SEQUENTIAL POLYPEPTIDE SYNTHESIS

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INTRODUCTION

Sequential polypeptides are polymers of two or more amino acids in a specific sequence that is repeated throughout the polymer. As a polyamino acid, they may be used as models[1,2] for more complex structural or catalytic proteins (enzymes). Sequential polypeptides, however, add a dimension to homopolymers and copolymers since they permit the placement of specific side chains at specific locations on a polyamide backbone. This allows one to describe their physical and chemical properties not only in terms of their peptide backbone, but also in terms of side-chain interactions. These features of sequential polypeptides have led researchers to synthesize a large number of these polymers. Table 1 lists some examples of sequential polypeptides and the proteins for which they are models.

Generally, sequential polypeptides are prepared by polymerizing an oligopeptide. The oligopeptide, henceforth referred to as the monomer unit, consists of the amino acids in the sequence desired in the polymer. Chemically, therefore, there are two major steps in the preparation of sequential polypeptides: (1) the synthesis of the monomer unit, and (2) polymerization of the monomer units. In some instances, sequential polypeptides of defined length have been prepared.[26,28] In such cases the polymerization step is replaced by several fragment condensations of the appropriately blocked monomer units. For example, Sakakibara et al.[28] have their C-terminal peptide fragment attached to the Merrifield polymer[29] during their condensation steps.

As in all peptide synthesis, the aim is to obtain the desired product in a high yield with no racemization. In polymerization reactions a high yield ideally means a quantitative amount of material of a narrow range of a high degree of polymerization. A critical review of sequential polypeptide synthesis must therefore concentrate on the various steps and factors involved in the polymer preparation and how they affect the yield, the degree of polymerization, and the degree of racemization. There are several methods of measurement of the latter two parameters and a discussion of them is included.

The synthesis of sequential polypeptides has been reviewed by Katchalski and Sela in their general review of polyamino acids.[30] More recently, De Tar[31] and Jones[32-34] have discussed the

TABLE 1

Sequential Polypeptides as Protein Models

Protein	Sequential polypeptide[a]	Reference
Paramyosin	Leu-Glu(OH)-Lys(H)-Ala-Glu(OH)-Ser(H)-Gly	3
Silk fibroin	Ala-Ala-Gly	4
(Bombyx moir)	Ala-Gly-Ala-Gly-Ser(H)-Gly	5
	Ala-Gly	6, 7, 8
(Argidae)	Glu(NH_2)-Ala	9
Collagen	Pro-Ala-Gly	10, 11
	Pro-Hyp(H)-Gly	12, 13, 14
	Ser-Pro-Gly	15
	Ala-Pro-Gly	16
	Ala-Gly-thiazolidine-4-carboxylic	17
	Ala-Glu(OEt)-Gly	18
Histones	Gly-Lys(Tos)-Lys(Tos)	19
Proteases	Glu(OH)-Ser(H)-Gly	20
	Hyp(H)-Ser(H)-Gly	21
	His(H)-Ala-Glu(OH)	22
Trans-membrane channel	Ala-Ala-Gly	23
Antibacterial	Leu-Orn(H)-Leu	24
Antigen	Tyr(H)-Glu(OH)-Ala-Gly	25
	Tyr(H)-Ala-Glu(OH)	26
poly Glutathione	Glu(β-Ala-Cys(H))OH	27

[a]The sequence listed may not be the sequence of the monomer which was polymerized.

synthesis of the titled polymers. The sequential polypeptides synthesized each year are listed in the annual Chemical Society publication "Amino acids, Peptides and Proteins."[34]

PEPTIDE SYNTHESIS

In both stages of sequential polypeptide synthesis, that is the monomer unit synthesis and polymerization, the reaction of utmost concern is peptide-bond formation. Peptide-bond formation and the field of peptide synthesis in general may be considered as one. The development of blocking groups decreased side reactions, thus increasing yields and purity of products. The development of coupling agents increased yields and decreased racemization during peptide-bond formation. Many laboratories today are examining in detail the mechanisms involved in the various steps of peptide synthesis, changing this field of chemistry from one of art to one of science. This review is not intended to cover the whole realm of peptide synthesis. The subject is covered admirably in several books[35-38] and recent reviews.[34,39]

The peptide bond is formed due to the nucleophilic attack of an amine at the carbonyl carbon atom (Figure 1). The more acidic the conjugated acid of X (Figure 1) the better X is as a leaving group. The ability X has as a leaving group determines the extent to which the tetrahedral intermediate (II, Figure 1) converts to products (III, Figure 1). The rate limiting step, however, is the initial nucleophilic attack (I → II).* To promote peptide-bond formation, therefore, either the nucleophilicity of the amine or the electrophilicity of the carbonyl carbon atom must be increased. The ability of X to make the carbonyl carbon atom more electrophilic is also related to the acidity of HX.[42] Since X, as OH, is a poor leaving group and a poor electron withdrawer (pKa for H_2O is about 14),[43] RCOX (Figure 1) where X is something other than OH must be synthe-

*When the attacking nucleophile is a secondary amine, pyrrolidine, and piperidine, the rate limiting step in aminolysis is the decay of the tetrahedral intermediate.[40,41]

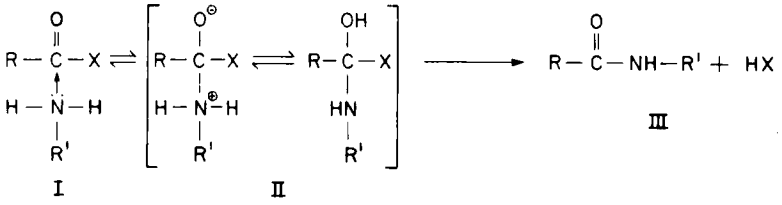

FIGURE 1. Schematic representation of reaction pathway in peptide-bond formation.

sized. The latter compound need not be isolated as it may be formed during the coupling reaction.

The relationship of acidity of HX and the ability for RCOX to undergo aminolysis is linear up to a point[42,44] and levels off beyond this region. The levelling off of the rate of aminolysis is due to the limited number of collisions which occur between the reacting species.[44] These findings indicate that preparations of RCOX where the conjugated acid of X is very acidic need not be pursued, since the coupling reaction will not proceed any faster and with respect to racemization (see below) an acidic HX is a disadvantage.

In sequential polypeptide synthesis there is one reagent which is believed to act by increasing the nucleophilicity of the amine. The reagent is tetraethylpyrophosphate.[45] The mechanism for peptide-bond formation with tetraethylpyrophosphate also permits it to react with free carboxyls and thus act as an acidic X. The actual reaction pathway depends on the order of mixing of reactants.[46]

An alternative approach to peptide-bond formation is to design a bifunctional X (Figure 1). It would be moderately electronegative, making the carbonyl carbon atom more electronegative, and contained on it would be a basic group. The basic group on X would act as a base catalyst on the attacking amine, increasing the amine's nucleophilicity. Several of the latter type of X groups are available and are used in many laboratories in the preparation of sequential polypeptides.

Of utmost concern to all synthetic peptide chemists is the problem of racemization. In sequential polypeptide synthesis the problem may be worse. A polymerization reaction where say each coupling step is 99% racemic free would yield as a product a polymer, e.g., of degree of polymerization of 20 with only 82% optically pure amino acids (0.99^{20}). Since the optically inverted amino acids formed during the polymerization reaction are randomly distributed among the polymers and within the polymer chains (Reference 38, p. 74), purification of the optically pure polymer is an impossibility with existing techniques.

Racemization in peptide synthesis proceeds by two mechanisms:[47,48] (1) via oxazolone-ring formation, and (2) α-carbon proton abstraction. A third postulated mechanism of racemization, that of β-elimination of S-benzyl cysteine to dehydroalanine with subsequent reformation of the S-benzyl-cysteine peptide, has been disproven.[49,50] Isotope exchange experiments have shown that the mechanism for racemization for S-benzyl-cysteine peptides is α-proton abstraction and that racemization by this mechanism occurs without any exchange of protons with the surrounding medium (*isoracemization*,[50,52] *enolization*[53]). In mechanism (1) there are 2 distinct steps, oxazolone-ring formation and racemization (Figure 2). Oxazolone-ring formation is: (a) the rate determining step, (b) a unimolecular reaction (c) specific-base catalyzed,[54] (d) a nucleophilic attack by the acyl oxygen (anion) at the carbonyl carbon atom, (e) dependent on the acidity of X, since it must be a good leaving group and it must contribute electrophilicity to the carbonyl carbon atom to which it is attached, and (f) dependent on the α-amino group having a proton on it.* The racemization step is a general base catalyzed one. Both compounds I and II (Figure 2) are susceptible to aminolysis and alcoholysis. During peptide-bond formation, compound I is believed to be the reactive species. Thus, a proper choice of X may lead to racemic free peptide synthesis ($k_{-2} > k_3$, Figure 2). In the model studies of Kovacs et al.[56] the best ratio for rate of peptide-bond formation versus rate of racemization was where HX was pentachlorophenol (pKa = 5.3[57]).

*This is not a rigid requirement, since N-acyl-N-methyl amino acids can racemize via an oxazolonium salt.[55]

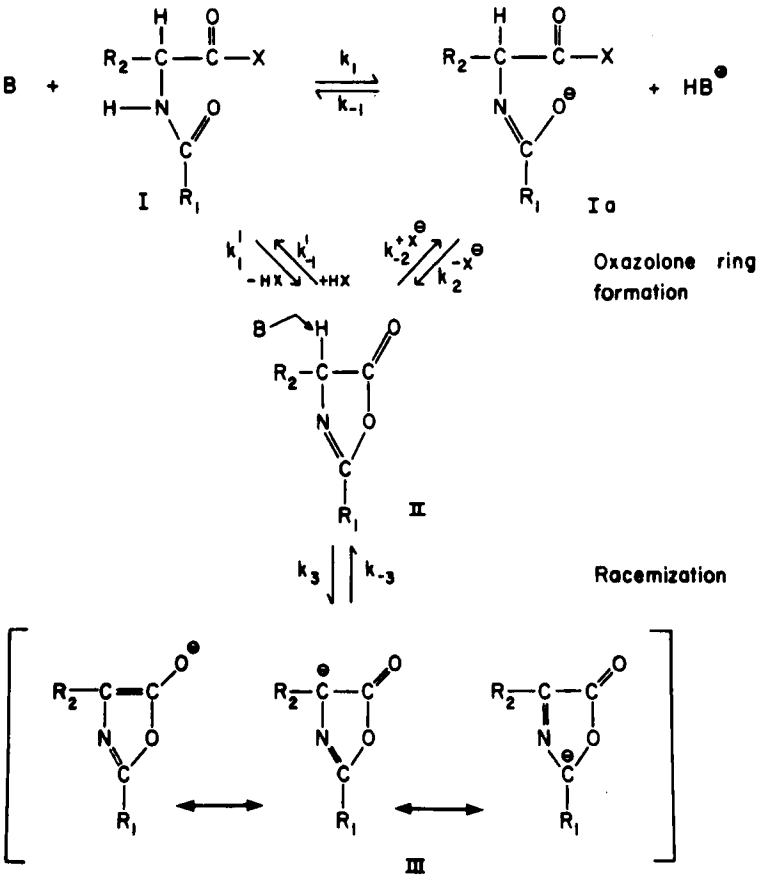

FIGURE 2. The mechanism of racemization via oxazolone-ring formation.

The synthesis of RCOX (Figure 1) in some instances proceeds via an oxazolone intermediate,[57] that is HX (or X^-) released in going from I (or Ia) to II is different from the HX (or X^-) going from II to I (or Ia). As with peptide-bond formation, the preparation of RCOX requires conditions where k_{-1} (or k_{-2}) is greater than k_3.

Accordingly, it may be seen why the extent of racemization during peptide synthesis is affected by the solvent, the type and concentration of base present, the counterion to the base, temperature, the nucleophilicity of the carbonyl oxygen on the acyl group attached to the amino group, the amino acid, the leaving group X, the bulkiness of the attacking amine, and the nucleophilicity of the attacking amine.[48,58,59]

MONOMER SYNTHESIS

Table 2 lists the agents which have been used in the last 10 years in the preparation of sequential polypeptides. Monomer preparation for polymerization by condensing agents requires that the N-terminal amino group and the C-terminal carboxyl group be free. The other polymerizing procedure uses the monomer ester (or hydrazide or chloride) salt (hydrochloride, hydrobromide, trifluoroacetate, etc.). Thus, although the monomer synthesis is independent of the method of polymerization, the choice of blocking groups for the N-terminal amino group and the C-terminal carboxyl group will be important to the eventual polymerization procedure.

Figure 3 illustrates four possible routes to the preparation of the monomer from the blocked form to the form to be used in the polymerization stage. Route 1 is used in the preparation of the monomers for polymerization by the condensation agents. Although the deblocking is shown by one step,[76] it may equally well be a two step process with the C-terminal blocking group (Z, Figure 3) being removed first.[77] Route 2 is a popular route to active ester sequential polypeptide synthesis.[78] This route in comparison with routes 3 and 4 is

TABLE 2

Polymerizing Agents

Agent number	Agent	Abbreviation	Reference
	Condensing agents		
1	Dicyclohexylcarbodiimide	DCC	60
2	DCC plus N-hydroxysuccinimide in catalytic amounts	DCC/HOSu	61
3	DCC plus 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine in catalytic amounts	DCC/HOBn	61
4	DCC plus 1-hydroxy-benzotriazole in catalytic amounts	DCC/HOBt	61
5	N-Cyclohexyl-N′-[β-(N-methylmorpholinium)ethyl]-carbodiimide *p*-sulfonate	CMCI	6
6	CMCI plus HOSu in catalytic amounts	CMCI/HOSu	6
7	Tetraethylpyrophosphate	TEPP	62
8	Bis-*o*-phenylenepyrophosphite	BPP	15
9	iso-Butylchloroformate	iBuOCOCl	63
	Active esters		
10	*p*-Nitrophenyl ester	-ONp	64
11	2,4,6-Trichlorophenyl ester	-OTcp(2,4,6)	65
12	2,4,5-Trichlorophenyl ester	-OTcp(2,4,5)	66
13	Pentachlorophenyl ester	-OPcp	67
14	Pentafluorophenyl ester	-POfp	68
15	3-Hydroxypyridine ester	-OPy	68
16	2-Isobutyl-4-nitro-6-methyl-3-hydroxypyridine ester	-OPy(IBNM)	69
17	4-(Methylsulfonyl)phenyl ester	$-OMSO_2P$	70
	Esters which may participate in intramolecular base catalysis		
18	N-Hydroxysuccinimide ester	-OSu	71
19	8-Hydroxyquinoline ester	-OQu	68
20	*o*-Hydroxyphenyl ester	-OPOH	72
21	N-Ethyl-3,5-dichlorosolicylamide ester	EDCSA	73
	Other methods		
22	Peptide hydrazide in the presence of iodine	$-NHNH_2/I_2$	74
23	Acid chloride	-Cl	75

longer and may be more time consuming. In some instances Z is an alkyl ester whose removal by base hydrolysis may result in some racemization.[79] To circumvent these latter two disadvantages, some laboratories have carried out the monomer synthesis without protection of the C-terminal carboxyl.[80] The coupling reactions in the modified route 2 monomer synthesis are generally active ester coupling and are carried out in organic-aqueous mixtures.[70,80] In some instances these reactions may be carried out in anhydrous solvents.[81]

