49	S2 deliver, waste open	Vacuum, line flush	46	
50	S2 deliver, waste open	Vacuum	20	2.700
51	S2 deliver, waste open	Line flush (pressurize)	4	
52	S2 deliver, waste open	Vacuum	20	
53	Line flush, waste open	Vacuum	20	
54	Vacuum, line flush	S4 vent and pressurize; line flush (pressurize)	4	
55	Vacuum, line flush	S4 deliver, vent flask	9	0.250
56	Vacuum, line flush	Line flush, vent flask	5	
57	Vacuum, line flush	Argon, vent flask	5	
58	Vacuum, line flush	Line flush; transfer to collector	15	
59	Vacuum, line flush	S4 deliver, vent flask	2	0.050
60	Vacuum, line flush	Line flush, vent flask	5	
61	Vacuum, line flush	Argon, vent flask	5	
62	Vacuum, line flush	Line flush; transfer to collector	10	
63	Line flush (pressurize)	Line flush; transfer to collector	2	
64	Vacuum	Line flush; transfer to collector	2	
65	Vacuum	S4 deliver, vent flask	45	1.000
66	Vacuum	Line flush; waste open	15	
67	Line flush (pressurize)	Collector step; Argon (pressurize)	1	
68	Line flush (pressurize)	Argon (preasurize)	1	
69	Vacuum	Argon; line flush; vent flask	90	
70	R3 vent and pressurize; line flush (pressurize)	Argon; line flush, vent flask	4	
71	R3 deliver, waste open (cleavage)	Vacuum	250	5 cc/min (gas)
72	Line flush, waste open	Vacuum	16	
73	Line flush, waste open	Line flush (pressurize)	4	
74	Vacuum	Argon; line flush, vent flask	30	
75	Line flush (pressurize)	Argon; line flush, vent flask	2	
76	S3 vent and pressurize; line flush, waste open	Argon; line flush, vent flask	2	
77	S3 deliver, transfer to flask	Argon; line flush, vent flask	7	0.025
78	Precipitation	Argon; line flush, vent flask	40	
79	S3 deliver, transfer to flask	Argon; line flush, vent flask	15	0.050
80	Equilibration	Argon; line flush, vent flask	60	
81	S3 deliver, transfer to flask	Argon; line flush, vent flask	14	0.050
82	Equilibration	Argon; line flush, vent flask	50	
83	Line flush; transfer to flask	Argon; line flush, vent flask	10	0.050
84	Vacuum, line flush	Argon; line flush, vent flask	70	
85		Vacuum	40	