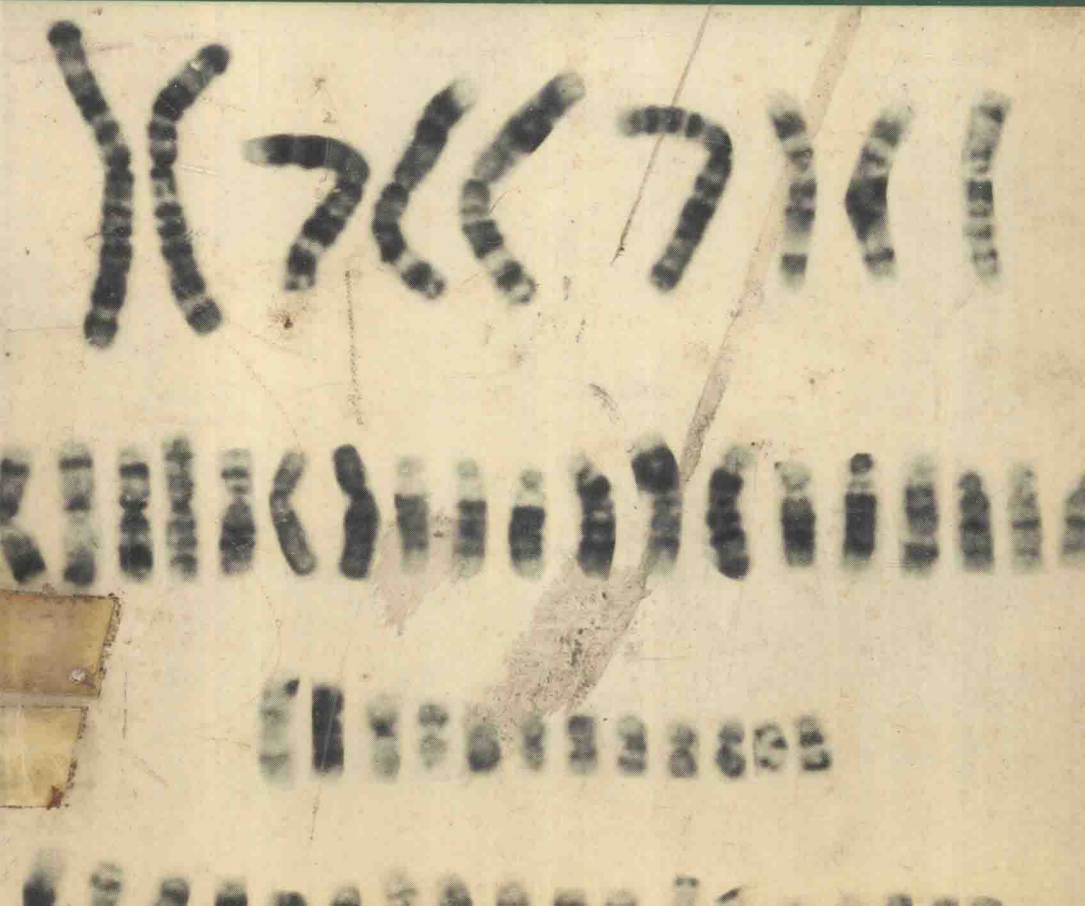
NEW TECHNIQUES IN BIOPHYSICS AND CELL BIOLOGY

Volume 3

Edited by R. H. Pain & B. J. Smith



New Techniques in Biophysics and Cell Biology

Volume 3

Edited by

R. H. Pain

Department of Biochemistry,
University of Newcastle-upon-Tyne

B. J. Smith

Cell Genetics Group,
Imperial Cancer Research Fund Laboratories, London

A Wiley-Interscience Publication

JOHN WILEY & SONS

LONDON · NEW YORK · SYDNEY · TORONTO

Library of Congress catalog card number 72—8611
ISBN 0 471 01814 7

Typeset in IBM Century by Preface Ltd, Salisbury, Wilts
and printed in Great Britain by The Pitman Press, Bath

