Peptide Synthesis by the Soluble-polymer Technique

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This report delineates the conditions necessary for the successful use of the soluble-polymer method of peptide synthesis and illustrates it with the preparation of a pentapeptide.

ONE of the major causes of low yields in classical peptide synthesis is the loss during physical manipulations involved in purification, and it is because of the elimination of this problem that the Merrifield solid-phase method [1] is so successful. This method has been used in the synthesis of bradykinin,[2] L-methionyl-L-lysylbradykinin,[3] isoleucine-angiotensin II,[4] bradykininylbradykinin,[5] and the B chain of bovine insulin.[6] Many modifications and improvements have been made to the original method, including the use of more easily removable protecting groups [2,5,7] and the automation of the procedure.[7]

In 1965 Shemyakin described the use of a soluble-polymer system for the synthesis of peptides; [8] he used polystyrene of average molecular weight 200,000 to synthesize a tetrapeptide.

The method was claimed to be superior to the insoluble-polymer method because of the greater probability of quantitative reaction at all sites; in the Merrifield method the rate and extent of formation of peptide bonds and the removal of protecting groups depend on the permeability of the polymer.

We were attracted to this method during studies of the synthesis of a portion of a naturally occurring peptide, and the problems encountered and overcome in the application of the technique to the elaboration of small linear peptides are described below with reference to the synthesis of L-alanyl-L-isoleucyl-*N*(ω)-nitro-L-arginyl-L-seryl-L-alanine.

Polystyrene (Dow styron 678) of molecular weight 230,000—250,000 was chosen for the polymer support; it was soluble in most of the common organic solvents and insoluble in water and the lower molecular weight alcohols. The polymer was desulphurized to remove sulphur-containing free-radical terminating agents, which would cause catalyst poisoning and extensive coloration of products during chloromethylation.

We planned to attach the *C*-terminal amino-acid to the polymer by chloromethylation followed by reaction of the triethylammonium salt of the *N*-protected amino-acid with the benzyl chloride grouping, but problems were immediately encountered when stannic chloride-catalyzed reaction of chloromethyl methyl ether with polystyrene in the absence of a solvent yielded a gelatinous insoluble product. The generation of this material was attributed to cross-polymerization by a stannic chloride-catalyzed intermolecular Friedel-Crafts reaction involving the benzyl chloride residues. Even at low concentrations of the polymer in carbon tetrachloride, cross-linking still occurred, as shown by a gradual increase in viscosity of the reaction mixture.

To eliminate this problem a study of the chloromethylation reaction was made in which the relative amounts of the reactants were varied. A kinetic study is illustrated in Figure 1, showing that the chlorine

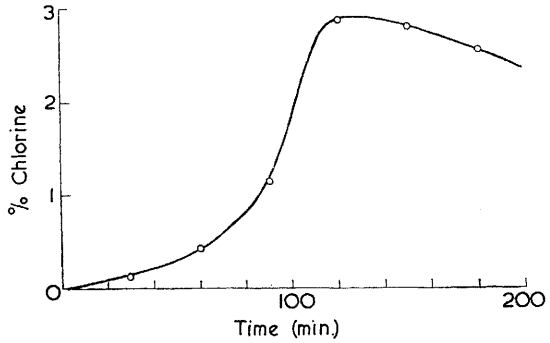

FIGURE 1 The chloromethylation of polystyrene

content reaches a maximum after 2 hr. and then decreases gradually as cross-polymerization proceeds. From these studies the best conditions were found for the preparation of a soluble chloromethylated polystyrene with a very low degree of cross-linking.

