# Solvation of the Polymer Matrix. Source of Truncated and Deletion Sequences in Solid Phase Synthesis[1]

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An automated procedure utilizing hydrogen chloride-36 for monitoring the free amine in automated solid phase synthesis was developed. Discrepancies were found between the values determined by this procedure and those from amino acid analysis in the synthesis of a peptide, residues 63–74, of acyl carrier protein. These results led to a hypothesis of dynamic solvation changes of the polymer matrix as synthesis proceeds. The effects of chain termination by acetylation were also in agreement with the hypothesis. Dynamic solvation changes of the polymer matrix leads to the sequence-dependent problems of solid phase synthesis, both truncated and deletion sequences. It may also be responsible for difficulties encountered with monitoring procedures and with attempts to terminate unreacted peptide chains. Based on these observations, a modified procedure of solid phase peptide synthesis was developed which significantly improved the synthesis of residues 63–74 of acyl carrier protein.

Since the introduction of the solid phase method for peptide synthesis by Merrifield in 1962,[5] an enormous number of peptides have been prepared.[6] A more striking achievement, however, has been the synthesis by the solid phase procedure of several large proteins with high biological activity, *e.g.*, ribonuclease A,[7] fragment $P_2$ of *Staphylococcus aureus* nuclease T,[8] soybean trypsin inhibitor,[9] and acyl carrier protein.[10]

Despite these impressive achievements, many peptides have not been prepared in an adequate yield and the cause of such failures is still not clear despite extensive studies of the sequence-dependent[11] problems of solid phase synthesis.[6,12–15] Most failures in the method have been attributed to incomplete coupling and deprotection steps.[6,16–21] Fortunately, a wide variety of methods has been established for the formation of a peptide bond,[18] and recently several new resin supports have been developed.[6a] One could expect, therefore, that many of these synthetic problems could be overcome by a judicious choice of the polymeric support, side chain and $\alpha$-amino protecting groups, the method of removal of the $\alpha$-amino protecting group, and the reagent used for mediating the coupling reaction. Some recent studies[16] have indicated that difficult coupling reactions may be facilitated by the use of a mixed solvent or the addition of urea to the reaction mixture.

The formation of truncated peptides[22] by incomplete coupling and deprotection steps is a problem in that the overall yield of the synthesis is decreased. The regrowth of partially complete sequences, with the formation of deletion sequences,[23] poses a much greater threat to the success of the synthesis of a large molecule, as the separation of the desired peptide from a large number of very similar products may be beyond the scope of present methods of protein purification. Bayer[12] has established that such deletion sequences can occur in specialized cases, and by analogy it has been suggested that the larger synthetic proteins must contain such products. Also the formation of deletion sequences places a severe limit on the size of the peptide that can be synthesized.[13,24]

Two approaches which have been taken to reduce the occurrence of deletion sequences are either to increase the yield of the coupling reactions, by multiple couplings, for example, or to terminate truncated sequences by treatment of the resin, after completion of the coupling reaction, with a very reactive acylating reagent, *e.g.*, acetic anhydride[14,25] or other reagents.[26–28] It has yet to be demonstrated, however, that such reagents, because of their reactivity, will not cause side

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(11) It has been established that these failures are not necessarily associated with a particular amino acid residue, and therefore are described as sequence-dependent problems.

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(20) J. Scotchler, R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).

(21) K. Lubke, "Peptides 1969," North-Holland Publishing Co., New York, N. Y., 1971, pp 154–156.

(22) A truncated peptide is defined as a peptide which becomes unavailable for reaction at some stage in the synthesis and does not add any further amino acids.

(23) A deletion sequence is defined as a truncated peptide which resumes growth at some later stage in the synthesis.

(24) (a) For example, the synthesis of human growth hormone (188 amino acids) would require an average coupling yield of 99.5% if the desired protein was to be the major product of the synthesis. Such calculations, however, are based on the assumption that all truncated sequences become deletion sequences and grow at the same rate as the correct sequence. (b) J. M. A. Baas, H. C. Beyerman, B. van de Graff, and E. W. B. de Leer, "Peptides 1969," North-Holland Publishing Co., New York, N. Y., 1971, pp 173–176.

(25) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963).

(26) L. B. Markley and L. C. Dorman, *Tetrahedron Lett.*, 1787 (1970).

(27) T. Wieland, C. Birr, and H. Wissenbach, *Angew. Chem., Int. Ed. Engl.*, **8**, 764 (1969).

(28) H. Wissmann and R. Geiger, *ibid.*, **9**, 909 (1970).

reactions that are deleterious to the synthesis, *e.g.*, acetic anhydride.[29] Also it is unclear whether the amino groups which are resistant to coupling will react with the terminating reagent.

Before these variations in procedure can be rationally applied, it is necessary to have a reliable means of quantitation for both the coupling and deprotection steps, and in fact numerous assay procedures have been reported,[6a] most of which depend on monitoring the free amino group. The automation of the solid phase synthetic method has added the further requirements that the analytical procedure must be rapid enough in order to monitor and perhaps control the progress of the automated synthesizer. At this stage, the potentiometric method of Brunfeldt[30] is the procedure which seems most applicable for use with an automated synthesizer.

Little progress has been made in understanding the cause of the sequence-dependent problems of solid phase synthesis, although several possible explanations have been advanced. The effect of steric hindrance of the amino terminus of the growing peptide chain has often been discussed with relevance to solid phase synthesis.[15,17,21] The environment of the peptide can be dramatically effected by the solvent used, as it has been found that only solvents which swell the resin and provide a reasonably polar environment will allow an efficient coupling reaction, *e.g.*, dimethylformamide and chloroform, but not benzene.[25] Since both the polymerization of the polystyrene support and the chloromethylation reaction are random processes, one could expect a heterogenous environment due to the random distribution of cross-links and reactive sites so that some reactive sites would be more sensitive than others to steric hindrance.

It has been noted[12] that solid phase synthesis is a heterogenous reaction and as such depends on the rate of diffusion of reagents in the resin. One could expect, however, that diffusion should normally be a rapid process, as the swollen beads contain 80–90% solvent.[25]

In a recent review,[6a] it was stressed that as a synthesis proceeds the physical properties of the resin reflect the change from that of hydrophobic polystyrene to that of a mixed polystyrene–protein matrix. One might then expect a change, perhaps dramatic, in local solvation of the heterogeneous polymer matrix as the synthesis proceeds. If the solvation was decreased, then the accessibility of the heterogenous population of sites could also decrease with a consequent drop in the yield. It was difficult to evaluate such a concept because of the lack of published examples of this phenomenon; in fact only two well-documented examples have been described.[6a,20]

At the beginning of our investigations on acyl carrier protein, it became clear that the synthesis of the initial sequence, residues 74–63 (see Figure 1), was a good example of the sequence-dependent problems of solid phase synthesis—the yield of growing peptide at the end of the sequence was only 20% of the initial glycine value. It was decided to examine monitoring procedures and methods for minimizing truncated and deletion sequences before proceeding further. The synthesis was then repeated under a variety of conditions and followed by analysis of the yield of both the coupling and deprotection steps.

