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REVIEW ARTICLE

Recent Trends in the Synthesis of Linear Peptides

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Synthetic peptides with an ordered sequence of amino acids are of great interest as biologically active substances, as test materials for probing the details of biochemical processes, and as models with which to study the chemical and physical properties of proteins. The synthesis of peptides is also of great importance to confirm structures deduced for naturally occurring peptides and fragments obtained from protein hydrolysates.

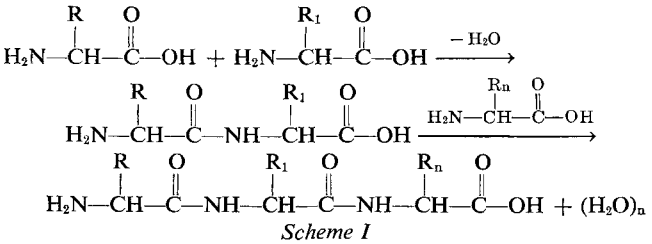
Insulin, which produces hypoglycemia, is perhaps one of the most frequently used peptide hormones in medicine. Other peptide hormones of therapeutic interest are adrenocorticotropic hormone (ACTH), oxytocin, vasopressin, melanocyte-stimulating hormones (α- and β-MSH), angiotensin, bradykinin, glucagon, *etc.* Another important class of biologically active peptides includes the antibiotics such as tyrocidins, gramicidin, bacitracin, valinomycin, polymyxin, and actinomycin-D which are produced by lower organisms such as bacteria and fungi.

Since the turn of this century, synthetic peptide chemistry has undergone periods of varying activity. After the excellent contributions of Fischer (1), who may well be considered as the father of the present day concept of step-by-step synthesis of peptides, a rather slow period of development followed. The next breakthrough came with the introduction of the carbobenzoxy protecting group in 1932 by Bergmann (2) who, with his collaborators, used this protecting group in combination with acid chloride and azide methods to synthesize a wide range of relatively small peptides which were used for the studies of the substrate specificity of enzymes. The next major triumph of synthetic peptide chemistry was the synthesis of the posterior pituitary hormone in 1953 by du Vigneaud *et al.* (3). This opened a new era. The rapid advances and continuous refinement in techniques of isolation, purification, sequence determination of the peptides and proteins, and availability of a rich choice of protecting and coupling methods led to the synthesis of a large number of pure peptides with intricate structures. The synthesis of peptide hormones such as angiotensin (4), bradykinin (5), eledoisin (6), α-MSH (7), β-MSH (8), gastrin (9), ACTH (10), insulin (11), secretin (12), glucagon (13), thyrocalcitonin (14), and many others is a remarkable achievement on the part of peptide chemists. In addition to commercial synthesis of corticotropin, angiotensin, and many other hormones, a number of hormone analogs with more potency than the natural hormones have been synthesized (15, 16).

The tremendous growth of interest in the synthesis of peptides is well reflected in ever increasing publications in this field. So rich is the literature concerning the synthesis of peptides that it is barely possible to include every development in a review article of this size. Fortunately, there have been a number of timely reviews and books in this field which have provided peptide researchers with necessary information. An excellent review article by Fruton (17) marked the begin-

ning of monographs in the field of peptides. This was followed by a number of reviews by Wieland (18–22) and many others (23–29). "Chemistry of Amino Acids" in three volumes by Greenstein and Winitz (30), published in 1961, "Peptides" in two volumes by Schroder and Lubke (31), in 1965, and "Peptide Synthesis" by Bodanszky and Ondetti (32), in 1966, provide a comprehensive literature survey of the progress in the synthesis of peptides. The scope of this review therefore will be limited to a brief survey of the existing methods frequently used in peptide synthesis. The contributions of modified and new methods will be discussed in some detail along with some of the problems associated with their use in the synthesis of peptides.

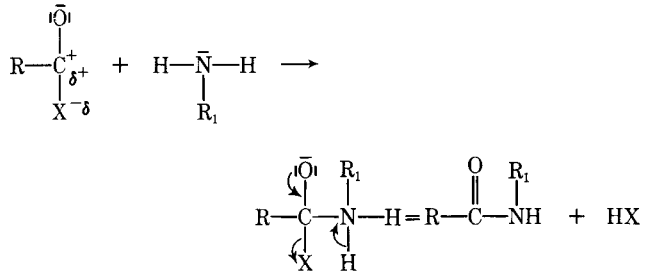
In essence, peptide and protein molecules consist of amino acids which are linked to each other by amide bonds to form what may be called "peptide chains." The amide bond formation involves the elimination of water; this can be brought about either by elevated temperatures, or by suitable activation of carboxyl or amino groups (Scheme I).



Scheme I

Elevated temperature peptide synthesis has not been the choice approach because of extensive racemization. However, the recent work of Fox, who has successfully used thermal condensation, will be briefly discussed at a later stage.

The synthesis of pure peptides requires the protection of all functional groups not expected to participate in the desired reaction. The carboxyl activation which is most frequently used in the formation of the peptide bond involves the introduction of a negative group, X, which will augment the electrophilic character of the carboxyl carbon atom. This facilitates the attack of a nucleophilic amino group to form the peptide bond (Scheme II).



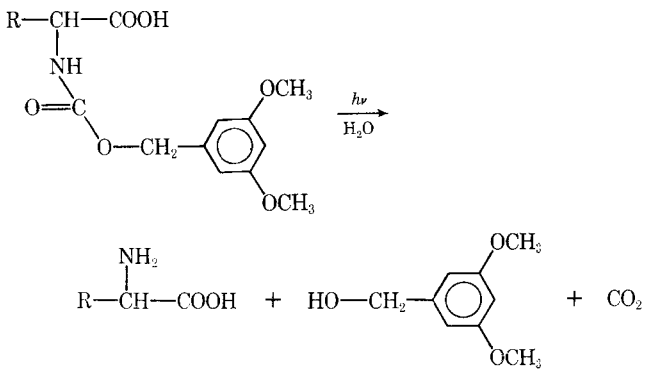
Scheme II

The peptide bond formation through the activation of the amino group has met with relatively less success; however, suitable selection of esters to block the carboxyl of amino acids may make them better reactive partners. For example, the positive inductive effect of tertiary butyl esters would make the free electron pair of amino nitrogen more reactive, when compared to methyl, ethyl, or benzyl esters. In general, a peptide synthesis can be accomplished with the proper selection of (*a*) amino or *N*-protecting groups; (*b*) carboxyl or *C*-protecting groups: and (*c*) carboxyl-activating groups. When the incorporation of polyfunctional amino acids is required in the peptide chain, the ω-groups, which may give rise to side reactions, must also be protected by suitable blocking groups. The protecting groups must be readily and selectively removed during or at the end of the synthesis.

AMINO-PROTECTING GROUPS

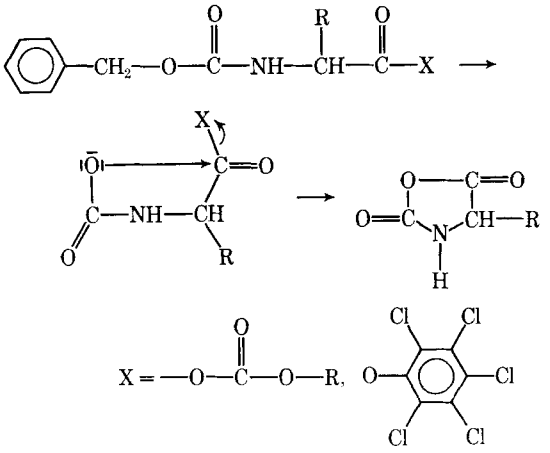
A detailed review on the most important amino protecting groups was recently published by Boissonnas (33).

Benzyloxycarbonyl and Related Groups—The carbobenzoxy or more appropriately the benzyloxycarbonyl (Z) group which was introduced in 1932 by Bergmann and Zervas (2), has been and still is responsible for the major work done in the peptide synthesis field. This versatile group can be conveniently introduced by the treatment of benzyloxycarbonyl chloride with the corresponding amino acid in aqueous alkaline solution. These derivatives are stable compounds and the protecting group can be easily removed by a variety of methods. The most frequently used conditions for the cleavage of benzyloxycarbonyl group are the hydrogenolysis (2) and the treatment of hydrobromic acid and acetic acid (34). The other deprotecting conditions which may be employed are reduction with sodium in liquid ammonia (35) and hydrogen chloride in ethanol (36). Barltrop and Schofield (37) have reported another approach for the removal of this protecting group by means of UV irradiation. Photochemical solvolysis is considerably enhanced by *m*-methoxyl substitution in the benzene ring. The use of 3,5-dimethoxybenzyloxycarbonyl as an amino-protecting group and the deprotection by irradiation of this group from several amino acids and a dipeptide derivative was reported by Chamberlin (38) (Scheme III).



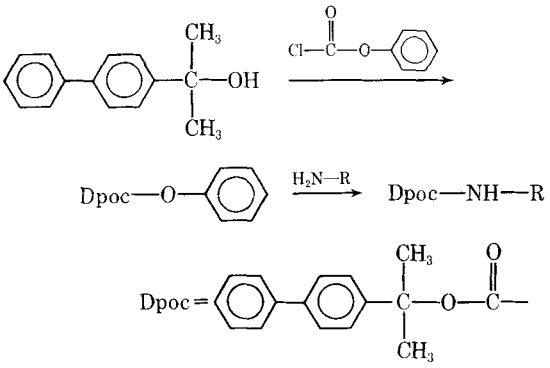
Scheme III

The removal of the benzyloxycarbonyl group by hydrobromic acid probably follows the protonation of the carbonyl oxygen as the first step followed by fission of the benzyl-oxygen bond. The carbamic acid intermediates so formed undergo decarboxylation with the liberation of the amino group (39). The benzyloxycarbonyl group is fairly stable to alkaline conditions required for the removal of methyl, ethyl, or the benzyl



Scheme IV

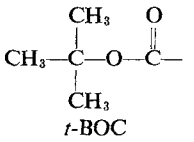
esters which are frequently used for carboxyl protection. However, the formation of urea or hydantoin derivatives may be encountered when the *N*-benzyloxycarbonyl peptides, particularly in the peptides where glycine is next to the amino acid which is protected by the benzyloxycarbonyl group, are treated with alkali (40). The formation of *N*-carboxy anhydrides (Leuch's anhydride) is another serious limitation with the use of benzyloxycarbonyl groups particularly when they are used in combination with acid chlorides (41). The formation of Leuch's anhydride *via* the carbamic acid derivatives was recently confirmed when the *C*-activated *N*-benzyloxycarbonyl amino acids were subjected to catalytic hydrogenation under anhydrous conditions (42) (Scheme IV).



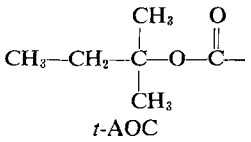
Scheme V

A number of substituted benzyloxycarbonyl and other urethan-type protecting groups have been used frequently for amino protection. The electron-releasing substituents in the benzyl group normally increase the rate of cleavage while the electron-attracting groups increase their stability towards acids. The substituted benzyloxycarbonyl groups which have been successfully used in the synthesis of peptides include *p*-bromo (43), *p*-chloro (44), *p*-nitro (45), and *p*-methoxy (46) derivatives. In a comparative study of different *p*-substituted benzyloxycarbonyl glycine ethyl esters, it was observed that the *p*-methoxy derivatives were cleaved five to ten times faster than the other derivatives on treatment with hydrogen bromide in acetic acid (47). β-Phenylazo and *p*-(*p'*-methoxyphenylazo)benzyloxycarbonyl derivatives (48) which afford the colored compounds have been used relatively less frequently. Sieber and Iselin (49) suggested the use of 2-(*p*-diphenyl)-isopropyloxycarbonyl (Dpoc) group for amino protection. This group has an interesting feature; it can be removed under mild acid condition. The Dpoc group can be easily introduced into amino acids by reacting either the mixed carbonate or the azide of Dpoc with esters or salts of amino acids (Scheme V).

Among the modified urethan derivatives, *tert*-butyloxycarbonyl (*t*-BOC) has come to be a considerably important amino-protecting group (46, 50). The removal of this protecting group is accomplished with considerable ease with 90% trifluoroacetic acid. While this group is reasonably sensitive to acidic conditions, it can still be used in combination with even more acid-sensitive groups such as trityl group. The advantage of this fact has been applied in planning the synthesis of longer peptide chains. *tert*-BOC amino acids can be prepared by using *tert*-butylazidoformate (51), *tert*-butyl-*p*-nitrophenyl carbonate (52), *tert*-butyl-pentachlorophenyl carbonate (53), or *tert*-butyl fluoroformate (54) as condensing agents.



t-BOC

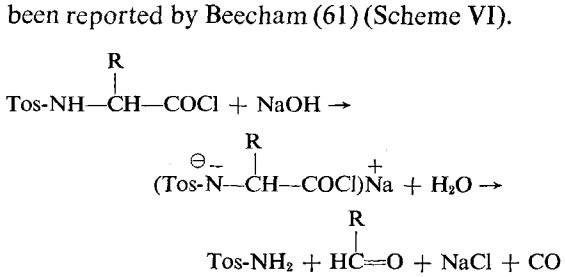


t-AOC

The use of *tert*-amyloxycarbonyl (*t*-AOC) group for amino protection has been suggested by Sakakibara (55). This protecting group has no particular advantage over the *t*-BOC group with the exception that *t*-AOC-protected derivatives are relatively less soluble in water and can be easily extracted with organic solvents from the aqueous reaction media. Deprotection of the *t*-AOC group can be achieved under the same conditions used for the removal of the *t*-BOC group.

p-Toluenesulfonyl (Tosyl) Group 

For a period of time this group enjoyed considerable popularity and even today affords a good protection for ω-amino functions, particularly in the case of lysine (56). The main limitations for the tosyl-protecting group is that α-tosyl amino acids generally cannot be coupled through the mixed anhydride method or through their active esters (57). Usually the acid chloride (58), azide (59), and tetraethyl pyrophosphite (60) methods have been used for the synthesis of peptides with α-tosyl protection. The ready decomposition of tosyl amino acid chlorides in alkaline solutions to toluenesulfonamide, probably because of the high reactivity of amide nitrogen, has been reported by Beecham (61) (Scheme VI).

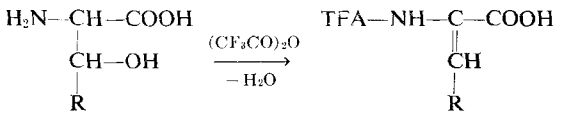


Scheme VI

The tosyl group is remarkably stable to the commonly

used methods for the cleavage of other protecting groups. The method of choice for the removal of the tosyl group is reduction with sodium in liquid ammonia, which was suggested by du Vigneaud (62). The mechanism of deprotection which was reinvestigated by Kovacs (63) appears to proceed by the formation of sulfite and toluene instead of thiocresol as previously reported. There are recent conflicting reports which indicate that during reduction, in addition to the removal of tosyl group, cleavage of the peptide chain between certain amino acids is also possible (64, 65). In the case of carboxyl-activated δ-tosyl ornithine derivatives, the easy six-membered lactam formation poses an additional problem in the use of tosyl group (66).

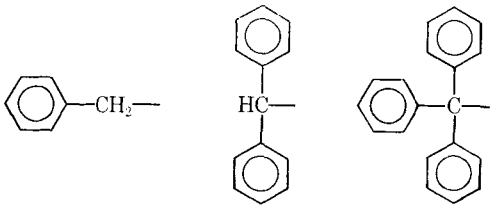
Trifluoroacetyl (TFA) Group (CF_3CO—)—This group which was introduced by Weygand (67) has a remarkable advantage in that it can be cleaved by very mild alkali hydrolysis; however, a number of problems are associated with the use of this group. Some of the serious factors which have contributed to the relative lack of interest in this protecting group are (*a*) possibilities of racemization both at the amino protection and at the peptide bond formation stage; (*b*) problems in the removal of this group from larger peptides; and (*c*) difficulties encountered in the introduction of TFA group, particularly in serine and threonine (Scheme VII).



Scheme VII

Because of high vapor pressures, TFA-protected amino acid and peptide esters can be easily sublimed in high vacuum (68). This offers new applications for the quantitative determination of amino acids in protein hydrolysates. The gas chromatographic separation of diastereomeric TFA dipeptide esters provides a useful tool in determining the degree of racemization during peptide synthesis (69).