One disadvantage which route 2 carries with it that is not shared by routes 3 and 4 is the danger of racemization upon synthesis of the active ester of the blocked monomer (step 2 – route 2).[82] Unless the C-terminal amino acid is glycine or proline, the activation of the blocked monomer may result in oxazolone formation and thus racemization. In route 3, the backing-off procedure,[83] the C-terminal amino acid is blocked via the active ester (-X). The synthesis of the C-terminal ester is via the Nα-urethane-(e.g., Boc[20] or Cbz[84]) or Nα-Nps-amino acid.[85] The use of

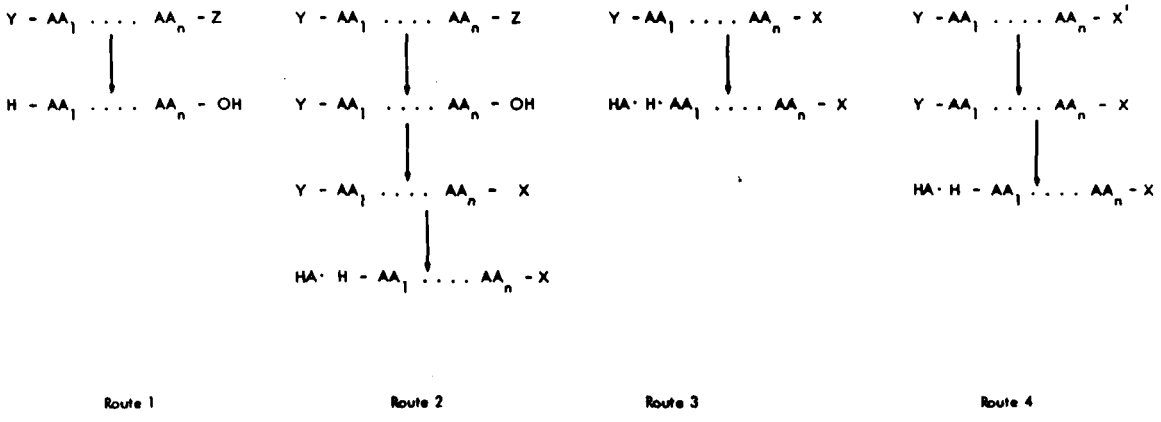

FIGURE 3. Four routes to the synthesis of the monomer unit in preparation for the polymerization step.

route 3, however, limits the types of coupling reactions that may be used in the monomer synthesis, that is the coupling reaction should proceed at a rate much faster than the aminolysis of the active ester. To date, this has generally meant coupling by the mixed anhydride[4] or carbodiimide[82] method. Generally, route 3 yields of active monomer are poor, although as may be expected, this is dependent on -X, the active ester.[86] More recently, laboratories using this route have worked out conditions which minimized side reactions and thus, increased yields.[19,20]

Route 4 is a modification of route 3. The C-terminal blocking group, -X′, is the active ester, -X, in a modified form which may be readily activated.[70] In some instances the conversion of -X′ to -X simultaneously removes the N-terminal blocking group, Y.[11] To date, *o*-hydroxyphenyl ester (agent number 20, Table 2) and 4-(methylsulfonyl)phenyl ester (agent number 17, Table 2) are the active esters which are amenable to route 4 monomer synthesis. Polymerizing agent number 17 in the inactive form is 4-(methylthio)phenyl ester and is activated by *m*-chloroperoxybenzoic acid. Since the activation process is oxidation, this method of polymerization will be limited to those monomer units not containing oxidizable amino acids.

The oxygen of the *o*-hydroxy of polymerizing agent number 20 acts as a base catalyst on the attacking amine. The inactive form of this ester is where the *o*-hydroxy is benzylated[11] or phenacylated.[87] Benzyloxyphenyl esters of Cbz-peptides have been difficult to crystallize.[70] Attempts at improving crystallinity by preparing methoxylated or halogenated benzyl analogues have generally been unsuccessful.[72] The phenacyl analogues of *o*-hydroxyphenyl esters, however, are reported to be easily crystallizable.[87,88] Activation of *o*-benzyloxyphenyl ester peptides occurs by catalytic hydrogenation or with hydrogen bromide in acetic acid. An *o*-(2,4-dichlorobenzyloxy)phenyl ester of Cbz-tripeptide could not be debenzylated under either of these procedures.[72] The activation of the *o*-phenacyloxyphenyl esters is with zinc powder in acetic acid.

The most important factor in terms of yield and degree of polymerization in polyamino acid[1] and sequential polypeptide[31] synthesis is the purity of the active monomer. The more easily a compound crystallizes, the better the chances are of obtaining the material pure. Thus, in choosing blocking groups for the α-amino and the side-chain functional groups, crystallizable blocking agents should be chosen; e.g., Cbz-peptides of N-hydroxysuccinimide[75] and pentachlorophenyl[89] esters are readily crystallizable.

The synthesis of the blocked monomer unit uses the general principles of peptide synthesis, namely, elongation of the peptide chain from the C-terminal amino acid to the N-terminal amino acid in a stepwise fashion.[90] The amino acid being added to the growing chain should not be an acyl-amino acid to prevent oxazolone formation. Trifunctional amino acids should have their side chains blocked. The latter is not always followed for serine,[12] hydroxyproline,[69] tyrosine,[62] and histidine.[91] Protection of carboxyl side chains of aspartic acid with methyl ester has resulted in intramolecular imide formation during the polymerization step.[82]

Since methyl esters are removed by base hydrolysis, 88% racemization was obtained on the deprotection step of poly (α-methyl-β-L-aspartate).[60] With N-terminal glutamic acid peptides a carboxyl blocking group labile to aminolysis may result in pyroglutamic acid formation during the polymerization step.[20,60] These last few comments illustrate that the selection of side-chain blocking groups is important in both the preparation of the monomer unit and in obtaining the final product.

A sequential polypeptide with the repeating unit containing n amino acids may be prepared from n possible monomers, with only the N- and C-terminal region differing, e.g., if $n = 3$, poly (AA_1-AA_2-AA_3) may be prepared from H-AA_1-AA_2-AA_3-X, H-AA_2-AA_3-AA_1-X, or H-AA_3-AA_1-AA_2-X. Since amino acids differ in their racemizability[56] and their coupling reactivity,[59,73] one may suspect that the amino acid at the N-terminal and the C-terminal will affect the product's degree of polymerization and racemization.

In conclusion, the preparation of the monomer for the polymerization stage requires careful planning in terms of route of synthesis, selection of blocking groups, and the actual sequence of amino acids.

POLYMER SYNTHESIS

The general procedure for polymerization is to prepare a concentrated solution of the monomer. In some instances dissolving the monomer requires warming. Upon addition of a condensing agent or base the polymerization begins. After some period of time, the polymer is isolated. The isolation procedures are variable and depend on the laboratory and the polymer being prepared. These work-up procedures are generally attempts to rid the polymer preparation of cyclic peptides due to intramolecular aminolysis. Some of the more vigorous work-up procedures will naturally lower yields, as some of the polymers of lower degree polymerization will be removed with the cyclic peptides. Thus, yield of polymer, one criterion of a satisfactory polymerization procedure, is not only dependent on the procedure but also on the laboratory preparing the polymer (e.g., two laboratories using the same procedure in preparing poly (Glu(OEt)-Gly) report 87% yield[92] and very low yield[93]). This makes comparison of polymerization procedures by their polymer yield a poor method. These data are, however, listed in the tables comparing the various factors of sequential polypeptide synthesis.

The other criteria of good polymerization methods, degree of polymerization[12,94] and degree of nonracemization[11] are dependent on the method of measurement. This further adds to the confusion of comparison of polymerization procedures between laboratories. The degree of polymerization, in some instances, may have been falsely reported since unknowingly the sequential polypeptide had aggregated.[10]

Although there is one report where a noncrystalline monomer yielded a polymer of a higher degree of polymerization than a crystalline monomer,[95] the rule generally is as described earlier, crystalline material yields larger polymers. In comparing two polymerizing agents, sequences of peptides, or other factors of sequential polypeptide synthesis, the purity of the monomer, for this review, has been assumed to be absolute. Since the purity of the monomer, the work-up procedure, and the methods of measurement of degree of polymerization and racemization vary among laboratories, those reports wherein one laboratory carried out comparative studies are worth a separate reading.[6,7,19,60,61,63,68,70,71,75,77,78,85,89,92,96-103] The results drawn here from sequential polypeptide synthesized by various laboratories are in sound agreement with those reported in the latter articles.

The method of fragment condensation[26,28,104] for the preparation of sequential polypeptides of defined length will not be used in the comparison of sequential polypeptides prepared by linear polymerization. The solid-phase method used in the laboratory of Sakakibara[14,28,105,106] has been very successful in the preparation of collagen models.[14,107,108] The latter method has been expanded to include sequential polypeptides of defined length interspersed with a sequence of amino acids,[109,112] or sequential polypeptide of a defined length interspersed between blocks of homopolyamino acids.[111] To date, the degree of polymerization of sequential polypeptides prepared by the solid-phase fragment condensation method has been limited to 20.[8,28]

1. Effect of Monomer Length

An examination of compounds 1 to 6 in Table

3 indicates that the number of residues in the monomer is not a factor in the degree of polymerization or yield. Although preparation numbers 7 to 15 do not show a clear relationship, a trend appears to be forming in preparations 7, 11, and 15. The polytripeptide, 11, is larger than the polydipeptide, 7. Polytetrapeptide, 15, is smaller than the other two polymers. These results may be explained by noting that dipeptides have a propensity to cyclize to diketopiperazines.[78] The cyclization reaction would lower the concentrations of reactive species which in turn would lower the degree of polymerization. With larger monomer units, the degree of polymerization is lowered because the growing polymer precipitates out of solution earlier. The precipitation of polymers during the polymerization reaction would be expected to be the chief terminating factor. Theoretically, one would expect that a sequential polydipeptide with a degree of polymerization of 40 could equally well be prepared from a tetrapeptide if the degree of polymerization were 20.[78]

Monomer units of 6 or more amino acids generally have lower degrees of polymerization than shorter monomer units.[3,114] In some instances solubilization of the monomer unit in the polymerization solvent is the problem. Stewart[101] showed that by changing the sequence of a hexapeptide solubility was improved and polymerization proceeded where previously it had not.

2. Effect of N-terminal Amino Acid of Monomer

The α-amino group of the N-terminal amino acid is the attacking nucleophile. Although the nucleophilicity of the α-amino groups of amino acids is dependent on the amino acid,[56] it appears that polymerization reactions are not as sensitive. N-Alkyl α-amino acids show definitely lower yields of polymer. (Compare compounds 7, 8, and 9 to 1 through 6, and 15 to 10 through 14 in Table 4.) Table 4 indicates that as the N-terminal side chain is lengthened or becomes bulkier, the polymerization reaction becomes more difficult (compare preparations 10 through 15). Steric hindrance of

TABLE 3

Effect of Monomer Length on Polymerization[a]

Preparation No.	Sequential polypeptide[b]	Yield (%)	D.P./method[c]	Reference
1	Glu(OBzl)-Gly	78	27–54/A	20
2	Glu(OEt)-Gly-Gly		25/DNP	18, 112
3	Glu(OBzl)-Ser(H)-Gly	45	15–38/DNP,A,Y	20
4	Glu(OEt)-Glu(OEt)-Gly	95	30–50/ONP	101, 112
5	$[Glu(OEt)]_3$-Gly	95	30–50/ONP	101
6	$[Glu(OEt)]_4$-Gly	96	30–50/ONP	101
7	Ala-Gly		94/L.S.	93
8	Ala-Gly-Gly	98	$3/[m]_D^{25}$	6
9	Ala-Gly-Gly		41/ONP; 69/DNP	18
10	Ala-Gly-Gly		162/[η]	113
11	Ala-Ala-Gly		176/[η]	113
12	Ala-Glu(OEt)-Gly		29/ONP; 55/L.S.	18
13	Ala-Pro-Gly	60	24 and 62/A	86
14	Ala-Pro-Gly	41	11/Y	12
15	Ala-Ala-Ala-Gly		74/[η]	113

[a] Polymerization was via the *p*-nitrophenyl active ester method.
[b] The sequential polypeptide listed is in the sequence that the monomer unit was in.
[c] Degree of polymerization (D.P.) was calculated from the molecular weight of the polymer product. The methods were A, Archibald's sedimentation velocity; DNP, colorimetrically from the amount of dinitrophenylamino acid obtained on hydrolysis of the dinitrophenylpeptide; Y, sedimentation equilibrium method of Yphantis; ONP, the amount of *p*-nitrophenol released on base hydrolysis, measured spectrophotometrically; L.S., light scattering; $[m]_D^{25}$ – molar rotation of light based on the amount of rotation at the sodium D-line; [η]-intrinsic viscosity. For discussion on reliability of methods, see Section 5.

TABLE 4

Effect of N-terminal Amino Acid of Monomer Unit on Polymerization[a]

Preparation no.	Sequential polypeptide[b]	Yield (%)	D.P.[d]	Reference
1(a)	Ala-Gly	50	94/V.S.	115
(b)	Ala-Gly		94/L.S.	93
2(a)	Ser(H)-Gly		12.5/DNP	116
(b)	Ser(H)-Gly		121/L.S.	93
3	Cys(Bzl)-Gly	65		117
4	Phe-Gly			118
5	Lys(Cbz)-Gly	97		96
6	Glu(OBzl)-Gly	78	27–54/A	20
7	Sarc-Gly	67		119
8	Pro-Gly	24	92/Y	12
9	Hyp(H)-Gly	7	75/Y	12
10(a)	Ala-Gly-Gly		41/ONP	18
(b)	Ala-Gly-Gly	80	$8[m]_D^{25}$	6
(c)	Ala-Gly-Gly		$162/[\eta]$	113
(d)	Ala-Gly-Gly	93	$3/[m]_D^{25}$	6
11	Cys(Bzl)-Gly-Gly	40		92
12	His(H)-Gly-Gly			118
13	Asp(OCH_3)-Gly-Gly	30–70	$8–33/[\eta]$	82
14	Glu(OEt)-Gly-Gly		17/ONP	18, 112
15	Pro-Gly-Gly	39		92

[a]Polymerization was via the *p*-nitrophenyl active ester method.
[b]The sequential polypeptide was synthesized from the monomer unit in the sequence shown.
[c]Degree of polymerization was calculated from the molecular weight of the polymer product. The methods were as in Table 3, footnote c and V. S., Van Slyke nitrogen determination.

the α-carbon substituent to the aminolysis reaction (Figure 1) may explain the latter correlation. Alternatively, the α-carbon, side-chain effect may simply be due to a decreased solubility effect. The longer side chain decreases the solubility of the growing polymer resulting in precipitation. This decreases the yield and degree of polymerization of the sequential polypeptides.