For the successful use of the soluble-support method it must be possible to precipitate the polymer quantitatively and in suitable physical form after each reaction step. We eventually found that precipitation of polystyrene derivatives was best achieved by pouring a 10% solution in dimethylformamide into aqueous N-sodium chloride solution. The efficiency of removal of low molecular weight compounds by precipitation and washing was monitored by treating *O*-benzyl-*N*-t-butoxycarbonyl-L-seryl-L-alanylpolystyrene with the mixed anhydride from *N*(ω)-nitro-*N*-t-butoxycarbonyl-L-arginine and isobutyl chloroformate under the conditions of actual peptide bond formation. The polymer was precipitated and washed and the peptide cleaved from the support and chromatographed on an ion-exchange column. Fractions absorbing at 269 mμ were

[1] R. B. Merrifield, *J. Amer. Chem. Soc.*, 1963, **85**, 2149.
[2] R. B. Merrifield, *J. Amer. Chem. Soc.*, 1964, **86**, 304; *Biochemistry*, 1964, **3**, 1385.
[3] R. B. Merrifield, *J. Org. Chem.*, 1964, **29**, 3100.
[4] G. R. Marshall and R. B. Merrifield, *Biochemistry*, 1965, **4**, 2394.
[5] V. A. Najjar and R. B. Merrifield, *Biochemistry*, 1966, **5**, 3765.
[6] A. Marglin and R. B. Merrifield, *J. Amer. Chem. Soc.*, 1966, **88**, 5051.
[7] R. B. Merrifield, *Science*, 1965, **150**, 178; R. B. Merrifield and J. M. Stewart, *Nature*, 1965, **207**, 522; R. B. Merrifield, J. M. Stewart, and N. Jermberg, *Analyt. Chem.*, 1966, **38**, 1905.
[8] M. M. Shemyakin, Yu. A. Ovchinikov, A. A. Kinyunshkin, and I. V. Kozhevnikova, *Tetrahedron Letters*, 1965, 2323.

collected; t.l.c. showed $N(\omega)$-nitro-L-arginine to be present to the extent of 1% of that originally added. After this finding, two precipitations were carried out for each polymeric intermediate.

Attachment of the *C*-terminal amino-acid to the polymer support by heating a solution of the triethylammonium salt of an *N*-t-butoxycarbonylamino-acid under reflux with chloromethylpolystyrene in dioxane for 12 hr.[8] gave a product still containing 1·12% chlorine. Increasing the reaction time to 24 hr. gave an insoluble cross-polymerized product which was obviously generated by gradual thermal decomposition of the t-butoxycarbonyl groups and subsequent alkylation of the unmasked amino-groups by residual benzyl chloride residues. A more efficient substitution was achieved by heating the sodium salt of *N*-t-butoxycarbonyl-L-alanine in dimethylformamide solution with chloromethylpolystyrene for 9 hr. at 100° to give a product containing only 0·30% chlorine. Because use of the high temperature carried the risk of cross-polymerization, the rate of ester formation at room temperature was studied. The degree of substitution was assessed by comparing the intensities of the 1720 cm.$^{-1}$ benzyl ester absorption and the 1600 cm.$^{-1}$ aromatic absorption; Figure 2 shows that

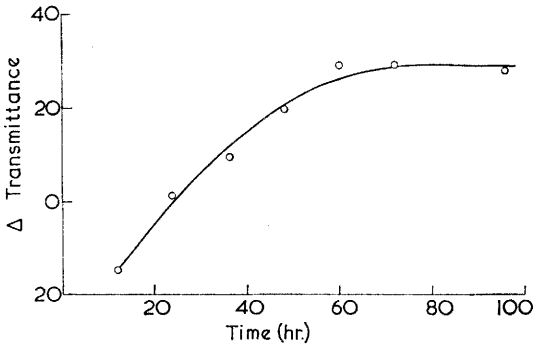

FIGURE 2 Rate of formation of *N*-t-butoxycarbonyl-L-alanylpolystyrene; Δ transmittance represents the difference between transmittance at 1600 and 1720 cm.$^{-1}$

maximum substitution is achieved after 80 hr., to yield an amino-acid-substituted polystyrene containing 0·43% chlorine.

The best degree of substitution of amino-acid on the polymer support was about 0·5 mmole/g. At higher substitution values derivatives such as amine hydrochlorides tended to be sparingly soluble.

