AN APPROACH TO THE SEMISYNTHESIS

OF

ACYL CARRIER PROTEIN

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ABSTRACT

The purpose of this study was to prepare several semisynthetic analogues of the acyl carrier protein of E. coli, using the Merrifield solid-phase method for the preparation of the synthetic 1-6 hexapeptide in the protected form. It was hoped that the synthesis of these analogues, and their evaluation by the $^{14}CO_2$ assay and by other methods, might contribute to the study of protein structure and function in general, and those of ACP in particular.

The strategy chosen for the synthesis of the protected 1-6 hexapeptide was to employ acid-stable protecting groups for glutamic, aspartic, and arginine residues, and to cleave the completed peptide from the resin in a protected form using HBr in acetic acid. The p-nitrobenzyl group was chosen for the protection of acidic amino acid side-chains, as this group has often been stated to be stable to HBr in acetic acid.

In the course of this work, however, it became obvious that the stability of the p-nitrobenzyl group to HBr in acetic acid, while greater than that of other benzyl esters, was not sufficient to allow the convenient preparation of peptides protected with this blocking group. Cleavage of the protecting group occurred to an appreciable extent, and thus the product was contaminated with a mixture of deprotected peptides.

Other disadvantages of the p-nitrobenzyl group were also encountered during this study. In particular, problems

of instability were encountered in the preparation of protected amino acid intermediates for peptide synthesis. The p-nitrobenzyl group was also found to give low coupling yields during synthesis, and poor solubility during purification, to amino acids and peptides protected with it. It is clear that the problems of instability and insolubility associated with p-nitrobenzyl ester protection were aggravated by the fact that many of the target peptides bore two p-nitrobenzyl groups per molecule.

The coupling of a crude hexapeptide to the native 7-77 peptide yielded a product which gave some activity in the $^{14}CO_2$ assay after extensive purification of the semisynthetic protein. This result, together with amino acid analysis of the crude peptide, implies that the desired protected hexapeptide composed a significant proportion of the peptide after cleavage from the resin. The insolubility conferred by the p-nitrobenzyl protecting group, however, presumably caused the target peptide to be selectively lost from the mixture during the purification procedures, in favour of deprotected peptides and deletion peptides having greater solubility.

For this reason, the major products which were purified from the crude cleaved peptides were pentapeptides lacking one glutamic acid residue bearing the p-nitrobenzyl protecting group.

These pentapeptides, when coupled to the native 7-77 peptide, gave products which were inactive in the $^{14}CO_2$ assay. This result is interesting, in that it suggests that both Glu^4 and Glu^5 are essential to the interaction between

the 1-6 and 7-77 peptides which maintains the active conformation of ACP. However, these data must be treated as tentative until an active semisynthetic ACP satisfying analytical criteria is prepared by this method. The results obtained by assaying the purified semisynthetic ACP prepared from a crude hexapeptide suggest that a fully-active semisynthetic ACP could be prepared by this method, but they cannot be regarded as conclusive in the absence of supporting analytical data.

The approach to semisynthesis chosen for this study appears to be basically sound, but new acid-stable protecting groups for carboxyl functions are clearly required if the method is to become generally useful.

ACKNOWLEDGEMENTS

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INTRODUCTION

As scientists study the created world, it is their task not only to analyse each object into its component parts, but also to consider the way in which these parts interact to carry out the functions of the object as a whole. In the case of protein molecules, the protein chemist seeks to investigate not only the chemical structure of each molecular species, but also the role which each part of the protein plays in the mechanism of action of the entire molecule. Two types of involvement of parts of a protein molecule in the function of that molecule have been distinguished. The first type is where a functional group takes a direct part in chemical interactions of the active site with substrates, receptors, cofactors, or membranes. The second is an indirect role, where a functional group helps to maintain the three-dimensional structure of the molecule necessary to give the optimal configuration of the active site.

The study of protein structure and the study of protein function are thus complementary and interdependent activities. Complete knowledge of the amino acid sequence of a protein, and even of its crystal structure, could in theory be attained without any understanding of the mechanism by which the protein acts. In practice, however, structure and function are usually investigated in parallel, and each study assists the other.

One of the most useful methods for studying the relationship between the structure and function of a protein

has been the preparation of modified forms of that protein. If a certain functional group is suspected of involvement in the mechanism of the protein, then efforts are made to alter that functional group. If the activity of the protein is unaffected by the modification, it can be concluded that the group was playing no immediate part in the activity. If modifying the functional group does influence the protein's activity, the inference is drawn that the modified group had been directly or indirectly involved in the mechanism of action of the protein.

The preparation of modified forms of a protein can be achieved either by total chemical synthesis of analogues of the molecule, or by derivatisation of functional groups in the native protein. The intention is always to prepare a derivative of the protein where a specific modification has been introduced into a known site (or a small number of sites) with the molecule. To this end, the chemistry of peptide synthesis[1] and specific modification by chemical or enzymatic methods[2] have been extensively studied.

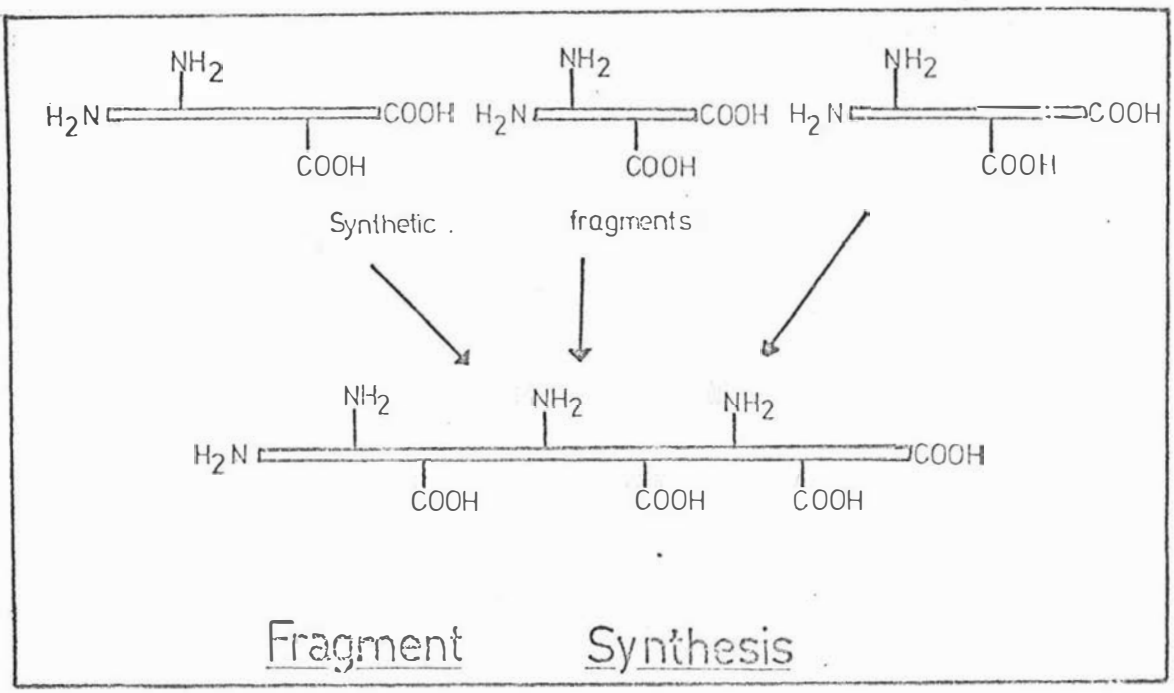
These techniques become increasingly difficult to apply, however, with larger proteins. Although total chemical syntheses have been reported for proteins as large as ribonuclease[3] and the acyl carrier protein of *E. coli*[4], the synthesis of a peptide containing more than 50 amino acid residues is a very laborious task, and the yield and purity of the final product are usually low. Specific modification of the functional groups of a larger protein also tends to be more difficult to achieve. In a large protein, each type of residue is likely to occur many times within the sequence.

Consequently it is difficult to find a method which will introduce only one modification into the molecule. In addition, the commonly-used reagents and enzymes lack specificity, and tend to react with more than one amino acid. Thus an attempt to modify a protein may well produce a mixture of products, which must then be separated and characterised.

For these reasons the techniques of fragment synthesis and semisynthesis[5] have often been suggested for the preparation of analogues of a protein. These methods involve the synthesis of a protein in the form of a number of small peptides, each of which can be purified to homogeneity, before they are joined to form the final product. In the case of semisynthesis, one or more of these fragments is prepared from the native protein, which has been isolated from biological materials (see Figure 1.). The principal advantage of semisynthesis is that it often requires a much smaller effort to isolate a large pure peptide from a natural source than to synthesise a large pure peptide by chemical means.

Thus semisynthesis could lead to the preparation of a large number of analogues of a protein more quickly and easily than any other available technique. In spite of the fact that the advantages of semisynthesis are well known[5], and that many of the necessary techniques have already been developed, applications of semisynthesis to the problems of biochemistry have been few in number[6].

The crucial step in fragment synthesis and semisynthesis is the formation of peptide bonds between the appropriate α-amino and α-carboxyl groups to form the complete peptide



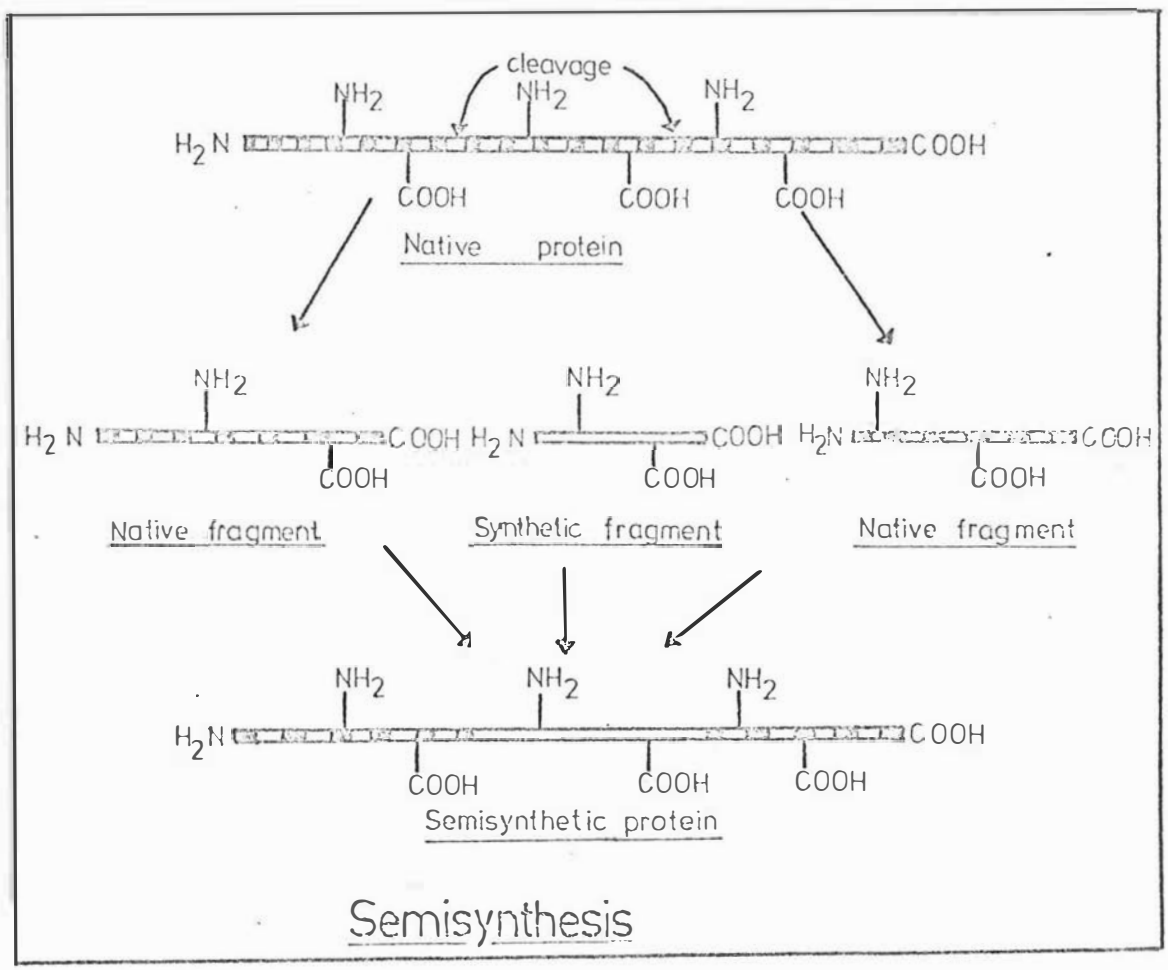


FIGURE 1 Simplified Outline of Fragment Synthesis and Semisynthesis

chain. In order that these reactions proceed in a specific manner, it is necessary, in all but the most specialised cases, that all other amino and carboxyl groups in the component peptides be protected by blocking groups which can be removed at the end of the synthesis. (See Figure 2). Thus both native and synthetic peptides must be prepared in a fully or partly-protected form, depending on the requirements of the particular protein synthesis which is to be attempted.

The solid-phase synthesis of Merrifield[7] has several advantages for the synthesis of peptides and proteins, in that it is rapid, and it lends itself to automation. Consequently much effort has been expended in adapting the techniques of solid-phase peptide synthesis to the preparation of protected peptides, for use in fragment synthesis or semi-synthesis.

The Merrifield method of peptide synthesis, where the growing peptide chain is attached throughout the synthesis to an insoluble polymeric support, is outlined in Figure 3. The final step in this procedure, the cleavage of the benzyl ester linkage which attaches the completed peptide to the insoluble polystyrene support, constitutes the main obstacle to the preparation of protected peptides by the Merrifield method. The conditions of the cleavage are somewhat vigorous (liquid HF, 0^{o}, 30 mins; or 33% BHr in acetic acid, 20^{o}, 30 mins), and most of the commonly-used protecting groups are removed by these reagents.

To avoid this difficulty, a variety of modifications of the technique have been introduced[8-22], in order that the

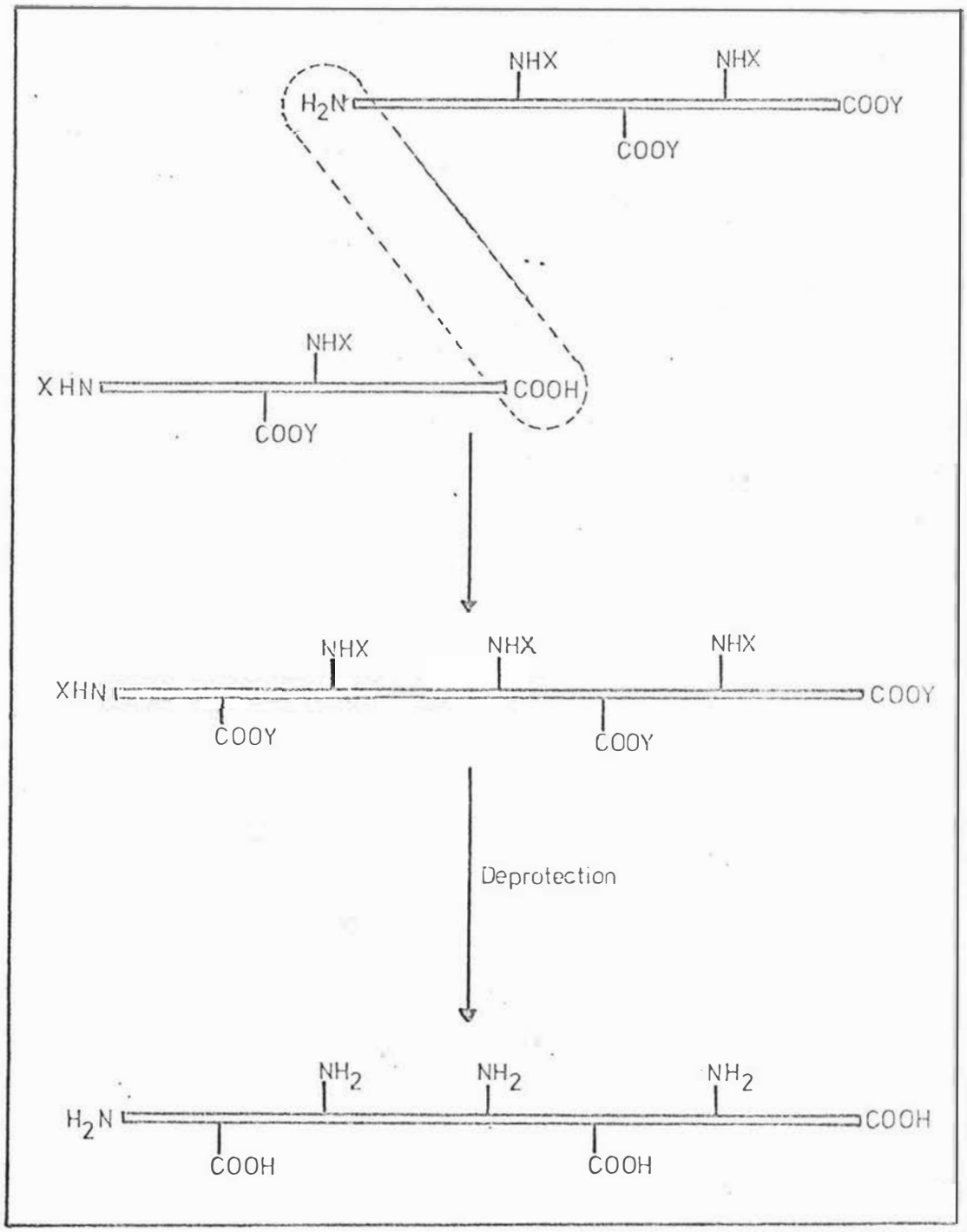


FIGURE 2 The use of blocking groups in fragment synthesis and semisynthesis to achieve specific peptide bond formation

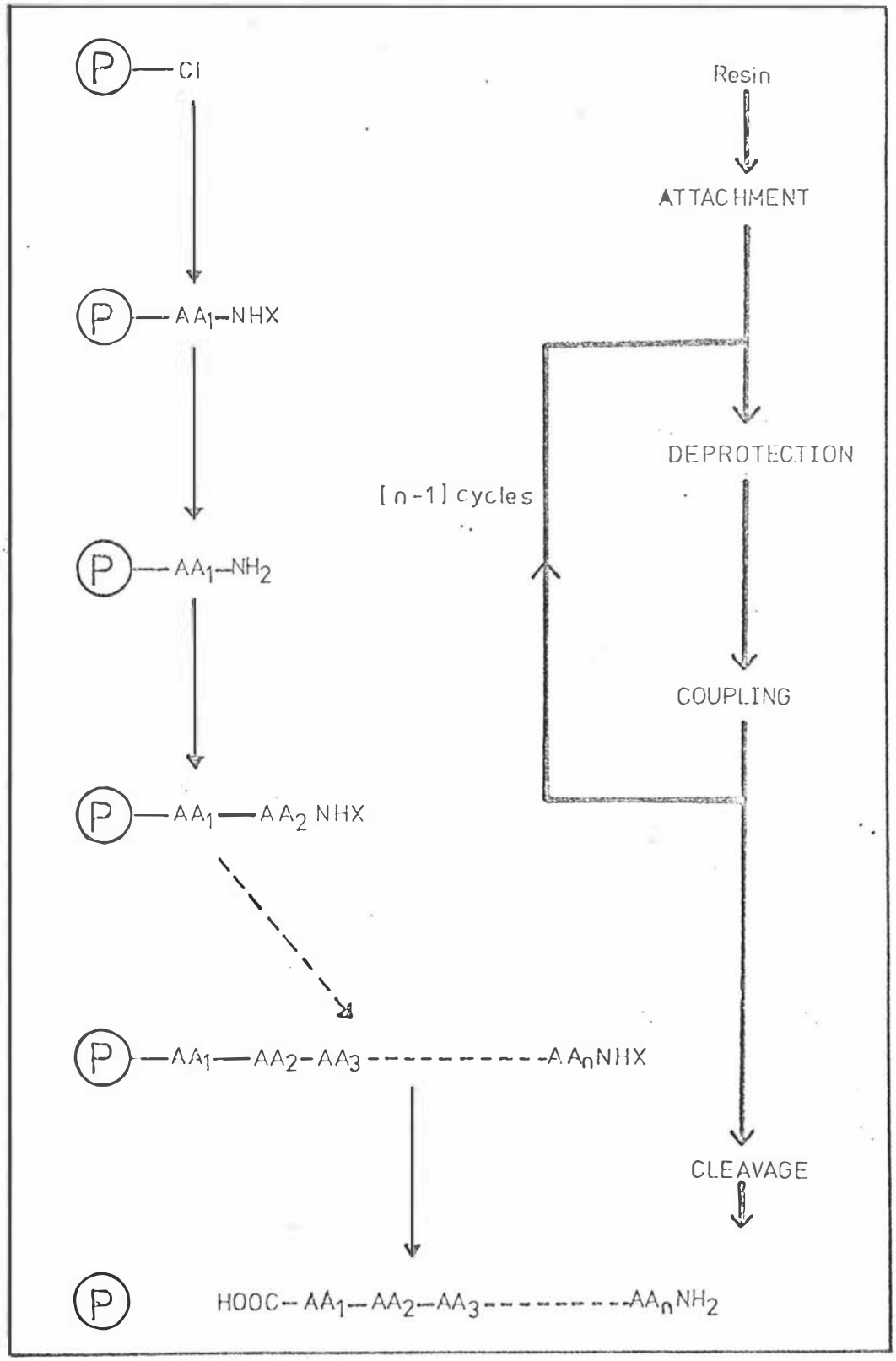


FIGURE 3 Merrifield solid-phase peptide synthesis

final peptide-resin can be cleaved under conditions mild enough not to remove the side-chain protecting groups. For example, the resin has been modified to permit the cleavage of the peptide chain from its support by photolysis[21], mild acid cleavage[9,10], oxidation[11,12,14], or alkylation[13]. None of these alternative methods has found a wide application. Most of the published approaches to solid-phase fragment synthesis involve changes in the standard procedures of synthesis. These new procedures are not only time-consuming, but also contain difficulties which would make them unsuitable for routine use, or limitations which would restrict their applicability to a wide range of syntheses.

An attractive approach to the solid-phase synthesis of protected peptides is to use standard methods of peptide synthesis with the exception that the side chains are protected by acid-stable groups. A range of protecting groups is known which are stable to HBr in acetic acid; for example, Lys(TFA), Arg(NO_2), His(DNP), Cys(Acm). If a suitable acid-stable group could be found for carboxyl protection, and the amino terminus was protected by trifluoroacetylation, HBr cleavage would release a peptide with only one free functional group, the carboxyl-terminal COOH, which could be used to form an active ester for fragment condensation.

For this reason a variety of blocking groups for carboxyl protection was considered in this study for their stability to acidolytic cleavage. The most promising were the substituted benzyl esters, and the preparation and stability of the p-nitrobenzyl and p-chlorobenzyl side-chain esters of aspartic and glutamic acids were studied in some depth. A

new and facile method[23] for the synthesis of these esters was introduced, and the commercial applications of this procedure are currently under development.

The p-chlorobenzyl esters of aspartic and glutamic acids were found to be insufficiently stable to HBr in acetic acid to permit the preparation of protected peptides using this blocking group. As a benefit not directly related to the present study, however, the p-chlorobenzyl ester group was found to be more stable to trifluoroacetic acid than the benzyl esters which are commonly used for Asp and Glu side-chain protection. Trifluoroacetic acid is the reagent used to each deprotection step of the Merrifield procedure (Figure 3) to remove the t-butyloxycarbonyl blocking group which is employed for temporary protection of the α-amino group. Consequently the p-chlorobenzyl esters of Asp and Glu are being marketed as derivatives which are less likely to decompose under the repeated trifluoroacetic acid treatments necessary during the synthesis of a large peptide. The use of this protecting group appears to limit the amount of premature side-chain deprotection, and hence the number of branched-chain impurities in the final product.

The p-nitrobenzyl esters of Asp and Glu have been reported to be stable to HBr in acetic acid[24]. Initial experiments with these derivatives showed that they were, indeed, more stable to this reagent than any of the other protecting groups which were evaluated. Later results, however, demonstrated that this stability was insufficient to allow the convenient use of the p-nitrobenzyl ester for the protection of peptides during cleavage from the resin by HBr

in acetic acid. There were also difficulties during the synthesis of the amino acids derivatives, and the peptides protected by p-nitrobenzyl esters were found to have very limited solubility in common solvents. These two problems, of instability during synthesis of a peptide and cleavage from the resin, and insolubility of the protected product, impose severe limitations on the use of this protecting group for the synthesis of protected peptides.

In spite of these difficulties, however, some success was obtained using the p-nitrobenzyl ester as a side-chain protecting group for glutamic acid in the semisynthesis of several analogues of the acyl carrier protein of E. coli.

Acyl carrier protein[25] (ACP) has a number of properties which make it suitable as a model system for the study of the techniques of semisynthesis. It is a small protein, and yet it interacts in a very specific manner with at least 12 different enzymes of lipid biosynthesis. The structure of ACP is very important to its activity, and small modifications in the molecule produce drastic changes in its reaction with many of its enzymes[26]. The protein is also very stable (for example, it is not destroyed by boiling in 0.1M acid), and hence it requires no special precautions to retain its activity while manipulations are carried out.

The fatty acid synthetase of bacteria differs from that of higher organisms in that it is easily dissociated into component enzymes, each of which catalyses a single reaction of the biosynthesis of fatty acids (see Figure 4). One of the earliest observations in this field was made by Lynen[27], who reported that acetoacetate, an intermediate in the

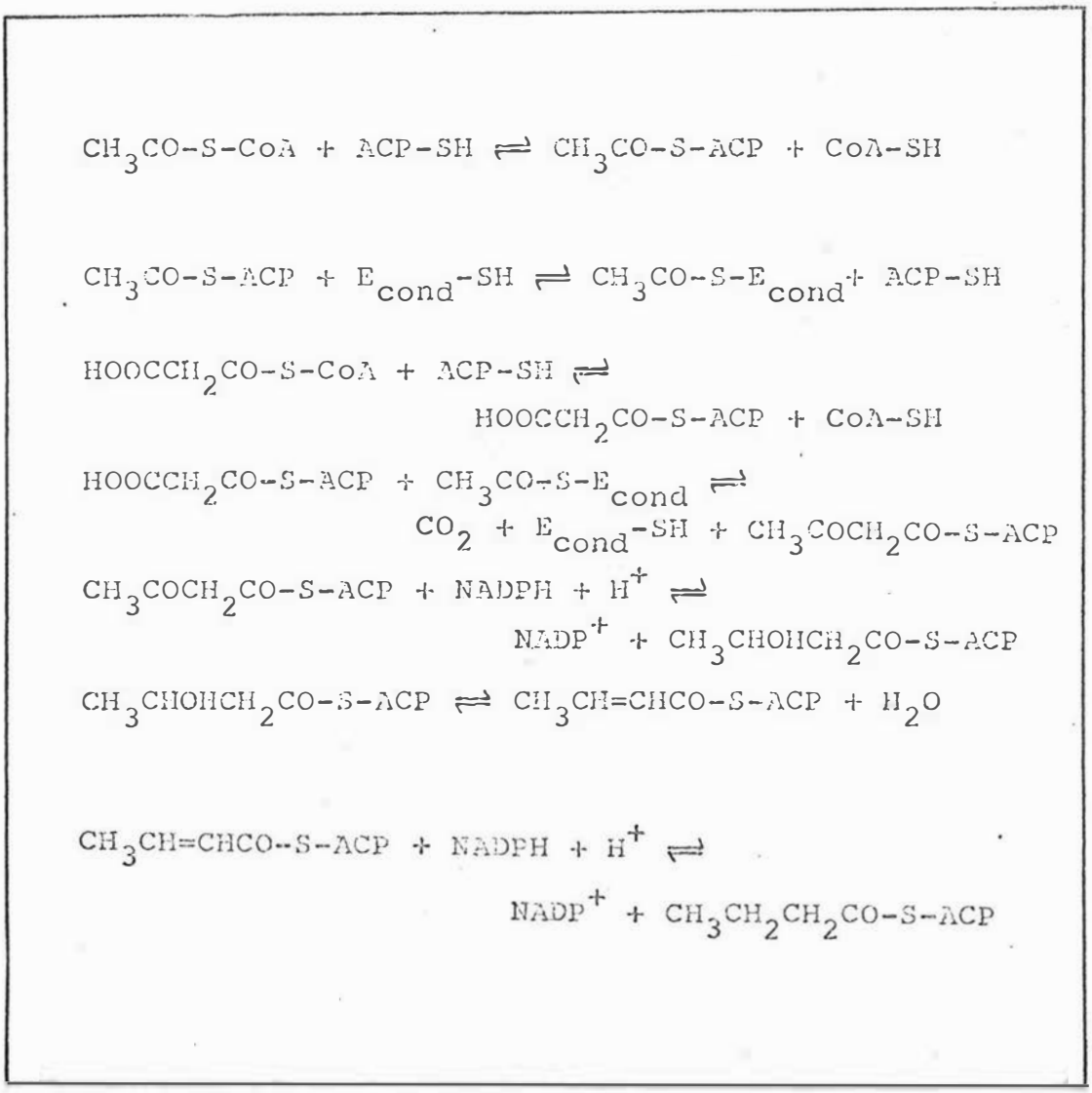


FIGURE 4 The reactions of fatty acid synthesis

synthesis of fatty acids, appeared to be bound to a protein in the yeast fatty acid synthetase complex. This led to the demonstration[28] of a component of the bacterial fatty acid synthesising system which was a small acidic protein which contained a phosphopantetheine prosthetic group, to which fatty acids were attached throughout their synthesis by a thioester linkage. This protein was subsequently designated ACP.

