KINETIC STUDIES FOR THE PRODUCTION OF

SELECTED HORMONES BY SOLID

PHASE SYNTHESIS

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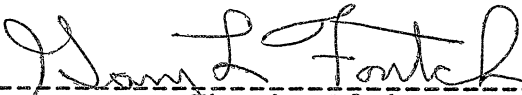Oklahoma State University

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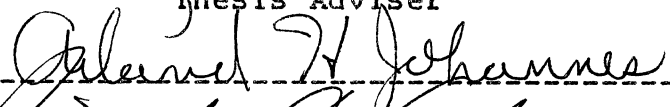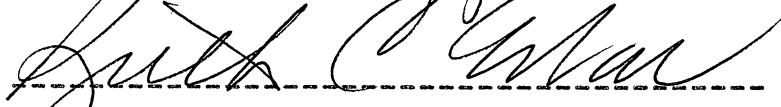the Degree of
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Dedicated to my parents

Yushin Wang and Jingwen Li

KINETIC STUDIES FOR THE PRODUCTION OF
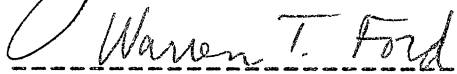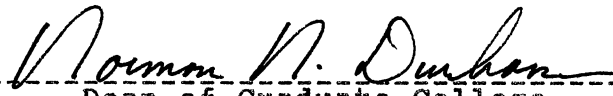
SELECTED HORMONES BY SOLID

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PREFACE

This study is conducted to learn more about the reaction kinetics of solid phase peptide synthesis. Reaction rates have been measured for the production of Bradykinins, Angiotensins and Endorphins by solid phase synthesis. Reaction kinetic models are found to be a function of temperature, concentrations, amino acid attached, amino terminus anchored to peptide fragment, chain length of peptide fragment, resin characteristics, mixing efficiency, and others. Reaction mechanisms are proposed for this heterogeneous, non-elementary reaction.

My interest in this field was initiated by my adviser, Dr. G. L. Foutch, who has always been more than ready to provide valuable assistance and has done the majority of the work in providing me advice and in proofreading this manuscript. I would like to individually thank each of my committee members, Dr. A. Johannes, Dr. R. Erbar and Dr. W. Ford for their concern in the whole process as well as for their time and effort in reading the rough draft of this thesis and providing me with valuable suggestions for further improvement. Also, I would like to thank Dr. M. Seapan, who had been a committee member, for his help.

My parents, my wife's parents and my wife have my deepest appreciation for their love and support.

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NOMENCLATURE AND ABBREVIATIONS

A   amino terminus on peptide fragment

AB   coupling product

Ala   alanine

Arg   arginine

B   free amino acid monomer

Bhoc   benzhydryloxycarbonyl

Boc   tert-butyloxycarbonyl

Bpoc   biphenylisopropyloxycarbonyl

B_2 amino symmetrical anhydride

B_2A^* intermediate

Bzl   benzyl

C   general expression for concentration

C_A concentration of component A, mole/l

C_{A0} initial concentration of component A, mole/l

C_{B0} initial concentration of component B_2, mole/l

CBZ   carbobenzoxy

DCC   dicyclohexylcarbodiimide

DCM   dichloromethane

DCU   dicyclohexylurea

Ddz   2-(3,5-dimethoxyphenyl)propyl(2)oxycarbonyl

DMF   dimethylformamide

DVB   divinylbenzene

E   apparent activation energy, kcal/mole

Gly	glycine
His	histidine
Ile	isoleucine
im	imidazole
K	general expression for reaction rate constant
K_1	diffusion rate in positive direction
K_2	diffusion rate in reverse direction
K_3	chemical reaction rate constant
K_1'	combined constant in forward diffusion direction
K_f	pseudo first-order reaction rate constant, s^{-1}
K_o	frequency factor in Arrhenius' law
K_s	apparent second order reaction rate constant, 1/mole s
Leu	leucine
Lys	lysine
M	mole ratio of initial loading components
N	amino
NCA	N-carboxyanhydride
NMR	nuclear magnetic resonance
NPS	o-nitrophenylsulfenyl
Pam	phenylacetamidomethyl
Phe	phenylalanine
Ppoc	2-phenylpropyl(2)oxycarbonyl
Pro	proline
R	gas constant, kcal/mole ^{o}K
R', R"	side chain on amino acid
r_A	rate of reaction respect to component A, mole/l s
Ser	serine

SPPS   solid phase peptide synthesis

t   time, s

T   temperature, ^{o}K

TEA   triethylamine

TFA   trifluoroacetic acid

TFE   trifluoroethanol

Thr   threonine

Tos   4-toluenesulfonyl

Tyr   tyrosine

UV   ultra-violet (spectrophotometer)

Val   valine

X_A reacted mole fraction of component A

[ ]   concentration

CHAPTER I

INTRODUCTION

Classical Solid Phase Peptide Synthesis (SPPS) has been studied for many years. This method was introduced by R. B. Merrifield in 1963 (Merrifield, 1963). By that time, the "classical" solution methods were used for the chemical assembly of amino acid to form peptides. Unfortunately, these methods were laborious, time-consumed, and skill-intensive. SPPS provided a method to overcome these problems.

The basic idea of the solid-phase approach involves covalent attachment of the growing peptide chain to an insoluble polymeric support, so that un-reacted soluble reagents can be removed by simple filtration and washing without manipulative losses. Those laborious intermediate purifications need in the solution syntheses are thus eliminated. This strategy can be extended to a peptide of any desired sequence in one suitably designed reactor. After that, a reagent can be applied to cleave the crude peptide from the support under conditions that are minimally destructive towards sensitive residues in the sequence, and liberate the finished peptide into solution. Theoretically, all the reactions involved in the synthesis should be

carried to 100% completion, so that a homogeneous product can be obtained. Finally, prudent purification and scrupulous characterization of the synthetic peptide product must be performed to ensure and verify that the desired structure is indeed the one obtained.

Current commercial solid phase synthesis of peptides produces various sequences, 5-20 amino acids in length, in kg quantities per year. This method has truly revolutionized the entire peptide field, and its influence has spread to other areas, such as oligonucleotide synthesis, with equally dramatic results. There are many research aspects in SPPS, including apparatus, resins, protection schemes, anchoring regiments, coupling conditions, monitoring techniques, cleavage procedures and purifications. Also, some researchers focus their attention on kinetic characteristics like coupling rate, chain length effect and temperature influence. However, despite an understanding of the underlying chemistry, reaction kinetic information on SPPS is limited. The reaction kinetic model and reaction mechanism are still unclear, while the suitable kinetic information is essential in guiding large-scale reactor design and process operation of SPPS.

Kinetic studies on SPPS have been performed in our laboratory for several years. Several investigators in succession have been involved. The previous work (Chen and Foutch, 1989a) showed a simple shifting order model for poly-phenylalanine and poly-serine with resin cross-linked

with 2% divinylbenzene (DVB). Variation with chain length and temperature occurred. Some other factors, such as mole ratio of amino symmetrical anhydride to amino acid terminus on peptide fragment, percentage of DVB crosslinking, and mixing rate of bulk phase, were tested and discussed qualitatively. However, these studies were mainly limited to some poly-amino acid sequences. Real hormone synthesis is much more complicated with more factors involved which make the kinetic study more difficult.

The research work presented in this thesis addresses the kinetics for the production of selected hormones by SPPS. The experiments determine reaction rate as a function of peptide chain length, symmetrical anhydride concentration, specific amino acid being reacted, peptide fragment anchored to the resin, reaction temperature, and polymer support type. The selected hormones are Bradykinins, Angiotensins and Endorphins. As a first step, the changing amino symmetrical anhydride concentration during the coupling reaction was continuously measured by Ultra-Violet/Visible Spectrophotometer (UV) monitoring. Experiments are triplicated to establish consistent and reliable results. Concentration, temperature and other factors are tested over the ranges of interest. The second step is to model the experimental data in order to obtain kinetic parameters describing the production of these peptides. All variables mentioned above are considered in the models. The third step is to propose reaction

mechanisms for this production by considering both the chemical characteristics and the kinetic properties.