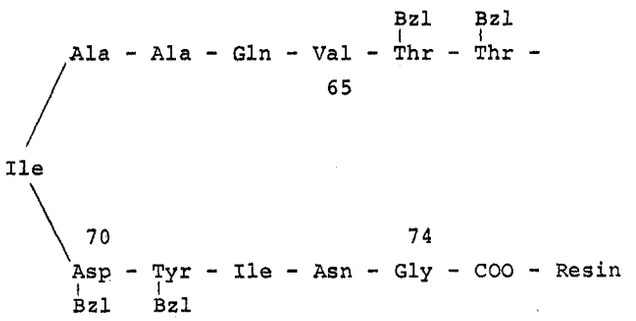


Figure 1.—Fully protected partial sequence 74–63 of acyl carrier protein.

For these analytical studies, the method developed by Dorman[31] was adapted so that the progress of an automated synthesis could be followed. From these studies, a modified synthetic procedure was derived that overcame to a considerable extent the problems originally observed in the synthesis of this peptide, and which was subsequently applied to the synthesis of acyl carrier protein. Of more general significance was the evidence which indicated that the difficulties observed in this synthesis can be attributed to changes in solvation of the peptide resin at different stages in the synthesis, which give rise to both truncated and deletion sequences. With change in the accessibility of the free amino group, monitoring methods must be used with extreme caution.

## Results and Discussion

The sequence 74–63 of ACP (see Figure 1) was synthesized by the stepwise addition of suitably protected amino acids to 0.66 mmol of *tert*-butyloxycarbonyl (BOC)-glycine (0.33 mmol of amino acid per gram of peptide resin) esterified to a 1% cross-linked polystyrene resin support. The procedures used are similar to those described by Merrifield for solid phase synthesis,[32] and the specific details are described in the Experimental Section.

Although the synthesis of the initial C-terminal sequence of ACP, 74–63, would appear straightforward, early attempts to synthesize this peptide by standard procedures were discouraging (Figure 2). The addition of asparagine$_{73}$, isoleucine$_{72}$, and tyrosine$_{71}$ gave a progressive decrease in the yield of the growing peptide chain, which implies truncation. Steric hindrance of the coupling of amino acids with bulky protecting groups should be greatest at the beginning of the synthesis,[33] and this could account for these initial difficulties, especially in the case of isoleucine, with its bulky isobutyl side chain.

The most significant feature of the growth profile of the sequence 74–63 is the dramatic increase in yield for

(29) J. Halstrom, K. Brunfeldt, and K. Kovacs, *Experientia*, **27**, 17 (1971).

(30) K. Brunfeldt, P. Roepstorff, and J. Thomsen, "Peptides 1969," North-Holland Publishing Co., New York, N. Y., 1971, pp 148–153.

(31) L. C. Dorman, *Tetrahedron Lett.*, 2319 (1969).

(32) (a) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); (b) R. B. Merrifield, *Advan. Enzymol.*, **32**, 221 (1969).

(33) The initial amino acid, glycine, was attached to the resin under conditions that are much more vigorous (100°, 24 hr) than one uses for peptide bond formation. It could be expected therefore, that some of the reaction sites would be located in the more hindered regions of the resin matrix. This conclusion is supported by the frequent observation that cleavage of the peptide from the resin releases significant quantities of the initial amino acid.

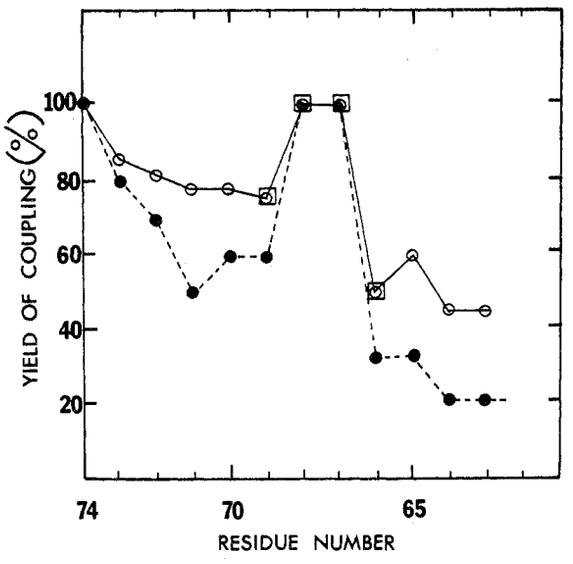


Figure 2.—The growth profile for the synthesis of the sequence 74–63 of ACP by two different synthetic procedures. The yield of each synthesis was determined by amino acid analysis of the peptide resin after the addition of each amino acid. The data present are the average of two syntheses in which the standard solid phase synthetic procedures were used (●) and four syntheses in which the modified procedures were used (○). All duplicate syntheses gave essentially the same results. If the resin peptide was acetylated after the addition of $Ile_{69}$, and then the synthesis was continued as for the modified procedure, the incorporation of the subsequent residues is shown by the points □.

the addition of $alanine_{68}$ and $alanine_{67}$, which implies regrowth and formation of deletion sequences. However, the next amino acid, glutamine, was incorporated with a much lower yield, which again implied truncation.

It was clear at this stage that the synthesis had to be followed by some analytical technique which allowed quantitation of the yield of both the coupling and deprotection steps. The Dorman[31] procedure was chosen, which measures the amount of chloride bound to the resin by conversion of free amino groups to their corresponding hydrochlorides on treatment of the resin with pyridine hydrochloride. The chloride is displaced from the resin with triethylamine and measured by titration. However, it was decided that the use of chloride-36 would greatly increase both the speed and sensitivity of the assay. Instead of a laborious titration procedure, the chloride could be simply measured by radioactivity, while a very small amount of bound chloride (as would be expected for an efficient coupling reaction) could be accurately determined by a suitable increase in the specific radioactivity of the chloride-36. Table I indicates that both methods of chloride determination gave the same values for BOC and deprotected glycine resin, and, therefore, the chloride-36 procedure was used in all subsequent assays.