ACP can easily be prepared from extracts of E. coli[29]. The steps involve removal of nucleic acids from the cell extract, and precipitation of protein by ammonium sulphate to 80% of saturation. The 80% ammonium sulphate supernatant, containing the ACP, is acidified; the ACP is recovered in the precipitate, which is then chromatographed on DEAE-cellulose, and finally on DEAE-Sephadex. The protein appears to be homogeneous on DEAE-Sephadex chromatography, polyacrylamide disk gel electrophoresis, and ultracentrifugal analysis. Modifications of this procedure have yielded homogenous preparations of ACP from a variety of other species[30].

ACP from E. coli exhibits properties expected of a globular protein. The amino acid sequence[31] is given in Figure 5. The prosthetic group was established to be 4'-phosphopantetheine[32] and to be attached by a phosphodiester linkage to serine 36 of the protein sequence (see Figure 5). The protein has 22 acidic residues and only 6 basic residues out of a total of 77. Although the protein has yet to be crystallised, and consequently its three-dimensional structure is unknown, it is clear that there must be a preponderance of acidic groups on the surface of the protein.

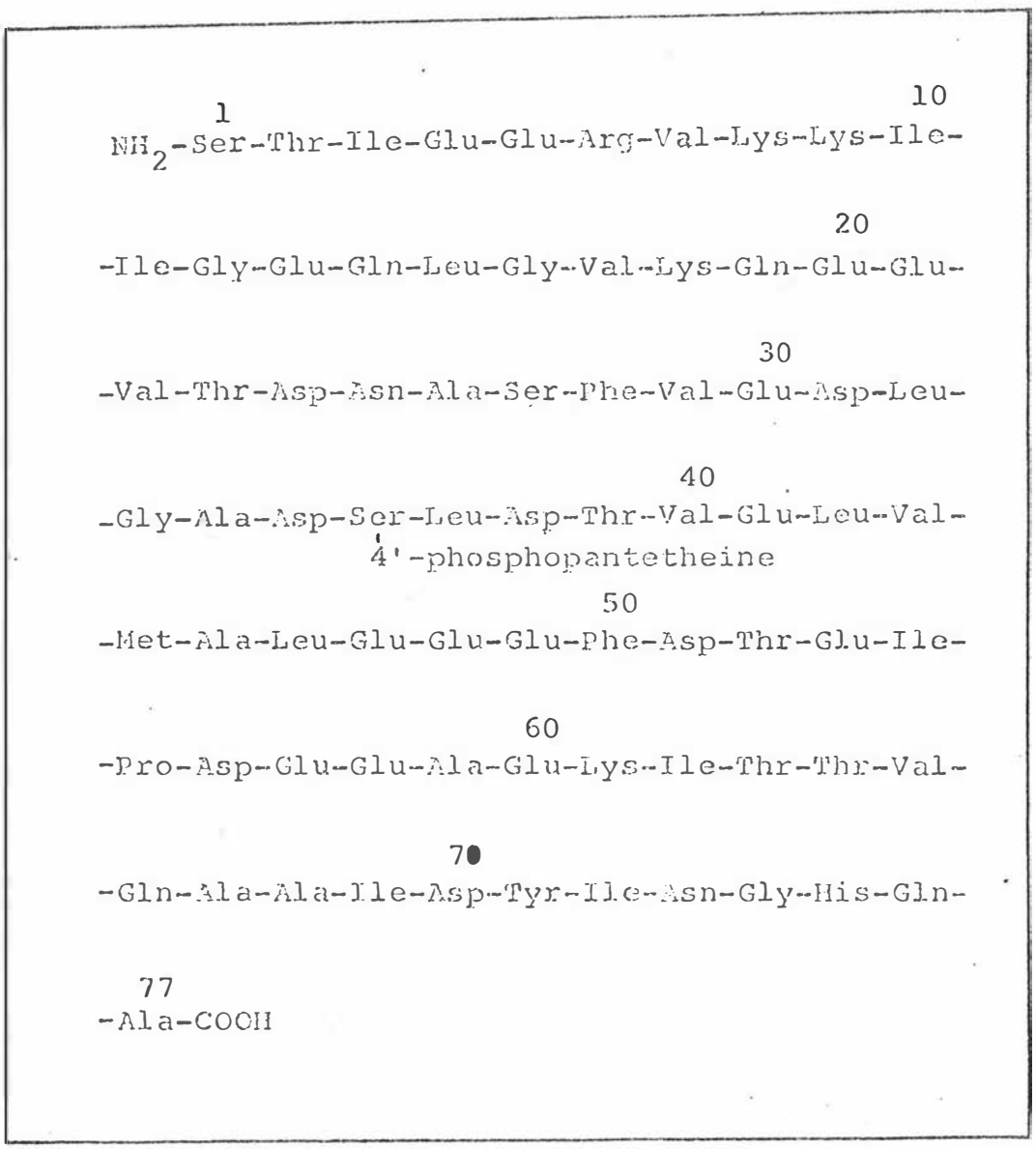


FIGURE 5 The amino acid sequence of the acyl carrier protein of E. coli (from Vanaman et al, Reference 31)

In the absence of X-ray crystallographic data, other techniques have been employed in attempts to understand the properties of ACP. Both total chemical synthesis[4] and chemical and enzymatic modification[33-37] have been carried out on ACP, and the modified proteins have been investigated for their activity with some or all of the many enzymes for which ACP is a substrate. Carboxypeptidase A treatment[33] which removed up to three residues from the C-terminus of the protein did not affect its activity in the $^{14}CO_2$ exchange assay.[29] Acetylation of the four lysine residues and the amino terminus of ACP did not reduce the activity of the protein, although its stability to thermal denaturation was slightly reduced.[34] Nitration of the single tyrosine residue or alkylation of the single methionine[35] did not abolish the activity of ACP, although the rates of individual reactions of lipid synthesis were altered. The tryptic peptide comprising residues 19-61[33] and the cyanogen bromide peptide comprising residues 1-44[25a] were found to be totally inactive.

Modification of as few as two carboxyl groups[35-37] was sufficient to inactivate the protein, but the modification of 11 of the 22 carboxylates could be carried out without causing extensive changes in the structure of the molecule as measured by its optical rotatory dispersion.[35] In contrast, the acetylation of the N-terminus and all of the lysine ε-amino groups caused a large change in the structure of the protein,[35] but its activity in the $^{14}CO_2$ assay was not diminished[34] provided that a sufficient salt concentration was present in the assay mixture to compensate for the loss in charge caused by acetylation.[37] The two examples given in this paragraph illustrate the two types of modification

which can be distinguished: that in which the reactivity of the molecule is affected by disordering the structure of the molecule, and that where functional groups in the active site or sites are destroyed.

Synthesis of the acyl carrier protein with Arg^6 present as N^G-nitroarginine led to a product with activity in the assay reaction.[38] Similarly, modification of Arg^6 by a trimer of butane-2,3-dione did not abolish the activity of ACP.[37] However, the ($norleucine^6$) derivative was inactive.[38] The role of Arg^6 is unclear, especially when it is considered that the acyl carrier proteins from *Clostridium butyricum*[30f] and spinach[30a] do not contain arginine. (As the amino acid sequences of these acyl carrier proteins have not been determined, it is not known what amino acid replaces Arg^6.)

Acetylation of all four ε-amino groups and the N-terminus, and tryptic cleavage of the acetylated ACP at the single arginine residue, produce the 7-77 peptide, which is devoid of both structure and activity. No activity could be produced by combining the 1-6 and 7-77 peptides under a variety of conditions.[34] It appears likely that part or all of the N-terminal hexapeptide is participating in interactions which make a major contribution to the conformational stability of the molecule. The differential acetylation experiments of Prescott *et al*[39] and the resistance of ACP to aminopeptidase digestion[35] are interpreted to mean that the amino terminus of ACP is buried within the interior of the molecule, which is consistent with its taking part in maintaining the overall structure of the protein.

Synthesis of the analogues of ACP comprising residues

2-74, 3-74, 4-74, 5-74 and 6-74[38] revealed that omission of successive residues from the N-terminus resulted in a steady decline in activity, and that the analogue lacking the 1-5 pentapeptide was essentially inactive (see Figure 6). The N-terminal hexapeptide of ACP is highly functionalised, containing two hydroxyl, a guanidino , and two carboxyl groups in the side-chains of six residues. It seems likely that all of these functional groups, as well as the hydrophobic Ile^3 side-chain, play a part in maintaining the structure of ACP. Valuable information on the relationship between structure and activity of this protein, and on protein-protein interactions in general, could be obtained by the preparation of analogues containing variations in the first six amino acids.

Acyl carrier protein is an ideal candidate for the preparation of analogues by semisynthesis. Apart from the general advantages of semisynthesis mentioned earlier, ACP has two specific advantages as a model system. First, a peptide whose presence is essential to the protein's activity is found at the N-terminus of the protein, and hence its replacement by semisynthesis involves the joining of only two peptides, rather than three as in the general case (see Figure 1). In addition, as acetylated ACP is fully active in the $^{14}CO_2$ exchange assay,[34] it is not necessary to use temporary blocking groups for the N-terminal and lysine ε-amino groups. All amino groups can be permanently blocked by acetylation. Tryptic cleavage of acetylated ACP then releases the acetylated 7-77 peptide, which has only one free amino group.

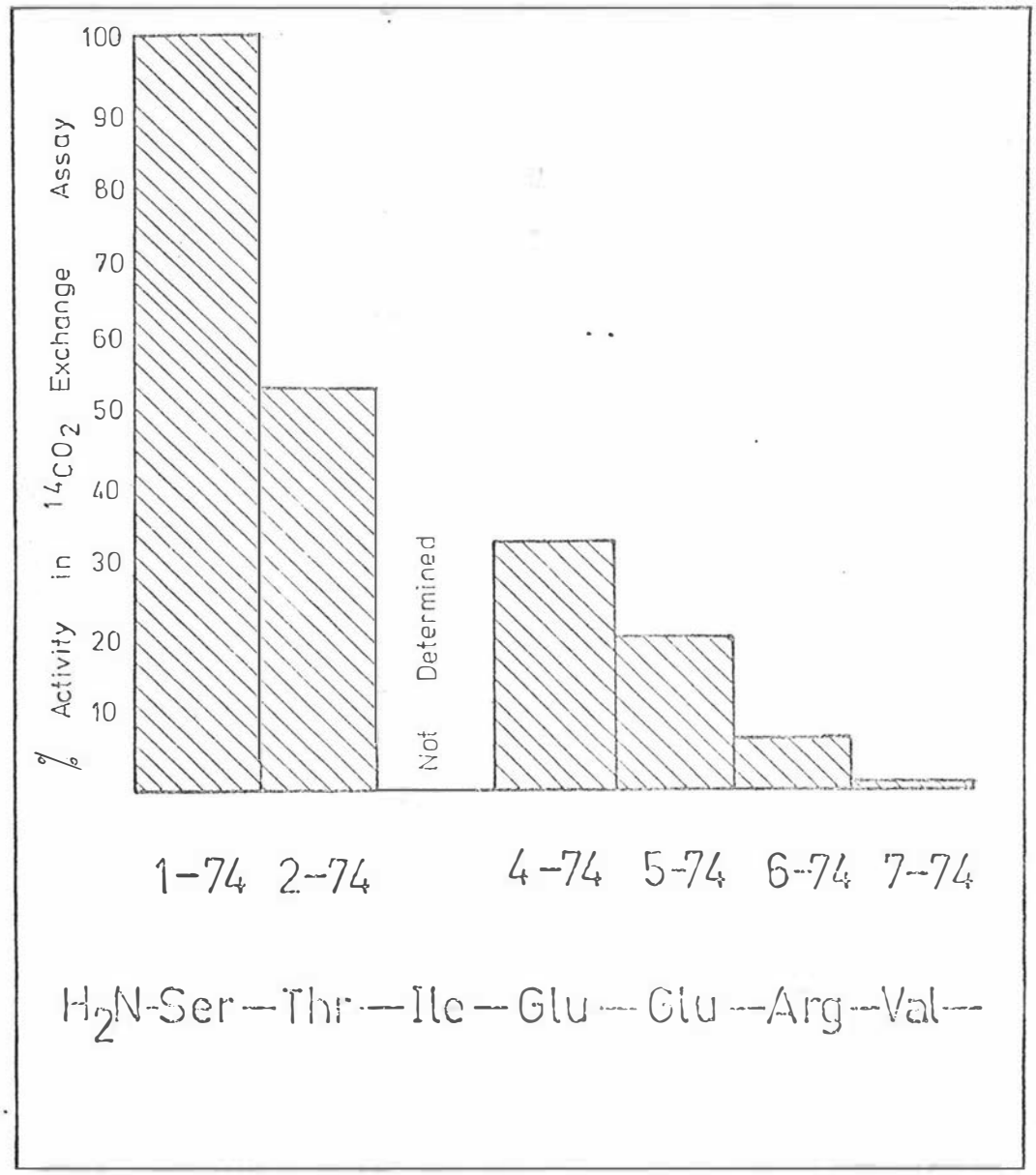


FIGURE 6 The Activity of Synthetic Analogues of ACP in the $^{14}CO_2$ assay. Activities relative to the 1-74 peptide as 100% (from Ref. 38)

If the coupling reaction between the 1-6 and 7-77 peptides is carried out using preformed active esters, the carboxyl-protecting groups of the N-terminal hexapeptide can be removed before coupling is performed. Specificity of reaction is ensured by the fact that only one carboxyl group has been activated. The absence of any temporary blocking groups on the peptides involved in the final coupling step minimises the problems of insolubility which are often found with fully-protected peptides, and obviates the necessity for a final deprotection reaction to be carried out on the semisynthetic protein. The semisynthetic protein is expected to assume the conformation of native ACP readily, as the denaturation of ACP is known to be a reversible process[40]. The simplified semisynthetic procedure which is possible is shown in Figure 7.

Following this pattern, the semisynthesis of acyl carrier protein and of several analogues containing variations in the sequence of the N-terminal hexapeptide was initiated. The acetylated 7-77 peptide was available from E. coli ACP using the published procedures for isolation, purification[29], acetylation, and tryptic cleavage[34]. The active-site thiol group was protected by formation of the DTNB derivative[41].

The protected form of the 1-6 hexapeptide and several analogues were synthesised by the solid-phase method, using the p-nitrobenzyl ester derivatives of Glu and Asp, and Arg^6 protected as N^G-nitroarginine. The N-terminal amino groups of the peptides were blocked by acetylation, and the peptides were cleaved from the resin with HBr in acetic acid. The

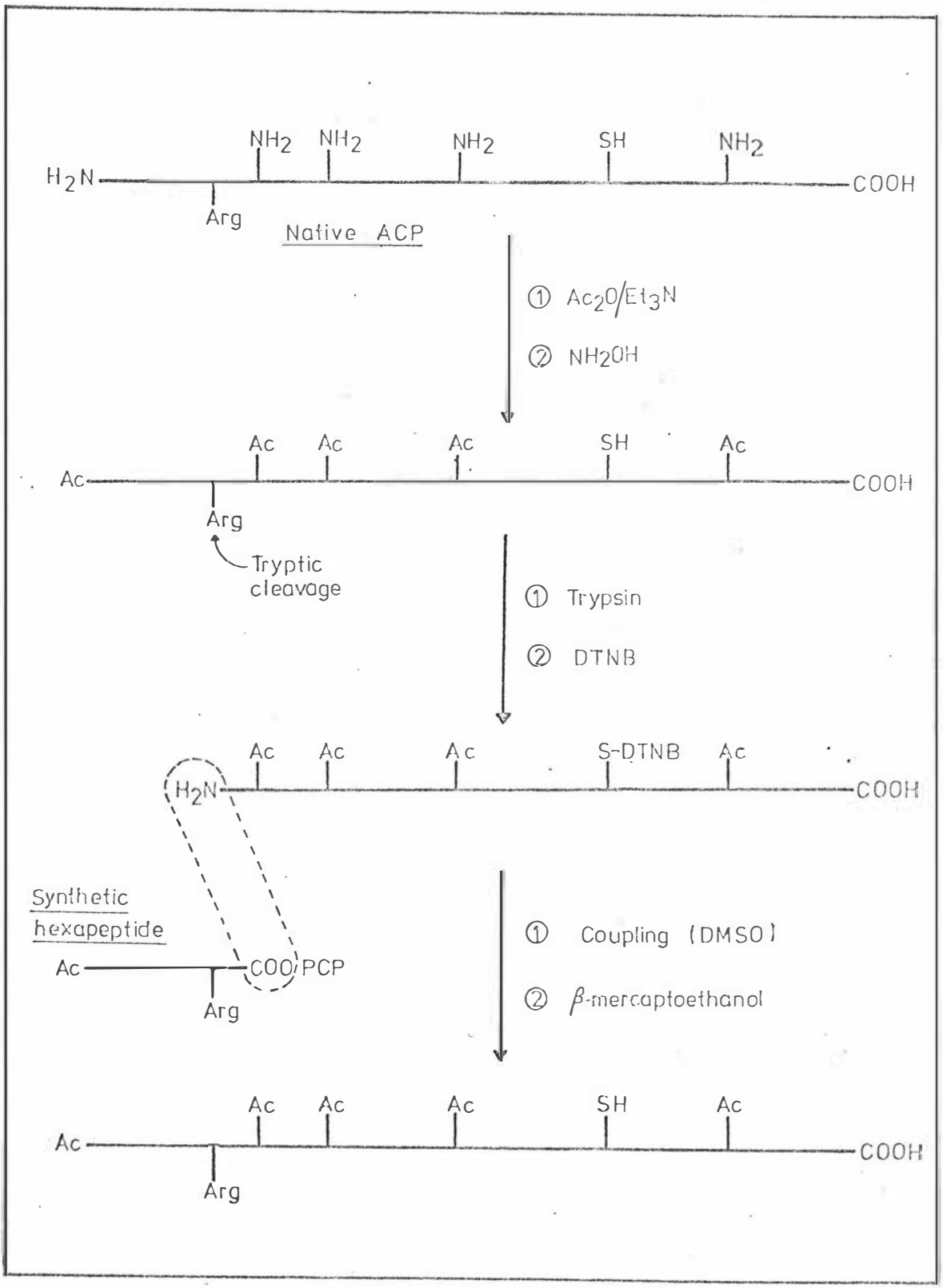


FIGURE 7 The semisynthesis of ACP

C-terminal carboxyl group of the peptide was activated by formation of the pentachlorophenol ester[42], and all temporary protecting groups were removed by hydrogenation.

The synthetic 1-6 hexapeptide and the native 7-77 tryptic peptide were then coupled in solution. The thiol protecting group was removed by reduction with β-mercapto-ethanol, and the product, semisynthetic acetylated ACP, was assayed by the $^{14}CO_2$ exchange assay.

SECTION 1

SUBSTITUTED BENZYL ESTERS AS SIDE-CHAIN PROTECTING GROUPS IN SOLID-PHASE PEPTIDE SYNTHESIS

Protection of the side-chain carboxyl groups of aspartic and glutamic acids in peptide synthesis has been most commonly achieved by benzyl esters.[43] This protection is very suitable, in that it is fairly stable to the conditions of peptide synthesis, and it can be removed at the end of the synthesis by strongly acidic or reducing conditions.[44]

There are important reasons, however, for seeking alternative carboxyl-protecting groups, for it has been shown[45-47] that benzyl esters are not completely stable to the conditions commonly used to remove the t-Boc[48,49] group during peptide synthesis. This lability gives rise to a cumulative loss of side-chain protection, and increases the possibility of branching of the peptide chain, particularly during long syntheses.

An important use of more stable carboxyl-protecting groups is in the synthesis of protected peptides, which can be used in fragment synthesis and semisynthesis of proteins.[50-54] In an attempt to develop a simple method for the preparation of protected peptides which could be used for the semisynthesis of ACP, it was decided to examine the synthesis of a fully protected peptide using standard solid-phase techniques. If the side-chains and amino terminus of a peptide were blocked by groups stable to acidolysis, the

synthesis could be performed on a chloromethylated resin, with the usual t-Boc group for α-amino protection, and using HBr in acetic acid for the cleavage of the peptide from the resin.

Two acid-stable carboxyl-protecting groups were evaluated: the p-chlorobenzyl ester as a group of moderately increased stability for use in longer syntheses, and the p-nitrobenzyl ester as a much more stable group for the synthesis of protected peptides with HBr cleavage from the resin. A new facile method was developed for the preparation of the substituted benzyl esters of aspartic and glutamic acids. This procedure involves the copper-catalysed hydrolysis of the corresponding amino acids diesters (see Figure 8), and is superior to previously published methods both in simplicity and in yield of the products. A report of the preparation of these esters was accepted for publication as a communication to the Journal of Organic Chemistry[23], as was a later paper[55] giving the evidence presented in this section for the suitability of these compounds for use in solid-phase peptide synthesis.

The criteria for a more stable side-chain protecting group are that it should be easily prepared, that it should be significantly more stable to the conditions of peptide synthesis than benzyl ester protection, and that it should be readily removed at the end of the synthesis. The acid stability of the benzyl ester group can be conveniently increased by the introduction of electron-withdrawing substituents into the aromatic ring. For example, Merrifield[55] investigated the use of chlorinated benzyl-

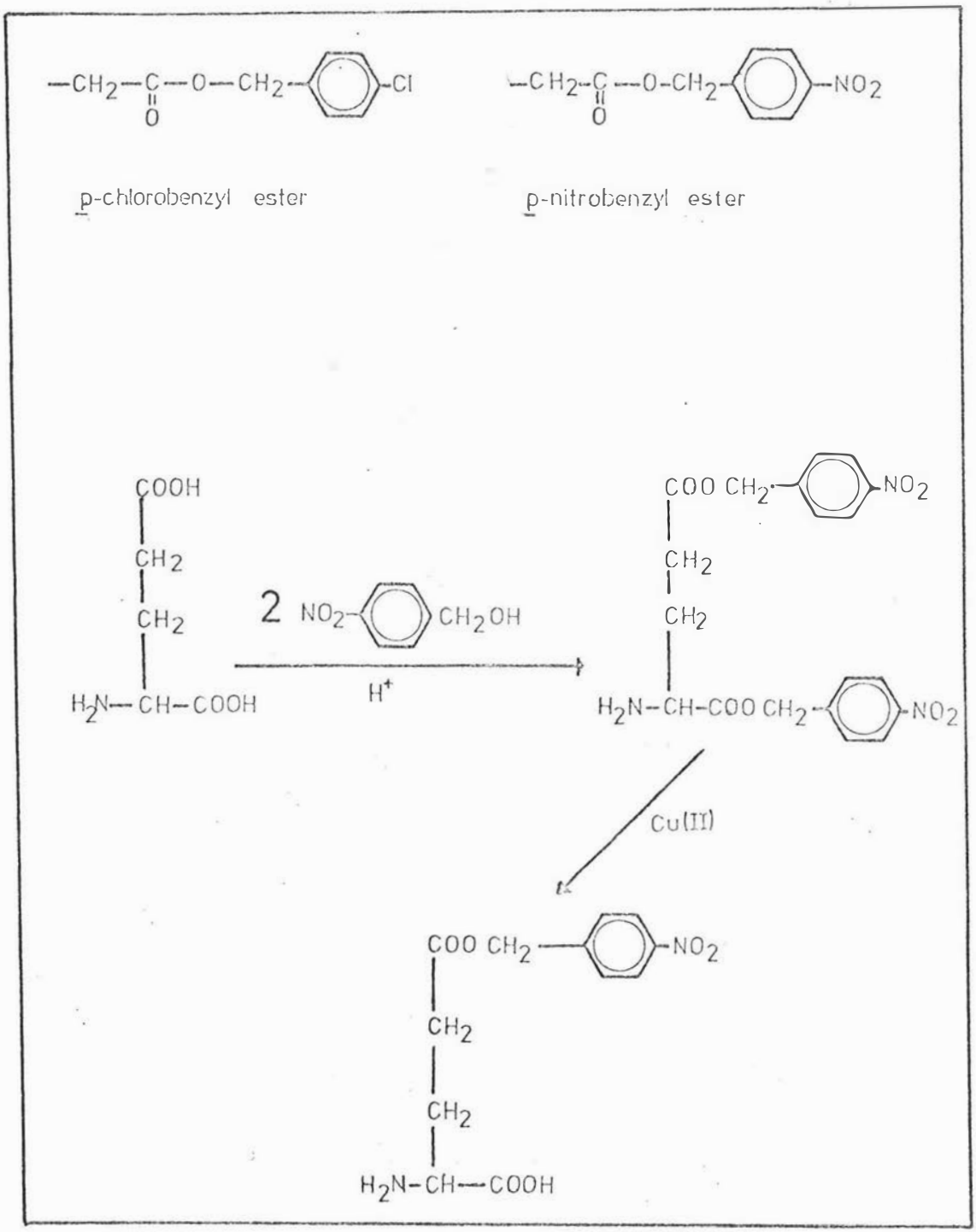


FIGURE 8 Simplified outline of the synthesis of substituted γ-monoesters of glutamic acid by the copper-catalysed hydrolysis of the corresponding diesters

oxycarbonyl (Z) groups for the protection of lysine residues, and found that (4-Cl-Z) Lys was three times more stable than Z-Lys to trifluoroacetic acid in dichloromethane (1:1). Other chlorinated derivatives were found to be even more stable, some to the point of being difficult to remove by HF cleavage. Similarly, Li[57,58] employed the 4-bromobenzyl ester for the protection of Glu residues, and claimed it to be four times as stable as the benzyl ester, but detailed evidence as to the suitability of this protecting group in peptide synthesis was not presented.

Following the above criteria, the p-chlorobenzyl ester was selected for investigation. It can be readily prepared by the copper-catalysed hydrolysis method developed during the course of this work[23], and the starting material, p-chlorobenzyl alcohol, is commercially available. The stability of this ester to trifluoroacetic acid (TFA) hydrolysis was studied, and compared with the stability of the benzyl ester. In order to provide a measurable extent of hydrolysis in a reasonable time, the studies were performed at 45°C. The samples from these hydrolyses were analysed by high-pressure liquid chromatography (HPLC) on a Porasil silica column with potassium phosphate buffer (pH 4.0, 0.02 M) as the eluent. It should be noted than an aqueous buffer system was used in conjunction with a silica column. The aqueous system was found to minimise tailing of the benzyl esters, a frequent problem in the analysis of polar compounds on silica columns.

The p-chlorobenzyl ester was found to be approximately twice as stable as the benzyl ester to hydrolysis by

TFA-CH_2Cl_2 (1:1) at 45°C. From the temperature dependence of Hammett factors[59] it can be predicted that this stability difference will be significantly greater at room temperature. The p-chlorobenzyl ester was also found to be completely removed by liquid HF at 0°C for 30 minutes, and can therefore be recommended for the synthesis of large peptides. Although the data of Merrifield[45] imply that benzyl ester protection of Asp and Glu is adequate for moderate-sized peptides, it should be emphasised that such protection is inadequate for small acidic proteins such as the acyl carrier protein (ACP) from E. coli. This protein contains 14 glutamic and 7 aspartic acid residues out of a total of 77. Using the method of Merrifield[45] it can be calculated that in a synthesis of ACP using benzyl ester protection of Asp and Glu, 5.6% of the chains would be prematurely deprotected at a glutamic acid residue and 2.7% at an aspartic residue. These side reactions should be reduced to an acceptable level by the use of a more stable protecting group such p-chlorobenzyl.

