Studies on Sequencing of Peptides from the Carboxyl Terminus by Using the Thiocyanate Method

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We report on our experience in applying the thiocyanate method developed by Stark (1968) (*Biochemistry* 7, 1796–1807) to the sequencing of short peptides from the carboxyl end in free solution. Yields fell to very low levels after three cycles of degradation. The method was time-consuming because of the filtration and freeze-drying stages involved. To overcome these problems, peptides were attached to modified polystyrene polymers for sequential degradation in the solid phase, and a maximum of six amino acids was determined. Also, ribonuclease was attached to active-ester glass beads and sequential degradation was carried out to determine six amino acids at the C-terminal end of this protein.

The linear sequencing of proteins and peptides has been successfully carried out in recent years mainly by the Edman degradation procedure, which operates from the N-terminus (Edman, 1950). The reactions carried out in free solution were automated (Edman & Begg, 1967). However, short peptides are better sequenced manually by the dansyl-Edman method (Gray, 1967). By using the general methods of Merrifield (1963) for the preparation of polymers and the attachment of peptides, it was shown that the Edman degradation could be applied to peptides attached to a solid support (Laursen, 1966; Schellenberger *et al.*, 1967; Dijkstra *et al.*, 1967). Laursen (1971) reported on an automatic peptide sequencer using the Edman degradation, with the peptide attached in the solid phase. This method has been widely used (see Laursen, 1975).

No comparable progress has been achieved from the C-terminus. Only the C-terminal amino acid has been identified by hydrazinolysis (Akabori *et al.*, 1952) and by selective ^3H labelling (Matsuo *et al.*, 1966; Baba *et al.*, 1972). There are many problems involved in the use of carboxypeptidases for determining more than the single C-terminal amino acid of a protein (Ambler, 1967*a,b*). The most successful enzymic method appears to be that of Hermodson *et al.* (1972), who determined the sequence of five amino acids at the C-terminus of monkey amyloid protein A by using yeast proteinase C.

Schlack & Kumpf (1926) originally proposed a chemical method of sequencing from the C-terminus. This involved the formation of a peptidyl thiohydant-

oin which was subsequently cleaved, and the method was used to a limited extent by other workers (Waley & Watson, 1951; Baptist & Bull, 1953; Fox *et al.*, 1955). Stark (1968) made detailed studies of the reactions involved and showed that typically two or three stages of degradation could be achieved with peptides, although in the best case six stages were possible. In his work the amino acid thiohydantoin derived from the C-terminus was determined by difference analysis. Cromwell & Stark (1969) extended this method to protein. They used t.l.c. to identify the amino acid thiohydantoin directly. In addition they described a procedure for hydrolysing the thiohydantoin to its parent amino acid, which was then analysed. Using large quantities of ribonuclease and papain (100 μmol), Yamashita & Ishikawa (1971) used the thiocyanate method to determine the sequence of 10 C-terminal amino acids in ribonuclease and 14 in papain. They claimed that about 10 amino acids from the C-termini of polypeptides could be analysed, starting with 0.01–0.1 μmol of material (Yamashita & Ishikawa, 1972). No details were given. Short peptides attached to glass beads were similarly sequenced (Williams & Kassell, 1975).

The mass spectra were reported for some amino acid thiohydantoins (Suzuki *et al.*, 1972; Rangarajan *et al.*, 1973) and for their trimethylsilyl derivatives (Rangarajan *et al.*, 1973). Rangarajan *et al.* (1973) were the first to show that g.l.c. could be used for the identification of these compounds. Rangarajan & Darbre (1975) reported on the two-dimensional t.l.c. of amino acid thiohydantoins and they also showed that these compounds could be determined indirectly with g.l.c. by hydrolysis of and derivative formation from the parent amino acids. This paper is on studies made by the methods of Stark (1968) and Cromwell

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& Stark (1969) with the reactions in free solution and also sequential-degradation studies in which the peptide was attached to a solid phase. Preliminary reports of some of this work have been published (Rangarajan & Darbre, 1974; Darbre & Rangarajan, 1975).