New Techniques in Biophysics and Cell Biology

Volume 3

Contributors to volume 3

JOHN BRIDGEN	Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.
C. M. DOBSON	The Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, U. K.
A. M. M. van EGMOND-COWAN	Department of Human Genetics, University of Leiden, Wassenaarseweg 72, Leiden, The Netherlands.
D. M. GLOVER	Department of Biochemistry, Imperial College, South Kensington, London SW7, U.K.
RICHARD O. HYNES	Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, U.S.A.
B. A. LEVINE	The Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, U.K.
P. L. PEARSON	Department of Human Genetics, University of Leiden, Wassenaarseweg 72, Leiden, The Netherlands.
P. M. A. SHERWOOD	Department of Inorganic Chemistry, Unversity of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

Contents

CHAPTER 1

Solid-phase techniques in protein sequencing

John Bridgen
Medical Research Council,
Laboratory of Molecular Biology,
Hills Road, Cambridge CB2 2QH, U.K.

I INTRODUCTION

The solid-phase method of sequencing involves the covalent attachment of the peptide or protein to an inert support. The coupled material is then packed into a column and the reagents and solvents used for the Edman degradation pumped through in the normal sequence. The advantages of the method are the ease with which it may be automated and the fact that no losses of peptide material occur during the sequencing process.

Although the principles of automatically determining the amino acid

sequence of a peptide or protein attached to an inert support were first described by Laursen in 1966, the method is still not as commonly used as the automatic liquid-phase technique of Edman and Begg (1967). The reasons for this, which are essentially chemical, arise from problems associated with the attachment methods and the development of suitable supports for larger molecules. Considerable progress has recently been made in overcoming these problems and, now that commercial versions of the solid-phase sequencer are available, the method is rapidly increasing in popularity.

The technique offers a number of potential advantages over the spinning-cup methods, notably in cost, simplicity and the ease with which smaller peptides may be sequenced (cf. Bridgen, 1974).

II. THE INERT SUPPORT

The essential properties of the support are: that it should be inert to the reagents and solvents used during the sequencing process, that it should be capable of being highly substituted with functional groups and that it should have the correct physical properties, particularly with regard to flow and swelling characteristics. To date only two types of support — polystyrene and porous glass — have been successfully utilized.

A. Polystyrene-Based Resins

These are based on 1% cross-linked polystyrene of -400 mesh, e.g. Bio-Beads SX-1 (Bio-Rad). The degree of cross-linking is the minimum necessary to give sufficient mechanical strength, while the mesh size is a compromise between maximum surface area and problems associated with keeping smaller particles in the reaction column.

The two polystyrene derivatives in current use are aminopolystyrene (AP) (Laursen *et al.*, 1972) and triethylenetetramine polystyrene (TETA) (Horn and Laursen, 1973). The preparation of the AP-resin is a two-step procedure consisting of a nitration step as described by Laursen (1971) for the preparation of nitrochloromethyl polystyrene, followed by a reduction with $SnCl_2$ (see the procedure outlined for the reduction of the older nitroethylenediamine resin (Laursen, 1971). The product is stable and should appear mid-brown in colour. The resin may be evaluated by its swelling properties in dimethylformamide (DMF) — suitable batches should swell to approximately four times the dry volume. The resin should also give a very strong positive test for amino groups (Kaiser *et al.*, 1970) and should be capable of coupling 'test peptides' (normally the B-chain of insulin) at substitutions of 1 — 4 nmols/mg resin.