The results of this research will expand the knowledge of reaction kinetics involved, and allow for improved design and operation of potential industrial scale SPPS facilities.

CHAPTER II

LITERATURE REVIEWS

Reaction Kinetics

General Kinetic Study

There are many factors which can affect the reaction kinetics of SPPS thereby making a kinetic study difficult. The reaction is a heterogeneous one. Based on the properties of insoluble resin-bound peptide chain, soluble amino acid reactant, and solvent, the kinetics may vary. Some researchers may even get contradictory conclusions with the same reactants involved. So far, there is a lack of authoritative kinetic theory in this area. Merrifield (1984) gave second order rate constants for Bpoc-amino acid anhydrides as reactants. The effect of resin and amino acid structure on kinetics and coupling efficiency was discussed. Based on his observations, the Beta-structured amino acids, such as Ile and Val, react measurably slower. The aminomethyl-resin, Ala-resin, is more active with a derivative than the Alpha-amine of an amino acid ester, Val-resin. He measured both pseudo first order and second order reaction rates and observed ideal kinetics to at least 90% completion. It was found that the second order reaction

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rate model was obeyed over the range of 10^{-6} to 10^{-3} mole/liter concentration, but deviated at higher concentration. These results suggested that diffusion or mass transfer might become partially rate limiting under actual coupling conditions where the concentration is 0.05 to 0.2 mole/liter. Merrifield found no measurable effect on the efficiency of the couplings with his arbitrary tetra-peptides. However, the effect of chain length on kinetic data was not discussed. All the kinetic data were limited to a single attachment.

Rudinger and Buetzer (1975) proposed a reaction rate model for the rate of disappearance of the soluble reactant, in which rate constants are composed of a so called "true" second-order coefficient with some combination coefficients of concentration and volume in the "reaction" and "bulk" phases. This model shows that the reaction is not elementary. The reaction rate constant is only an "apparent" one. Unfortunately, the authors did not have any discussion about reaction mechanisms. Also, their rate constants were one order of magnitude lower than those of Merrifield.

Periyasamy and Ford (1985) studied the effect of DVB crosslinkage on the rate of exchange of solvent in and out of swollen crosslinked polystyrene beads. They noticed that the first-order exchange rate constants decrease about three fold as the DVB crosslink percentage increases from 1% to 20%. This information can be used for the discussion of

diffusion effects.

In previous OSU kinetic study (Chen, 1988), the bulk and film diffusions were not considered as limiting factors since the reaction rates did not depend on the speed of stirring of the slurry of polystyrene beads in the solution. This conclusion was further obtained quantitatively by Pickup and co-workers (Pickup et al., 1990). They reported the first direct measurements of the self-diffusion coefficients of SPPS coupling reagent by the NMR pulsed-gradient spin-echo method. Their results showed that the time required for self-diffusion was only 0.1-0.2 times of the total coupling reaction. They therefore concluded that the diffusion was not rate-limited. However, they did not distinguish the film-diffusion and intra-particle diffusion. What they called "self-diffusion" is essentially the bulk diffusion and film-diffusion. The intra-particle diffusion is important for the final 10% reaction since there are substantial deviations to slower reaction rates during that period (Merrifield, 1984).

The general phenomenon about DVB percentage on reaction rate is that the resin with lower DVB crosslinkage has higher reaction rate. Pan and Morawetz (1980) explained this phenomenon chemically: the micro-Brownian motion makes the medium uniform and leads to similar reaction rates between chemically similar groups. However, the authors thought that high crosslinking introduces a severe restriction to mobility, and a spatial variation of the

local polarity may cause a variation of rate constant.

The apparent half-times and first-order rate constants for some amino acids in aminolysis of polymeric activated esters were tested by Gut and Davidovich (1976). It was found that the bulky side chains of valine and isoleucine, and the secondary amino group of proline, may cause a reaction slow-down. The retardation effect of aliphatic residues unbranched at Beta-carbon atoms is slow, such as those in leucine and lysine. And the alkyl substituents have the lowest retardation effect. But the fact that alanine deviates from this series may be due to a different course of the reaction curve. In the authors' experimental data, the apparent first order reaction rate constant decreases during the course of the reaction, indicating that the reactions are not truly first order. Unfortunately, the authors did not give any positive answers about reaction kinetic models except for their negative conclusion of first order reaction.

Amino Acid Blocking Group

Ragnarsson et al. (1974) studied the influence of different protecting groups like Boc, Bpoc, Bhoc, and Ppoc on reaction rate. They noticed that the bulky groups can cause a rise in steric hindrance or a reduction of amino acid derivative penetration into the interior of the resin. Christensen et al. (1980) made a comparison of the coupling rate among some Boc-, Bpoc-, and Ddz-amino acid derivatives

activated with DCC in SPPS. Contrary to Ragnarsson's observation, Christensen and co-workers thought that the coupling times are almost identical irrespective of the applied type of amino acid derivative in their three types of tested blocking groups. The only exceptional case in their hexa-peptides was that the Boc-Val coupled apparently slower than Bpoc-Val and Ddz-Val. The Boc-group is the choice for Alpha-amino protection in 95% of all SPPS. The Bpoc-group is very labile and the Ddz-group is very acid labile also. However these two blocking groups have less importance compared with Boc-group in SPPS (Erickson and Merrifield, 1976).

Temperature

Some researchers studied the effect of temperature on coupling efficiency. For example, Tam (1985) found that when temperature increased from room temperature to about 50 ^{o}C, the coupling yield increased about 1.7%. This enhancement of coupling efficiency at elevated temperature by symmetrical anhydride in polar solvent may provide an indirect evidence for the physical nature of the coupling difficulties, such as physical aggregation due to hydrogen bounding, and an useful solution to overcome the refractory non-reactive problem in the coupling reaction. But the elevation of temperature is limited by the boiling point or vapor pressure of the solvent. In Tam's experiment, 1-methyl-2-pyrrolodinone was used instead of DCM in order to

operate the experiment at higher temperature (DCM has a boiling point of 39.8 $^\circ$C at normal atmospheric pressure).

Peptide Fragment

Live and Kent (1983) examined a number of peptide resins in DCM and DMF and found that in general the attached peptide chains show freedom of motion at a rate comparable to those for free molecules in solution, implying good solvation of the peptide. Some difficulties, formerly attributed to physical inadequacies of the resin, have been shown to have their origins in chemical side reactions. This increases the attention about the role of the anchored peptide in reactivity and kinetics. Live and Kent indicated that synthesized peptides have an aggregation tendency in a poor solvent. This mechanism suggested a straightforward remedy to the difficulty to employ a solvent such as DMF which can accept hydrogen bonds and displace the peptide-peptide interaction.

Amino Acid

In terms of the effect of amino acid type on coupling rate, Ragnarsson et al. (1971) noticed, as some other researchers did, the differences in reactivity for different amino acids. They assumed these differences were due to steric hinderance. Glycine had the highest reactivity of the 16 amino acids used, Ile and Val had the lowest reactivity and are known to be sterically hindered. The

authors also tried to find the effect of different amino
acid-resin combinations by using Val-resin and Ala-resin.
Unfortunately, with their large experiment error, they could
not see much difference between these amino-acyls.

The size of the side chain can strongly influence the
coupling reaction rate, Gut (1973). He chose two model
penta-peptides for experiments. The two were identical
except for the amino-acid resin ester. When reaction half-
times were compared he noticed that the carboxyl terminal
amino acid affects reactivity of the adjacent amino group.
When Val was attached to Phe-resin, its reaction half-time
was longer than that to Gly-resin. Although his
experimental data was limited, this half-time observation
indicates a marked steric influence which is much more
obvious than when using the solution method.