It was found necessary to modify some of the washes that were used in the original Dorman procedure so that the chloride-36 could be accurately measured. In Table II, the two procedures are compared and it is clear that the modifications do not effect the amount of bound chloride. Dimethylformamide was not a suitable solvent to remove the excess pyridine hydrochloride, presumably due to traces of dimethylamine in the solvent which displaced some of the bound chloride, and as a consequence the level of chloride-36 in the

TABLE I

COMPARISON OF METHODS USED FOR THE MEASUREMENT OF CHLORIDE[a]

| | Chloridimeter | $^{31}Cl$ radioactivity |
|---|---|---|
| BOC-Gly resin | 30 | 26 |
| Total recovery, % | 98 | 103 |
| Gly resin | 762 | 725 |
| Total recovery, % | 98 | 97 |

[a] Gly resin (0.66 mmol) was treated with pyridine hydrochloride (7.5 mmol). The excess chloride was removed by a series of washes and the bound chloride was displaced with triethylamine (see Experimental Section). The chloride was then determined by titration of the chloride with a chloridimeter or by measurement of the $^{36}Cl$ radioactivity as described in the Experimental Section. Each value in the table is an average of three determinations (error for both assays was ±5%).

TABLE II

DORMAN ANALYSIS OF DEPROTECTED GLYCINE RESIN[a]

| Original procedure | | | Modified procedure | | |
|---|---|---|---|---|---|
| No. of wash | Reagent | $^{36}Cl$ dpm in wash | No. of wash | Reagent | $^{36}Cl$ dpm in wash |
| 1 | Pyr HCl[b] | 29,420 | 1a | Pyr HCl | 28,580 |
| 2 | $CH_2Cl_2$ | 12,643 | 2a | $CH_2Cl_2$ | 13,195 |
| 3 | $CH_2Cl_2$ | 4,898 | 3a | $CH_2Cl_2$ | 4,956 |
| 4 | $CH_2Cl_2$ | 933 | 4a | $CH_2Cl_2$ | 741 |
| 5 | DMF | 612 | 5a | *t*-BuOH | 906 |
| 6 | DMF | 194 | 6a | *t*-BuOH | 486 |
| 7 | DMF | 115 | 7a | *t*-BuOH | 131 |
| 8 | DMF | 120 | 8a | *t*-BuOH | 23 |
| 9 | DMF | 90 | 9a | $CH_2Cl_2$ | 20 |
| 10 | DMF | 70 | 10a | $CH_2Cl_2$ | 0 |
| 11 | DMF | 80 | 11a | $CH_2Cl_2$ | 0 |
| 12 | $Et_3N$[c] | 4,906 | 12a | $Et_3N$[d] | 3,370 |
| 13 | DMF | 587 | 13a | $Et_3N$ | 1,835 |
| 14 | DMF | 306 | 14a | $Et_3N$ | 306 |
| 15 | DMF | 63 | 15a | $CH_2Cl_2$ | 257 |
| | | | 16a | $CH_2Cl_2$ | 61 |
| | | | 17a | $CH_2Cl_2$ | 23 |
| Total dpm 1–15 | | 55,037 | | | 54,890 |
| Total dpm 12–17 | | 5,862 | | | 5,852 |
| Recovery of total $^{36}Cl$, % | | 100 | | | 100 |

[a] Gly resin (0.66 mmol) was treated with pyridine hydrochloride (7.5 mmol, $1.4 \times 10^6$ dpm). In two determinations the resin was treated with the washes 1–15 and 1a–17a, each wash was collected in a 25-ml volumetric flask, and the sample was made up to the mark with dichloromethane. A 1-ml sample was counted for $^{36}Cl$ radioactivity. [b] The abbreviations used in the diagram are Pyr HCl = pyridine hydrochloride, $CH_2Cl_2$ = dichloromethane, DMF = dimethylformamide, $Et_3N$ = triethylamine. [c] 10% (v/v) triethylamine dissolved in dimethylformamide. [d] 3% (v/v) triethylamine dissolved in dichloromethane.

washes would not fall to zero (see washes 5–11). This problem was solved by the substitution of an alcohol wash in steps 5a–8a. The resin was then washed with dichloromethane (steps 9a–11a) to prevent loss of peptide from the resin by transesterification caused by traces of alcohol present contaminating the triethylamine wash.[34] Triethylamine, at the concentrations used by Dorman, was found to severely quench the counting of chlorine-36. However, it was found that three washes of a lower concentration of triethylamine were sufficient to displace all of the bound chloride, and allow satisfactory counting of the sample (steps 12a–14a).

(34) The possibility of this side reaction was reduced further by the substitution of the more sterically hindered *tert*-butyl alcohol for ethanol in steps 5a–8a.

The assay was found to be quite reproducible and, in all cases, the recovery of chloride-36 was excellent (98%). Moreover, the procedure was easily adapted to monitor an automated synthesis. An automated synthesizer[35] was modified to accommodate a fraction collector, which was used to collect the effluent from the reaction flask. In order to quantitate effluent collection, it was necessary to drain by nitrogen pressure rather than by vacuum filtration. The machine was readily programmed to execute a Dorman analysis after each coupling and deprotection step, especially as pyridine hydrochloride was the only reagent that was not already used in the synthetic procedure. Washes 1a–11a and 12a–17a (Table II) were collected in separate containers and a sample of each was then counted. The volume and pH of the fractions collected were used as a check on the operation of the synthesizer. The results of the analyses performed during the synthesis of the peptide 74–67 of ACP are shown in Table III.

TABLE III
CHLORIDE BINDING DATA[a] FOR THE SYNTHESIS OF THE PEPTIDE 74–67

| Residue | 1st Coupling in $CH_2Cl_2$ | 2nd Coupling in $CH_2Cl_2$ | 3rd Coupling in $CH_2Cl_2$–DMF (1:1) | Acetylation[b] | 1st Deprotection[b] | 2nd Deprotection[b] |
|---|---|---|---|---|---|---|
| 74 Gly | 38[c] | | | | 782[f] | |
| 73 Asn[d] | 300 | 240 | | | 675 | |
| 72 Ile | 362 | 370 | 304 | | 765 | |
| 71 Tyr[d,e] | 431 | 411 | 430 | 380 | 390 | 415 |
| 70 Asp | 84 | 88 | | 178 | 360 | |
| 69 Ile | 186 | 187 | | | 374 | |
| 68 Ala | | 47 | | | 267 | 320 |
| 67 Ala | 106 | 92 | | | 275 | |

[a] Expressed as μmoles of free amino groups as measured by the Dorman analysis. [b] As described in the Experimental Section. [c] Result of analysis of the initial *t*-BOC-glycine resin. [d] Both active ester couplings were carried out in DMF. [e] Dorman analysis after a fourth coupling in benzene gave 425 μ*M* of chloride bound. The peptide 74–67 of ACP (Figure 1) was synthesized using the standard procedure described in the Experimental Section. The yield of each coupling reaction was estimated by the procedure of Dorman. [f] The amino acid analysis of the BOC-glycine resin indicated that 660 μmol of amino acid was esterified to the resin.

