Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

Solid Phase Peptide Synthesis

John Morrow Stewart[a]
[a] Department of Biochemistry, University of Colorado School of Medicine Denver, Colorado

To cite this Article Stewart, John Morrow(1976) 'Solid Phase Peptide Synthesis', Journal of Macromolecular Science, Part A, 10: 1, 259 — 288
To link to this Article: DOI: 10.1080/00222337608068099
URL: http://dx.doi.org/10.1080/00222337608068099

J. MACROMOL. SCI.--CHEM., A10(1&2), pp. 259-288 (1976)

SOLID PHASE PEPTIDE SYNTHESIS

John Morrow Stewart

Department of Biochemistry
University of Colorado School of Medicine
Denver, Colorado 80220

The last two decades have been an era of rapid progress in peptide research. This era was begun by the work of Sanger on the amino acid sequence determination of insulin and by du Vigneaud on the structure determination and synthesis of oxytocin. This period has seen impressive progress in the structure elucidation and synthesis of many peptides of natural origin and of great biological significance, as well as in methods for sequence determination and chemical synthesis of peptides [1-4]. Perfection of techniques and instruments for automatic determination of the amino acid sequence of peptides and proteins has made possible a greatly broadened understanding of genetics and evolution as well as the more chemical areas of mechanism of action of enzymes and hormones, and physical chemistry of peptides and proteins. Effective methods of peptide synthesis are crucial to progress in this area, because only by synthesis can adequate amounts of important peptides be made available for chemical, biological, and physical studies, as well as for exploration of the structure-function aspects of biological molecules. In general, progress in peptide synthesis has lagged far behind that in amino acid sequence determination. This is not surprising since effective peptide synthesis requires a very sophisticated system of selectively removable protecting groups for functions of the amino acids involved, and the synthesis of a large

heteropolymer of defined sequence requires near perfection of each one of the many steps of the assembly. The classical approach to peptide synthesis, using standard organic chemical methods of synthesis and purification of intermediates, has yielded impressive results during these two decades. However, the special problems associated with the assembly of large molecules make staggering investments in time and materials necessary for the synthesis of large peptides or proteins by classical methods.

Fourteen years ago, in an attempt to alleviate some of these problems, Merrifield introduced solid phase peptide synthesis [5]. In this method the amino acid which will form the carboxyl-terminal residue in the desired peptide sequence is attached by a covalent ester bond to an insoluble support which serves as a carrier for the peptide as well as a blocking group for the carboxyl function throughout the synthesis. During this attachment the amino group of the C-terminal amino acid is protected by a selectively removable blocking group, and all amino acid side-chain functions are blocked by protecting groups which will be stable throughout the synthesis but can be removed at the end without damage to the peptide chain. From this point the operations involved in peptide synthesis by the solid phase method are application of a reagent to remove the amine blocking group and coupling of a new N-protected amino acid. Reaction conditions are chosen so that both of these reactions will be forced, as nearly as possible, quantitatively to completion. All excess reagents and by-products should be removable from the resin-supported peptide by washing with appropriate solvents. If this goal of complete reactions can be achieved, a homogeneous macromolecule of defined amino acid sequence can be synthesized, and the complex operations of purification at the intermediate stages of classical solution peptide synthesis can be completely avoided. Moreover, all of the operations of synthesis after attachment of the first amino acid to the resin involve merely selection and addition of a solvent or solution, agitation with the resin for a suitable period of time, and removal of the liquid by filtration. Suitable equipment has been designed and constructed to carry out

all of these operations automatically [6], and at least two commercial versions of such automatic instruments are available [7]. After the desired amino acid sequence has been assembled on the resin, a reagent is applied which will cleave the peptide from the resin and remove the protecting groups from the peptide. The rationale of solid phase peptide synthesis (SPPS) is given in Fig. 1. Using currently available resin supports, amino acid derivatives, equipment, and technology [8], several very impressive syntheses have been achieved [9]. Perhaps the most spectacular of these was the synthesis by Gutte and Merrifield of a protein with the properties of ribonuclease A [10]. Significant milestones in the progress of SPPS are listed in Table 1. Solid phase peptide synthesis is clearly one of the most important and imaginative applications of grafts on polymers ever to be described. It is also safe to say that its ultimate scope and potential can only be imagined at the present time.

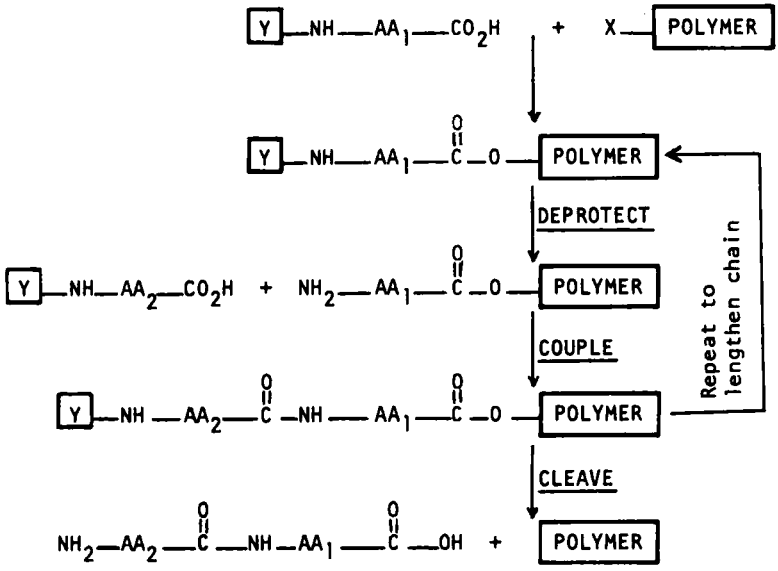

Fig. 1. The principle of solid phase peptide synthesis.

TABLE 1

Progress of Solid Phase Synthesis

Date	Peptide	Amino Acids
1963	A tetrapeptide	4
1964	Bradykinin	9
1965	Angiotensin II	8
1966	Insulin	21 + 30
1968	Clostridium ferredoxin	55
1969	Ribonuclease A	124
1969	Phe59 cytochrome c	104
1970	Pancreatic trypsin inhibitor	58
1971	"Human growth hormone"	188
1971	Parathormone 1-34	34
1971	Lysozyme	129
1971	Acyl carrier protein	74
1971	Salmon calcitonin	32-NH_2
1973	Human ACTH	39

In this review I shall not attempt to cover in detail the chemistry, methodology, and achievements of SPPS, as this has already been done several times, most recently by Meinhofer [9]. His review serves as an excellent summary of the method and the literature, including earlier reviews. On the contrary, after a brief introduction to the chemistry of SPPS, I shall concentrate on several problem areas which need application of the talents of organic chemists, and in particular on those problems associated with the resin support. I shall also give my personal opinions and suggestions for future developments.