Cross-polymerization proved to be a serious problem, since even 0·1% was sufficient to cause insolubility in organic solvents. The first sign of cross-polymerization was an increase in viscosity of the reaction solution due to gel formation.[9] Such solutions always gave products insoluble in organic solvents.

The facility with which cross-polymerization occurred is shown by the following examples. A sample of L-alanylpolystyrene containing 0·4% benzylic chlorine per g. polymer, obtained by neutralization of the hydrochloride in dimethylformamide solution at room temperature, quickly separated from solution, presumably owing to alkylation of the free amino-groups by residual benzyl chloride groupings in other polymer molecules. Treatment of *N*-butoxycarbonyl-L-seryl-L-alanylpolystyrene (Va) with a saturated solution of hydrogen chloride in tetrahydrofuran at 0° caused precipitation after a few min. This is attributed to benzyl ether formation between the free hydroxy-group of L-serine and the excess of benzyl chloride residues of the polymer support. Alkylation of the terminal amino-group can be ruled out in this strongly acidic medium.

Because residual benzylic chlorine atoms were causing unavoidable cross-polymerization, methods for their removal were investigated. Heating *N*-t-butoxycarbonyl-L-alanylpolystrene (II) under reflux in dimethylformamide containing morpholine or piperidine for 12 hr. reduced the chlorine content from 2·17 to 0·11%, whereas elemental analysis indicated no chlorine to be present after treatment with sodium acetate in dimethylformamide at 100° for 24 hr. Merrifield replaced the excess of benzylic chlorine atoms with acetoxy-groups by use of triethylammonium acetate.[1]

Attempts at cleavage of the t-butoxycarbonyl protecting groups by passing hydrogen chloride through a solution of the protected peptide–polymer in dimethylformamide at room temperature [8] were inefficient because of the rapid decomposition of dimethylformamide. Cleavage was found to be complete in 1 min. on treatment with tetrahydrofuran saturated with hydrogen chloride at 0°, following which cleavage reactions were run for 15—20 min. to ensure their completion. It was also found that the *N*-t-butoxycarbonyl group was stable in less than N-acidic tetrahydrofuran solutions.

In order to demonstrate the general applicability of

[9] See R. W. Lenz, 'Organic Chemistry of Synthetic High Polymers,' Interscience, New York, 1967, p. 693.

the soluble-support method, L-alanyl-L-alanine (IV) was prepared from *N*-t-butoxycarbonyl-L-alanine and L-alanylpolystyrene by various coupling methods; the time taken for complete amide-bond formation was followed by comparing the intensities of the amide-carbonyl absorption at 1690 cm.$^{-1}$ and the benzyl ester-carbonyl absorption at 1740 cm.$^{-1}$. The most convenient method consisted of addition of the mixed anhydride of *N*-t-butoxycarbonyl-L-alanine and isobutylcarbonic acid in tetrahydrofuran to L-alanylpolystyrene in dimethylformamide; reaction was complete in 30 min. compared with *ca.* 1 hr. when dicyclohexylcarbodi-imide was used with [10] or without *N*-hydroxysuccinimide. The mixed anhydride method also had the advantage of low temperature, which would be expected to minimize cross-polymerization. L-Alanyl-L-alanine was also successfully prepared by use of *N*-ethyl-*S*-phenylisoxazolium 3-sulphonate as the coupling agent in dimethylformamide.

A trace of L-alanine was always detected in the products from these experiments and could arise from a small degree of cross-linking between the amino-groups of deprotected L-alanine residues and benzylic chlorine atoms so producing *C*,*N*-blocked L-alanine molecules, which would be released under the cleavage conditions. Alternatively, incomplete purification by one precipitation would leave t-butoxycarbonyl-L-alanine occluded in the polymer and this would be converted into L-alanine during the cleavage reaction. The latter was supported by the fact that an additional precipitation markedly lowered the proportion of L-alanine in the product.