Materials and Methods

Materials

Chemicals. Hexafluoroacetone sesquihydrate and *N*-methylmorpholine were purchased from Aldrich Chemical Co. (New Road, Gillingham, Dorset SP8 4JL, U.K.); amino acids, dicyclohexylcarbodi-imide, ethanolamine, methyl isothiocyanate, β -mercaptoethanol, KNO_3 , sodium acetate, NaH_2PO_4 , Na_2HPO_4 , granulated tin, triethylamine, trifluoroacetic acid, trifluoroacetic anhydride and urea were from BDH Chemicals (Poole, Dorset, U.K.); 1,4-dioxan, pyridine and ammonium thiocyanate were from Fisons Scientific Apparatus (Loughborough, Leics., U.K.); acetic anhydride was from Hopkin and Williams (P.O. Box 1, Romford, Essex, U.K.); BBOT [2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen] was from Koch-Light (Colnbrook, Bucks., U.K.); 12*M*-HCl was from E. Merck (Darmstadt, Germany); *NO*-bis(trimethylsilyl)trifluoroacetamide and peptides were from Sigma Chemical Co. (P.O. Box 14508, St. Louis, MO, U.S.A.); polystyrene-*p*-divinylbenzene polymer (1% cross-linked) Biobeads S-X1 (200–400 mesh) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.); Sephadex G-25 (medium and fine grades) was from Pharmacia (Uppsala, Sweden) and *N*-hydroxysuccinimide-controlled-pore glass (pore diam. 55 nm; particle diam. 125–177 μm) was from Pierce Chemical Co. (Box 117, Rockford, IL 61105, U.S.A.). Tetra- and hexa-peptides were a gift from the Wellcome Research Laboratories (Beckenham, Kent BR3 3BS, U.K.) through the courtesy of Dr. A. W. Phillips.

G.l.c. materials. Stationary phases and support materials were purchased as follows: Dexsil 300 GC (Analabs, North Haven, CT 06473, U.S.A.), Chromosorb W, 80–100 mesh (Field Instruments, Richmond, Surrey, U.K.), and Silicone OV-17 (Phase Separations Ltd., Queensferry, W. Lothian, Scotland, U.K.).

Apparatus. G.l.c. was carried out with a Pye 104 model 24 gas chromatograph fitted with dual-flame ionization detectors. Speedomax W 1 mV 1s (Leeds and Northrup, Wharfedale Road, Tyseley, Birmingham, U.K.) strip-chart recorders were used. Peak areas were determined with Kent Chromalog 2 (Kent Instruments, Luton, Beds., U.K.) and Vidar Autolab 6300 (Anachem, 636 Hitchin Road, Luton, Beds., U.K.) digital integrators. Nitrogen (99.9% 'white spot' from British Oxygen Co., East Lane, North

Wembley, Middx. HA9 7PT, U.K., and high-purity oxygen-free from Air Products, New Malden, Surrey, U.K.) was used as carrier gas. The glass g.l.c. column (3.25 m $\times$ 2.5 mm internal diam.) was packed with HP Chromosorb W 80–100 mesh, coated with 1% (w/w) Dexsil 300 GC or with 5% (w/w) OV-17 (Rangarajan *et al.*, 1973).

Methods

Methylthiocarbamoyl polymer with γ -amino-*n*-butyric acid side chain. This was prepared from polystyrene polymer (1% cross-linked) by the stages shown previously (Darbre & Rangarajan, 1975). The carboxylic acid polymer I (Table 2) (15 g) was mixed intimately with 30 g of KNO_3 with a pestle and mortar and added slowly with stirring to 35 ml of ice-cold conc. H_2SO_4 . The temperature was kept below 8°C. After addition was complete, the mixture was heated to 90°C for 30 min. The reaction mixture was cooled in ice and 500 ml of ice-cold water added. The polymer was filtered off, washed with water until the washings were pH 3–4 (pH paper), then with methanol and ether, and dried. There was a weight gain of about 40% and the polymer II was pale yellowish-brown. The nitro groups were reduced by adding 30 g of granulated tin slowly to a well-stirred suspension of 15 g of polymer in 165 ml of conc. HCl (Mann & Saunders, 1964). The reaction was kept under control by cooling when necessary. The mixture was finally heated at 80°C for 2 h until all the tin had dissolved. The dark-brown polymer III was filtered off, washed first with 1 *M*-HCl to remove tin salts, then with water, methanol and ether, and dried. The amino groups were blocked by stirring at 40°C for 4 h 10 g of polymer in 35 ml of pyridine *N*-methylmorpholine trifluoroacetate buffer at pH 9.5 with the addition of 10 ml of methyl isothiocyanate (Laursen, 1971). The methylthiocarbamoyl polymer IV was filtered off, washed alternately with dimethylformamide and methanol until the washings were colourless and finally with ether, and dried.