Triphenylmethyl (Trityl) and Related Groups—

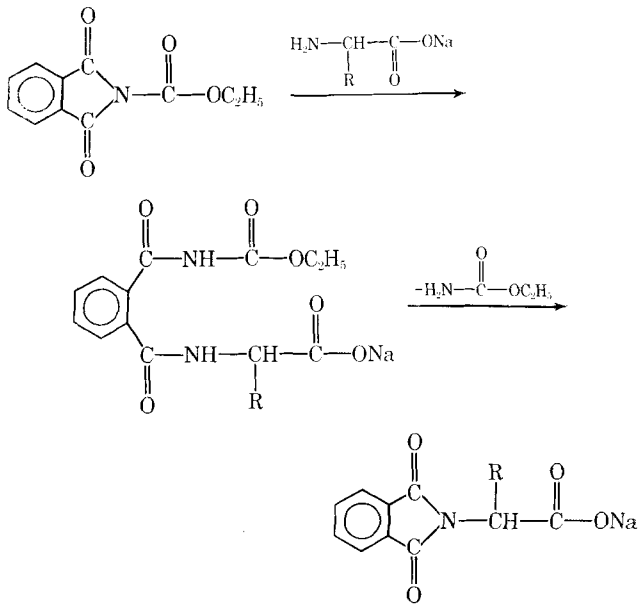


benzyl benzhydryl trityl

The trityl group is peculiar, in that while it is very easily removed from a peptide, it is quite difficult to introduce this group to the amino acids. Tritylation which is normally carried out with trityl chloride proceeds in rather low yields; however, the yields may be improved to a certain extent by using diethylamine instead of triethylamine as an acid acceptor in the reaction (70). The easiest tritylation is that of amino acid esters. However, the saponification of the ester which necessarily follows offers serious problems. The lack of hydrolysis may well be attributed to the bulky trityl group. The tritylglycine ethyl ester and the tritylalanine methyl ester are perhaps the only esters which can be saponified at room temperature under normal conditions (70). This may be explained by the lack of an elaborate side chain in these amino acids which would permit the nucleophilic attack by the hydroxyl group. The trityl group can be removed with acetic acid even in the presence of *tert*-butyloxycarbonyl group and this selective detritylation was used by Schwyzer for the synthesis of an ACTH sequence (71). The trityl group can also be removed by catalytic hydrogenation. Under controlled conditions of hydrogenation, benzyl esters may be removed in preference to trityl groups as in the case of trityl amino acid benzyl esters (72). This procedure may find use in the preparation of trityl amino acids.

Benzyl and dibenzyl protections for the amino group have met with little success because of their stability in acids and considerable resistance to catalytic hydrogenation. The benzhydryl group, which has been so far used only for the protection of sulfhydryl and carboxyl groups, holds good promise for amino protection as the properties of this group are between those of benzyl and trityl groups.

Phthalyl Group—This group which was introduced into peptide synthesis in the late forties (73, 74) until recently did not enjoy popularity because of difficulties encountered during phthalylation. However with the elegant conditions introduced by Nefkens (75), this protecting group is gaining considerable attention. Phthalylation, according to Nefken's procedure, is carried out using *N*-ethoxycarbonylphthalimide in dilute sodium carbonate solution. The mechanism suggested for the reaction assumes the opening of the five-membered ring and subsequent formation of the phthalylamino acid with the elimination of urethan (Scheme VIII).

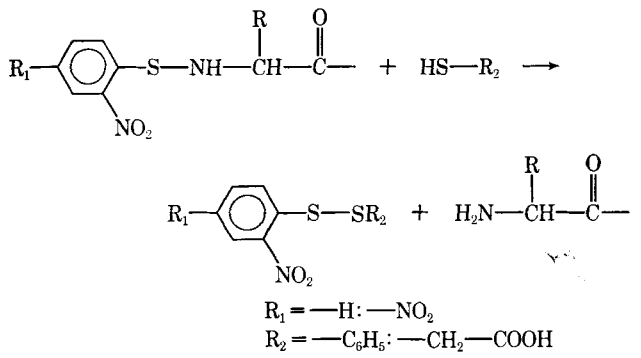


Scheme VIII

The phthalyl group has recently been used for the selective protection of the ω-amino group of lysine (72) and of ornithine (76). The deprotection of this group is achieved only by hydrazinolysis. Since the phthalyl group is not stable to alkali, the ester group used for

carboxyl protection in the synthesis of peptides with phthalyl protection must be hydrolyzed under acidic conditions. A combination of phthalyl and *tert*-butyl groups is quite suitable. A useful method for the introduction of phthalyl protection with ethoxyacetylene and phthalic acid was reported recently (77). *N*-protected derivatives were prepared from amino acids or their esters, both in anhydrous and in aqueous solution.

Arylsulfenyl and Related Groups—These groups were introduced by Zervas *et al.* (78) and provide an excellent method for amino protection. Tritylsulfenyl and arylsulfenyl amino acids, with electron-withdrawing substituents in the ring are rapidly cleaved by mild acid treatment. The *o*-nitrophenylsulfenyl (NPS) group, which has been the group of choice, is selectively removed when the *tert*-butyl ester is present. This group can be also removed by Raney nickel (79) and this provides a useful approach to avoid the partial cleavage of the other acid-labile groups present. However, the benzyloxycarbonyl group is removed under the same conditions. Raney-nickel cleavage of NPS group fails when applied to sulfur-containing peptides. Other useful deprotecting agents for the NPS group which were suggested by the Scoffone laboratories are thioglycolic acid and thiophenol (80). A disulfide is formed in addition to the free amino acid or peptide (Scheme IX).

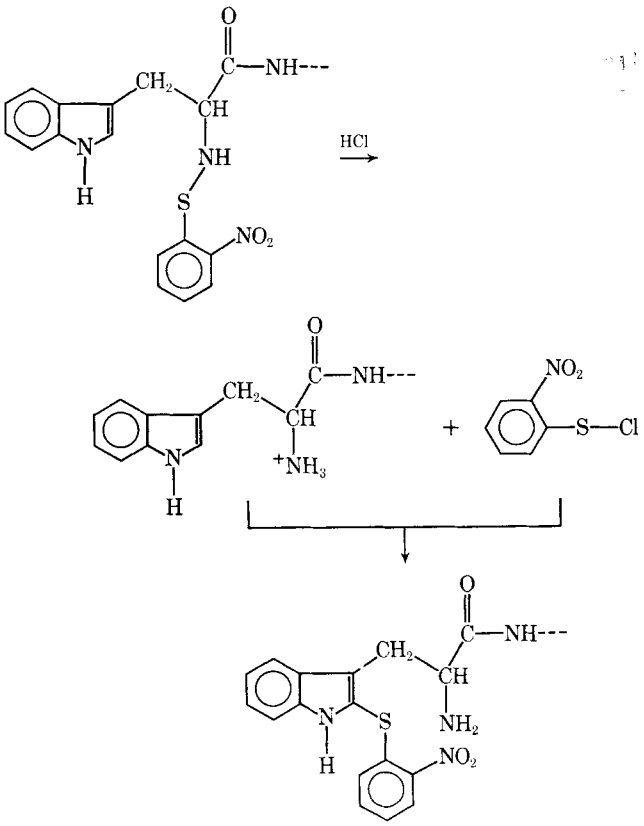


R_1 = —H: —NO_2
R_2 = —C_6H_5: —CH_2—COOH

Scheme IX

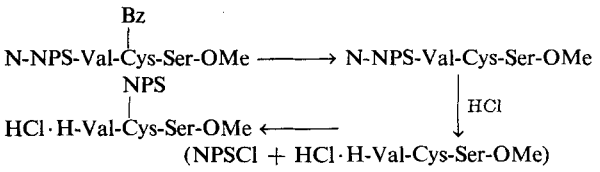
Various other reagents have been reported for the cleavage of the NPS group (81). The NPS derivatives of amino acids generally are not easily obtainable in a crystalline form, and their purification and characterization is often carried out by preparing the corresponding dicyclohexylammonium salts. Fontana *et al.* (82) suggested the use of the 2,4-dinitrophenylsulfenyl (DNPS) group which gave easily crystallizable compounds with a higher melting point when compared with the corresponding NPS derivatives. The DNPS group can be cleaved with the same conditions as those used for the NPS group. When the NPS or DNPS are cleaved with acid from tryptophan peptides, the sulfenyl halide formed during the removal step reacts quantitatively at the 2-position of the indole ring (80). This reaction mechanism has been discussed by Wunsch (83) (Scheme X).

The use of sulfenyl halides has been successfully extended as modifying reagents for polypeptides and proteins. Tryptophan was converted to 2-hydroxytryptophan through an intermediate 2-thio-aryl-tryptophan derivative (84). When the *N*-*o*-nitrophenylsulfenyl



Scheme X

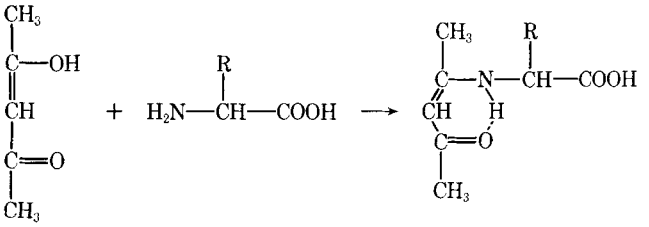
group is removed from cysteine peptides bearing free thiol groups by means of hydrogen chloride in methanol or in nonpolar solvents, an N → S transfer of the NPS group takes place to give the corresponding S-NPS derivative (85) (Scheme XI).



Scheme XI

Therefore, during the preparation of cysteine peptides using *N*-NPS derivatives of amino acids and *S*-protected cysteines, the removal of NPS-group must be done either in *S*-protected cysteine peptides or in the oxidized -*S*-*S*-peptides, *i.e.*, in the cystine peptides.

Tritylsulfenyl amino acids have a remarkable advantage over trityl amino acids in that they can be coupled conveniently by the mixed anhydride or *p*-nitrophenyl ester methods. Trityl amino acids do not



Scheme XII

undergo coupling with these methods because of steric hindrance.

Schiff Bases—Schiff bases are normally quite unstable and cannot be used as protecting groups. However, Schiff bases which can be stabilized by hydrogen bonds by the use of 1,3-dicarbonyl compounds (86) or by hydroxy derivatives (87) provide suitable protecting groups in the synthesis of peptides (Scheme XII).

CARBOXYL PROTECTION

The primary consideration for the substitution of the carboxyl group is the release of the amino acid from its zwitterion state rather than actual protection. The protecting groups most commonly used have been limited to methyl, ethyl, benzyl, *tert*-butyl, and *p*-methoxy-benzyl esters. Several new carboxyl groups have come to the limelight in recent years.

Methyl and Ethyl Esters—These esters are normally prepared by introducing dry hydrogen chloride into a suspension of the amino acid in absolute methanol or ethanol. The corresponding amino acid esters are obtained as hydrochlorides. Another convenient approach is the use of thionyl chloride (88) or acetyl chloride (89). Methyl or ethyl esters are removed during or after the synthesis of peptides by alkaline hydrolysis which generally produces a number of problems such as racemization, transpeptidation, and formation of urea or hydantoin derivatives. Alkali-labile *N*-protecting groups, such as phthaloyl, have to be excluded when *C*-protection is carried out by methyl or ethyl esters. Cleavage of sensitive amide bonds is another possibility when alkali is used for the removal of these esters. Removal of methyl or ethyl *C*-protection becomes more difficult as the number of amino acids increase in the peptide chains. Dilute hydrochloric acid in aqueous acetone (73) and lithium halides in pyridine (90) have been recommended as alternative methods for the removal of the methyl or ethyl *C*-protection.

Benzyl Esters—The main advantage of benzyl esters over methyl or ethyl esters is their easy removal by hydrogenolysis. These esters can be prepared in satisfactory yields by using other catalysts rather than hydrochloric acid. Polyphosphoric acid (91), *p*-toluenesulfonic acid (92), and benzene sulfonic acid (93) have been successfully used for benzylation. Water formed during the reaction is removed by azeotropic distillation. The catalytic hydrogenation of benzyl esters in ethanol may be accompanied by transesterification and formation of the ethyl ester. This can be prevented by the use of *tert*-butanol as solvent (94). Benzyl esters cannot be cleaved selectively by hydrogenation in the presence of a benzyloxycarbonyl group; however, they may be hydrogenated selectively in the presence of trityl groups. Benzyl esters are removable by hydrogen bromide and by sodium in liquid ammonia. Benzyloxycarbonyl groups in the presence of these esters may be selectively removed by controlled treatment with hydrogen bromide in glacial acetic acid (95) or in nitromethane (96). Catalytic hydrogenation of benzyl esters in large peptides is generally quite difficult.

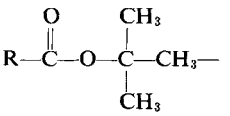
***p*-Nitrobenzyl Esters**—These esters which can be prepared by the modification of esterification methods (97) used for the preparation of benzyl esters have a definite advantage in that they are stable to hydrogen bromide–acetic acid reagent; therefore, these esters allow the selective removal of the benzyloxycarbonyl group. They are removed easily by hydrogenolysis. The derivatives of *p*-nitrobenzyl esters are generally higher melting compounds and can be easily crystallized. Another significant advantage is the α-esterification when the benzyloxycarbonyl-aspartic anhydride is treated with *p*-nitro benzyl alcohol under anhydrous conditions (98).

***p*-Methoxybenzyl Esters**—*p*-Methoxybenzyl esters which were first reported by Weygand and Hunger (99) have not gained great popularity mainly because of the difficulties encountered during their preparation. However, Stewart (100) has suggested a convenient method for the preparation of *p*-methoxybenzyl esters by imidazole-promoted condensation of the corresponding *p*-nitrophenyl esters with *p*-methoxybenzyl alcohol. Since these esters are more sensitive to acid reagents, their future use as carboxyl protecting agents seems to be imminent.

Recently, Weygand *et al.* (101) reported an extensive study on the kinetics of different methoxy-substituted benzyl esters and found 2,4-dimethoxy and 2,4,6-trimethoxy benzyl esters as suitable protecting groups for amides such as asparagine and glutamine. These esters are stable to catalytic hydrogenation and methanolic hydrochloric acid, but can be conveniently removed by trifluoroacetic acid.

Methyl-Substituted Benzyl Esters—The use of 2,4,6-trimethylbenzyl and pentamethylbenzyl groups for carboxy protection was recently reported by Stewart (102). The advantage of these esters over benzyl esters is their rapid cleavage by hydrogen bromide in acetic acid under conditions which result in complete removal of the benzyloxycarbonyl group.

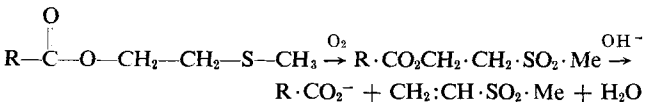
Tertiary Butyl Esters



The introduction of these esters has been a significant contribution in the synthesis of peptides (103). They are cleaved with extreme ease by acid reagents probably by the formation of the carbonium ion. The free *tert*-butyl esters of most amino acids are stable and distillable liquids, and normally do not undergo self-condensation to form diketopiperazine derivatives. They are very stable to hydrogenation and less sensitive to alkali treatment. These esters have been particularly useful when the incorporation of monoamino dicarboxylic acids is desired in the peptide chains. Among the methods for their removal, the treatment with trifluoroacetic acid is perhaps most suitable (104).

β-Methylthioethyl Esters—The use of β-methylthioethyl esters for the protection of carboxyl group is a novel approach which was suggested by Rydon (105). Their utility in the synthesis of peptides is based on the observations that the meththiodides and sulfones derived from β-methylthioethyl esters of amino acids and peptides are split to the parent carboxylic acids under extremely mild alkaline conditions. In the initial reports, their removal was suggested by first converting

these esters into the meththiodides by the action of methyliodide, and subsequent treatment of the meththiodides with aqueous alkali solution at room temperature. The free carboxylic acid is regenerated along with the formation of dimethylvinylsulfoniumiodide. The above method for deprotection failed with a series of *N*-*tert*-butoxycarbonylglutamic acid peptides in which the side chain carboxyl group was protected as benzyl ester because of contamination with considerable amounts of side products, which led to difficulties during purification. In a recent communication, Rydon (106) has proposed a new method for the removal of these esters. The new approach involves the oxidation of methylthioethyl esters with ammonium molybdate to the corresponding sulfones which, in turn, can be readily split at room temperature in mild alkaline solutions (Scheme XIII).

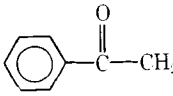


Scheme XIII

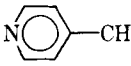
Amides and Substituted Hydrazides—The hydrazide group itself is not suitable for carboxyl protection because an amino group cannot be acylated selectively in its presence. The hydrazides used are therefore blocked with suitable amino protecting groups in order to prevent the side reactions (107, 108). These include benzyloxycarbonyl, *tert*-butyloxycarbonyl, and trityl hydrazides. The cleavage of hydrazide bond to form the free acid is not possible; therefore, this type of carboxyl protection is suitable only for the synthesis of intermediates which can be converted into reactive derivatives such as azides.