The yield of each coupling step was also followed by amino acid analysis and the results of the two analytical techniques are compared in Figure 3.

The two measurements agreed well for the addition of the first three amino acids, but after the addition of aspartic$_{70}$ there was a sharp drop in the amount of bound chloride, although the incorporation of aspartic$_{70}$ had not increased. Furthermore, the analysis of each amino acid addition and deprotection was complicated by a background of bound chloride,[36] which was superimposed on the amount of *chloride bound to* the free amino groups, although the value would remain constant after repeated couplings of that particular residue (see Table III). A disturbing feature was that, as the synthesis proceeded, the amount of chloride bound after deprotection showed a steady decrease (see Figure 2). The monitoring of the synthesis of the sequence 74–63 was repeated four times on different preparations with

(35) Based on the design of R. B. Merrifield, J. M. Stewart, and N. Jernberg, *Anal. Chem.*, **38**, 1905 (1966).

(36) This background is presumably due to binding of chloride by the resin, perhaps, by continued formation of quaternary amine by exposure of the residual chloromethyl groups to triethylamine.

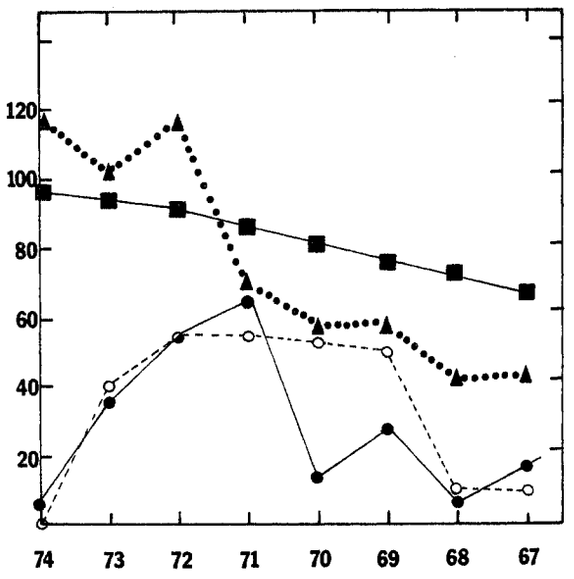


Figure 3.—The yield of the synthesis of peptide 74–63 as measured by amino acid analysis. The values recorded in the graph were an average of three separate syntheses which gave essentially the same results and are expressed as per cent of total free amine converted to the hydrochloride. The analyses were carried out as described in the Experimental Section. The amount of free amine after deprotection was determined by Dorman analysis (▲). The free amine by Dorman analysis (●) after double coupling is compared with the free amine which should be present (○) based on amino acid analysis. The per cent polystyrene (■) present in the polymer–peptide matrix is also shown.

essentially the same results. The constant background observed after several additions of a particular acid suggests that this effect is caused by a change in the properties of the polymer rather than a random accumulation of by-products from the coupling reaction.[37] A similar background effect due to the resin was also found by Beyerman[38] when *N*-(2-$^{14}$*C*-BOC) amino acids were used to follow the progress of a coupling reaction, or $^{35}$*S*-sulfuric acid was used to determine the peptide content.

In Figure 3, it can be seen that the amount of chloride bound by the deprotected peptide decreased as the synthesis proceeded. Bayer noted a similar effect in the synthesis of ferredoxin.[39] This decrease could be caused by the cleavage of the peptide from the resin during the deprotection steps, or the pyridine hydrochloride wash in the case of the Dorman analysis, or by a decrease in the background of bound chloride as the properties of the resin change.

One of the problems of the Dorman analysis is that repeated treatment of the resin peptide with pyridine hydrochloride may cause cleavage of some of the peptide from the resin or other undesirable side reactions due to the acidic nature of this reagent. In Figure 4, the yield of the synthesis of the peptide 74–69 is compared with an identical synthesis that has been analyzed at each coupling and deprotection step with the Dor-

(37) For example, dicyclohexylurea and other by-products have been observed to give a positive test with ninhydrin reagent.

(38) H. C. Beyerman, P. R. M. van der Kamp, E. W. B. de Leer, W. Maassen van den Brink, J. H. Parmentier, and J. Westerling, "Peptides 1971," North-Holland Publishing Co., New York, N. Y., 1972.

(39) E. Bayer, G. Jung, and H. Hagenmeier, *Tetrahedron Lett.*, 4853 (1968).

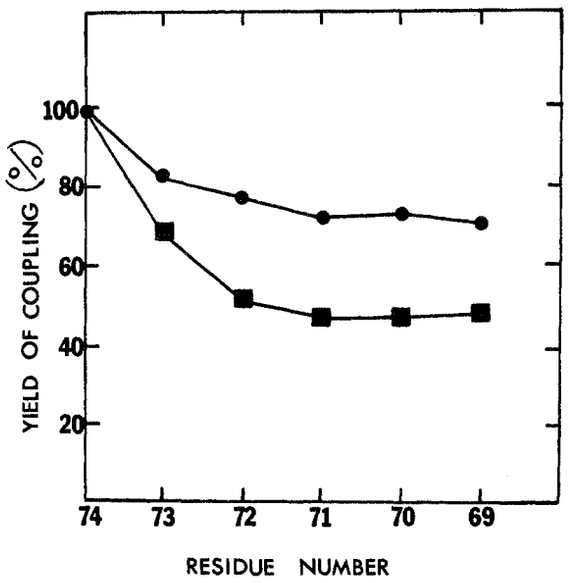


Figure 4.—The effect of the Dorman analysis on the yield of the synthesis of the sequence 74–69 of ACP. The yield of each synthesis was determined by amino acid analysis of the peptide resin after the addition of each amino acid. The data presented are the average of three syntheses that have been followed by the Dorman analysis (■) and four unmonitored syntheses (●). All duplicate syntheses gave essentially the same results. Both procedures involved the same number of coupling and deprotection steps.

man method. It is clear that the analytical procedure has caused a significant drop in the yield of the synthesis.