With L-alanylpolystyrene, three methods of cleavage from the polymer were found to be applicable: (*a*) passing hydrogen bromide into a suspension of the substituted polymer in trifluoroacetic acid,[8] (*b*) passing hydrogen bromide into a solution of the polymer in benzene, and (*c*) heating the peptide–polymer with anhydrous hydrazine in dimethylformamide. In all cases the residual polymer after precipitation and washing was devoid of i.r. absorption attributable to the amino-acid.

In order to determine if serious racemization had occurred, a sample of L-alanylpolystyrene prepared by the best methods described was cleaved with hydrogen bromide in trifluoroacetic acid, and the L-alanine was purified by crystallization. The specific rotation of the sample was the same at four wavelengths as that of the original L-alanine, thus indicating that racemization was minimal during one synthetic cycle.

With the experimental problems adequately resolved the technique could now be applied in synthesis; the two peptides chosen as a test were *N*(ω)-nitro-L-arginyl-L-seryl-L-alanine (VII) and L-alanyl-L-isoleucyl-*N*(ω)-nitro-L-arginyl-L-seryl-L-alanine (VIII), representing units 122—124 and 120—124 respectively of the tobacco mosaic virus protein.[11] These peptides were considered suitable since they contain L-arginine and L-serine, which present some difficulty in synthetic sequences and because use of the *N*(ω)-nitro-protecting group of arginine allows chromatographic separations to be followed spectroscopically.

To synthesise the tripeptide, *N*-t-butoxycarbonyl-L-alanylpolystyrene (II) was prepared from *N*-t-butoxycarbonyl-L-alanine, sodium carbonate, and chloromethylpolystyrene at room temperature, and the protecting group was removed with tetrahydrofuran saturated with hydrogen chloride. To the resulting L-alanylpolystyrene was added the mixed anhydride from *O*-benzyl-*N*-t-butoxycarbonyl-L-serine and isobutyl chloroformate to yield *O*-benzyl-*N*-t-butoxycarbonyl-L-seryl-L-alanylpolystyrene (VI), which was treated with tetrahydrofuran–hydrogen chloride to remove the *N*-protecting group. The tripeptide–polymer, *N*(ω)-nitro-*N*-t-butoxycarbonyl-L-arginyl-*O*-benzyl-L-seryl-L-alanylpolystyrene (VI) was prepared by one additional mixed anhydride reaction and the partially protected tripeptide was cleaved from the polymer with trifluoroacetic acid–hydrogen bromide and chromatographed on a weakly acidic Rexyn 102 (H) ion-exchange column in N-aqueous acetic acid. The major component was homogeneous by t.l.c. in four solvent systems, and elemental analysis was consistent with the structure of *N*(ω)-nitro-L-arginyl-L-seryl-L-alanine acetate dihydrate. The minor component of the separation was *N*(ω)-nitro-L-arginine.

The pentapeptide, L-alanyl-L-isoleucyl-*N*(ω)-nitro-L-arginyl-L-seryl-L-alanine (VIII) was synthesized from the tripeptide–polymer by successive additions of *N*-t-butoxycarbonyl-L-isoleucine hemihydrate and *N*-t-butoxycarbonyl-L-alanine, both as mixed anhydrides of isobutylcarbonic acid, followed in each case by removal of the t-butoxycarbonyl group. The pentapeptide was removed from the support as described above and chromatographed on Rexyn 102 (H) by use of aqueous acetic acid with a gradient elution technique. Four components were isolated in the ratio 2·3 : 15·0 : 1·6 : 1·0;* the second was rechromatographed to yield a product homogeneous by t.l.c. in four solvent systems. Elemental analysis was consistent with the structure L-alanyl-L-isoleucyl-*N*(ω)-nitro-L-arginyl-L-seryl-L-alanine (VIII).

The tripeptide and pentapeptide were synthesised in 4 and 6 days respectively, although final purification of the products required considerably longer.

Compared with the soluble-polymer technique, the insoluble-polymer method has the advantage of easier manipulation, which means that more amino-acids can be added in a specific time and adaption to an automated technique is easier.[7] The time factor diminishes in importance when preparation of protected amino-acids and final purification is considered. Both techniques

* The three impurities contain *N*(ω)-nitro-L-arginine and are probably formed as a result of incomplete removal of *N*(ω)-nitro-L-arginine at the tripeptide stage.