THE CHEMISTRY OF PEPTIDE SYNTHESIS

The basic chemical operation in peptide synthesis is the formation of amide bonds. This very simple operation is complicated by several factors. Outstanding among these are the polyfunctional

nature of amino acids, the problems associated with repetitive operations to synthesize macromolcules, the insolubility of the intermediates obtained, and the difficulty of purification of these intermediates.

All amino acids contain at least two functional groups, the amine and carboxyl functions, and for selective peptide synthesis one of these groups in each component of a reaction mixture must be blocked by a selectively removable group. In addition, many amino acids contain reactive side-chain functional groups which must be blocked during the synthesis. The blocking groups for these side-chain functions must be stable enough to remain intact throughout all synthetic operations, but must be fully labile to some reagent which can remove them quantitatively at the end of the synthesis without damage to the peptide chain. The proper design of a system of blocking groups is one of the major considerations in peptide synthesis, since several categories of groups must be available with different orders of stability. This is true both of classical and solid phase methods of synthesis. Closely associated with this problem is that of strategy of synthesis. There are several ways in which a large peptide chain may be assembled. For example, amino acids may be added one at a time, from one end or the other of the chain. This is the stepwise approach. Alternatively, small peptides may be synthesized and then these small peptide fragments may be combined into a large final product; this is the fragment condensation approach. Each of these methods has its advantages and disadvantages, which will not be discussed here, but have been adequately treated in reviews. Most large peptides synthesized by classical solution methods have been synthesized by the fragment condensation approach, while solid phase synthesis is a special case of the stepwise method in which the polymeric resin support serves also as blocking group for the carboxyl of the C-terminal amino acid, and amino acids are added one at a time to this residue bound to the polymer.

As amino acids with blocked side-chain functional groups are assembled into a peptide, the molecular weight increases rapidly

and the solubility of the product in organic solvents usually shows a concomitant decrease. Once the peptide has reached any significant size, only powerful solvents such as dimethylformamide can be used as reaction media. In many cases even this is not adequate, and insolubility of intermediate products frequently causes very serious problems in solution methods of peptide synthesis. Failure to obtain complete reaction in coupling steps is a particularly onerous problem in fragment condensation methods in solution after the fragments have reached large size. The molar concentrations of the reactive groups are very low, and these groups are prevented from effectively finding each other by the large size and complex folding of the attached peptide chains. The resulting low yields cause serious losses of very expensive large-molecule intermediates. On the other hand, if the stepwise approach is used, physical and chemical properties of the large-molecule starting material and the product of a given coupling reaction may not be sufficiently different to allow effective purification, although coupling reactions can usually be more effectively forced to completion by use of a large excess of the small-molecule reactant.

All of these problems combine to make the prospect of synthesis of a large (50 amino acids or more) peptide or of a protein (100 amino acids or more) by solution methods a very forbidding one. Solid phase synthesis offers an approach to a solution of some of these problems, and is at the present time clearly the only practical method for synthesis of molecules in these size ranges.

THE CHEMISTRY OF SOLID PHASE PEPTIDE SYNTHESIS

The chemistry of the most commonly used system of SPPS, that introduced by Merrifield [11], is shown in Fig. 2. The support most widely used in SPPS so far has been a copolymer of styrene and divinylbenzene (DVB). Early work was done with a 2% DVB polymer, while recently a 1% cross-linked resin has been found to be more satisfactory. These resins swell very extensively in the solvents used for the synthesis. This is a crucially important point, since the synthesis takes place throughout the resin beads. There is hope

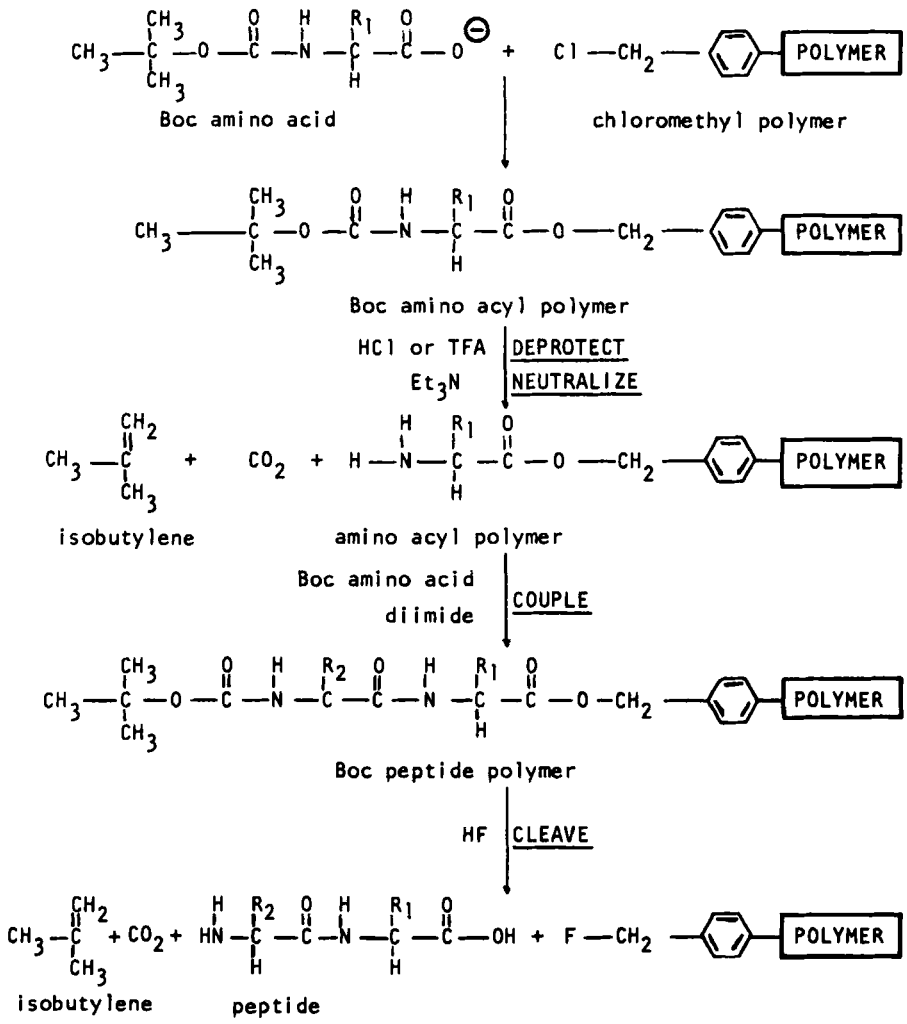

Fig. 2. Chemistry of the standard system of solid phase peptide synthesis.