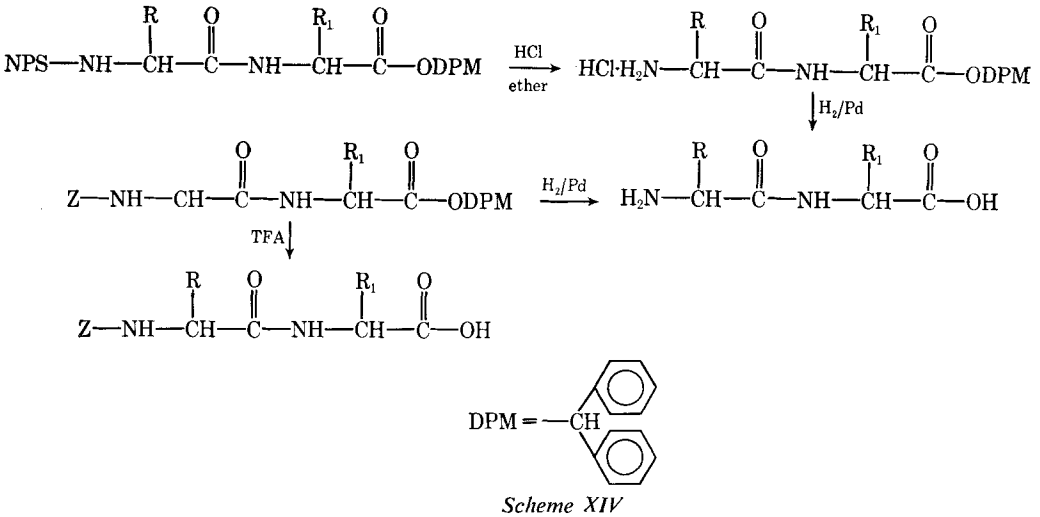
Phthalimidomethyl Esters—This carboxyl-protecting group introduced by Nefkens (109) has an attractive feature in that it is stable to catalytic hydrogenation, but can be removed under a number of conditions such as the following: hydrogen chloride in organic solvents, diethylamine in ethanol, dilute aqueous sodium hydroxide, and hydrazine.

Diphenylmethyl Esters (Benzhydryl)—Significant progress was made in the laboratories of Zervas (110), Fruton (111), and Hiskey (112) by the introduction of benzhydryl esters. This group can be conveniently removed by hydrogen chloride (or hydrogen bromide) in nonpolar solvents or by hydrogenolysis. With the introduction of NPS amino-protecting groups, the use of these esters is guaranteed a secure place for carboxyl protection in the synthesis of peptides. The NPS group can be selectively removed in the presence of the benzhydryl group (113) (Scheme XIV).

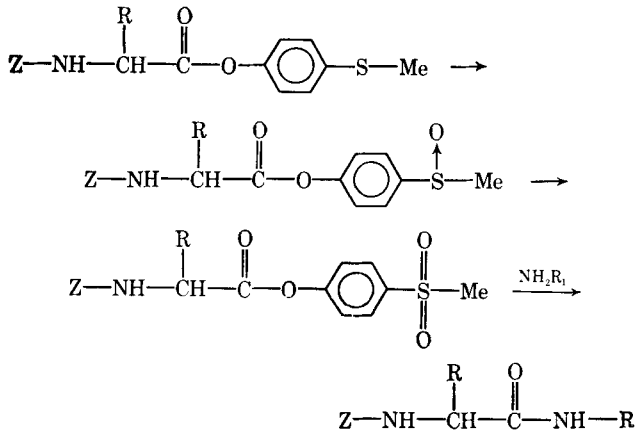
Phenacyl Esters (PAC)  —The phenacyl group differs from the tertiary butyl and diphenylmethyl ester groups in that it is stable to hydrogen chloride or hydrogen bromide even when high concentrations of these reagents are used. This group can be removed by catalytic hydrogenation. A combination of diphenylmethyl and phenacyl ester protection as outlined by Zervas (114) offers a convenient approach for the incorporation of monoamino dicarboxylic acid in the peptide chains.

4-(Methylthio)phenyl Esters (MTP)—This ester for carboxyl protection (115) may be considered as an extension of Rydon's approach (105). The interesting feature of this ester is that it can be directly converted by oxidation to the sulfoxide, 4-(methylsulfinyl)phenyl ester, and further oxidation of the sulfoxide leads to the formation of the sulfone, 4-(methylsulfonyl)phenyl active ester, which can be used for coupling in the synthesis of peptides (Scheme XV).

4-Picolyl Esters  —An ingenious method of blocking the carboxyl group with 4-picolyl esters was recently suggested from the laboratories of Young (116). These esters, in addition to being good protecting groups, also provide a basic "handle" when incorporated into the *C*-terminal residue and facilitate the isolation of the growing peptide after each coupling step. The 4-picolyl esters at the termination of the synthesis can be removed by catalytic hydrogenation or by electrolytic reduction. The usefulness of this procedure for the lengthening of the peptide chain from *C*-



Scheme XIV



Scheme XV

terminal residues of amino acid was demonstrated by the synthesis of Val[5]-Angiotensin II.

A related method using *p*-dimethylazobenzyl esters has also been reported (117).

Salt Formation—The protection of the carboxyl group by salt formation represents the least elaborate approach. In order to avoid protonation of the amino group in amino acids, a suitably strong base such as sodium hydroxide or organic bases such as triethylamine, tributylamine, or dicyclohexylamine may be used. The relative lack of success of this approach is due to low yields and the difficulties encountered during the purification of the reaction products. However, recently a number of active esters of *N*-protected amino acids have been used successfully for coupling with amino acids and peptides that were *C*-protected by suitable salt formation (118, 119).

PROTECTION OF ω-GROUPS IN THE AMINO ACIDS

Excellent surveys enumerating the masking of ω-groups during the synthesis of peptides have been reported (31, 32).

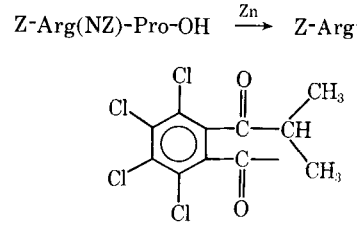
The selective formation of α-esters by the ring opening of the *N*-benzyloxycarbonyl glutamic anhydride by the corresponding alcohols was indeed a big contribution by Gibian and Klieger (120). The selective ring opening to form α-esters in the case of *N*-protected aspartic anhydride is relatively more difficult; however, the reaction of *p*-nitrobenzyl alcohol with *N*-benzyloxycarbonyl aspartic anhydride usually results in the formation of α-*p*-nitrobenzyl ester (121). β-Carboxyl can then be protected by a tertiary butyl ester. Another very useful approach for selective protection of carboxyl groups of glutamic acid by diphenylmethyl and phenacyl esters has been suggested by Zervas (114).

Serine and threonine can be incorporated in the peptide chain with their free hydroxy groups; however, in order to limit any possibility of side reactions it is advantageous to suitably mask the hydroxyl group.

A number of derivatives with suitable protection for the hydroxyl groups of serine and threonine have been reported (32). Among the most promising of the masking groups for the hydroxy function of serine and threonine is the 2,2,2-trifluoro-1-benzyloxycarbonylaminoethyl (Z-TF) group suggested by Weygand. This group can be cleaved by catalytic hydrogenation or with hydrogen bromide in acetic acid. A very useful combination of this hydroxyl-protecting group with *p*-methoxybenzyloxycarbonyl and tertiary butyloxycarbonyl as amino protection has been suggested (122).

The weakly basic imidazole ring in the side chain of histidine poses a number of problems and it is desirable to provide suitable protection for the imidazole ring in the synthesis of peptides incorporating histidine. Benzyl (123), benzyloxycarbonyl (124), and trityl (125) groups for imidazole protection have been successfully used in combination with a variety of amino-protecting groups for the synthesis of histidine peptides. Two very attractive protecting groups, 2,2,2-trifluoro-1-benzyloxycarbonylaminoethyl (Z-TF) and 2,2,2-trifluoro-1-*tert*-butyloxycarbonylaminoethyl (BOC-TF) for the imino group of histidine, appear to be of particular interest (126). The first-mentioned protecting group (Z-TF) can be removed by catalytic hydrogenation while the latter (BOC-TF), which is stable to hydrogenation, can be conveniently cleaved with trifluoroacetic acid. A combination of these groups with pentachlorophenyl active esters for lengthening the peptide chains and for the synthesis of sequential polypeptides incorporating histidine has shown promising results in the author's laboratories. The use of adamantyloxycarbonyl (AdOC) group was suggested for amino protection (127) and this group has been successfully employed by Wunsch (13) for histidine protection during the synthesis of glucagon.

Arginine, which contains a guanidine group of an unusually basic nature, can be incorporated if suitable protonation is available. In order to reduce the basic character of the guanidine group, the classical approach is to convert this group into the nitroguanidine derivatives. Zervas has used the tribenzyloxycarbonyl and tritrityl derivatives of arginine (128) in the synthesis of peptides. It is interesting to observe that one acyl group, the ω-benzyloxycarbonyl group, is removable by hydrogen bromide in acetic acid. However, all three benzyloxycarbonyl groups are labile to hydrogenolysis. The α-trityl group forms tritrityl arginine which can also be selectively removed, thereby offering a useful ditrityl arginine derivative for lengthening the peptide chain from *C*-terminal residue. The trityl group in the guanido moiety is exceptionally stable to hydrogenolysis and can be cleaved only by hydrogen bromide in acetic acid. The idea of using derivatives of ornithine throughout the synthesis and to convert ornithine to arginine by guanylation at a suitable stage was proposed by Fruton (129) and was successfully applied by Bodanszky (130) for the synthesis of biologically active peptides. The tosyl group has also been used for the protection of



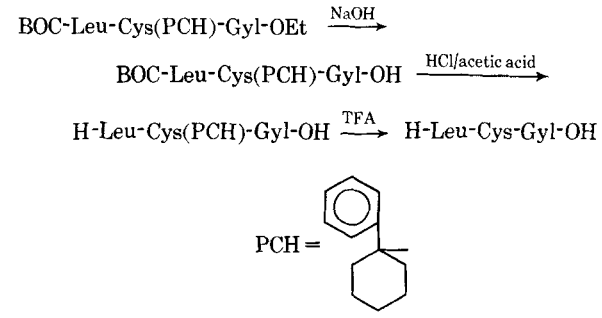
(TIP)

Scheme XVI

the guanido group in arginine. While the α-amino tosyl group can be conveniently removed by electrolytic reduction, the removal of tosyl protection from ω-tosyl arginine under the same conditions is relatively difficult, particularly in longer peptides. In addition to nitration, protonation, tosylation, and other methods, the use of the tetrachloroisopropyloxyphthaloyl (TIP) group and the *p*-nitrobenzyloxycarbonyl (NZ) group for the protection the of guanido group in arginine has been suggested (131, 132). An interesting method for the removal of the nitro or *p*-nitrobenzyloxycarbonyl guanido-protecting groups by acidic zinc reduction has been suggested by Pless and Guttmann (133) even in larger peptides. This method leaves the α-amino benzyloxycarbonyl group intact (Scheme XVI).

The sulfhydryl group in cysteine is highly reactive and must be afforded suitable protection. The *S*-benzyl group which is cleaved by sodium in liquid ammonia was suggested by du Vigneaud (134) and has remained the group of choice. However, in order to provide the sulfhydryl-protecting groups which can be removed under relatively milder conditions, an extensive search was undertaken in various laboratories. A number of *SH*-protecting groups such as acyl, trityl, benzhydryl, benzyloxycarbonyl, and *p*-methoxybenzyloxycarbonyl have been suggested. All these groups can be selectively removed under mild conditions without affecting sensitive parts of the molecule and especially the already existing -*S*-*S*-bridge. Another approach is the incorporation of *o*-tosyl serine (135) or β-chloroalanine (136) residues into a peptide chain and their subsequent conversion into *S*-protected cysteine residues by nucleophilic displacement reactions. The use of the acetamidomethyl group for the protection of the *SH* group of cysteine has been suggested recently (137). This protecting group is stable under conditions which cause the removal of commonly employed acid-labile substituents, but can be easily cleaved with two equivalents of Hg at pH 4.

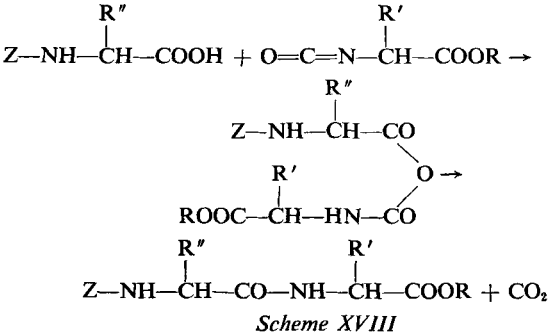
A series of *tert*-arylalkyl groups such as 1-phenylcyclohexyl (PCH), for *SH* protection of cysteine were reported by Konig (138). Because of the stability of these groups toward HCl/acetic acid, HBr/acetic acid and hydrazide, they allow the selective removal of *N*-benzyloxycarbonyl, *N*-*tert*-butyloxycarbonyl, and *N*-phthalyl groups. Trifluoroacetic acid is the reagent of choice for the cleavage of PCH and related protecting groups (Scheme XVII).



Scheme XVII

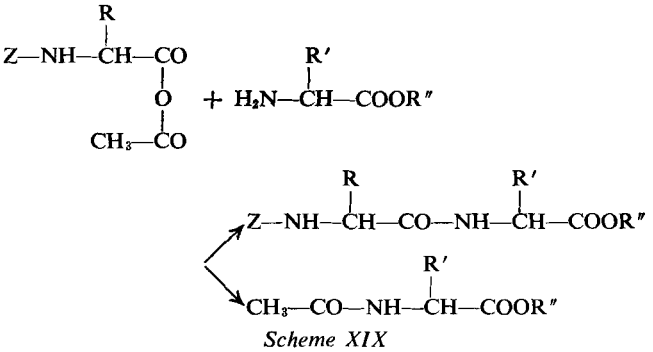
FORMATION OF THE PEPTIDE BOND

Since the turn of this century, a number of classical methods joining the amino acids through amide bonds have been developed for the formation of well defined peptides. In a broad sense, a peptide bond may be formed either by the activation of an amino group or by the activation of a carboxyl group. The amino group is capable of amide formation by way of its free electron pair. The nucleophilic character of the amino group would be expected to increase with the substitution of electron-releasing groups, thereby allowing easy formation of the amide bond. This approach has not been fully explored. However, a number of reactive derivatives of the amines have been reported for the formation of peptide bonds. The actual coupling may be visualized to proceed through the activated carboxyl component. For example, isocyanates, which are prepared from amino ester hydrochlorides and phosgene as reactive amino derivatives (139), form a peptide bond with *N*-protected amino acids or peptides through a mixed anhydride of a carbamic acid (Scheme XVIII).



Scheme XVIII

Other methods based on reactive amine derivatives which have been used in the synthesis of peptides include phosphazo (140), phosphorus acid esters (141), arsenious acid esters (142), and phosphorus pentoxide (143). While these methods have a definite place in peptide chemistry, the bulk of peptide synthesis involves the peptide bond formation through the activation of the carboxyl group.

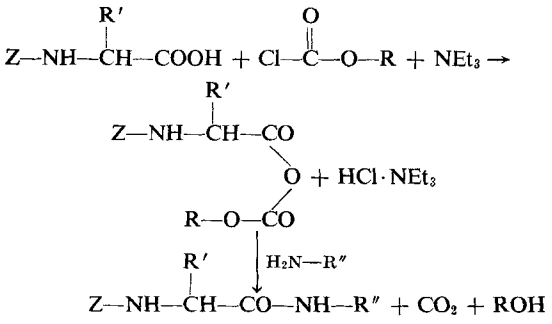


Scheme XIX

Peptide Synthesis by Activation of the Carboxyl Group—*Mixed Anhydride Methods*—The actual application of the mixed anhydride method in the synthesis of peptides was made in the laboratories of Wieland (144). One of the main considerations in the selection of anhydride-forming acid, or the "partner" acid as more appropriately termed by Bodanszky and Ondetti (32), is the presence of an electron-releasing structure.

This will limit the formation of *N*-acetyl amino acids or peptides during coupling (Scheme XIX).

The acids with low dissocation constants and long, branched aliphatic chains are more useful and lead to selective formation of peptide bonds. Introduction of carbonic acid–half ester–half chlorides, where the alkoxy group provides the electrons, is a tremendous improvement in mixed anhydride method. Ethylchloroformate (145) and isobutylchloroformate (146) are the reagents of choice for the formation of mixed anhydrides of *N*-protected amino acids and peptides (Scheme XX).