[10] F. Weygand, D Hoffmann, and E. Wuensch, *Z. Naturforsch*, 1966, **216**, 426.

[11] M. L. Anson, K. Bailey, and J. T. Edsall, *Adv. Protein Chem.*, 1963, **18**, 1.

have a considerable time advantage over conventional methods.

On the other hand, the soluble-polymer method has the advantage of much greater flexibility, since a wider variety of coupling techniques can be used. Recently it was reported [12,13] that the efficiency of addition to an insoluble peptide–polymer decreases after the second or third amino-acid, presumably as a result of solvation effects at the reaction sites. This problem is not encountered in the soluble-polymer method although the peptide–polymer does change its solubility characteristics to some extent as the *N*-terminal amino-acid is varied. It will be of interest to study the extension of the soluble-polymer method to the elaboration of longer-chain peptides carrying a wide variety of *N*-terminal amino-acids.

EXPERIMENTAL

M.p.s were recorded with a Kofler hot-stage apparatus. Elemental microanalyses were performed by Mr. C. F. Geiger, Ontario, California. I.r. spectra were obtained with a Baird-Atomic spectrophotometer by Dr. R. Hill of this department and with a Perkin-Elmer 137B spectrophotometer. U.v. spectra were recorded with either a Perkin-Elmer 4000 or a Beckman DU spectrophotometer. A Bellingham and Stanley Pepol 60 polarimeter was used for measuring optical rotations. Thin-layer chromatograms were run on plates (45 × 75 mm.) with silica gel G (Merck) as adsorbent, unless otherwise specified, and were developed with the following solvent systems (ratios % w/w): * (A) 95% ethanol–water (63 : 37), (B) n-propanol–water (64 : 36), (C) n-butanol–acetic acid–water (60 : 20 : 20), (D) n-propanol–34% ammonia (67 : 33), (E) 95% ethanol–34% ammonia (77 : 23), (F) butan-2-one–pyridine–water–acetic acid (70 : 15 : 15 : 2), and (G) chloroform–methanol–acetic acid (90 : 9 : 1). Amino-acid and peptide spots were detected by spraying with ninhydrin solution followed by heating at 100° for 1 min. *N*-t-Butoxycarbonyl derivatives were treated briefly with gaseous hydrogen chloride before detection in this manner. A Waring Blender was used for homogenization of precipitated polymer suspensions.

Sulphur-free Polystyrene.—To a solution of polystyrene (150 g.; Dow Styron 678, K27, clear #7, average molecular weight 230,000—250,000) in dry dioxan (1 l.) was added Raney nickel (W-7) [from alloy (125 g.)].[14] The suspension was stirred at 25° for 3 hr. and under reflux for 12 hr. It was then cooled and the catalyst was filtered off and washed with dioxan. The filtrate and washings were poured into water (10 l.) and the precipitate was homogenized with water (2 l.), filtered off, and washed successively with water and methanol; yield of sulphur-free polystyrene powder (dried 24 hr. *in vacuo* at 25°) 138 g.

Chloromethylpolystyrene (I).—Polystyrene (25 g.) was dissolved in carbon tetrachloride (190 ml.) by brief heating and the cooled solution was treated successively with chloromethyl methyl ether (25 ml.) and stannic chloride (anhydrous; 2·0 ml.). After 2 hr. the mixture was poured into a large volume of methanol and the collected precipitate was washed successively with large quantities of methyl and ethyl alcohols. It was then dissolved in dimethylformamide (125 ml.) and precipitated in water (5 l.). The collected precipitate was homogenized with water (500 ml.), filtered off again, and washed successively with large amounts of water and methyl alcohol. The product was dried for 24 hr. at 75° to yield chloromethylpolystyrene (23·5 g.) (Found: Cl, 2·25%).

A kinetic study was carried out by withdrawing samples (2 ml.) at definite times, working up, and submitting for chlorine analysis. The results are shown in Figure 1.