γ -Amino-*n*-butyric acid (1.5 mmol) was converted into its methyl ester hydrochloride form by causing it to react with 5.0 ml of methanolic 4 *M*-HCl at 70°C for 1.5 h in a stoppered tube (Islam & Darbre, 1972). The contents were taken to dryness on a rotary evaporator, and, after addition of further methanol, taken to dryness a second time to remove any excess of HCl. The γ -amino-*n*-butyric acid methyl ester hydrochloride was dissolved in 3.0 ml of dry dimethylformamide and 200 μl of triethylamine was added slowly to liberate the free base. The methylthiocarbamoyl polymer (IV) (1.0 g) was suspended in 7.0 ml of dry dimethylformamide and allowed to swell for about 20 min, then 50 μl of triethylamine was added followed by the solution of γ -amino-*n*-butyric acid methyl ester (1.5 mmol) containing dicyclohexylcarbodi-imide (1.6 mmol) (Sheehan &

Hess, 1955). The reaction mixture was stirred at room temperature (20°C) for 18 h in a stoppered vessel. The light-brown polymer with attached γ -amino-*n*-butyric acid methyl ester was filtered off and washed successively with dimethylformamide, methanol and ether, and dried.

The methyl ester groups were hydrolysed by stirring 1 g of the polymer with 10 ml of 0.8 M-NaOH in methanol/acetone solution (1:1, v/v) at room temperature for 4 h. The solution was adjusted to about pH 3.0 with 1 M-HCl and the polymer V was filtered off, washed with water, methanol and ether, and dried. The amount of attached γ -amino-*n*-butyric acid was determined after hydrolysing 25 mg of the polymer with aq. 6 M-HCl *in vacuo* at 110°C for 24 h. The *N*-trifluoroacetyl methyl ester derivative was prepared and determined by g.l.c.

Attachment of peptide to methylthiocarbamoyl γ -amino-*n*-butyric acid polymer. The peptide methyl ester hydrochloride was dissolved in 0.5 ml of dimethylformamide and 30 μ l of triethylamine added to liberate the free base. About 800 mg of polymer was allowed to swell in 4.0 ml of dimethylformamide for about 20 min, when the peptide methyl ester solution was added, followed by a solution of dicyclohexylcarbodi-imide (0.2 mmol) in dimethylformamide. The mixture was stirred at room temperature for 18 h. The polymer was filtered off, washed extensively with dimethylformamide and methanol, and dried. The peptide methyl ester was hydrolysed as described for γ -amino-*n*-butyric acid methyl ester.

Attachment of protein to glass beads. The *N*-hydroxysuccinimide-controlled-pore glass beads were used as purchased (Darbre & Rangarajan, 1975). The buffers were prepared from 0.1 M-sodium acetate and 0.1 M-acetic acid or 0.1 M-NaH₂PO₄ and 0.1 M-Na₂HPO₄. The protein dissolved in the buffer solution (5–7 mg/ml) was cooled to 4°C, and, for every 5 ml of solution, between 0.5 g and 1.0 g of the active glass beads was added. The vessel was degassed in the cold and gently rocked at 0–4°C for 4 h. Magnetic stirring caused fracturing of the beads. The beads were washed with ice-cold buffer on a sintered-glass funnel and any remaining active ester groups were allowed to react with 1.0 M-ethanolamine in the same buffer solution at room temperature for 2 h. The beads were filtered off and washed with buffer, then deionized water, and finally 1,4-dioxan and methanol. They were dried with a stream of N₂ gas.