3. Effect of C-terminal Amino Acid of Monomer

The relationship of polymerizability of a monomer relative to its C-terminal amino acid is not clear. Table 5 indicates that alanine as a C-terminal is slightly better than glycine in terms of yield and degree of polymerization (compare preparation 2 to 1, 9 to 8, and 12 to 11 in Table 5). Glycyl C-terminal peptides, in turn, yield better polymers than other amino acids. Hydroxyproline appears to be an excellent C-terminal amino acid (Table 5, preparation 7), but the high figures for degree of polymerization quoted may be due to cyclic material present in the preparation. Hydroxyproline should act the same as proline except with possibly a slight improvement in solubility of the growing polymer chain.

4. Effect of Intermediate Amino Acids of Monomer

The observation by Shibnev et al.[98] that proline or hydroxyproline as an intermediate amino acid in monomer units give lower yields of polymer than other amino acids is not apparent. Comparison of preparation 3 to 1 and 2 (Table 6) or preparation 16 to 13, 14, and 15 (Table 6) shows no marked change in degree of polymerization or yield of polymer when imino acids rather than amino acids are internal in monomer units. Alanine as an intermediate amino acid appears to be better than glycine (in Table 6 compare preparations 13 and 14) in terms of yield of polymer. Addition of further bulkiness to the α-carbon, however, results in decreasing polymerizability (in Table 6 compare preparations 14 to 15, 5 to 6, 10 to 11, and 11 to 12). If the α-carbon

TABLE 5

The Effect of the C-terminal Amino Acid of the Monomer Unit on Polymerization

Preparation no.	Sequential polypeptide[a]	Yield (%)	D.P.[b]	Reference
1(a)	Gly-Pro-Gly	21	24/V.S.	68
(b)	Gly-Pro-Gly	31	28/A	104
2	Gly-Pro-Ala		71/A	104
3	Gly-Pro-Lys(Tos)		16–23/V.S.	120
4	Gly-Pro-Glu(OBzl)	30	6/V.S.	121
5	Gly-Pro-Gly	50	71/V.S.	68
6	Gly-Pro-Pro		38–40/A	122
7	Gly-Pro-Hyp(H)	57	374/V.S.	68
8	Gly-Pro-Gly	70	53/V.S.	68, 113
9	Gly-Pro-Ala	31	89/I.R.	123
10	Gly-Pro-Leu	81	15–22/I.R.	123
11	Gly-Pro-Gly	42	38/V.S.	68
12	Gly-Pro-Ala	23	19/ONP	98
13	Gly-Pro-Lys(Tos)		16–23/	120

[a]Polymerization was via the *p*-nitrophenyl (preparation no. 1 to 4), pentachlorophenyl (preparation no. 5 to 7), N-hydroxysuccinimide (preparation no. 8 to 10) and 2,4,6-trichlorophenyl (preparation no. 11 to 13) ester methods.
[b]As in Tables 3 and 4, footnote c and I.R., infrared.

TABLE 6

Effect of the Intermediate Amino Acid of a Monomer on Polymerization[a]

Preparation no.	Sequential polypeptide	Yield (%)	D.P.[b]	Reference
1	Gly-Ser(H)-Gly		16–23/DNP	116
2	Gly-Asp(Im)-Gly	31	28/A	82
3	Gly-Pro-Gly	55	24/DNP	104
4	Asp(OCH_3)-Gly-Gly	70	26/[η]	82
5	Asp(OCH_3)-Ser(H)-Gly	75	37/A	116
6	Asp(OCH_3)-Ser(Ac)-Gly		18/A	116
7	Glu(OEt)-Gly-Gly		25/DNP	18, 112
8	Glu(OBzl)-Ser(H)-Gly	45	15–38/DNP,A,Y	20
9	Glu(OEt)-Glu(OEt)-Gly	95	30–50/ONP	101, 112
10	Glu(OMe)-Ser(Ac)-Glu(OMe)	100	15–20/ONP	124
11	Glu(OEt)-Cys(Bzl)-Glu(OEt)	92		92, 95
12	Glu(OEt)-Val-Glu(OEt)	92		92, 125
13	Pro-Gly-Gly	39		92
14	Pro-Ala-Gly	81	55/A	10
15	Pro-Ser(H)-Gly	65	66–87/A	10
16	Pro-Hyp(H)-Gly	59	56/DNP	12

[a]As in Table 4, footnote a.
[b]Calculated from molecular weight as described in Tables 3, 4, and 5.

side chain of the intermediate amino acid has an ester or an amide in it (in Table 6 preparations 2 and 9), the product obtained is better than would be expected from the bulkiness of these side chains (in Table 6 compare preparations 8 and 9).

The relationship of polymerizability with the intermediate amino acid's α-carbon bulk does not appear to hold under all conditions. Two alternative explanations are (a) there is a correlation between the hydrophobicity of the side chain with the monomer units polymerizability or (b) the relationship of polymerizability with the intermediate amino acid lies not in the side chain itself, but is rather dependent on the effect the amino acid has on the secondary structure of the polymer.[126,128] If the growing polymer will take up helical structures then both the nucleophilic amine and the active carboxyl groups should be available for coupling reactions. On the other hand, randomly coiled polymers and polymers with β-structure will, on a time-average basis, have one of their reactive ends buried. This effect of lowering the concentration of one of the reactive species would naturally result in lower yields or shorter polymers.

Table 7 lists four polymers that were prepared under identical conditions. The only difference between the starting monomer units was the optical activity of the terminal amino acids. Both chemical reactivity and solubility of side chains cannot be a factor, since the amino acids of the four monomer units have identical physical and chemical reactivity. The differences in yield and degree of polymerization may, however, be attributed to the secondary structure of the growing sequential polpeptide. The conformation of the growing L-L polypeptide would probably be comparable to the growing D-D polypeptide and similarly, the L-D sequential polypeptide would have a comparable conformation to the D-L polypeptide (the optical sense of the similar conformations may be expected to be opposite). The yield and degree of polymerization of the polymers in Table 7 bear this out.

5. Effect of Polymerizing Agent

Of all the variables in the polymerization step the relationship between the polymerizing agent and the degree of polymerization and yield is the best (Table 8). If one considers the product of polymer yield and its degree of polymerization as a measurement of the polymerizing agent's ability to promote sequential polypeptide synthesis and if the value for N-hydroxysuccinimide ester is taken to be 100, then Table 9 is a numerical correlation between polymerizing agent and its ability.

The results of Table 8 are in agreement with the model studies of Kovacs et al.[56] with the exception of pentafluorophenyl ester. Carbobenzoxyamino acid pentafluorophenyl ester reacts with valine methyl ester faster than any other active ester. In sequential polypeptide synthesis the pentafluorophenyl ester shows approximately 10% of the ability of N-hydroxysuccinimide ester

TABLE 7

Dependency of Polymerization Results on the Secondary Structure of the Growing Sequential Polypeptide

Preparation[a] no.	Monomer sequence	Code[b]	Yield (%)	D.P.[c]
1	Ala-Gly-Pro	L-L	70	40_n, 62_w
2	D-Ala-Gly-D-Pro	D-D	53	40_n, 56_w
3[d]	Ala-Gly-D-Pro	L-D	41.5	10_n, 16_w
4	D-Ala-Gly-Pro	D-L	30	11_n, 19_w

[a]The monomer pentachlorophenyl ester hydrobromide salt at 1.4 *M* in dimethylsulfoxide, in the presence of 2 equivalents of N-methylmorpholine was polymerized for 3 days at room temperature.[129]

[b]Code use in text for discussion purposes.

[c]Degree of polymerization determined from number-average (n) and weight-average (w) molecular weight as determined from gel chromatography off a Biol-Gel P-150 column.[94]

[d]Reaction period was 4 days.

TABLE 8

Effect of Polymerizing Agent on Polymerization Step in Sequential Polypeptide Synthesis

Preparation no.	Agent	Yield (%)	D.P.[a]	Reference
		$(Ala\text{-}Gly)_n$		
1	-OPfp	30–40	94/-	130
2	-OPcp	60–80		131
3	-ONp	80	94/L.S.	92, 93
4	-OTcp(2,4,6)	60		7, 65
		$(Gly\text{-}Pro\text{-}Gly)_n$		
5	-OPfp	14	8/V.S.	68
6	-ONSu	70	53/V.S.	68, 123
7	-OPcp	50	71/V.S.	68
8	-OTcp(2,4,5)	53	43/V.S.	99, 132
9	-ONp	31	28/A	104
10	-OTcp(2,4,6)	42	38/V.S.	68
11	-OQu	20	14/V.S.	68
12	-OPy	76	20/V.S.	68
13	-OPy(IBNM)		14/V.S.	69
		$(Glu(OB)\text{-}Glu(OB)\text{-}Glu(OB))_n$[b]		
14	-ONSu	54	43_n, 57_w/G-150[c]	103
15	-OPcp		38/[η]	87
16	-OPOH	50	17/[η]	87
17	DCC	44	12/G-75	77
		$(Pro\text{-}Ala\text{-}Gly)_n$		
18	-ONSu	16		129
19	-OPcp	34		129
20	-ONp	22		129
21	TEPP	2	49/G-75	133
22	DCC/HONSu	<1	20/G-50	61
23	DCC/HOBn	<1	20/G-50	61
24	DCC/HOBT	<1	18/G-50	61
		$(Pro\text{-}Ser(H)\text{-}Gly)_n$		
25	-OPcp	100	14/V.S.	134
26	-ONp	65	66–87/A	10
27	TEPP	56	28/G-25	15
28	BPP	21	7/G-25	15
29	DCC/HONSu	32	21/G-25	15
30	DCC/HOBn	40	13/G-50	61

[a]Degree of polymerization calculated as described in Tables 3 to 6. G-25, G-50, G-75, and G-100 refer to degree of polymerization determined by gel filtration from Sephadex G-25, G-50, G-75, and G-150, respectively.

[b]The sequence of amino acids in the monomer was DLD for preparation 14; LLL for preparations 15 and 16; and LLD for preparation 17. The blocking group, B, for the γ-carboxyl was the benzyl ester except for preparation 17 which had the *t*-butyl ester.

[c]The subscripts *n* and *w* refer to number average and weight average degree of polymerization as calculated from the respective molecular weights.

TABLE 9

Polymerization Factor

Agent[a] no.	Polymerizing agent	Polymerizing factor
18	-ONSu	100
13	-OPcp	>95
12	-OTcp(2,4,5)	60
11	-OTcp(2,4,6)	43
15	-OPy	40
10	-ONp	30
20	-OPOH	25
19	-OQu	10
14	-OPfp	9
7	TEPP	8
2	DCC/HONSu	7
3	DCC/HOBn	6
1	DCC	5
8	BPP	<1

[a]Agent number corresponds to the number listed in Table 2.

in polymerization. The results of Table 8 are also in agreement with the study of Shibnev et al.[68] The polymerizing agents listed in Table 2 that are not included in Table 9 fall in the 10 and below polymerization factor region. These polymerization factors need not be absolute since other variables in the polymerization step would naturally affect both the degree of polymerization and the yield (e.g., compare preparation 26 to 25 in Table 8).

6. Effect of Solvent

Table 8 lists some selected polymerization reactions. A more complete list of synthesized sequential polypeptides would not give the polymerization factors of Table 9 if other variables of the polymerization step were not kept constant. One such variable is the solvent of polymerization. The solvent should have the following properties: (a) it should allow ionic species formation necessary in the coupling step (Figure 1), (b) solubilize large polypeptides, and (c) not react with the activated monomer. Since water and methanol would hydrolyze or alcoholyze the active monomer species, the degree of polymerization in such solvents would be expected to be low (in Table 10 compare preparations 10 to 11, 16 and 17 to 12 through 15, and 18 to 19). Solvents which would accommodate all these properties are dimethylformamide, dimethylsulphoxide, dioxane, tetrahydrofuran, hexafluoroacetone, pyridine,[15] methyl acetonitrile,[77] 1-methyl-2-pyrrolidone, diethylphosphite,[62] dimethylacetamide,[115] and hexamethylphosphotriamide. The first five solvents have been used in polymerization reactions with the active ester and bifunctional polymerizing agents. The other solvents are generally used with the condensing agent polymerizations. Solvents which have low dielectric constants, that is they do not readily permit ion formation, have also been used in sequential polypeptide synthesis. Benzene, light petroleum ether, chloroform,[135] and methylene chloride[61] are examples of such solvents (Table 10, preparations 11, 20, and 21).

Dimethylformamide and dimethylsulphoxide are the solvents most commonly used in the polymerization step, since they generally give the best yields and degree of polymerization of polymer. In some instances, however, the solvent of polymerization to give the best results will depend on the monomer unit[78] (in Table 10 compare preparations 6 and 7 to 12 and 13), the method of polymerization[15,61] (compare preparations 3 and 4 to 11 and 19 in Table 10), and in all instances the initial concentration of monomer unit. The effect of solvent on condensing agent polymerization has been examined carefully by Heidemann and his collaborators.[15,61]

7. Effect of Monomer Concentration

Monomer units in an activated form at a concentration of 0.01 *M* or less will almost exclusively form cyclic peptides (p. 271, Reference 36). In order to polymerize, the monomer unit concentration must be greater than 0.01 *M*. The dependency of degree of polymerization and yield is linearly related to the initial concentration of the monomer up to a certain value (Table 11). This relationship depends on the successful intermolecular reaction over the intramolecular reaction. Above a certain monomer concentration, this relationship falls off, since the amount of solvent available to solvate the growing polymer chain decreases. The result is lower or constant degrees of polymerization with increasing monomer concentration.