Although many approaches to the synthesis of protected peptides have been suggested, involving alternative resins and cleavage techniques[8-22], none has found a wide application.

One disadvantage is that many of the modified resins involve lengthy syntheses[9,10,14,15,21]. More significantly, the effect of these modifications on the properties of the resin has not been evaluated, so that the routine use of such resins must await further evaluation of their behaviour, particularly in longer syntheses. It has been observed that for mild, selective cleavage procedures, yields can decrease

with larger peptides[22,60], so that these methods may not be suitable for the synthesis of large protected peptides.

Most of the published approaches to fragment synthesis involve changes in the standard procedures of synthesis. These new procedures are not only time consuming, but also often contain difficulties which would make them unsuitable for routine use, or limitations which would restrict their applicability to a wide range of syntheses. For example, resins which form a labile bond to the first amino acid require the use of even more labile α-amino protection during the synthesis[14,15], which is inconvenient for routine use. Resins where the bond to the peptide chain is activated in the penultimate step involve treating the peptide with such reagents as peroxides or methyl iodide[11-13]. This activation step is not compatible with peptides which contain Trp, Cys or Met, and may also cause racemisation[61].

An attractive approach to the solid-phase synthesis of protected peptides is to use standard methods of peptide synthesis with the exception that the side chains are protected by acid-stable groups. Such stable blocking groups are known for most side-chain functional groups, with the exception of the carboxyl groups of aspartic and glutamic acids. If a suitable acid-stable group could be found for carboxyl protection, and the amino terminus was protected by trifluoroacetylation, HBr cleavage would release a peptide with only one free functional group, the carboxyl-terminal COOH, which could be used to form an active ester for fragment condensation.

For this reason the stability of p-chlorobenzyl and

p-nitrobenzyl esters to HBr cleavage was investigated. The p-chlorobenzyl ester was found to be only twice as stable to HBr as the benzyl ester, and this stability was not sufficient for the isolation of peptides protected with this group. The p-nitrobenzyl ester, however, was 50 times as stable as the benzyl ester when the amino acid was not involved in peptide bonds. On the basis of these results, the p-nitrobenzyl ester was chosen for the side-chain protection of aspartic and glutamic acids in the synthesis of a protected model peptide and the protected 1-6 hexapeptide from ACP. In both cases the product was cleaved from the resin with HBr/acetic acid.

The stability of p-nitrobenzyl esters to HBr cleavage is well known[24a,b], although quantitative data on this stability do not appear to have been reported, and little use appears to have been made of this property in peptide syntheses[24c,d]. Some of the reasons for the apparent reluctance of peptide chemists to employ this protecting group emerged during this study. Although the p-nitrobenzyl esters of the free amino acids, Asp and Glu, were very stable to HBr/acetic acid as reported here, yet when these amino acids were present as part of a peptide chain the stabilities of the p-nitrobenzyl side-chain esters were drastically reduced. Both in the model peptide, Gly-Glu(OBzl p-NO_2)-Ala, and in the ACP 1-6 hexapeptide, Ac-Ser-Thr-Ile-Glu(OBzl p-NO_2)-Glu(OBzl p-NO_2)-Arg(NO_2)OH, the p-nitrobenzyl groups were found to be removed at a much greater rate than expected. A further complication was that rearrangement of the target peptide appeared to be occurring, so that a large number of

products were formed instead of the desired single cleavage product.

The poor stability of this protecting group resulted in cleaved peptides which were contaminated by substantial amounts of deprotected and rearranged products. The separation of these peptide mixtures was further complicated by their low solubility, which also appeared to be a direct result of the use of the p-nitrobenzyl protecting group.

PART A

Synthesis of the Diesters of Glutamic Acid

The substituted benzyl diesters of glutamic acid were prepared by the method of Shields, McGregor and Carpenter[62,63]. This method involves the azeotropic distillation of a mixture of the amino acid and an excess of the appropriate substituted benzyl alcohol in the presence of a strong acid catalyst. During the distillation, water is removed from the condensate, initially by a Dean & Stark apparatus, and later, as the reaction nears completion, by passage through a bed of drying material. Either benzene or carbon tetrachloride was found to be satisfactory as the solvent, and either p-toluenesulphonic acid or benzenesulphonic acid as the catalyst. The product, an amino acid dibenzyl ester salt, precipitated on cooling of the reaction mixture, and was recrystallised from ethanol.

Materials and Methods

p-Chlorobenzyl alcohol and p-nitrobenzyl alcohol (reagent grade) were supplied by Fluka. All solvents were redistilled before use.

In a typical synthesis, glutamic acid (147g, 1 mole), p-chlorobenzyl alcohol (570g, 4 moles) and benzenesulphonic acid (174g, 1.1 moles) were suspended in carbon tetrachloride (1.5l). The heterogeneous mixture was heated under reflux with removal of water by azeotropic distillation. After 5 hours, and the removal of 40 cm^3 of water, the reaction

mixture solidified. The solid was broken up, and distillation was continued with calcium chloride beads in the Dean & Stark apparatus. The reaction was terminated after 23 hours. The product precipitated on cooling. It was washed with diethyl ether, and recrystallised from boiling ethanol (1.5l): yield 52lg (94%). The reaction times, yields, and melting point of the diesters of glutamic acid are shown in Table I.

TABLE I The Synthesis of Glutamic Acid Diesters

Ester	Time	Yield	M.Pt.
$Glu(OBzl)_2HTs$	10 hrs	69%*	115-117°
$Glu(OBzl\ \underline{p}Cl)_2HBs$	23 hrs	94%	
$Glu(OBzl\ \underline{p}NO_2)_2HTs$	41 hrs	88%	121-124°

* The lower yield of the unsubstituted benzyl ester is thought to be due to its higher solubility in the solvents used for crystallisation and recrystallisation.

PART B

Synthesis of the γ-Monoesters of Glutamic Acid

The substituted benzyl esters of glutamic acid were prepared by selective hydrolysis of the corresponding diester in the presence of Cu(II) (see Figure 9). The appropriate amino acid diester salt was dissolved in aqueous ethanol or aqueous dioxan in the presence of a three-fold excess of copper sulphate. The pH was then raised to 8.0, and maintained there for the required time. The pH was then lowered to 3.0, and the copper complex of the monoester could be isolated by filtration.

The copper complex was then decomposed with boiling aqueous EDTA by the method of Ledger and Stewart[64]. The product precipitated on cooling, and could be recrystallised from boiling water if necessary.

Hydrogen sulphide could also be used to decompose the complex, giving a precipitate of copper sulphide. This procedure, however, was found to give drastically lower yields, perhaps due to trapping of the product within the extremely insoluble copper sulphide precipitate.

Attempts were made to monitor the progress of the hydrolysis reaction by measuring the base addition required to maintain the pH of the reaction mixture at 8.0. However, the rate of base uptake was found to bear little relationship to the rate of the reaction. It possibly reflects the formation of insoluble salts such as $Cu(OH)_2$.

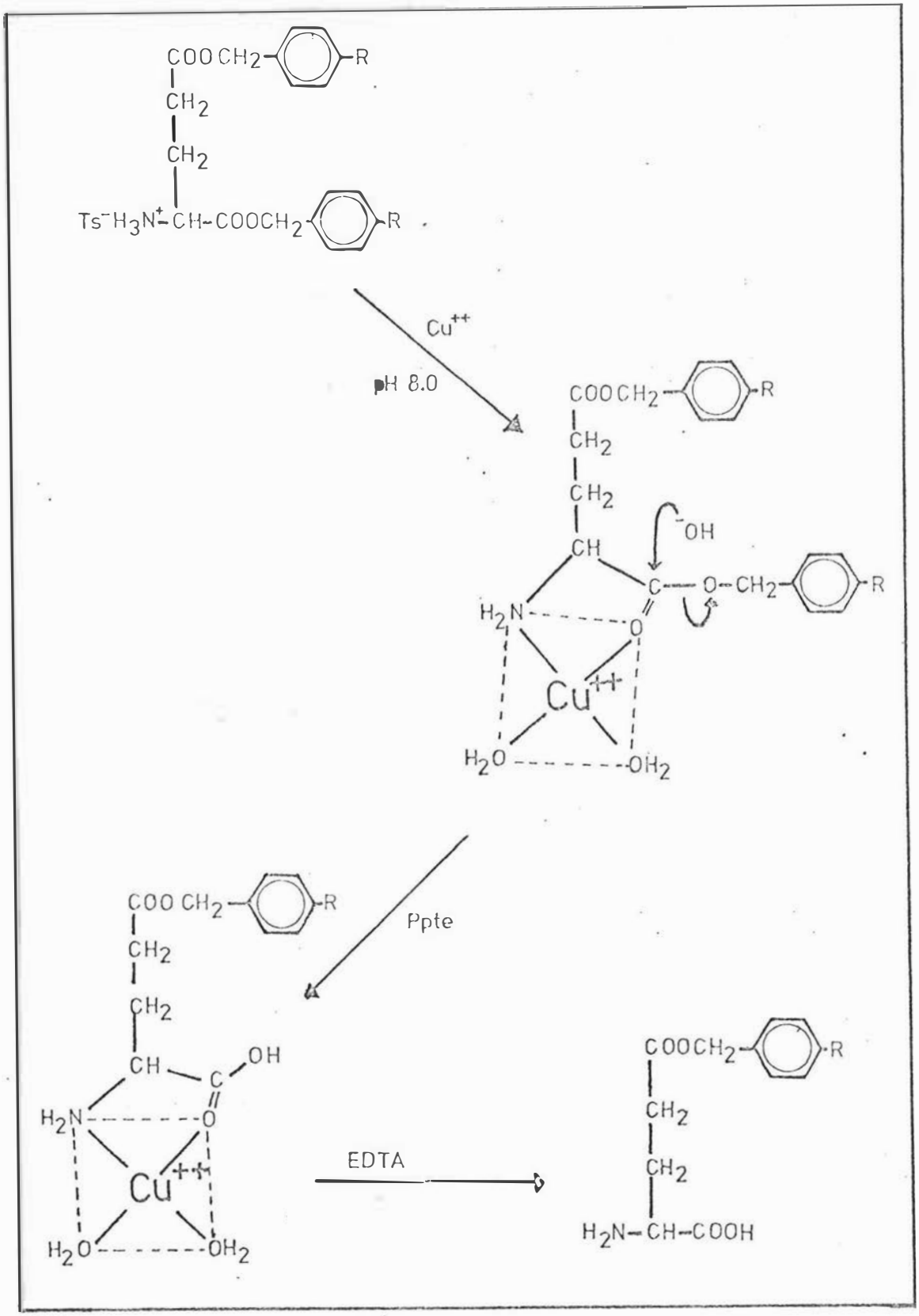


FIGURE 9 Copper-catalysed hydrolysis of glutamic acid diesters

The rate of the copper hydrolysis reaction could, however, be measured by quantitative TLC of aliquots of the reaction mixture. The rates of hydrolysis of a variety of esters were measured in this way, in order to elucidate the mechanism of the hydrolysis reaction.

Methods

Synthesis of glutamic acid γ-benzyl ester

In a typical copper hydrolysis, glutamic acid dibenzyl ester p-toluenesulphonate (10g, 20 mmol) was dissolved in ethanol (140 cm^3) and aqueous $CuSO_4.5H_2O$ (20g, 80 mmol in water, 350 cm^3) was added. The pH was raised to 8.0 with 1M NaOH, and the solution was maintained at that pH and 32°C for 60 minutes. The pH was then lowered to 3.0 with 3M HCl and the precipitate of the copper complex of Glu γ(OBzl)[49] was filtered off and washed with water, ethanol and ether. Ethylenediaminetetraacetic acid disodium salt (7.8g, 21 mmol) in 100 cm^3 of water was added, the solution was boiled and filtered, and on cooling, glutamic acid γ-benzyl ester precipitated out. The product was collected by filtration and washed with water, ethanol and ether: yield 3.5g (14.8 mmol, 74%): m.pt 169-170°; $(\alpha)_D^{22}$ +19.3° (c 5.49, acetic acid) (lit. m.pt 169-170°; $(\alpha)_D^{22}$ +19.2° [64]).

The yields for various esters of glutamic and aspartic acids are given in Table II.

TABLE II Yield of Asp and Glu Monoesters Prepared by the Copper-Catalysed Hydrolysis of Corresponding Diesters

Ester	Crude Yield* mol%	Yield of recrystd. ester, mol%	m.pt °C	Lit.
Glu(OBzl)	95	74	169-170	169-170[65]
Glu(OBzl p-Cl)	93	54(a)	169-170	176[66]
Glu(OBzl p-NO_2)	87	54(a)	158-159(b)	171-172[64]
Glu(OMe)	99		180[66]	182[66,67]
Glu(OEt)	96		192-194	194[67]
Asp(OBzl)	98	67	220-222	221[68]
Asp(OBzl p-Cl)	98	83	208-210	208[68]
Asp(OBzl p-NO_2)	97	88	193-195	189-190[64]
Asp(OMe)	98		188-190(c)	191-193(c)[67]

* Measured by amino acid analysis of an aliquot of the reaction mixture

(a) The reduced yield of these esters is possibly due to their very low solubility in all common solvents.

(b) It has been observed that this compound may show more than one distinct melting point, presumably because of the existence of several crystalline forms.

(c) As hydrochloride.

Measurement of the Rate of Hydrolysis

A variety of diesters of glutamic acid were hydrolysed by the above procedure. Samples of the reaction mixture were quenched with dilute acid, treated with EDTA, and chromatographed on silica gel plates, using a 1-butanol: acetic acid : pyridine : water (15:3:10:12) solvent. The γ-ester spots were visualised with ninhydrin, and the ninhydrin colour was eluted with ethanol and measured at 250 nm (see Figure 10). The rates of reaction are given in Table III.

TABLE III Rate Constants for the Cu(II)-Catalysed Hydrolysis of Glutamic Acid Diesters

Diester	Rate,* min^{-1}
Glu(OBzl p-NO_2)$_2$	0.54
Glu(OBzl)$_2$	0.54
Glu(OBzl p-Cl)$_2$	0.14
Glu(OMe)$_2$	0.070
Glu(OEt)$_2$	0.067
Glu(OEt 2-Cl)$_2$	0.050

* pH 8, 32°

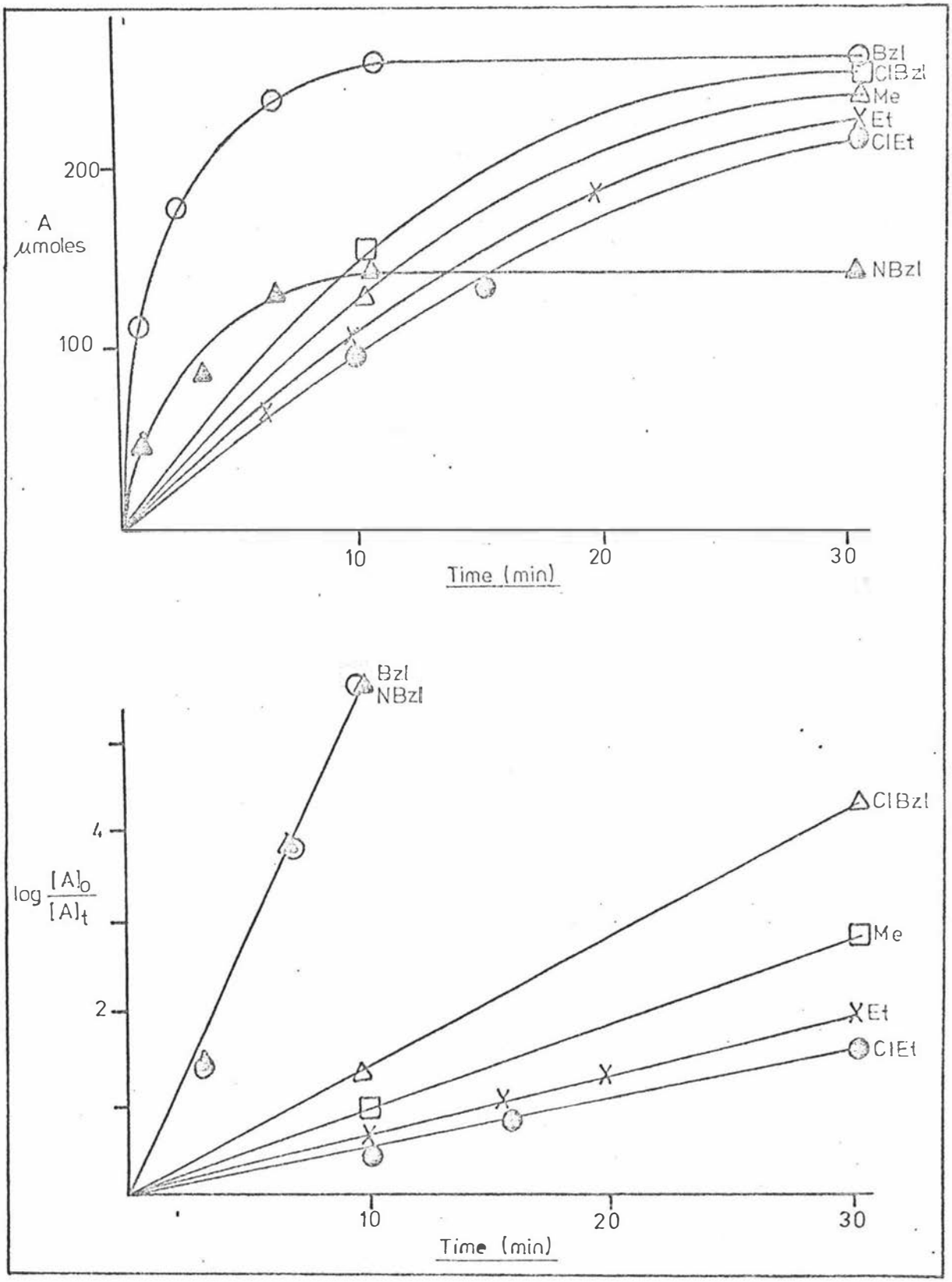


FIGURE 10 The rates of copper-catalysed hydrolysis for substituted benzyl esters of glutamic acid

Discussion

Terashima et al[69] have proposed a structure for the copper complexes of aspartic and glutamic acids where both the amino nitrogen and one of the carboxyl oxygens are coordinated to the copper atom only if a five-membered ring is formed. This proposal was confirmed by the absence in the hydrolysis product of any trace of the α-monoesters of aspartic and glutamic acid or of the free amino acids. Hydrolysis of the β or γ-ester to give these products would require formation of a six or seven-membered ring respectively (see Figure 11).

The mechanism of the copper-catalysed hydrolysis of amino acid esters has been suggested[70] to proceed by OH^- attack on the carbonyl group of the copper coordinated ester linkage. If this is the case, the rate of hydrolysis should be increased by electron-withdrawing substituents on the ester group. To test this hypothesis, the rate of the copper hydrolysis reaction for various glutamic acid diesters was measured (see Table III).

The rates are consistent with the mechanism proposed, in that the ethyl ester reacts more slowly, and the benzyl ester more rapidly, than the methyl ester. Esters substituted with chlorine, however, react more slowly than do unsubstituted esters, in spite of the electron-withdrawing nature of the chlorine moiety. This anomaly is probably due to the large volume occupied by the chlorine atom, as reactions of amino acid copper complexes appear to be very susceptible to steric hindrance[69].

The yields obtained in the hydrolysis of the p-nitro

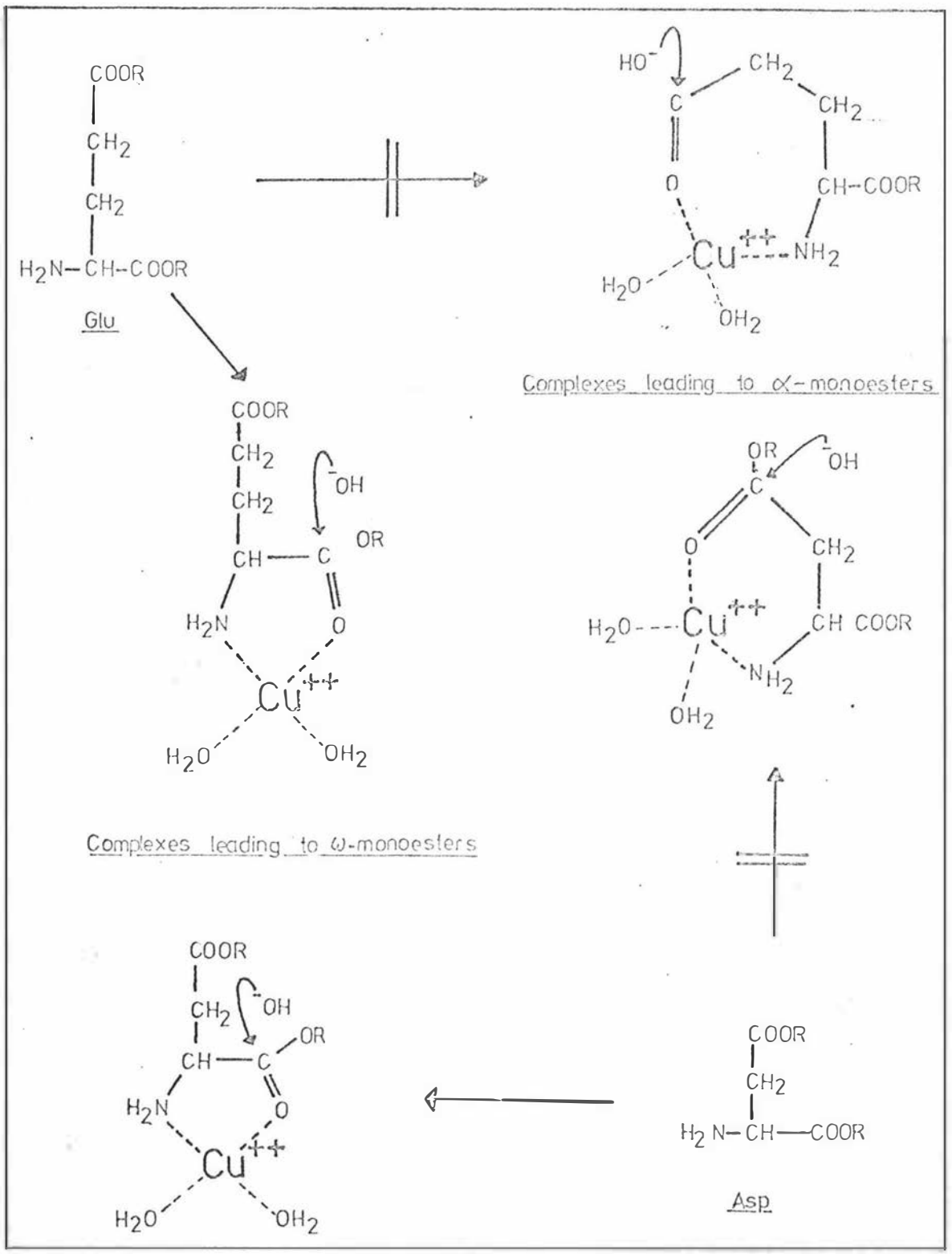


FIGURE 11 Favoured and non-favoured complexes between Cu(II) and glutamic and aspartic acid diesters

benzyl ester of glutamic acid were very variable, and usually lower than those for the corresponding benzyl or p-chlorobenzyl esters. That this poor yield was not due to incomplete reaction of the starting material could be shown by repeating the hydrolysis on the same sample. In a case where the initial yield was 44% based on the starting diester, repeating the hydrolysis on the same sample gave only a further 4% yield. Thin-layer chromatography revealed that the starting material was in fact disappearing, but that no ninhydrin-positive materials other than the product were appearing. It was concluded that the starting diester was decomposing to compounds with no free amino groups. It seems likely that these were pyroglutamic acid or derivatives of pyroglutamic acid. In an attempt to minimise the side-reactions of the p-nitrobenzyl ester, the hydrolysis reaction was carried out at pH 6.0, but this did not improve the yield of the reaction. No attempt was made to isolate or identify the unwanted byproducts, as the yield was adequate for the present studies, and for future work it seemed more promising to seek side-chain protecting groups which were less labile. Several derivatives which seem likely to have greater stability (such as the trifluoromethylbenzyl ester) are currently under study.

PART C

Synthesis of t-Boc Derivatives

For the synthesis of the tert-butyloxycarbonyl (t-Boc) derivatives of the substituted benzyl monoesters of aspartic acids, the method of Stewart and Young[71], employing t-Boc azide as acylating agent, was used. The method of Ragnarsson et al[72], employing t-butylphenylcarbonate, was also successful with the benzyl esters of aspartic and glutamic acids, but with substituted benzyl esters the yields were less than with the t-Boc azide method. The products obtained from the substituted benzyl esters with this method were also less pure, to the extent that they could not be satisfactorily purified by crystallisation or silica column chromatography. This was felt to be due to decomposition of the substituted benzyl ester under the more basic conditions of Ragnarsson's method, which uses tetramethylguanidine as base in place of triethylamine, the base used in the t-Boc azide method.

In the case of the p-nitrobenzyl ester, it could be shown that exposure to basic conditions, including an attempt at column chromatography on alumina, gave extensive degradation of the product and concommitant appearance of a yellow compound. This yellow derivative did not have a free amino group, and an amino group could not be liberated by exposure to HCl vapour.

This reduced stability to basic conditions was felt to be a disadvantage of the p-nitrobenzyl ester. For future studies it is recommended that other derivatives be sought

which could replace the p-nitrobenzyl group for the protection of the side-chain carboxyl groups of aspartic and glutamic residues.

Materials and Methods

t-Boc azide was synthesised by the method of Carpino et al[73], and was used without distillation[74]. Tetramethylguanidine was purchased from Eastman, and t-butylphenylcarbonate from Koch-Light Laboratories.

(I) t-Boc Azide Method

Glutamic acid γ-p-nitrobenzyl ester (2.82g, 0.01 mol) was suspended in dimethylsulphoxide (50 ml). Triethylamine (2.7 ml, 2.0g, 0.02 mol) was added, and the mixture stirred for three days at room temperature, by which time it was homogeneous. The solution was then diluted with three volumes of water and extracted three times with hexane to remove any unreacted azide. The aqueous solution was chilled, acidified to pH 3, and extracted three times with ether. The ether extract was washed with water, dried over anyhdrous magnesium sulphate, and evaporated under reduced pressure. The product was obtained as a yellow oil (3.6g, 94%) which crystallised on standing. The product was homogeneous by TLC on silica gel plates with 1-butanol : pyridine : water (2:2:1, R_f = 0.72) and was used without further purification.

The t-Boc p-chlorobenzyl ester of glutamic acid was synthesised by the same procedure, and was isolated as an oil which crystallised on standing overnight to give the product in 63% yield. The product was recrystallised from ether-petroleum ether: m.pt 80-81°C, TLC (1-butanol :

pyridine: water, 2:2:1) R_f 0.82; (ethanol : aqueous NH_3, 9.1) R_f 0.78. Anal. Calc for $C_{17}H_{22}ClNO_6$ (371.41): C,54.98; H,5.97; N,3.77; Cl,9.55. Found: C,54.91; H,5.84; N,4.10; Cl,9.50.

(II) t-Butylphenylcarbonate Method[72]

Glutamic acid γ-p-nitrobenzyl ester (10g, 35.4 mmol) was suspended in dimethylsulphoxide (50 ml). Tetramethylguanidine (4.1g, 35.4 mmol) and t-butylphenylcarbonate (7.8g, 39.1 mmol) were added slowly over 10 minutes to the stirred suspension. After 20 hours the reaction was almost complete as estimated by TLC. Water (80 ml) was added, and the solution was extracted with hexane three times. The pH of the aqueous solution was then lowered to 3.0 with 10% sulphuric acid, and the solution was extracted three times with ethyl acetate. The ethyl acetate extract was washed with water three times, dried over anhydrous magnesium sulphate, and evaporated under pressure. The product was obtained as a brown oil (7.1g, 53%). The product co-chromatographed with an authentic sample of glutamic acid γ-p-nitrobenzyl ester (prepared by the t-Boc azide method) during TLC on silica gel plates using 1-butanol : pyridine : water (2:2:1, R_f = 0.72). However, it also contained significant amounts of a yellow compound which migrated at the solvent front in the above TLC system, and which could not be removed by repeated crystallisation and silica column chromatography.

PART D

Stability of Substituted Benzyl Monoesters of Glutamic Acid to Acidolytic Cleavage

The γ-benzyl, γ-p-chlorobenzyl, and γ-p-nitrobenzyl esters of glutamic acid were investigated for their stabilities to trifluoroactic acid (TFA) in dichloromethane (1:1), to HBr in acetic acid (33% w/w) and to liquid HF at 0^{o}. The rate of acidolytic cleavage was estimated in the case of TFA by analytical HPLC, and in the case of HBr by semi-quantitative TLC. In the case of HF, the qualitative presence or absence of cleavage was detected by TLC.