of obtaining a homogeneous product from a synthesis only if all the reactions go fully to completion. In order to achieve this goal, reagents must be able to penetrate freely into the swollen resin matrix. The polystyrene is functionalized by chloromethylation,

and the chloromethyl polymer is caused to react with a salt of the N-protected amino acid. The most common protecting group for the amino function is the tertiary butyloxycarbonyl (Boc) group. Reaction of the salt (usually with triethylamine) of the Boc amino acid with the chloromethyl polystyrene attaches the amino acid to the resin as an ester. This resin ester, in effect a substituted benzyl ester, is fairly stable to all of the reactions used in the synthesis. The Boc protecting group can be completely removed in most cases without loss of an excessive amount of the first amino acid (and resulting peptide) from the resin. However, in the synthesis of large peptides, or proteins, loss of the peptide from the resin by acidolysis can be a significant problem. Anhydrous strong acid in a suitable medium (such as HCl in dioxane or trifluoroacetic acid in chloroform or dichloromethane) will remove the Boc group. The protonated form of the amino group on the aminoacyl polymer is converted to the free base by treatment with a tertiary amine, usually triethylamine, and the next Boc amino acid is coupled to the amino group. The most commonly used coupling reagent is dicyclohexylcarbodiimide. This is a satisfactory procedure for coupling all amino acids except glutamine and asparagine. In these cases active esters are usually used to avoid the side reaction of dehydration of the omega amide to a nitrile (and incorporation of this derivative into the peptide). Following the coupling reaction, excess Boc amino acid and by-products can be washed out of the polymer suspension, leaving the pure peptide attached to the polymer. An excess (usually 2.5 to 5 equivalents) of each new Boc amino acid and coupling agent must be used to help assure complete coupling. The cycle of deprotection, neutralization, and coupling of the new amino acid is repeated as necessary to assemble the desired peptide chain on the polymer. Finally, the peptide is removed from the polymer by treatment with a stronger acid, usually anhydrous HF, or HBr in trifluoroacetic acid. The advantage of HF is that it also simultaneously removes all of the commonly used blocking groups from side-chain functions of the peptide, yielding the free peptide in one step. When HBr is used for cleavage of

the peptide from the resin, some further step must often be used to remove certain blocking groups. It is noteworthy that all of the reactions of the synthesis and cleavage take place under anhydrous conditions, thus assuring that no hydrolysis of the peptide chain can occur. Further details of the chemistry of solid phase synthesis can be obtained from any of the reviews on the subject [8,9].

Several different kinds of peptide derivatives can be obtained from the synthetic peptide-resin. The different kinds of cleavage commonly used and the derivatives formed are shown in Fig. 3. Other types of linkage of peptide to the resin support have been developed and used for certain special purposes. One area that has received special emphasis is the synthesis of peptide amides. This is important since so many peptide hormones are amides. This fact, coupled with the problems which arise in ammonolysis from the resin of peptides containing glutamic and aspartic acids, has led to the development of resins which yield peptide amides directly by treatment with HF. The most widely used of these is the benzhydrylamine (BHA) resin [12], in which the additional activation of the added phenyl group on the carbon bearing the amine function is adequate to enable cleavage of the C-N bond by HF (Fig. 4). Although this resin has been successfully used for the synthesis of a number of peptide amides, certain peptides, notably those with carboxyl-terminal phenylalanine, are not completely removed from the resin by the normal HF treatment. Clearly a more labile type of peptide-resin link is needed for synthesis of some peptide amides. We investigated this problem and found [13] that the ease of removal of peptides from the BHA resin depends on the nature of the C-terminal amino acid of the peptide. Histidine peptides appear to be removed most easily, with glycine, glutamic acid, threonine, proline, methionine, valine, leucine, and phenylalanine peptides being increasingly more stable. Hardly any of the amide of angiotensin II (terminating in phenylalanine) could be removed from the BHA resin by standard HF treatment (0°, 30 min).

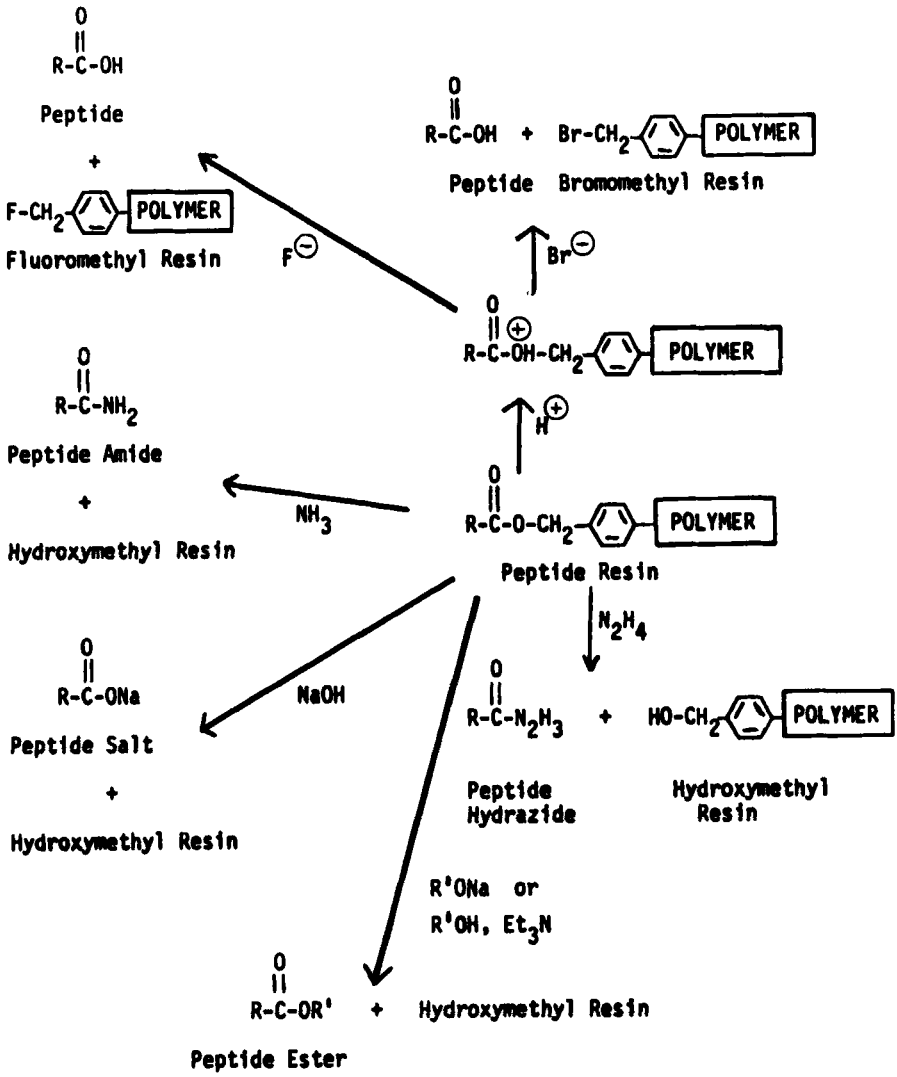

Fig. 3. Cleavage of peptides from the resin.