Morawetz (1979) studied the influence of the chain
backbone on side chain reactivity and found that some
factors might lead to a significant difference between
reaction rates of functional groups attached to polymer
chains and analogous low molecular weight reagents. One
factor was the energetic interactions between a polymer and
a low molecular weight reagent which might either
concentrate or deplete their reaction rates with functional
groups appended to the polymer. Another factor was the
polymer backbone. This backbone made a contribution of
solubility to the effective solvent medium in its immediate
neighborhood. If this is true, then the reaction rate will

be sensitive to solubility effects. The polymeric reagent will then behave differently from the low molecular weight analog. The third factor was a group with a bulky reagent. If a group attached to a polymer is to react with a bulky reagent, there will be a steric restraint due to the chain backbone.

Side Reaction

Although many researchers worry about the completion of reaction and the purity of final polypeptide, the product purity in SPPS is usually quite satisfactory. Some evaluation work about the purity was done by Tregear (1974). The author used two similar model peptides which were identical except for one mid-way attachment. With a thin-layer chromatography, the author found incomplete deprotection of the Boc-glutamine occurred in varying degrees at one step in one peptide while not detected in another peptide. This finding may be related to the sequence difference in the position immediately prior to the glutamine residue. In the first sequence, the previous amino acid was phenylalanine; in the second, it was leucine. Generally, peptides showed 95% purity by sequence analysis. This result shows that it is indeed possible to synthesize and isolate peptides of good purity within the 30-35 amino-acid range. It is also apparent that a satisfactory sequence analysis is an essential criterion in establishing the homogeneity of the synthetic peptide. Of course, more

effort should be made to identify the side-products formed during a solid phase synthesis so that appropriate steps can be taken to avoid their formation and increase the yield of the desired sequence.

The formation of peptides with one or more internal amino acids missing is perhaps the most serious practical shortcoming of SPPS for the synthesis of long peptides. The major reason deletion peptides are produced is due to a chemical side reaction. The side reaction is believed to be caused by aldehyde functionalities on the resin support forming a Schiff's base with the free amino group of the growing peptide chain, which resists acylation under normal condition. This chemical mechanism was proved by Kent (1983). He used quantitative ninhydrin monitoring, quantitative Edman degradation and direct chromatographic analysis methods to evaluate the amount of deletion peptides formed. It seems that if resin containing minimal amounts of aldehydes is used, a long peptide chains can be routinely assembled by the stepwise solid phase method with minimal levels of deletions. Mitchell et al. (1978) found that aminomethyl-resin prepared using a strong acid Friedel-Crafts catalyst was quite satisfactory.

Kinetics of First Attachment

Resins with anchored first amino acids are now commercial products. The method for this first attachment is different from subsequent attachments. The kinetics may

also differ. Essentially the resin is reacted with some salt (i.e. cesium salt) of the amino acid and finished in one step. Raymond and Scott (1987) proposed a mechanism for this reaction. They assumed that the amino acid salt in the liquid phase first goes reversibly to the gel phase and then forms a covalent-bound with amino acid in the gel phase. The sequence is mass transfer followed by chemical reaction. The authors modeled the reactions as a set of non-linear first order ordinary differential equations. Instead of using a steady state approximation, they solved the equations by a numerical method (fifth order Runge-Kutta) and obtained the rate constants. The final results indicated a second order, irreversible gel phase reaction. They believed that there was some maximum point for amino acid concentration above which the amino acid provides no increase in yield. Although the reaction rate increased with increasing temperature, the amino acid might begin to degrade with long coupling times at high temperature. When they used their kinetic data to fit their pseudo first-order rate model, they could not get consistent results. This was explained by mass transfer limitations before the amino acid reaches the active chloromethyl sites within the gel phase.

Solid Support in SPPS

The polymer support (resin) is very important in SPPS. Most of the work in this area involves a crosslinked polymer to which appropriate functional groups are attached,

allowing easy separation of the polymer from an excess of
the reagent in the solution phase and a simple isolation of
the reaction product. Yet, in spite of its many successes,
polypeptide synthesis on cross-linked polymeric supports
suffers from a serious problem. Since the intermediates
during the build up of the polypeptide chain are not
isolated and purified, each reaction step must go to
completion if the final product is not to be contaminated by
deletion polypeptides. In a synthesis of a polypeptide with
40 amino acid residues, a conversion of 99% in each step of
the synthesis would lead to 55% of polypeptides having the
wrong amino acid sequence.

Many factors can effect the completion of reaction.
Different blocking groups and different crosslinkages for
polymer supports are known to be important. In order to
study the properties of the polymer support, Pan and
Morawetz (1980) analyzed some acyl-aromatic amine residues
in linear and cross-linked polymers by acetic and butyric
anhydrides. It was found that the polymers crosslinked
differently such as with ethylene dimethacrylate,
hexamethylene, divinylbenzene, methylene bisacyrylamide,
ethylene bisacrylamide, have different first order rate
constants. This indicates that one cross-linker may be
superior over the others. In contrast, Pan and Morawetz
thought that the reaction conversion was remarkably
insensitive to the cross-link density in the gel. According
to their observation, methyl acrylate-aminostyrene

copolymers containing 1 and 15 wt.% of a cross-linking agent
had a very similar conversion. The effect of steric
hindrance then should be excluded. The authors also
mentioned the difference in the kinetic behavior of the
reactive residues attached to linear and cross-linked
polymers. The polymer with no cross-linkage followed first
order behavior up to a conversion of 99%, while those for
cross-linked gels deviated significantly from first order
reaction at a conversion of about 80%, and the final stages
of the conversion were extremely slow. Their observations
are further proof of the superiority of lower crosslinkage.

Despite the advantages of resins with low crosslinkage,
there may be increased handling difficulty because the
strength of resin decreases with the decreasing crosslink
percentage. Considering this dual character, the resin
support used is quite often a polystyrene suspension polymer
cross-linked with 1% DVB. A cross-linkage of 2% was used in
the earlier experiments, whereas 0.5% cross-linked beads
were found to be too frail in an ordinary apparatus (Barany
et al., 1987).

Unfavorable polymer effects due to interaction between
the crosslinked support and the attached peptide chain have
been considered as the source of chain-length dependent
coupling problem in SPPS. However, Mutter et al. (1985)
thought that conformational transitions of the growing
peptide chain might cause a dramatic decrease in the
reactivity, and this is revealed to be a general source of

trouble. Based on their opinion, the conformational transitions might be the origin of both incomplete coupling reaction and inefficient analytical control. In light of their results, the polymer-support used in SPPS must exhibit maximum swelling in solvents while guaranteeing optimum solvation of the peptide chain at the same time. Consequently, a major step forward in overcoming unfavorable conformational effects in SPPS may be to change from truly solid phase condition towards some kind of "liquid-phase" condition. This may result in some investigation for some crosslinked polymers with an infinite degree of swelling, i.e. the number of crosslinks approaching zero.

Many kinds of polymers can be used as the solid support in SPPS. The earliest and the most broadly used is copolymer of styrene with varying degrees of crosslinked divinylbenzene, which were first used by Merrifield in 1963. The degree of DVB crosslinkage will determine the extent of swelling, the effective pore size and the mechanical stability of the beads. These properties, in turn, will determine the suitability of the supports for SPPS. In the early Merrifield's method (1963), it was thought that 1% cross-linked beads were too fragile and became disrupted during the synthesis making filtration difficult. On the other hand, the high cross-linkage was too rigid to permit easy penetration of reagents, causing slower and less complete reactions. Therefore, Merrifield chose 2% cross-linked resin for his synthesis. With the development of

improved resin production techniques, 1% cross-linked beads
are now suitable, and have been used for most of the cases.
Previous lab work at OSU (Chen and Foutch, 1989b) showed
that the reaction rates with 1% cross-linked beads was
higher than those with 2% cross-linking.