The intramolecular reaction, that is monomer cyclization, requires the peptide backbone in a certain conformation. Since the solvent will have an effect on the polypeptide conformation,[127] the region of linear dependency of degree of

TABLE 10

Solvent Effect on Monomer Polymerization[a]

Polymer	Preparation no.	Solvent[b]	Yield (%)	D.P.	References
		-ONp			
$(Ala\text{-}Gly\text{-}Gly)_n$	1	H_2O	77	$5/[m]_D^{25}$	6
	2	60% LiBr	94	$3/[m]_D^{25}$	6
	3	DMF	98	$3/[m]_D^{25}$	6
	4	DMSO	80	$3/[m]_D^{25}$	6
	5	HMPT	93	$3/[m]_D^{25}$	6
$[Asp(OCH_3)\text{-}Gly\text{-}Gly]_n$	6	DMF	60	$14/[\eta]$	82
	7	DMSO	65	$23/[\eta]$	82
	8	MP	65	$33/[\eta]$	82
	9	HMPT	50	$17/[\eta]$	82
		-OPcp			
$(Ala\text{-}Gly\text{-}Gly)_n$	10	H_2O	89	7/[m]	6
	11	Benzene	93	43/[m]	6
$[Glu(Gly)OBu]_n$	12	DMF	27	15/Y	63
	13	DMSO	26	10/Y	63
	14	HFA	26	5/Y	63
	15	DMF	52	10/Y	63
	16	8 *M* Urea	71	$8[\eta]$	63
	17[c]	MeOH	25	$8/[\eta]$	63
		-ONSu			
$(Ala\text{-}Gly\text{-}Gly)_n$	18	H_2O	75	$11/[m]_D^{25}$	6
	19	DMSO	80	$62/[m]_D^{25}$	6
$[[D\text{-}Glu(OBzl)]_4\text{-}Leu]_n$	20	*Light petroleum*	40		78
	21	Benzene	58		78
	22	THF	48		78
	23	Dioxane	51		78

[a]Polymerization reaction for $(Ala\text{-}Gly\text{-}Gly)_n$ via the -ONp and -ONSu method was carried out in the presence of one equivalent triethylamine (TEA) for 48 hr. The -OPcp preparation was carried out in the presence of 2.5 equivalents of TEA also for 48 hr. $[Asp(OCH_3)\text{-}Gly\text{-}Gly]_n$ was prepared in the presence of one equivalent of TEA overnight. $[Glu(Gly)OBu]_n$ was prepared in 3 days in the presence of TEA, two equivalents (preparation no. 12 to 14) or 1.5 equivalents (preparation no. 15 to 17). $[[D\text{-}Glu(OBzl)]_4\text{-}Leu]_n$ was prepared in 4 days at room temperature in the presence of one equivalent of TEA.

[b]Concentrations of monomer unit at start of polymerizing reaction were: preparation no. 1, 2, 5, and 18, 5 *M*; preparation no. 19, 2.5 *M*; preparation no. 3, 4, and 5, 2.2 *M*; preparation 11, 2 *M*; preparation 7, 8, and 9, 1.8 *M*; preparation 6, 1.1 *M*; the remaining preparations were at 1 *M*. Abbreviations: DMF, dimethylformamide; DMSO, dimethylsulfoxide; HMPT, hexamethylphosphotriamide; MP, 1-methyl-3-pyrolidone; HFA, hexafluoroacetone; THF, tetrahydrofuran; and TEA, triethylamine.

[c]Reaction time was 1 day.

TABLE 11

Effect of Initial Monomer Concentration on Its Polymerization[a]

Preparation no.	Concentration (*M*)	Yield (%)	D.P.
1	0.66	40	21/[η]
2	0.83	45	33/[η]
3	0.90		40/DNP
4	1.55	70	26/[η]
5	1.8	65	23/[η]
6	2.4		30/[η]

[a]The hydrobromide salt of Asp(OCH_3)-Gly-Gly-ONp was dissolved in dimethylsulfoxide in the given molar concentrations. Polymerization was initiated with equivalent of TEA and carried out overnight at room temperature.[82]

polymerization with concentration will depend on the solvent. The studies of Hardy, Rydon, and Thompson[78] on the polymerization of H-[D-Glu (OBzl)]$_x$-Leu-ONSu hydrochloride salt have shown this. When the monomer unit is an odd number of amino acids long (x = 2 or 4), benzene is a better solvent than tetrahydrofuran. When the monomer unit is an even number of amino acids long (x = 1 or 3), tetrahydrofuran is the better solvent in terms of relative ratio of polymer synthesis to cyclization. Under optimal polymerizing conditions the yield of polymer is approximately equal for any value of x, except when x = 2 in which case the major product in any solvent or concentration is the cyclohexapeptide. Optimal polymerizing conditions also yield polymer whose degree of polymerization is relatively constant (for x = 1 or 2, degree of polymerization is approximately 40; for x = 3 or 4, the degree of polymerization is approximately 32). It appears, therefore, that under optimal solvent conditions both the yield and the degree of polymerization are independent of the monomer, but are only dependent on the polymerization agent.

It was noted earlier that above a certain initial concentration of monomer unit the degree of polymerization and yield of polymer became constant (compare preparations 5 and 6 to 1 through 4 in Table 11). Precipitation due to aggregation was explained as the cause of the poor polymerization. This may be partially reversed by an addition of solvent periodically during the course of the reaction.[4,6,10,12,20,71,78,80,85,86,88,103,116,136] The question of when to add the extra solvent and the effect of the addition of solvent on the yield and degree of polymerization of the sequential polypeptide has not been examined.

In most publications on sequential polypeptide synthesis and in the author's experience it is noted that within a very few minutes after the addition of the organic base to the polymerization mixture, the solution becomes very viscous. Ramachandran et al.[102] assumed the material precipitating out at the start of the reaction to be triethylamine hydrochloride. The precipitating monomer counter-ion-base complex may initiate the precipitation of the growing sequential polypeptide, thus terminating its growth. Alternatively, the high concentration of salt formed at the start of the reactions may stimulate polymer precipitation by a salting out effect. The extra step that Ramachandran et al.[102] put into their polymerization of H-Tyr(Bzl)-Ala-Glu(OBzl)-ONSu, of filtering off the triethylamine hydrochloride, yielded them excellent results (100% yield of polymer; degree of polymerization of one fraction was 173).

The difference between polymerization reactions where the solution is diluted during the course of the reaction versus dilution right at the start of the reaction is that after the reaction has progressed some the intramolecular reaction is unlikely because of the length of the polypeptide. One method of insuring early polymer formation and at the same time preventing cyclization reaction is by adding at the start of the reaction a trace amount of active ester "poison." The "poison" is glycine methyl or ethyl ester[137,138] and is added at approximately 0.1% of the molar concentration of the active monomer. The addition of glycine alkyl ester to the polymerization reaction mixture has permitted Johnson and his collaborators to prepare under dilute conditions (0.1 *M* or less of active monomer) several polytetrapeptides in relatively good yield[25,70,137-150] with excellent degrees of polymerization, e.g., 230.[145]

8. Effect of Base

Polymerization of the monomer unit by methods other than the use of condensing agents requires the presence of a base. Generally, triethylamine is the base used to neutralize the acid, HA (Figure 3). In order to keep the α-amino group nucleophilic the acid HX (Figure 1) formed during

the course of the reaction needs to be neutralized in some instances. Thus, polymerization with pentachlorophenyl esters requires two or more equivalents of base to be added at the start of the reaction (preparations 14 and 15 in Table 12).

The number of equivalents of base added to initiate the polymerizing reaction is generally equal to the number of equivalents of the monomer unit. Increasing the equivalent ratio of base to monomer unit appears to lower both the yield and degree of polymerization of the sequential polypeptide (in Table 12 compare preparations 1 to 5, 6, 7, and 8 and 9). The excess base may also subject the polymer to racemization by the proton abstraction and oxazolone mechanism. Monomer units with α-methyl esters of aspartic acid undergo imide formation in the presence of excess base.[82] There are at least two examples where during the polymerization the diluent contained both the solvent and triethylamine.[10,100] From the evidence of Table 12 and the above discussion it would not seem advantageous to increase the ratio of base to monomer during the course of the reaction.

If the acid released in peptide bond formation (HX, Figure 1) is not too acidic and under the conditions of polymerization exists in part in an ionized form then X^-, the ionized alcohol of the active ester, may act as a base. There are two examples where this philosophy has been used: a 2,4,5-trichlorophenyl ester polymerization[153] and a *p*-nitrophenyl ester polymerization.[68]

The observation that *p*-nitrophenoxide ion may act as a base in monomer *p*-nitrophenyl ester polymerization prompted DeTar et al.[116] to use the sodium salt of the alcohol as the base to initiate polymerization. The results they obtained favored *p*-nitrophenoxide as the base over triethylamine (in Table 12 compare preparation 10 to 11).

N-Methyl morpholine, a base which has been shown to decrease the tendency of racemization during acyl peptide coupling reactions,[58] has been employed in a few cases as the base in sequential polypeptide synthesis. The polymer was obtained in poor yield and had a lower degree of polymerization than when triethylamine was used (in Table 12, compare preparations 12 to 13 and 14 to 15).*

9. Reaction Time and Temperature

The effect of length of time of polymerization and reaction temperature has only been investigated systemically in one system, the polymerization of H-Pro-Ala-Gly-OH with DCC in the presence of catalysts HONSu, HOBn, and HOBt.[61] The results indicate that increasing the duration of the reaction from 24 to 232 hr at 20°C has no marked effect on the yield, but does increase the degree of polymerization. Similarly, increasing the temperature of the reaction mixture from −20°C to 20°C increases the degree of polymerization after 232 hr without affecting the yield markedly. A similar conclusion may be drawn from the studies of Johnson and his collaborators on the preparation of (Phe-Glu(OH)-Ala-Gly)$_n$-Gly-OMe[141] or -l-C^{14}-Gly-OEt.[142] The former polymer prepared over a period of 7 days was approximately 10 times larger than the latter preparation prepared in 3 days. Some of the increase in the size of polymer may be due to the free γ-carboxyl in the 7-day preparation, whereas the 3-day preparation had the γ-carboxyl as a *t*-butyl ester.

From the brief evidence cited above, it appears that the initiation of polymerization occurs early in the course of the reaction and further mixing time will only lengthen polymer chains. This means fragment condensation occurs shortly after the polymerization reaction begins. Temperature, which will affect all coupling reactions, affects the fragment condensation step more. The fragments longer than the monomer units need to collide more times in order that the α-amino of one fragment finds the activated carboxyl of a second fragment. A rise in the temperature will increase the number of collisions of the fragments and the monomer units. The relative increase in collisions with temperature will favor the fragments. The result is an increase in degree of polymerization without a relative change in yield of polymer.

10. Effect of Counterion of Salt Form of Monomer

The blocked activated monomer (Figure 3, Y-AA$_1$ . . . AA$_n$-X) in several instances may have more than one method of deprotection. The deprotection results in the salt form of the

*In dimethylsulfoxide N-methylmorpholine salts are more soluble than triethylamine salts. In some instances the former base has of necessity been the one of choice.[81]

TABLE 12

Effect of Base on Sequential Polypeptide Synthesis

Preparation no.	Base	Equivalent base to monomer	Yield (%)	D.P.	References
		$[Glu(Gly)OBu]_n$[a]			
1	TEA	1.5	52	10/Y	63
2	TEA	2.0	27	15/Y	63
3	TEA	2.5	35	62/Y	63
4	TEA	2.55	32	15/Y	63
5	TEA	3	42	11/Y	63
		$[Lys(Cbz)\text{-}Ala\text{-}Ala]_n$[b]			
6	TEA	1	81	22/V.S.	151, 152
7	TEA	2.5	70	8/V.S.	151, 152
		$[Lys(Cbz)\text{-}Lys(Cbz)\text{-}Gly]_n$[b]			
8	TEA	1	52	10/V.S.	151, 152
9	TEA	2.5	46	9/V.S.	151, 152
		$[Asp(OCH_3)\text{-}Ser(H)\text{-}Gly]_n$[c]			
10	TEA	1.25	84	21/DNP; 18/A	116
11	NaONP	1	75	33/DNP; 37/A	116
		$[Pro\text{-}Ala\text{-}Gly]_n$[d]			
12	TEA	2.0	81	55/A	10
13	NMM	4.5	22		129
		$[\text{-}Glu[Asp(\text{-})OBu]OBu]_n$[e]			
14	TEA	3.1	50	86/Y	97
15	NMM	2.2	64	29/Y	97

[a]Polymerization of 1 *M* monomer pentachlorophenyl ester in dimethylformamide at room temperature.

[b]Polymerization of 1 *M* monomer 2,4,5-trichlorophenyl ester in dimethylformamide at room temperature.

[c]Polymerization of monomer *p*-nitrophenyl ester in dimethylsulfoxide with TEA using 1.2 *M* monomer with further dilution to 0.6 *M* for 4 days; NaONp using 1 *M* monomer for 50 hr.

[d]Polymerization of monomer *p*-nitrophenyl ester in dimethylsulfoxide with TEA using 1.5 *M* monomer for 20 hr at room temperature with further dilution to 0.5 *M* using dimethylsulfoxide, and one equivalent of triethylamine and continued polymerization for 21 hr at 50°C; N-Methylmorpholine (NMM) using 0.9 *M* monomer for 4 days at room temperature.

[e]Polymerization at 0.8 *M* monomer pentachlorophenyl ester in dimethylformamide at room temperature.

activated monomer ($HA \cdot H\text{-}AA_1 \ldots AA_n\text{-}X$, Figure 3). The choice of deprotection and therefore the salt form of the monomer may affect the result of the polymerization. Burichenko et al.[154] prepared poly (Gly-Lys(Tos)-Gly) via the pentachlorophenyl and 2,4,5-trichlorophenyl ester. When the monomer was in the hydrochloride salt form the polymer had a greater degree of polymerization than when the monomer was a hydrobromide salt for both esters. The larger polymer may have been due to a more crystalline hydrochloride tripeptide ester. Frequently, hydrobromide peptides are hygroscopic and difficult to crystallize.[101] In some instances both halide salts of the peptide esters are hygroscopic. When this occurs, the hydrohalide salt may be changed to the *p*-toluenesulphonate salt.[96]

In some instances the choice of deprotection may not be applicable to the polymer preparations. Shibnev et al.[155] attempted to prepare the hydrochloride salt of H-Gly-Ser(H)-Pro-OPcp by hydrogenation of the carbobenzoxy tripeptide ester in the presence of one equivalent of HCl. The yields were low. Treatment of the blocked tripeptide ester with hydrogen bromide in glacial acetic acid gave the hydrobromide salt in 90% yield. Similarly, one may assume that Poroshin and his collaborators could not use the same form of deprotection in their comparative studies of the affect of polymerizing agents on polymerization of H-Gly-Pro-Gly-OH.[68,98,99,125,156,157]

The counterion of the salt form of a peptide ester may indirectly affect the polymerization of the monomer. It may increase or decrease the solubility of the monomer unit in the solvent of polymerization. The solubility of the salt form of the base formed from the counterion and the free base at the initiation of the polymerization reaction will affect the size and yield of the polymer product (see Section 8). The role of the acid of the peptide salt (HA, Figure 3) as an acid catalyst of peptide-bond formation during polymerization is highly unlikely.[63]

11. Racemization

Above all criteria one would suspect that the criterion of optical purity of polymer would be the one of greatest concern. Yet in over 400 sequential polypeptides synthesized in the past 10 years only 65 of them have a description of their optical purity. A possible reason for the few reports on optical purity of sequential polypeptides is perhaps the belief that the methods used are free of the risk of racemization. In those polymer preparations where a test for optical purity was made, the values found were generally within 5% of absolute purity and were within the region of sensitivity of the method of measurement.