The rate of cleavage of these esters by TFA at ambient temperature was extremely slow, and consequently the investigation was conducted at 45^{o}.

Methods

(I) Stability of Monoesters to Trifluoroacetic Acid Cleavage

The equipment used for high-pressure liquid chromatography is described in Section 2 C(II).

Samples of the monoester (1mg) were dissolved in trifluoroacetic acid-dichloromethane (1:1, 0.1 cm^3) and held at 45^{o}C in sealed tubes. At predetermined time intervals a tube was dried with a stream of N_2 and the residue dissolved in potassium phosphate buffer, 0.02M, pH 4.0 (25 cm^3). These solutions were examined by HPLC on a Corasil I pellicular silica column (2 ft x 0.125 in.), eluting with the potassium phosphate buffer at 4 cm^3/min and 2300 psi. The elution was monitored by UV absorption at 210 nm and

calibrated with standard samples. The results and a typical chromatogram are shown in Figure 12.

The rates of deprotection with TFA-CH_2Cl_2 (1:1) at 45°C were Glu(γ-OBzl) 6.6 x 10 $^3min^{-1}$, Glu(γ-OBzl p-Cl) 4.1 x 10 $^3min^{-1}$. Hence the p-chlorobenzyl ester is 1.6 times as stable to trifluoroacetic acid under these conditions as is the benzyl ester.

(II) Stability of Monoesters to HBr-Acetic Acid

The monoester (500 mg) was suspended in HBr in acetic acid (33% w/w, 25 cm^3) and allowed to react at room temperature. Aliquots were taken at various times, dried with a stream of N_2, and dissolved in water (3 cm^3). A portion of this solution (10 µl) was spotted onto silica gel plates which were developed with 1-butanol-pyridine-acetic acid-water (15:10:3:12) and visualised with ninhydrin. The spots corresponding to glutamic acid and the monoester were cut out and eluted with boiling ethanol, and the absorbance of the ninhydrin colour read at 565 nm (Glu) or 520 nm (monoester) and compared with standards. The results are given in Figure 13. The heterogeneity of the reaction mixture made rigorous kinetic analysis difficult, and therefore the time at which the glutamic acid reached a stable maximum concentration was taken as the completion of the reaction: Glu(γ-OBzl) 1.5h, Glu(γ-OBzl p-Cl) 3h, Glu(γ-OBzl p-NO_2) 48h.

(III) Stability of Monoesters to HF Cleavage

The monoester (100 mg) was suspended in liquid HF (10 cm^3) for 30 minutes at 0°C in the HF line. The HF was

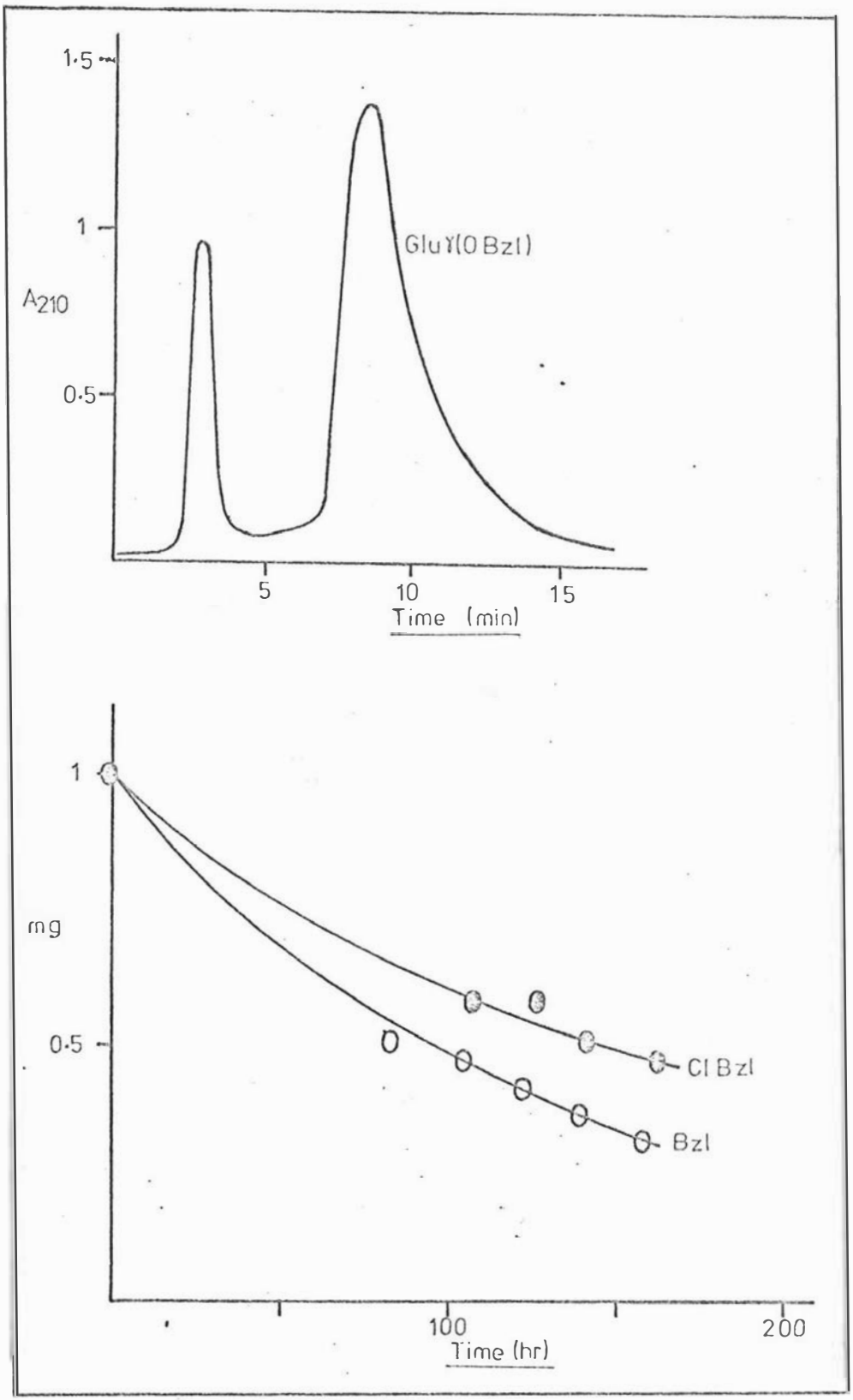


FIGURE 12 High-pressure liquid chromatography of glutamic acid γ-benzyl ester, and the rate of hydrolysis of Glu(OBzl) and Glu(OBzl p-Cl) by trifluoroacetic acid

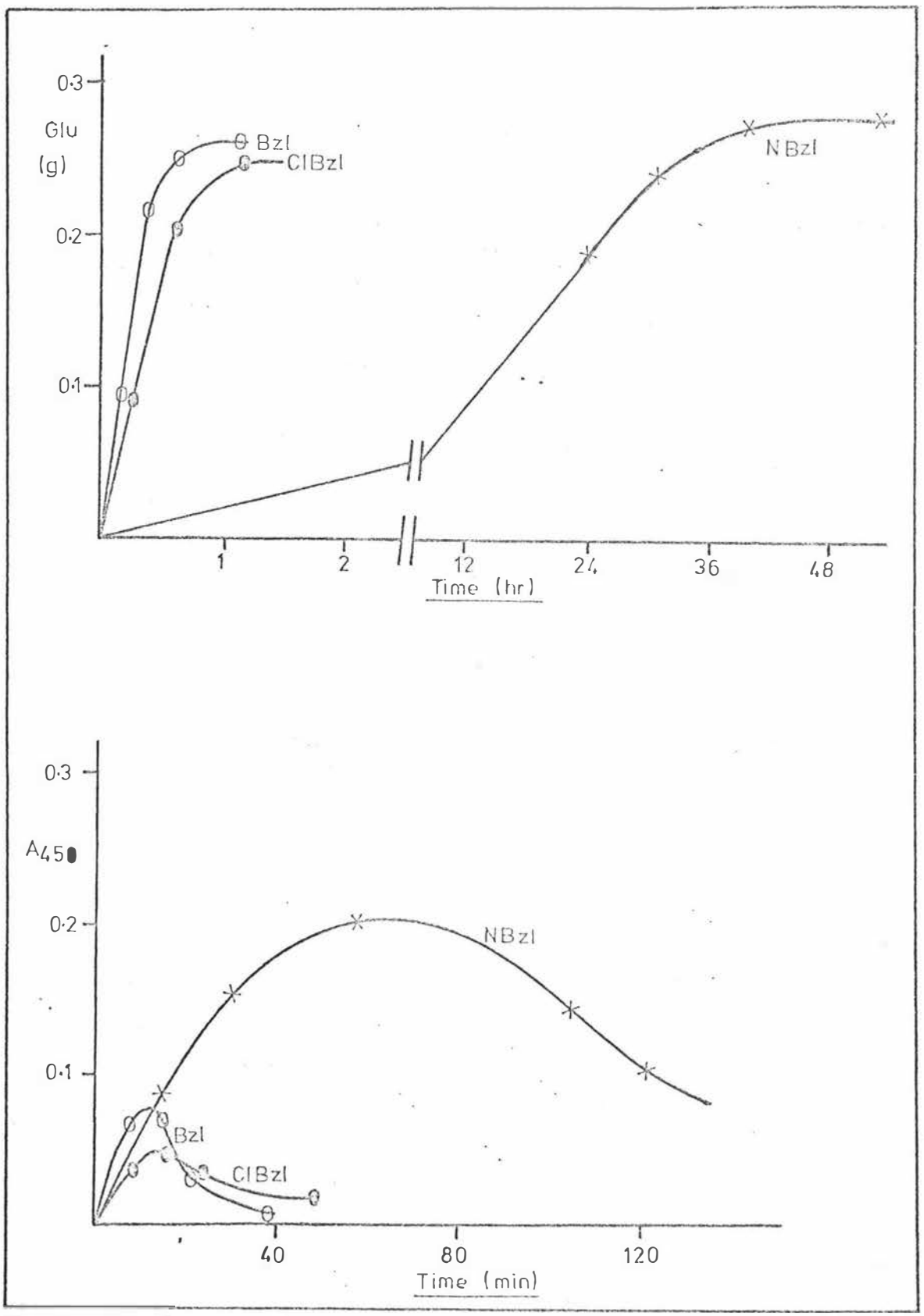


FIGURE 13 The stability of the substituted benzyl esters of glutamic acid and of glutamyl peptides to HBr-acetic acid

(Upper) Release of glutamic acid from its substituted benzyl monoesters

(Lower) Release of protected peptides from the resin and their subsequent hydrolysis

then evaporated off under reduced pressure, and the residue dissolved in water (100 cm^3). Aliquots of this solution (50 µl) were chromatographed on silica TLC plates with 1-butanol : pyridine : water (2:2:1). Both the benzyl and p-chlorobenzyl esters were completely cleaved by HF under these conditions. The p-nitrobenzyl ester was stable, and no free glutamic acid was detected by TLC, confirming the observation of Marglin[75].

PART E

Synthesis of Protected Tripeptides

Model tripeptides incorporating a glutamic acid residue having the side-chain carboxyl group protected by benzyl, p-chlorobenzyl, or p-nitrobenzyl esters were synthesised in order to investigate the stability of these groups to HBr in acetic acid.

Materials and Methods

Synthesis of Protected Tripeptides

Peptides of the sequence H-Gly-Glu(OBzl-X)-Ala-OH, where X is H, p-Cl, or p-NO_2, were synthesised by standard solid-phase techniques[76]. t-Boc-alanine substituted polystyrene-1% divinylbenzene resin (1g, 660 μmol/g) was used in each case. The t-Boc group was used for α-amino protection, and 50% trifluoroacetic acid in dichloromethane was used for deprotection. The coupling reagent used was dicyclohexyl-carbodiimide.

Portions of the peptide-resins (500 mg) were cleaved with HBr in acetic acid (33% w/w, 20 cm^3). Samples (1 cm^3) were taken at various time intervals, dried with a stream of N_2, dissolved in water (0.5 cm^3), and analysed by high - voltage paper electrophoresis at pH 6.5 and 2.1 in pyridine-acetic acid-formic acid buffers. The electrophoretograms were visualised with ninhydrin and the appropriate spots cut out and eluted with boiling ethanol. The absorbances of the ninhydrin colours were read at 450 nm (for protected peptides) or 400 nm (for deprotected peptides). The results are shown in Figure 14.

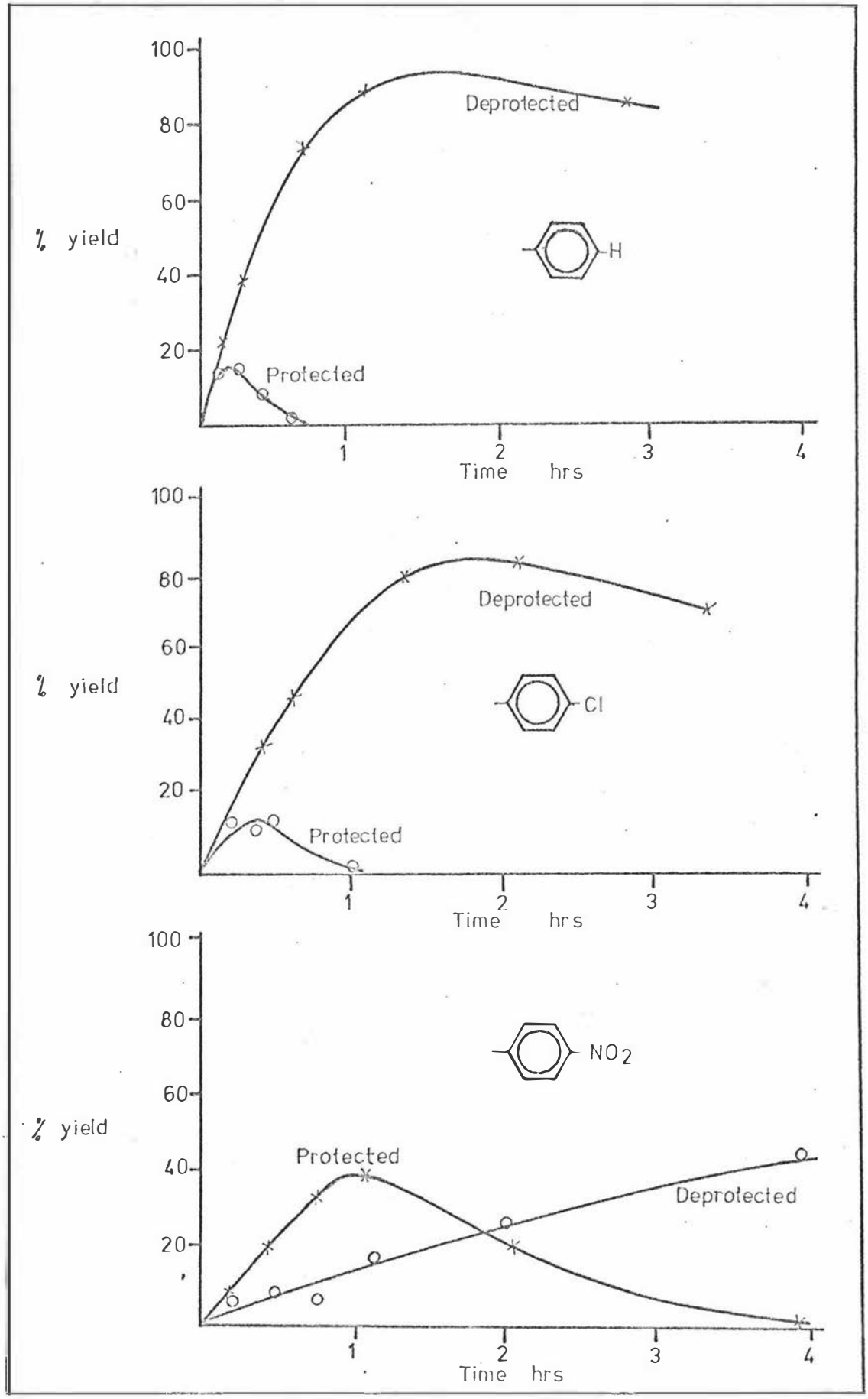


FIGURE 14 HBr-acetic acid cleavage of the protected tripeptides H-Gly-Glu(OBzl-X)-Ala-OH from the resin (where X = H, p-Cl and p-NO_2). Quantitation of protected and deprotected forms.

In addition to the desired peptide which was estimated in this way, a variety of other products also appears in the reaction mixture within a few minutes of the beginning of the cleavage. (See Figure 15). The first additional product to appear was purified by preparative electrophoresis, and was shown to have one net negative charge at pH 6.5, but to have the same amino acid composition as the desired peptide. It was therefore concluded to arise by loss of side-chain protection of the glutamic acid side-chain. Two other peptides, isolated by preparative electrophoresis, were shown to have the same amino acid composition as the target protected peptide, and a fourth did not contain glutamic acid after hydrolysis. From the amino acid composition and electrophoretic mobility of these cleavage products, it was postulated that both intramolecular rearrangement and peptide bond cleavage were occurring to a significant extent with cleavage times longer than one hour.

The tripeptide H-Gly-Glu(OBzl p-NO_2)-Ala-OH was prepared by HBr cleavage of the appropriate peptide-resin (509 mg) with HBr in acetic acid (33% w/w, 7 cm^3) for one hour at room temperature. The cleavage products were evaporated under reduced pressure and the residue containing the peptide was dissolved in 50% acetic acid (3 cm^3) and purified by gel filtration on a Sephadex G10 column (26 x 270 mm) in the same solvent. The peptide was further purified by ion exchange chromatography on a Sephadex SP-C25-120 column (16 x 95 mm) with an ammonium acetate buffer (pH 4.5, 0.01 M). The peptide was eluted from the ion exchange column as a single symmetrical peak, and gave

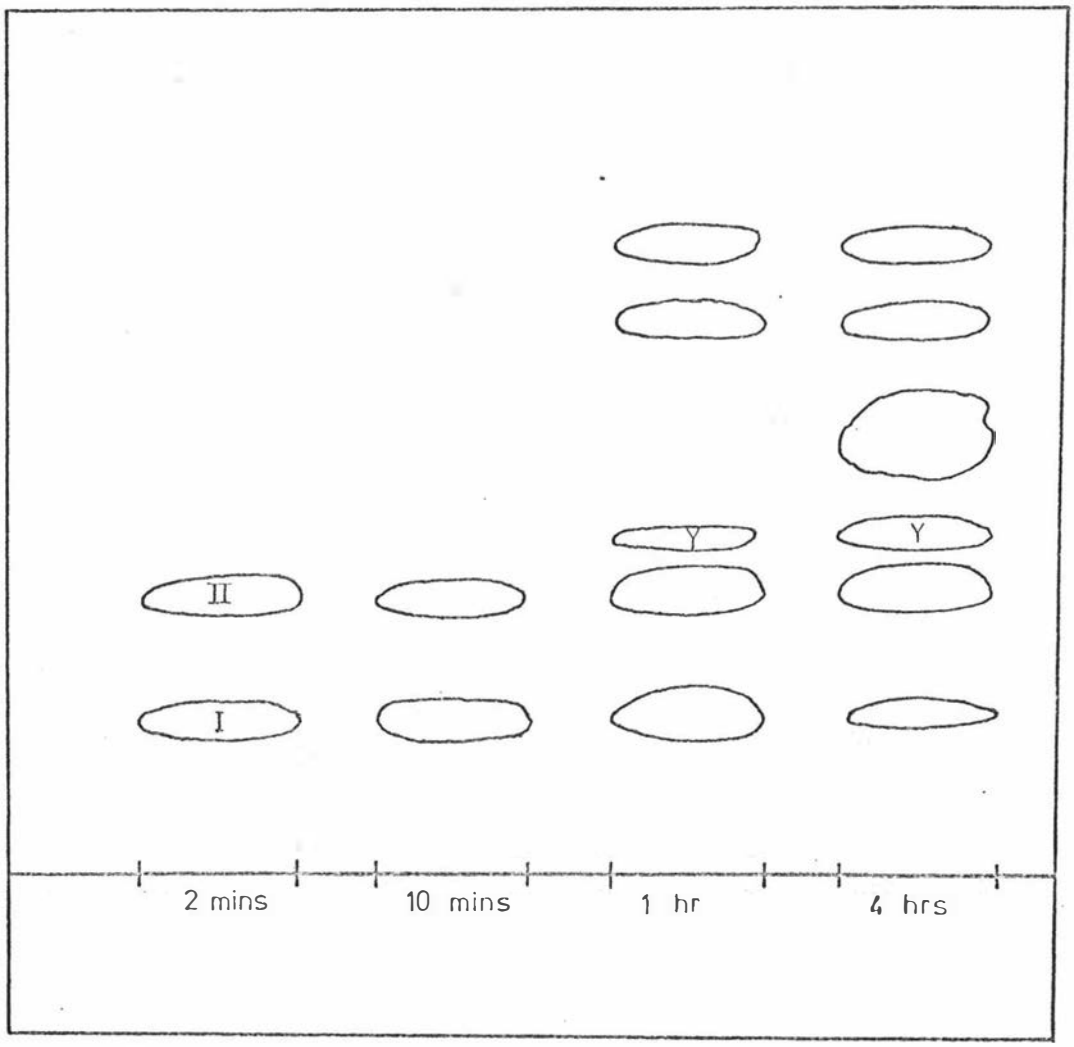


FIGURE 15 HBr/acetic acid cleavage of the protected tripeptide H-Gly-Glu)OBzl p-NO)-Ala-OH from the resin. Paper electrophoresis of cleavage products
I = H-Gly-Glu(OBzl p-NO_2)-Ala-OH
II = H-Gly-Glu-Ala-OH
Visualised with ninhydrin (all spots red/purple with the exception of spots marked Y, which were yellow)

only one spot on high-voltage paper electrophoresis at pH 6.5. The pooled peak was desalted on a Sephadex G10 column (26 x 270 mm) in 50% acetic acid, and lyophilised. The peptide was recrystallised from ethanol-ether to give a white crystalline powder (12 mg, 13% based on picric acid analysis of the resin peptide[77]), m.pt 190.5-192°C. Amino acid analysis after acid hydrolysis (HCl-propionic acid, 1:1, for two hours at 130°C) gave Gly 1.1, Glu 1.0, Ala 1.1. A satisfactory elemental analysis could not be obtained, possibly because of the extremely hygroscopic nature of the peptide. Consequently the material was acetylated to increase the volatility[78] and submitted for mass spectrometry. Although the sample gave no molecular ion, a fragment was observed at m/e 345.0932 consistent with the loss of two water molecules from the desired tripeptide, and all further fragment peaks were consistent with the proposed structure. The peptide was homogeneous on high-voltage paper electrophoresis at pH 2.1 and 6.5 (R_f 0.75 and 0.0 respectively, relative to glutamic acid).

Discussion

The benzyl and p-chlorobenzyl protecting groups were not expected to be stable to HBr in acetic acid. However, it has been reported often in the literature[24] that the p-nitrobenzyl group is completely stable to HBr. Consequently the above results (Figure 13) which show the complete removal of p-nitrobenzyl protection within a few hours exposure to HBr in acetic acid, and a low yield of protected peptide even when the HBr cleavage was limited in duration, were seen to be anomalous. It was thought that the presence of glycine

as the next amino acid in the peptide chain might have increased the rate of molecular rearrangement, and hence accelerated the loss of side-chain protection. In peptides where the amino acids adjacent to the glutamic residues had bulky side-chains, it was postulated that p-nitrobenzyl ester protection might be more stable to HBr cleavage.

Although rearrangements were occurring during cleavage of the above protected tripeptide from the resin with HBr/ acetic acid, a significant yield of pure tripeptide was isolated. The yield was expected to be even better in the case of the ACP 1-6 hexapeptide, where the glutamic acid residues are not adjacent to glycine in the peptide chain. For this reason the p-nitrobenzyl group was used for the synthesis of several analogues of the 1-6 hexapeptide from the acyl carrier protein of E. coli as detailed in the next section.

SECTION 2

SYNTHESIS AND PURIFICATION OF THE FULLY-DEPROTECTED ACTIVE ESTER DERIVATIVES OF THE ACP 1-6 HEXAPEPTIDE AND SEVERAL ANALOGUES

The p-nitrobenzyl group was chosen for the protection of the side-chain carboxyl groups of aspartic and glutamic acid residues during the synthesis of these peptides. According to published reports[24] this group should be completely stable to HBr in acetic acid, the reagent used to cleave the finished peptide from the resin. The stability of these esters was borne out in the tests in Section 1(D) on the side-chain esters of glutamic acid. As the free amino acid was stable in its protected form, the assumption was made that this would also be true when the glutamic acid was involved in peptide bonds with both α-amino and α-carboxyl groups. Consequently the result in Section 1(E), which showed that the side-chain protecting group in Gly-Glu(OBzl p-NO_2)-Ala was hydrolysed at an appreciable rate, was dismissed as an anomaly due to the presence of glycine as the amino acid next to the glutamic residue in the peptide. The first measurements of the stability of the protected ACP 1-6 hexapeptide analogues were carried out using the UV absorption of the cleavage reaction mixture, and these results were interpreted to show that the protecting groups used were relatively stable to HBr in acetic acid. Closer examination by high-voltage paper electrophoresis and column chromatography, however, showed that the desired product

was in all cases being rapidly degraded to a variety of peptides with greater numbers of free carboxyl groups. The presence of significant amounts of free p-nitrobenzyl alcohol was also detected during chromatography of the cleaved peptides. p-Nitrobenzyl alcohol could only be formed during the cleavage reaction by the acidolysis of side-chain protecting groups. It became obvious that the cleavage of side-chain protecting groups from Asp and Gly by HBr in acetic acid proceeds much more rapidly when these amino acids are in a peptide chain than when they are present as free amino acids (Figure 13).

This result is in contrast to the cleavage of these protecting groups by trifluoroacetic acid, where it has been shown[57] that the cleavage proceeds much more rapidly for the free amino acids than when α-amino and α-carboxyl groups are involved in peptide bonds. However, a similar effect has been demonstrated[79] with the p-chlorobenzyl esters of Asp and Glu, in that solvolysis with HBr in acetic acid occurred more rapidly on peptide esters than on esters of the free amino acids. It seems reasonable to speculate that protecting-group cleavage is occurring by a different mechanism in the presence of a good nucleophile such as Br^- than with a poor nucleophile such as CF_3COO^-.

Although the yields of protected peptides isolated by these methods were lower than anticipated, enough material was available from the cleavage reactions to permit the purification of a sufficient quantity of several of the analogues of the ACP 1-6 hexapeptide for use in the semi-synthetic coupling reaction. The great sensitivity of the

ACP assay[29] meant that only very small amounts of each hexapeptide analogue were required.

Two further disadvantages of the use of p-nitrobenzyl esters for the protection of acidic amino acid residues became apparent during the purification of the peptides bearing this protecting group. The first is that glutamic and aspartic acids protected in their side-chain carboxyl groups with this ester, appear to couple in very poor yield to the growing peptide chain during solid-phase peptide synthesis. The reasons for this low yield are obscure: they possibly relate to absorption of the p-nitrobenzyl group to the polystyrene resin matrix, thus preventing the free diffusion of reactants within the resin; or to side-reactions of the activated amino acid which render the carboxyl group unavailable for reaction.

The second disadvantage of the use of p-nitrobenzyl esters is that p-nitrobenzyl peptide derivatives are extremely insoluble in most solvents. The solubility problems encountered in the purification of large fully-protected peptides are well-known[80], and several solvent systems have been developed in which these compounds have good solubility. These are typically aprotic polar solvents such as DMF, N-methylpyrrolidone[80] or DMSO. The low volatility of these solvents, however, hinders the subsequent isolation of the purified products. Another goal of peptide chemists is the development of side-chain protecting groups which increase the solubility of the protected peptide. Unfortunately, it appears that the p-nitrobenzyl group is the reverse: a protecting group which decreases the solubility of the

peptide. In the case of the ACP 1-6 hexapeptide analogues protected by p-nitrobenzyl groups on the side-chain carboxyl groups, the insolubility of these peptides severely limited the number of chromatographic techniques which could be used for their purification. The problems caused by the instability and insolubility associated with the use of the p-nitrobenzyl protecting group were presumably aggravated by the fact that many of the target peptides contained two of these groups per molecule. Isolation of the protected hexapeptides could not be achieved by any of the column chromatographic methods examined. The use of preparative high-pressure liquid chromatography was necessary for preparation of milligram amounts of these peptides in a pure state.