There have been contradictory views about the nature of
polymer-supported reactions. Some investigators suggested
that the cross-linked polystyrene is relatively rigid in
nature and that reactive moieties on the polymer support
remain isolated from one another (Regen and Lee, 1974).
This leads to a concept of site isolation where a specific
reactive site on a polymeric support remains completely
inert toward inter-molecular reactions. In contrast, others
suggested that solvent-swollen cross-linked polystyrene
chains have significant molecular mobility (Rebek and Trend,
1979). This internal flexibility of chains should
facilitate the inter-site reaction between peptide chains
throughout a swollen polymer gel. Sarin et al. (1983)
believed that under the condition normally employed in SPPS,
potential inter-site reactions between different peptide
chains in the swollen gel should occur with easy and should
be driven to completion if there was no competing reaction.
Their opinion was consistent with that of Rebek's, and
stated the advantage of good swollen solid support. Sarin
et al. (1980) tested the volumes of swollen peptide-resins
in DCM and DMF during the course of synthesis. Based on
their experimental data, it was clear that the swelling

properties of the peptide-resin were influenced by each of the components in the system. During the course of peptide synthesis on a cross-linked polystyrene support, the solvation was initially a property of the polymer that made up the resin. While at the end of a long synthesis it was strongly influenced by the protected peptide, which would be more highly solvated than if it was not covalently attached to a loosely cross-linked polymer network. Sarin and co-workers observed that the DCM was a better swelling solvent than DMF for polystyrene, whereas DMF was a better solvating medium for protected peptide. Also, they concluded that the initial volume of the swollen un-substituted resin bead was not the final volume of the swollen highly loaded peptide-resin bead, which could be much larger and could contain substantially more solvent. In their experiment, no maximum swollen volume had been reached, and the space potentially available for peptide chain growth within the swollen beads actually increased rather than being gradually filled by peptide. Their observations can be used to discuss the role of intra-particle diffusion. Based on the same consideration as Sarin's, Kent and Merrifield (1980) concluded that many of the problems leading to poor synthesis were due to simple organic chemical side reactions rather than intra-particle steric hindering. They thought that the resin actually helps reduce the synthetic difficulties encountered with peptides which tend to self-aggregate. They emphasized the role of crosslinked resin

supports as enhancing solvation and reactivity. The authors
tested five different resin supports used in SPPS, Bzl(s-
DVB), Pam(S-DVB), Pam(Kel-F-g-styrene), Pam(polyarylamide)$_w$
and Pam(polyacrylamide)$_g$. After comparing the reaction
completion of these resins, they found that the four
crosslinked resins all gave the same average levels of
deletion peptide formation. The best resin among them was
Pam(s-DVB), which gave the best coupling synthesis. The
non-crosslinked Pam(Kel-F-g-styrene) resin was significantly
poorer than the other syntheses. These data showed that
both polystyrene and polyacrylamide crosslinked resins
worked well, and were superior to non-crosslinked resins.

Although the most common solid support used in SPPS is
Boc-polystyrene-co-divinylbenzene, this resin is not
completely stable under the acidic conditions required to
remove the Boc group. Acidolysis of the benzyl ester is
undesirable during stepwise assembly of the peptide because
the yield of crude peptide obtained at the end of the
synthesis is thereby decreased. In addition, the production
of new hydroxymethyl sites due to premature release of
peptide chains from the resin can result in late initiation
of peptides and formation of deletion peptides lacking one
or more residues at the carboxyl terminus. In order to
overcome these drawbacks, many researchers have tried new
resins (Mitchell et al. 1976; Goddard et al., 1988; Mergler
et al., 1988; Calas et al., 1984; Findeis and Kaiser, 1989).
As a summary of this section, polymer support plays an

important role in SPPS. Many polymers have proved suitable for polypeptide synthesis. However, the most common used commercial resin still is Boc-polystyrene-co-DVB.

Chain Length Effect

One of the limitations of SPPS is the possibility of creating failure and truncated sequences which cannot be separated or even analytically distinguished from the desired sequence. Theoretically the number of failure peptides increases with increasing chain length. Only 100% yield in every coupling step can prevent the formation of these undesired sequences. The question then is, how to drive every coupling step close to complete, and what factors cause incomplete and/or side reaction? Bayer et al. (1970) had a general feeling that there must be resin-imposed steric limitations to stepwise SPPS. Their experiments showed that peptide sequences such as Arg-His, Arg-Arg, or Arg-Lys, had much lower yields and more failure sequences.

Efficiency is another debatable point. Some workers feel that reactions will be less efficient close to the polymer backbone, while others feel that there will be significant declines in yield as the peptide is elongated, due to temporary steric occupation of peptide chains within the polymer network. Some workers (i.e. Sarin et al., 1984) even do not suggest the correlation between efficiency and chain length.

Sarin et al. (1984) investigated the relationship of chain length to synthetic efficiency. They designed a tetra-peptide model and found no significant effect of chain length on the synthetic efficiency. They discovered that there was no spatial limitation to the growth of a peptide chain in lightly cross-linked polystyrene resin beads with a very high content of peptide. Based on their opinion, the space available for the growing peptide chain on the highly loaded, swollen peptide-resin was larger than the initial volume of the swollen un-substituted resin bead. The favorable solvation and availability of sufficient space for the growing peptide chains was used to explain the high synthetic efficiency observed in their study. With the observation of high synthetic efficiency, even up to 60 residues, they claimed a lack of intrinsic limitations to stepwise solid-phase synthesis over an extreme range of peptide loading. The poor synthetic results obtained in certain instances are believed by some workers to have chemical rather than resin-related physical explanations. But, the authors did not analyze the composition of their products. If the products were composed of desired sequence, failure sequence and truncated sequence, the reaction would hardly be called "highly efficient" because the definition must be with respect to the desired product.

Hooper et al. (1985) tried another monitoring technique for completeness of a SPPS reaction. The principle was based on pKa changing with increasing chain length. In

amino terminus deprotection and condensation with the next amino acid, reactions usually proceed by nucleophilic acyl substitution where the amino terminus acts as a nucleophile. Reaction rates are directly proportional to amino terminus nucleophilicity. Electron release from the peptide to the amino terminus determines the amino terminus nucleophilicity. Measuring the amino terminus pKa indicates both electron density and reactivity. Noticing this principle, Hooper and co-workers (1985) set up a model peptide and measured the amino terminus pKa value at every attachment. They found that as peptide chain length increased from 1 to 4 amino acids, pKa went from less than 9.9 to 11.37 in DCM, a pKa increase of at least 1.47. This change showed nucleophilicity increasing, therefore, the amino terminus pKa might be lower with incomplete coupling.

Most model peptides used for testing chain length effect have been short. However, SPPS is a complicated reaction and sequence-dependent incomplete coupling may not be just in one direction. Based on this hypothesis, Meister and Kent (1983) made several longer peptides. They monitored the completeness of each attachment. Their data showed that the coupling of sterically hindered amino acids, such as Thr(Bzl), Ile and Val, were more often incomplete no matter what position they were in the peptide sequence. They were unable to correlate incomplete coupling with known properties of the N-terminal amino acids involved. The major point in Meister and Kent's work was a pronounced

maximum tendency to couple incompletely around 15 residues long. This tendency dropped off rapidly after that. No incomplete reactions were observed for couplings to a chain of 21 or more residues in length. They interpreted this as the ability of some resin-bound peptides to form intermolecular hydrogen-bonded aggregates in poor solvents since they could not find the same phenomenon when the peptide was synthesized in DMF, a much better polar and hydrogen-bonding solvent. This intermolecular aggregation on chain length might have maximum tendency at about 15 residues. In order to overcome this problem, they suggested an adequate feedback control of the synthesis.

Testing Methods

A coupling time of 2 to 4 hours and even longer is normally used in SPPS. By monitoring the coupling reaction with a suitable test, it is possible to determine when acylation is complete. The time for the synthesis can thus be minimized.