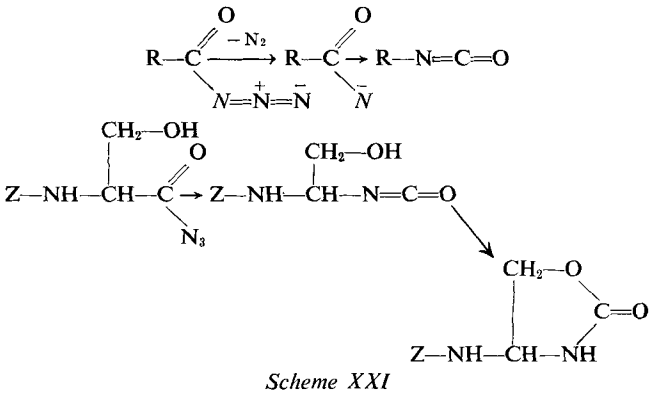
Scheme XX

Easy removal of the by-products greatly facilitates the isolation of the desired peptide from the mixed anhydride reaction mixture. The scope and limitations of this method have been discussed by Albertson (147). One of the most serious limitations is the danger of racemization when the *N*-protection for peptides used in mixed anhydride coupling is other than benzyloxycarbonyl or *tert*-butyloxycarbonyl. Anderson *et al.* (148) have reported an extensive reevaluation of the conditions used for mixed anhydride coupling. The tertiary base used for mixed anhydride formation is not merely a hydrogen chloride acceptor, but it first reacts with the chloroformate to form a quaternary compound which then reacts with the carboxyl group. The degree of racemization is dependent on the basicity of the amine used. The use of weaker bases such as *N*-methylmorpholine has been recommended. The best results are obtained with isobutylchloroformate, and the solvents of choice for mixed anhydride coupling are ethyl acetate and tetrahydrofuran.

N-Acyl amino acids, after activation with isobutylchloroformate, are used for the coupling reaction without the isolation of the active intermediate; however Anderson (148) has reported the isolation of *N*-phthalyl alanine mixed anhydride with isobutyl carbonic acid. This is perhaps the first time that a reactive derivative of a mixed anhydride has been isolated. In the author's experience, a combination of modified mixed anhydride and pentachlorophenyl active ester methods provides a useful approach for lengthening the peptide chains of *N*-protected, *C*-activated peptide units from *C*-terminal residues of amino acids (149). The synthesis of these peptide units without any significant racemization is of considerable importance for the preparation of sequential polypeptides. Another approach for the preparation of mixed anhydrides is by the use of diphenylphosphoric acid (150) which is particularly useful for the activation of hindered trityl amino acids. A large variety of "partner acids" such as derivatives of phosphoric, phosphorous, and sulfuric acids, and aliphatic or aromatic sulfonic acids which are employed for the formation of mixed anhydrides have been reviewed (32).

Azide Method—Despite a number of disadvantages, this method, which was introduced by Curtius (151), is very highly regarded in the synthesis of peptides, mainly because racemization during the coupling reaction is not observed. The azides are unstable compounds which are used without isolation and are prepared *via* the crystalline hydrazides of *N*-protected amino acid or peptide esters.

The formation of isocyanates due to the rearrangement of azides may lead to the formation of urea derivatives, and cyclic urethans in serine-containing peptides (152) (Scheme XXI).

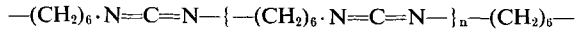


Scheme XXI

The preparation of hydrazides *via* hydrazinolysis is not possible with phthalyl or trifluoroacetyl *N*-protected amino acid or peptide esters. Hofmann (153) has suggested the use of protected hydrazides, and this approach has been used by Weygand (154) for the synthesis of very useful intermediates for lengthening the peptide chains both from the amino and carboxyl terminal residues of amino acids.

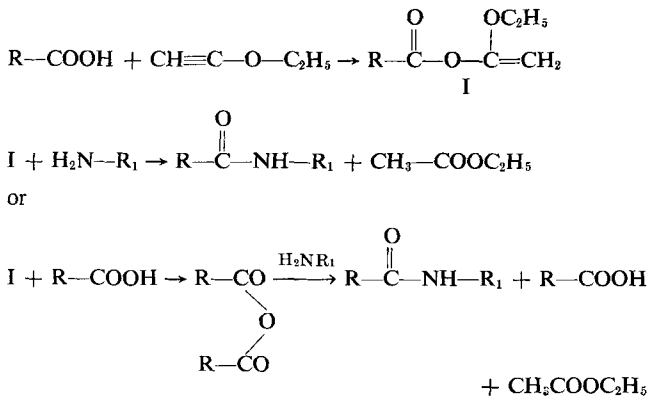
Carbodiimides—Among the most frequently used carbodiimides is the *N,N'*-dicyclohexylcarbodiimide which was introduced by Sheehan and Hess (155). This method is perhaps the most popular method of peptide synthesis and the mechanism of possible routes of acylation with the aid of carbodiimides has been discussed in detail by Khorana (156). When a dialkylcarbodiimide is allowed to react with a carboxyl group, an *o*-acylurea is first formed which in turn reacts readily with nucleophiles. One of the disadvantages of this method is the rearrangement of the reactive intermediate *o*-acylurea to a more stable *N*-acylurea which is only slightly susceptible to reactions with nucleophiles. Another disadvantage is the difficulty of the removal of *N,N'*-dicyclohexylurea which is formed as a by-product. To meet this difficulty, dialkylcarbodiimides such as *N*-ethyl, *N'*-(γ-dimethylaminopropyl) carbodiimide which yield water soluble ureas have been devised (157). The use of insoluble polycarbodiimides such as polyhexamethylene carbodiimide, which allows easy purification of reaction products, has been suggested by Frankel (158) (Scheme XXII).

The use of an interesting coupling reagent, ethoxyacetylene, was reported by Arens (159). The coupling



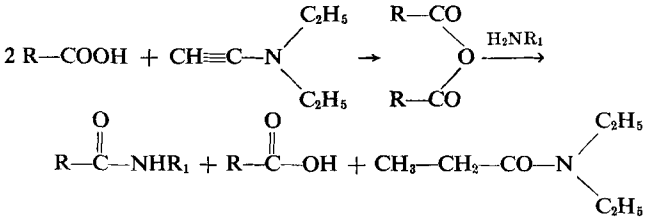
Scheme XXII

with this reagent has an attractive feature in that the by-product ethyl acetate does not interfere with the isolation of the desired product. The peptide bond formation may proceed either directly, by the reaction to amino component to the addition product of carboxylic acid and ethoxyacetylene, or through the formation of symmetrical anhydride (Scheme XXIII).



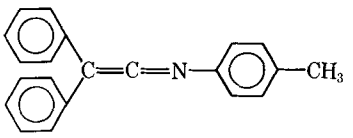
Scheme XXIII

Buyle and Viehe (160) introduced inamines such as dimethylamino *tert*-butylacetylene or dimethylaminophenyl acetylene as coupling reagents. These types of inamines are also capable of forming symmetrical anhydrides; and, Weygand (161) has used symmetrical anhydrides formed by diethylaminomethyl acetylene for the synthesis from *C*-terminal residue of amino acids of a protected nonpeptide. The by-product, propionic acid diethyl amide, is soluble in petroleum ether and can be easily removed (Scheme XXIV).



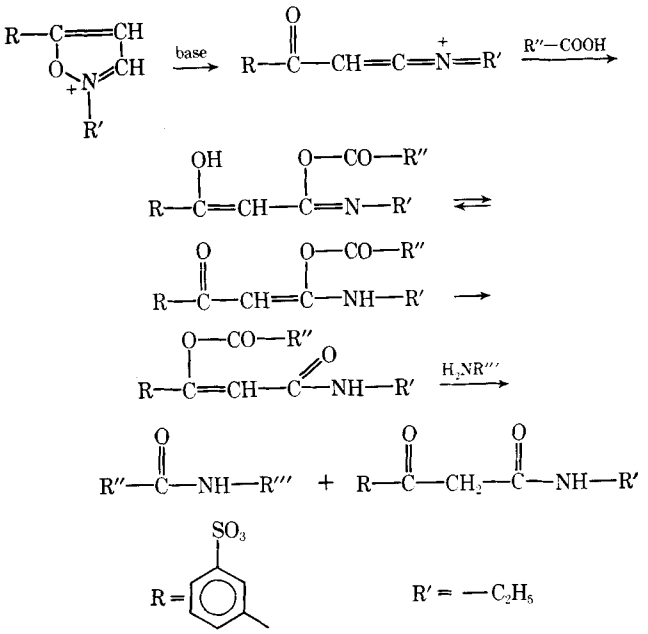
Scheme XXIV

Ketenamines such as diphenyl ketene *p*-tolylimine (Scheme XXV) have been used in the synthesis of peptides (162) and in a sense, the mechanism of their reaction closely resembles that of isoxazoliums salts which were introduced by Woodward (163, 164) a few years later.



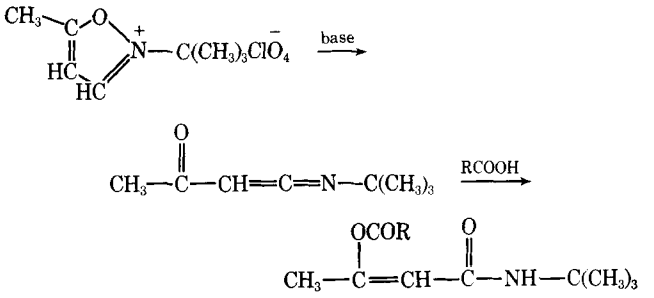
Scheme XXV

Woodward's reagent, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate, reacts with a base to form a ketimine which adds to the carboxyl group and a tautomeric enol ester is formed. This is followed by a rearrangement involving an acyl migration to the actual activated enol ester derivative (Scheme XXVI).



Scheme XXVI

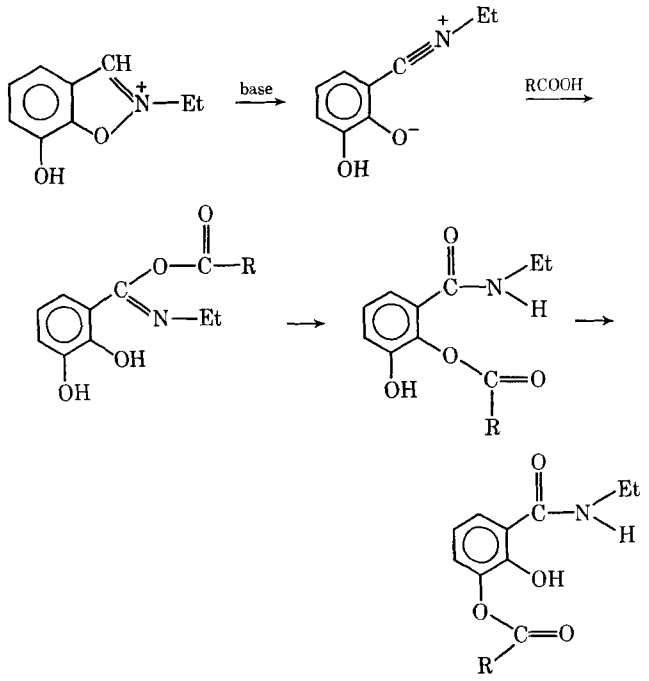
Although high yields are obtained during coupling with Woodward's reagent under optimum conditions, lower yields result if the addition of amine component to active enol ester is delayed. This is perhaps due to intramolecular O → N acyl migration. Woodward and Woodman (165) have proposed the solution to this problem by using bulky *N-tert*-butyl-substituent which gives a stable enol ester. A new coupling reagent, *N-tert*-butyl-5-methylisoxazolium perchlorate, has been suggested, which in addition to forming stable activated enol esters, also makes possible the isolation of the intermediate ketoketenimes (Scheme XXVII).



Scheme XXVII

Another isoxazolium cation, 2-ethyl-7-hydroxybenzisoxazolium fluoroborate, which seems to be quite promising was proposed by Kemp (119). The interesting feature of this coupling reagent is the remarkable base stability of the activated 3-acyloxy-2-hydroxy-*N*-ethylbenzamide derivatives, and this should make them useful for coupling with salts of amino acids (Scheme XXVIII). Attachment of electron-withdrawing substituents in the benzene ring of benzisoxazolium cation such as in the case of 5,7-dichloro-2-ethyl benzisoxazolium salt, has been suggested for a possible increase in the activity of the intermediate active esters (166) (Scheme XXIX).

Belleau and Malek (167) have used *N*-ethoxycarbonyl-2-ethoxy 1,2-dihydroquinoline (EEDQ) as a

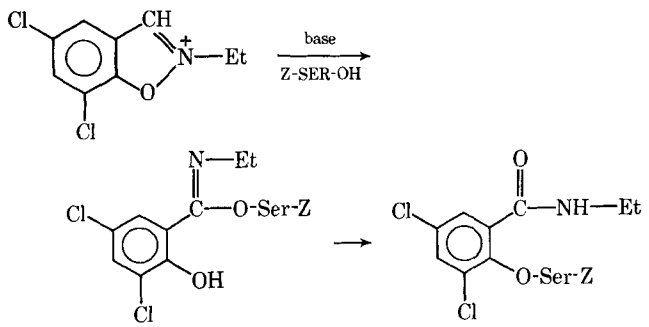


Scheme XXVIII

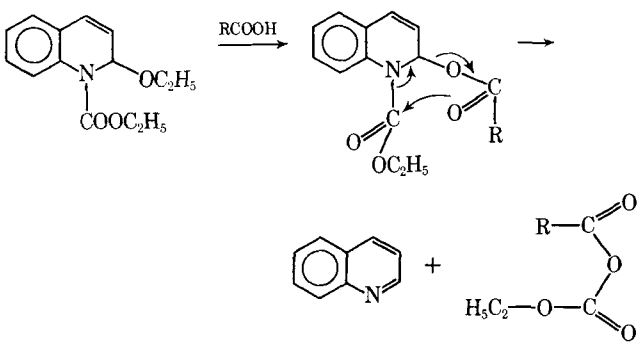
coupling reagent. The actual activation proceeds *via* the formation of the mixed anhydride which is preceded by the displacement of the 2-ethoxy substituent by carboxylic acids. Slow formation but rapid consumption of the active derivative should minimize the side reactions (Scheme XXX).

Reactive *N*-acyl Derivatives—The transfer of acyl residues from derivatives of N^{im}-acylhistidine to amino groups, and thus the possibility of carboxyl activation by imidazolide formation, has been described by several authors (168). Sheehan found that the intramolecular amide (active lactam) of *N*-α-*p*-nitrobenzyloxycarbonyl histidine reacts with benzylamine to form *N*-α-*p*-nitrobenzyloxycarbonyl histidine benzylamide (169). This, along with the contributions from Staab (170), Anderson (171), and Beyerman (172), have led to a number of useful coupling reagents such as *N,N'*-carbonyldiimidazole, thionyldiimidazole, and carbonyl dipyrazole. The reaction proceeds through the formation of acyl derivatives which couple with the amino group to form the peptide bond. The liberated imidazole has a beneficial catalytic effect on the rate of the reaction (Scheme XXXI).