Despite the limitations, some generalizations may be made on factors affecting degree of racemization of sequential polypeptides during the polymerization step. Solvent, dilution of monomer unit, and proportion and type of base affect racemization during peptide-bond formation (see Section 2). In addition, the amount of racemization depends on (a) the optical configuration of the N- and C-terminal amino acid (in Table 13 compare preparations 9 to 8 and 1 to 2 or 3), (b) the length of the monomer,[78] (c) the sequence of the monomer,[71] and (d) the polymerizing agent (Table 13).[87] Polymerization with condensing agents results in more racemization than polymerization with active esters (in Table 13 compare preparations 1 through 3 to 4). *o*-Hydroxyphenol esters of peptides result in no racemization during polymerization (in Table 13 compare preparation 5 to 6 and 7). N-Hydroxysuccinimide esters of peptides produce no[78,158] or very little (about 2%)[71] racemization in the polymers prepared from them.

Kovacs et al.[73] have carried out model studies on the rate of coupling and racemization during peptide synthesis. Their results indicate the order of decreasing ratio of coupling rate over racemization rate is pentafluorophenyl ester > pentachlorophenyl ester > N-hydroxysuccinimide ester > 2,4,5-trichlorophenyl ester > *p*-nitrophenyl ester. From Table 9 it appears that polymerization does not follow this order. From the 65 polymers that have been tested for racemization the order for lowest to highest racemization reported for polymerizing agent is *o*-hydroxyphenyl ester > N-hydroxysuccinimide ester > *p*-nitrophenyl ester > pentachlorophenyl ester (poly γ-benzy-L-glutamate prepared from H-Glu(OBz)-Glu(OBz)-Glu(OBz)-OPcp was less than 10% optically pure).[87]

12. Cyclization

The major reaction that competes with polymerization is cyclization. The formation of diketopiperazine from dipeptides, cyclohexapeptides from tripeptides and cyclotetra- and pentapeptides

TABLE 13

Racemization During Polymerization[a]

Preparation no.	Peptide	Agent	Optical purity (%)[b]	Reference
1	$[Glu(OBzl)\text{-}Glu(OBzl)]_n$	DCC	$87/H^+$	77
2	$[Glu(OBzl)\text{-}D\text{-}Glu(OBzl)]_n$	DCC	$94H^+$	77
3	$[D\text{-}Glu(OBzl)\text{-}Glu(OBzl)]_n$	DCC	$94/H^+$	77
4	$[D\text{-}Glu(OBzl)\text{-}Glu(OBzl)]_n$	-ONP	$98\text{–}99/[\alpha]_D$	85
5	$[D\text{-}Glu(OBzl)\text{-}Glu(OBzl)]_n$	-OPCP	$96\text{–}97.5/[\alpha]_D$	85
6	$(Gly\text{-}Gly\text{-}Phe)_n$	-OPOH	$100/H^+$	72
7	$(Gly\text{-}Gly\text{-}Phe)_n$	-OPCP	$92\text{–}95/[\alpha]_{223}$	73
8	$(Gly\text{-}Gly\text{-}Phe)_n$	-ONP	$100/H^+$	84
9	$(Gly\text{-}Gly\text{-}D\text{-}Phe)_n$	-ONP	$95/[\alpha]_{223}$	73

[a]For details of polymerization reaction see original publication.
[b]Methods of measurement of optical purity were H^+-acid hydrolysis of polymer followed by optical rotation measurement of amino acid mixture; $[\alpha]_D$ – optical rotation of polymer at sodium D-line compared to a nonracemicly prepared polymer; $[\alpha]_{223}$ – optical rotation of polymer at 223 nm compared to a nonracemicly prepared polymer.

have in some instances been isolated and quantitated.[78] The discussion to this point has centered on optimization of polymerization. These conditions would in most instances work against the cyclization reaction.

In addition to the concentration of the monomer unit at the start of the polymerization reaction (see Section 7), the variable that stands out the most in affecting cyclization versus polymerization is the length of the monomer unit. Generally, dipeptides give poor yields of polymer, e.g., various preparations of poly (Ala-Gly).[6,7,65,67,92,93,130,131] This generalization, however, does not apply to dipeptides whose amino acids have opposite optical configurations.[77,78,103]

The polymerizing agent will also be important in determining whether cyclization or polymerization proceeds. Yamamoto and Noguchi[115] prepared poly (Try(H)-Glu(OH)) via the pentachlorophenyl ester of the dipeptide. Trudelle,[88] however, prepared the polymer only from the tetrapeptide *o*-hydroxyphenyl ester. The latter preparation yielded a much longer polymer (16,000 molecular weight average) than the former preparation (2,500 molecular weight average).

ANALYTICAL TOOLS

1. Degree of Polymerization

Generally, the molecular weight of sequential polypeptides is reported. From a standpoint of efficiency of a polymerization reaction, the degree of polymerization is more informative. The sequential polypeptides referred to in the various tables of this review are, therefore, reported in degrees of polymerization and were calculated from the polymer's molecular weight.

The molecular weight of sequential polypeptides may be determined chemically or physically (Table 14). The chemical method of determining molecular weight is based on the reactivity of the N-terminal amino group or the C-terminal carboxy group. With any chemical method the molecular weight obtained is the number average value. These values may be high if the polymer preparations contain any cyclic peptide material. With polymers containing glutamic acid, as the N-terminal amino acid, there is the added danger that pyrrolidone ring formation may have occurred and N-terminal chemical methods of molecular weight determination may be too high.[20] In general, the drawback to chemical methods of molecular weight determination is the question of availability of the terminal functional groups for reaction (methods 1 to 4) or ionization (methods 5 and 7).

Methods 2 to 4 (Table 14) form colored products. The amount of product is determined spectrophotometrically, light absorption with methods 2 and 4, and fluorescence with method 3. The absorption or fluorescence reading with method 2 or 3 may be made on either the modified peptide or modified amino acid (obtained on acid hydrolysis).

Method 7 (Table 14) for molecular weight

TABLE 14

Methods of Determining Molecular Weight

Method no.	Reactive group	Method[a]	Sample Reference
		Chemical	
1	N-terminal amino	HONO (Van Slyke)	7
2		DNFB (Sanger)	20
3		Dansyl chloride	75
4		Ninhydrin	62
5		$HClO_4$/acetic acid	115
6	C-terminal carboxy	*p*-nitrophenol	101
7		NaOMe	62
		Physical	
	Property	Method	
8	Spectroscopic	Infrared	123
9		Optical rotation	6
10		Nuclear magnetic resonance	118
11	Colligative	Osmotic pressure	12
12	Hydrodynamic	Light scattering	18
13		Sedimentation velocity (Archibald)	20
14		Sedimentation equilibrium (Yphantis)	20
15		Viscosity	114
16	Permeability	Gel filtration	129
17		Membrane flow	142
18	Polyelectrolyte	Ion-exchange chromatography	158
19	Radioactivity	C^{14}-terminal Gly alkyl ester	144

[a]Names in parentheses are the more commonly used to identify method or reagent.

determination may be used only with those sequential polypeptides prepared via the *p*-nitrophenyl ester method (agent 10, Table 2). The polymer product is subjected to mild basic conditions and the release of *p*-nitrophenoxide ion is followed spectrophotometrically. Naturally, it is assumed that all the polymers have a C-terminal *p*-nitrophenyl ester amino acid. Cyclic material or sequential polypeptides with a free C-terminal carboxyl group in the polymer preparation would lead to molecular weight estimates higher than the real value.

All the chemical methods of molecular weight determination are time dependent, since one must be assured that all possible functional groups that could react do react. With methods 1 to 4 and 6, this is not a problem. With the titration methods (5 and 7) the change in color at the equivalence end-point may not be sharp, i.e., with the addition of titrant a change in solution color is observed, which reverts back to the original color within several seconds. The reappearance of the original solution color may be due to solubilization of polymer or due to solubilization of carbon dioxide from the air. The exact polymer end-point is not clear and therefore an exact number average molecular weight is not available.

Of the various chemical methods of molecular weight analysis of sequential polypeptides, the titration methods (5 and 7) are the most rapid and the dansyl chloride method (3, Table 14) is the most sensitive. The radioactivity method (19, Table 14), although not chemical, does measure the number of terminal carboxyls in the polymer preparation. This easy method does suffer from the drawbacks of peptide terminal analysis, i.e.,

the question of cyclic peptides. To date, it has only been used by Johnson and his collaborators.[144] The radioactive method of molecular weight determination is dependent on the presence of the radioactive glycyl-alkyl ester in the reactions mixture during the polymerization step.

The spectroscopic methods of molecular weight analysis (methods 8 to 10, Table 14) depend on a difference in optical properties of an intermediate amino acid or bond versus the terminal one. These methods yield degree of polymerization rather than molecular weights. The infrared spectroscopic method is based on the measurement of the ratio of the intensities of the amide I bands (1,650 cm^{-1}), which are proportional to the number of peptide bonds and the band of the end peptide group (~1,740 cm^{-1}).[68] The NMR method, which has only been used on poly (Asp(OCH_3)-Gly-Gly), depends on the relative intensity of the internal methyl ester protons (τ=3.88) versus the external methyl ester protons (τ=3.93). Method 9 (Table 14) depends on the difference in optical rotation of the sodium D-line between the terminal and internal [$(Ala)_x$-$(Gly)_y$] peptide.[6] For x = 1, y = 2, the specific residue optical rotation internally, $[m_i]_D$, is -111°, and externally, $[m_t]_D$, is +73°. The degree of polymerization, n, is

$$n = \frac{[m_t]_D - [m_i]_D}{[m]_D - [m_i]_D}, \qquad (1)$$

where $[m]_D$ is the specific residue optical rotation of the polymer preparation.[159] As in the chemical methods of molecular weight determination, the presence of cyclic peptides in the polymer preparation would give too high values. The universal use of optical procedures for molecular weight determination is not likely, since the spectroscopic properties of polymers are dependent on the polymer's secondary structure.[160]

The applicability of osmotic pressure (method 11, Table 14) is limited by the membrane pore size and its stability to the solvent in which the polymer will dissolve. Low molecular weight polymers would traverse the membrane and not contribute to the osmotic pressure of the polymer solution.[161] The number average molecular weight by osmotic pressure may, therefore, be too high.

The drawbacks to determination of molecular weight by light-scattering (method 12, Table 14) are the number of measurements necessary, the danger of dust particles in solution, and the assumption of a specific tertiary structure.[162] The popular methods of sedimentation velocity[163] and sedimentation equilibrium[164] are limited by the number of solvents that may be used. The possibility of polymer aggregation[30] complicates the calculation of the weight average molecular weights by these ultracentrifugal methods.[163] In addition, with method 13 (Table 14) the partial specific volume necessary to calculate the molecular weight is generally approximated from the amino acid composition of the polymers. The use of viscosity to determine the molecular weights of polymers requires prior knowledge of the relationship of the polymer size with its reduced viscosity (e.g., poly γ-benzyl-L-glutamate).[165] As a general method of molecular weight determination of sequential polypeptides, viscosity is not useful. It is useful as a relatively quick method of comparing the efficiency (in terms of degree of polymerization) of polymerizing conditions on a specific monomer unit.[129]

Yaron and his collaborators[158,166] have been able to purify sequential polypeptides of defined length by the use of ion-exchange chromatography (method 18, Table 14). Up to 14 to 16 degrees of polymerization the resolution appears satisfactory;[168] however, larger polymers will require refinement of the technique.[158] The use of the polyelectrolyte properties of sequential polypeptides as a method for molecular weight determination is an area which requires further investigation.

Methods 16 to 18 (Table 14) are procedures where the synthetic sequential polypeptides are fractionated. For methods 16 and 18, the position of elution off the column determines the molecular weight of the polymer.[94,166] For method 17, various membranes are used to separate the sequential polypeptides into fractions of fixed molecular weight regions. Of the three methods gel filtration has found the widest use. Using a column of one of the Sephadex G or Bio-Gel P gels calibrated with protein and polypeptides believed to be of similar shape to the sequential polypeptide being investigated and under the chromatographic conditions for elution of the polymer, an elution pattern may be obtained which describes both the size and the amount of each polymer.[94] Cyclic peptides need not be removed

since their elution is confined to specific regions near the column volume.[78]

With most of the methods of molecular weight determination the polymer should be in solution. The solvent for solubilization may eliminate several of the methods of Table 14. Polyamino acids in the presence of indicator alone have had their molecular weights determined by the titration methods (5 and 7, Table 14).[30] With some sequential polypeptides, nonaqueous titration may be the only method of molecular weight determination.

2. Degree of Racemization

Generally, the method of measurement is to hydrolyze the polymer and measure the optical rotation of the hydrolysate. The result is compared to an appropriate mixture of amino acids which have undergone the conditions of hydrolysis.[20] Alternatively, the amino acids obtained on hydrolysis of the polymer are subjected to enzymatic degradation. The amino acids insensitive to the enzyme are analyzed chromatographically.[78] An alternative enzymatic method is to subject the sequential polypeptide to an aminopeptidase.[10] If any amino acid has undergone inversion, hydrolysis will terminate and peptides will be found chromatographically. A third method is to compare the optical rotation of the same sequential polypeptides prepared in a test system and by a known racemization-free method.[73,85,87,103] Comparison of the optical rotation of the polymers at 223 nm[73] is more sensitive than at the sodium D-line.[87] An apparently extremely sensitive method of racemization detection is the one used by Yaron et al.[158] Here the trifluoroacetyl O-methyl ester of the C-terminal amino acid (of the monomer) obtained after acid hydrolysis of the sequential polypeptides is gas chromatographed. The stationary phase containing CF_3CO-Phe-Ala-Leu cyclohexyl ester permits the separation of the L and D enantiomorphs.[167]

FUTURE DEVELOPMENTS

New blocking groups and coupling agents will continue to be reported. Development in the former area will improve the crystallinity of monomer units and/or ease of deprotection. New coupling agents of the active ester type (10 to 17, Table 2), the bifunctional ester type (18 to 21, Table 2), or the activatable ester type (17 and 20, Table 2) will be prepared. Some of these agents may have the racemization-free coupling properties of *o*-hydroxyphenol and the polymerization properties of N-hydroxysuccinimide or pentachlorophenol. The development of new catalysts may revitalize polymerization of peptides by condensing agents. This form of polymerization is desirable in the preparation of sequential polypeptides where the monomer unit is not a synthetic peptide, but is a purified biological peptide. The latter polymers may be invaluable in the preparation of antibodies to naturally occurring peptides.

From the lengthy discussion of the various factors important in the polymerization step (part 4) it is clear that the conclusions drawn were vague. Perhaps the reason for this vagueness is that the conclusions were made empirically. Future experiments on variables in the polymerization step of sequential polypeptides synthesis should be based on a role for each variable. Ideally, the relationship between the variable and the polymer product may be written as a mathematical expression and the results of the experiments would follow the mathematical expression. In this manner the preparation of sequential polypeptides of desired length would become a *fait accompli* and not a trial and error procedure as it is today.