There are many methods to test the completeness of coupling in SPPS. Gut and Rudinger (1968) mentioned that early researchers usually demonstrated the purity of a sample by a careful comparison of a host of its physical, chemical and biological properties with those of the natural material. It seems, however, that the day has come to synthesize large synthetic polypeptides. We can no longer use natural material as a crutch to demonstrate or measure

their purity. Najjar and Merrifield (1966) gave a method for quantitative determination of completeness of solid phase peptide coupling reactions. This involves sacrifice of a small sample of the resin-peptide for hydrolysis and subsequent amino acid analysis. This method requires a considerable amount of time. Gut and Rudinger (1968) gave another quantitative measurement method which involved a spectroscopic measurement of a soluble product formed in the reaction, e.g. measurement of p-nitrophenol formed in p-nitrophenyl ester coupling. A serious drawback of their method is that it is obviously limited to active ester coupling and even further limited to active esters containing chromophoric leaving groups. In 1969, Dorman (1969) proposed a non-destructive method for the determination of completeness of coupling reactions. In his method, the resin-peptide was treated with pyridine hydrochloride to form a hydrochloride of any uncoupled resin amine, followed by combination with dilute nitric acid. Finally the equivalents of required nitrate represented the amount of uncoupled resin amine. The success of this method rested on the ability of pyridine hydrochloride to form the hydrochloride of uncoupled resin amine quantitatively without causing premature removal of Boc protective groups on the coupled amino acid. Also, the o-nitrophenylsulfenyl (NPS) protective group was cleaved at a substantial rate by the reagent, thereby precluding the use of this pyridine hydrochloride solution with the NPS protective group.

Another method of detecting reaction completion is the
picrylsulfonic acid (2,4,6-trinitrobenzene sulfonic acid)
test, which was developed by Hancock and Battersby (1975),
and further tested qualitatively by Stahl et al. (1979).
This micro-test provides a rapid, simple method which has a
high sensitivity to uncoupled amino groups on the resin, and
does not react with by-products from the coupling reaction.
The method can detect as low as 3 nmol of free amino groups
per milligram of resin. The basic principle is that the
reagent, picrylsulfonic acid, can react rapidly with the
amino resin to form an orange-red trinitrophenlated
derivative. A very positive test (little coupling) is
indicated by a bright red to a faint yellow color for almost
complete coupling. This method has been used extensively by
many researchers because of its advantages: rapid, easily
distinguished, and insensitive to by-products.

Christensen (1979) provided a qualitative test for
monitoring coupling completeness in SPPS by using chloranil
(2,3,5,6-Tetrachloro-1,4-benzoquinone). If free amino
groups are present, a green to blue color would develop on
resin beads in less than five minutes in this method. The
sensitivity for detection is quite high. However, when
histidine was present, the result might be blurred. This
method can be used when the N-terminal acid residue is
proline which is not detectable by another common used
method, the ninhydrin test (Kaiser et al., 1970). Although
the ninhydrin test have been widely used as a convenient

guide to the progress of a coupling reaction, it is only semi-quantitative because the amount of amine is estimated visually from the intensity of a blue color found in the solution and on the resin beads. Sarin et al. (1981) used this test for their Pam-resin support and observed that the color was present in solution while the beads were colorless. They proposed a mechanism for the side reaction leading to the previously observed blue beads and found a way to overcome it. They called their work the quantitative ninhydrin monitoring method. Their method can be used for measuring either the total number of growing peptide chains on the solid support or the number of un-reacted amino groups remaining after the coupling reaction is completed. This method still suffers as a synthetic monitoring tool because of the length of time required to complete the test due to drying and accurately weighing the resin peptide samples, the need for a highly skilled technician to obtain reproducible results, and a lack of sensitivity for completeness of deprotection. In order to overcome these, Kalbag et al. (1985) provided another testing method by which both deprotection and coupling in SPPS can be quantitatively monitored. The basic principle of this method is to utilize the heat lability of Boc protecting groups. This group can yield isobutene and carbon dioxide on pyrolysis. In order to facilitate the quantification of their method, the authors developed a special solid phase support which contained an isopropyl ester moiety as an

internal standard. When this resin was pyrolysed with an attached Boc-amino acid residue, a mixture of propene and isobutene was produced. The amount of propene and isobutene can be quantitatively compared by some instrument, such as gas chromatography. The efficiency of the coupling reaction as well as deprotection can thus be indicated.

As a summary, every testing method in SPPS has its own advantages and disadvantages. People should choose a proper method based on their product requirements, facility availability, reaction property and other considerations.

Synthetic Methods

The standard procedure for SPPS was described by Stewart and Young in great detail (Stewart and Young, 1984). Washing, deprotection, neutralization, coupling and other steps were all discussed. But, since SPPS involves complex chemistry and chemical synthesis, special attention must be paid to individual synthesis. Corley et al. (1972) provided a modification of the Merrifield solid phase method for the synthesis of Bradykinin. They shortened the period of coupling and deprotection. Also, a methanol wash, which caused dramatic shrinking of the resin support, was inserted. Supposedly this methanol wash could decrease contamination by residual solvent and dissolve reagent from the previous step. In this early study, the authors still used amino acid and DCC for coupling other than amino symmetrical anhydrides. Hodges and Merrifield (1974)

constructed an instrumental method which automatically monitored the progress of SPPS. Their work could be one of the earliest studies of on-line SPPS. The UV instrument they used could measure total peptide chains and the number of un-reacted peptide chains after the coupling reaction. Yamashiro et al. (1976) added 1,2,2-trifluoroethanol (TFE) to the coupling reaction so that the coupling proceeded in 20 % TFE in DCM. They found that the average coupling efficiency for their model peptide was increased to 99.7% instead of 98.3% for non-TFE. They explained this favorable effect of TFE on coupling by their visual observation that 20% TFE in DCM swelled the peptide-resin to approximately twice the volume of peptide-resin in DCM along. But the use of TFE could bring additional purification problems and side-reactions. The authors did not discuss this possibility.

Yamashiro and Li (1978) synthesized an ovine Beta-lipotropin with a linear structure of 91 amino acid residues by solid phase method. The final product was found to be indistinguishable from the natural hormone. With this long chain product, they paid special attention to the side chain protection group for individual amino acids The side chain of tryptophan was protected with a formal group in view of its susceptibility to destruction under conditions of repeated acidolysis of Boc groups. For histidine, a benzyloxycarbonyl group was used. While for threonine, serine and glutamic acid, very stable p-halo-benzyl

protecting groups were used through residue 66 with benzyl protection thereafter. Their work further proved the importance of chemistry in SPPS.

Industrial-scale SPPS requires large amounts of expansive reagents and solvents. Realized this problem, Patchornik (1987) developed a method in which the reagent was bound to the polymer. This was realized by binding cross-linked polystyrene to good leaving groups such as nitrophenol. The protected amino acid residue was then attached to this leaving group and allowed an unprotected amino terminus of a soluble amino acid to react with that protected residue. This synthesis differed from Merrifield's in that the growing peptide was in solution phase. Then the polymer could be regenerated and re-used. The reagent was also recyclable. But the peptide yields were typically lower than that of classical SPPS. Other limitations were the difficulty in automation of the synthesis and the additional cost in preparation of insoluble polymeric acyl-transfer reagents.

Merrifield (1986) predicted new improvements in SPPS. One prediction was that the insertion of an acetamidomethyl group between the benzyl ester and the polystyrene matrix would increase the stability of the benzyl ester to trifluoroacetic acid by a factor of 25 to 400. When such a linkage was finally constructed, it would be more stable. Another prediction was an alternative protecting group strategy which made use of an orthogonal system in which the

N, C and the side-chain groups represented three different classes of compounds that would be cleaved by three different reactions. In this way, any one of the functional groups can be removed selectively in the presence of the other two. The yield of desired product can thus be increased.