Monitoring amount of peptide or protein attached to solid phase. The amount of coupling to polymer and controlled-pore glass beads was determined by hydrolysing a weighed sample of the solid phase *in vacuo* in a Rotaflow stopcock (Darbre, 1971) with aq. 6 M-HCl containing 1 mM- β -mercaptoethanol and 0.1% (w/v) phenol at 110°C for 24, 48 and 72 h. The dried amino acid residue was esterified and trifluoroacetylated for determination of the amino acids by

g.l.c., with norleucine as internal standard (Darbre & Islam, 1968; Islam & Darbre, 1969, 1972).

In some experiments the attachment to glass beads was determined by the disappearance of protein from the medium by the method of Lowry *et al.* (1951). Changes in the u.v. absorbance of the medium were not considered to be valid as a method of following protein attachment (Cuatrecasas & Parikh, 1972). *N*-Hydroxysuccinimide is released as a result of both the coupling reaction and hydrolysis of the active ester by water. Also, protein in the solution contributes to the total absorbance.

Sequential degradation of peptides attached to polymer. Ammonium thiocyanate was recrystallized from ethanol, and acetic acid and acetic anhydride were redistilled (see Stark, 1968). A solution of ammonium thiocyanate (2.0 mmol) in acetic anhydride (26 mmol) and acetic acid (6.0 mmol) was added to 100 mg of polymer (about 0.4 μ mol of attached peptide) in a small stoppered vessel and stirred at 80°C for 1.5 h. Excess of acetic anhydride was hydrolysed by adding 3.0 ml of water, and the polymer with the attached peptidyl thiohydantoin was filtered off through a sintered-glass funnel, washed with water, then with acetone, and dried.

The peptidyl-thiohydantoin attached to the polymer was cleaved with aq. 12 M-HCl at 30°C for 1.5 h. The HCl solution was removed through a sintered-glass funnel and the polymer was washed with a few drops of water to remove excess of HCl. Any remaining HCl was removed with an acetone wash to prevent any methyl ester formation. Because not all thiohydantoin were soluble in acetone, the polymer was then stirred with 3.0 ml of methanol at room temperature for 30 min. This was sucked off and the polymer further washed with methanol and dried. The aqueous filtrate was taken to dryness and then the acetone and methanol filtrates were added and taken to dryness. The amino acid thiohydantoin was identified by g.l.c. (Rangarajan *et al.*, 1973) or t.l.c. (Rangarajan & Darbre, 1975).

Sequential degradation of proteins attached to glass beads. About 500–750 mg of glass beads containing 1 μ mol of attached protein was treated with 1 ml of hexafluoroacetone trihydrate prepared by distillation from the sesquihydrate (Burkhardt & Wilcox, 1967), followed by a freshly prepared solution of ammonium thiocyanate (2.0 mmol) in a mixture of acetic anhydride (54 mmol) and acetic acid (11.0 mmol). The stoppered flask was gently rocked in a shaking water bath at 50°C for 2 h. A further 2.0 mmol of solid ammonium thiocyanate was added and shaking continued at 50°C for 18 h. The beads were filtered off through a sintered-glass funnel, washed exhaustively with aq. 50% (v/v) acetic acid and then with water until the washings were colourless. The beads were finally washed with 1,4-dioxan and transferred to a dry conical flask. Cleavage was carried out by shaking

for 1 h at room temperature with 2 ml of 12M-HCl. The glass beads were then taken to dryness with a stream of N_2 gas. Acetone (3.0 ml) was added to extract any remaining HCl. The extract was dried down with N_2 gas. The beads were extracted three times with 1.0 ml portions of methanol and all the extracts were combined. The combined dried residue was analysed by g.l.c. or t.l.c.