Active Esters—The synthesis of peptides *via* the aminolysis of activated esters was pioneered with the

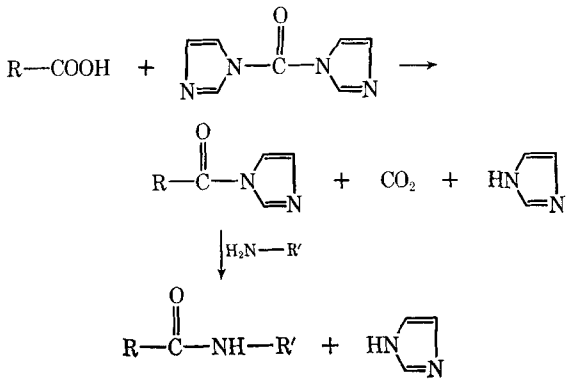


Scheme XXIX

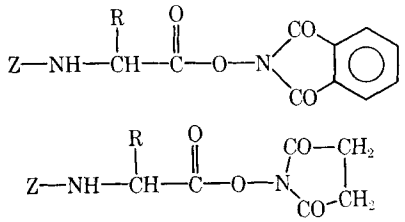


Scheme XXX

use of phenylthiol esters by Wieland (173). One of the main advantages with the use of active esters is the possibility of isolation and purification of the reactive intermediates. These esters have relatively less energy when compared to other activating methods such as mixed anhydride or DCC, and therefore normally do not acylate weaker nucleophiles such as the OH group in hydroxy or phenolic amino acids. A large number of methyl esters with different negative substituents were examined by Schwyzer, and as a result, cyanomethyl esters were introduced in the synthesis of peptides (174). Bodanszky embarked on an elaborate study of phenyl esters, in which the already pronounced electron attraction of the phenyl group was further enhanced by appropriate substituents, and recommended the use of *p*-nitrophenyl esters (175), which indeed was a valuable service to the peptide chemists. Schwyzer's group, shortly afterwards, described the preparation of a series of negatively substituted aryl esters, among them the very interesting *p*-methanesulfonylphenyl ester (176). The use of halogen-substituted phenyl esters such as 2,4,5-trichlorophenyl and pentachlorophenyl ester was suggested by Kupryszewski (177). A detailed kinetic study of various active esters was reported by Stick and Leemann (178). The use of pentachlorophenyl esters has been reinvestigated by Kovacs and this author as a versatile tool for the synthesis of peptides and sequential polypeptides (179). The pentachlorophenyl active esters have the following advantages: (*a*) they are one of the most active esters; (*b*) they are generally higher melting compounds than other active esters, which leads to their easy crystallization and purification; (*c*) they are conveniently prepared without any significant racemization by *N,N'*-dicyclo-



Scheme XXXI

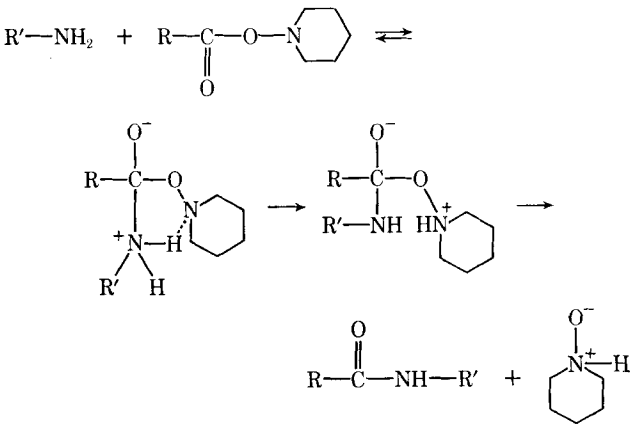


Scheme XXXII

hexylcarbodiimide (DCC) method; (*d*) they are stable to controlled hydrogenation conditions and make an excellent combination with *N*-benzyloxycarbonyl and *tert*-butyl protecting groups when the incorporation of trifunctional amino acids in the peptides is desired. Pentachlorophenyl esters of *N*-protected amino acids also couple in satisfactory yields with amino acids and peptides *C*-protected by suitable salt formation (118).

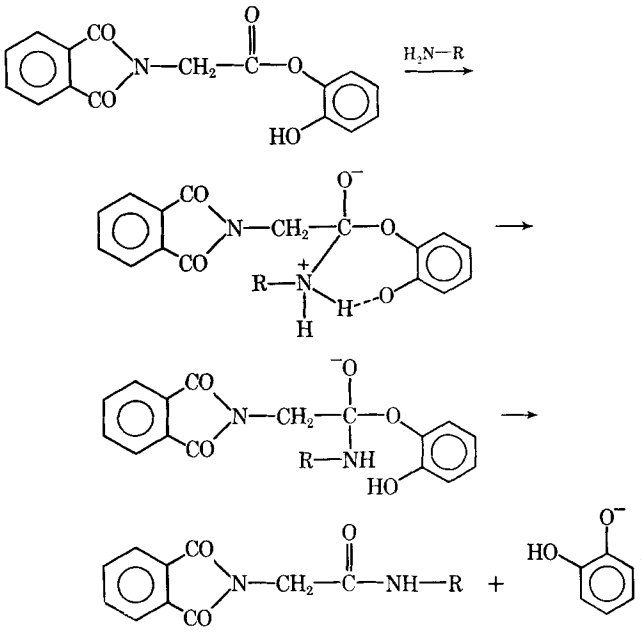
Further search for active esters led Nefkens (180) to the use of *N*-hydroxyphthalimide esters which were shortly followed by the introduction of analogous esters namely the *N*-hydroxysuccinimide esters from the laboratories of Anderson (181) (Scheme XXXII). During the past few years, the latter esters have received considerable attention mainly due to the easy removal of the by-product of acylation, *N*-hydroxysuccinimide, by water.

Another type of active esters which have already proven their worth in the synthesis of peptides, and which hold a bright future, are the reactive derivatives of *o*-acylhydroxylamine such as the esters of *N*-hydroxypiperidine introduced by Young (182). The coupling through these esters proceeds without any significant racemization. This may be attributed to less chances for oxazolone formation because of the ability of the heterocyclic nitrogen atom to share and then accept the proton from the incoming amine. The condensation is accelerated on account of the stability through hydrogen bonding of the transition complex of the adduct; and further, the leaving group will be a tautomer of 1-hydroxypiperidine rather than the unstable anion (183) (Scheme XXXIII).



Scheme XXXIII

Closely related to *N*-hydroxypiperidine esters are the oxine esters which also undergo coupling without racemization, and their reaction mechanism seems to be analogous to 1-piperidyl esters (184). The idea of stabilization of the transition complex through hydrogen bonding and therefore limiting the possibility of oxazolone formation during coupling has been extended in the use of *o*-hydroxyphenyl esters. Their initial success in the synthesis of peptides without any racemization (183) will certainly lead to the development of more versatile active esters (Scheme XXXIV).



Scheme XXXIV

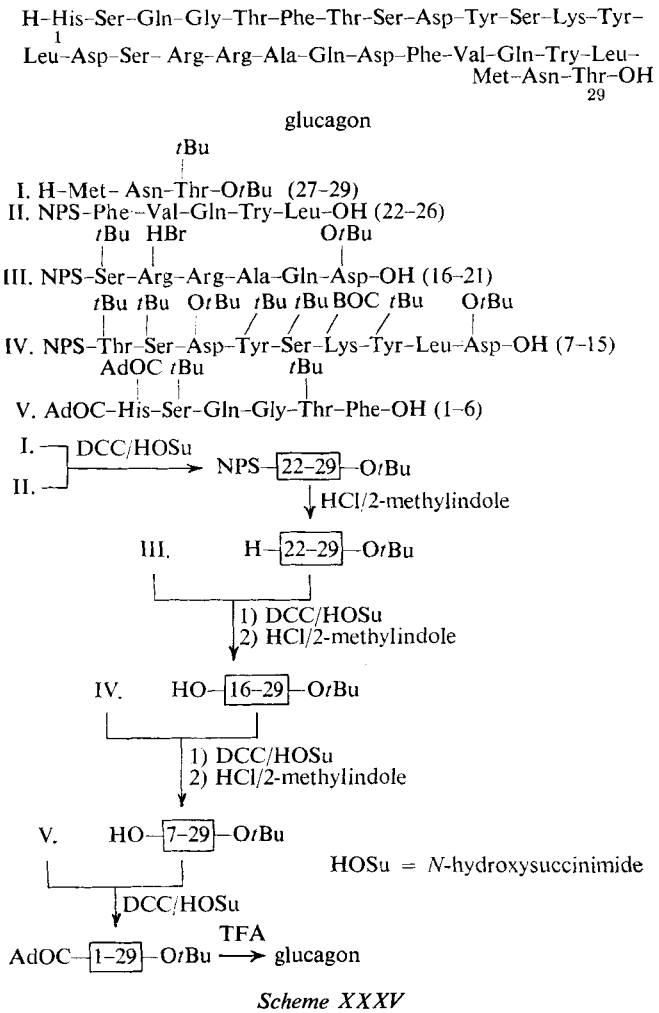
Young (185) prepared the acyl amino acid esters of 2-hydroxypyridine and of 2-mercaptopyridine (SPyr). Esters of the latter (2-pyridylthiolesters) react very rapidly and exothermically with amino esters, giving high yields of protected peptides. A notable feature of these esters (SPyr) is the easy coupling with strongly hindered amino acids such as valine, isoleucine, and α-aminoisobutyric acid (186).

Active esters can be prepared by the condensation of the phenols or corresponding components with *N*-protected amino acids and peptides by a number of coupling methods. Most frequently used are the dicyclohexyl carbodiimide and mixed anhydride methods. A number of active esters of hydroxyamino acids with *N*-α-NPS and *o*-*tert*-butyl protection have been recently reported (187). These intermediates should be quite useful in lengthening the peptide chains. By using isopropyl alcohol, Walter *et al.* (188) were able to prepare previously unreported NPS-protected glutamine and asparagine *N*-hydroxysuccinimide esters. In certain cases, the rate of aminolysis by active esters may be accelerated by the use of suitable catalysts such as imidazoles and 2-hydroxypyridine.

During the preparation of active esters, the *C*-terminal amino acid residue of the peptide chain may undergo racemization; however, with the use of controlled experimental conditions, this problem can be overcome. Formation of the peptide bond is normally associated with the partial loss of optical purity of the amino acid whose carboxyl is activated. This problem may be avoided by lengthening the peptide chains from *C*-terminal residues of amino acids, in which case the

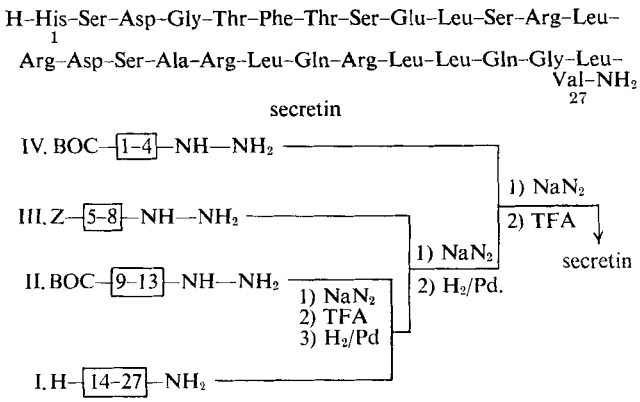
activated component would always be a monomer and, hence, less prone to oxazolone formation. The procedure of fragment condensation, with suitable activation methods and selection of the fragments in which the *C*-terminal residue is glycine or proline, has been very rewarding to limit the degree of racemization during the peptide synthesis. To cite only a few examples, glucagon, secretin, and thyrocalcitonin were recently synthesized by fragment condensation approach.

Glucagon—The synthesis of the pancreatic hormone, glucagon, was achieved by the condensation of five fragments (13). A combination of NPS, and *tert*-butyl and benzyloxycarbonyl groups were used throughout the synthesis. *N*-Terminal histidine was used as *Nα,N*-(im)-di-adamantyloxycarbonyl histidine and the peptide bond formation and condensation of the fragments was carried out mainly with DCC/HOSu method. The following fragments were synthesized and then condensed stepwise (Scheme XXXV).



Scheme XXXV

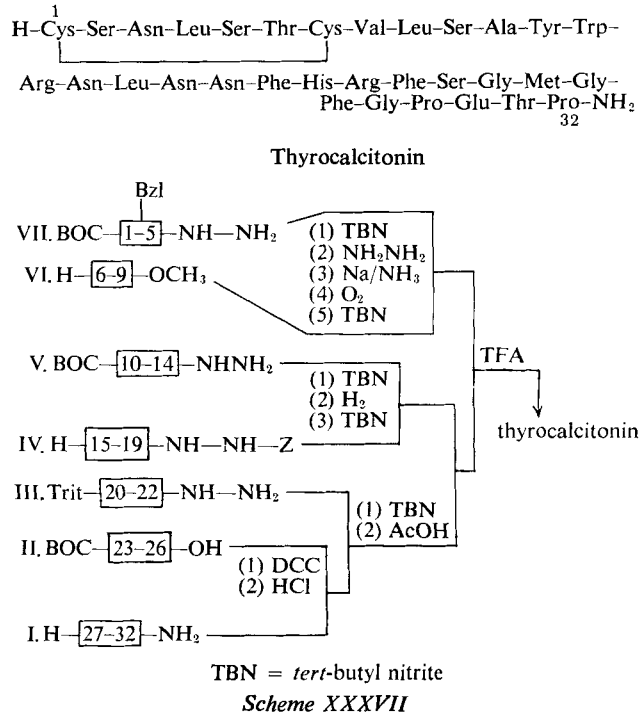
Secretin—The structure of secretin resembles glucagon a greal deal. The presence of four arginine residues in secretin imparts a strong basic character to the molecule. The *C*-terminal amino acid valine is present in the amide form. Synthesis of secretin was accomplished in a stepwise manner, starting from *C*-terminal amino acid



Scheme XXXVI

(189). The other approach was the stepwise synthesis of *C*-terminal tetradecapeptide, and this was attached to three fragments of the *N*-terminal half of the molecule, using the stepwise condensation approach (12). Benzyloxycarbonyl and BOC groups were predominantly used for amino protection. Serine and arginine residues were incorporated as *o*-benzyl and nitro derivatives respectively. Both of these protections were removed by hydrogenation after each fragment condensation. The peptide bonds were generally formed with *p*-nitrophenyl esters and 2,4-dinitrophenyl esters (for arginine and threonine). The fragments were condensed *via* the azide method (Scheme XXXVI).

Thyrocalcitonin—In thyrocalcitonin which is a calcium-regulating hormone, the *C*-terminal amino acid proline is present in the amide form, and cysteines at positions one and seven are joined by a disulfide bridge. The synthesis of thyrocalcitonin was achieved by stepwise condensation of seven fragments (14). Benzyl protection was used for sulfhydral groups and the disulfide bridge was formed at the nonapeptide stage. Serine residues were incorporated without OH



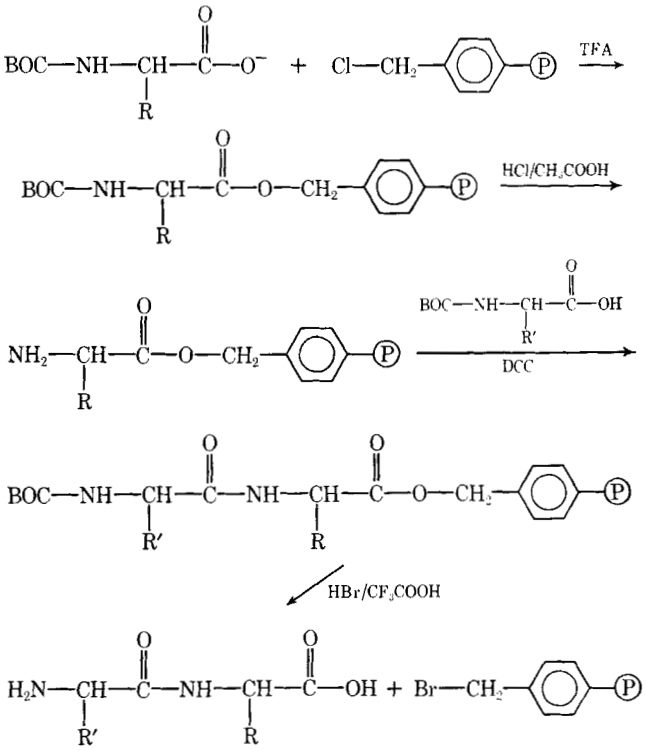
TBN = *tert*-butyl nitrite

Scheme XXXVII

protection. BOC protection was predominantly used for amino groups and the coupling reactions were mainly carried out by azide methods. Di-Z-histidine was used for the preparation of the tripeptide III and was converted to the dityrosyl derivative before coupling for the formation of the tridecapeptide amide (Scheme XXXVII).

Peptide Synthesis with the Help of Polymeric Support—The isolation and purification of each intermediate during the building of a peptide chain is of utmost importance to limit the extent of racemization in the final product. However, the development of newer techniques and the modification of conventional methods has inspired the synthesis of the peptides without the isolation of intermediates. An apparent advantage of the approach is the tremendous saving of time which is normally required for the stepwise purification. Knorre and Shubina (190) have elongated the peptide chain at the carboxyl end by coupling an aqueous solution of formyl amino acid with an amino acid ester by means of water-soluble carbodiimides. The reaction mixture upon passing through resin columns is freed from reactant and reagents. The purified product, thus obtained, is saponified and is used directly for the next step in the synthesis. A protected hexapeptide was synthesized with less than 5% racemization. Sheehan (191) used a somewhat similar approach but extended the peptide chain from the amino end. The technique proved quite successful for the synthesis of a protected heptapeptide.