TETA resin is also prepared by a two-step procedure. Initially the chloromethyl derivative is prepared and this is then treated with triethylenetetramine prior to use. It is important that the chloromethylation reaction is carefully controlled since high degrees of substitution lead to poor swelling properties. Between 0.75 and 1.5 mEq. of chloromethyl groups per gram of resin is satisfactory. Our procedure for preparing this resin is as follows:

10 g of dioxane-washed polystyrene (Bio-Beads SX-1, -400 mesh, Bio-Rad Laboratories, Ltd.) is stirred for 1 hr at room temperature in 50 ml $CHCl_3$. The mixture is cooled to 0° and a solution of 1.5 ml anhydrous $SnCl_4$ in 40 ml of chloromethyl methyl ether is slowly added with stirring. Stirring is continued at 0° for a further 30 min and the mixture is then filtered through a sintered-glass funnel, washed with 1 litre of dioxane-3*M*-HCl (3:1, v/v) and then with water followed by methanol. In each case time should be allowed for the wash solvent to penetrate the beads. The resin is dried overnight *in vacuo*.

Since the degree of chloromethylation will depend on the source and purity of the chemicals used, it is important to perform a chlorine analysis on the dry resin. This may be performed by a modification of the Volhard method (Stewart and Young, 1969), after heating the resin (100 mg) in 1.5 ml pyridine for 1 hr at 100 °C.

The degree of chloromethylation can be controlled by changing either the volume of $SnCl_4$ added or the time and temperature of the reaction. Chloromethylated polystyrene may be purchased in 200—400 mesh (e.g. Bio-Rad) at various degrees of substitution. These resins appear perfectly adequate, at least for coupling smaller peptides.

Chloromethylated polystyrene is stable and should only be converted to the relatively unstable TETA derivative in small batches. Normally one month's supply is converted to the amino derivative at a time by heating with triethylenetetramine (Horn and Laursen, 1973) and this is then stored at −20 °C. TETA resin may be qualitatively assayed by the ninhydrin method (Kaiser *et al.*, 1970). As the resin ages, a significant quantity of blue colour will be found free in the supernatant and the development of this colour may be used as a guide to the usefulness of the resin. Because of the gradual loss of amino groups TETA resin should be washed with DMF before use.

B. Porous Glass Supports

Porous glass is an inert support with the advantage that, unlike polystyrene-based supports, it has no differential swelling properties in different media. Thus coupling reactions may be carried out in aqueous solvents and problems of solubility of proteins and large peptides in organic solvents are avoided. The supports are also considerably easier to produce than their polystyrene counterparts. Two derivatives are in

common use — 3-aminopropyl glass (APG) (Robinson *et al.*, 1971; Wachter *et al.*, 1973) and *N*-(2-aminoethyl)-3-aminopropyl glass (AEAPG) (Bridgen *et al.*, 1975). These are both made by incubating the glass beads (Corning CPG-10, 200—400 mesh, 75 Å pore diameter) with a 2% (v/v) solution of 3-aminopropyltrimethoxysilane or *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (Pierce) in acetone at 45 °C for 24 hr (Robinson *et al.*, 1971). The derivatized beads are then filtered and thoroughly washed with acetone and methanol before drying *in vacuo*.

Of the various pore diameters available, the 75 Å size appears to give the best results (Bridgen, 1975), not only in terms of the degree of substitution, which presumably arises from the increased surface area, but also in terms of the repetitive yield found during sequencing. However, for larger proteins (mol. wt. >25—30,000) the 120 Å pore diameter support appears to be superior.

These resins may be assayed with ninhydrin in the same manner as the polystyrene derivatives and should be stored at —20 °C to minimize oxidation or hydrolysis of the Si—O bond. In particular the AEAPG support appears to break down in the same manner as the TETA resin.

C. Applications

TETA and AEAPG supports both contain activated primary amines and are suitable for coupling cyanogen bromide fragments (see below) where the more reactive amino group is important. For reasons which are not yet clear the AP and APG supports often give better results when coupling is via peptide or protein amino groups. In all cases the glass derivatives are used for proteins and large fragments (> 30 residues) since they give higher coupling yields and more efficient degradation for these molecules than is found for the corresponding polystyrene supports (Wachter *et al.*, 1973; Machleidt *et al.*, 1973). However, porous glass based supports are unsuitable for coupling small peptides, owing possibly to partial adsorption of peptide onto the porous glass without covalent attachment.