Identification of amino acid thiohydantoins. A Pye Series 104 gas chromatograph, model 24, was used as previously described (Rangarajan *et al.*, 1973) with Dexsil 300 GC or OV-17 stationary phases. Bibenzyl was included in the final sample for g.l.c. and the relative retention time was used for the identification of amino acid thiohydantoin. In this work mass spectrometry was not used. Amino acid thiohydantoins were also determined indirectly by g.l.c. after hydrolysis (Rangarajan & Darbre, 1975).

The two-dimensional t.l.c. of amino acid thiohydantoins was previously described (Rangarajan & Darbre, 1975). Since that report we have used BBOT as a fluorescent indicator, which gives a much lighter background than butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole].

Results and Discussion

Reactions in free solution

Stark (1968) showed the probable chemical reaction steps that occurred when, first, the terminal carboxyl group of a peptide reacted with thiocyanate in the presence of acetic acid and acetic anhydride to form the peptidyl thiohydantoin and, secondly, when the amino acid thiohydantoin corresponding to the C-terminal amino acid was cleaved from the peptide chain. He was the first worker to consider the minimum conditions required for achieving complete reaction.

We studied some of the procedures advocated by Stark (1968) and Cromwell & Stark (1969). The Sephadex G-25 separations were found to be as described by these authors. Only proteinyl-thiohydantoins were completely resolved from the peaks caused by salt and degradation products. These were recovered by freeze-drying the peak tubes (total volume about 100 ml). The dried residue was cleaved by reaction with 1 ml of 12M-HCl at room temperature for 1 h and taken to dryness with a stream of N_2 gas, and then subjected to Sephadex G-25 filtration as before. The amino acid thiohydantoin was detected by its E_{262} . Truncated proteins were detected by their E_{280} , and truncated peptides were detected with ninhydrin. The peak tubes corresponding to the amino acid thiohydantoin were pooled (approx. 90 ml) and freeze-dried. The residue was taken up in methanol for analysis by g.l.c. (Rangarajan *et al.*, 1973) or t.l.c. (Rangarajan & Darbre, 1975). In some cases the

amino acid thiohydantoin was hydrolysed, and the parent amino acid was then determined by g.l.c. (Rangarajan & Darbre, 1975).

The yield of amino acid thiohydantoin was about 70% and decreased with subsequent degradation cycles. Stark (1968) did not quote yields, but gave molar ratios of amino acids. The possibility that the low yields were due to the inaccessibility of carboxyl groups was explored by adding either 8M-urea or sodium dodecyl sulphate to the reaction mixture when preparing the proteinyl-thiohydantoin. No reaction occurred in the presence of 8M-urea, and there could be several reasons for this.

(i) It was reported by Newallis & Rumanowski (1964) that urea formed adducts with fluoroacetones, of the type $NH_2-CO-NH-C(OH)(CF_3)_2$. It is possible that urea formed an adduct with the hexafluoroacetone present, thereby causing precipitation of the protein and hence inhibiting the reactions.

(ii) Carboxylic acid amides can be prepared by heating the corresponding acid with urea (Cherbuliez & Landolt, 1946; Rahman, 1956; Guthrie & Rabjohn, 1957). This could have occurred between urea and acetic acid.

(iii) Bengen & Schlenk (1949) and von Schlenk (1949) described the formation of inclusion or clathrate compounds between urea and a wide variety of organic molecules. This might have occurred.

(iv) Preferential acetylation of urea might have led to the formation of ureides.

Sodium dodecyl sulphate was added to the protein solution (in 1.0 ml of hexafluoroacetone trihydrate and 0.2 ml of water) in a ratio of 3:1 (w/w). The solution was heated to 100°C for 2 min (Weber *et al.*, 1972) before the proteinyl thiohydantoin was prepared. With lysozyme and ribonuclease the yield of amino acid thiohydantoin in the fourth cycle was approx. 10%, as calculated from the absorption (molar extinction coefficient 1.75×10^4 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ in acid; Cromwell & Stark, 1969). Ribonuclease A, reduced and carboxymethylated, was taken through four stages of sequential degradation with similar results.