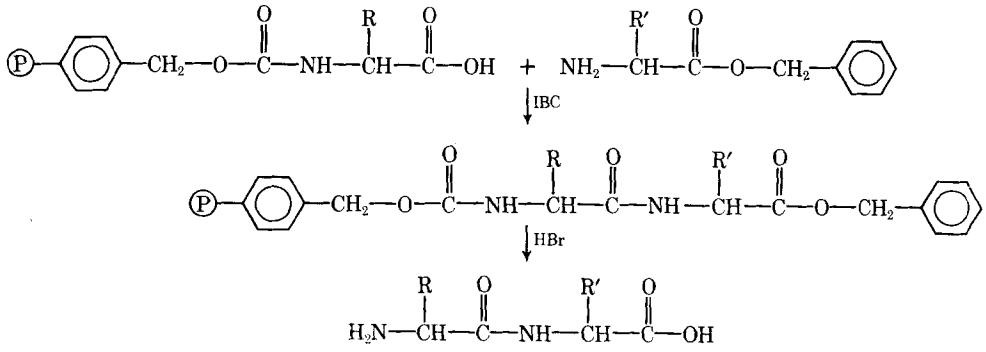
The above two approaches were only the beginning of the new ventures into less conventional approaches in the synthesis of peptides. In 1963, Merrifield (192) proposed an ingenious idea for lengthening the peptide chains by providing an insoluble polymeric support to the *C*-terminal residue of amino acids and extending the peptide chains by stepwise addition of succeeding amino acids. In this approach, which is called solid phase method for peptide synthesis, the growing peptide chain is at all times during the synthesis firmly bound by a covalent linkage to a solid particle and is, therefore, completely insoluble in all the reaction solvents. This allows separation of the peptide chain from soluble starting materials and by-products by filtration and thorough washings. Thus the intermediate peptides are purified, not by the usual recrystallization procedures, but by dissolving away the impurities. This greatly simplifies the manipulations and shortens the time required for the synthesis of peptides. After the desired sequence is assembled, the peptide is removed from the solid support. For a supporting solid, a copolymer of 98% styrene and 2% divinyl benzene in the form of small (20–80μ) beads is chloromethylated and the product is attached to the *N*-protected amino acid to give a substituted benzyl ester derivative. The amino group was originally protected by the benzyloxycarbonyl group but it was replaced by the *tert*-butyloxycarbonyl group and recently by *o*-nitrophenylsulfenyl group (193). The advantage of the last two groups is the easy removal by relatively mild acid conditions which minimizes the danger of cleavage of the peptide chain from the resin during the synthesis. After deprotection of the amino group, the next protected amino acid is coupled by DCC or active ester methods



Scheme XXXVIII

in solvents having high dielectric constants, such as methylene chloride or dimethylformamide (Scheme XXXVIII).

The selection of benzyl ester linkage can be justified on the basis of its stability to various synthetic reactions and easy cleavage by anhydrous hydrogen bromide at the end of the synthesis. The reactions outlined in Eq. 38 constitute one cycle in which a dipeptide is formed. However, additional cycles may be carried out in the same way, one at a time, until the desired sequence of amino acids in the peptide is completed. One of the most critical requirements for a successful synthesis, using a polymeric support is the quantitative coupling reaction. Unless this can be achieved at each step of the synthesis, a chain which does not react at one step will have a chance to react at each of the later stages and will lead therefore to undesired peptides with different amino acid composition. An excess of activated component, a longer period of reaction time, and the use of low cross-linked, high swelling resin generally leads to complete coupling. Of course the potential difficulty in the final purification after the peptide is removed from the resin must be accepted in return for convenience and speed in building up the peptide chain. In case DCC is used for activation, the possibilities for the loss of excess reagent due to the formation of *N*-acylurea must be considered. A suitable selection of active esters (194) for coupling to resin attached amino acids or peptides would eliminate this side reaction, and would also permit the incorporation of hydroxy amino acids without any protection on hydroxyl group. Active ester coupling has been of particular use in the case of glutamine or asparagine incorporation on account of the dehydration of carboxamide groups in the side chains of these amino acids when DCC is used. In the case of peptides incorporating aspartyl



IBC = isobutylchloroformate

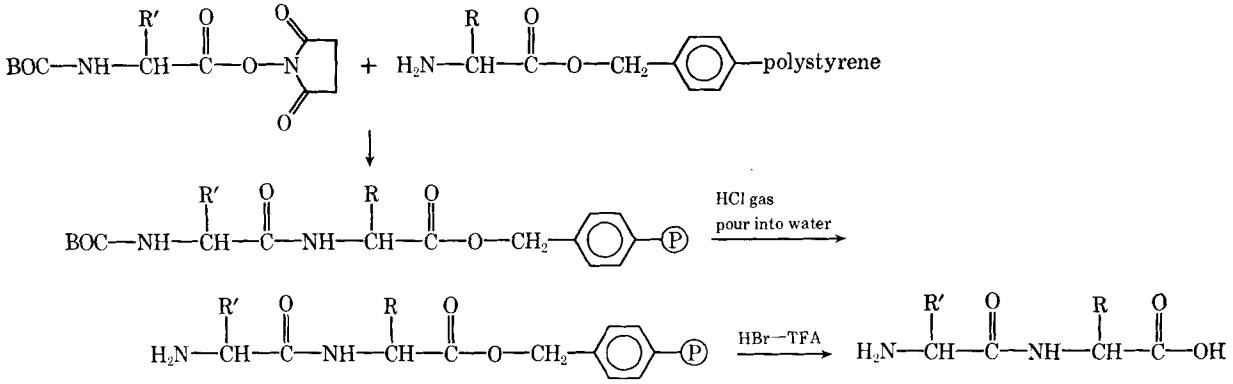
Scheme XXXIX

or glutamyl residues with side chain carboxyl esterified, transpeptidation normally does not occur under the conditions used during synthesis by solid phase method. However, the treatment of HBr-trifluoroacetic acid, which is used to liberate the peptide from the resin support, may lead to the formation of amino succinyl derivatives in the case of peptides containing β-benzyl aspartyl-seryl residues. The opening of the imide derivatives may produce undesirable peptides with β-aspartyl linkages. Bumpus has reported the difficulties encountered during the formation of arginylproline bond under the conditions of the solid phase method using DCC (195). For the synthesis of peptides where the *C*-terminal amino acid occurs as an amide such as in oxytocin, the desired peptides, after the synthesis, are cleaved from the resin by ammonolysis in methanol. Although this approach has been quite successful with smaller peptides, particularly those with glycine as *C*-terminal amino acid, a considerable degree of difficulty was encountered during the ammonolytic cleavage of secretin where the *C*-terminal valine could not be removed from the resin as an amide (196).

Letsinger and Kornet (197) suggested as a modification to Merrifield's approach the use of "popcorn polymer" of polystyrene with a very low degree of cross-linking by divinylbenzene. In addition, the *N*-terminal amino acid was the anchoring group with the resin and the chain was extended by stepwise incorporation of subsequent amino acids. This approach has not gained much popularity mainly because of the possibility of racemization which is normally associated with the lengthening of the peptide chains from the *N*-terminal residues of amino acids (Scheme XXXIX).

A very interesting idea was proposed by Shemyakin (198) in which the insoluble cross-linked polystyrene was substituted by a soluble polymeric support. This offers the apparent advantage of having all reactions carried out in solution, thereby eliminating the problem of permeability or diffusion. The polymeric support was an emulsion polystyrene of average molecular weight of 200,000. The coupling of amino acid and peptide derivatives of oxymethyl-polystyrene was carried out with hydroxysuccinimide esters of BOC-protected amino acids. This approach may prove to be a very effective synthetic tool if certain problems can be overcome, such as formation of viscous gum when the linear polystyrene is thrown out of solution by pouring it into water (Scheme XL).

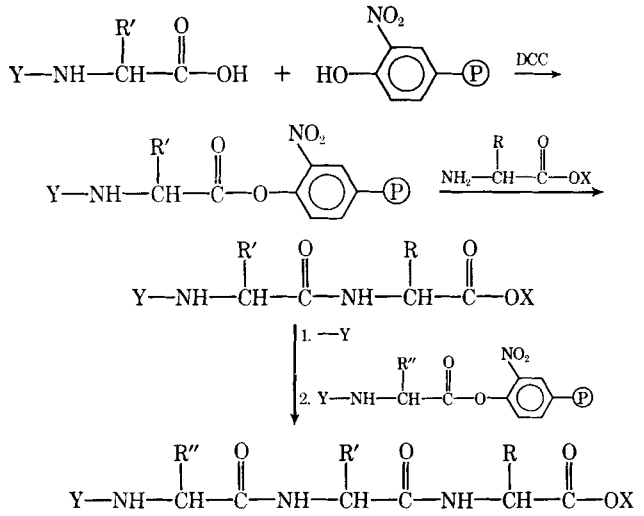
One of the main concerns in the solid phase peptide synthesis is that the purification of intermediates cannot be effected. At about the same time, a new approach was suggested from the laboratories of Katchalski (199) and Wieland (200). Where in Merrifield's method, it is the peptide which is bound to the insoluble carrier and the *N*-blocked amino acid-activated derivative is added while in solution, in the new approach a solution of free peptide ester is added to an insoluble polymer-supported, *N*-blocked amino acid-active ester. The reaction between peptide ester and active amino acid ester can be carried out in the presence of a large excess of insoluble active ester, and the unreacted reagent (active ester) can be readily removed by filtration or centrifugation and washing. The new peptide remains in solution and can be suitably purified before it is reacted with the next insoluble active ester



Scheme XL

component. Katchalski proposed a number of insoluble polymer-active esters. However, best use was made of DCC coupling of *N*-protected amino acids or peptides with a polymer made from 4-hydroxy-3-nitrostyrene cross-linked with 4% divinylbenzene (Scheme XLI). Another very interesting feature of this approach is the application of these insoluble polymer-active esters in the synthesis of cyclic peptides. The use of insoluble polymer-active esters attached to peptides limits the intermolecular condensation and facilitates intramolecular aminolysis, thereby avoiding the formation of linear polymers; cyclic peptides of high purity are isolated in good yields (201) (Scheme XLII).

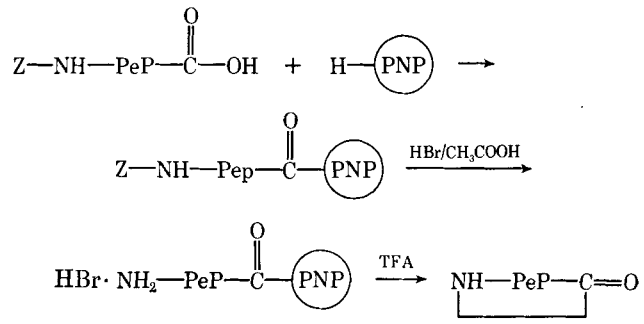
Wieland utilized formaldehyde cross-linked *p,p'*-dihydroxydiphenylsulfone as a starting material for the synthesis of the corresponding insoluble *N*-blocked amino acid active esters (200) (Scheme XLIII).



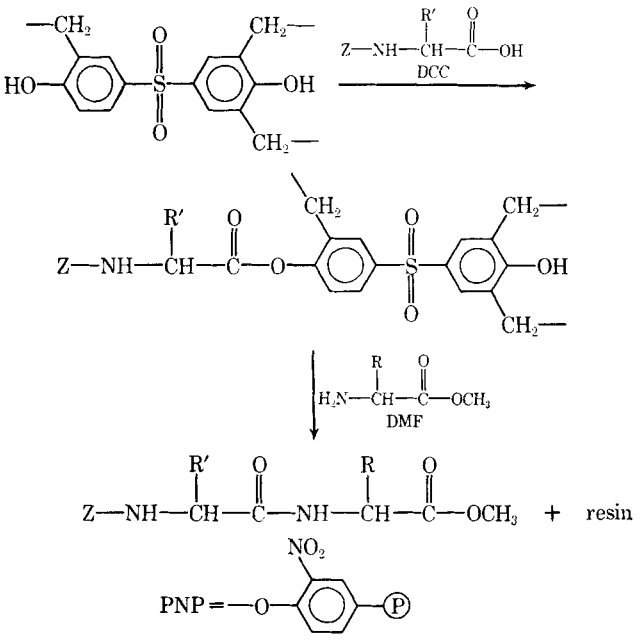
Y = amino protection X = carboxyl protection

Scheme XLI

The synthesis of peptides with polymeric active esters was also investigated by Blout (202). BOC-amino acid *N*-hydroxysuccinimide active ester derivatives of copoly(ethylene-*N*-hydroxymaleimide) were used as insoluble active ester reagents. For the preparation of these esters, copoly (ethylenemaleic anhydride) was condensed with hydroxylamine hydrochloride, and the resulting *N*-hydroxy polymer derivative was coupled either by mixed anhydride or DCC methods with BOC-amino acids (Scheme XLIV).



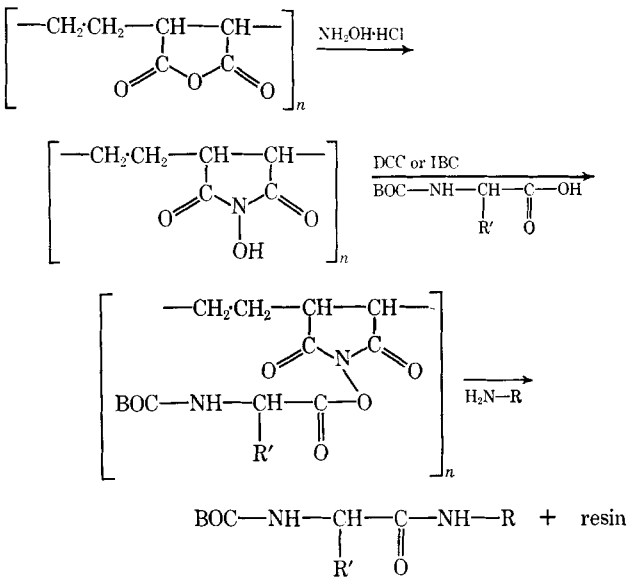
Scheme XLII



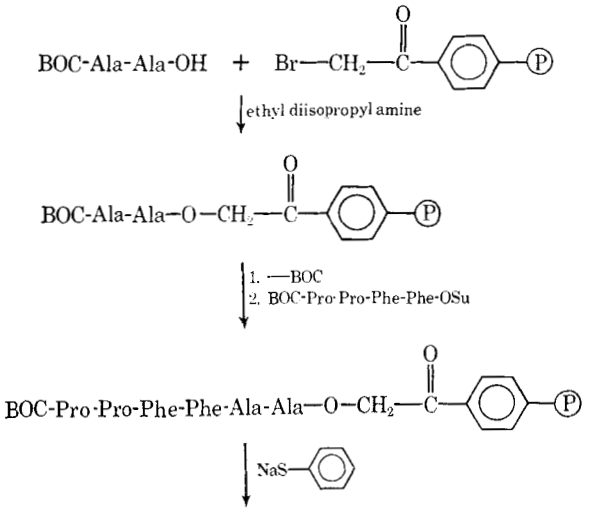
Scheme XLIII

The substitution of activated amino acids in the polymers previously mentioned can be varied by appropriate adjustment of the *N*-protected amino acid : polymer molar ratio. Satisfactory results are obtained when 1–2 mmole of activated amino acid is bound to each gram of the resin.

Weygand (203, 204) investigated the possibilities for the coupling of the *N*-protected peptides with amino acids or peptides anchored to polymers. The advantage of this approach is the availability of a relatively larger number of peptide intermediates, which can be properly characterized before incorporation in the peptide chain. Dipeptides, *N*-protected by BOC, were reacted with the bromoacetyl derivative of polystyrene; and, after the removal of *N*-protection from the resulting dipeptide polymer derivatives, coupling was carried



Scheme XLIV



Scheme XLV

out with *N*-protected di- or tetrapeptide *N*-hydroxysuccinimide esters. Selective removal of the peptides from the polymer was achieved by sodium thiophenolate, and the *N*-protected peptides so obtained were free from racemization (Scheme XLV).

While the above approach which has also been used by Anfinsen (205) is indeed a very useful modification in solid phase synthesis of peptides, the proper evaluation of this will depend on the successful synthesis of larger peptides.

The success of the solid phase method and new approaches using various forms of solid supports is apparent from the synthesis of a large number of biologically active peptides such as bradykinin, oxytocin, angiotensin, insulin, *etc.*, and their analogs. However, the most frequently used approach is still the one which was originally suggested by Merrifield, and in order to accelerate the synthesis of peptides, a simple automated apparatus has been designed (206). It consists of a reaction vessel where the actual synthesis takes place and devices which transfer reagents and solvents into and out of the vessel. The entire series of events is controlled by a programmer which can be arranged in advance to direct the various operations of the instrument. The automated process produces a protected peptide chain attached to the solid polymer support, and after cleavage from the resin and deprotection, the peptide is suitably purified.

It was apparent, from the onset of the introduction of the solid-phase method, that the synthesis of larger peptides and proteins would become feasible, and this was indeed demonstrated by the synthesis of a protein. A linear polypeptide of 124 amino acid residues, with the sequence of bovine pancreatic ribonuclease A (RNase A), was synthesized by Merrifield using 369 chemical reactions and 11,931 steps of the automated peptide synthesis machine without any intermediate isolation steps (207). Anhydrous hydrogen fluoride was used for the single step cleavage of the peptide from resin and simultaneous removal of all protecting groups.