Classical SPPS is usually carried out at low initial matrix loading, typically 0.1-0.3 mmole of peptide per gram of support. The possibility of using the maximum possible matrix loading routinely is attractive. This would lead to a synthesis of much larger amounts of peptide using a given amount of support. It would also lead to improved sensitivity in the monitoring of reactions in the solid phase, enhanced coupling rates during peptide bond formation, and economies in the use of reagents and washing solvents. Epton et al. (1987) developed a novel approach for these purposes. Their approach utilized a phenolic bead-from core network at an initial matrix loading of 5 mmole per gram. With ultra-high load solid phase synthesis of peptides, much larger amounts of peptide might be synthesized in a given reaction volume. The increased peptide concentration in all coupling steps meant that there was less need to use large excesses of coupling reagents. There was also considerable economy in the use of washing solvents. But the ultra-high load gel networks need careful handing to avoid shearing and fracture. Also the solvent swelling properties are less predicable. This new approach

has to be tested for the synthesis of large polypeptides.

Large Scale Production

Experience over the past decades has shown that the Merrifield method can be successfully scaled up. (Groginsky, 1986; Meienhofer, 1984). Currently, production runs up to 500 grams of resin are fairly common. The major concerns in the design of large scale synthesizers and reactors may include instrument design factors, process factors and chemistry factors. Most commercial instruments have incorporated much more sophisticated electronic programs but have been designed for discontinuous process with the same chemistry. In some cases a continuous process has been developed successfully (Jonczyk and Meienhofer, 1983; Frank and Doring, 1988; Cameron et al., 1987; Sheppard, 1983; Dryland and Sheppard, 1986; Kent and Hood, 1985). These researchers gave direction for the design and development of large-scale SPPS although most of their work was laboratory-scale. Edelstein et al. (1986) described a pilot-scale SPPS reactor capable of obtaining 100 grams purified peptide per synthesis. Their multi-reactor system could make four different peptides simultaneously. In their design, they emphasized the highly versatile automated system. They noticed that the identifying physico-chemical parameters between laboratory-scale and large-scale, such as shear requirements, flow patterns and reaction kinetic requirements, were important to the development of solid

phase reactors. Also, it should be realized that no system for peptide synthesis is really practicable without highly advanced separation equipment. Hundreds of equilibrium stages are necessary for the partitioning of inevitably significant fractions of impurities which accompany any method of peptide synthesis.

Edelstein et al. (1989) summarized some scale-up developments in SPPS. They predicted that peptide synthesis scale-up was entering a period of intense activity. Many pharmaceutical companies bring peptide products out of discovery phase into development. Currently, a scale of 100-500 grams per synthesis has been reported. But the commercialization of the equipment needed for large-scale, most-effective and reliable synthesis is still at a primitive stage. The cost of equipment is very high and the methods are logistically difficult. These give engineers a challenging task.

Currently, large-scale SPPS is either done manually, lessening financial benefits and reliability, or performed on custom equipment other than commercial facilities. In order to develop a successful automated process-scale solid phase synthesizer, expertise in mechanical engineering, chemical engineering, electronics and process control must be blended with in-depth understanding of SPPS and process automation principles. The key questions here, and the ones toward which very little effort has been expanded, are the kinetics of the main chemical reactions and the optimal

design of a large-scale facility (Kg/batch) based on suitable kinetic data.

Previous OSU Research

The kinetic study of SPPS has continued for several years at OSU. The earliest work concerned constructing and testing an experimental apparatus. (Dietrich, 1987). The original design used pre-saturated nitrogen for mixing. Poly-phenylalanine was used as the test peptide. The reaction temperature was fixed. The amino acids with a compatible ultraviolet spectra were measured using a flow cell and a spectrophotometer. The kinetic data were analyzed by plotting a logarithmic curve of reaction rate vs. amino terminus concentration. The purpose of doing this was to find the apparent reaction order. But the data contained significant scatter from the rate plot since a small bump could cause a large slope change. The data obtained showed that the reaction order changed during the course of reaction. The lower order behavior was observed at high amino terminus concentration, while higher order was observed when concentration decreased.

Another OSU kinetic work (Chen, 1988) utilized the apparatus described above but used mechanical stirring instead of nitrogen bubbling for mixing. The modal peptides were poly-serine and poly-phenylalanine, occasionally with Bradykinin. The resin used was polystyrene with 2% cross-linked DVB, occasionally with 1% DVB. Some factors, such as

temperature, chain length, excess mole ratio, mixing rate and percentage of DVB, to reaction rate were discussed. A simple zero-first shifting order model was proposed. But the reaction mechanism was unclear. Also the major work was limited to some poly-amino acid sequences which are less attractive than real hormones. Finally, the effect of temperature was limited to two temperatures. This, of course, is not enough to get a quantitative conclusion. However, these works lead to a starting point for the kinetic study presented in this thesis.

Babbrah (1990) created a mass balance model within the solid particle. The reaction concentration was predicted by this model. Intra-particle diffusion and chemical reaction were both considered. Some deviation was observed between the numerical solution from this theoretical model and the experimental result from Chen's data, indicating that the diffusion might be important in this process. The diffusion phenomenon will be further discussed in Chapter V of this thesis.

CHAPTER III

MATERIALS AND METHODS

Experimental Materials

Eight model hormones were synthesized by SPPS during this study. These hormones are: Bradykinin, $[Tyr_8]$-Bradykinin, $[Tyr]$-Bradykinin, Angiotensin III, $[Val_4]$-Angiotensin III, Alpha-Neoendorphin, Beta-Neoendorphin, and $[Arg_8]$-Alpha-Neoendorphin. All these hormones are relatively short peptides, with chain length of 7 to 10 amino acids. Bradykinin has been synthesized and discussed by other researchers (Chen, 1988; Corley et al., 1972); the rest have not been discussed broadly. Eleven amino acids are involved in coupling these peptides. These amino acids are: Arginine, Glycine, Histidine, Isoleucine, Leucine, Proline, Serine, Lysine, Phenylalanine, Tyrosine, and Valine. The N-terminal blocking group for all of these amino acids is tert-butyl-oxycarbonyl (Boc). While the C-terminal (side chain functional group) blocking groups vary for different amino acids. The Boc group is a labile protecting group, while the side chain blocking groups are much more stable. The synthesis depends on the differential sensitivity of these two classes of protecting groups to acid, which should be greater than 1000:1 generally. The

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Boc group is completely removed by some dilute solutions of strong acids, with minimal loss of the anchoring bond or of the other protecting groups. Particularly, the amino acids with blocking groups used in this study are: Boc-N-tosyl-L-Arginine, Boc-L-Glycine, Boc-N-im-tosyl-L-Histidine, Boc-L-Isoleucine, Boc-L-Leucine, Boc-L-Proline, Boc-o-benzyl-L-Serine, Boc-N-(2-chloro-CBZ)-L-Lysine, Boc-L-Phenylalanine, Boc-o-benzyl-L-Tyrosine, and Boc-L-Valine. All these chemicals are obtained from SIGMA Chemical Co. The histidine with this form can result in a final deblocking problem for the hormones, but will not affect this study since the final deblocking is not performed in this project.

Another problem amino acid is arginine. The symmetrical anhydride coupling reaction can not be used for Boc-Arg(tos) due to undesired insertion reactions (Stewart and Young, 1984). That is, more than one arginine may be added to the peptide in a single coupling step. Therefore, if arginine is located at the end of a peptide, it is simply omitted. Otherwise, some other amino acids will be used instead. Fortunately, the resin with the first blocked amino acid attached is a commercial product. Some properties of arginine can still be studied when it functions as the anchored amino terminus. It should be noticed that all the amino acids used are optically pure L-amino acid derivatives. The most satisfactory and broadly used solid phase support is a gel prepared by suspension copolymerization of styrene with 1 percent m-divinylbenzene

as a cross-linking agent. Polystyrene with the first amino acid anchored is used throughout this study. The resulting spherical beads have 40-70 μm in diameter, with an average of 50 μm. This geometrical size is observed by electron microscopy in this project. In organic solvents, such as dichlomethane, the resin beads swell to five or six times their original dry volume. The resin is markedly hydrophobic, while the peptide chain is fundamentally hydrophilic. The amino acid resin esters used in this study are: Boc-N-tosyl-L-Arginine resin, Boc-L-Proline resin, Boc-L-Phenylalanine resin, and Boc-N-(2-chloro-CBZ)-L-Lysine resin. These resins are obtained from SIGMA Co.