High-Pressure Liquid Chromatography (HPLC) is steadily increasing in popularity as a rapid and sensitive tool for the analysis and purification of a wide variety of chemical compounds. The technique has been made possible by the development of new column packing materials based on silica. These column packings are available in very small and accurately defined particle sizes, and consequently mass transfer rates into and out of the particles are very high. This confers on these column packings a very high resolving power, but also a high resistance to solvent flow through the column. A second advantage of silica-based packings, however, is that they are very resistant to deformation, even under high pressures. It has therefore been possible to use pumping systems and sample injectors which are capable of producing and withstanding pressures of up to 6,000 psi. With such systems, liquid chromatography has advanced

dramatically in speed and resolving power.

Peptide chemists have been slow to use the techniques of HPLC within their field of specialisation. The earliest packing materials were of underivatised silica, and these gave poor results with such polar materials as peptides and peptide derivatives. The interaction of polar materials with silanol (-Si-OH) functions on the silica particles leads to pronounced tailing of the eluted peaks, and consequently a very poor resolution of mixtures of compounds[81]. It was not until the development of bonded reversed-phase columns, where all silanol functions are blocked by reaction with an organic silane, that small-particle silica packings became useful for the analysis and purification of peptides.

There have been very few reported separations of underivatised peptides by HPLC. The most important contributions to the reversed-phase chromatography of peptides have been those of Frei *et al*[82], Burgus and Rivier[83], Chang *et al*[84], and Hancock *et al*[85]. The preparative separation of protected peptides on underivatised silica columns has been reported by Meienhofer *et al*[81], but their work was limited by the severe problems of band tailing mentioned above. Kikta and Grushka[86] have reported the separation of peptides and amino acids on a column composed of peptides bonded to a silica matrix. The efficiency of their columns, however, was very low, and this was thought to be due to low rates of mass transfer into these bonded phases.

Consequently the work reported here is novel, both in that it involves the analysis of protected peptides by reverse-phase HPLC, and in that it involves the preparative

HPLC of these peptide derivatives. Using the methods and columns described in Section 2(CII), 5-10 mg of a crude protected peptide could be analysed and separated within a few hours to give pure products which could not be achieved by any of the other techniques which were attempted (see Figure 26).

The three disadvantages of the p-nitrobenzyl protecting group, its poor stability to HBr in acetic acid, the insolubility it confers on the peptides protected by it, and the low coupling yields experienced with the use of this group during peptide synthesis, combined to result in a difficult purification task. A number of contaminants of the peptide are generated both by incomplete coupling steps and by breakdown of the protected peptide during cleavage from the resin, and the resulting mixture of peptides is of limited solubility. It is an impressive tribute to the power of the HPLC technique that it was able to resolve these peptide mixtures easily, quickly, and with minimal sample pre-treatment. The amount of purified peptide obtained from a single 30-minute chromatographic procedure (1-2 mg) is sufficient for the preparation of 10-20 mg of semisynthetic ACP, for which the sensitivity of the assay is 0.1-0.5 µg. Thus the capacity of the technique was more than adequate for the solution of the separation problems created by the use of the p-nitrobenzyl protecting group.

Although these peptide mixtures could be resolved in this way, the success or failure of this approach to semi-synthesis is dependent on the desired peptide's being a major component of the mixture. Unfortunately, it appears

that from each peptide synthesis, the major product isolated was in fact a pentapeptide lacking one of the amino acid residues which were protected by the p-nitrobenzyl group.

The reason for this phenomenon is thought to be two-fold. In the first place, the low couping yield of amino acids bearing the p-nitrobenzyl moiety would result in a significant number of the peptide chains' being synthesised without this residue. Secondly, the insolubility of the p-nitrobenzyl residue would cause peptides bearing this residue to be selectively lost during purification procedures.

It appears likely that the correct peptide sequence may have been present in the crude peptide after cleavage from the resin. The amino acid analysis of the crude peptide showed that it contained a satisfactory proportion of glutamic acid. When this crude peptide was coupled to the 7-77 peptide by the methods given in later sections, extensive purification of the semisynthetic product yielded a material which was active in the $^{14}CO_2$ assay. Purification of the crude hexapeptide, however, gave in reasonable yield only components lacking one protected glutamic acid residue. Whatever proportion of the crude peptide mixture consisted of the desired protected peptide, it appears that this was selectively lost during purification in favour of deletion peptides and deprotected peptides with greater solubility. None of the components isolated in greatest yield from the mixture, when purified, and coupled to the 7-77 peptide, displayed any activity in the $^{14}CO_2$ assay, even after several steps of purification of the semisynthetic product.

PART A

Synthesis of the t-Boc Arg(NO_2) Resin

It was attempted to use the caesium salt method of Gisin[87] for the attachment of the first amino acid to the resin, as this method was claimed to give both higher coupling yields and also purer synthetic products. The caesium salt method was indeed found to give very high coupling yields, as measured by amino acid analysis and picric acid binding[77]. The resin obtained by this method, however, was found to have unsatisfactory physical properties. In particular, it became extremely sticky after the attachment of the second amino acid. This property of the resin resulted in very low coupling yields for the addition of the third and subsequent amino acids, and also made filtration of solvents from the resin suspension during the synthesis a very slow process.

Similar difficulties have been encountered by other workers using the caesium salt method for the synthesis of resins bearing t-Boc Arg(NO_2)[88] and other amino acids[89].

The reason for the stickiness of the resin encountered here is not clear. One suggestion is that some caesium becomes bound to the resin during the attachment of the first amino acid, and it is not removed by the DMF/water, DMF, and ethanol washes prescribed in the method of Gisin. If so, it is conceivable that further washes with a different series of solvents might remove this bound caesium to produce a resin giving better results in peptide synthesis. The matter was not pursued, however, and a resin was used which

had been made by the more conventional method of Merrifield[90].

Materials and Methods

t-Boc Arg(NO_2) and chloromethyl resin were obtained from Schwarz-Mann. The resin was floated twice in dichloromethane to remove fines, and dried over P_2O_5.

t-Boc Arg(NO_2)-resin which had been prepared by the method of Merrifield[90] was a gift from Dr W.S. Hancock. DMF was distilled from calcium hydride, and stored over molecular sieves. All other solvents were redistilled before use.

t-Boc Arg(NO_2) (5.85g, 18.3 mmol) was dissolved in ethanol (50 ml) and water (10 ml), and the pH of the solution (as measured by a glass electrode) was raised to 7.0 with caesium bicarbonate (1 M). The solution was dried on a rotary evaporator, and the oil obtained was dried over P_2O_5 in a vacuum dessicator (0.1 mm Hg) for three days. The oil was dissolved in dry DMF (200 ml) and chloromethyl resin (10g, 15.3 mmol) was added. The suspension was maintained at 60°C for 21 days.

At this time the resin was collected by filtration and washed with chloroform, DMF, DMF:H_2O (9:1), DMF, and ethanol (three times with each solvent). The resin was dried over P_2O_5, and the amount of amino acid substituted on the resin was determined by picric acid binding[77] and amino acid analysis of the HBr/acetic acid cleavage of an aliquot of the resin (see Part B). The results were: picric acid binding, 550 umoles/g; amino acid analysis of the HBr/acetic acid-cleaved product, 686 umoles/g, no detectable Arg or Orn.

PART B

Synthesis of the Protected ACP 1-6 Hexapeptide and Cleavage From the Resin

The synthesis was performed by conventional solid-phase techniques[76]. The t-Boc group was used for the protection of α-amino functions, and deprotection was accomplished using 50% (v/v) trifluoroacetic acid in dichloromethane. Coupling reactions were performed using amino acid symmetrical anhydrides, which were formed *in situ*. Each coupling was performed twice in each of two different solvents, dichloromethane and dichloromethane:DMF (1:1). A typical coupling cycle is shown in Figure 16.

The α-amino group of the final serine residue was acetylated with acetic anhydride in the presence of triethylamine before cleavage of the peptide from the resin. The cleavage was performed using 33% HBr in acetic acid. Extended cleavage times were thought to be inadvisable on the basis of the results given in section 2(A), and therefore cleavages were limited to two 20-minute periods on any resin sample. A summary diagram of the synthesis is shown in Figure 16.

Materials and Methods

Solid-phase peptide synthesis was performed on a Schwarz-Mann automated peptide synthesiser. All solvents were analytical grade, or purified as in a previous study[76]. t-Boc amino acids were obtained from Schwarz-Mann. A typical coupling cycle is shown in Figure 17, and the procedure for

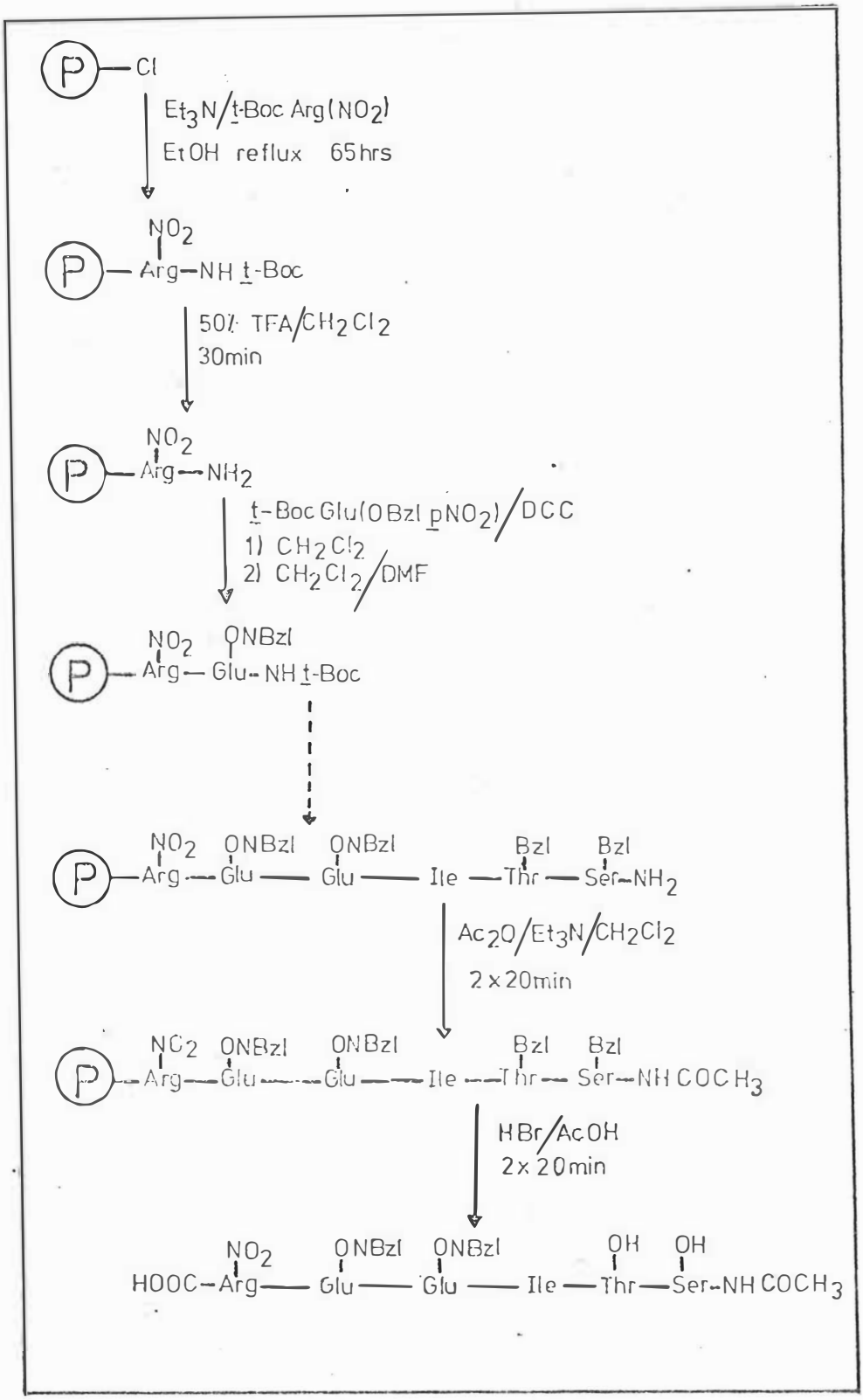


FIGURE 16 Synthesis of the protected form of the ACP 1-6 Hexapeptide

Solvent	Time	Number
Deprotect		
CH_2Cl_2	1'	3
TFA/CH_2Cl_2(1:1)	5'	1
" " (3:1)	30'	1
CH_2Cl_2	1'	3
EtOH	1'	3
CH_2Cl_2	1'	3
Neutralise		
Et_3N/CH_2Cl_2(10%)	5'	2
CH_2Cl_2	1'	6
Couple		
Boc-AA/CH_2Cl_2	5'	Not drained
DCC/CH_2Cl_2(0.5 mole)	60'	Not drained
DCC/CH_2Cl_2(0.5 mole)	60'	drain

The above three steps were repeated once. The coupling of the third amino acid in the peptide is performed in the reverse order; the DCC is added before the amino acid, in order to minimise diketopiperazine formation.

FIGURE 17. Typical coupling cycle for addition of one amino acid residue to the resin-bound peptide chain

a picric acid analysis in Figure 18.

The final picric acid analysis gave 225 umol (80% yield). The peptide-resin was acetylated in the synthesis vessel with a 50-fold excess of each of acetic acid and triethylamine, in dichloromethane as solvent. The peptide-resin was shaken in this mixture for 30 minutes at room temperature, filtered, and washed with dichloromethane (3x), ethanol (3x), and dichloromethane (3x). The acetylation and washes were then repeated.

The peptide was then cleaved without removal of the resin from the synthesis vessel. The cleavage was performed with 33% HBr in acetic acid, and was repeated once. Each cleavage consisted of a 20-minute reaction with HBr/acetic acid, and the appropriate washes, which are detailed in Figure 19. Each cleavage product was evaporated to dryness as soon as possible on a rotary evaporator, using a water-bath temperature of 40^{o}.

Solvent	Time	Number
Deprotect		
CH_2Cl_2	1'	3
TFA/CH_2Cl_2(1:1)	5'	1
TFA/CH_2Cl_2(3:1)	30'	1
CH_2Cl_2	1'	3
EtOH	1'	3
CH_2Cl_2	1'	3
Neutralise		
Et_3N/CH_2Cl_2(10%)	5'	2
CH_2Cl_2	1'	6
Couple		
Picric acid/CH_2Cl_2(sat)	5'	2
CH_2Cl_2	1'	5
Elute		
Et_3N/CH_2Cl_2(10%)	5'	4
CH_2Cl_2	1'	3
EtOH	1'	4

The washes from the last eleven steps were collected, diluted appropriately, and the absorbance at 358 nm was measured.

FIGURE 18 Picric acid analysis of the amount of free amino groups on a resin-bound amino acid or peptide

Solvent	Time	Number
HBr/acetic acid (33%) (manual addition)	30'	1
TFA/CH_2Cl_2(1:1)	5'	3
CH_2Cl_2	1'	4

The above washes were pooled, and the solvents were evaporated under reduced pressure.

HBr/acetic acid (33%) (manual addition)	30'	1
TFA/CH_2Cl_2(1:1)	5'	3
CH_2Cl_2	1'	4

The above washes were pooled with the residue from the first cleavage, and the solvents removed by evaporation under reduced pressure. Acetone was added to the residue, and evaporated under reduced pressure, three times to remove traces of acetic acid.

FIGURE 19 Procedure for cleavage of a protected peptide from the resin using HBr/acetic acid

PART C

Purification of the Protected ACP 1-6 Hexapeptide

(I) Column Chromatography

The low solubility of this protected peptide severely restricted the number of chromatographic methods available for its purification, and many methods were attempted unsuccessfully.

The initial trials were performed on the nonacetylated peptide. The first attempted purification utilised gel filtration on Sephadex G25, with 50% acetic acid as the eluant. This process removed at least two contaminating non-peptide substances, as determined by U.V. absorption and high-voltage paper electrophoresis of aliquots of the column effluent. However, the solubility of the protected peptide in this solvent was less than 0.1 mg/ml, which would have rendered the process too cumbersome for routine use. The peptide at this point gave a satisfactory amino acid analysis after acid hydrolysis, but showed extensive heterogeneity on high-voltage paper electrophoresis at pH 6.5 and 2.1

The next step in a routine purification would be ion exchange chromatography, and this was attempted on the non-acetylated peptide using both Dowex 50W-X12 with aqueous buffers at pH 4.5 or 2.1, and Bio-Rad AG1-X2 at pH 5.0 or 8.5. The solubility of the protected peptide in these solvents was very low, and satisfactory separations could not be obtained.

It was decided at this stage that, since the peptide with a free amino terminus was not particularly soluble in aqueous solvents, subsequent trials would use the N-acetylated peptide. It was hoped that acetylation would confer greater solubility in organic solvents.

Silica column chromatography using a variety of solvents, and partition chromatography on Sephadex G-25 using n-butanol:acetic acid:water (4:1:5), both gave a partial resolution of several components in the synthetic peptide, but complete purification of the product could not be obtained.

The most successful form of column chromatography attempted was that on Sephadex LH-20. Using methanol or DMF as the mobile phase gave almost no resolution of the various impurities. The use of 80% methanol/water, chloroform : hexane : methanol (15:4:1) or chloroform : methanol (9:1) gave better separations of peptide mixtures, but the solubility of the crude peptides in these solvents was inadequate.

Mixtures of ethyl acetate and methanol were the most satisfactory eluants tested with Sephadex LH-20 columns. Adjustment of the proportions of these solvents to give the most useful compromise between resolving power and solubility resulted in a final eluant of 18% methanol in ethyl acetate. A Sephadex LH-20 column with this eluant was used routinely for the preliminary separation of protected peptides from non-peptide contaminants. The products obtained from this column were soluble in a wider variety of solvents, but were still heterogeneous as shown by high-voltage paper electrophoresis.

Materials and Methods

All solvents were redistilled before use. The absorbance at 280 nm of column effluents was measured by a Uvicord monitor. High-voltage paper electrophoresis was performed on Whatman No. 1 paper in pyridine-acetic acid-formic acid buffers, pH 6.5 and 2.1. TLC was performed on Eastman precoated silica-gel plates. Peptides were visualised with ninhydrin or the peptide spray[91].

(a) Gel Filtration in 50% Acetic Acid

The crude non-acetylated peptide was fractionated on a 306 x 25 mm column of Sephadex G-25 eluted with 50% acetic acid. The position of the salt peak was monitored by a conductivity meter. The chromatogram is shown in Figure 20. High-voltage paper electrophoresis revealed that the product was located in fraction I. Fraction II contained free p-nitrobenzyl alcohol, as determined by U.V. spectroscopy, and by high-pressure liquid chromatography on an alumina column using ethanol : acetic acid : water (6:3:1) as eluant. Amino acid analysis of an aliquot of fraction I gave (Arg + Orn)[92] 1.1, Glu 2.15, Ile 1.0, Thr 0.7, Ser 0.7. The low values obtained for serine and threonine are presumably due to their destruction during acid hydrolysis in the presence of N^G-nitroarginine[92].

(b) Ion Exchange Chromatography

Using peak I from the previous column, ion exchange chromatography was attempted using a 105 x 16 mm column of Dowex 50W-X12, and 0.01 M phosphate buffer pH 4.5 or

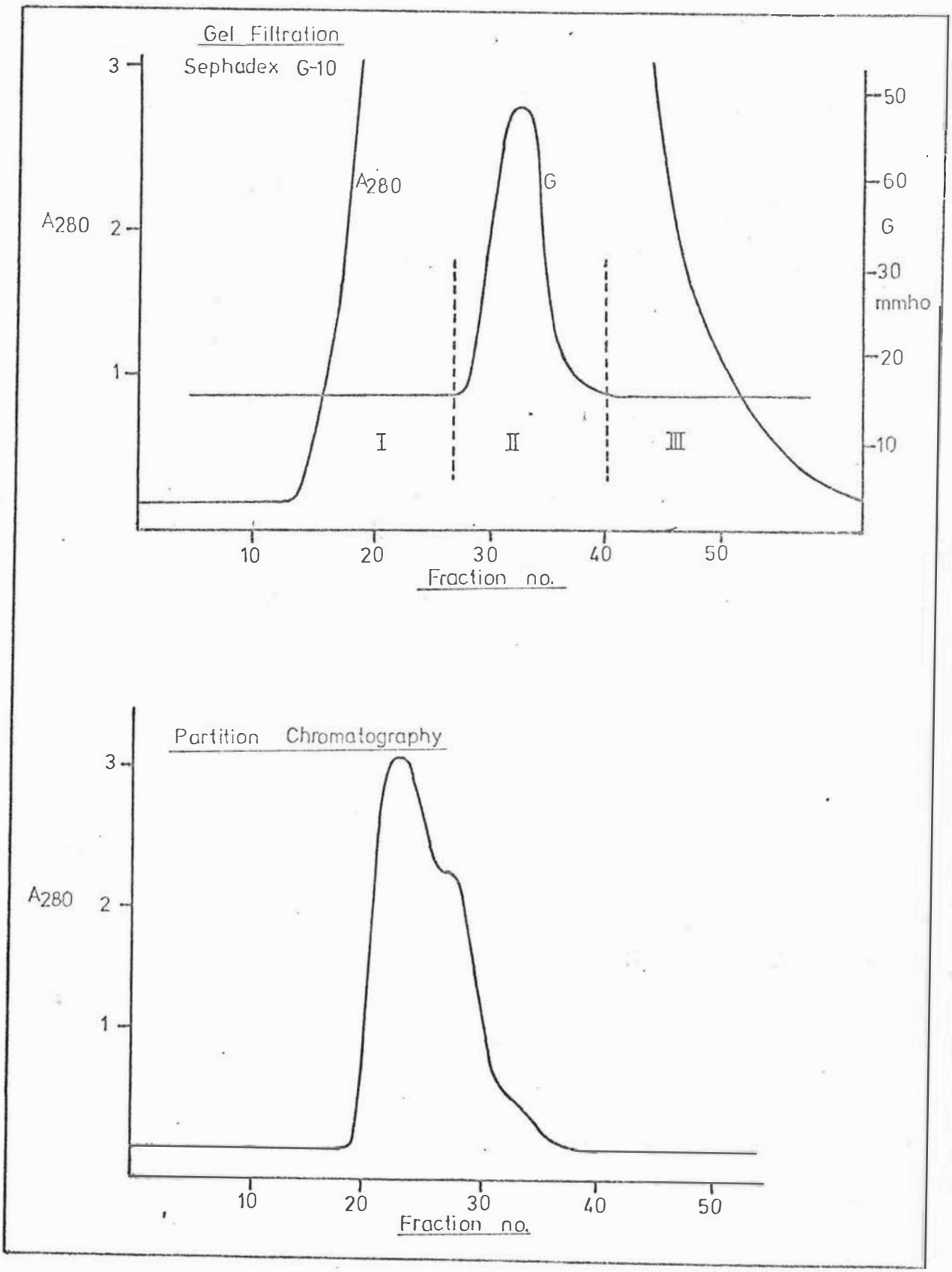


FIGURE 20 Gel filtration and partition chromatography of the protected form of the ACP 1-6 hexapeptide

0.1 M pyridine-acetic acid-formic acid buffers of pH 3.5 and 2.1. Successful purifications were not obtained, and the crude peptides were extremely insoluble in these aqueous buffers. Better solubility was obtained in 0.05 M ammonium acetate buffer pH 8.5, but an attempt at ion exchange chromatography on Bio-Rad AG1-X2 in this buffer was not successful.

(c) Silica Column Chromatography

The crude acetylated peptide was loaded onto a 427 x 30 mm column of silica gel (BDH, 60-120 mesh) in ethyl acetate, and eluted with n-butanol : pyridine : water (2:2:1). Some separation of non-peptide constituents was obtained, but the fully-protected and partly deprotected peptides were found to co-chromatograph in this system. Thin-layer chromatography on silica gel plates with ethanol : aqueous NH_3 (9:1), methanol, and ethanol : triethylamine (9:1) revealed that no useful separations could be obtained with these solvents.

(d) Partition Chromatography

Partition chromatography on a 308 x 25 mm column of Sephadex G-25 was performed by the method of Yamashiro[93] using the two-phase system n-butanol : acetic acid : water (4:1:1). Only partial resolution of the crude acetylated peptide was obtained. The resulting chromatogram is shown in Figure 20.

(e) Sephadex LH-20 Chromatography.

The crude acetylated peptide was fractionated on a 310 x 25 mm column of Sephadex LH-20 using a variety of solvents. The resulting chromatogram is shown for two representative mixtures in Figure 21. The following solvents gave good resolution of the components of the crude peptide mixtures, but poor solubilities of these peptides: 80% MeOH-water, chloroform : hexane : methanol (15:4:1), chloroform : methanol (9:1), and ethyl acetate : methanol (9:1). A typical separation of this type, using chloroform : hexane : methanol (15:4:1) is shown in Figure 21. The following solvents gave good solubilities to the crude peptide mixtures, but the separations obtained were inadequate: MeOH, DMF and ethyl acetate : methanol (2:1 and 3:1). A typical separation of this type, using ethyl acetate : methanol (2:1) is shown in Figure 21.

The most useful separation was obtained from the LH-20 column using 18% methanol in ethyl acetate as the eluant. This gave a useful compromise between resolving power and solubility; a typical separation is shown in Figure 22. High-voltage paper electrophoresis and analytical HPLC (see Section 2(D)) revealed that peak II on this chromatograph contained the major peptide in a partially purified form.

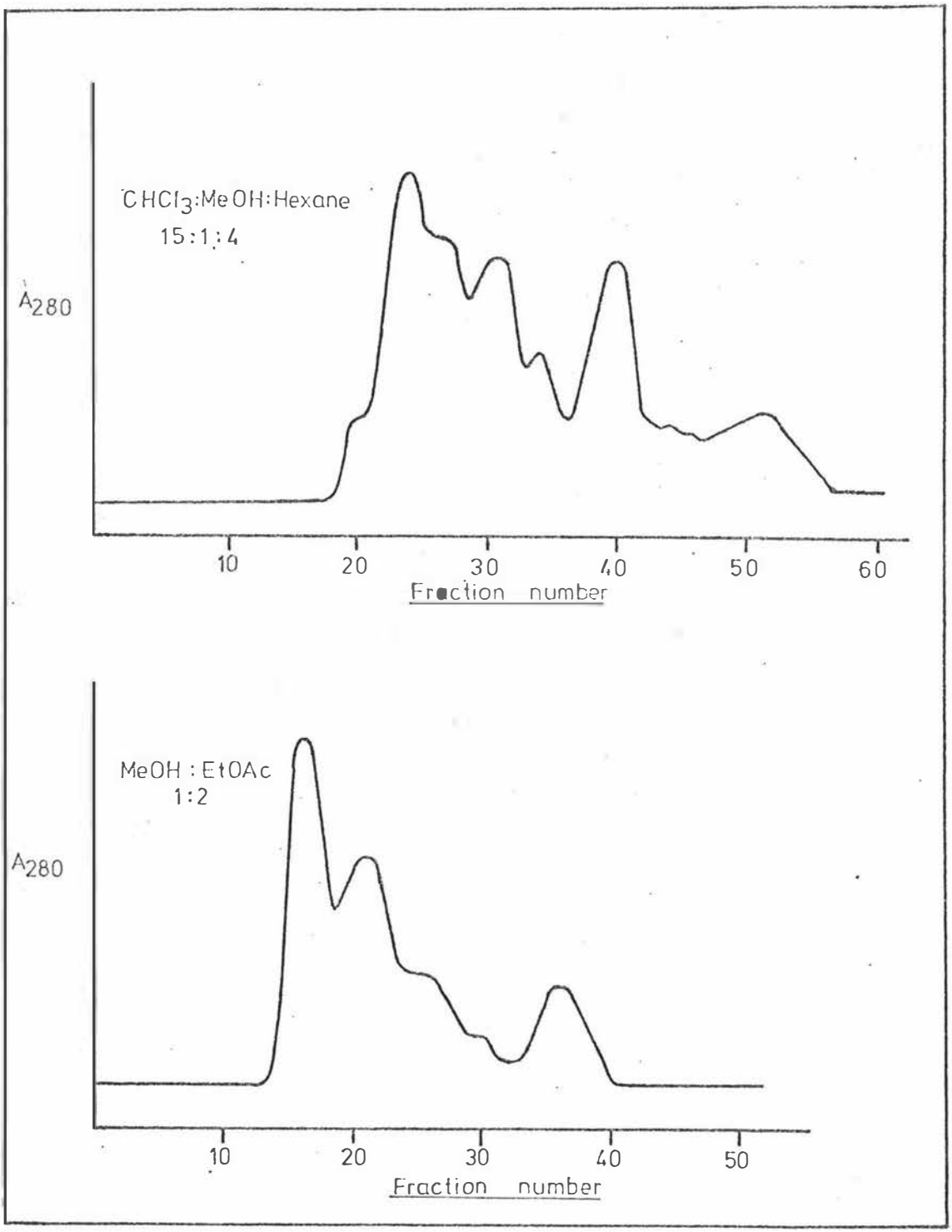


FIGURE 21 Sephadex LH-20 chromatography of the protected form of the ACP 1-6 hexapeptide. Typical unsatisfactory separations.