Use of higher temperature and longer time for HF cleavage is not satisfactory because of harmful side reactions. Of the several resin types examined as potential solutions to this problem, the

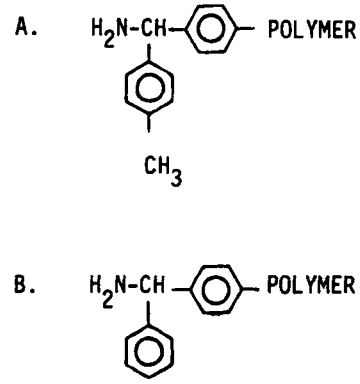

Fig. 4. The benzhydrylamine (A) and p-methylbenzhydrylamine (B) resins for synthesis of peptide maides.

p-methylbenzhydrylamine (MBHA) resin (Fig. 4) appears to be a useful complement to the BHA resin. It does not seem that a single amine resin will offer the requisite stability to Boc deprotection reagents while maintaining sufficient HF lability. When used with Boc alpha protection, MBHA is suitable for all short peptides and long peptides having labile C-terminal residues. MBHA should be satisfactory for synthesis of all peptides if an alpha blocking group more liable than Boc is used.

Some investigators have worked on the solid phase synthesis of small peptides which would subsequently be coupled together in solution (or even on solid phase resins) to synthesize larger peptides. This was the goal of the hydrazine cleavage of the peptide-resin indicated in Fig. 3. A better approach to this problem is probably that of Wang and Merrifield [14], who synthesized a resin carrying the equivalent of Boc hydrazide groups on the polymer (Fig. 5), which after peptide synthesis would yield protected peptide hydrazides directly by acidolysis. These could be converted to azides for direct coupling to other peptides.

PROBLEMS IN SOLID PHASE SYNTHESIS

In spite of the impressive progress made in solid phase synthesis in the decade since its introduction, many serious

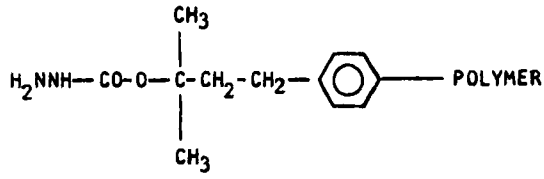

Fig. 5. The resin for synthesis of protected peptide hydrazides.

problems still remain to be overcome. The desirable goal of 100% reaction at each stage has not been generally attainable, although in certain cases (e.g., 98% yield of a decapeptide [15]) the mark has been missed by only a very narrow margin. In the case cited, the average coupling yield must have been at least 99.93%. Many of the peptides listed in Table 1, particularly the large ones, were obtained only as very impure materials. At the present time reliance must be placed on purification of the final product if one hopes to obtain homogeneous material. While this is feasible for small peptides, it is not realistic if one hopes to synthesize pure proteins (chains of more than 100 amino acids) or large peptides. Currently available purification techniques are clearly not selective enough to separate, for example, a synthetic chain of the size of ribonuclease (124 amino acids) from a 123 amino acid chain in which the deletion was a simple aliphatic amino acid such as glycine or alanine. While some peptide chemists believe it will be possible to synthesize essentially homogeneous large peptides by SPPS when sufficient improvement has been made in blocking groups and resin supports, others feel that this is not a realistic goal, and that pure products can be obtained only by combination of small peptides into larger chains. In this latter approach, the small fragments can be purified exhaustively, and then when they are coupled together to provide the larger peptides the differences between the desired product and the incorrect products will be sufficiently large that available purification techniques can offer some hope for achieving homogeneity.

Other difficulties have arisen in the synthesis of some peptides because of unexpected idiosyncrasies in the behavior of the peptide. These difficulties appear to be sequence dependent and are probably due to interactions of one part of the peptide chain with another or with the resin matrix, and cause certain sequences to show unusual difficulty of removal of blocking groups or coupling of protected amino acids. Some of these problems may well be improved by new types of blocking groups for the various functional groups of amino acids, or by new types of coupling reactions. Other problems are clearly due to the nature of the polymeric support used and to interactions between the peptide chain and the support, and demonstrate the need for improved types of supports.

PROBLEMS ASSOCIATED WITH THE PEPTIDE CHAIN

A suitable system of stable and labile groups for the various functions of the amino acids is an essential requirement for any kind of peptide synthesis. The "stable" groups should be completely stable throughout the synthetic operations and should be removable at the end of the synthesis by means of a reagent which will cause no harm to the peptide itself. In SPPS these stable groups are the blocking groups for the side-chain functions and the bond attaching the carboxyl-terminal amino acid to the resin. The "labile" blocking group is that which protects the α-amino function of each amino acid as it is coupled onto the peptide and is removed before attachment of the next amino acid. This labile blocking group must be completely removable by a reagent which will neither remove any of the other "stable" blocking groups nor cleave peptide from the resin. These requirements are not fulfilled completely by the system of SPPS chemistry shown in Fig. 2. An illustration will point up these problems.

Perhaps the most outstanding achievement of the solid phase method was the synthesis by Gutte and Merrifield [10] of a protein (124 amino acid residues) with ribonuclease activity (Table 1).

During the synthesis of this protein it became apparent that losses of the peptide from the resin at each step were significant, so that at the end of the synthesis only 16% of the original amino acid substituted on the resin still remained. Furthermore, the crude product contained a very large proportion of shorter peptides. These can arise either when peptide chains fail to continue to grow ("truncation sequences") because of failure of complete removal of the labile blocking (Boc) group, or of failure to achieve further coupling of amino acids to a chain, or when chains do not grow for a time and then resume growth ("deletion sequences"). Deletion sequences can arise at places in the peptide chain where either deprotection or coupling reactions are very slow if insufficient time is allowed for their completion, or if truncated sequences become again reactive at a later stage of the synthesis. Non-reactivity of a peptide chain on the resin is apparently due to peptide-peptide or peptide-resin steric interactions. As the chain grows, the microenvironment of the peptides changes, and truncated ends previously hindered may again become available due to changes in the secondary or tertiary structure of neighboring peptide chains on the resin. Another problem encountered in the synthesis of ribonuclease and other large molecules was slow removal of side-chain "stable" blocking groups during the synthesis, exposing the reactive groups with consequence branching of the peptide chain and contamination of the product with molecules having molecular weights larger than that of the desired product.

With respect to loss of peptide from the resin, evidently one needs for this kind of synthesis a greater difference in the stability of the α-blocking group and the link attaching the peptide to the resin. A solution to this problem might be found either in the use of more labile α-blocking groups or in the use of a more stable bond linking the peptide to the resin. The latter approach is not practical since the currently used HF treatment for removal of peptides from the resin is apparently too harsh for some peptides,

and will probably be undesirable for most proteins. Several α-blocking groups are available which are more labile than the Boc group, but there are also problems associated with their use. The so-called Bpoc group (see Ref. 9, p. 111) is extremely labile and has been used in a number of syntheses, but this greater lability makes the derivatives very unstable and inconvenient to use. Recently Birr [16] has introduced the Ddz group, of a reactivity intermediate between Boc and Bpoc, which he feels is labile enough to have most of the advantages of Bpoc protection without the extreme instability. The very mild acid needed for complete removal of either of these groups should not cause significant loss of peptide from the resin. Several research groups, including my own, are currently working on other labile blocking groups and believe that the α,2,4,5-tetramethylbenzyloxycarbonyl (TmZ) group offers an ideal combination of properties [13]. The TmZ group is cleaved acidolytically 4200 times as fast as the Boc group, 1/5 as fast as the DdZ group, and 1/30 as fast as the Bpoc group. Removal of the TmZ group by 0.5% trifluoroacetic acid can be monitored photometrically, and the optical activity of the group should help assure optical purity of the amino acids used in synthesis.