Another factor studied was the instability of amino acid thiohydantoins under the column conditions used. Valine thiohydantoin in 50% acetic acid was passed through a column (54 cm $\times$ 1.6 cm internal diameter) of Sephadex G-25 (fine beads). In two experiments the recoveries were 7.0 and 8.0%, compared with control values in which the valine thiohydantoin was kept in 50% acetic acid under N_2 but was not passed through the column. Because it was not possible to work under an N_2 atmosphere, reducing agents were used. Ascorbic acid, cysteine, dithiothreitol and thioglycolic acid were discarded because they gave interfering peaks with g.l.c. Dithiobutane was obnoxious and not fully studied. In the presence of either glutathione or β -mercaptoethanol (2 mm), the recovery of valine thiohydantoin from the Sephadex

Table 1. Amounts of peptides attached to methyloxycarbonyl chloride polymer

Results are the means of triplicate determinations.

Peptide	Amount of peptide (μmol)	Amount of polymer (mg)	Amount of peptide attached/100mg of polymer (μmol)	Attachment yield (%)
Glycyl-alanine	100	250	38	95
Glycyl-leucine	100	150	60	90
Glycyl-leucine	3	150	1.80	90
Valyl-leucine	80	200	34	85
Methionyl-alanine	60	200	26	86
Glycyl-alanyl-leucine	4.5	200	2.1	93
Glutathione (reduced)	100	150	50	75
Glutathione (reduced)	3	200	1.4	93
Tetrapeptide*	3	200	0.12	8
Hexapeptide†	3	200	0.14	9

* Leucyl-glycyl-alanyl-phenylalanine.

† Leucyl-alanyl-valyl-glycyl-phenylalanyl-glycine.

Table 2. Swelling ratios (V_s/V_D) in different solvents of polystyrene-*p*-divinylbenzene polymer (1% cross-linked) modified with substituent groups V_D is the volume of dry polymer and V_s the volume of polymer after 2h in solvent (Laursen, 1971).

Polymer	Substituent groups	Dimethyl-formamide	1,4-Dioxan	Acetic anhydride	Acetic acid	Acetone	6M-HCl (aq.)	12M-HCl (aq.)
I	-CO ₂ H	5.0	5.6	1.3	1.0	4.0	1.1	1.0
II	-CO ₂ H	5.4	1.8	2.3	1.3	2.6	1.1	1.2
	-NO ₂							
III	-CO ₂ H	4.1	1.8	2.0	1.6	2.5	1.9	1.7
	-NH ₂							
IV	-CO ₂ H	3.0	3.5	2.2	1.3	2.7	2.1	1.9
	-NH-CS-NH-CH ₃							
V	-CO-NH-[CH ₂] ₃ -CO ₂ H	3.2	2.9	2.5	1.4	2.7	2.2	1.9
	-NH-CS-NH-CH ₃							

Table 3. Hydrolysis of *N*-acetyl-leucine thiohydantoin at room temperature

Means of duplicate results are given.

Acid	$10^3 \times k$ (s ⁻¹)	$t_{\frac{1}{2}}$ (s)
6M-HCl in 1,4-dioxan	1.6	424
10M-HCl in 1,4-dioxan	6.2	112
12M-HCl, aq.	8.3	84

column was 100% when compared with the control value.

In the absence of a reducing agent ribonuclease gave a yield of about 60% for valine thiohydantoin in the first cycle, 40% for serine in the second and 30% for alanine in the third cycle. However, when the two Sephadex separations were carried out in the presence of β -mercaptoethanol (2mm) the corresponding yields were approx. 80, 80 and 50%. However, a low yield of approx. 10% was still obtained in the fourth cycle. The

low yield was unlikely to be caused by incomplete cleavage at each stage, because this would have resulted in carry-over visible on the g.l.c. record. This was not seen. Yamashita & Ishikawa (1971) also reported dramatically lowered yields of amino acid thiohydantoin with ribonuclease (at cycle 4) and with lysozyme (at cycle 3).