N-t-Butoxycarbonyl-L-alanylpolystyrene (II).—A mixture of *N*-t-butoxycarbonyl-L-alanine [15] (6·40 g., 0·034 mole), sodium carbonate (1·79 g., 0·017 mole), and chloromethylpolystyrene (15 g.; 3·31% Cl) in dimethylformamide (150 ml.) was stirred at 25° for 7 days. Addition to water (2 l.) caused precipitation of a solid, which was collected, homogenized with water (500 ml.), filtered off again, washed with water and methanol, and air-dried. A solution of the product in dry dioxan (300 ml.) was filtered to remove a trace of insoluble material, and the concentrated solution (100 ml.) was poured into water. The precipitate was collected, homogenized with water (500 ml.), filtered off again, washed with water and methanol, and dried *in vacuo* at 25° for 24 hr. to yield a solid (14·9 g.), $\nu_{max.}$ (KBr) 1740, 1160 (benzyl ester), and 1720 (urethane) cm.$^{-1}$ (Found: Cl, 0·46; N, 1·36%.) (0·54 mmole L-alanine per g. polymer, based on the more accurate figure for chlorine).

A kinetic study under these conditions was followed by i.r. spectroscopy. The results (Figure 2) indicate maximum substitution of *N*-t-butoxycarbonyl-L-alanine for chlorine in 72 hr.

Removal of Residual Benzylic Chlorine.—A solution of *N*-t-butoxycarbonyl-L-alanylpolystyrene (II) (15 g.; 1·72% Cl) in dimethylformamide (150 ml.) containing sodium acetate (24·6 g., 0·3 mole) was heated with stirring for 24 hr. at 105°. The cooled solution was then added to methanol (3 l.). The precipitate was collected, homogenized with methanol (200 ml.), filtered off, washed with methanol, dried, dissolved in dimethylformamide, and precipitated in water (4 l.). The precipitate was collected, homogenized with water (1 l.), filtered off, and washed successively with water and methanol; yield (dried *in vacuo* at 25° for 24 hr. 14·8 g. (Found: Cl, 0·00%).†

L-Alanylpolystyrene Hydrochloride (III).—*N*-t-Butoxycarbonyl-L-alanylpolystyrene (II) (10 g.) was dissolved in tetrahydrofuran (100 ml.) saturated with hydrogen chloride at 0°. After 15 min. the solution was poured into N-aqueous sodium chloride to give a precipitate which was homogenized with water (200 ml.), filtered off, and washed with water. The product (9·8 g.) was dried *in vacuo* at 25° for 24 hr., $\nu_{max.}$ ($CHCl_3$) 2440 ($\overset{+}{N}H_3$), 1740, and 1160 (benzyl ester) cm.$^{-1}$.

* Systems (A)—(F) have been described by A. T. James and L. J. Morris, 'New Biochemical Separations,' D. van Nostrand Co., Ltd., London, 1964, p. 136.

† Although no chlorine could be detected by the Carius method, mild cross-polymerization did occur under vigorous conditions thus indicating the presence of a trace.

[12] G. R. Marshall, Abstracts of A.C.S. Meeting, San Francisco, April 1968.

[13] B. S. Wildi and J. H. Johnson, Abstracts of A.C.S. Meeting, San Francisco, April 1968.

[14] H. R. Billica and H. Adkins, *Org. Synth.*, Col. Vol. III, 1955, p. 176.

[15] R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, 1959, **42**, 2622.

Cleavage from the Polymer Support.—(*a*) Hydrogen bromide was passed through a stirred suspension of *N*-t-butoxycarbonyl-L-alanylpolystyrene (II) (1·0 g.; 0·64 mmole L-alanine per g. polymer) in trifluoroacetic acid (15 ml.) for 150 min. at 25°. After purging with nitrogen, the suspension was filtered and the solid was washed with trifluoroacetic acid and dried *in vacuo* at 25° for 24 hr.; lack of i.r. absorption at 1740, 1160, and 1720 cm.$^{-1}$ showed complete cleavage from the polymer.