The use of a modifiable link for attachment of the peptide to the resin has been proposed by several investigators, and has been called the "Safety-Catch Principle" by Sheppard [17]. In this approach the first amino acid is attached to the resin by a bond which is quite stable. At the end of the synthesis, the bond is modified selectively by a chemical reaction to another form which can be very easily cleaved. One approach to such a method was described by Marshall and Liener, and by Flanigan and Marshall, who used a thioether bond in the peptide-resin link. After the synthesis the thioether was oxidized to a sulfone which activated the ester bond linking the peptide to the support and made it quite labile. This example suffers from the need to use an oxidative reagent, and is not generally applicable, but it illustrates the principle. Different types of "safety-catch" links have been

proposed by others; the most promising may be the use of acid-catalyzed elimination of a tertiary alcohol to labilize the attaching link before cleavage [18].

The problem of loss of side-chain blocking groups during synthesis appears to be much improved at the present time over the situation prevailing in the "standard" SPPS system [8] due to the introduction of more stable blocking groups to replace those that had been found to be too labile [19]. The introduction of blocking groups for the amides of asparagine and glutamine is a significant improvement because it allows diimide-mediated coupling reactions to be used for these amino acids. In one case this led to a 20% increase in the yield of a simple peptide. It is not certain yet what the ideal blocking group for the amides will be. Allowing for the fact that blocking groups can be abnormally stable in certain sequences, the proposed benzhydryl group may be too stable for glutamine. A more labile group, such as xanthydryl, may be more useful at the present time. It does allow the use of diimide coupling reactions with attendant yield increases, but is so labile that it is removed at the first acidolytic step for deblocking Boc groups. Thus it may not effectively protect against glutamine cyclization or acidolytic deamidation, two other problems of these amino acids. A blocking group of intermediate stability is needed. Another amino acid which can still give problems is histidine. The best derivative at the present time is toluenesulfonyl (Tosyl) histidine, but the Tosyl group appears to be marginally stable for some purposes. For example, Boc Tosyl histidine must be kept as the salt for storage at room temperature; after liberation of the acid it should be stored in the freezer. Synthesis of C-terminal histidine peptides using this derivative should be done on hydroxymethyl resin, due to base-catalyzed detosylation when coupling is attempted in the usual way with chloromethyl resin. The Tosyl group is currently the best blocking group for the guanidino group of arginine, but the requirement for use of HF for cleavage is a difficulty for very large-scale synthesis. The new boron tris-trifluoroacetate

cleavage reagent [20] may offer a solution to this problem. The nitro blocking group is not acceptable due to degradation of nitroarginine to ornithine. A new approach is needed for methionine, which can cause problems in some syntheses. The sulfoxide has seen some application as a protecting derivative, but cannot always be reduced readily. In all of these cases, work is needed on development of new blocking groups.

The basic cause of the problems in currently used blocking groups is that the system depends on quantitative (rate) differences in ease of cleavage of the different bonds in the same reaction, namely acidolysis. What is needed is for all the side-chain blocking groups and the peptide-resin link to be susceptible to a type of cleavage reagent that is qualitatively different from that used for removal of the labile (α-amino blocking) group. The specifically labilized "safety-catch" peptide-resin link mentioned above would be a first approximation to this principle. This area and the ones discussed in the preceding paragraph urgently need the attention of skilled organic chemists who also understand the peculiar problems of peptide synthesis.

There is need for general improvement of coupling reactions in SPPS. Dicyclohexylcarbodiimide (DCC) has been by far the most widely used condensing agent in SPPS, largely because of the speed of DCC-mediated coupling reactions and the convenience of its use. With the advent of blocking groups for asparagine and glutamine, it can be used with all amino acids. Racemization has not generally been a problem except with histidine or im-benzyl histidine (Tosyl histidine is satisfactory). Particularly when used with an additive such as hydroxybenzotriazole [21], it may be the condensing agent of choice for SPPS. Some investigators have claimed improved yields in SPPS through the use of Boc amino acid symmetrical anhydrides, even though it has also been claimed that the symmetrical anhydride is the actual acylating agent in solid phase coupling reactions mediated by DCC. At the moment there is no clear explanation for this, although one might be found in possible harmful effects of the

dicyclohexyl urea which precipitates in the resin and has been thought by some persons to cause occlusion of otherwise reactive peptide amino groups. This has been offered as the explanation for the observed greater efficacy of double coupling over single coupling with prolonged times for obtaining complete SPPS coupling reactions. In the double coupling procedure the two coupling reactions were separated by washing with a solvent which will dissolve and remove the urea. Repetition of the triethylamine neutralization step is usually effective for this purpose, and should always be done before repetition of the coupling reaction. Whether the actual acylating agent in DCC-mediated couplings is the symmetrical anhydride or the O-acyl isourea, either of these is a very bulky reagent, and some new type of coupling agent of very small size might lead to significant improvement of SPPS coupling reactions.

In order to evaluate difficulties such as those described above, one must have methods for accurate monitoring of the deprotection and coupling reactions [22]. At the present time, no fully satisfactory solution to this problem is available. Monitoring of deprotection is a very difficult problem, as the last fraction of a percent of undeprotected amino group must be determined in the presence of a large amount of free amino groups. Neither methods based on estimation of amount of blocking group removed or amount of free amino group produced on the resin appear to have the requisite sensitivity, although they may be useful to warn the operator of a gross failure of the deblocking reaction. The use of radioactive Boc groups has been suggested, and may have the requisite sensitivity, but this appears to be a very expensive and impractical approach. Titration of amino groups formed in the deprotection reaction has been used by some to monitor deprotection, but some peptide chemists feel that operations such as this may have harmful effects on the peptide and cause the monitoring process to be partially self-defeating. Better success has been obtained in monitoring of coupling reactions, for in this case one goes to an end point of zero and

small residual amounts of uncoupled amino groups are theoretically easier to determine. The widely used ninhydrin method lacks sensitivity, the naphthaldehyde method is slow, and the very sensitive fluorescamine method [23] does not detect proline amino groups and appears to suffer from spurious fluorescence, especially when used on the standard Merrifield resin. The use of xanthydryl asparagine and glutamine also causes problems for fluorescamine monitoring, since the xanthydryl group is fluorescent. Improvements are needed in both these types of monitoring.