In several publications of the synthesis of sequential polypeptides, the authors have alluded to the fact that using polymers without proper definition of purity and size as models for biological proteins is invalid. Optical purity measurements will require more sensitive methods of measurement of racemization. These will have to include the amino acids other than the monomer C-terminal. Methods, such as those used in analyses of racemization during peptide-bond formation (Reference 168 and references cited therein), will need to be used analytically for sequential polypeptide synthesis. Presently, analyses of the size of polymer preparations are probably best described by gel filtration methods. This method allows for a description of both the number average and weight average molecular weight.[94] The problem of time and sample size is a disadvantage to this method of polymer size analyses. Polyacrylamide gel electrophoresis,[169] a popular method presently used in protein molecular weight determination, has yet to be used for sequential polypeptide analyses. This latter

method coupled with densitometer readings may give the same data as the gel filtration method.

The advantages that sequential polypeptides have over polyamino acids have been discussed. Such advantages warrant the use of sequential polypeptides as models for proteins. Newly discovered proteins and new protein functions will continue the demand for sequential polypeptides. Hopefully, clarification and explanation of the roles of various factors in sequential polypeptide synthesis will allow the researcher in the future to fill these demands.

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APPENDIX

Abbreviations

All amino acids are L unless designated otherwise. Amino acids and substituted amino acids follow the recommendations of IUPAC-IUB commission of Biochemical Nomenclature Symbols of Amino-Acid Derivatives and Peptides (*Eur. J. Biochem.*, 27, 201, 1972). Other abbreviations used are defined in the tables or the footnotes to the tables.

SELECTED REFERENCES

Brack, A. and Spach, G., Synthesis and conformation of periodic copolypeptides of L-alanine and glycine, *Biopolymers*, 11, 563, 1972.

Cowell, R. D. and Jones, J. H., Sequential polypeptide synthesis. Part 1. Use of mono-esters of catechol in the synthesis of sequential polypeptides, *J. Chem. Soc.* (C), 1082, 1971.

DeTar, D. F., The active ester synthesis of sequence peptide polymers, in *Peptides*, Beyerman, H. C., van de Linde, A., and van den Brink, W. M., Eds., North-Holland Publishing, Amsterdam, 1964, 125.

Hardy, P. M., Rydon, H. N., and Thompson, R. C., Polypeptides. Part XVII. The synthesis of some sequential polypeptides of γ-benzyl-D-glutamate and L-leucine, *J. Chem. Soc. (Lond.), Perkin I*, 5, 1972.

Heidemann, E. and Meisel, H. D., Die polykondensation von tripeptiden nach der "Eintopfmethode.," *Die Makromolekulare Chemie*, 168, 1, 1973.

Johnson, B. J., Linear polypeptides of a known repeating sequence of aminoacids. Synthesis of poly(L-glutamyl-L-glutamyl-L-alanyl-glycyl) [1-^{14}C] glycine ethyl ester, *J. Chem. Soc.*, (C), 1412, 1969.

Kovacs, J., Giannotti, R., and Kapoor, A., Polypeptides with known repeating sequence of amino acids. Synthesis of poly-L-glutamyl-L-alanyl-L-glutamic acid and polyglycyl-L-phenylalanine through pentachlorophenyl active ester, *J. Am. Chem. Soc.*, 88, 2282, 1966.

Lorenzi, G. P., Doyle, B. B., and Blout, E. R., Synthesis of polypeptides and oligopeptides with the repeating sequence L-alanyl-L-prolylglycine, *Biochemistry*, 10, 3046, 1971.

Ramachandran, J., Berger, A., and Katchalski, E., Synthesis and physicochemical properties in aqueous solution of the sequential polypeptide poly (Tyr-Ala-Glu), *Biopolymers*, 10, 1829, 1971.

Shibnev, V. A., Chuvaeva, T. P., and Poroshin, K. T., Use of various activated esters in the synthesis of polypeptides of regular structure and their comparative evaluation, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 121, 1970.

Stewart, F. H. C., The synthesis and polymerization of peptide *p*-nitrophenyl esters, *Aust. J. Chem.*, 18, 887, 1965.

Yaron, A., Tal (Turkeltaub), N. and Berger, A., Synthesis and conformation of poly (L-lysyl-L-alanyl-L-alanyl), a sequence-ordered water-soluble copolymer, *Biopolymers*, 11, 2461, 1972.

REFERENCES

1. **Katchalski, E. and Sela, M.,** Biological properties of poly-α-amino acids, *Adv. Protein Chem.*, 14, 392, 1959.
2. **Katchalski, E., Sela, M., Silman, H. I., and Berger, A.,** Polyamino acids as protein models, in *The Proteins*, Vol. 2, Neurath, H., Ed., Academic Press, New York, 405, 1964.
3. **Cowell, R. D. and Jones, J. H.,** Sequential polypeptides. Part 3. The synthesis of two polyheptapeptides with functional side-chains, *J. Chem. Soc. (Lond.), Perkin I*, 1819, 1972.
4. **Doyle, B. B., Traub, W., Lorenzi, G. P., Brown, F. R., III, and Blout, E. R.,** Synthesis and structural investigation of poly (L-alanyl-L-alanylglycine), *J. Mol. Biol.*, 51, 59, 1970.
5. **Stewart, F. H. C.,** Poly-L-alanylglycyl-L-alanylglycyl-L-serylglycine: A synthetic model of Bombyx mori silk fibroin, *Aust. J. Chem.*, 19, 489, 1966.
6. **Brack, A. and Spach, G.,** Synthesis and conformation of periodic copolypeptides of L-alanine and glycine, *Biopolymers*, 11, 586, 1972.
7. **Poroshin, K. T., Chuvaeva, T. P., and Shibnev, V. A.,** Conditions for the polycondensation of activated esters of peptides illustrated by a model of silk fibroin, *Dokl. Akad. Nauk Tadzh. SSR*, 12(7), 28, 1969.
8. **Ebihara, S. and Kishida, Y.,** Synthesis of oligo-L-alanylglycine by the solid phase peptide synthesis, *Nippon Kagaku Zasshi*, 90, 823, 1969.
9. **Jones, J. H. and Walker, J.,** Sequential polypeptides. Part 7. The synthesis of poly-(L-glutaminyl-L-alanine), a model of the silks produced by sawflies of the family Argidae, *J. Chem. Soc. (Lond.), Perkin I*, 2926, 1972.
10. **Brown, F. R., III, Di Corato, A., Lorenzi, G. P., and Blout, E. R.,** Synthesis and structural studies of two collagen analogues: Poly(L-Prolyl-L-Seryl-Glycyl) and Poly(L-Prolyl-L-Alanyl-Glycyl), *J. Mol. Biol.*, 63, 85, 1972.
11. **Jones, J. H.,** Racemization-free sequential polypeptide synthesis, *Chem. Commun. (J. Chem. Soc., Sect. D)*, 1436, 1969.
12. **De Tar, D. F., Albers, R. J., and Gilmore, F.,** Synthesis of sequence peptide polymers related to collagen, *J. Org. Chem.*, 37, 4380, 1972.
13. **Poroshin, K. T., Chuvaeva, T. P., and Shibnev, V. A.,** Use of activated esters for synthesizing polytripeptide $(Gly\text{-}Pro\text{-}Hyp)_n$ as a model of collagen structure, *Dokl. Akad. Nauk Tadzh. SSR*, 12(8), 24, 1969.
14. **Sakakibara, S., Inouye, K., Shudo, K., Kishida, Y., Kobayashi, Y., and Prockop, D. J.,** Synthesis of $(Pro\text{-}Hyp\text{-}Gly)_n$ of defined molecular weights. Evidence for the stabilization of collagen triple helix by hydroxyproline, *Biochim. Biophys. Acta*, 303, 202, 1973.
15. **Heidemann, E. and Nil, H. W.,** Synthetische polypeptide der sequentztypen $(Glycin\text{-}L\text{-}Prolin\text{-}L\text{-}Serin)_n$ und (Glycin-L-Serin-L-Prolin), *Z. Naturforsch. Teil. B.*, 24, 851, 1969.
16. **Segal, D. M. and Traub, W.,** Polymers of tripeptides as collagen models. VI. Synthesis and structural investigation of poly(L-alanyl-L-prolylglycine), *J. Mol. Biol.*, 43, 496, 1969.
17. **Fairweather, R. and Jones, J. H.,** Sequential polypeptides. Part 6. The synthesis of some sequential polypeptide collagen models containing proline analogues, *J. Chem. Soc. (Lond.), Perkin I*, 2481, 1972.
18. **Anderson, J. M., Rippon, W. B., and Walton, A. G.,** Model polytripeptides for collagen, *Biochem. Biophys. Res. Commun.*, 39, 808, 1970.
19. **Poroshin, K. T., Burichenko, V. K., Maryash, L. J., and Shibnev, V. A.,** Pentachlorophenyl and 2,4,5-trichlorophenyl esters of gly-lys(Nε-tos)-lys(Nε-tos) and their polymerization, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 1276, 1971.
20. **De Tar, D. F. and Vajda, T.,** Sequence peptide polymers. II. Poly Glu(OH)-Gly Poly, poly Glu(OH)-Ser(H)-Gly, and their benzyl ester, *J. Am. Chem. Soc.*, 89, 1004, 1967.
21. **Khalikov, Sh. Kh., Poroshin, K. T., and Shibnev, V. A.,** Preparation of polypeptides with stable conformation as possible models of esterase activity, *Dokl. Akad. Nauk Tadzh. SSR*, 11(9), 28, 1968.
22. **Goren, H. J., Katchalski, E., and Fridkin, M.,** A model for acid-base catalysis in proteolytic enzymes, *9th International Congress of Biochemistry*, 89 (Abst. 2L12), 1973.
23. **Goodall, M. C. and Urry, D. W.,** A synthetic transmembrane channel, *Biochim. Biophys. Acta*, 291, 317, 1973.
24. **Fridkin, M., Frenkel, A., and Ariely, S.,** Poly(L-leucyl-L-ornithyl-L-leucine) and its grandization product: Synthesis and antibacterial activity, *Biopolymers*, 8, 668, 1969.
25. **Johnson, B. J.,** Carboxyl protection using salt formation for the synthesis of linear sequential polypeptides: Synthesis of poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester, *J. Pharm. Sci.*, 59, 1850, 1970.
26. **Schechter, B., Schechter, I., Ramachandran, J., Conway-Jacobs, A., and Sela, M.,** The synthesis and circular diochroism of a series of peptides possessing the structure $(L\text{-}Tyrosyl\text{-}L\text{-}Alanyl\text{-}L\text{-}Glutamyl)_n$, *Eur. J. Biochem.*, 20, 308, 1971.
27. **Kovacs, J., Kalita, C., and Ghatak, U. R.,** Synthesis of polyglutathione, polyaspartathione, and related sequential polypeptides, *J. Org. Chem.*, 37, 30, 1972.
28. **Sakakibara, S., Kishida, Y., Kikuchi, Y., Sakai, R., and Kakiuchi, K.,** Synthesis of poly-(L-prolyl-L-prolylglycyl) of defined molecular weights, *Bull. Chem. Soc. Jap.*, 41, 1273, 1968.
29. **Merrifield, R. B.,** Solid phase peptide synthesis. I. The synthesis of a tetrapeptide, *J. Am. Chem. Soc.*, 85, 2149, 1963.

30. **Katchalski, E. and Sela, M.,** Synthesis and chemical properties of poly-α-amino acids, *Adv. Protein Chem.*, 13, 243, 1958.
31. **De Tar, D. F.,** The active ester synthesis of sequence peptide polymers, *Peptides,* Beyerman, H. C., Van de Linde, A., and Van den Brink, W. M., Eds., North-Holland Publishing, Amsterdam, 1967, 125.
32. **Jones, J. H.,** Peptide synthesis, in *Amino Acids, Peptides and Proteins,* Vol. 1, Young, G. T., Ed., The Chemical Society, London, 1969, chap. 3.
33. **Jones, J. H.,** Peptide synthesis, in *Amino Acids, Peptides and Proteins,* Vol. 2, Young, G. T., Ed., The Chemical Society, London, 1970, chap. 3.
34. **Jones, J. H.,** Peptide synthesis, in *Amino Acids, Peptides and Proteins,* Vol. 3, Young, G. T., Ed., The Chemical Society, London, 1971, chap. 3.
35. **Greenstein, J. P. and Winitz, M.,** *Chemistry of the Amino Acids,* Vol. 2, John Wiley Sons, New York, 1961, chap. 10.
36. **Schröder, E. and Lubke, K.,** *The Peptides,* Vol. 1, Academic Press, New York, 1965.
37. **Bodanszky, M. and Ondetti, R. M.,** *Peptide Synthesis,* Wiley-Interscience, New York, 1965.
38. **Law, H. D.,** *The Organic Chemistry of Peptides,* Wiley-Interscience, New York, 1970.
39. **Patchornik, A. and Fridkin, M.,** Peptide synthesis, *Annu. Rev. Biochem.*, 42, 1973, in press.
40. **Menger, F. M. and Smith, J. H.,** Mechanism of ester aminolyses in aprotic solvents, *J. Am. Chem. Soc.*, 94, 3824, 1972.
41. **Menger, F. M. and Vitale, A. C.,** Anion-catalyzed ester aminolyses in a hydrocarbon solvent, *J. Am. Chem. Soc.*, 95, 4931, 1973.
42. **Pless, J. and Boissonnas, R. A.,** Über die geschwindigkeit der aminolyse von verschiedenen neuen, aktivierten, N-geschützen α-aminosaüre-phenylestern, imebesondere 2,4,5-trichlorophenylestern, *Helv. Chim. Acta,* 46, 1609, 1963.
43. **Izatt, R. M. and Christensen, J. J.,** Heats of proton ionization, pK, and related thermodynamic quantities, in *Handbook of Biochemistry, Selected Data for Molecular Biology,* Sober, H. A., Ed., The Chemical Rubber Co., Cleveland, 1970, J-167.
44. **Brenner, M.,** Critical evaluation of coupling methods, in *Peptides,* Beyerman, H. C., Van de Linde, A., and Van den Brink, W. M., Eds., North-Holland Publishing, Amsterdam, 1967, 1.
45. **Anderson, G. W., Blodinger, J., and Welcher, A. D.,** Tetraethylpyrophosphite as a reagent for peptide synthesis, *J. Am. Chem. Soc.*, 74, 5309, 1952.
46. **Anderson, G. W.,** New approaches to peptide synthesis, *Ann. N.Y. Acad. Sci.*, 88, 676, 1960.
47. **Young, G. T.,** The mechanism of racemization during peptide synthesis, in *Peptides,* Beyerman, H. C., Van de Linde, A., and Van den Brink, W. M., Eds., North-Holland Publishing, Amsterdam, 1967, 55.
48. **Goodman, M. and Glaser, C.,** Racemization mechanisms in peptide synthesis, in *Peptides: Chemistry and Biology,* Weinstein, B. and Lande, S., Eds., Marcel Dekker, Inc., New York, 1970, 267.
49. **Kovacs, J., Mayers, G. L., Johnson, R. H., and Ghatak, U. R.,** Racemization studies in peptide chemistry. Re-investigation of the "β-elimination-readdition" mechanism of N-benzyloxycarbonyl-S-benzylcysteine derivatives, *J. Chem. Soc. Chem. Commun.*, 1066, 1968.
50. **Kovacs, J., Mayers, G. L., Johnson, R. H., and Ghatak, U. R.,** On the racemization of amino acid active ester, in *Peptides: Chemistry and Biochemistry,* Weinstein, B. and Lande, S., Eds., Marcel Dekker, New York, 337, 1970.
51. **Mayers, G. L. and Kovacs, J.,** Investigations on the mechanism of racemization of N-benzyloxycarbonyl-S-benzyl-L-cysteine active esters in non-polar solvents, *J. Chem. Soc. Chem. Commun.*, 1145, 1970.
52. **Kovacs, J., Cartegiano, H., Covers, R. E., and Mayers, G. L.,** Isoracemization of N-carbobenzoxyamino acid ester derivatives, *J. Am. Chem. Soc.*, 93, 1541, 1971.
53. **Kemp, D. S. and Rebek, J., Jr.,** Peptide racemization mechanism. A kinetic isotope effect as a means of distinguishing enolization from oxazolone formation, *J. Am. Chem. Soc.*, 92, 5792, 1970.
54. **Kemp, D. S. and Chien, S. W.,** Specific base catalysis of azlactone formation, *J. Am. Chem. Soc.*, 89, 2245, 1967.
55. **McDermott, J. R. and Benoiton, N. L.,** N-Methylamino acids in peptide synthesis. IV. Racemization and yields in peptide-bond formation, *Can. J. Chem.*, 51, 2562, 1973.
56. **Kovacs, J., Davis, E. J., Johnson, R. H. Cortegiano, H., and Roberts, J.,** Studies on the rate of racemization and coupling of N-benzyloxy-carbonylamino acid and peptide active esters, in *Chemistry and Biology of Peptides,* Meienhofer, J., Ed., Ann Arbor Science Publishers, Ann Arbor, 1972, 359.
57. **Kovacs, J., Kisfaludy, L., Ceprini, M. Q., and Johnson, R. H.,** Investigations on the stereospecificity of peptide active phenyl ester formations and coupling, *Tetrahedron,* 25, 2555, 1969.
58. **Anderson, G. W.,** Racemization control in the synthesis of peptides by the mixed carbonic anhydride and dicyclohexylcarbodiimide methods, in *Peptides: Chemistry and Biochemistry,* Weinstein, B. and Lande, S., Eds., Marcel Dekker, New York, 1970, 255.
59. **Kemp, D. S.,** Mechanisms of peptide coupling reactions, *Peptides 1971,* Nesvadba, H., Ed., North-Holland Publishing, Amsterdam, 1973, 1.
60. **Hardy, P.M., Haylock, J. C., and Rydon, H. N.,** Polypeptides. Part 18. Syntheses of poly-(β-aspartic acid) and poly-(γ-glutamic acid) and their benzyl esters, *J. Chem. Soc. (Lond.), Perkin I,* 605, 1972.