The filtrate was evaporated *in vacuo* to leave an oil which was partitioned between water (20 ml.) and ether (20 ml.). The aqueous layer was washed with ether and passed through a column (15 ml. wet) of Amberlite IR-45 anion exchange resin (analytical grade), and the eluant was lyophilized to give a solid, identical with L-alanine in t.l.c. systems [solvent (A) R_F 0·45, (B) 0·38, (E) 0·39].

(*b*) A solution of *N*-t-butoxycarbonyl-L-alanylpolystyrene (II) (1·0 g., 0·64 mmole L-alanine per g. polymer) in anhydrous benzene (25 ml.) was stirred at 25° for 150 min. with addition of hydrogen bromide. The solution was purged with nitrogen followed by evaporation to a glass, which was dissolved in dimethylformamide (5 ml.). Addition to water (50 ml.) gave a solid which was purified as above. The aqueous filtrate was treated as in (*a*) to yield a solid, homogeneous by t.l.c. and of the same mobility as L-alanine.

L-Alanyl-L-alanine (IV).—To a solution of *N*-t-butoxycarbonyl-L-alanine (0·755 g., 4·0 mmoles) and triethylamine (0·404 g., 4·0 mmoles) in tetrahydrofuran (5 ml.) at −15° was added a solution of isobutyl chloroformate (0·545 g., 4·0 mmoles) in tetrahydrofuran (3 ml.) at 0° and the mixture was stirred for 15 min. at −15° before it was added to L-alanylpolystyrene hydrochloride (III) (1·0 g.; 0·40 mmole L-alanine per g. polymer) in tetrahydrofuran (10 ml.) containing triethylamine (0·101 g., 1·0 mmole; added 30 sec. previously) at 0°.

Reaction proceeded at 0° for 2 hr. and then at 25° for 6 hr. after which the mixture was poured into aqueous N-sodium chloride (125 ml.). The precipitate was homogenized with water (100 ml.), filtered off, washed with water and methanol, and dried *in vacuo* at 25° for 12 hr.; $\nu_{max.}$ ($CHCl_3$) 1740, 1160 (benzyl ester), 1720 (urethane), and 1690 (amide) cm.$^{-1}$. The product was dissolved in benzene (25 ml.) and treated with hydrogen bromide to liberate the dipeptide. Two ninhydrin-positive spots were detected on t.l.c. in system (F). The major spot (R_F 0·68) was identical with L-alanyl-L-alanine (IV) (Mann Research Laboratories, New York) and the minor spot (R_F 0·40) with L-alanine.

N(ω)-Nitro-L-arginyl-L-seryl-L-alanine (VII).—To a solution of *O*-benzyl-*N*-t-butoxycarbonyl-L-serine (2·95 g., 10 mmoles) and triethylamine (1·01 g., 10 mmoles) at −15° in tetrahydrofuran (7 ml.) was added isobutyl chloroformate (1·366 g., 10 mmoles) in tetrahydrofuran (3 ml.). After 15 min. at −15°, the solution was added with stirring to L-alanylpolystyrene hydrochloride (III) (10·0 g., 0·58 mmole L-alanine per g. polymer) in dimethylformamide containing triethylamine (1·01 g., 10 mmoles); reaction proceeded for 2 hr. at 0° and 6 hr. at 25°. The product was precipitated in N-aqueous sodium chloride, homogenized with water, washed with water and methanol, and dried at 25° for 5 hr. *in vacuo*; $\nu_{max.}$ (CCl_4) 1740, 1160 (benzyl ester), 1720 (urethane), and 1690 (amide) cm.$^{-1}$. The polymer was dissolved in tetrahydrofuran (100 ml.) saturated with hydrogen chloride at 0° and stirred for 15 min. at 0° before precipitation in water, homogenization with water, and washing with water. The product (10·6 g.) was dried for 5 hr. at 25°; $\nu_{max.}$ ($CHCl_3$) 2240 ($\overset{+}{N}H_3$), 1740, 1160 (benzyl ester), and 1690 (amide) cm.$^{-1}$.