The method of attachment of the first amino acid to the conventional Merrifield chloromethyl polystyrene resin has recently been shown to be a source of problems in SPPS. The chloromethyl groups are usually present on the resin at a much higher concentration than that desired for the first amino acid. Therefore, these groups are not usually completely replaced during the amino acid attachment step. Originally, because he was worried about alkylation of peptide chains by chloromethyl groups on the resin, Merrifield converted all of the remaining chloromethyl groups to acetoxy groups. Subsequently it was found that good yields of peptides could be obtained without this added step, and it was usually omitted. However, in some cases where investigators have looked carefully at the amount of amino groups on the resin throughout a synthesis, loss of amine not attributable to loss of peptide from the resin could be observed. There have also been cases of sudden catastrophic chain termination, notably when neutralized peptide-resins (after the triethylamine step) were left standing for extended periods of time. A logical explanation for these observations is alkylation of the peptide amino group by residual chloromethyl groups. Another difficulty with residual chloromethyl groups is that they are slowly converted to quaternary groups by the neutralizing amine at each step of the synthesis, particularly if triethylamine is used. These quaternary groups, along with those already present from coupling the initial amino acid to the chloromethyl resin, confer undesirable ion-exchange properties upon

the polymer. Rather than try to avoid this side reaction by use of the expensive diisopropylethylamine (on which people may skimp due to the expense and risk incomplete neutralization--a situation made even worse by the hindered structure of the amine), it would appear to be more logical to avoid both these problems by elimination of residual chloromethyl groups. This can be done either by Merrifield's original method of conversion to acetoxy groups after attachment of the first amino acid or preferably by using a different method of attachment of the first amino acid to the resin. During the conventional method of attachment of the first amino acid by reaction of the triethylammonium salt of the Boc amino acid with the chloromethyl resin, some triethylamine reacts with chloromethyl groups to form quaternary ammonium groups on the resin. These, as well as the additional quaternary ammonium groups formed gradually during the synthesis, can cause serious problems. If either acetic or trifluoroacetic acids is used in the deprotection step, some amount of these acids may be strongly bound to the quaternary ammonium groups by ion exchange and resist complete removal by the amine neutralization step. Subsequent use of diimide coupling agents may activate these acids and cause termination of the peptide chain by acetylation or trifluoracetylation. Both of these side reactions have been observed. It has been suggested by some investigators that the superior results they have obtained by the use of preformed Boc amino acid symmetrical anhydrides in the coupling reaction are due to lack of activation of residual trifluoroacetic acid groups on the resin by DCC. Quaternary ammonium groups on the resin also interfere with some kinds of monitoring during the synthesis. Such monitoring is essential for effective synthesis of long peptide chains. A logical solution to all these problems would appear to be avoidance of direct use of chloromethyl resin for attachment of the first amino acid. Two alternate attachment techniques that have been suggested (see Ref. 8) are the use of hydroxymethyl resins or sulfonium resins. One of these methods should probably be chosen for the synthesis of long peptides.

Alternatively the first amino acid could be attached to the chloromethyl resin by the Loffet procedure [24], followed by conversion of all remaining chloromethyl groups to acetoxy or other innocuous groups before proceeding with the synthesis. Recent observations in several laboratories that synthesis of a particular peptide sequence on a benzhydrylamine resin may give a far better overall result than synthesis of the same sequence on the classical Merrifield resin may be due to the fact that no quaternary groups exist or can be formed on the former.

PROBLEMS ASSOCIATED WITH THE POLYMERIC SUPPORT

In many cases of failure to achieve quantitative reactions in SPPS it is difficult to differentiate clearly between those problems caused primarily by the peptide sequence and those caused primarily by the nature of the resin used as the support for the peptide. In certain cases, however, the latter cause seems to be clearly responsible. An early example of this type of problem was encountered by Merrifield in the synthesis of angiotensinyl bradykinin. At that time both angiotensin and bradykinin had been satisfactorily synthesized on 2% cross-linked resins using HCl in glacial acetic acid as the reagent for the deprotection. However, when the synthesis of the longer chain was attempted, in which the sequences of angiotensin and bradykinin were added together, a difficulty was encountered during the second half of the synthesis. This difficulty was failure to remove the Boc group completely at one step of the synthesis. The problem was overcome by making two changes. First, the cross-linking of the resin was reduced to 1%, giving a product which swells much more extensively and which apparently allows better penetration of reagents into the resin. Second, the reagent for deprotection was changed from HCl-HOAc to HCl in dioxane. Whereas the previous reagent does not swell the resin extensively, the dioxane reagent swells the resin to the maximum attainable with any solvent. Four years later a claim was made in the literature that a group of peptides could not be synthesized by solid phase because of failure to deprotect. It was later shown that this

difficulty could be overcome when reagents were used which swelled the resin maximally and when the resin cross-linkage was reduced to 1%. Very recently a 0.5% cross-linked resin has been described by Birr [18], who has said that it improves the synthesis of certain peptides. It is not yet possible to give a complete evaluation of this resin. This new resin swells twice as much as the 1% cross-linked resin in dichloromethane, but is very gelatinous and appears to require special equipment for handling.

The logical ultimate development in this direction would be the use of a noncross-linked resin. However, linear plystyrene is soluble in the solvents used for synthesis, and serious chemical and mechanical problems arise in handling such material. Some work has been done in this area, the most recent being a proposal for use of polyvinyl alcohol as the polymeric support [25], with ultrafiltration as the means for separating the peptide-polymer from low molecular weight materials. The use of such soluble polymeric carriers introduces several new problems and greatly complicates the mechanical operations of synthesis. These materials have not as yet received extensive application or thorough evaluation.

One probable cause for discrepancies in attempts to compare SPPS results from different laboratories or even from different batches of resins is the lack of precise knowledge by the chemist of the exact degree of cross-linking of the resin he is using at any time. Due to the different reactivities of styrene and divinylbenzene, the cross-links are probably concentrated near the core of the resin beads; this may actually be a good thing for SPPS. What is more important and probably even less appreciated is that the chloromethylation reaction is an effective means for cross-linking styrene-DVB polymers. Patterson has stated [26] that the common Dowex 2-X8 (nominally 8% cross-linked) is synthesized by chloromethylation and subsequent aminolysis of a styrene-2% DVB polymer; the additional cross-linking is introduced in the chloromethylation step. Other chemists [27], attempting to use soluble linear polystyrene as a carrier for peptide synthesis,

found that it was very difficult to chloromethylate this polymer without cross-linking and consequent insolubilization. They also found that cross-links were introduced during the neutralization steps of the peptide synthesis (chain termination?!). During the chloromethylation reaction, the chlorine content of the resin first rose and then fell as the chloromethyl groups already introduced reacted with other aromatic nuclei to establish cross-links.