The variable background of bound chloride makes it difficult to use the Dorman analysis to follow the yield of a synthesis, unless the coupling of a particular amino acid is repeated until the amount of bound chloride remains constant. Although this background has been observed elsewhere,[38,40,41] several studies have not noted this problem.[14,31] This difference, however, may be due to the wide variation in properties that has been observed for different resin preparations.[7,42] Dorman[31] used a hydroxymethyl resin and did not comment on any background due to the resin binding chloride. Beyerman[38] noted that a chloromethyl resin, which had been treated with triethylamine, took up a considerable amount of $^{35}S$-sulfuric acid, while a hydroxymethylated resin, or a chloromethylated resin in which the esterification of the amino acid was carried out with sodium carbonate instead of triethylamine, did not react with $^{35}S$-sulfuric acid. Therefore, if the correct resin is chosen, the problem of a variable background may be overcome. The procedure should still be used with caution, however, as it can lower the yield of the synthesis (see above).

The variable background of bound chloride observed during the synthesis can be understood only if one considers that the solvation of the resin changes with the nature of the peptide–polystyrene matrix. For example, the sharp decrease in the amount of chloride bound by the resin after the addition of aspartic$_{70}$ (see Figure 3) can be explained by a decrease in solvation of the resin with a corresponding loss of chloride binding sites. These observations are consistent with studies on the rate of release of various amino acids, esterified to a chloromethyl resin, with acidic hydrolysis in a variety of solvents.[16] It was found that some sites on the resin were less accessible than others, and the effect of improper solvation during hydrolysis was to close off completely the less accessible sites, rather than generally decrease the rate of reaction at all sites by similar amounts.

The hydrophobic nature of the polystyrene resin requires that a relatively nonpolar solvent is used to allow swelling of the resin and penetration of the reagents into the matrix. As the length of the peptide chain increases, however, one would expect the requirement of a polar solvent for correct solvation of the peptide chains. In fact, in two published examples,[6a,20] a sudden drop in yield of the synthesis was overcome by the use of a polar solvent, and these studies led to the proposal that the use of a mixture of dichloromethane and dimethylformamide might be used for difficult amino acid additions. In Table III, the chloride binding data indicated that the use of this solvent mixture gave an increased incorporation of isoleucine$_{72}$.

This problem of a variable background of chloride (see Table III) could be explained if the resin contained a constant number of binding sites for chloride whose exposure was variable. If the degree of solvation of the resin changed significantly after the addition of a particular amino acid, then one would expect a change in the measured number of binding sites. If this assumption is correct, it should be possible to expose some of the buried sites by treatment of the resin with a series of washes which alternately shrink and swell the polymer matrix. The availability of previously buried sites could be readily tested by treatment of the resin with triethylamine, which should displace some further chloride from the resin. The development of the modified Dorman procedure was ideally suited for this purpose, as the use of chloride-36 allowed the measurement of very small quantities of chloride.

Three such studies at different stages of the synthesis are shown in Table IV and, in each case, it was found that further chloride-36 could be displaced from the resin after a series of shrink and swell washes. The amount of chloride trapped by the resin was not sufficient to affect the overall recovery of chloride in the assay (maximum amount of chloride released by the extra washes was only 1–5% of the total chloride added to the resin), but it was sufficient to prevent the accurate determination of small amounts of free amino groups. The same result was obtained if the original method of Dorman was followed (washes 1–15 in Table II) so that this effect was not a result of our modifications to the washing procedure.

It is clear from the growth profile of the peptide 74–63 (see Figure 2), synthesized by standard procedures for solid phase synthesis, that the synthesis has several problems which lead to a very low yield of the completed peptide. The analytical studies (see Table III) indicated, however, that variation in the conditions of coupling and deprotection could significantly improve the yield of the synthesis. More significantly, the studies described in Table IV indicated that a swell–shrink–swell wash cycle could expose buried functional

(40) R. B. Merrifield, unpublished observations.
(41) B. Mehlis, W. Fischer, and H. Niedrich, "Peptides 1969," North-Holland Publishing Co., New York, N. Y., 1971, pp 146–147.
(42) We have noted different backgrounds for glycine resins which were obtained commercially and those that were prepared in our laboratory.

TABLE IV

REEXPOSURE OF CHLORIDE BINDING SITES AT DIFFERENT STAGES OF THE SYNTHESIS OF THE PEPTIDE 74–67[a]

| Number of wash | Reagent | BOC-Gly$_{74}$ $^{36}$Cl dpm in washes | BOC-Ala$_{67}$ $^{36}$Cl dpm in washes | Deprotected Ala$_{67}$ $^{36}$Cl in washes |
|---|---|---|---|---|
| 12 | $Et_3N$ | 206 | 427 | 1,809 |
| 13 | $Et_3N$ | 65 | 167 | 241 |
| 14 | $Et_3N$ | 10 | 43 | |
| 15 | $CH_2Cl_2$ | 5 | 25 | |
| 16 | $CH_2Cl_2$ | | 16 | |
| 17 | $CH_2Cl_2$ | | | |
| 18 | *t*-BuOH | | | |
| 19 | *t*-BuOH | | | |
| 20 | *t*-BuOH | | | |
| 21 | $CH_2Cl_2$ | | | |
| 22 | $CH_2Cl_2$ | | | |
| 23 | $CH_2Cl_2$ | | | |
| 24 | $Et_3N$ | 73 | 213 | 630 |
| 25 | $Et_3N$ | 16 | 75 | 112 |
| 26 | $CH_2Cl_2$ | | 20 | 19 |
| Total dpm in wash 11–15 | | 286 | 778 | 2,050 |
| Total dpm in wash 23–25 | | 89 | 308 | 761 |
| % of dpm in wash 23–25 relative to wash 11–15 | | 31 | 40 | 37 |

[a] The resin peptide was treated with pyridine hydrochloride (7.5 mmol, $1.4 \times 10^6$ dpm) and the excess pyridine hydrochloride was removed by the same washes (1a–11a) as described in Table II. All available chloride was then displaced by the washes 12–17 as shown in this table. The resin was then subjected to washes 18–23 in an attempt to expose buried regions of the resin that had bound chloride, and in fact the second triethylamine treatment washes 24–26 did liberate more chloride. Samples of the washes were counted for chloride-36 in the manner described for Table II.