The uses of the various types of support are summarized in Figure 1.1.

D. New Supports

It is clearly unsatisfactory to have so many different supports and we have attempted to produce resins which are suitable for all applications. Polyacrylamide-based derivatives have the disadvantage that they are hydrolysed by the trifluoracetic acid used in the cleavage step of the degradation. However, a new polydimethyl-acrylamide derivative has been produced (Atherton *et al.*, 1976) which is reasonably stable and initial results with this support are promising. An alternative procedure

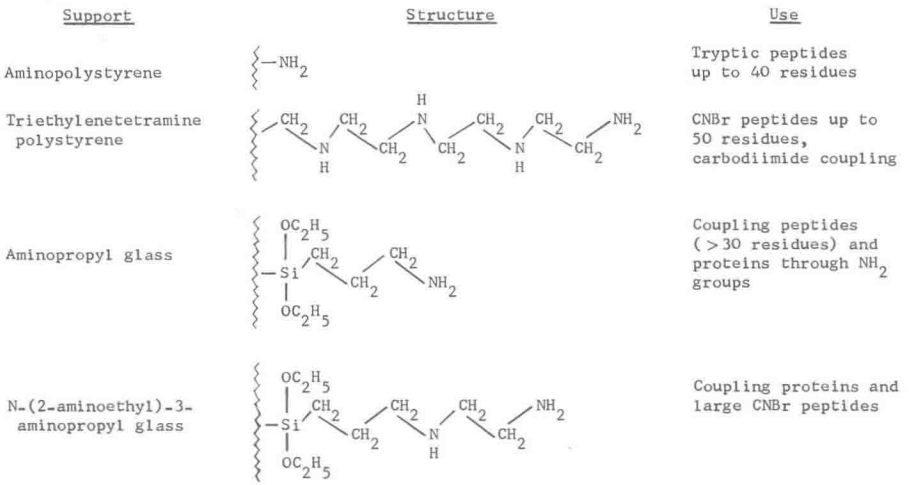


Figure 1.1. Supports used for solid-phase sequencing and their applications. These supports are available commercially (LKB-Biochrom; Pierce).

is to use a graft copolymer or pellicular support in which a thin layer of one polymer, e.g. polystyrene, is coated on another polymer, e.g. Teflon, or inert support, e.g. glass. Initial experiments (Bridgen, unpublished) with a specially prepared amino derivative of Pellionex (polystyrene on glass, Reeve-Angel, Ltd.) indicate that the degree of substitution is too low for these materials to be generally useful.

III. COUPLING METHODS

Having prepared the support, the next step is to attach the peptide or protein covalently. Of the three methods in general use, two (Sections A and B below) work very well most of the time and one (Section C) works tolerably well for about half of the time.

A. The Diisothiocyanate Method

The principle here is to cross-link peptide α- and ϵ-NH_2 groups with resin amino groups using the bifunctional reagent, phenylene-diisothiocyanate (DITC). Coupling procedures have been described in detail by Laursen *et al.* (1972) for polystyrene and by Machleidt *et al.* (1973) for glass supports. Clearly, an important requirement of this procedure is to avoid intramolecular or peptide—peptide cross-linking. Laursen *et al.* (1972) advocate activation of the peptide with a 50-fold molar excess of DITC over peptide amino groups. A better procedure (Machleidt *et al.*, 1973) which can be used for the porous glass

derivatives is to activate the support with a large excess of DITC and then remove the excess by washing with DMF. The method is suitable for any peptide or protein with a C-terminal or internal lysine residue. The N-terminal amino acid remains attached to the resin and should be identified before sequencing commences. Similarly, lysine residues remain attached via their ϵ-NH_2 group and will appear as gaps in the sequence. Sequencing continues until the final lysine residue, when the remainder of the peptide chain becomes detached from the resin.