The significance of this for standard SPPS is that chemists should be very careful in conducting the chloromethylation reaction to see that it is done under the most gentle conditions possible for introduction of the requisite number of chloromethyl groups. It is obviously not possible to know about the conditions of chloromethylation used in the production of commercial resins, but a high chlorine content is unnecessary and may mean that the reaction was pushed vigorously, with concomitant cross-linking. Moreover, a low chlorine content may mean that the reaction was done under such vigorous conditions that most of the chloromethyl groups introduced were used up in cross-linking reactions.

The best measure of cross-linking of chloromethylated resins is the degree to which they swell in a solvent such as dichloromethane. Some guidelines for such estimation have been published by Fankhauser and Brenner [28]. The chemist would be wise to check each new batch of commercial resin in this way before using it in synthesis.

Another probable cause of confusion in interpretation of results in SPPS is the fact that the usual polystyrene-DVB beads contain a significant amount of linear polystyrene trapped in the cross-linked matrix. These linear chains may be chloromethylated and participate in peptide synthesis, only to dissolve gradually in the reaction solvents and be lost, along with their load of peptide. To avoid the loss, the beads can be extracted with hot trifluoroacetic acid before use. This treatment may remove as much as 20% of the weight of some batches of beads.

Since many of the difficulties encountered in SPPS appear to be related to penetration of reagents into the polymer matrix,

several investigators have tried supports which are nonporous and in which the reactive sites are limited to the surface. A pellicular layer of polystyrene coated on glass beads has been suggested, but appears to lack the requisite mechanical stability, and additionally did not appear to offer significant chemical advantages. Glass beads have also served as carrier for peptide synthesis in systems involving covalent linkage of the functional group to the silicon of the glass particles. At the present time there has been little evaluation of such systems. Another system which has received some attention consists of "linear" polystyrene grafted onto Kel-F particles. This type of carrier has been used for several syntheses but does not appear to offer the needed improvements in the system. One of the problems with this resin may be that the added polystyrene is not truly linear, or that the added layer is too thick. Other investigators have reported on work with macroporous, macroreticular, and "popcorn" types of polymers. All of these surface-limited supports suffer from the disadvantage of having very low capacity.

SOME SUGGESTIONS FOR NEW POLYMERIC SUPPORTS

An ideal insoluble support for solid phase peptide synthesis would have several characteristics.

1. The support should have good mechanical properties. It should be possible to conduct manipulations such as agitation and filtration without mechanical degradation of the polymer or excessive filtration times.

2. The support should be chemically compatible with a good system of peptide synthesis. The chemist should be able to use a variety of kinds of links for attaching the peptide to the resin and of blocking groups for the amine function as well as side-chain functions. For example, a glass support is not compatible with the use of HF unless one visualizes complete dissolution of the glass as a means of liberating the peptides.

3. The support should not cause problems due to steric interactions with the peptide chain. This probably means that a very

low degree of cross-linkage, or no crosslinkage at all, will be necessary.

4. A high level of substitution should be possible. This is desirable so that huge amounts of support need not be handled in order to synthesize a reasonable amount of peptide. This is difficult to attain in systems having the reaction restricted to the surface of the material, and division of the support into particles small enough to have a very large surface will probably cause serious mechanical problems in handling.

5. The matrix and the peptide chain should probably be solvated to a similar degree by solvents suitable for reaction media. This requirement may mean that polystyrene-based resins are not ultimately desirable, and that new types of polymer need to be examined. A suitable reaction medium should probably be protic, and should be helix-breaking for protected peptides.

It is difficult to visualize any conventional polymer which would meet all of these criteria, and new polymeric supports will probably need to be designed and synthesized. One novel suggestion was that solid phase synthesis might be carried out in continuous belts, films, or fibers of support material, using automatic processing equipment.

It has been suggested by Sheppard [17] that a major difficulty in solid phase synthesis may be the different nature of the polymeric support and the peptide chain which has grown on the support. For successful synthesis, presumably both the polymer carrier and the peptide chain should be fully extended and solvated (Fig. 6A). The polystyrene matrix usually used is nonpolar and swells in nonpolar solvents. On the contrary, the peptide chain is polar and should be expanded by polar solvents which may collapse the polystyrene matrix (Fig. 6B). The nonpolar solvents which expand the matrix may collapse the peptide chain (Fig. 6C), thus causing many of the serious problems, apparently due to steric interaction, which have been observed. Following his own suggestion, Sheppard [29] has examined cross-linked polyacrylamide as a carrier for SPPS and has reported that the synthesis of one peptide which had been

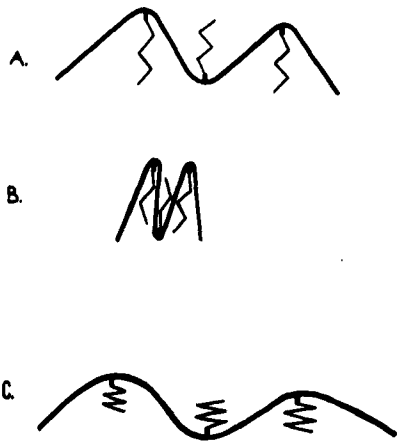

Fig. 6. Peptide-resin interactions in solid phase peptide synthesis (A) The ideal case, with both polymer matrix and peptide chain extended. (B) In polar solvents, with the hydrophilic peptide chain extended but the matrix collapsed. (C) In nonpolar solvents, with the matrix expanded but the peptide collapsed.

very difficult in the conventional SPPS system was much improved. Polar solvents were used throughout the synthesis. It has been observed that the nature of the resin beads changes during a synthesis. After attachment of several amino acids the conventional resin no longer swells as extensively in nonpolar solvents such as dichloromethane and chloroform. It has been found that with certain peptide resins, coupling reactions which could not be forced to completion in dichloromethane as solvent did go to completion when dimethylformamide or urea was added to the reaction mixture. Recently we found do significant change in swelling of a benzhydrylamine resin after assembly of a long peptide chain.

Perhaps a useful approach to a solution of some of the resin-associated problems of SPPS would be to synthesize a new type of carrier polymer such as that depicted schematically in Fig. 7.

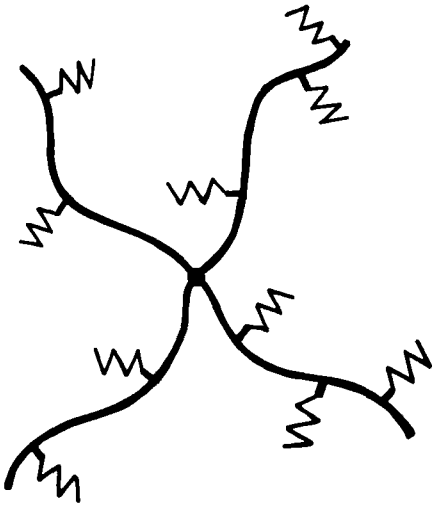

Fig. 7. A proposed ideal support resin for solid phase peptide synthesis.