The solvent used in this study is analytical reagent grade DCM. This solvent swells the resin effectively but does not solvate the peptide chains very well. In such a solvent, the peptide chain might collapse upon itself and no longer be reactive. Another solvent, DMF, can solvate peptide chains and also swell polystyrene resin. But it gives some problems during synthesis, because there will be some acyl urea which is not active and represents undesirable loss of activated Boc-amino acid from the coupling reaction when using DCC. The chain collapse is not critical for short chains. DCM is used throughout this study. DMF is used only when there is a solvation problem with DCM, and with special caution during synthesis.

Compared with direct amino acid coupling, symmetrical anhydrides frequently give dramatically increased reaction

rates in difficult SPPS coupling reactions. Another advantage of anhydride coupling is that the peptide chain cannot be terminated by DCC-activation of any residual TFA remaining in the resin at the coupling step. The symmetrical anhydride is obtained by using two equivalents of Boc-amino acid with one equivalent of 1,3-Dicyclohexylcarbodiimide (DCC) which is supplied by Aldrich Chemical Company.

The deprotection of amino acid terminal is accomplished by using reagent grade trifluroacetic acid (TFA) provided by Fisher Scientific. Impurities in TFA can be harmful in peptide synthesis. A scavenger in the TFA reagent is recommended to protect the reactive groups on the amino acid from any harmful substance and also prevent any oxidative effect of the TFA on the peptide. The scavenger used in this study is indole from Eastman Kodak Company.

Neutralization of peptide-resins is achieved by treatment with reagent grade triethylamine (TEA) from Fisher Scientific. This material should be used fresh. Amine with faint red color means that it is either not fresh or contaminated, and should not be used anymore.

Experimental Strategy

SPPS begins with an insoluble resin, which is functioned with a chloromethyl group. The first amino acid is blocked at one end and at reactive side-chain groups, and anchored to the resin by a stable covalent bond. The end

blocking group is then removed and the second amino acid is added. In a similar way, the subsequent units are combined stepwise until the entire peptide has been assembled. The detailed scheme is given by Stewart and Young (1984). The first attachment and the final deblocking are not studied in this project.

The synthesis scheme is shown in Figure 1. In this strategy, three major chemical reactions are involved. The first reaction is deblocking Boc by using dilute solution of TFA in DCM. Some carbon dioxide is liberated during this deprotection step. In the laboratory device, this small quantity of carbon dioxide should not interfere. But for large batches of resin, the vessel must be vented after most of the carbon dioxide has been evolved. Another by-product is isobutylene which is soluble in DCM and can be washed out. The second reaction involves the neutralization of the deblocked end by a dilute solution of TEA in DCM. When acidolysis is used to deprotect peptides, the newly formed amino group is left as a salt of the deprotecting acid. This salt must be neutralized to yield a free amino group. The coupling reaction is then ready to carry out. Between these three major chemical reactions, there is a series of washing steps by DCM to remove soluble excess reactants and impurities.

Experimental Procedures

The detailed procedure for the whole experiment is

```
              R'
              |
Resin-COO-CH-NH-Boc

              |
              |   TFA (Deprotection)
              ↓

              R'
              |
Resin-COO-CH-NH₃  ⁺

              |
              |   TEA (Neutralization)
              ↓

              R'
              |
Resin-COO-CH-NH₂

              |              R"
              |              |
              |   DCC + HOOC-CH-NH-Boc   (Coupling)
              ↓

              R'            R"
              |             |
Resin-COO-CH-NH-CO-CH-NH-Boc   (Dipeptide)
```

Figure 1.   A Simplified Scheme of SPPS

described as follow. (Adapted from Stewart and Young (1984),
Dietrich (1986) and Chen (1988).)

Step 1. Symmetrical Anhydride Preparation

The basic chemical reaction in symmetrical anhydride
preparation is

2 Amino Acid + 1 DCC = 1 Symmetrical Anhydride + 1 DCU

DCU is a precipitate. In order to prevent the deposition of
this precipitate inside the reactor, the symmetrical
anhydride is prepared outside the reactor. DCC is first
weighed and dissolved into an exactly measured amount of DCM
to get a DCC solution in standard concentration. The amount
of amino acid is based on the requirement of experimental
mole ratio. In this study, a mole ratio of symmetrical
anhydride to reactive amino terminal on the resin is 1.5:1.
One mole symmetrical anhydride needs two moles of amino acid
based on stoichiometrical relation. The desired amount of
amino acid is weighed in a small vial. A certain quantity
of DCM is added into the vial. With careful shaking, the
amino acid will be totally dissolved in DCM to get a
transparent solution. Then a stoichiometric amount of DCC
is added into this solution. The measurement of DCC should
be as accurate as possible for improving UV absorption. The
vial then is left in a freezer for at least one hour with
occasionally shaking.

Step 2. Resin Preparation

The resin is weighed based on concentration
requirements of the experiment. The mole quantity of amino

terminus in unit weight of resin is labeled by manufacturer. The weighed resin is added to the reactor. By adding a certain amount of DCM, this resin is allowed to swell in the DCM for at least 5 minutes. Usually, the volume of DCM added is based on a rule of one gram resin roughly with 15 ml DCM. This rule of thumb is also used throughout in following steps, except for coupling which requires exact volume based on the requirements of the experiment concentration.

Step 3. Prewashing

The swollen resin is washed by DCM three times. Each washing takes about 2 minutes. Occasional stirring is required in order to get complete mixing and efficient washing. The DCM is then completely drained by a mini-pump after every washing.

Step 4. Deprotection

Deprotection is accomplished by a solution of TFA in DCM. The volume ratio of TFA to DCM is 1:3. This solution is prepared in advance. A small quantity of indole (about 1 mg/ml) is added to the solution. The reagent is allowed to stand overnight before use. It will gradually become purple because of indole. Prior to deprotection, the resin is washed by TFA/DCM reagent for about 2 minutes. The system is totally drained afterward. The reagent is then added to the system again and allow to stay in the reactor for at least 30 minutes with occasionally stirring.

Step 5. DCM Washing 1

After deprotection is finished, DCM is used to wash the entire system. About 4 to 6 washings are needed. Since the indole in the TFA reagent gives the resin a purple color, completely washing can be easily assured by colorless DCM solvent in the reactor. Occasionally stirring is recommended.

Step 6. Neutralization

The neutralization is performed by freshly prepared TEA/DCM solution. The volume ratio of TEA to DCM is 1:9. The neutralization is done two times, each has a length of 2 minutes. Since the entire system at this stage is very water sensitive, it is necessary to place a drying tube on the tope of the reactor (containing calcium chloride) in order to absorb moisture. This drying tube is used in later steps also.

Step 7. DCM Washing 2

The neutralized reactant is washed by DCM 4 to 6 times. Make sure that the resin is suspended in solvent uniformly. Some resins, especially Phe-resin, tend to stick at the bottom of the reactor. Stirring is necessary. Sometimes, back-flush by running the pump in reverse is very helpful to prevent the sticky problem. It has been observed that the non-uniform reaction environment can result in a failed experiment.

Step 8. Instrument Setting

The UV and chart recorder are warmed up in advance. The UV is set to a desired wavelength based on the

individual amino acid requirement. Two quartz cells are filled with DCM and inserted into UV compartments. The absorbance is then zeroed. The disturbances from the DCM and the cell are canceled. The front cell now is ready to fill with symmetrical anhydride solution to get an initial reading. The recorder is set to a suitable chart paper speed. The remote control is set to "on." The pen is adjusted to the zero point on the paper. All these adjustments are performed during the washing period 2 mentioned above.

Step 9. Temperature Control

The temperature control device is turned on and water circulates through the reactor jacket. With lower temperature experiments, ice is added to the controller and the circulator. The temperature is read from thermocouple meter. After stable temperature is reached, the reactor is ready for the coupling step.