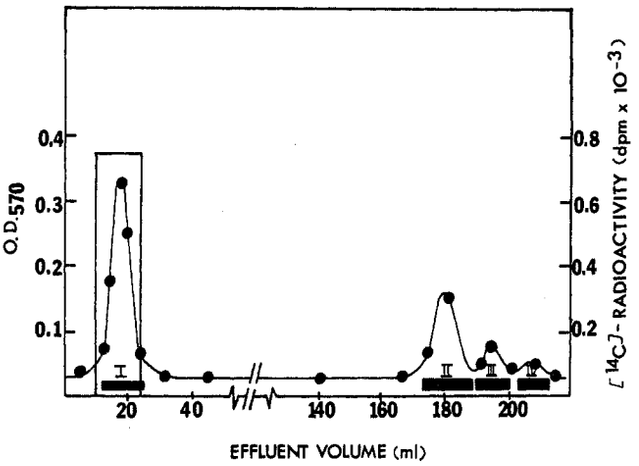


Figure 5.—Separation of the products from the synthesis of the sequence 74–69 of ACP. This figure shows the separation on a Dowex 50-X8 column (0.9 × 17 cm) of the products from the synthesis of the peptide 74–69. The column was developed at 30 ml/hr with a pyridine acetate gradient at 55°, and 6-ml fractions were collected. The progress of the column was monitored by ninhydrin analysis (570 mm) of the alkaline hydrolysate of a sample of each fraction. A portion (6%) of each fraction was checked for $^{14}$C radioactivity.

groups. These considerations led to the development of a modified synthetic procedure.

The chloride binding data described in Table III suggested that the coupling of isoleucine$_{72}$ was improved if the coupling reaction was carried out in a mixed solvent of dichloromethane and dimethylformamide, while the yield of addition of asparagine$_{73}$ was also increased if the coupling reaction was repeated. A second deprotection after the addition of tyrosine$_{71}$ and alanine$_{68}$ increased the amount of free amine as detected by the chloride binding measurements. If these data were an accurate estimate of the amino groups that were available for a coupling reaction, then it was clear that persistent efforts to ensure reaction at the less accessible sites on the resin were necessary to achieve a good yield in the synthesis.

In an attempt to meet these conditions, all coupling reactions were repeated with a 1:1 mixture of dichloromethane and dimethylformamide as the solvent, and the deprotection step was repeated in an attempt to ensure complete deblocking of the peptide chain. Also the swell–shrink–swell wash was used between all coupling and deprotection steps (the exact sequence of washes used is described in the Experimental Section).

The sequence 74–63 of ACP was then resynthesized with this new procedure (see Experimental Section) and the progress of the synthesis was followed by amino acid analysis. As is shown in Figure 2, the modifications were successful, as the yield of completed peptide at the end of the synthesis was doubled.

However, the decrease in yield of the synthesis for the addition of asparagine$_{73}$, isoleucine$_{72}$, and tyrosine$_{71}$, as well as the regrowth of partially complete sequences after the addition of alanine$_{68}$ and alanine$_{67}$ was still evident (see Figure 2). It was decided that these problems would serve as an excellent test for the effectiveness of acetylation as a reagent for the termination of incomplete sequences. If the peptide resin was acetylated after the addition of tyrosine$_{71}$ and the peptide cleaved from the resin, one could expect to obtain *N*-acetylglycine, *N*-acetylaspartylglycine, and *N*-acetylisoleucylaspartylglycine. Alternatively, if the resin was acetylated after the addition of Ile$_{69}$, then the yield of addition for Ala$_{68}$ and Ala$_{67}$ should not be greater than that of isoleucine$_{69}$.

The peptide 74–63 (Gly-Asn-Ile-Tyr-Asp-Ile-Ala-Ala-Gln-Val-Thr-Thr) was synthesized with an identical procedure used for the synthesis depicted in Figure 2 except that the peptide resin (0.3 mmol) was acetylated with a large excess of $^{14}$*C*-acetic anhydride (3 mmol, $5.4 \times 10^6$ dpm/mmol) for 20 min after the coupling of isoleucine$_{69}$. Despite this treatment, the synthesis gave the same regrowth of incomplete peptides with the coupling of alanine$_{68}$ and alanine$_{67}$.

Peptide 74–63 (Gly-Asn-Ile-Tyr-Asp-Ile) was synthesized as before, except that acetylation with $^{14}$*C*-acetic anhydride was performed after the addition of tyrosine$_{71}$. A sample of the peptide resin (50 mg, 16 μmol of glycine esterified to the resin) was cleaved by a HBr and trifluoroacetic acid treatment and the product was isolated (see Experimental Section). The peptides were chromatographed on Dowex 50-X8 and the column was monitored by ninhydrin analysis after alkaline hydrolysis of a sample of each fraction (see Figure 5). The absorbance at 570 nm indicated that four fractions were present and these were numbered consecutively by Roman numerals. A portion (6%) was checked for $^{14}$C radioactivity while the rest was subjected to an acid hydrolysis and the amino acid content was determined (see Table V). The analysis in-

TABLE V
COMPOSITION OF THE MIXTURE OF PEPTIDES FROM THE SYNTHESIS OF THE SEQUENCE 74–69 OF ACP[a]

| Peak no. | Amino acid composition, μmol | | | | | |
|---|---|---|---|---|---|---|
| | Gly | Asp | Ile | Tyr | Peptide[b] | $^{14}$C dpm |
| I | 1.0 | 2.0 | 0.81 | 0.79 | 125 | 1,500[c] |
| II | 1.0 | 0.1 | | | 38 | |
| III | 1.0 | 0.95 | 0.75 | | 17.5 | |
| IV | 1.0 | 0.98 | 0.15 | | 3.1 | |
| Resin peptide[d] | 1.0 | 1.41 | 1.36 | 0.6 | 306 | |

[a] Peptide 74–69 (0.3 mmol), which had been acetylated with $^{14}C$-acetic anhydride (3 m$M$, 5.2 × $10^3$ dpm/μmol) after the addition of $Ile_{72}$, was cleaved from the resin and fractionated on Dowex 50-8 (see results and Figure 4). The amino acid composition and $^{14}C$ radioactivity of the pooled peaks were then determined. [b] Based on μmoles of glycine. [c] This corresponds to the acetylation of 2.3 μmol of peptide. [d] The amino acid analysis of the resin peptide at different stages of the synthesis indicated that amino acids were added with the following yields: $Asn_{73}$, 78%; $Ile_{72}$, 71%; $Tyr_{71}$, 59%; $Asp_{70}$, 63%; $Ile_{69}$, 65%.

dicated that peak I consisted of the complete peptide 74–69 and acetylated peptides while peaks II–IV were the products of incomplete coupling of $asparagine_{73}$, $isoleucine_{72}$, and $tyrosine_{71}$, *i.e.*, Gly, Gly-Asx, and Gly-Asx-Ile, respectively. The quantities of these peptides corresponded closely to the values of truncated sequences expected from the amino acid analysis of the peptide resin, and the recovery of material was good (60% of the glycine esterified to the resin was recovered from the Dowex 50 column). The finding that only a small fraction (4%) of the incomplete peptides present in this sample had been terminated with the acetic anhydride treatment is consistent with the hypothesis that incomplete coupling and/or deprotection reflects sites which are not solvated by the reagents and may be inaccessible to chain-terminating reagents or to titration as well as to coupling.