Small arginine peptides may also be attached after deguanidation with 50% hydrazine at 70 °C for 15 min (Morris *et al.*, 1973) which converts the arginine residue to ornithine. This is then coupled via the δ-NH_2 group. Side reactions include possible hydrazide formation at asparagine and glutamine side chains with subsequent attachment to the resin. If the peptide does not contain these residues, the time for the hydrazinolysis may be increased to ensure complete reaction.

B. Attachment of CNBr Fragments

After cyanogen bromide cleavage all of the fragments produced, except the C-terminal fregment, will have a homoserine residue at the carboxy-terminus. After conversion to the lactone by incubation in trifluoroacetic acid, the lactone may be coupled directly to TETA polystyrene (Horn and Laursen, 1973) or to AEAPG (Bridgen, 1975).

The method is both mechanically and chemically simple and all residues of the peptide, including the C-terminal homoserine, may be identified.

Coupling yields vary from 100% for small fragments to 20%—30% for larger molecules.

C. Attachment through Carboxyl Groups

The original carbodiimide methods (e.g. Laursen, 1971) coupled all of the peptide carboxyl groups to the resin with the result that glutamic acid residues remained attached and were not identified. In addition, the degradation was blocked whenever an aspartic acid residue was encountered. This was not especially useful and a new method (Previero *et al.*, 1973) which promised the specific attachment through the peptide C-terminal carboxyl group using a N,N′ disubstituted carbodiimide was especially welcome.

However, in our hands, coupling yields (>60%) tend to be lower than by other methods and some peptides do not couple at all. The success

of this method may depend on one's ability to completely remove extraneous nucleophiles during the activation procedure.

IV. INSTRUMENTATION

The first automatic solid-phase sequencer was described by Laursen (1971) and only detailed changes have since been made to this original design. A schematic diagram of such an instrument is shown in Figure 1.2. Essentially the peptide resin, diluted with glass beads if polystyrene derivatives are used, is packed into a jacketed column and attached to the instrument. Reagents or solvents are pumped through the column according to a pre-punched programme tape and the effluent is directed either to a waste bottle or to a fraction collector. A more detailed description is given by Laursen *et al.* (1975).

At the time of writing there are four commercial versions of

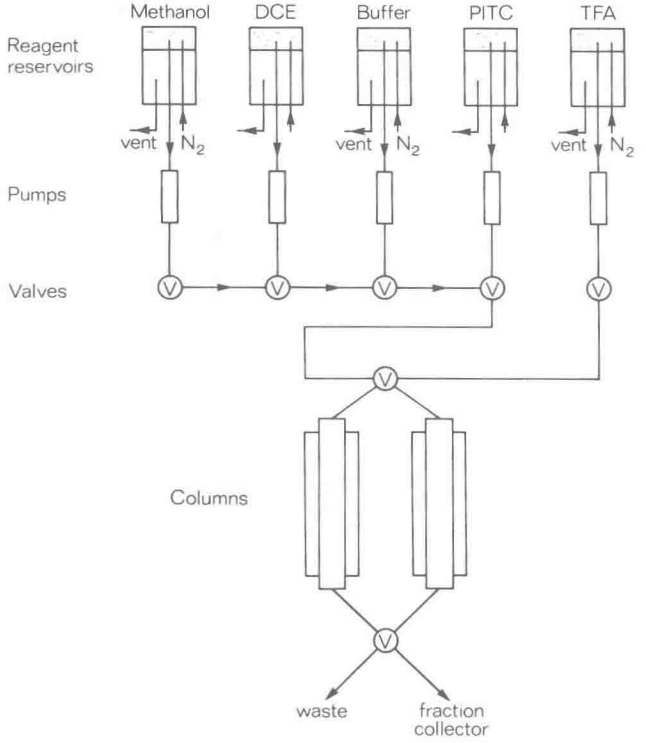


Figure1.2. A schematic diagram of a two-column solid-phase sequencer

solid-phase sequencer available

Anachem SPA 2400	Anachem Ltd., 20a North Street, Luton, U.K.
LKB 4020	LKB-Biochrom Ltd., Science Park, Cambridge, U.K.
Sequemat 16K	Sequemat Inc., Watertown, Mass. U.S.A.
Socosi PS 300	Socosi, St. Maur, France.