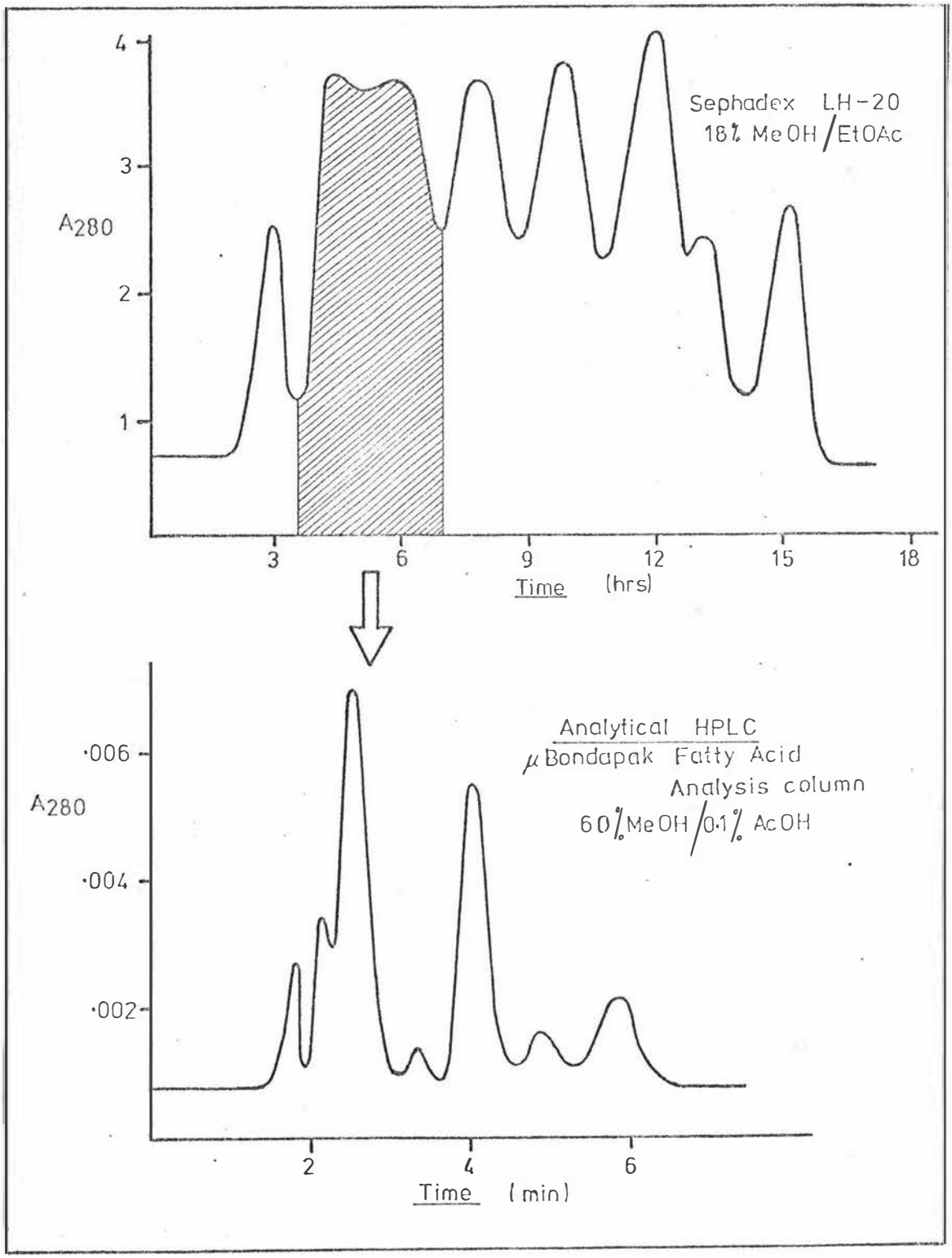


FIGURE 22 Sephadex LH-20 chromatography of the protected form of the ACP 1-6 hexapeptide, and analytical HPLC of the major peptide peak

(II) High-Pressure Liquid Chromatography

Three columns were tested for the purification of the above partly purified peptides.

(a) Waters C18-Porasil B (Octadecyl-silyl silica : bonded reversed-phase partition chromatography column)

This column did not give sufficient retention of these compounds in water-methanol mixtures containing 0.25% acetic acid to give useful separations. (Acetic acid was added to the mobile phase to ensure that free carboxyl groups on the peptides were fully protonated, and thus that the peptides were retained for as long as possible by interaction with the non-polar column packing.) Even when the methanol concentration was as low as 48%, the peptides were essentially unretained by the column (see Figure 23). The use of lower concentrations of methanol was not possible, as the partly purified peptide did not have sufficient solubility in such solvents.

(b) Waters AX-Corasil (Silica column with bonded anion-exchange functions)

This column was investigated with a variety of mobile phases, having methanol concentrations of from 48% to 80%, and pH from 3.15 to 6.0. The compounds eluted as very broad peaks from the column, and no useful separations could be obtained (see Figure 23).

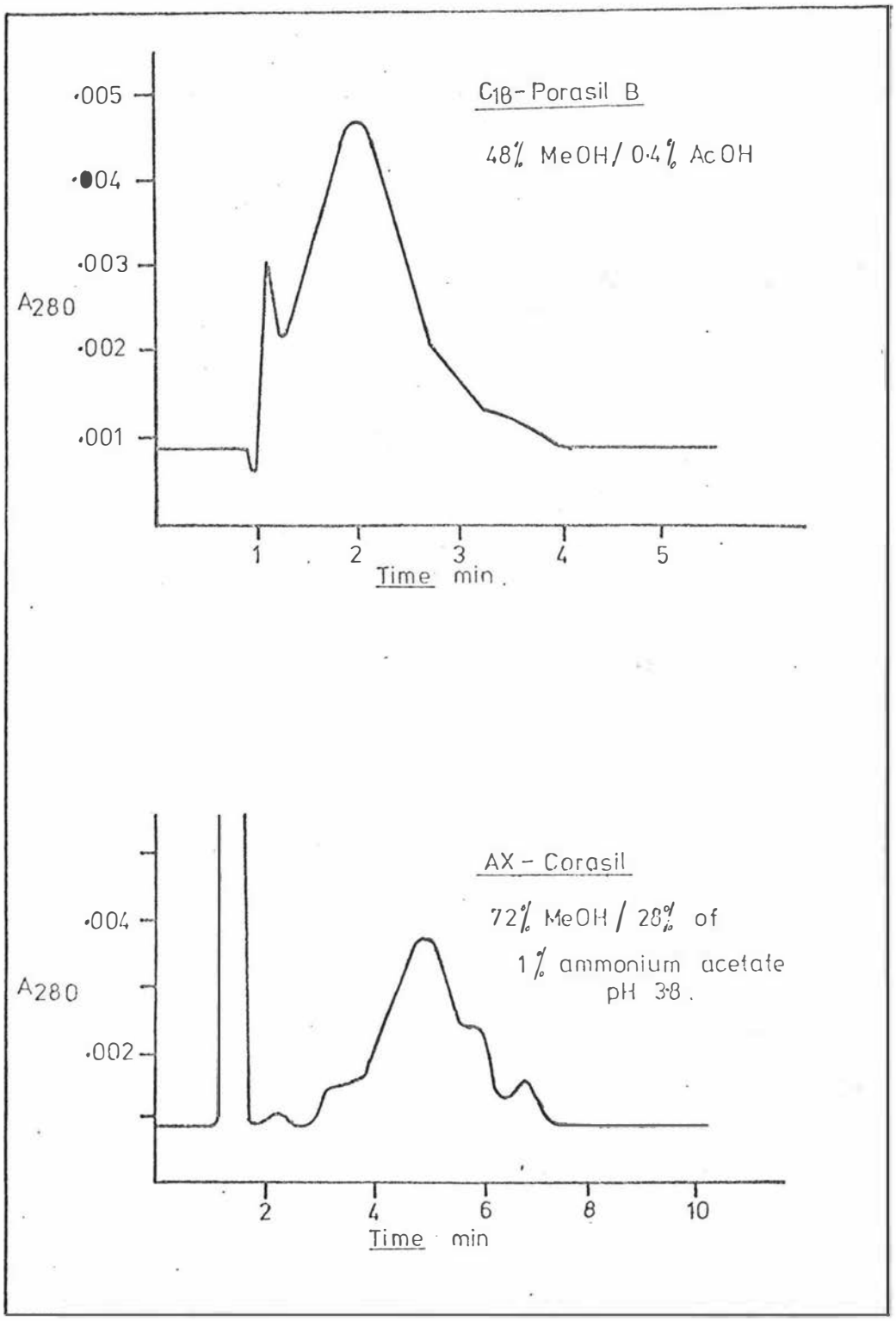


FIGURE 23 Analytical HPLC of protected peptides. Unsuccessful separation conditions

(c) Waters μ-Bondapak Fatty Acid Analysis Column

(Alkyphenyl silica column: microparticulate bonded reversed-phase partition chromatography column)

This column was investigated using mobile phases containing from 48% to 90% methanol, and varying concentrations of phosphoric or acetic acids. The most satisfactory separations were obtained at 60% methanol containing 0.25% acetic acid (e.g. Figure 22).

These separations were transferred to a preparative scale, using two 9mm x 60cm columns of Waters Bondapak Phenyl-Porasil B. This packing material is a large-particle form (37-50 micron) of that used in the Fatty Acid Analysis column. The larger particle size reduces the resolution available with this material compared with the 10 micron microparticulate packing, but it enables the use of much larger columns, and these can be packed by simple manual techniques. This material is also fully porous, and thus larger sample masses can be applied without degrading the resolution of the column.

The column resolution of a preparative column can be effectively increased by recycling the column effluent through the column again. In theory this recycling process can be repeated almost indefinitely, to give any required degree of resolving power. The ability of recycling to multiply the column resolution was verified by the use of a test substance (see Figure 24) but the resolution of the column set used was sufficient for most of the peptide separation problems encountered,

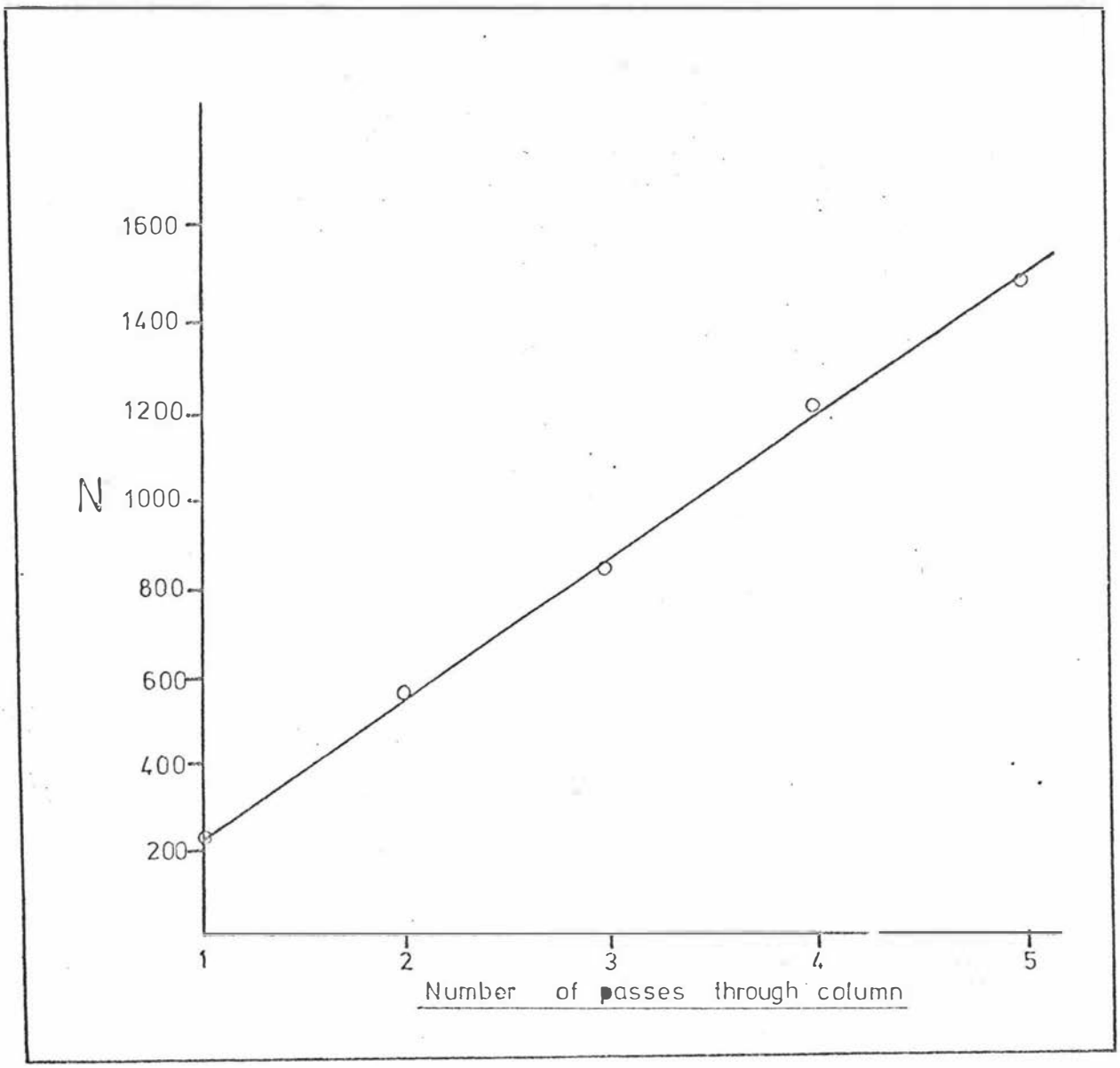


FIGURE 24 The effect of recycling on the resolution of the column set used for preparative HPLC
Test substance = nitrobenzene

without the use of recycling.

The resolution of the column set (measured as the number of theoretical plates, N) was found to be dramatically increased, and tailing of the eluted peaks virtually abolished, by silanising of the stationary phase before packing of the column. The packing material as received from the manufacturer contains a small number of residual silanol groups, which must be blocked by reaction with hexamethyldisilazane to obtain the maximum performance from the packed column.

The greatest sample mass which can be applied to a column without degrading the resolution obtained varies with the sample. Using the column set given in the Materials and Methods section, between 1 and 10 mg of any of the samples encountered in this work could be purified in a single run.

A variety of peaks was collected from HPLC fractionation of each crude peptide mixture. The peptides were identified in each case by retention time, by acid hydrolysis and amino acid analysis, and by high-voltage paper electrophoresis at pH 6.5. In each resin-peptide HBr/acetic acid cleavage, the major product isolated by LH-20 chromatography (see Figure 22) was found to consist of a mixture of two compounds of N^{G}-nitroarginine. These presumably arise from attachment of this amino acid to the resin in sites which are too inaccessible to permit the attachment of a growing peptide chain during synthesis.

The second major peptide isolated in each case was found to be the pentapeptide lacking one of the residues protected by a p-nitrobenzyl group. The target peptide presumably formed one of a very large number of minor peptide products in each mixture. The probable reasons for this situation are given in the Introduction to this section; its result was to make the isolation of the correct peptide from each cleavage product impracticable. The technique of preparative HPLC, however, was completely sufficient for the isolation of any protected peptide from these cleavage mixtures that was present in a large enough proportion to make the task worthwhile. The peptides isolated by this method were judged to be homogeneous when analysed by analytical HPLC and high-voltage paper electrophoresis.

Materials and Methods

High-pressure liquid chromatography was performed using a locally-constructed chromatograph incorporating two Waters model 6000 pumps, a Waters model 660 controller, a Cecil model 212 variable-wavelength U.V. monitor and a Linear Instruments Co. 2-channel chart recorder. Packed columns of C18-Porasil B and AX-Corasil (2 mm x 61 cm) and a μ-Bondapak Fatty Acid Analysis column (4 mm x 30 cm) were supplied by Waters Associates Ltd.

Waters Bondapak Phenyl-Porasil B was silanised by treatment with hexamethyldisilazane (HMDS) to remove unreacted silanol groups. The packing material (75 ml) was placed in a glass column and washed with 5 column volumes of 5% HMDS in

dry toluene. The column was then washed with toluene and methanol.

The absence of any residual silanol groups on the packing material after silanisation was verified by passing a solution of Methyl Red in methanol through the glass column. Any unblocked acidic groups would have given a yellow colouration with this indicator. The packing material was removed from the column and dried over P_2O_5. Two preparative columns (7 mm x 60 cm) were filled with this material by the tap-fill method[94].

Analytical columns were eluted at 1.5 ml/min, and preparative columns at 5.0 ml/min. Plate counts were determined on the μ-Bondapak Fatty Acid Analysis column (N = 6,000 plates/meter) and the Bondapak Phenyl-porasil B preparative column (N = 520 plates/1.2 m) using nitrobenzene as the test substance, and 60% methanol-water containing 0.25% acetic acid as the eluant.

The peptide samples used were partly purified peptide mixtures obtained from the Sephadex LH-20 column as in Part I of this section. They were dissolved in the appropriate mobile phase at a concentration of approximately 1 mg/ml.

(a) Waters C18-Porasil B

This column was eluted with various water-methanol mixtures containing acetic acid. The best separation obtained with this column was using 48% methanol containing 0.4% acetic acid. A typical chromatogram is shown in Figure 23.

(b) Waters AX-Corasil

This column was eluted with various aqueous methanol solutions, buffered at pHs from 3.15 to 6.0. The best separation obtained with this column was using 72% methanol/1% ammonium acetate buffer pH 3.8. A typical chromatogram is shown in Figure 23.

(c) Waters μ-Bondapak Fatty Acid Analysis

This column was eluted with various aqueous methanol mixtures containing from 99.9% to 48% methanol and 0.03% to 1% phosphoric acid or 0.03% to 6% acetic acid. The best results in both resolution and solubility of the crude peptide mixtures were obtained with an eluant composed of 60% methanol and 0.1% acetic acid. A typical chromatogram is shown in Figure 22.

(d) Waters Phenyl-Porasil B Preparative Column

This column set was eluted with aqueous methanol solutions containing 30%, 40%, or 50% methanol and 1% acetic acid. For each sample, the methanol concentration was selected which gave adequate solubility of the sample, combined with a retention time on the column which was neither so short as to prevent the resolution of components of the sample, nor so long as to be wasteful of the mobile phase. The samples were dissolved in the mobile phase. For the initial evaluation of a chromatographic separation, 5-10 μl of a sample containing approximately 1 mg/ml was injected: for a preparative separation, up to 2 ml (the

maximum volume of the sample injector) was loaded.

The samples used were pooled fractions from the Sephadex LH-20 column from which the solvent had been removed by evaporation under reduced pressure. After chromatography on this preparative column set, the appropriate fractions were concentrated in the same way and analysed by high-voltage paper electrophoresis, acid hydrolysis and amino acid analysis, and HPLC on the μ-Bondapak Fatty Acid Analysis column using a mobile phase containing 50% methanol and 1% acetic acid. The peptide products were judged to be homogeneous by these criteria (see Figure 25).

Acid hydrolysis was performed in 6 M HCl for 16 hours. For the major peptide product, the results were: $(Arg + Orn)^{92}$ 1.4, Glu 1.0, Ile 0.88, Ser 0.86, and Thr 0.88. It was apparent from this composition, and from the mobility of the peptide on high-voltage paper electrophoresis at pH 6.5, that this peptide was in fact a pentapeptide lacking one of the Glu (OBzl $\underline{p}$-NO_2) residues. Several of the minor peptide components were also examined, but the fully protected hexapeptide could not be located.

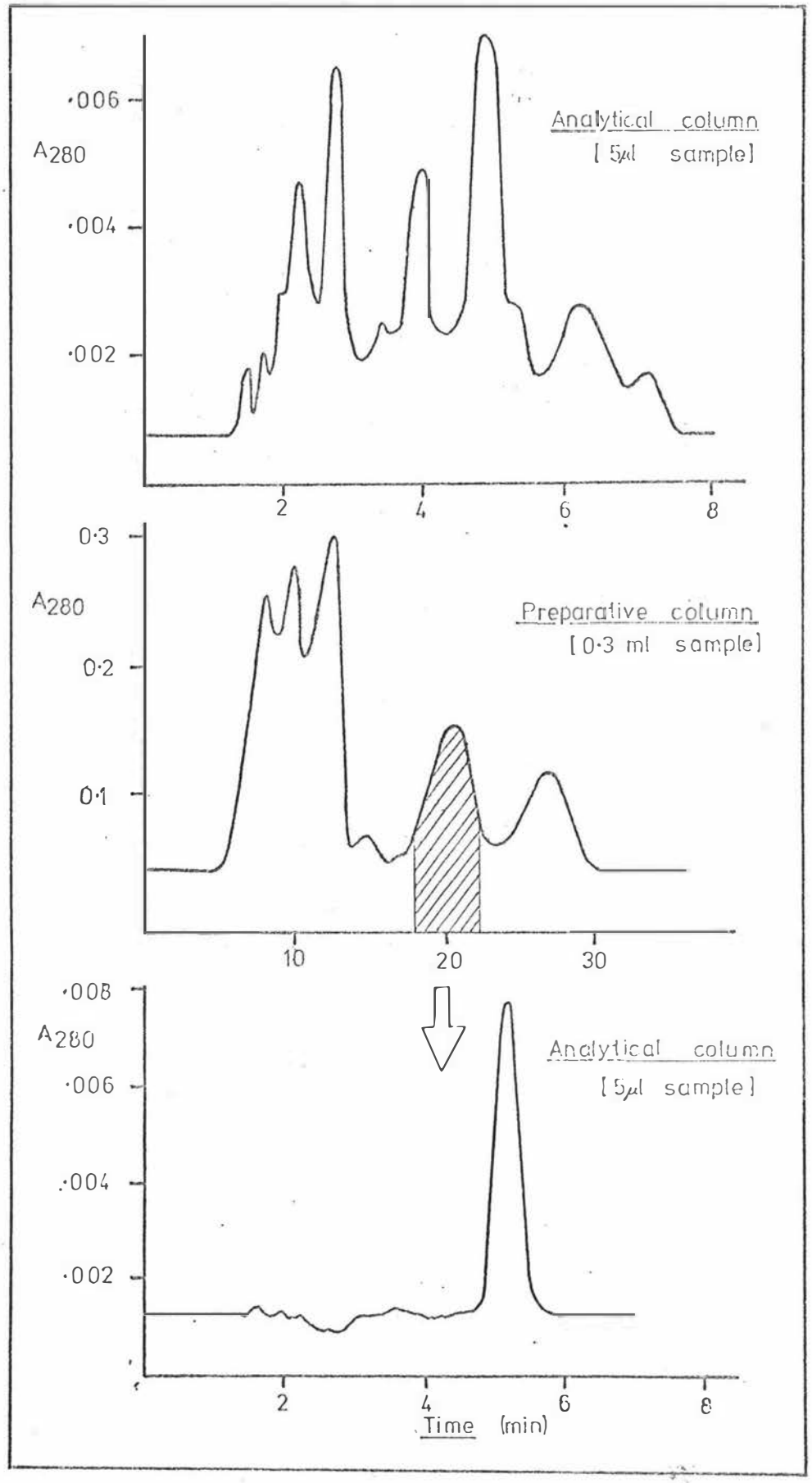


FIGURE 25 Purification of a protected peptide by preparative HPLC

PART D

Preparation of Analogues of the Protected ACP 1-6 Hexapeptide

The synthesis of the following analogues of the ACP 1-6 hexapeptide in fully-protected form was attempted by the methods of Section 2(B): [Gln^4] ACP 1-6, [Gln^5] ACP 1-6, [Asp^4] ACP 1-6, [Asp^5] ACP 1-6, [Ala^1Ala^2] ACP 1-6, [Glu^3Ile^4] ACP 1-6, and [$Glu^3Glu^4Ile^5$] ACP 1-6. These analogues were selected from the very large number of possible variations because they involved modifications of functional groups on the peptide which may play a part in stabilising the active conformation of the entire ACP molecule.

These derivatives were purified using the procedures developed for the ACP 1-6 hexapeptide: Sephadex LH-20 chromatography in 18% methanol in ethyl acetate, followed by preparative HPLC on Bondapak Phenyl-Porasil B in 50% methanol-water containing 1% acetic acid. The purity of the products was examined by analytical HPLC, high-voltage paper electrophoresis, and acid hydrolysis and amino acid analysis.

In no case was the protected peptide of the correct amino acid composition isolated as a major product of the HBr/acetic acid cleavage from the synthesis resin. From several of the above analogues, however, pentapeptides lacking a Glu(OBzl p-NO_2) residue were isolated and purified to homogeneity. Two of these analogues (from the [Gln^4], and [Asp^4]ACP 1-6 syntheses) were prepared in sufficient quantities to enable the preparation of the

corresponding ACP analogues. Amino acid analyses after acid hydrolysis of the protected hexapeptide active esters gave the following results: from the [Gln^4] synthesis, (Arg + Orn)[92] 1.00, Glu 1.06, Ile 0.66, Thr 0.78, Ser 0.84; from the [Asp^4] synthesis, (Arg + Orn)[92] 1.00, Glu 0.92, Ile 1.08, Thr 0.67, Ser 0.63. The low values found for serine and threonine are presumably due to destruction of these amino acids during hydrolysis in the presence of N^G-nitroarginine.[92]

PART E

Preparation and Purification of the Pentachlorophenyl Active Esters of the Protected Hexapeptides

The pentachlorophenyl active esters of the protected peptides were formed by the "crystalline complex" method of Kovacs *et al.*[42] This method uses a 3:1 complex of pentachlorophenol (PCP) and dicyclohexylcarbodiimide (DCC), and is claimed to give the PCP esters of peptide esters without causing racemisation of the C-terminal amino acid.

Materials and Methods

The crystalline PCP:DCC complex was prepared by the method of Kovacs *et al*[42] and recrystallised from ether/hexane.

In a typical procedure, [Asp^5]ACP 1-6 (5 mg, 5 µmol) was dissolved in ethyl acetate (1 ml) and the PCP-DCC complex (5 mg, 5 µmol) was added. The reaction was allowed to proceed in darkness for 16 hours at room temperature, and then quenched by the addition of an excess (20 µl) of acetic acid. The ethyl acetate solution of the product was extracted with water, 0.1 M HCl, water, saturated sodium bicarbonate, and finally water, and dried over anhydrous magnesium sulphate. The solvent was evaporated in a stream of air, and the solid product was extracted with fresh ethyl acetate. The ethyl acetate was evaporated under reduced pressure, and the product was obtained as an oil (6.1 mg, 89%). The product was homogeneous on TLC (silica, ethyl acetate, visualised with I_2 vapour: R_f 0.65) and on analytical HPLC (µ-Bondapak Fatty Acid Analysis column, 50% methanol/1% acetic acid, 1.5ml/min: t_R 3.0 min).

PART F

Deprotection of the Protected Peptide Active Esters

In order to establish the optimal conditions for removal by hydrogenation of the N^G-nitroarginine protecting group without destruction of the active ester, the hydrogenation of the model compounds pentachlorophenol (PCP) and t-butyloxycarbonyl-N^G-nitroarginine pentachlorophenyl ester (t-Boc Arg(NO_2) PCP) was investigated. The latter compound was synthesised by the method of Kovacs et al.[42b] The compound could not be obtained in analytically pure form even after silica column chromatography and repeated crystallisation. This was thought to be due to the presence of the lactam or other decomposition products, as mentioned by Kovacs et al. In spite of the impurities present in this model compound, hydrogenation studies were proceeded with, and some useful information was obtained.