Each total degradation cycle included two Sephadex column separations with freeze-drying of column effluents, and this in our hands required 3½ days. Because we were unable to determine more than three C-terminal amino acids with the proteins studied, it was decided to apply the method in the solid phase.

Solid-phase sequencing

The early experiments with solid-phase attachment of peptides and subsequent sequencing were made with a methyloxycarbonyl chloride polymer (Darbre & Rangarajan, 1975). The attachment yields were only satisfactory with di- and tri-peptides, as shown

in Table 1, but this polymer was easily prepared and useful for studies of the sequencing reactions.

A modified polystyrene-*p*-divinylbenzene (1% cross-linked) polymer was prepared by the stages (I-V) shown in Table 2. The permeability of polymers to solvents was determined by the method of Laursen (1971), and the swelling ratios are given in Table 2.

Methods for cleaving the polymer-attached peptidyl thiohydantoin were studied. Acetohydroxamic acid in pyridine solution (Stark, 1968) was not used because of the instability of amino acid thiohydantoin in the reaction mixture. Yamashita (1971) used Amberlite IR-210 (H⁺ form) for cleavage in free solution, but this was not applicable here.

The rate of conversion of acylamino acid thiohydantoin into amino acid thiohydantoin was studied spectrophotometrically by using *N*-acetyl-leucine thiohydantoin as a model. Standard mixtures in ethanol of *N*-acetyl-leucine thiohydantoin (0.066 mM) and leucine thiohydantoin (0.066 mM) in various proportions were prepared and their absorbance ratios at 280 nm and 265 nm determined. These gave a linear relationship, which was used to study the hydrolysis of *N*-acetyl-leucine thiohydantoin. The results in Table 3 give the equilibrium constant and the half-life of *N*-acetyl-leucine thiohydantoin for three concentrations of acid. Aq. 12M-HCl showed the highest speed of reaction, and this was the agent used for cleaving

peptidyl-thiohydantoin attached to the solid phase. Stark (1968) reported on the deacylation of *N*-acetylalanine thiohydantoin with both nucleophilic reagents and acids at 25°C. He quoted for aq. 10M-HCl, $t_4 = 102$ s, which is within the range in Table 3.

Peptides were attached to the methylcarbamoyl γ -amino-*n*-butyric acid polymer and the yields are shown in Table 4. The attachment yields of the di- and tri-peptide were similar to those shown in Table 1, but the tetra- and hexa-peptides were attached to a much greater extent (90%). This improvement was ascribed to the increased polarity of the polymer allowing easier accessibility of reactive sites in the solvent used, and also to the introduction of a spacer group. Laursen (1971) methylthiocarbamoylated free amino groups after attachment of peptide to the polymer. The hexapeptide in Table 1 (0.4 μ mol) was attached to the methylthiocarbamoyl γ -amino-*n*-butyric acid polymer (100 mg) and sequentially degraded. The results are shown in Fig. 1, where each of the amino acids was identified by g.l.c. or t.l.c. Valine in cycle 4 was determined after hydrolysis of the thiohydantoin (Rangarajan & Darbre, 1975). Extraneous g.l.c. peaks (as shown in Fig. 1, cycle 5) were ignored because they did not give retention times relative to that of the internal standard corresponding to any known derivative. Absolute retention times are not useful in g.l.c. for identification.

Table 4. Amount of peptide attached to methylthiocarbamoyl γ -amino-*n*-butyric acid polymer

Means of duplicate results are given.

Peptide	Amount of peptide (μ mol)	Amount of polymer (mg)	Amount of peptide attached/50mg of polymer (μ mol)	Attachment yield (%)
Glycyl-leucine	4	800	0.24	95
Glycyl-alanyl-leucine	4	800	0.21	84
Tetrapeptide*	5	1000	0.22	90
Hexapeptide*	4	800	0.22	90

* As in Table 1.