To *O*-benzyl-L-seryl-L-alanylpolystyrene hydrochloride (10·6 g.) was added *N*-t-butoxycarbonyl-*N*(ω)-nitro-L-arginine (3·20 g., 10 mmoles) as its mixed anhydride with isobutylcarbonic acid exactly as described above. The substituted polymer (10·5 g.). isolated in the same way, was treated with hydrogen chloride–tetrahydrofuran to give *N*(ω)-nitro-L-arginyl-*O*-benzyl-L-seryl-L-alanylpolystyrene hydrochloride; no urethane carbonyl peak at 1720 cm.$^{-1}$.

A sample (2·5 g.) was cleaved by trifluoracetic acid–hydrogen bromide to yield the tripeptide (140 mg.), 26% based on 0·58 mmole L-alanine per g. polymer, which was dissolved in N-aqueous acetic acid (1 ml.) and chromatographed on a column of Rexyn 102(H^+) [16] (2 × 90 cm.; 100—200 mesh). The column was developed with N acetic acid at a flow rate of 15 ml./hr. The chromatography was followed by measuring the absorbance of fractions (10 ml.) at 269 mμ. Fractions 26—31 contained *N*(ω)-nitro-L-arginine; fractions 70—90 containing the tripeptide were combined and lyophilized to yield *N(ω)-nitro-L-arginyl-L-seryl-L-alanine acetate* (VII) (37 mg.), $\lambda_{max.}$ (N-AcOH) 269 mμ (ε 6500), homogeneous by t.l.c. layer in four systems [(system (A) R_F 0·78, (B) 0·45, (D) 0·64, (F) 0·41] (Found: C, 35·3; H, 6·6; N, 20·6. $C_{14}H_{27}N_7O_9,2H_2O$ requires C, 35·5; H, 6·6; N, 20·7%).

L-Alanyl-L-isoleucyl-N(ω)-nitro-L-arginyl-L-seryl-L-alanine.—A sample of *N*(ω)-nitro-L-arginyl-*O*-benzyl-L-seryl-L-alanylpolystyrene hydrochloride (5·0 g.) was treated successively with *N*-t-butoxycarbonyl-L-isoleucine hemihydrate (1·20 g., 5·0 mmoles) and *N*-t-butoxycarbonyl-L-alanine (0·95 g., 5·0 mmoles) by the methods described for synthesis of the tripeptide. The coupling and cleavage reactions were followed by i.r. spectroscopy. Final cleavage from the support was achieved with trifluoroacetic acid–hydrogen bromide, to yield the pentapeptide (380 mg., 42% based on 0·58 mmole L-alanine per g. polymer).

A sample (150 mg.) was dissolved in N-aqueous acetic acid (1 ml.) and chromatographed on a column of Rexyn 102(H^+) exchange resin, as described above. After collection of 15 fractions, a gradient elution technique was used in which 30% aqueous acetic acid was added to N-acetic acid. The separation was followed by u.v. spectroscopy (269 mμ). Four components containing the *N*(ω)-nitro-L-arginyl residue were detected; fractions 85—95 were combined and lyophilized to give the amorphous major product (84 mg.) which was redissolved in aqueous acetic acid and subjected to gradient elution separation as above (5 ml. fractions). Fractions 170—220 contained the only detectable component *L-alanyl-L-isoleucyl-N(ω)-nitro-L-arginyl-L-seryl-L-alanine acetate* (56 mg.), $\lambda_{max.}$ (N-AcOH) 269 mμ (ε 5300), homogeneous on t.l.c. in four systems [system (A) R_F 0·85, (B) 0·48, (D) 0·81, (G) 0·55] (Found: C, 41·9; H, 7·1; N, 19·1. $C_{22}H_{43}N_9O_{11},2H_2O$ requires C, 42·0; H, 7·2; N, 19·2%).

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[16] J. Inczedy, 'Analytical Applications of Ion Exchangers, Pergamon, New York, 1966, p. 81—82.