The properties of anhydrous hydrogen fluoride (HF) as a reagent for the acidolysis of various protecting groups have been studied by Sakakibara (208). Amino acids with various protecting groups, particularly the nitro group in nitroarginine, *tert*-butyl group in *S-tert*-butyl cysteine and many other protecting groups can be conveniently removed by HF at low temperatures in the presence of anisole. When carefully handled, HF should prove to be an effective tool for planning the strategy in the synthesis of peptides.

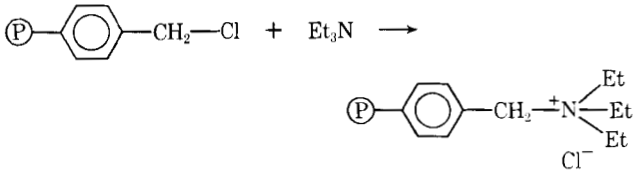
Iselin suggested the use of hydrazine for the cleavage of peptides from polymer support (209). Hydrazinolysis leaves the *N*-protection intact, and the cleaved peptide is isolated as hydrazide which can be directly coupled to another peptide after conversion into the azide. The same approach has been used by Anfinsen for the synthesis of water-insoluble *N*-protected peptide hydrazides which do not contain ω-protected aspartic or glutamic acid residues or other hydrazine-labile groups (210).

The possibility of a side reaction during esterification of the *C*-terminal amino acid with chloromethylated resin has been reported by Beyerman (211). This side reaction probably occurs between the free triethylamine and chloromethyl groups producing a quaternary ammonium compound (Scheme XLVI).

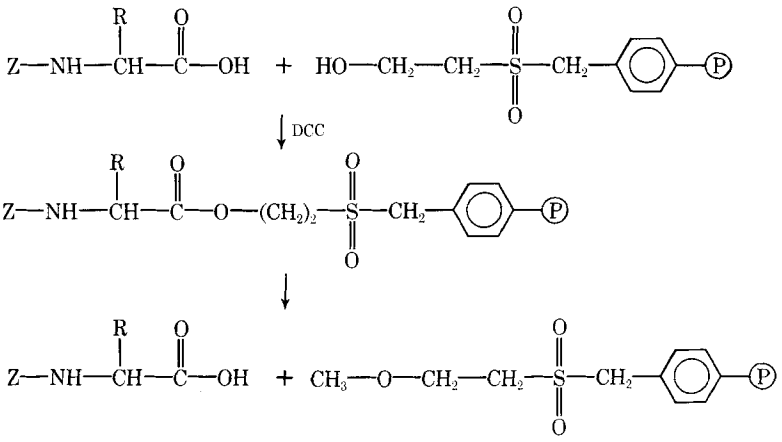
The *N*-protected *C*-terminal amino acid may be attached to the polymeric support by DCC method, if the chloromethylated polymer is first reacted with thioglycol and the resulting thioether is oxidized with perbenzoic acid. The *N*-protected peptides attached to the modified solid support can be selectively cleaved from the resin without affecting the *N*-protected group (212) (Scheme XLVII).

Solid phase method of peptide synthesis has been extended in the synthesis of polymers with different sequence and size. Different applications in the synthesis of oligo-nucleotides have been reported (213). Oligomers of nylon (214) and polypeptides with an ordered sequence of amino acids, such as H(prolyl-glycyl-glycyl)-OH (215), and H(prolyl-prolyl-glycyl)-OH (216) have been successfully synthesized with solid support. Recently, Rothe (217) reported the synthesis of different-sized oligomers of epsilon amino caproic acid by using dimeric and tetrameric units for the chain growth. Coupling of these units with the polymer-supported chain was carried out with the aid of diester chlorides of phosphorus acid instead of DCC.

The reverse of the peptide synthesis by solid phase, *i.e.*, the degradation of the peptide on a polymeric support using the Edmann method, has been suggested by two different groups. Stark's (218) procedure involves attaching the peptide by its *N*-terminal residue to a support of polystyryl isothiocyanate, cyclizing, isolating and analyzing the residual peptide, and reattaching the peptide to the resin. Repetition of these



Scheme XLVI

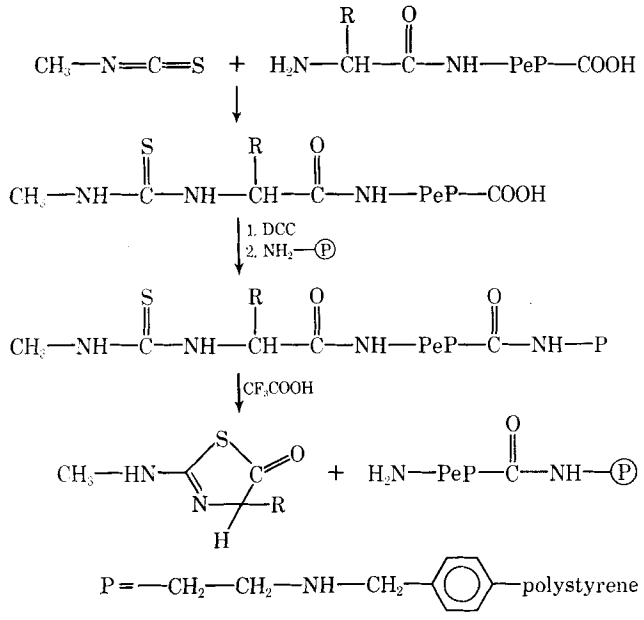


Scheme XLVII

steps leads to the establishment of the sequence of amino acid residues in the peptide chain. In the method proposed by Laursen (219), the peptide is attached by its *C*-terminal amino acid and the degradation is performed in the usual manner. The liberated thiazolinone is removed in each cycle simply by filtration (Scheme XLVIII).

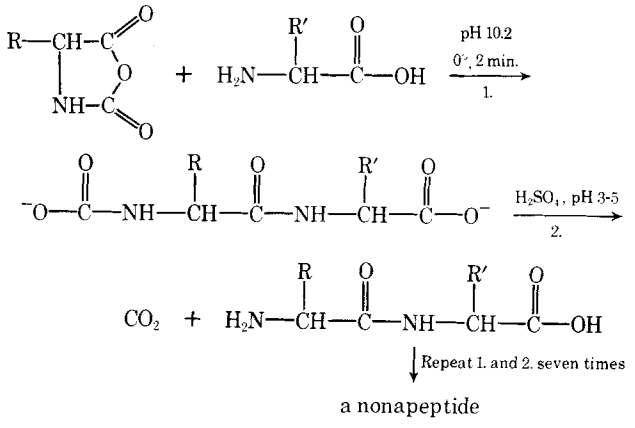
A closely related approach, using a combination of Edmann degradation on polymeric support and mass-spectrometric methods, has been suggested by Weygand (220) for the determination of the extent of amino acid residues incorporated in the growing peptide chains attached to the resin supports.

Leuch's Anhydrides—*N*-Carboxy-α-amino acid anhydrides (Leuch's anhydrides, NCA's) have been widely used in polymerization reactions (221) in anhydrous media for the synthesis of homopeptides and the random synthesis of heteropeptides. While the controlled synthesis of heteropeptides *via* Leuch's anhydrides has been attempted by many laboratories, relatively little success has been achieved in the use of this method. Bartlett (222), on the basis of his studies with NCA's of various amino acids, reported that the reaction cannot be



Scheme XLVIII

controlled adequately to provide a useful general method for peptide synthesis in water. Grant (223) proposed the limited use of Leuch's anhydrides for the formation of dipeptides in water at pH 5, particularly when the reacting amine is a much weaker base than the amine resulting from the synthesis. The real breakthrough in the utilization of NCA's in peptide synthesis came with the development of suitable reaction condition by Denkewalter *et al.* (224). They proposed the addition of solid NCA directly to an amino acid at 0–2°, generally at pH 10.2, with rapid mixing of the reactants, thus minimizing side reactions. Rapid mixing, in the case of relatively insoluble NCA's, may enhance the rate of dissolution of the NCA thereby reducing the possibility for carbamate exchange which leads to the formation by overactivation of tripeptides, and also to inactivation of the starting amino acids. Overactivation of more soluble NCA's *via* their anions is also limited by rapid mixing, thus preventing the formation of oligomers. Carbamate stability increases with pH, but at a pH above 10.5, hydrolysis of NCA becomes an important side reaction leading to the formation of hydantoic acid, most likely through NCA anion. The peptide carbamate is decarboxylated at pH 3–5 to produce the free peptide which can be immediately extended in length by repetition of the process with a new *N*-carboxyanhydride, or can be first isolated and purified before continuing with the synthesis. Racemization was not detected during the synthesis of peptides *via* NCA's; this method, used under careful



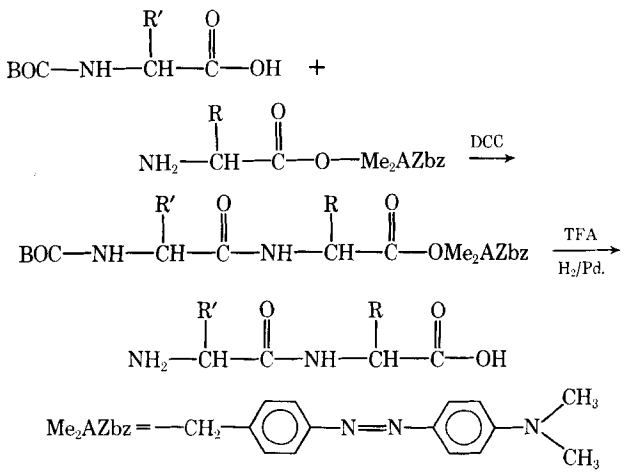
Scheme XLIX

conditions and in combination with other more conventional methods, should prove to be of great value in the synthesis of larger peptides (Scheme XLIX).

The extraordinary rapidity of the NCA method, which is particularly useful in the sequential preparation of higher peptides without isolation of intermediates, is indeed a very interesting feature of this method. Another significant advantage of the NCA method is that the reactions are carried out in aqueous medium and with the exception of the ϵ-amino group of lysine and sulfhydryl group of cysteine, polyfunctional amino acids used as a nucleophile can be used without any protection of the third functionality. NCA's with the exception of those of proline and glycine are quite stable when stored at low temperatures with protection from moisture.

Since the thiocarbamate salts proved to be more stable than the corresponding carbamate salts, the use of sulfur analogs of NCA's, the 2,5-thiazolidinediones (*N*-thiocarboxyanhydrides, NTA's), in the peptide bond formation has been suggested (225). NTA's usually give higher yields in the peptide synthesis. The optimal pH for their reaction is lower, *i.e.*, 9–9.5. Low pH usually favors the desired aminolysis of an anhydride over hydrolysis. NTA of glycine does not form the isocyanate which is characteristic of glycine NCA; therefore, the chances of hydantoic acid formation as an undesirable side reaction are eliminated with the use of the NTA of glycine. There is another interesting difference between the NCA and the NTA of unprotected histidine. While the NCA of histidine undergoes intramolecular imidazole catalyzed isocyanate formation, this reaction is relatively less favored in the case of the NTA of histidine; therefore NTA of histidine is more suitable for the incorporation of histidine in histidyl peptides.

The tremendous scope of the NCA and NTA methods in the synthesis of larger peptides was demonstrated by a group of workers at Merck (226) when they applied these approaches, in combination with the *N*-hydroxysuccinimide and azide methods, toward the successful synthesis of a protein (*S*-protein) composed of 104 amino acids. The fragment condensation approach was used to attach 19 fragments. About 40% of the bonds



Scheme L

in the peptide fragments were formed through the use of NCA's and NTA's; the remainder with *N*-hydroxysuccinimide active esters. The azide method was used throughout for the condensation of fragments which contained 6-17 amino acids. The acetamido methyl group which is resistant to HF at 0° was used for the protection of sulfhydryls of eight cysteines, and acid labile BOC-protecting group was employed as a temporary blocking group of the growing peptide chain. All the protecting groups at the end of the synthesis were removed by HF still leaving the cysteines protected by the acetamido methyl group. After removal of sulfhydryl protections by mercuric acetate, the deprotected tetrahectapeptide was oxidized at pH 6.5 in the presence of mercaptoethanol to form four disulfide bridges. The resulting *S*-protein combined with the *S*-peptide to produce the enzyme activity.

Synthesis of Peptides with the Aid of Synthetic Polysaccharide[1]—Wieland (227, 228) has suggested the use of cationic benzyl ester derivatives for stepwise lengthening of the peptide chains from *C*-terminal residues of amino acids. These derivatives are strongly adsorbed on columns of sulfoethyl-(SE)-polysaccharide, and can be purified by rinsing of the colored zones with methanol. The azoesters of the peptide derivatives can be easily removed at the end of the synthesis by catalytic hydrogenation (Scheme L).

Synthesis of Polypeptides—Practically all the methods which are used for the stepwise synthesis of oligopeptides have been tried for the synthesis of polypeptides with an ordered sequence of amino acids. High molecular-weight polypeptides with known repeating sequences of amino acids, especially those which are water-soluble and contain polyfunctional amino acids, are of special interest. These polypeptides would be expected to resemble proteins more closely than those consisting of only difunctional amino acids. Polymerization of the selected sequences of amino acids in proteins can be of considerable help in the conformational studies and also to explore possible biological activities. The polymerization with the help of active esters such as pentachlorophenyl esters (179) has been quite useful and recently, in the author's laboratories, a number of sequential polypeptides incorporating histidine and serine at suitable distances have been synthesized *via* pentachlorophenyl esters. These polymers possess a definite pattern of activity comparable to the proteolytic hydrolysis of chymotrypsin. Further studies with polypeptides incorporating amino acids around the active site of enzymes are under progress and may provide interesting data for the catalytic activity of certain enzymes.

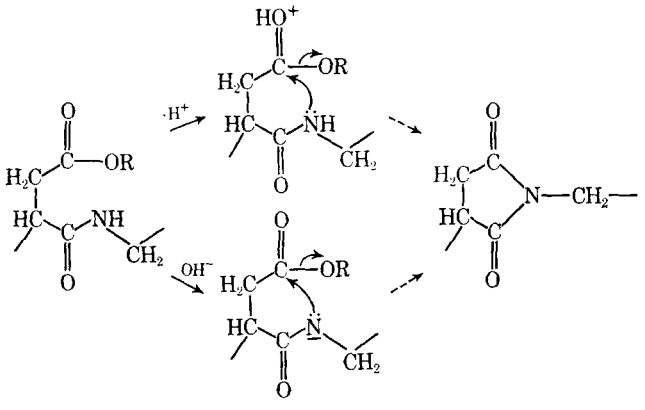
Fox (229) has introduced a rather unconventional but extremely interesting approach for the synthesis of polymers by thermal condensation. In this type of polymerization instead of "prespecified" sequences, the polymers obtained are of "predetermined" sequences. The thermal method of polymerization would lead to the polymers in which reacted α-amino acids are largely racemized. For the synthesis of thermal poly-

[1] Sephadex.

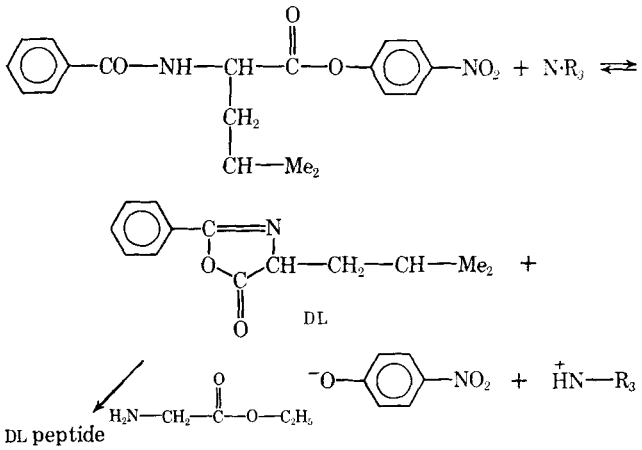
mers, the best results are obtained when sufficient proportions of aspartic acid, glutamic acid, or lysine are heated to 170° for 6 hr. with 16 other amino acids common to proteins. The molecular weights of these polymers are in the range of 4000 to 10,000. Some proportion of each of the amino acids common to protein is found in the polymers synthesized by thermal condensation, and the term "octadecatonic anhydropolymers" has been suggested to designate these polymers. It is of great interest that when six amino acids, that is glutamic acid, glycine, arginine, histidine, phenylalanine, and tryptophan which are considered as a part of the center of the active site in melanocyte stimulating hormone, were subjected to thermal polymerization, the resulting polymer exhibited MSH-activity (230). This kind of study may provide a new type of approach to identification of necessary or sufficient amino acid residues in active sites of protein hormones.