Materials and Methods

Hydrogenations were performed in a Parr pressure hydrogenator at 50 psi of hydrogen pressure. The catalyst, 10% palladium on charcoal, was supplied by Fluka.

t-Boc Arg(NO_2)PCP was prepared by the method of Kovacs et al,[42b] and partially purified by silica column chromatography using diethyl ether as the eluant. Even after repeated crystallisation from carbon tetrachloride-hexane, the compound was found to be impure, as judged by melting point range, TLC (silica, chloroform : acetone 9:1) and elemental analysis.

The hydrogenation of pentachlorophenol was followed spectrophotometrically by removing samples at varying times and plotting their U.V. spectra in the region 280-320 nm. The solvents used in the hydrogenation were:

(1) methanol containing 1 mole HCl per mole PCP,

(2) methanol/acetic acid 10:1,

(3) methanol/acetic acid 10:1 containing 1 mole HCl per mole PCP,

(4) methanol/acetic acid 10:1 containing 5 moles HCl per mole PCP.

In each case the U.V. maximum at 305 nm gradually moved to a small maximum at 295 nm, and then disappeared completely (see Figure 26). The time for this reaction to be completed was approximately 24 hours under these conditions, but even after three hours the reaction had proceeded appreciably. Mass spectral analysis of the reaction mixture at this point showed the presence of tetra- and trichlorophenols in addition to pentachlorophenol. It was obvious that chlorine was being reduced from the phenol ring as hydrogen chloride.

Kovacs *et al*[42a] had also observed this phenomenon, but had stated that the addition of 1 mole of dry HCl had prevented it entirely. However, their hydrogenations were performed at atmospheric pressure and for a much shorter time (15 minutes), as they merely wished to remove the benzyloxycarbonyl group. It is probable that the removal of chlorine was still proceeding under their conditions of hydrogenation, but at the much slower rate observed here.

In order to maintain the reactivity of the PCP active ester, it was therefore necessary to limit hydrogenations to

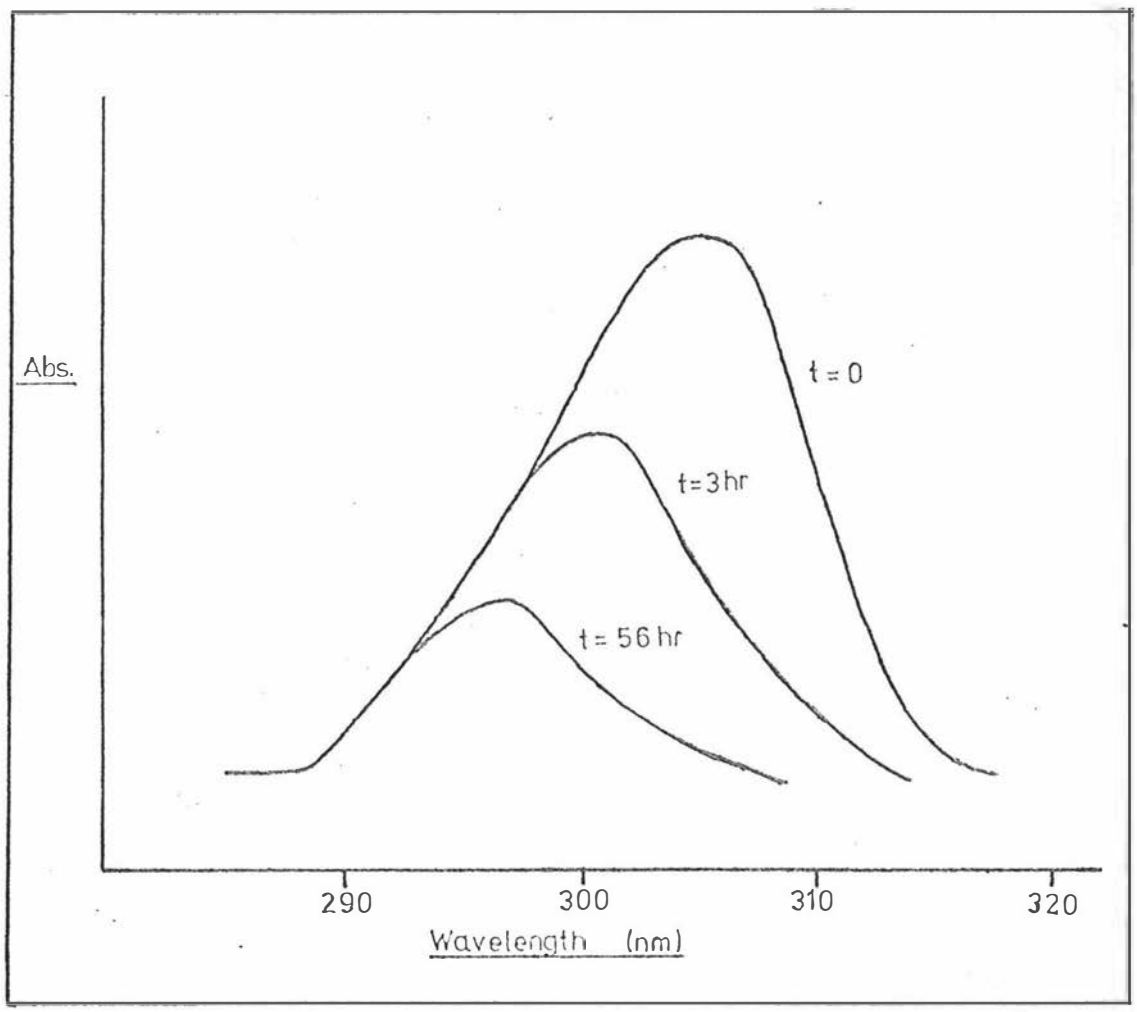


FIGURE 26 The U.V. spectrum of pentachlorophenol during catalytic hydrogenation

three hours under these conditions. HCl was concluded to have no advantage over acetic acid for the protection of PCP esters, and was omitted from the hydrogenation reactions of the peptide active esters.

The hydrogenation of crude t-Boc Arg(NO_2)PCP was monitored by its U.V. absorption at 275 nm. In all of the above solvent systems containing methanol, acetic acid and HCl, the hydrogenation was complete within 30 minutes, the shortest time interval examined.

The hydroxylamine assay of Lipmann and Tuttle[95] revealed that the active ester was still intact at 56½ hours, although by this time it contained a significant amount of tetra- and trichlorophenyl esters as shown by mass spectral analysis.

The conditions chosen for hydrogenation of the protected peptide pentachlorophenyl esters were: methanol/acetic acid (20:1), equal weights of 10% palladium on charcoal and the protected peptide, 50 psi hydrogen in a Parr hydrogenation apparatus for three hours. In all cases, this was sufficient completely to reduce the absorbance of the solution at 275 nm to zero.

At the conclusion of the hydrogenation, the catalyst was washed three times with 1% aqueous acetic acid. The solutions were pooled, stripped, and taken up in 1% acetic acid. This solution was then extracted three times with ethyl acetate, and the aqueous phase was lyophilised and stored at $0^{o}C$ until required for coupling to the native 7-77 peptide. The yield of deprotected peptide active ester was typically 80%, based on the weight of purified protected peptide acid used to form the active ester.

SECTION 3

ISOLATION OF NATIVE 7-77 PEPTIDE, SEMISYNTHETIC COUPLING, AND ASSAY OF PRODUCTS

Acyl carrier protein was prepared from frozen E. coli strain B paste, by the method of Majerus, Alberts and Vagelos,[29] with a few minor modifications.

Approximately 5 kg of E. coli strain B cell paste was grown in a "Fermacell" fermenter on a continuous-flow basis. A minimal medium containing only glucose and salts were used, in order to limit the costs involved. An E. coli auxotroph deficient in β-alanine synthesis was also grown on a medium supplemented with (^{14}C)-β-alanine. Alberts and Vagelos[96] have shown that most of the labelled β-alanine under these conditions is incorporated into the prosthetic group of ACP. In this way a small amount of ^{14}C-labelled ACP was obtained for use in monitoring the purification of larger quantities of unlabelled ACP.

Acetylation and tryptic cleavage of ACP were carried out by the method of Majerus,[34] except that slightly more vigorous conditions were employed for the acetylation to ensure that all lysine residues were completely blocked. The acetylated protein was treated with neutral hydroxylamine to remove acetyl groups from the active-site thiol group as well as from the tyrosine and histidine residues. The acetylation was carried out using (^{3}H) acetic anhydride, to facilitate the location of the product during later

chromatographic procedures. The active-site thiol group was converted to the DTNB derivative[41] to prevent its oxidation during the coupling reaction.

The native acetylated 7-77 peptide and the synthetic 1-6 peptide active ester were coupled in a variety of solvents, and the products were examined for activity in the $^{14}CO_2$ exchange assay.[29,97] The only product with measurable activity was obtained by coupling a crude hexapeptide mixture with the 7-77 peptide, and purifying the resulting semi-synthetic ACP by gel filtration and ion exchange chromatography. When purified peptides were coupled, however, no activity could be detected in the products, even after purification of the semisynthetic proteins by similar procedures.

Amino acid analysis of the various purified semisynthetic products revealed that the coupling reaction was most successful when performed in mixed organic/aqueous solvents. The use of dimethylsulphoxide alone, while giving good solubility to the reactants, resulted in low coupling yields (as determined by the amount of arginine incorporated into the semisynthetic protein). It was also felt that the oxidising properties of this solvent[98] were a disadvantage. Similarly the use of aqueous sodium bicarbonate (0.1 M, pH 8.15),[99] gave a low yield of semisynthetic product.

The most successful coupling reactions were performed in a mixed solvent containing 50% N-methylpyrrolidone and 20 mM potassium phosphate buffer pH 7.0. The use of N-methylpyrrolidone alone failed to give adequate solubility to the 7-77 peptide. The mixed solvent, however, gave good

yields of coupling of the 7-77 peptide to either of two pentapeptides purified from crude hexapeptide mixtures, the [des Glu^5] and [Gln^{4}des Glu^5] ACP 1-6 hexapeptides. Neither of the resulting analogues had any measurable activity in the $^{14}CO_2$ exchange assay.

In view of the observation that less than one mole of arginine was incorporated per mole of 7-77 peptide coupled, it was felt to be necessary to verify that unreacted 7-77 peptide would not inhibit the assay. It was found that a 75-fold excess of 7-77 peptide over ACP did not significantly inhibit the assay reaction. Consequently the assay was felt to be capable of detecting active semisynthetic products even when they were present as less than 5% of the amount of unreacted 7-77 peptide remaining.

The fact that the N-terminal amino acid of the 7-77 peptide was valine probably contributed to the failure of the coupling reactions to go to completion even in the most successful case. The bulky side-chain of the valine residue would be expected to slow peptide bond formation by steric hindrance.

Materials and Methods

All medium constituents were reagent grade. (^{14}C)β-alanine was obtained from International Chemical and Nuclear Ltd. Silicone Antifoam A Concentrate was obtained from Sigma. "Dextrostix" glucose estimation papers were supplied by Ames. Ammonium sulphate (specially low in heavy metals) was obtained from BDH. Dialysis tubing was obtained from Union Carbide, and prepared by the method of Vanaman et al.[100] Streptomycin sulphate was supplied by Glaxo Laboratories,

Palmerston North.

Caproic anhydride was prepared by the method of Gerard and Thrush,[101] and caproyl pantetheine was prepared by the method of Simon and Shemin.[102] Malonyl pantetheine was prepared by the method of Trams and Brady.[103] Acid anhydrides and acyl thioesters were assayed by conversion to the corresponding hydroxamic acids.[95] Radioactivity was determined using Bray's scintillation solvent[104] in a Packard 2002 scintillation counter.

Sodium (^{14}C) bicarbonate was obtained from New England Nuclear, and (^{3}H) acetic anhydride was purchased from the Radiochemical Centre, Amersham. Trypsin (DCC treated) and trypsin inhibitor (soybean type II) were obtained from Sigma. 1 M neutral hydroxylamine was prepared by mixing equal volumes of 4 M hydroxylamine hydrochloride and 3.5 M NaOH immediately before use, and diluting with an equal volume of distilled water.

Samples for amino acid analysis were hydrolysed in 6 M HCl for 16 hours at 110°C in sealed tubes.

Organisms

E. coli strain B was provided by the Department of Microbiology and Genetics, Massey University. *E. coli* strain M-99, a β-alanine auxotroph, was the gift of Dr W. Nulty, Washington University, St. Louis, Mo.

Media

(1) Starter Medium

KH_2PO_4	0.1%	$MgSO_4.7H_2O$	0.07%
K_2HPO_4	0.1%	Na_3citrate	0.05%
NaCl	0.1%	glucose	0.5%
$(NH_4)_2SO_4$	0.4%		

(2) Maintaining Medium

K_2HPO_4	0.5%	$(NH_4)_2SO_4$	0.2%
NaH_2PO_4	0.2%	$MgSO_4.7H_2O$	0.07%
NaCl	0.1%	glucose	0.5%

Media were autoclaved in 1 l flasks or 50 l aluminium vessels for one hour at 15 psi. In all cases the glucose solution was autoclaved separately.

PART A

Growth of E. coli Strain B and Strain M-99

One-litre flasks of the starter medium were innoculated from the initial agar culture, and incubated with agitation on a gyratory shaker at 30°C until the absorbance at 420 nm of the cell suspensension reached 2.0. These flasks were then used to innoculate the fermenter culture.

The "Fermacell" fermenter was operated at a constant volume of 20 l and at 25°C. A 20 l batch of the starter medium was autoclaved and innoculated with the above 1 l cell suspension. Agitation and aeration of the fermenter were commenced, and continued for approximately 8 hours until the absorbance at 420 nm of the cell suspension became constant at about 4.0. From this time the maintaining medium was pumped into the fermenter at 7-8 l/hr to maintain the A_{420} of the culture at its maximum value. The cell suspension was harvested at the same rate by the use of a level probe and associated controls, and a pump, to maintain the fermenter volume constant at 20 l. The absorbance of the cell culture was monitored by a Hitachi spectrophotometer, and the pH by a Radiometer pH meter, both equipped with simple flow-through cells. The residual glucose in the harvested culture was estimated by "Dextrostix" glucose estimation papers.

The cells were harvested in a Sharples continuous-flow cetrifuge at 30,000 rpm and a flow rate of 20 l/hr, and the cell paste was collected and frozen in polythene bottles. The yield was found to be approximately 850 g/day.

Where necessary, foaming of the "Fermacell" culture was

eliminated by automatic addition of silicone antifoam, controlled by a conduction foam probe in the fermenter. With the minimal medium which was employed, however, foaming was not found to be a serious problem.

Cells of E. coli strain M-99 were grown in flasks containing 3 l of the starter medium with 0.5% glucose as carbon source. This medium was supplemented with 0.75 μM (^{14}C)-β-alanine (8 μCi/μmole). The flasks were incubated at 30^{o} with shaking in a gyratory shaker, and the cells were harvested by centrifugation at 8,000 rpm for 25 minutes. The yield was found to be approximately 1 g/l, which is appreciably lower than that for E. coli strain B under similar conditions.

PART B

Isolation and Purification of Acyl Carrier Protein

All steps in the isolation procedure were performed at 4°C. In a typical procedure, 200 g of cell paste was thawed at 0°C and suspended in an equal volume of 10 mM triethanolamine-HCl buffer pH 7.5 containing 10 mM β-mercaptoethanol and 1 mM EDTA using a glass homogeniser. The cells were then ruptured by two passes through a French pressure cell at 8000 psi. The extract was diluted to a volume of 1.2 l using the above triethanolamine-HCl buffer, and subjected to centrifugation at 12,000 g for 30 minutes.

The protein concentration of the supernatant was determined by the biuret method,[105] and 10 ml of a 10% solution of streptomycin sulphate was added per gram of protein. (Typically there was approximately 20 g of protein at this stage.) The precipitate was removed by centrifugation at 12,000 g for 10 minutes. Solid ammonium sulphate (47.6 g per 100 ml) was then added to the supernatant, and the solution was stirred at 0°C for 15 minutes. The precipitate was removed by centrifugation at 12,000 g for 10 minutes. Trichloroacetic acid was added to the supernatant to a final concentration of 10% w/v, and the solution was stirred at room temperature for 20 minutes.

The precipitate of ACP was harvested by centrifugation at 12,000 g for 10 minutes, and resuspended in 50 mM imidazole-HCl buffer pH 7.0 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The pH of the solution was adjusted to 7.0 with 3.5 M NaOH, and the solution was homogenised

thoroughly in a glass homogeniser. The insoluble material was removed by centrifugation at 34,000 g for 10 minutes. Trichloroacetic acid was again added to the supernatant to a final concentration of 10% w/v, and the solution was stirred at room temperature for 20 minutes. The precipitate of ACP was collected in a bench centrifuge, and taken up in 50 mM imidazole-HCl buffer pH 7.0 containing 10 mM β-mercapto-ethanol and 1 mM EDTA. The pH was adjusted to 6.2 with 3.5 M NaOH, and the conductivity was adjusted by dilution with distilled water to be less than 6 mmho at $4^{o}C$. A small amount of ACP, isotopically labelled with (^{14}C)-β-alanine in the prosthetic group (1.2×10^{5} dpm) was added at this point to facilitate the identification of ACP during subsequent chromatographic procedures.

The solution of ACP was loaded on a 110 x 6 mm column of Whatman DE52 DEAE-cellulose, previously equilibrated with 10 mM potassium phosphate buffer pH 6.2 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The column was then eluted with 10 column volumes of this buffer in a linear gradient of LiCl (0.2-0.5 M). ACP was eluted at a conductivity of 21 mmho, which corresponds to an LiCl concentration of 0.36 M in this buffer. The eluate was collected in fractions of 100 drops each, and small aliquots (100 µl) were assayed for radioactivity using Bray's scintillation solvent in a Packard 2002 scintillation counter. A typical chromatogram is shown in Figure 27.

The appropriate tubes were pooled, diluted to a conductivity of 6 mmho at $4^{o}C$, and the pH was adjusted to 7.0 with 1 M NaOH. The solution was loaded onto a 105 x 16 mm

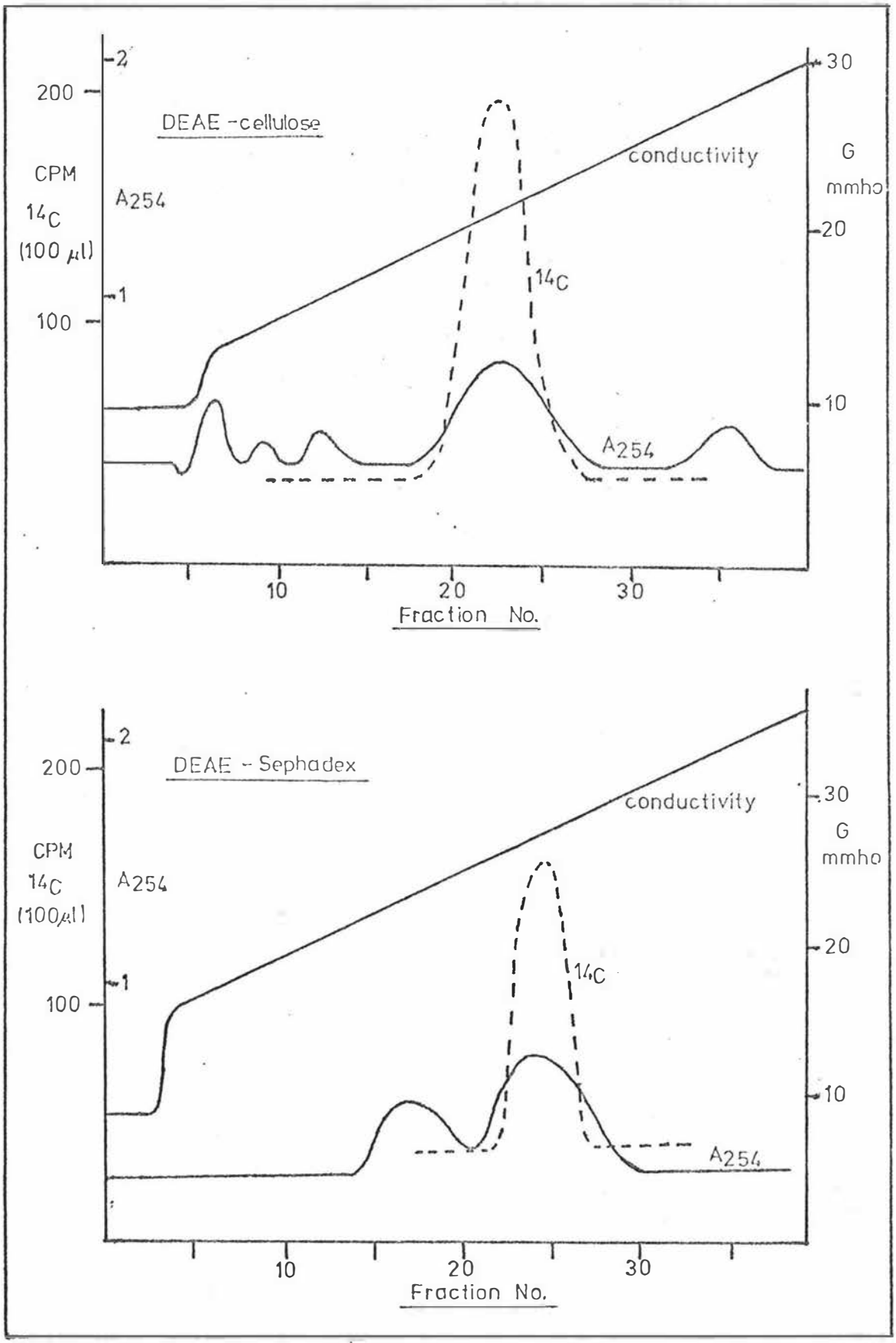


FIGURE 27 Ion-exchange chromatography of (^{14}C)-labelled ACP

column of DEAE-Sephadex A25, which had been previously equilibrated with 10 mM potassium phosphate pH 7.0 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The column was eluted with 10 column volumes of this buffer in a linear gradient of LiCl (0.30-0.55 M). ACP was eluted at a conductivity of 28 mmho, which corresponds to an LiCl concentration of 0.47 M in this buffer. Aliquots were assayed for radioactivity as for the DEAE-cellulose column. A typical chromatogram is shown in Figure 27.

The appropriate fractions were pooled and dialysed overnight against a single 2.5 l charge of 5 mM β-mercaptoethanol. The dialysed solution was concentrated by lyophilisation, and the ACP was precipitated by the addition of solid trichloroacetic acid to a final concentration of 5% w/v. The precipitate was washed with 5% TCA, and dissolved in 10 ml of 0.1 M Tris-HCl buffer pH 6.2 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The pH of the solution was adjusted to 6.2 with 1 M NaOH, and the solution was stored frozen at -20°C. The yield of protein from 200 g of *E. coli* paste was measured by the method of Lowry[106] to be 21 mg.

The ACP obtained from this procedure was found to be homogeneous by SDS-polyacrylamide electrophoresis. The amino acid composition, determined after hydrolysis in 6 M HCl, agreed well with the values determined by Vanaman *et al.*[107]

PART C

Acetylation and Tryptic Cleavage

In a typical reaction, ACP (32 mg, 3.2 μmoles) was dissolved in 0.1 M Tris-HCl buffer pH 8.0 (10 ml). (^{3}H) acetic anhydride (15 mg, 150 μmoles, 80 μCi/mmol) was added, and the reaction mixture was stirred at room temperature for 25 minutes. At this time a further 10 mg (100 μmoles) of acetic anhydride was added, and the reaction was allowed to proceed for 10 minutes. The pH was maintained at 8.0 throughout the reaction by the addition of 1 M NaOH.

The reaction was terminated by the addition of solid trichloroacetic acid to a final concentration of 5% w/v. The precipitate of acetyl ACP was collected in a bench centrifuge, and washed with 5% TCA. The precipitate was then dissolved in 1 M neutral hydroxylamine (6 ml) and the solution incubated at 30^{o}C for 30 minutes. The acetyl ACP was precipitated with TCA, and washed with 5% TCA solution.

The acetylated ACP was taken up in 0.1 M Tris-HCl buffer pH 8.0 containing 10 mM β-mercaptoethanol, and the pH was adjusted to 8.0 with 1 M NaOH. Trypsin (0.6 mg, 2% by weight of the amount of ACP to be digested) was added, and the solution was stirred at room temperature for 1 hour. Trypsin inhibitor (0.7 mg) was added to terminate the reaction, and the solution was loaded onto a Sephadex G25 column and eluted with 20 mM ammonium acetate pH 6.9. Fractions were collected and assayed for radioactivity as shown in Figure 28. The appropriate fractions containing the (^{3}H)-acetylated ACP 7-77 peptide were pooled, the pH of the solution was adjusted

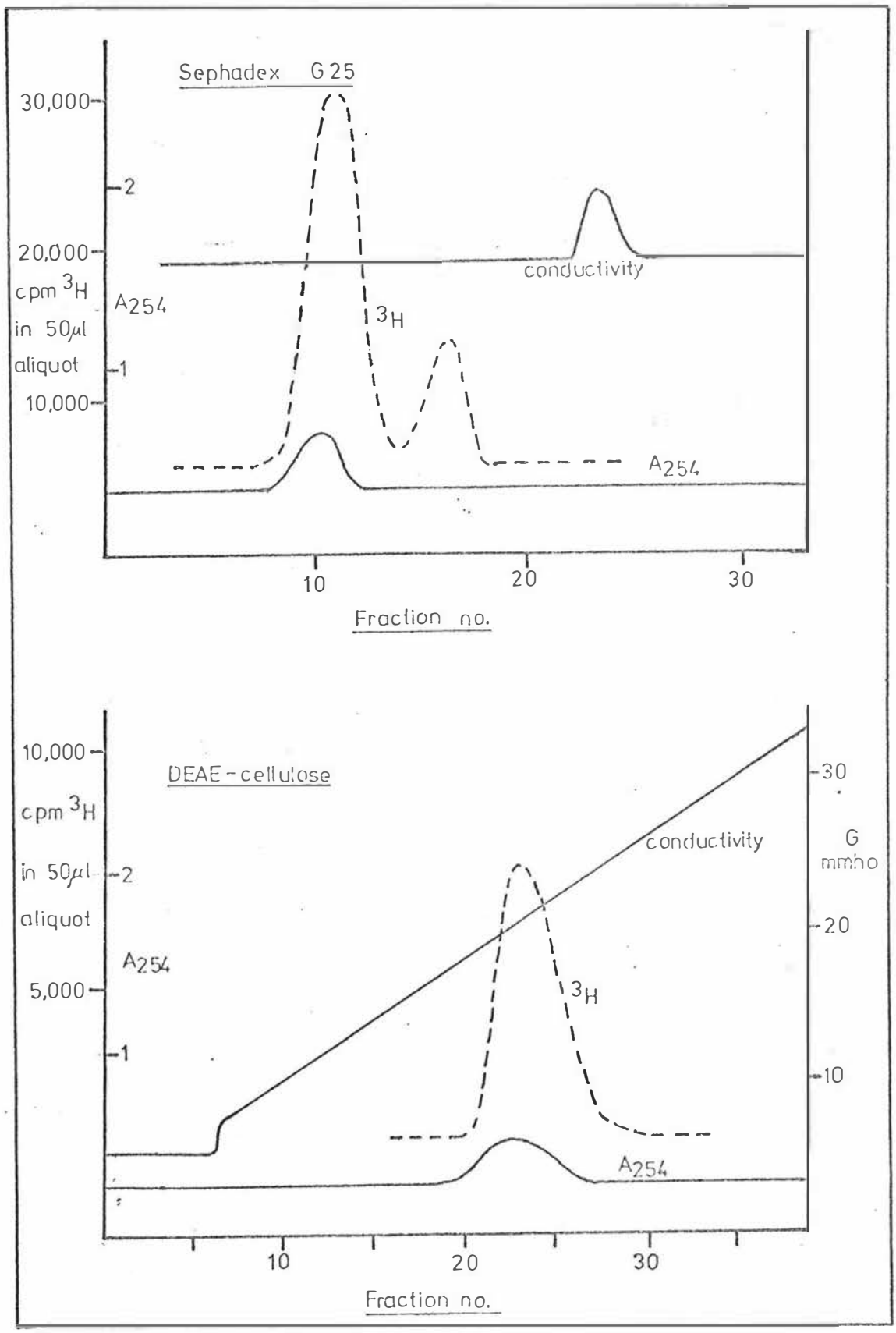


FIGURE 28 Gel filtration and ion-exchange chromatography on the ACP 7-77 peptide

to 6.2, and the solution was loaded onto a 110 x 16 mm column of Whatman DE52 DEAE-cellulose which had previously been equilibrated with 10 mM potassium phosphate buffer pH 6.2 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The column was eluted with 10 columns of the same buffer with a linear gradient of LiCl (0.2-0.5 M). The (^{3}H)-acetyl ACP 7-77 peptide was eluted at a conductivity of 21 mmho, which corresponds to an LiCl concentration of 0.36 M in this buffer. A typical chromatogram is shown in Figure 28.