Of these the Sequemat, LKB and Anachem instruments are roughly equivalent in design except that the latter two have variable speed pumps and are of bench-top construction. All three instruments have the capacity for two columns which allows the simultaneous degradation of two peptide samples. The Socosi PS 300, on the other hand, uses a reaction cell rather than a column with the reagents and solvents delivered by nitrogen pressure rather than pumps. It will be interesting to see whether this arrangement permits efficient washing of the resin. Only one peptide can be degraded at a time.

The LKB instrument currently offers greater flexibility in flow-rates and programming, which is important for incorporating new developments such as high sensitivity operation (Bridgen, 1976a,b) or automatic conversion (Bridgen, 1976c).

A comparison of the various instruments is shown in Table 1.1.

V. SEQUENCING

Before the degradation commences the resin-peptide has to be packed into the reaction column. Normally, a Chromatronix or Altex type of column (0.3 x 10—20 cm) is used. The dead-space from the column outlet to the lower end of the heating jacket is filled with glass beads (200—400 mesh, washed with HCl and methanol) and the peptide-bonded support then added. In the case of polystyrene supports the resin must be 'diluted' with glass beads (1 part resin to 19 parts glass beads) to avoid the swelling and shrinking effects caused by the passage of different solvents through this column. After filling to the top with glass beads the column is connected to the instrument, the heating jacket connected, and the degradation started. The chemistry of the Edman reaction is shown in Table 1.2.

Chemicals used for solid-phase sequencing are listed below.

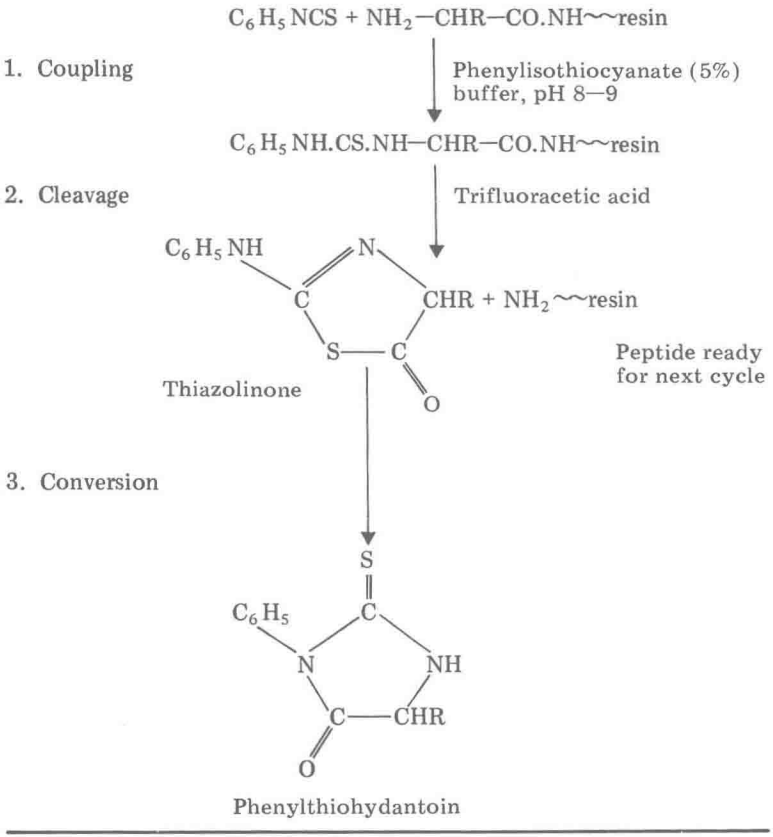
Solvent 1	Methanol
Solvent 2	Dichloroethane
Reagent 1	5% PITC in acetonitrile
Reagent 2	*N*-methylmorpholine buffer (Laursen, 1971)
Reagent 3	Trifluoroacetic acid