Cyclic Peptides—Most peptide antibiotics are cyclic in character and in contrast to proteins, contain both D and L amino acid residues. Certain amino acids common to proteins, *e.g.*, arginine, methionine, and histidine, occur rarely in peptide antibiotics. The cyclic peptide antibiotics include both homodetic and heterodetic structures. The synthesis of homodetic cyclic peptides is, in principle, the same as that of linear peptides. However, it is essential to work at the right dilution to suppress polycondensation as much as possible in favor of the formation of the intramolecular peptide bond. An interesting phenomenon, "doubling reaction," was reported by Schwyzer (231). If a linear cyclizable peptide with an odd number of amino acid residues, *e.g.*, a pentapeptide, is subjected to cyclization, the corresponding cyclic pentapeptide is not obtained, but rather, through dimerization, a cyclic decapeptide is obtained. A detailed classification of different types of cyclic peptides and specific synthetic methods for their preparation have been reviewed in detail during recent years (29, 31, 32).

The Problem of Transpeptidation Reactions—Intramolecular chemical transpeptidation of peptides and polypeptides with amino dicarboxylic acid residues has been observed in two different cases: (*a*) on peptides and polypeptides containing aspartyl or glutamyl residues in the peptide chains with free ω-carboxyl group; (*b*) on peptides and polypeptides with esterified



Scheme LI

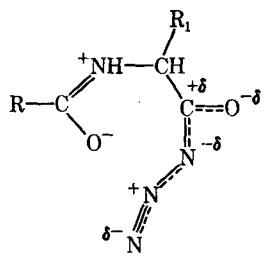


Scheme LII

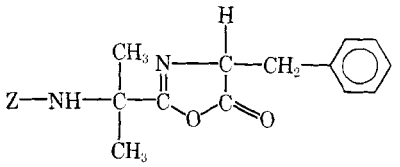
aspartyl and glutamyl residues under basic conditions. The formation of glutarimide derivatives from *N*-protected γ-glutamyl dipeptides and subsequent ring opening to yield α-peptides has been reviewed (179). In the case of the transpeptidation reactions with esterified glutamyl and aspartyl peptides, the imide ring formation is a well established base catalyzed reaction; and, to prevent this type of transpeptidation reaction, *tert*-butyl esters, which can be removed by acid treatment, provide a special advantage.

The formation of cyclic imide was observed as a side reaction in the synthesis of peptides containing the aspartyl-glycyl sequence when the β-carboxyl group of aspartic acid was esterified. The intermediates where the β-carboxyl group of the aspartyl residue is in its free unesterified form show no tendency to undergo cyclization either in the acid or alkaline conditions. This is perhaps due to the fact that alkoxy groups are better leaving groups than hydroxyls, and are therefore more easily displaced in the nucleophilic attack of the glycine nitrogen (232) (Scheme LI).

Racemization—Synthesis of biologically active peptides must proceed without affecting the configuration about any of the asymmetric centers of the components. A continuously increasing amount of attention is being devoted to the problem of racemization, and many laboratories are actively engaged in developing new approaches which would limit the degree of racemization during the synthesis of peptides. While there are many instances where racemization has been observed during the introduction or removal of protecting groups from amino acids or peptides, the most critical aspect, which has received the maximum attention, is the possibility of racemization during the peptide bond formation. Racemization may occur



Scheme LIII



Scheme LIV

either because of the formation of oxazolone (azalactone) intermediates or by the direct proton abstraction from the asymmetric alpha carbon. A recent discussion by Bodanszky and Ondetti (32) summarizes generally accepted beliefs about the loss of optical purity during the synthesis of peptides.

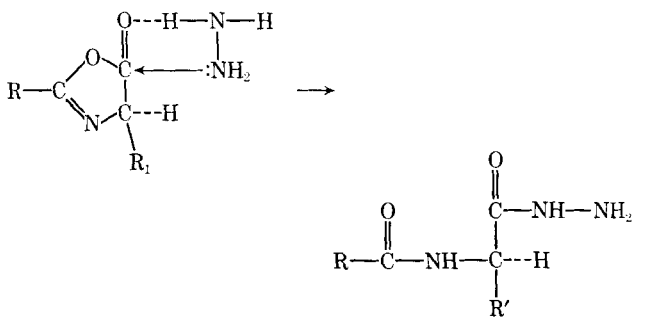
Mechanistic investigations of racemization began when it was shown by Young (233) that the reaction between benzoyl-L-leucine *p*-nitrophenyl ester and glycine ethyl ester, in the presence of a tertiary base, proceeds through the reversible formation, racemization, and coupling of the oxazolone (Scheme LII).

Independent of the above work, Goodman (234) reported the preparation of the optically active crystalline oxazolone from benzoyl-L-phenylalanine. On the basis of equilibrium studies of this oxazolone with various nucleophiles, it was concluded that during the coupling process a small steady state concentration of oxazolone can form which racemizes much faster than its ring opens. The reason for the racemization-free coupling of azide method may be attributed to the inhibition of bond movements (essential for proper conformation for oxazolone formation) due to the attraction between the negative charge developing on the amide oxygen and the positive charge on the central nitrogen atom of the azide group (235) (Scheme LVIII).

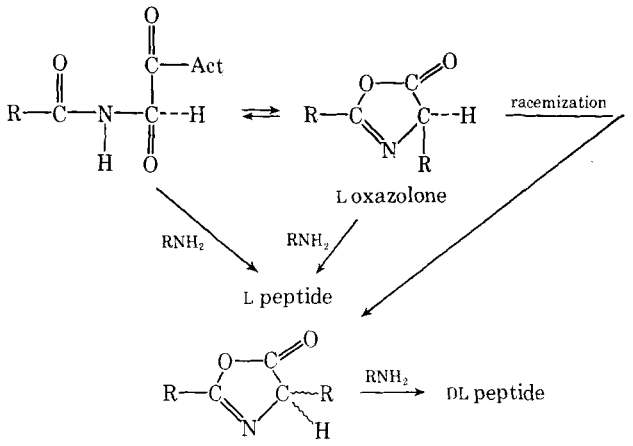
There is a clear dependence of oxazolone formation in the presence of base; Kemp (236) recently reported kinetic evidence which supports the presence of equilibrated amide anions as reactive intermediates leading to azalactone formation in the case of peptide activated esters.

Goodman's group (237) synthesized optically active 2-(1-benzyloxycarbonyl-amino-1′-methyl)ethyl-4-benzyloxazolone from benzyloxycarbonylaminoisobutyl-L-phenylalanine, and reported a number of interesting observations about racemization and ring opening reactions of this oxazolone with several amino acid esters (Scheme LIV).

Racemization of oxazolones may follow pseudo-first-order or second-order kinetics, depending on the attacking amino ester and the nature of the solvent. One of the most important factors which controls the



Scheme LV

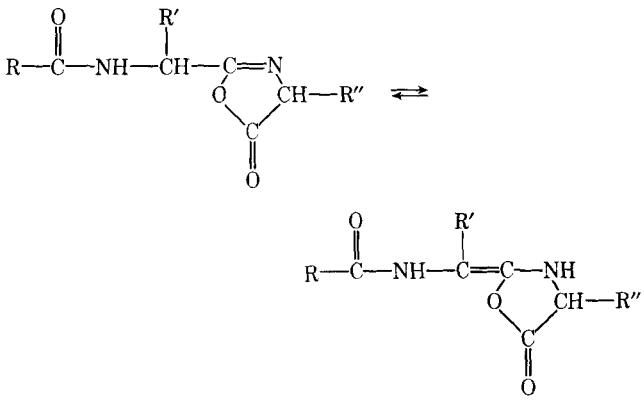


Scheme LVI

racemization process is the nucleophilicity-basicity ratio of the attacking nucleophile. Racemization rate can be accelerated by the presence of chlorides or phosphates which increase the ionic strength of the solution and facilitate the charge separation necessary for racemization. The coupling or ring-opening reaction is always a second-order reaction which is governed by the nucleophilicity of amino components in different solvents. The rate at which a reagent opens an oxazolone ring is a measure of the reagent's nucleophilicity, and the rate at which a reagent racemizes an oxazolone is a measure of reagent's basicity. Certain 1,2-dinucleophiles such as hydrazine which have high nucleophilicity-basicity ratio open the oxazolone ring without racemization (Scheme LV).

Elegant studies carried out by Young (238) on base-catalyzed racemization of various acyldipeptide *p*-nitrophenyl esters have, in fact, established that racemization of many peptide activated species proceeds through oxazolone during the coupling reaction (Scheme LVI).

The degree of racemization of peptide product will no doubt depend on the relative rates of each reaction. For example, coupling of acylpeptides by means of DCC can lead to racemization since this is an excellent reagent for the preparation of oxazolones. In the coupling reactions which proceed through oxazolone formation, the chances of racemization can be limited by the use of nonpolar solvents and by proper selection of the tertiary base. When an oxazolone is formed from the *C*-terminal residue of a peptide chain, the possi-



Scheme LVII

bility of proton abstraction from the alpha carbon of penultimate amino acid residue may lead to its partial racemization, due to rearrangement of the oxazolone (239) (Scheme LVII).

In the case of acetyl and benzoyl amino acids, oxazolones are formed very readily. This, perhaps, is the reason for the racemization in the case of benzoyl and formyl-L-leucine *p*-nitrophenyl esters. The tendency toward oxazolone formation decreases from benzoyl to acetyl to formyl, and this is indeed paralleled by a decrease in racemization during the coupling reactions. The oxazolone formation, in the case of urethan-protecting groups such as benzyloxycarbonyl-protected amino acids, is not favored, and this may be due to resonance stabilization due to the polarization of the carbonyl bond of benzyloxycarbonyl amino acids. The phthalyl-protected amino acids which do not undergo oxazolone formation also should be safe from racemization. However, racemization in the case of activated acyl amino acids such as benzyloxycarbonyl esters of β-cyanoalanine, derivatives of cysteine, and phthalyl-D-valine, which cannot form oxazolones, has been recorded. The mechanism of this type of racemization may be postulated mainly as a result of excellent studies by Liberek (240). Racemization in these cases most likely occurs by direct exchange of hydrogen at the asymmetric center, particularly when the activation is strong and when the acyl groups and the side chains are powerfully electrophilic.

It has been observed that *N*-benzyloxycarbonyl-*S*-benzyl cysteine esters racemize easily in the presence of base. This racemization may proceed either by resonance stabilization of the anion formed by α-hydrogen abstraction or by reversible β-elimination of the benzyl thiol moiety. The racemization of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine-*p*-nitrophenyl ester was attributed to base-catalyzed reversible β-elimination of good leaving benzyl thiol group (241, 242). However, in a recent study using benzyl (35S) thiol, Kovacs *et al.* were able to demonstrate that racemization in the case of *p*-nitrophenyl, as well as of the pentachlorophenyl esters of *N*-benzyloxycarbonyl-*S*-benzylcysteine, does not follow the β-elimination readdition mechanism (243).

On the basis of conformational studies of the amide bond, it has been suggested that oxazolone formation is possible only in the case of *trans*-conformation of the amide, since in the case of *cis* amides, the distance of oxygen atom of amide bond and carbon atom in carboxyl group is too far to facilitate the formation of oxazolones (244).

Detection of Racemization—There are several methods available for determining the optical purity of a peptide once it has been synthesized. However, these methods must be distinguished from different approaches in which synthetic model peptides are used to detect the degree of racemization during peptide bond formation. These approaches are based on the isolation of enantiomers by employing such techniques as countercurrent distribution, fractional crystallization and gas-liquid chromatography (GLC), paper and thin-layer chromatography. Most frequently used model peptides, in the fractional crystallization method, are Young's benzoyl-L-leucylglycine ethyl ester (245) and Anderson's benzyloxycarbonylglycyl-L-phenylalanylglycine ethyl ester (246). The formation of D-alloisoleucine from L-isoleucine has been observed by many authors, and the amounts of alloisoleucine and leucine can be quantitatively determined. This affords a good method to determine the extent of racemization during coupling reaction (247). The GLC separation of diastereoisomeric *N*-trifluoroacetyl dipeptide esters provides a good method for the detection of racemization (248); however, it entails a two-step chemical modification of the sample before analysis (replacement of *N*-protecting groups such as benzyloxycarbonyl or *tert*-butyloxycarbonyl by trifluoroacetyl residues). Halpern's findings that *tert*-butyloxycarbonyl amino acid amides can also be separated by GLC (249), and the separation of trifluoroacetyl tripeptide esters by Weygand by GLC (250) will provide further versatility to this method. Recent work on the preparation of diketopiperazines and their steric analysis by different analytical methods (251) may be useful for the determination of degree of racemization, particularly of dipeptides which do not yield volatile trifluoroacetyl derivatives suitable for gas chromatographic analysis.

A relatively new, though extremely promising approach, is the application of nuclear magnetic resonance (NMR) spectroscopy for the detection of racemization in peptides (252). A series of diastereoisomeric *N*-acylalanylphenylalanine methyl esters and *N*-acylphenylalanylalanine methyl esters have been shown to possess different NMR spectra. The methyl doublet signal in an L-L (or D-D) is at lower field than the equivalent signal for the D-L (or L-D) analog. This is most probably due to deshielding. The application of this property has been extended in determining the degree of racemization during the synthesis of a number of peptides with different *N*-acyl-protecting groups. The sensitivity of the NMR method was improved by the use of ^{13}C-H satellite peak of predominant L-L isomer as an internal standard (225).

In many cases, the biological activity of a natural peptide depends, to a large extent, on its optical purity, and the comparison of the biological activities of the natural and synthetic compounds can be used as a reasonable criteria for the optical purity of the synthetic compound. However, the exactitude of biological testing methods poses a serious limitation to this approach. The comparison of optical rotations of synthetic and natural peptides is useful to some extent for determining the optical purity; however, in the compounds with several asymmetric centers, identical values of optical rotation do not necessarily imply optical homogeneity. The most satisfactory method for the determination of steric homogeneity is the enzymatic hydrolysis (253). Renal amino peptidase can be used for total hydrolysis, and the analysis of the hydrolysate can be performed with L or D amino acid oxidase. The hydrolysis with stereo specific enzymes such as leucine amino peptidase, carboxyl peptidase A and B, trypsin, and chymotrypsin has been successfully used for the determination of optical purity. The complete disappearance of the original peptide established by electrophoresis or paper chromatography is evidence for the optical

purity of the peptide bond for which the enzyme is selective. Another method for the determination of the optical purity of the synthetic peptides is the total hydrolysis by acid; the comparison of the hydrolysate with a control containing the expected amino acid residues treated under the identical conditions, provides a guideline for optical purity. A serious drawback of the acid hydrolysis is that some free amino acids, when exposed to acid, may behave differently from the same moieties built in a peptide sequence.

CONCLUSION

A proper selection of protecting groups and coupling methods is one of the most important tasks during the synthesis of larger peptides. Both the classical and relatively less conventional methods have their advantages and disadvantages. It is hard to rationalize the scope and usefulness of one particular method without considering the utilities and refinements of many other techniques which are so important to the successful completion of the synthesis of peptides and polypeptides. The considerations must be given to the control of racemization, the maximum yields during coupling, and the product purification.

Wherever possible, the amino component should be first liberated and then added to the activated component. In addition to the proper selection of the solvents, the choice of hindered amines, such as tribenzylamine or the amines with suitable basicity such as *N*-methylmorpholine, further limit the degree of racemization in the reactions which require the use of a tertiary amine. The use of bifunctional catalysts which were developed by Beyerman is often helpful in accelerating the aminolysis of active esters without accelerating oxazolone formation.

The remarkable progress during the past few years in the techniques used in peptide synthesis strongly confirms that the aims which were once visualized for peptide synthesis, are indeed attainable. This has been demonstrated by the total synthesis of ribonuclease A. Although the synthetic peptide was relatively less active when compared with pure natural bovine pancreatic ribonuclease A, the complete synthesis of a protein molecule with true enzyme activity towards its natural substrate must be considered as one of the landmarks in the progress toward the continuous efforts for better understanding of biochemical processes. These present indications of the progress and refinement of the techniques used in the synthesis of peptides hold excellent promise for the future. The synthesis of larger quantities of biologically active peptides for medicine and the synthesis of intricate enzymes and complex proteins appear to be only a few steps away.

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The abbreviations used in this paper for amino acids and peptides are mainly those recommended in (*a*) "Peptides," *Proceedings of the Fifth, European Peptide Symposium Oxford, 1962*, G. T. Young, Ed., Pergamon Press, Oxford, 1963, p. 262, and (*b*) IUPAC-IUB Commission on Biochemical Nomenclature, abbreviated designation of amino acids derivatives and peptides. Tentative rules: *J. Biol. Chem.*, **241,** 527, 2491(1966); *Biochim. Biophys. Acta*, **121,** 1(1966).