The appropriate fractions were located by their radioactivity, pooled, and dialysed for nine hours against two changes of 10 mM β-mercaptoethanol. The solution was then lyophilised to dryness, and the product taken up in 0.1 M potassium phosphate pH 8.0. A ten-fold excess of dithiothreitol was added, and the solution allowed to stand at room temperature for 30 minutes to ensure complete reduction of the active-site thiol group. A three-fold excess of 5,5'-dithiobis(2-nitrobenzoic acid) over the total number of thiol groups in solution was added, and the resulting intensely yellow solution was chromatographed immediately on a Sephadex G25 column with 20 mM ammonium acetate pH 6.9 as the eluant.

The product was located by its radioactivity and by its absorbance at 280 and 254 nm. The appropriate fractions were pooled and lyophilised to yield 23 mg (70%) of DTNB-(^{3}H)-acetyl ACP 7-77 as a fluffy white powder.

An aliquot was hydrolysed in 6 M HCl at 110^{o}C for 16 hours and submitted for amino acid analysis. The results are shown in Figure 29, and are in reasonably close agreement

Lys	3.1 (4)	Phe	3.5 (2)
His	0.76(1)	Tyr	0.81(1)
Arg	0.1 (0)	Leu	4.1 (5)
Asp	8.7 (9)	Ile	5.6 (6)
Thr	4.5 (5)	Met	1.2 (1)
Ser	2.5 (2)	Val	6.1 (7)
Glu	15.5 (16)	Ala	6.5 (7)
Pro	1.3 (1)	Gly	3.6 (4)

FIGURE 29 Amino acid composition of the ACP 7-77 peptide. The figures in parentheses are the theoretical values, calculated from the data of Vanaman et al[107]

with the data of Vanaman et al.[107] The high value obtained for phenylalanine is possibly due to interference from unresolved components of the 4'-phosphopantetheine prosthetic group, such as β-alanine and 2-mercaptoethylamine.[108] N-terminal analysis by the dansyl method[109] revealed that valine was the sole amino-terminal residue, and that all lysine ε-amino groups were acetylated. The purified ACP 7-77 peptide was found to have less than 2% of the activity of native ACP in the $^{14}CO_2$ exchange assay as performed by the methods given in the next section.

The conditions employed for acetylation of ACP were more vigorous than those of Majerus.[34] These conditions were felt to be necessary in view of the fact that Majerus was obliged to use ion-exchange chromatography to remove from the desired 7-77 peptide a variety of smaller peptides, presumably resulting from tryptic cleavage at un-acetylated lysine residues. Even with the more vigorous acetylation used here, however, traces of these smaller peptides comprising up to 10% of the bound (^3H) acetyl groups, were produced and removed by the DEAE-cellulose column step.

PART D

Semisynthetic Coupling

In a typical coupling reaction, the DTNB derivative of the (^{3}H) acetylated ACP 7-77 peptide (1 mg, 0.1 μmoles, 0.03 μCi) and the ACP 1-6 hexapeptide analogue PCP active ester (0.3 mg, 0.3 μmoles) were suspended in N-methylpyrrolidone (500 μl) in a screw-capped reaction vial. Nitrogen gas was bubbled through the suspension, and 25 mM potassium phosphate buffer pH 7.0 (500 μl) was added. The reaction mixture was allowed to stand at room temperature for 16 hours, by which time it was completely homogeneous.

At that time, 0.1 M potassium phosphate buffer pH 8.0 (500 μl) and 1 M dithiothreitol (10 μl) were added in order to remove the DTNB protecting group. The resulting yellow solution was chromatographed on a Sephadex G25 column with 20 mM ammonium acetate pH 6.9 as the eluant. The product was located by its radioactivity. The appropriate fractions were pooled, the pH was adjusted to 6.2 with 1 M phosphoric acid, and the solution was loaded onto a 110 x 16 mm column of Whatman DE52 DEAE-cellulose which had previously been equilibrated with 10 mM potassium phosphate buffer pH 6.2 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The column was eluted with 10 column volumes of the same buffer in a linear gradient of LiCl (0.2-0.5 M). The product was eluted at a conductivity of 21 mmho, which corresponds to an LiCl concentration of 0.36 M in this buffer.

The product was located by its radioactivity, and the appropriate fractions were pooled and dialysed for nine hours

against two changes of 10 mM ß-mercaptoethanol. The solution was concentrated by lyophilisation, and reloaded onto the Sephadex G25 column in 25 mM ammonium acetate pH 6.9.

The product was located by its radioactivity, and the appropriate fractions were pooled and lyophilised to dryness twice to give the product (0.6 mg, 60%) as a white powder. A sample was hydrolysed and submitted for amino acid analysis, and the results are shown in Figure 30.

Using the above methods for coupling and purification of semisynthetic ACP analogues, the use of several different solvents was investigated in the coupling reaction. The amount of arginine incorporated into the product was taken as a measure of the coupling efficiency, as a chromatographic technique could not be found to separate ACP and the ACP 7-77 peptide. The results of these investigations are shown in Table IV. It can be seen that the most effective of these solvent mixtures is 50% N-methylpyrrolidone/20 mM potassium phosphate pH 7.0. The fact that the highest yield obtained was 0.60 is an indication that the coupling conditions have not yet been fully optimised. The N-terminal residue of the 7-77 peptide is valine, which is expected to slow the coupling reaction due to the steric hindrance of its bulky side-chain.

Lys	3.8 (4)	Phe	4.0 (2)
His	0.55(1)	Tyr	0.53(1)
Arg	0.60(1)	Leu	4.3 (5)
Asp	9.7 (9)	Ile	6.4 (7)
Thr	5.5 (6)	Met	1.4 (1)
Ser	3.0 (3)	Val	7.3 (7)
Glu	17.0(18)	Ala	6.7 (7)
Pro	1.7 (1)	Gly	4.7 (4)

FIGURE 30 Amino acid composition of semisynthetic ACP The values in parentheses are the theoretical values, from Vanaman et al[107]

TABLE IV Semisynthetic Coupling Yields Using Different Solvents

Solvent	Yield*
0.1 M $NaHCO_3$ pH 8.15	0.20
DMSO + 1 mole Et_3N	0.34
N-methylpyrrolidone + 1 mole Et_3N	0.53
N-methylpyrrolidone/25 mM potassium phosphate buffer pH 7.0 (1:1)	0.60

* Moles of arginine incorporated into the final product as purified by gel filtration and ion exchange chromatography. Coupling reactions carried out for 16 hours at room temperature.

In an earlier experiment, a crude synthetic preparation of the ACP 1-6 hexapeptide was coupled to the native 7-77 peptide in DMSO as solvent, and the product purified by the above procedure. The amount of product isolated was insufficient for amino acid analysis by the available procedures, but the $^{14}CO_2$ assay was performed on this sample as detailed in the next section.

PART E

The $^{14}CO_2$ Exchange Assay

Samples were assayed for ACP activity by the method of Alberts, Goldman and Vagelos.[29,97]

Synthesis of Caproic Anhydride[101]

Caproic acid (10 g, 86 mmol) and pyridine (6.8 g, 86 mmol) were dissolved in dry ether (30 ml) and cooled to -10°C. Thionyl chloride (5.1 g, 43 mmol in 5 ml dry ether) was added dropwise. The solid precipitate was filtered off, and the ether solution was dried over anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure, and the product was distilled under oil-pump vacuum (b.pt. 73° at 0.05 mm Hg) to yield 7.02 g (76%) of caproic anhydride. This compound was 99% pure as determined by conversion to the hydroxamic acid.[95] IR spectra showed the expected anhydride bands (1759, 1812 cm^{-1}) and demonstrated that the product was essentially free of caproic acid (1709 cm^{-1}).

Synthesis of Caproyl Pantetheine[102]

Pantethine (100 mg, 180 µmoles) was reduced with sodium borohydride (150 mg, 3 mmoles) in 1 M Tris-HCl buffer pH 9.0 at 0°C. Nitrogen gas was bubbled through the reaction mixture for 60 minutes. The pH was then lowered to 6.0 with 1 M HCl. The amount of free thiol groups was assayed with Ellman's reagent[41] (230 µmoles, 63% of theoretical yield).

The pH of the solution was raised to 8.0 with saturated sodium bicarbonate solution, caproic anhydride (0.21 g, 1 mmol) was added, and the reaction mixture was stirred at

0^{o} overnight. The pH of the solution was then lowered to 6.0, and the solution was extracted three times with ether. The ether was evaporated under reduced pressure, and the residue was taken up in water (10 ml). The aqueous layer was extracted with hexane, and stored at -10^{o}C. Assay by conversion to the corresponding hydroxamic acid showed that the concentration of caproyl pantetheine was 12 mM. The total yield was 120 µmoles (53%).

Synthesis of Malonyl Pantetheine[103]

Malonyl pantetheine was synthesised from malonic acid monothiophenyl ester, which was prepared by the DCC method of Khorana et al.[110]

Malonic acid (0.2 g, 2 mmol) and thiophenol (0.2 g, 2 mmol) were dissolved in DMF (5 ml) and stirred at 0^{o} C. DCC (1 g, 5 mmol) in DMF (5 ml) was added over a period of one hour, and the mixture was stirred for a further three hours at 0^{o}C. After the addition of 10 ml of water, the stirring was continued for a further 10 minutes. The precipitate of dicyclohexylurea was filtered off and washed with water. The aqueous solutions were pooled and acidified to pH 3.0, and extracted with three volumes of ether. The ether phase was washed with 0.01 M HCl (3 x), and water (3 x) and dried over anhydrous magnesium sulphate.

This ether solution contained 830 umole (42%) of malonic acid monothiophenyl ester, as determined by the absorbance of the solution at 237 nm.

Pantethine (200 mg, 360 µmoles) was dissolved in 1 M Tris-HCl buffer pH 9.0 at 0^{o}C, and nitrogen gas was bubbled

through the mixture. Sodium borohydride (260 mg, 6 mmoles) was added, and the reaction was allowed to proceed at 37°C for 20 minutes. The pH was then lowered to 7.0 with conc. HCl, and the precipitate of sodium borate was removed by centrifugation. Malonic acid monothiophenyl ester (830 μmoles) was added, and the pH was raised to 8.0 with 1 M potassium carbonate. Nitrogen gas was bubbled through the mixture, and the reaction was allowed to proceed at 0°C for six hours. The pH was then lowered to 2.0 with Dowex 50W-X2 resin. The resin was removed by decantation, and washed five times with water. The aqueous washes were pooled, and washed with ether.

Assay by conversion to the corresponding hydroxamic acid showed that the concentration of malonyl pantetheine was 50 mM. The total yield was 500 μmol (25% yield based on the initial malonic acid).

Preparation of Fraction A

Fraction A, a crude preparation from E. coli containing the enzymes of fatty acid synthesis, was prepared by the method of Majerus et al.[29] All operations were performed at 4°C.

E. coli paste (strain B, 25 g) was thawed at 4°C in 10 mM triethanolamine-HCl buffer pH 7.5 containing 10 mM β-mercaptoethanol (25 ml), and then suspended with the aid of a glass homogeniser. The cells were ruptured by two passes through a French pressure cell, and the suspension was diluted to 100 ml with the same buffer.

This suspension was centrifuged at 37,000 g for 30

minutes, and the precipitate was discarded. The supernatant protein concentration was assayed by the biuret method and found to be 25 mg/ml, and the supernatant was diluted with the above buffer until the protein concentration was 16 mg/ml. Streptomycin sulphate (2.5 g in 25 ml water) was added with stirring, and the precipitate formed was removed by centrifucation at 37,000 g for 10 minutes.

Solid ammonium sulphate (22.6 g per 100 ml) was added to the supernatant, the pH being maintained at 7.5 with 1 M NaOH. The solution was centrifuged at 37,000 g for 10 minutes. Solid ammonium sulphate (18.2 g per 100 ml) was added to the supernatant, and the solution was stirred at $4^{o}C$ for 15 minutes. The precipitate was collected by centrifugation at 37,000 g for 30 minutes, and redissolved in 10 mM triethanolamine-HCl buffer pH 7.5 containing 10 mM β-mercaptoethanol (100 ml).

Solid ammonium sulphate (43.6 g per 100 ml) was added, and the solution stirred at $4^{o}C$ for 15 minutes. The precipitate was collected by centrifugation at 37,000 g for 10 minutes, and redissolved in 30 ml of 50 mM triethanolamine-HCl buffer pH 7.5 containing 10 mM β-mercaptoethanol. This solution was stored in 3 ml aliquots at $-80^{o}C$ in a cryogenic freezer. Before assay, an aliquot was desalted on a 14 x 350 mm column of Sephadex G25, which was eluted with 10 mM potassium phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 1 mM EDTA. The protein concentration of this solution of Fraction A was determined by the biuret method to be typically 8 mg/ml, and 60 µl (approximately 500 µg) was used for each assay.

Assay of ACP Solutions

Assay mixtures contained the following reagents:

0.125 M imidazole-HCl pH 6.2	150 µl	18.7 µmoles
1 M ß-mercaptoethanol	10 µl	10 µmoles
12 mM caproyl pantetheine	4 µl	48 nmoles
50 mM malonyl pantetheine	4 µl	200 nmoles
Fraction A	60 µl	- -
0.25 M sodium (^{14}C) bicarbonate (0.2 µCi.umole)	35 µl	6.25 µmoles
ACP	0.1-0.5 µg	

These reagents were mixed in a 30 x 6 mm Durham tube. Incubation was carried out at 30°C for 40 minutes. The reaction was stopped by the addition of 25 µl of 10% perchloric acid, and the reaction mixture was transferred, with two water washes of 100 µl, to a scintillation vial. Bray's solution (5 ml) was added, and the vial was counted in a Packard 2002 scintillation counter.

A typical standard curve obtained with purified ACP is shown in Figure 31. For derivatives of ACP, the following results were obtained (relative to ACP as 100%): acetyl ACP, 90%; acetyl ACP 7-77 peptide, 0% (for quantities up to 25 µg); ACP in the presence of 25 µg of acetyl ACP 7-77 peptide, 100%. (See Figure 32).

The semisynthetic product obtained by coupling a crude synthetic ACP 1-6 hexapeptide with the native acetylated ACP 7-77 peptide, and purifying the resulting protein by the methods of Part IV of this section, gave a positive result in the $^{14}CO_2$ assay equivalent to 41% of the activity of

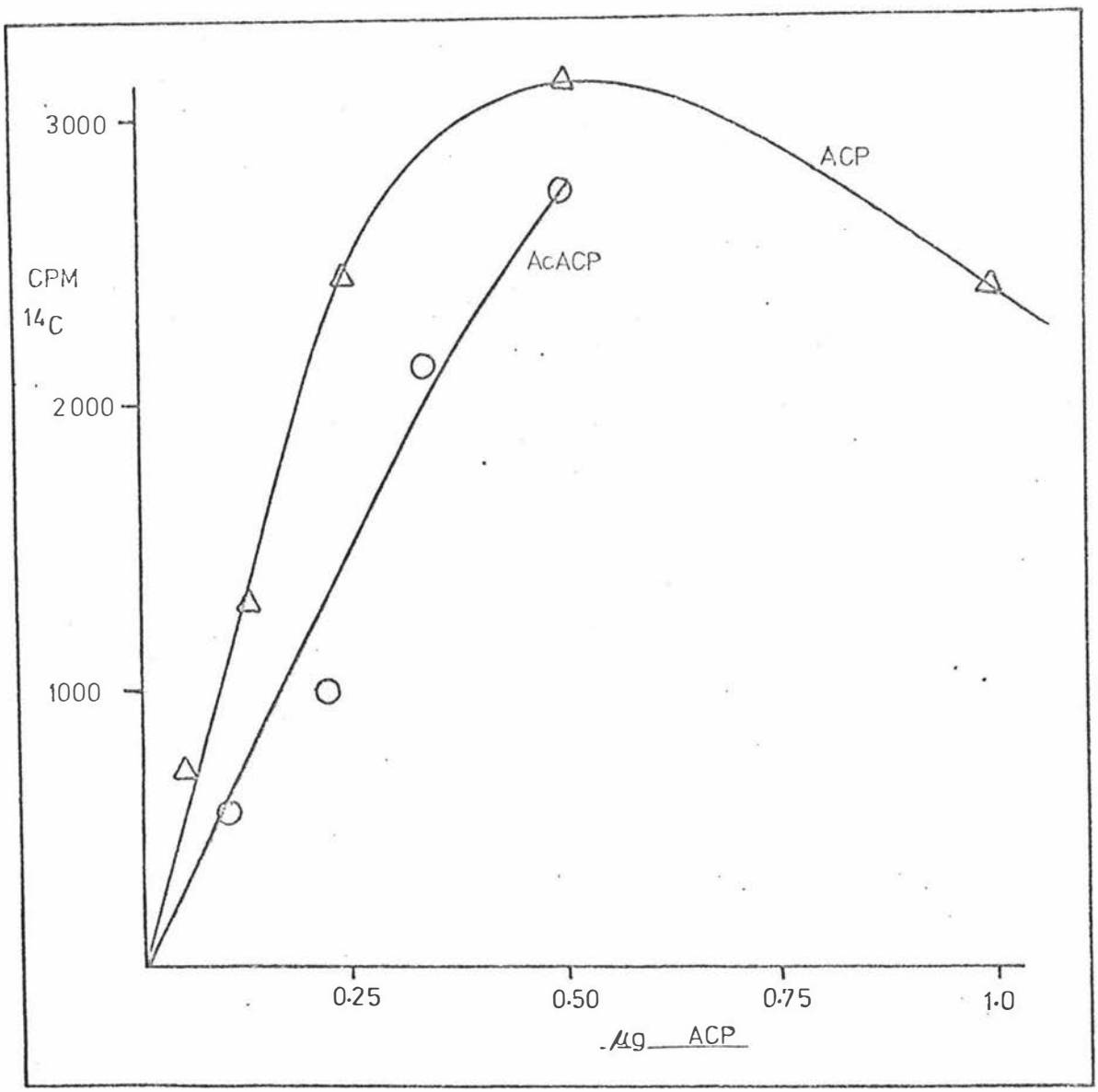


FIGURE 31 $^{14}CO_2$ assay of ACP and acetylated ACP

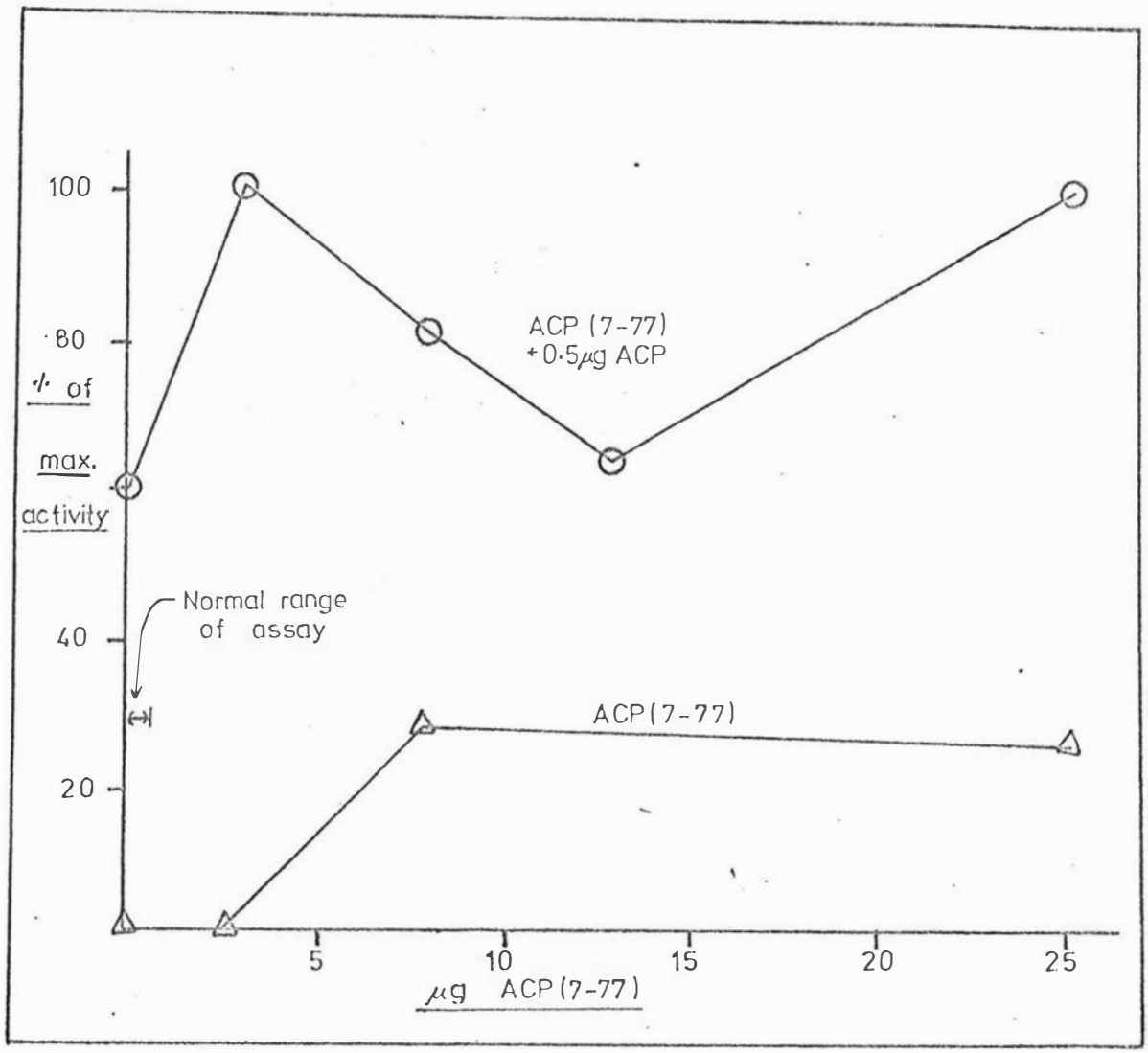


FIGURE 32 $^{14}CO_2$ assay of ACP (7-77) and of ACP in the presence of ACP (7-77)

native ACP (see Figure 33). The assay was inhibited by an excess of this protein, however, at a much lower level than is usual with native ACP preparations.

The inhibition was thought to be due to the presence of inactive forms of ACP which had not been removed by the purification procedures which were used. The combination of ion-exchange chromatography and gel filtration, while removing impurities differing greatly from ACP in size or charge, did not remove for example the unreacted 7-77 peptide. While the 7-77 peptide was shown not to inhibit the assay, it is possible that other inactive derivatives might do so.

Some damaged forms of the protein could conceivably arise from the oxidising properties of the dimethyl-sulphoxide coupling solvent which was used in this coupling reaction.[98] For example, the DTNB group was found to be completely removed from the active-site thiol group during coupling reactions performed in DMSO. It is possible that this thiol group was also partially and irreversibly oxidised during the coupling to give an inactive derivative which also inhibited the assay reaction.

In contrast to the results obtained with the crude hexapeptide, however, no activity could be obtained by coupling either of two purified pentapeptides, the [des Glu^5] and [Gln^4des Glu^5] ACP 1-6 hexapeptides, to the native 7-77 peptide. Purification of the resulting semisynthetic proteins by the procedures used for the product of the crude hexapeptide coupling failed to produce a sample with measurable activity in the $^{14}CO_2$ assay.

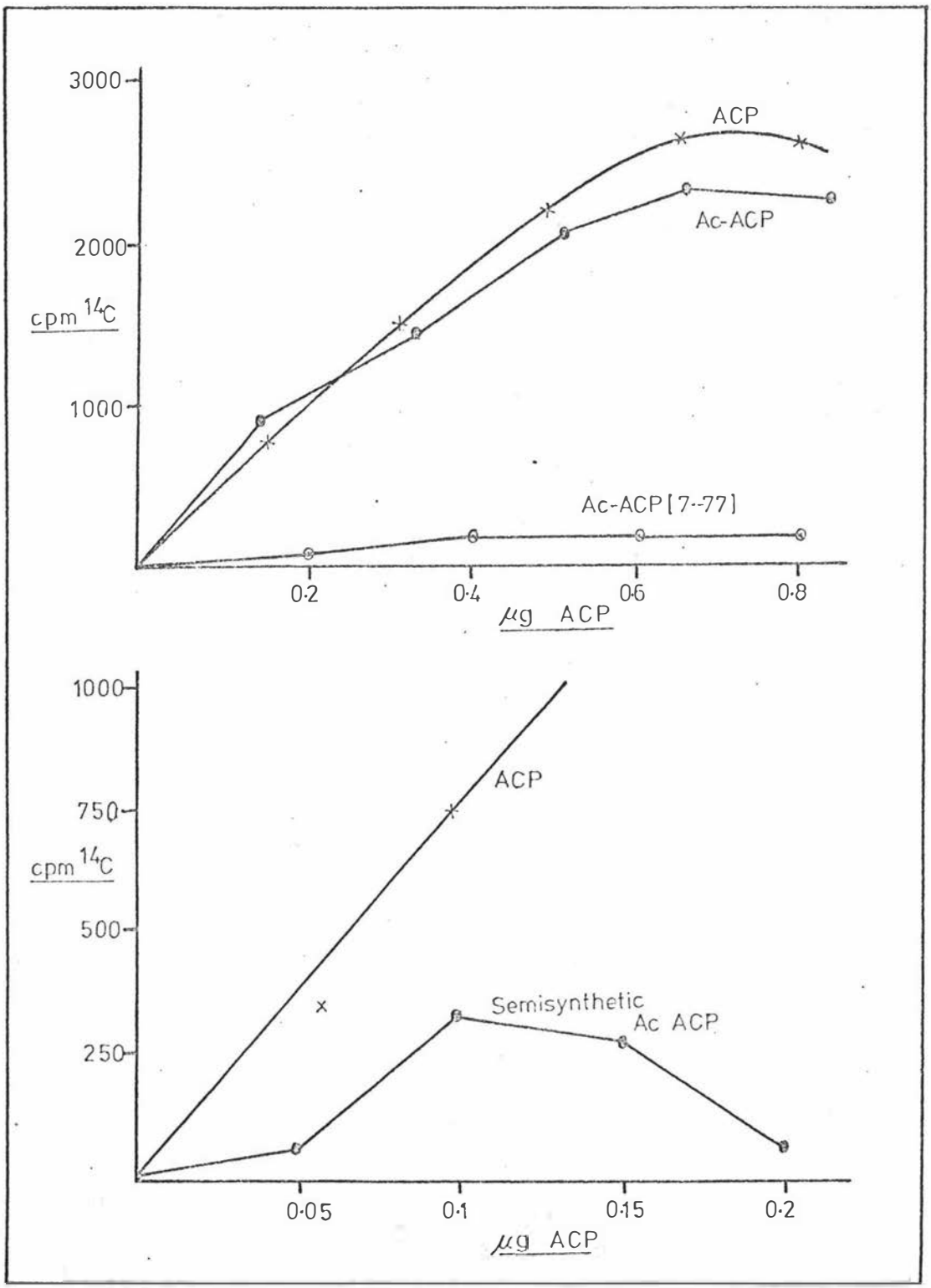


FIGURE 33 $^{14}CO_2$ assay of ACP, ACP derivatives, and semisynthetic ACP

CONCLUSION

The coupling of a crude hexapeptide to the native 7-77 peptide yielded a product which, after extensive purification, gave some activity in the $^{14}CO_2$ assay. This implies that the crude peptide contained a significant proportion of the desired protected hexapeptide. Amino acid analysis of the crude peptide also supports this suggestion. The insolubility conferred by the p-nitrobenzyl protecting group, however, presumably caused the target peptide to be selectively lost during the purification scheme, in favour of deprotected peptides and deletion peptides with fewer p-nitrobenzyl groups and hence greater solubility.

The pentapeptides which were purified and coupled to the native 7-77 peptide gave products which were inactive in the $^{14}CO_2$ assay. This result is interesting, in that it suggests that both Glu^4 and Glu^5 are essential to the interaction between the 1-6 and 7-77 peptides which maintains the active conformation of ACP. However, these data must be treated as tentative until an active ACP satisfying analytical criteria is prepared by this method. The prospects for a successful semisynthesis of ACP, using a modification of the methods employed in this study, appear to be good. New acid-stable protecting groups for carboxyl functions, however, are clearly required if this approach to semisynthesis is to become generally useful.

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