61. **Heidemann, E. and Meisel, H. D.,** Die polykondensation von tripeptiden nach der, "Eintopfmethode," *Die Makromolekulare Chemie*, 166, 1, 1973.
62. **Shibnev, V. A. and Lazareva, A. V.,** Use of tetraethylpyrophosphite for synthesizing polypeptides simulating the nonpolar region of the collagen molecule, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 398, 1969.
63. **Kovacs, J., Schmit, G. N., and Ghatak, U. R.,** Polypeptides with known repeating sequences of amino acids. Comparison of several methods used for the synthesis of poly-γ-D- and L-Glutamyl-glycine and investigation of its serological reaction with antianthrax immune serum, *Biopolymers*, 6, 817, 1968.
64. **Stewart, F. H. C.,** Synthesis of polydepsipeptides with regularly repeating unit sequences, *Aust. J. Chem.*, 22, 1291, 1969.
65. **Shibnev, V. A., Chuvaeva, T. P., Khalikov, Sh. Kh., and Poroshin, K. T.,** Use of activated esters in the synthesis of models related to the structure of silk fibroin, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 2409, 1970.
66. **Burichenko, V. K., Ovchinnikova, V. G., Dakhte, R. R., and Poroshin, K. T.,** Polypeptides with $[gly\text{-}gly\text{-}lys]_n$ sequences, *Dokl. Akad. Nauk Tadzh. SSR*, 14(3), 30, 1971.
67. **Brack, A., Caille, A., and Spach, G.,** Synthese und konformation regelmasiger copolymeres von alanin und glycine (Modellsubstanzen zum studium der seiden), *Monatshefte Chem. Verw. Teile Anderer Wiss.*, 103, 1604, 1972.
68. **Shibnev, V. A., Chuvaeva, T. P., and Poroshin, K. T.,** Use of various activated esters in the synthesis of polypeptides of regular structure and their comparative evaluation, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 121, 1970.
69. **Shibnev, V. A., Dyermaev, K. M., Chuvaeva, T. P., Smirnov, L. D., and Poroshin, K. T.,** Use of β-pyridol esters and their nitro derivatives in the synthesis of peptides and polypeptides with regulated composition, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 7, 1634, 1967.
70. **Johnson, B. J. and Rea, D. S.,** The 4-(methylthio)phenyl ester for the synthesis of polypeptides of a known repeating sequence of amino acids. Synthesis of poly-(L-lysylglycyl)glycine [1-^{14}C] ethyl ester hydrobromide, *Can. J. Chem.*, 48, 2509, 1970.
71. **Ali, A., Hardy, P. M., and Rydon, H. N.,** Polypeptides. Part 19. The synthesis of some sequential polypeptides of β-methyl aspartate and γ-methyl glutamate, *J. Chem. Soc. (Lond.), Perkin I*, 1070, 1972.
72. **Cowell, R. D. and Jones, J. H.,** Sequential polypeptide synthesis. Part 1. Use of mono-esters of catechol in the synthesis of sequential polypeptides, *J. Chem. Soc. Sect. C Org. Chem.*, 1082, 1971.
73. **Kovacs, J., Mayers, G. L., Johnson, R. H., Giannotti, R., Cortegiano, H., and Roberts, J.,** On the problem of racemization during the synthesis of sequential polypeptides, in *Progress in Peptide Research*, Lande, S., Ed., Gordon and Breach, New York, 1972, 185.
74. **Wolman, Y., Gallop, P. M., Patchornik, A., and Berger, A.,** Peptide synthesis via oxidative activation of acid hydrazides, *J. Am. Chem. Soc.*, 84, 1889, 1962.
75. **Ridge, B., Rydon, H. N., and Snell, C. R.,** Polypeptides. Part 21. Synthesis of some sequential macromolecular polypeptides of L-leucine and L-2-hydroxy-4-methylpentanoic acid, *J. Chem. Soc. (Lond.), Perkin I*, 2041, 1972.
76. **Heidemann, E. and Nil, H. W.,** Zur synthese von serinpeptiden, *Z. Naturforsch. Teil B.*, 24, 837, 1969.
77. **Marlborough, D. I. and Rydon, H. N.,** Polypeptides. Part 16. The synthesis of some diastereoisomeric poly-(γ-t-butyl-glutamates), *J. Chem. Soc. (Lond.), Perkin I*, 1, 1972.
78. **Hardy, P. M., Rydon, H. N., and Thompson, R. C.,** Polypeptides. Part 17. The synthesis of some sequential polypeptides of γ-benzyl D-glutamate and L-leucine, *J. Chem. Soc. (Lond.), Perkin I*, 5, 1972.
79. **Cowell, R. D. and Jones, J. H.,** Sequential polypeptides. Part 2. The preparation of two partially protected hexapeptides for use in sequential polypeptide synthesis, *J. Chem. Soc. (Lond.), Perkin I*, 1809, 1972.
80. **Ramachandran, J., Berger, A., and Katchalski, E.,** Synthesis and physicochemical properties in aqueous solution of the sequential polypeptide poly (Tyr-Ala-Glu), *Biopolymers*, 10, 1829, 1971.
81. **Jones, J. H.,** Personal communication.
82. **De Tar, D. F., Gauge, M., Honsberg, W., and Honsberg, U.,** Sequence peptide polymers. I. Polymers based on aspartic acid and glycine, *J. Am. Chem. Soc.*, 89, 988, 1967.
83. **Goodman, M. and Steuben, K. C.,** Peptide synthesis *via* amino acid active esters, *J. Am. Chem. Soc.*, 81, 3980, 1959.
84. **De Tar, D. F. and Estrin, N. F.,** An optically pure sequence peptide, poly (Gly-Gly-Phe), *Tetrahedron Lett.*, 5985, 1966.
85. **Heitz, F. and Spach, G.,** Synthesis and conformational study of alternating poly (γ-benzyl D,L-glutamates), *Macromolecules*, 4, 429, 1971.
86. **Lorenzi, G. P., Doyle, B. B., and Blout, E. R.,** Synthesis of polypeptides and oligopeptides with the repeating sequence L-Alanyl-L-prolyglycine, *Biochemistry*, 10, 3046, 1971.
87. **Trudelle, Y.,** Use of *o*-hydroxyphenyl esters for the preparation of an optically pure polytripeptide, *Chem. Commun. (J. Chem. Soc., Sect.D)*, 639, 1971.
88. **Trudelle, Y.,** Sequential polypeptides. Synthesis of poly (L-tryosyl-L-glutamyl-L-tyrosyl-L-glutamyl), poly (L-glutamyl-L-tyrosyl-L-glutamyl) and poly (L-glutamyl-L-glutamyl-L-tyrosyl-L-glutamyl) by use of catechol esters, *J. Chem. Soc. (Lond.), Perkin I*, 1001, 1973.
89. **Kovacs, J., Giannotti, R., and Kapoor, A.,** Polypeptides with known repeating sequence of amino acids. Synthesis of poly-L-glutamyl-L-alanyl-L-glutamic acid and polyglycyl-L-phenylalanine through pentachlorophenyl active ester, *J. Am. Chem. Soc.*, 88, 2282, 1966.

90. **Kapoor, A.,** Application of pentachlorophenyl active ester in the synthesis of peptides and sequential polypeptides from the C-terminal residues of amino acids, in *Peptides: Chemistry and Biochemistry,* Weinstein, B. and Lande, S., Eds., Marcel Dekker, Inc., New York, 1970, 17.
91. **Vajda, T.,** Synthesis of polypeptides with repeating sequences. Poly-L-glutamyl-L-histidyl-L-lysyl-L-tyrosine, *Acta Chim. Acad. Sci. Hung.,* 46, 221, 1965.
92. **Stewart, F. H. C.,** The synthesis and polymerization of peptide *p*-nitrophenyl esters, *Aust. J. Chem.,* 18, 887, 1965.
93. **Anderson, J. M., Chen, H. H., Rippon, W. B., and Walton, A. G.,** Characterization of 3 sequential polydipeptides containing glycine, *J. Mol. Biol.,* 67, 459, 1972.
94. **Fairweather, R., Jones, J. H., and Wilcox, J. K.,** A convenient gel chromatography method for determining the molecular weight distributions and averages of synthetic polypeptide collagen models, *J. Chromatogr.,* 67, 157, 1972.
95. **Fraser, R. D. B., Harrap, B. S., MacRae, T. P., Stewart, F. H. C., and Suzuki, E.,** Sequential polypeptides containing S-benzyl-L-cysteinyl and γ-ethyl-L-glutamyl residues, *J. Mol. Biol.,* 14, 423, 1965.
96. **Ledger, R. and Stewart, F. H. C.,** Sequential polypeptides containing γ-benzyl-L-glutamyl residues, *Aust. J. Chem.,* 20, 2509, 1967.
97. **Rodin, R. L. and Kovacs, J.,** Synthesis and conformational studies of poly-β-(γ-glutamyl)-aspartic acid, *Bioorganic Chem.,* 2, 65, 1972.
98. **Shibnev, V. A., Lisovenko, A. V., Chuvaeva, T. P., and Poroshin, K. T.,** Comparison of some methods used for synthesis of regular polypeptides, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk,* 2564, 1968.
99. **Shibnev, V. A., Chuvaeva, T. P., Martynova, G. A., and Poroshin, K. T.,** 2,4,5-Trichlorophenyl esters and their use in synthesis of polypeptides of regular structure, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk,* 637, 1961.
100. **Johnson, B. J.,** Polypeptides with a known repeating sequence of amino acids. Synthesis of poly (α- and γ-L-glutamyl-L-alanylglycine), *J. Chem. Soc.,* (C), 2638, 1967.
101. **Stewart, F. H. C.,** The preparation of sequential polypeptides containing glycyl and γ-ethyl-L-glutamyl residues, *Aust. J. Chem.,* 19, 2373, 1966.
102. **Poroshin, K. T., Zegelman, A. B., Yusupov, T. Yu., and Demyanik, G. N.,** Polycondensation of activated amino acid esters, *Dokl. Akad. Nauk Tadzh. SSSR,* 12(10), 28, 1969.
103. **Hardy, P. M., Rydon, H. N., and Storey, H. T.,** Polypeptides. Part 20. The synthesis of some diastereoisomeric poly-(γ-benzyl glutamates), *J. Chem. Soc. (Lond.), Perkin I,* 1523, 1972.
104. **Bloom, S. M., Dasgupta, S. K., Patel, R. P., and Blout, E. R.,** The synthesis of glycyl-L-prolyglycyl and glycl-L-prolyl-L-alanyl olgiopeptides and sequential polypeptides, *J. Am. Chem. Soc.,* 88, 2035, 1966.
105. **Sakakibara, S., Kishida, Y., and Aimoto, S.,** Crystallization of synthetic polypeptides with triple-helical structure, in *Chemistry and Biology of Peptides,* Meienhofer, J., Ed., Ann Arbor Science Publishers, Ann Arbor, 293, 1972.
106. **Okuyama, K., Tanaka, N., Ashida, T., Kakudo, M., Sakakibara, S., and Kishida, Y.,** An X-ray study of the synthetic polypeptide $(Pro\text{-}Pro\text{-}Gly)_{10}$, *J. Mol. Biol.,* 72, 571, 1972.
107. **Olsen, B. R., Berg, R. A., Sakakibara, S., Kishida, Y., and Prockop, D. J.,** The synthetic polytripeptides $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_{20}$ form micro-crystalline structures similar to segmental structures formed by collagen, *J. Mol. Biol.,* 57, 589, 1971.
108. **Sakakibara, S., Kishida, Y., Okuyama, K., Tanaka, N., Ashida, T., and Kakudo, M.,** Single crystals of $(Pro\text{-}Pro\text{-}Gly)_{10}$, a synthetic polypeptide model of collagen, *J. Mol. Biol.,* 65, 371, 1972.
109. **Kivirikko, K. I., Kishida, Y., Sakakibara, S., and Prockop, D. J.,** Hydroxylation of $(X\text{-}Pro\text{-}Gly)_n$ by protocollagen proline hydroxylase. Effect of chain length, helical conformation and amino acid sequence in the substrate, *Biochim. Biophys. Acta,* 271, 347, 1972.
110. **Kivirikko, K. I., Shudo, K., Sakakibara, S., and Prockop, D. J.,** Studies on protocollagen lysine hydroxylase. Hydroxylation of synthetic peptides and the stoichiometric decarboxylation of α-ketoglutarate, *Biochemistry,* 11, 122, 1972.
111. **Iio, T.,** Block sequential polypeptides of L-alanine and glycine with D,L-glutamic acid, *Biopolymers,* 10, 1583, 1971.
112. **Rippon, W. B., Chen, H. H., Anderson, J. M., and Walton, A. G.,** Characterization of two sequential polytripeptides containing glycine and ethyl-glutamate residues, *Biopolymers,* 11, 1411, 1972.
113. **Takahashi, S.,** The synthesis of regular copolymers of glycine and alanine, *Bull. Chem. Soc. Jap.,* 42, 521, 1969.
114. **Segal, D. M.,** Polymers of tripeptides as collagen models. VII. Synthesis and solution properties of four collagen-like polyhexapeptides, *J. Mol. Biol.,* 43, 497, 1969.
115. **Yamamoto, H. and Noguchi, J.,** Studies on the catalytic activity of poly-α-amino acids. II. Hydrolysis of *p*-nitrophenyl acetate by an interaction between hydroxyl and carboxyl groups of poly (L-Tyr-L-Glu), copoly (L-Tyr, L-Glu, L-Ala) or copoly (L-Tyr, L-Glu, L-Pro), *J. Biochem.,* 67, 103, 1970.
116. **De Tar, D. F., Rogers, F. F., Jr., and Bach, H.,** Sequence peptide polymers. III. Poly Asp(OH)-Ser(H)-Gly and other serine-containing polymers, *J. Am. Chem. Soc.,* 89, 3039, 1967.
117. **Stewart, F. H. C.,** The preparation of some sequential polypeptides by the *p*-nitrophenyl ester method, *Aust. J. Chem.,* 19, 1503, 1966.
118. **De Tar, D. F., Honsberg, W., Honsberg, U., Wieland, A., Gouge, M., Bach, H., Tahara, A., Brinigar, W. S., and Rogers, F. F., Jr.,** Synthesis of peptide polymers with repeating sequences, *J. Am. Chem. Soc.,* 85, 2873, 1965.