This type of material, called a "Hippie" resin by Rudinger, would have a dense, impermeable core, with long "hairs," or fibers, extending from it. These "hairs" should have reactive sites at suitable intervals for attachment of the first amino acid of the peptide chain. In this way the peptide chains would be able to grow freely outward from the center without encountering serious steric hindrance from a polymer matrix. The "whiskers" extending from the core might be composed of linear polystyrene chains, with the peptide attached in the usual way (Fig. 8), but more realistically should probably be relatively hydrophilic in nature so that they would be solvated by the same types of solvents which solvate the peptide chain. This might be achieved in a vinyl-type linear polymer by incorporating acrylamide groups (Fig. 9), or it might be necessary to go to a polyamide type of chain (Fig. 10). The reactive groups for attachment of the first amino acid should probably be incorporated into these whiskers during polymerization, rather than introduced later, as is the case with the present chloromethyl

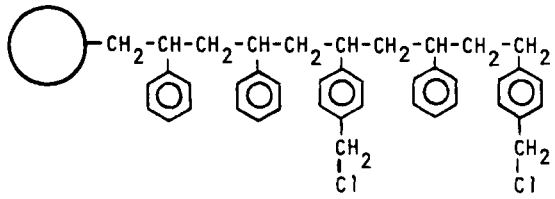

Fig. 8. Suggested polymer chains based on polystyrene.

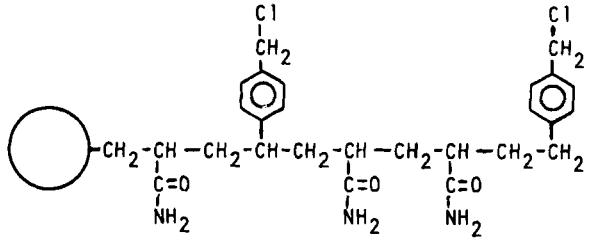

Fig. 9. Suggested polymer chains based on polyacrylamide.

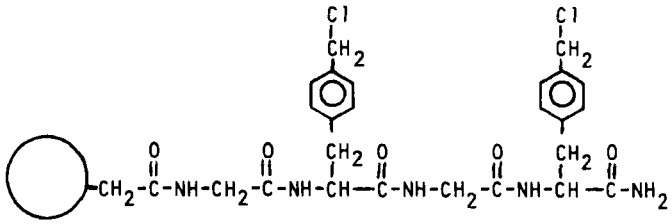

Fig. 10. Suggested polar polyamide resin chains.

resin. Perhaps some polymer chemist, reading this account of our problems and needs, will be inspired to design and synthesize such a polymer.

CONCLUSION

This account, which has concentrated on the present difficulties of SPPS, may give the reader a feeling of pessimism about the future of this method. It is by no means the intention of the

author to cause such a reaction. On the contrary, I am very enthusiastic about SPPS, and hasten to express the debt of gratitude I and all peptide chemists owe to Bruce Merrifield for the invention of this ingenious and important technique. I feel sure that eventually the wise men in Stockholm will see fit to honor him for this achievement, as others have already done. My real purpose has been to help the inexperienced and perhaps overenthusiastic follower of SPPS to avoid pitfalls that may lead to discouragement or ever bitter disillusionment, and to encourage organic and polymer chemists to work on the present problems of SPPS so that its full potential may be more rapidly realized. The future importance of peptide synthesis will surely exceed all our present expectations, possibly even our wildest pipe dreams. The areas of biological control, such as endocrinology and neurochemistry, offer many exciting prospects. Let us all work together, freely exchanging ideas and accomplishments, that we may rapidly solve the problems and meet the needs that only we can fulfill.

REFERENCES

[1] E. Wunch, ed., Syntheses von Peptiden, Theimie, Stuttgart, 1974, 2 vols.; E. Schröder and K. Lübke, The Peptides, Academic, New York, Vol. 1, 1965; Vol. 2, 1966.

[2] R. Walter and J. Meienhofer, eds., Peptides: Chemistry, Structure and Biology, Ann Arbor Science Publishers, Ann Arbor, Michigan, 1975, and previous volumes in this series.

[3] Y. Wolman, ed., Peptides 1974, Wiley, New York, 1975, and previous volumes in this series.

[4] G. T. Young, ed., Amino Acids, Peptides, and Proteins, Specialist Periodical Reports, The Chemical Society, London, Vol. 6, 1975 and earlier annual volumes.

[5] R. B. Merrifield, J. Am. Chem. Soc., 85, 2149 (1963).

[6] R. M. Merrifield, J. M. Stewart, and N. Jernberg, Anal. Chem., 38, 1905 (1966).

[7] Beckman Instruments, Palo Alto, California; Vega Engineering, Tucson, Arizona.

[8] J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, Freeman, San Francisco, 1969.

[9] J. Meienhofer, in Hormonal Proteins and Peptides (C. H. Li, ed.), Academic, New York, 1973, p. 46.

[10] B. Gutte and R. B. Merrifield, J. Biol. Chem., 246, 1922 (1971).

[11] R. B. Merrifield, Biochemistry, 3, 1385 (1964).

[12] P. G. Pietta, P. F. Cavallo, G. R. Marshall, and M. I. Pace, Gazz. Chim.Ital., 103, 483 (1973).

[13] G. R. Matsueda and J. M. Steward, Unpublished Data.

[14] S. S. Wang and R. B. Merrifield, J. Am. Chem. Soc., 91, 6488 (1969).

[15] T. Wieland, C. Birr, and F. Flor, Justus Liebigs Ann. Chem., 727, 130 (1969).

[16] C. Birr, Ibid., 763, 162 (1972).

[17] R. C. Sheppard, in Peptides 1971 (H. Nesvadba, ed.), North Holland, Amsterdam, 1973, p. 111.

[18] C. Birr, F. Flor, P. Fleckenstein, and T. Wieland, Ibid., p. 175.

[19] See papers by Erickson and Yamashiro in Chemistry and Biology of Peptides, (J. Meinhofer, ed.), Ann Arbor Science Publishers, Ann Arbor, Michigan, 1972.

[20] W. Bauer and J. Pless, in Ref. 2, p. 341.

[21] W. Konig and R. Geiger, Chem. Ber., 103, 2034 (1970).

[22] See Ref. 9, p. 169.

[23] S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigle, Science, 178, 871 (1972).

[24] A. Loffet, Int. J. Pept. Protein Res., 3, 297 (1971).

[25] E. Bayer and M. Mutter, Nature (London), 237, 512 (1972).

[26] J. H. Patterson, in Biochemical Aspects of Reactions on Solid Supports (G. Stark, ed.), Academic, New York, 1971, p. 189.

[27] B. Green and L. R. Garson, J. Chem. Soc., C, 1969, 401.

[28] P. Fankhauser and M. Brenner, in The Chemistry of Polypeptides (P. G. Katsoyannis, ed.), Plenum, New York, 1973, p. 389.

[29] E. Atherton, D. L. J. Clive, and R. C. Sheppard, J. Am. Chem. Soc., 97, 6584 (1975).