Table 5. Amount of protein attached to controlled-pore glass beads

Protein	Protein concn. (mg/ml)	Buffer	pH	Amount attached (mg of protein/g of glass beads)
Ribonuclease A	5	0.1 M-Acetate	6.2	22
	7	0.1 M-Acetate	6.2	29
	7	0.1 M-Acetate/6M-urea	6.2	27
	5	0.1 M-Phosphate	6.5	23
	7	0.1 M-Phosphate/6M-urea	6.5	25
	7	0.1 M-Phosphate	7.5	23
	7	0.1 M-Phosphate	7.9	19
	6.5	0.1 M-Acetate	6.2	21
Lysozyme	6.5	0.1 M-Acetate	6.2	20
Lysozyme (reduced and carboxymethylated)	6.5	0.1 M-Acetate	6.2	20

Fig. 1. Identification of six amino acid thiohydantoin after sequential degradation of polymer-bound hexapeptide

The g.l.c. instrument and Dexsil 300 GC column were described in the Materials and Methods section. N_2 gas flow rate 30 ml/min; sample size 1 μ l; attenuation 1×10^{-9} A for full-scale deflexion for valine and serine and 0.5×10^{-9} A for alanine; initial temperature 130°C programme at 2°C/min to 230°C. The hexapeptide (0.4 μ mol, Table 1) was degraded and for g.l.c. the total trimethylsilylated amino acid thiohydantoin from each cycle was dissolved in 40 μ l of pyridine/*NO*-bis-(trimethylsilyl)-trifluoroacetamide (1:1, v/v) containing bibenzyl internal standard (I.S.). In cycle 4 the thiohydantoin was hydrolysed and the trifluoroacetylvaline methyl ester was identified with norleucine internal standard. T.l.c. was previously described (Rangarajan & Darbre, 1975).

Fig. 2. Identification of six amino acid thiohydantoins obtained from the C-terminus of bovine pancreatic ribonuclease A attached to glass beads

Instrument and g.l.c. conditions were as in Fig. 1. Glass-bound ribonuclease ($1 \mu\text{mol}$) was sequentially degraded, and 1.6% of the total trimethylsilylated sample was injected on to the g.l.c. column, or 1.6% of the thiohydantoin solution in methanol was used for t.l.c.

No attempts were made at this stage to attach proteins to synthetic polymers. Laursen (1971) reported lowered attachment yields of insulin B chain (70%) to polymers, whereas the yield was nearly 100% for some smaller peptides. Instead we used commercial controlled-pore glass beads possessing both a spacer group and an active *N*-hydroxysuccinimide ester group. The amounts of protein coupled to the glass beads (Table 5) varied between 29mg/g at pH6.2 and 19mg/g at pH7.9. Cuatrecasas & Parikh (1972) reported that the *N*-hydroxysuccinimide ester of succinylated agarose reacted optimally with the α -amino group of L-alanine at pH8.5. The presence of 6M-urea did not increase ribonuclease attachment.

Ribonuclease A (1 μ mol) was attached to 500mg of glass beads and each of the six C-terminal amino acids was determined. Fig. 2 presents some of the proof of identification of the six C-terminal amino acids of ribonuclease, determined by g.l.c. and t.l.c. The lower yield of alanine in step 3 may have been due to the neighbouring amino acid, aspartic acid (Stark, 1968).

Williams & Kassell (1975) treated eight different peptides, possessing between two and five amino acids, with activated *N*-hydroxysuccinimide glass beads at pH8.3 and showed average attachment yields varying from 49 to 71%. These authors used subtractive analysis to follow each cycle of degradation.

In conclusion, it is believed that this is a useful carboxyl end-group method, which is being further studied to determine problems that might be encountered with some amino acids, such as aspartic acid and proline (Stark, 1968) and lysine (Williams & Kassell, 1975). The methyloxycarbonyl polymer was easily prepared and was useful for very short peptides, but a better range of peptides was attached to the methylthiocarbamoyl- γ -amino-*n*-butyric acid polymer. Work with polymer-attached protein was not carried out. The glass beads were used as purchased and, with our limited experience, ribonuclease and lysozyme were attached in good yield. The chemical reactions involved in the cyclization and cleavage were not difficult with the amino acids studied. Modifications in the procedure may be possible by using a column with continuous flow of solvents, as used by Laursen (1971).

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