Table 1.1 *Instrument parameters*

	Sample capacity	Cycle time (min)	Programmer unit	Pumps	Reaction unit	Fume extraction system	Size (cm)
Anachem SPA 2400	2	68	Punched tape	2-piston 3-syringe	Two columns	Partial	107 x 99 x 50
LKB 4026	2	65	Optical card reader	5-displacement	Two columns	Complete	118 x 70 x 50
Sequemat 16K	2	98	Punched tape	2-piston 3-syringe	Two columns	Partial	156 x 117 x 56
Socosi PS 300	1	146	Pinboard	N_2	Cell	Partial	185 x 65 x 60 + 50 x 60 x 50

Modification kits for high sensitivity operation and for automatic conversion are also offered by one manufacturer (LKB)

Table 1.2 Chemistry of the solid-phase Edman degradation



These chemicals do not need to have the exceptional purity necessary for spinning-cup sequencers. Suitable sources include Rathburn Chemicals, Walkerburn, Scotland, and Pierce Chemical Co., Rockford, Ill., U.S.A. Reagents and solvents are pumped through the column according to a pre-punched program tape in the sequence: methanol, buffer, buffer + PITC, buffer, methanol, dichloroethane, methanol, dichloroethane, trifluoroacetic acid, methanol. The effluent is directed into a waste bottle except for the trifluoroacetic acid and final methanol wash which are directed to the fraction collector. In two-column systems (Anachem, LKB, Sequemat) the reagents for the coupling reaction are directed through one column while TFA passes simultaneously through the second column. That is, one column runs half a cycle out of phase with respect to the other.

Table 1.3 Program times for sequencer steps (min)

	Wash 1	Coupling	Wash 2	Cleavage	Wash 3	Total time
Anachem SPA 2400	3	33	25	5	2	1 hr 8 min
LKB 4020	2	30	26	5	2	1 hr 5 min
Sequemat 16K	4	32	25	34	3	1 hr 38 min
Socosi PS 300	—	54	31	44	17	2 hr 26 min

Four published programs are shown in Table 1.3. Our standard procedure includes a fast TFA flow rate (0.15 ml/min) timed so that when the cleavage acid reaches the bottom of the column the pump is halted. After 3 min at 45 °C the released thiazolinone is washed into the fraction collector by a 4 min pulse of methanol. Another possible modification is the omission of the dichloroethane washes when porous glass supports are being used. Normally, one complete cycle of degradation takes between one and two hours. The mixture of TFA and methanol in each fraction collector tube is evaporated at 50 °C under a stream of N_2 and the thiazolinone converted into the more stable thiohydantoin by incubation in 1*M*-HCl or 20% TFA at 80 °C for 10 min. The phenylthiohydantoin (PTH) is then extracted with ethyl acetate (2 x 0.7 ml) or by washing through a Pasteur pipette containing Dowex-50(H^+). In the former case all of the PTH derivatives except those of histidine, arginine and cysteic acid are extracted while in the latter case the two basic derivatives are retained on the Dowex.

The PTHs are conveniently identified by thin layer chromatography (t.l.c.) but when larger molecules are being degraded semi-quantitative gas liquid chromatography (g.l.c.) or high pressure liquid chromatography (h.p.l.c.) methods may prove advantageous. For a complete review of the problem of PTH identification see Bridgen *et al.*, 1975.

VI. APPLICATIONS

The solid phase sequencer was originally developed as an automatic method for sequencing small peptides. However, the simplicity and low operating costs of the equipment have spurred on the development of methods for the degradation of larger molecules so that current applications range from small peptides to proteins. A list of some sequences obtained by the solid-phase method is given in Table 1.4 along with an indication of the quantities of material used. More recent references may be found in Bridgen (1976a) and in the *Proceedings of the International Conference on Solid-phase Methods in Protein Chemistry* (1975, Pierce Chem. Co.).

As well as conventional sequencing the coupling methods of the solid-phase technique may be exploited as selective purification