As the yield of addition of $isoleucine_{69}$ is only 60% of the value for the initial amino acid, one would expect that up to 40% of peptide chains would be available for acetylation, but this was obviously not the case, as all of the incomplete peptide chains coupled with both $alanine_{68}$ and $alanine_{67}$. Therefore, on deprotection of the α-amino group of the isoleucyl peptides, the solvation of peptide–polymer matrix must undergo a dramatic change so that the amino groups that were unavailable for acetylation can readily form a peptide bond. On the addition of $glutamine_{66}$, the yield then dropped to the level of the synthesis before the addition of $alanine_{68}$ and $alanine_{67}$.

Acetylation of the peptide resin after the addition of $tyrosine_{71}$ occurred with only a small fraction (4%) of the truncated peptides formed by the incomplete coupling of $asparagine_{73}$, $isoleucine_{72}$, and $tyrosine_{71}$ despite the use of a large excess (tenfold) of acetic anhydride (see Figure 5 and Table V). Bayer made a similar observation from studies with model peptides,[12] where it was found that only part of the uncoupled free amino groups could be acetylated.

Therefore, one cannot expect that repeated couplings of an amino acid will increase the yield of a difficult step if the unreactive amino groups are buried. This conclusion is supported by the data presented in Table III, where it is clear that four couplings of tyrosine, carried out in a variety of solvents, did not decrease the amount of free amino groups (as measured by the amount of bound chloride), although amino acid analysis after the four couplings indicated that tyrosine had reacted with only 60% of the peptide chains. Similar observations about the ineffectiveness of repeated couplings have been made in the synthesis of $(Leu\text{-}Ala)_6$[14] and of a tetrapeptide.[25] In the synthesis of lysozyme, it was found that repeated couplings resulted in the growth of peptides on partially deprotected side chains.[43]

The use of an insoluble support allows the solid phase method to have considerable advantages over protein synthesis carried out in solution, such as the facile removal of excess reagents, tremendous savings in time, and avoidance of the problems of insolubility of large fragments. At the same time, the use of a polymeric support introduces a new set of problems which still require extensive investigation before the solid phase method can be used routinely for any particular peptide sequence. If the use of a polystyrene support is to be completely successful, then an analytical method will have to be developed which allows the rapid determination of the yield of both coupling and deprotection reactions. This goal may be difficult to achieve, as the properties of the polymeric support may distort the analytical results, either by masking some of the functional groups or by trapping by-products of the coupling reaction which will react with the assay reagents. By a similar argument, the use of terminating reagents, such as acetic anhydride, has only limited application in stopping the regrowth of partially complete sequences. The problems of the solid phase synthetic procedure, however, cannot be so general or so serious as the various analytical studies[13,14,17] would suggest, because of the enormous number of peptides that have been synthesized successfully by the method. In fact, the success of the method would suggest that in most syntheses, truncated peptides, when formed, do not regrow to any significant extent during the rest of the synthesis.

An alternative to the problems of a polystyrene resin is the use of a special polymer in which a thin layer of styrene is localized on the surface of an inert bead. Several successful syntheses have been carried out with such a support either on Teflon[44] or on glass[45] and these achievements point to a possible solution to the sequence-dependent problems of solid phase synthesis. Other physical forms of polystyrene, such as the macroreticular resins,[46] should also be further investigated. A different approach to this problem proposed by Sheppard[15] would be the use of a polymer support whose solvation properties would be similar to that of the protected polypeptide which was being synthesized in order to minimize solvation charges as synthesis progresses. Once the problem of dynamic solvation changes is overcome, then monitoring procedures can be rationally applied to overcome the sequence-dependent problems which hinder the application of solid phase synthesis.

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## Experimental Section

**Reagents.**—*tert*-Butyloxycarbonyl (BOC)-amino acids and BOC-glycine which was esterified to a polystyrene–1% divinylbenzene resin were purchased from Schwarz BioResearch. The following side-chain blocking groups were used—aspartic acid, β-benzyl ester; threonine and tyrosine, benzyl ethers; glutamic acid, γ-benzyl ester—while the BOC group was used for α-amino protection. Hydrogen chloride-36 and $^{14}C$-acetic anhydride were purchased from New England Nuclear. All other chemicals, of reagent grade or better, were purchased from common sources.

A Packard liquid scintillation spectrometer, Model 544, was used for measurement of radioactivity. Amino acid analyses were measured with a Beckman Spinco amino acid analyzer, Model MS. Automated syntheses were performed on a synthesizer based on the design of Merrifield.[47] A Cotlove chloridometer was used in addition to Volhard titrations for chloride determinations.

**Preparation of the Pyridine Hydrochloride Reagent.**—Pyridine hydrochloride (0.3 mol) was dissolved in dichloromethane (1 l.) and the chloride content was checked by chloridimetry. Pyridine hydrochloride ($^{36}Cl$) was prepared in exactly the same manner except that $H^{36}Cl$ (25 μCi) was added to the solution to give a specific radioactivity of 183 dpm/μmol of chloride.

**Estimation of Bound Chloride.**—The peptide resin (2 g) was washed with dichloromethane (3 × 1 ml) and then treated for 15 min with 25 ml of the standard pyridine hydrochloride solution (0.3 *M*, measured with a volumetric pipette). The excess reagent was then removed by the following washes: (1) dichloromethane (3 × 20 ml, each for 2 min); (2) ethanol or *tert*-butyl alcohol[48] (3 × 20 ml, each for 2 min); (3) dichloromethane (4 × 20 ml, each for 2 min). All washes were removed by drying the resin under nitrogen pressure (1 min). Washes 1–3, as well as the remainder of the pyridine hydrochloride solution, were collected in a 250-ml volumetric flask made up to volume with dichloromethane and a sample was taken for chloride measurements. The amine hydrochloride, that had been formed by the pyridine hydrochloride treatment, was neutralized by two triethylamine–dichloromethane washes (1.5% triethylamine, 2 × 20 ml, each for 10 min). The peptide resin was then washed with dichloromethane (3 × 20 ml, each for 2 min). These washes were combined with triethylamine washes in a 100-ml volumetric flask and a sample was taken for chloride measurements.