119. **Stewart, F. H. C.,** Peptide synthesis with the N-nitroso derivatives of sarcosine and proline, *Aust. J. Chem.*, 22, 2451, 1969.
120. **Shibnev, V. A., Grechishko, V. S., and Poroshin, K. T.,** Synthesis and polymerization of activated esters of tripeptides involving N-tosylated lysine, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 2327, 1967.
121. **Poroshin, K. T., Chuvaeva, T. P., and Shibnev, V. A.,** Synthesis of glutamyl-containing polypeptides via *p*-nitrophenyl esters, *Dokl. Akad. Nauk Tadzh. SSR*, 12(11), 15, 1969.
122. **Shibnev, V. A., Finogenova, M. P., and Poroshin, K. T.,** Synthesis of a high-molecular-weight polytripeptide, $(\text{-gly-pro-pro-})_n$ with a collagen-type structure, *Dokl. Akad. Nauk SSSR*, 198(4), 862, 1971.
123. **Shibnev, V. A., Chuvaeva, T. P., Martynova, G. A., and Poroshin, K. T.,** N-hydroxysuccinimide esters in the synthesis of collagen-type structures, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 11, 2532, 1969.
124. **Stewart, F. H. C.,** Sequential polypeptides containing O-acetyl-L-seryl- and γ-methyl-L-glutamyl residues, *Aust. J. Chem.*, 21, 1935, 1968.
125. **Fraser, R. D. B., Harrap, B. S., MacRae, T. P., Stewart, F. H. C., and Suzuki, E.,** Sequential polypeptides containing L-valy and γ-methyl-L-glutamyl residues, *J. Mol. Biol.*, 12, 482, 1965.
126. **Ramachandran, G. N.,** Structure of collagen at the molecular level, in *Treatise on Collagen*, Vol. 1, Ramachandran, G. N., Ed., Academic Press, New York, 1967, 158.
127. **Scheraga, H. A. and Poland, D.,** in *Theory of Helix-Coil Transitions in Biopolymers*, Academic Press, New York, 1970.
128. **Fasman, G. D. and Chou, P. Y.,** Prediction of protein conformation. Calculated conformational parameter for amino acids in helical, β, and coil regions from proteins, *9th International Congress of Biochemistry* (Abstr.), 2i, 4, 70, 1973.
129. **Fairweather, R. and Jones, J. H.,** Sequential polypeptides. Part IV. The synthesis of poly-(L-alanyl-glycyl-L-proline) and its stereisomers, *J. Chem. Soc. (Lond.), Perkin I*, 1908, 1972.
130. **Shibnev, V. A. and Chuvaeva, T. P.,** Use of pentafluorophenyl esters in the synthesis of peptides and polypeptides of regular structures, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 954, 1967.
131. **Brack, A. and Spach, G.,** Nouvelles conformations de copolypeptides ordonnes a bas de L-alanine et de glycine, *C. R. Acad. Sci. [c] (Paris)*, 271, 916, 1970.
132. **Shibnev, V. A., Chuvaeva, T. P., and Poroshin, K. T.,** Synthesis of various activated tripeptide esters representing monomers for preparing models of collagen structure, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 2527, 1969.
133. **Heidemann, E. and Bernhardt, H. W.,** Synthetic polypeptides as models for collagen, *Nature*, 220, 1326, 1968.
134. **Shibnev, V. A., Khalikov, Sh.Kh., Finogenova, M. P., and Poroshin, K. T.,** Synthesis of polytripeptide $(\text{Pro-Ser-Gly})_n$ modeling the collagen-type of structure, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 2822, 1970.
135. **Dellacherie, E. and Neel, J.,** Synthesis and properties of a sequenced copolypeptide rich in methionine, poly (L-methionyl-L-methionyl-L-alanine), *Bull. Soc. Chim. Fr.*, 1218, 1969.
136. **Johnson, B. J.,** Polypeptides with a known repeating sequence of amino acids. Synthesis of poly-(L-lysyl-L-alanylglycine) hydrobromide, *J. Chem. Soc.*, (C), 3008, 1968.
137. **Johnson, B. J.,** Linear polypeptides of a known repeating sequence of amino acids. Synthesis of poly-(L-glutamyl-L-glutamyl-L-alanyl-glycyl) [1-^{14}C] glycine ethyl ester, *J. Chem. Soc.*, (C), 1412, 1969.
138. **Johnson, B. J. and Trask, E. G.,** Linear polypeptides of a known sequence of amino acids. Synthesis and immunochemical properties of poly (L-tyrosyl-L-glutamyl-glycyl-glycyl) glycine methyl ester, *J. Med. Chem.*, 14, 251, 1971.
139. **Johnson, B. J., Cheng, C., and Tsang, N.,** Inhibition studies on antibody to poly (L-tyrosyl-L-glutamyl-L-alanylglycyl) glycine-1-^{14}C ethyl ester. Synthesis and immunochemical properties of poly (L-alanyl-L-glutamyl-L-ananylglycyl) glycine-l-^{14}C ethyl ester, *J. Med. Chem.*, 15, 95, 1972.
140. **Johnson, B. J. and Chen, F.,** Linear polypeptide of a known sequence of amino acids: Poly-(L-phenylalanyl-L-glutamyl-L-alanylglycyl) glycine methyl ester, *J. Pharm. Sci.*, 60, 330, 1971.
141. **Johnson, B. J. and Cheng, C.,** Antigenic polypeptides. Synthesis and immunochemical studies of poly (L-phenylalanyl-L-glutamyl-L-alanylglycyl) glycine-l-^{14}C ethyl ester, *J. Med. Chem.*, 14, 1238, 1971.
142. **Johnson, B. J. and Cheng, C.,** Studies on the specificity of antibodies produced by linear antigenic polypeptides of a known primary structure. Synthesis and use of poly (L-tyrosyl-L-aspartyl-L-alanylglycyl) glycine methyl ester, *J. Med. Chem.*, 16, 415, 1973.
143. **Johnson, B. J.,** Conformation dependency of antibodies to poly (L-tyrosyl-L-glutamyl-L-alanylglycyl) glycine-1-^{14}C ethyl ester, *J. Pharm. Sci.*, 61, 1654, 1972.
144. **Johnson, B. J. and Trask, E. G.,** Linear polypeptides of a known repeating sequence of amino acids. Synthesis of poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl) [1-^{14}C] glycine ethyl ester, *J. Chem. Soc.*, (C), 2644, 1969.
145. **Johnson, B. J. and Chen, F.,** Linear polypeptides of a known primary structure. Synthesis and immunochemical studies of poly (O-methyl-L-tyrosyl-L-glutamyl-L-alanylglycyl) glycine-l-^{14}C ethyl ester, *J. Med. Chem.*, 14, 640, 1971.
146. **Johnson, B. J. and Cheng, C.,** Studies on the conformation dependency of antibody to poly (L-tyrosyl-L-glutamyl-L-alanylglycyl) glycine-l-^{14}C ethyl ester, *J. Med. Chem.*, 15, 423, 1972.

147. **Johnson, B. J. and Cheng, C.,** Specificity of antibodies produced by linear antigenic polypeptides of a known primary structure. Synthesis and use of poly (L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl) glycine methyl ester, *J. Pharm. Sci.*, in press.
148. **Johnson, B. J. and Trask, E. G.,** Linear polypeptides of a known repeating sequence of amino acids. Synthesis of poly-(L-tyrosyl-L-glutamyl-L-valylglycyl) [1-^{14}C] glycine ethyl ester, *J. Chem. Soc.*, (C), 2247, 1970.
149. **Johnson, B. J. and Tsang, N.,** Immunochemical studies on linear antigenic polypeptides with a known primary structure. Specificity of antibodies to poly (L-tyrosyl-L-glutamyl-L-alanyl-glycyl) alycine-1-^{14}C ethyl ester, *J. Med. Chem.*, 15, 488, 1972.
150. **Johnson, B. J.,** Linear polypeptides of a known primary structure. Synthesis and immunochemical studies of poly (L-glutamyl-L-tyrosyl-L-alanylglycyl) glycine-1-^{14}C ethyl ester, *J. Med. Chem.*, 14, 488, 1971.
151. **Shibnev, V. A. and Maryash, L. J.,** Synthetic analogues of histones and protamines. Part I. Synthesis of polypeptides containing terminal lysine and ornithine, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 435, 1973.
152. **Shibnev, V. A., Maryash, L. J., and Poroshin, K. T.,** Synthetic analogues of histones, *Doklad. Akad. Nauk SSSR*, 207(3), 625, 1972.
153. **Burichenko, V. K., Poroshin, K. T., Kasimova, G. F., and Shibnev, V. A.,** Synthesis of regular polypeptides containing C-terminal arginine, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 2596, 1972.
154. **Burichenko, V. K., Korycekina, N. I., and Poroshin, K. T.,** Preparation of regular polypeptides of the [Gly(Nε-Tos)Lys-Gly]$_n$ sequence, *Dokl. Akad. Nauk Tadzh. SSR*, 14(5), 16, 1971.
155. **Shibnev, V. A., Khalikov, Sh.Kh., Finogenova, M. P., and Poroshin, K. T.,** Synthesis of polypeptides (-gly-ser-hypro-)$_n$ and (-gly-ser-pro-)$_n$ with the aid of pentachlorophenyl esters, *Izv. Akad. Nauk. SSSR, Ser. Biol. Khim. Nauk*, 399, 1970.
156. **Shibnev, V. A., Poroshin, K. T., Chuvaeva, T. P., and Martynova, G. A.,** The use of γ-hydroxyquinoline peptide esters in the synthesis of polypeptides with regular structure, *Izv. Akad. Nauk SSSR, Ser. Biol. Khim. Nauk*, 1144, 1968.
157. **Poroshin, K. T., Chuvaeva, T. P., and Shibnev, V. A.,** Use of sublimation for purifying low molecular weight polypeptides, *Dokl. Akad. Nauk Tadzh. SSR*, 12(12), 21, 1969.
158. **Yaron, A., Tal(Turkeltaub), N., and Berger, A.,** Synthesis and conformation of poly (L-lysyl-L-alanyl-L-alanyl), a sequence-ordered water-soluble copolymer, *Biopolymers*, 11, 2461, 1972.
159. **Brach, A. and Spach, G.,** Application of different polycondensation methods in the preparation of poly (alanyl-diglycyl), in *Peptides*, Bricas, E., Ed., North-Holland Publishing, Amsterdam, 1968, 45.
160. **Lotan, N., Berger, A., and Katchalski, E.,** Conformation and conformational transitions of poly-α-amino acids in solution, *Ann. Rev. Biochem.*, 41, 869, 1972.
161. **Tanford, C.,** in *Physical Chemistry of Macromolecules*, John Wiley & Sons, New York, 1965, 210.
162. **Bier, M.,** Light-scattering measurements, in *Methods in Enzymology*, Vol. 4, Colowick, S. P. and Kaplan, N. O., Eds., Academic Press, New York, 1957, 147.
163. **Schachman, H. K.,** Ultracentrifugation, diffusion and viscometry, in *Methods in Enzymology*, Vol. 4, Colowick, S. P. and Kaplan, N. O., Eds., Academic Press, New York, 1957, 147.
164. **Yphantis, D. A.,** Equilibrium ultracentrifugation of dilute solutions, *Biochemistry*, 3, 297, 1964.
165. **Doty, P., Bradbury, J. H., and Holtzer, A. M.,** Polypeptides. IV. Molecular weight, configuration, and association of poly (γ-benzyl-L-glutamate) in solvents, *J. Am. Chem. Soc.*, 78, 947, 1956.
166. **Yaron, A., Dunham, E. K., and Schlossman, S. F.,** Synthesis and immunologic properties of the oligopeptide series: Lys$_n$-N$^\epsilon$-DNP-Lys and Lys$_n$-Ala$_3$-N$^\epsilon$-DNP-Lys, 1973, in press.
167. **Westley, J. W.,** The optical analysis of amino acid derivatives by gas chromatography, in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. 1, Marcel Dekker, New York, 1971, 1.
168. **Bosshard, H. R., Schechter, I., and Berger, A.,** A new method for the detection of racemization during peptide synthesis: Stereoselective hydrolysis of diastereomeric peptides by leucine aminopeptidase, *Helv. Chim. Acta*, 56, 717, 1973.
169. **Weber, K. and Osborn, M.,** The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis, *J. Biol. Chem.*, 244, 4406, 1969.