**Measurement of Chloride with the Chloridimeter.**—Although the chloridimeter was developed by Cotlove, *et al.*,[49] for the determination of chloride in serum and urine, it was found that the chloride was quantitatively extracted from dichloromethane into the aqueous phase under the conditions of the assay.

The sample (1 ml), dissolved in dichloromethane, was added to a mixture of 0.1 *M* nitric acid and 1.7 *M* acetic acid (3 ml) and gelatin reagent (0.2 ml). The assay mix was then added to the reaction vessel of the chloridimeter and the chloride concentration was determined. A sodium chloride solution (1.6 mg/l.) was used to calibrate the instrument, while the blank value was measured with dichloromethane (1 ml) and the assay mix (3.2 ml).

**Calculation of Chloride by $^{36}Cl$ Radioactivity.**—The sample (1 ml) was added to Bray's solution (10 ml) and the sample was counted in a Packard liquid scintillation spectrometer, Model 544, which had been programmed to present the data as disintegrations per minute of chloride-36. As several of the reagents used in the washes, particularly triethylamine and pyridine hydrochloride, caused strong quenching of the radioactivity, it was necessary to calibrate the program with quenching standards.

**Procedure for Acetylation of Incomplete Peptides.**—Peptide resin (2 g, 0.66 mmol) was treated with a solution of 3 mmol of $^{14}C$-acetic anhydride (specific radioactivity 5.4 × $10^3$ dpm/μmol) and 3 mmol of triethylamine dissolved in dichloromethane ($CH_2Cl_2$, 20 ml) for 20 min. It was found that the following washing procedures were necessary for adequate removal of the excess reagent (all washes 20 ml and for 2 min unless otherwise specified): 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$, 3 × triethylamine (1.5% v/v in $CH_2Cl_2$), 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$. All the washes were combined and an aliquot was counted for $^{14}C$ radioactivity to check that all of the excess acetic anhydride had been removed.

**Standard Procedure for the Coupling of BOC-Amino Acids.**—As both triethylamine and ethanol were used in each cycle of the synthesis, a small loss of peptide chain by transesterification may occur repeatedly during a long synthesis. To minimize this possibility, the sterically hindered alcohol, *tert*-butyl alcohol, was substituted for ethanol and the concentration of triethylamine was reduced from 10 to 3% (v/v). The *tert*-butyl alcohol was found to be efficient in shrinking the resin even when 5% $CH_2Cl_2$ was added to prevent freezing of the alcohol.

The following sequence of reactions was used to prepare the peptide resin for a coupling reaction (all washes 20 ml and 2 min unless otherwise specified): 3 × $CH_2Cl_2$, trifluoroacetic acid–$CH_2Cl_2$, (1:1, v/v, 20 ml, 2 min), 6 × $CH_2Cl_2$, 3 × triethylamine (3% v/v in $CH_2Cl_2$), 3 × $CH_2Cl_2$. The coupling step was carried out with a threefold excess of the appropriate amino acid (0.2 *M*) and dicyclohexylcarbodiimide (DDC, 0.2 *M*) as the coupling reagent, except for glutamine and asparagine, which were added in the same concentration as the *p*-nitrophenyl ester. All couplings were left for 6 hr, except for active esters which were coupled for 12 hr. After coupling the excess reagents were removed by 3 × $CH_2Cl_2$ washes.

**Modified Procedure for the Coupling of BOC-Amino Acids.**—The BOC group was removed and the peptide resin was prepared for coupling by the following sequence of washes (20 ml and for 2 min unless specified): 3 × $CH_2Cl_2$, trifluoroacetic acid–$CH_2Cl_2$ (1:1, 2 × 10 ml, 2 and 20 min), 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$, 2 × triethylamine (3%, v/v in $CH_2Cl_2$), 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$, 3 × dimethylformamide (only for couplings in that solvent). The coupling procedure was the same as described above, except that the time of reaction was reduced to 2 hr for DCC-mediated couplings. First, couplings were routinely carried out with dichloromethane as the solvent. The by-products from the reaction were removed by the following washes: 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$, 3 × triethylamine (3% v/v), 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$, 3 × dimethylformamide. The second coupling was carried out in a mixed solvent of dichloromethane and dimethylformamide (1:1) and left for 2 hr. The following washes then completed the procedure: 3 × dimethylformamide, 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$, 3 × triethylamine (3% v/v), 3 × $CH_2Cl_2$, 3 × *tert*-butyl alcohol, 3 × $CH_2Cl_2$.

**Preparation of Samples for Amino Acid Analysis.**—Peptide resin (2 mg) was hydrolyzed with a mixture of HCl (12 *N*) and propionic acid (1:1, 2 ml) for 2 hr at 130° according to the method of Scotchler, *et al.*[20] Free peptides were hydrolyzed with 6 *N* HCl in sealed, evacuated tubes for 24 hr at 110°.

**HBr–Trifluoroacetic Acid Cleavage of the Peptide from the Resin.**—Peptide resin (1 g) was added to a mixture of trifluoroacetic acid (30 ml) and anisole (0.3 ml) in a cleavage apparatus as described by Stewart, *et al.*[50] HBr bubbled through the solution for 30 min at 25°, and the trifluoroacetic acid was removed from the resin by filtration. The resin was then washed with trifluoroacetic acid (3 × 10 ml) and the filtrate was combined with these washes. The trifluoroacetic acid was immediately removed by evaporation under reduced pressure and the residue was dissolved in 0.01 *M* Tris-HCl, pH 7.3 (10 ml). The cleavage was then repeated on the peptide resin under exactly the same conditions as before, except that the time of reaction was increased to 1 hr. The two products were then combined.

**Registry No.**—Pyridine hydrochloride-$^{36}Cl$, 22069-61-0; hydrochloric-$^{36}Cl$ acid, 36640-18-3; sequence 74–63, 37746-85-3; sequence 74–69, 37746-86-4.

(47) R. B. Merrifield and J. M. Stewart, *Nature (London)*, **207**, 522 (1965).

(48) The *tert*-butyl alcohol used in this paper has 5% (v/v) dichloromethane added to prevent freezing.

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