Step 10. Initial Reading

The coupling reaction is usually quite fast. The half-life can be reached as quickly as 10 seconds. But the time-lag from the reactor to flow cell located in UV is about 10 seconds. Also, there is a finite reagent dumping period. The initial concentration reading will be lost. Increasing the revolution of the circulating pump can reduce the length of the time lag. But this will bring a problem of scattering the reading on chart paper so as to disturb the accuracy of kinetic information. A practical method is to

set some initial absorbance reading for symmetrical
anhydride solution before the symmetrical anhydride is
dumped into the reactor. The symmetrical anhydride in DCM
with DCU is first taken from the freezer and allowed to warm
to room temperature. The solution is dumped into a
graduated cylinder through Whatman #4 filter paper on which
the DCU precipitate is collected. Now the total reaction
volume of DCM is divided into two parts. One part is put
into reactor and allowed to start circulation. The stirrer
is left on. A small amount of DCM from the other portion is
placed into a vial. The rest is added to the graduated
cylinder which contains filtered symmetrical anhydride
solution. The same volume of DCM as that in the vial is
taken from the graduate cylinder, and added together with
the DCM in the vial. The criterion is that the solution
concentration in the vial equals the initial concentration
when all the solution is in the reactor. For example,
suppose the major reaction volume is 45 ml, then 20 ml DCM
is placed in the reactor and 2.5 ml to the vial. The volume
in the graduated cylinder totals 22.5 ml, which contains
symmetrical anhydride. Next, 2.5 ml of this solution is
taken from graduated cylinder and combined with the DCM in
the vial. The total volume is now 45 ml, and the solution
concentration in the vial is same as the initial
concentration when all the material outside the reactor is
dumped into the reactor at the same time. The front cell in
the UV is filled with the solution from the vial, and the UV

reading indicates the initial absorbance of the reactant.
After this reading, the solution in the vial and the cell
are combined with that in the graduated cylinder. The flow
cell is put into the front UV compartment and the instrument
is zeroed again. All these steps should be done as quickly
as possible in order to reduce the volume lost from
vaporization.

Step 11. Coupling

After making sure that the resin in the reactor is
mixed uniformly (back-flush if necessary), quickly turn on
chart speed control switch, and dump the symmetrical
anhydride solution into the reactor. The on-line UV
absorption curve is recorded by the chart paper
automatically. The success of monitoring relies mainly on
the uniformity of the entire reaction region. The
completion of the reaction is indicated by a horizontal line
on the chart paper.

Step 12. DCM Washing 3

After the coupling reaction is completed, the remaining
liquid in the loop is cleaned by back-flushing the pump.
The total reaction solution is then drained. Followed by
three DCM washes, each has about 2 minutes. The DCM for the
last wash can be left inside the reactor. The peptide-resin
is allowed to suspend in the DCM. The total synthesis for
one amino acid is completed and the system is ready for the
next synthesis.

Step 13. Product Testing

Except for checking the final horizontal line on the chart paper, some other methods are recommended to double check the completion of a coupling reaction. A convenient method is picrylsulfonic acid described by Stahl et al. (1979), which can give good qualitative results, and leading to a quantitative results. The picrylsulfonic acid is obtained from Aldrich Chemical Company. A very dilute solution of picrylsulfonic acid in DMF (about 1% Weight) is prepared freshly. In order to make sure the freshness of the DMF (does not contain any dimethyl amine, the hydrolysis product of the amide), some molecular sieve can be kept inside the DMF bottle. Another reagent which is needed in this test is 10% by volume diisopropylethylamine (from Sigma) in chloroform. A small amount of peptide-resin product (about 2 mg) is placed in a small test tube. Two drops of each reagent are added to the tube. After about 10 minutes at room temperature, 1 ml of ethanol is added. A magnifier (20X) is used to view the resin beads. The color of the resin beads can tell completeness of the reaction. A complete coupling is indicated by a faint yellow color, while little coupling is indicated by a bright red color.

Step 14. Material Balance

After the final coupling for the entire peptide, the peptide-resin is moved into a pre-weighted beaker. This beaker is then placed in a venting hood for overnight; followed by a further drying in an isothermal incubator. The final net weight of the peptide-resin is compared with

ideal weight. The product is kept for further analysis, such as electron microscopy.

Experimental Apparatus

All the coupling reactions are performed in a laboratory-scale water-jacketed glass reactor. Since many peptides adhere tightly to glass surfaces, it is necessary to treat the reactor by silanization before it is first used. The detailed procedure for silanization of glass was described by Stewart and Young (1984). Two reactors are operated in parallel and can be shifted in order to work with one UV detector. Each reactor has a capacity of about 100 ml, with a loading volume of 45 ml. The reactor has two openings at the top. When liquid is going through one opening, the other is also opened for easily dumping. At the bottom of the reactor, there is a filter which can allow liquid to pass through but will stop resin from flowing out. The speed-controlled stirrer is suspended from the top of the reactor, and operated at 200 rpm. This speed can be easily changed by a controller. The reactor is a closed system, thereby preventing solvent evaporation. Temperature control and monitoring are accomplished with a constant temperature bath and thermocouple. The temperature controller provided by Haake can control the temperature accurate to 1 $^{\circ}$C. The thermocouple is inserted inside the wall of the reactor. A multiple-way switch allows an easy connection of individual thermocouple to thermocouple meter.

The sampling liquid is circulated by a mini-pump through a
Teflon tube between the reactor and the flow cell. The flow
rate of sampling can be adjusted by the mini-pump. A three-
way stopcock is inserted in the loop, allowing fluid either
to the circulation loop or to the waste container. The
entire system is operated batchwise, but the monitoring of
the reaction dynamics is continuous. The final scheme is
shown in Figure 2.

Experimental Design

The experimental materials and the experimental
procedures described above are used to produce eight model
peptides. Considering the coupling difficulty of arginine
by symmetrical anhydride method, the final structure of
eight synthesized peptides are as follow.
Bradykinin-

Resin-Arg-Phe-Pro-Ser-Phe-Gly-Pro-Pro

[Tyr$_8$]-Bradykinin-

Resin-Arg-Tyr-Pro-Ser-Phe-Gly-Pro-Pro

[Tyr]-Bradykinin-

Resin-Arg-Phe-Pro-Ser-Phe-Gly-Pro-Pro-Phe-Tyr

Angiotensin III-

Resin-Phe-Pro-His-Ile-Tyr-Val

[Val$_4$]-Angiotensin III-

Resin-Phe-Pro-His-Val-Tyr-Val

Alpha-Neoendorphin-

Resin-Lys-Pro-Tyr-Lys-Phe-Leu-Phe-Gly-Gly-Tyr

Figure 2. The Experimental Set-up of SPPS

Beta-Neoendorphin-

    Resin-Pro-Tyr-Lys-Pro-Leu-Phe-Gly-Gly-Tyr

[Arg$_8$]-Alpha-Neoendorphin-

    Resin-Arg-Lys-Phe-Leu-Phe-Gly-Gly-Tyr

Experimentation has been limited to low excess of the Boc-amino symmetrical anhydrides. The mole ratio of symmetrical anhydride to amino acid terminus on the resin is 1.5:1. Reaction temperatures are mainly 78 $^\circ$F and 58 $^\circ$F. In order to obtain some quantitative effect of temperature on reaction kinetics, more temperatures are needed. In this study, 95 $^\circ$F and 45 $^\circ$F are also tested for some model peptides. The temperature chosen is restricted by the physical properties of solvent and cooling medium. In this experiment, the solvent is DCM and the cooling medium is water (could use something else). The DCM has a boiling point of less than 104 $^\circ$F at normal atmosphere pressure, while the circulation water will freeze at 32 $^\circ$F. The initial concentration of reactive sites on the resin lies between 0.013 and 0.021 mole/l. All eight model peptides have been synthesized under these operating conditions at least three times in order to get duplicated and comparable results. Some make-up experiments are needed whenever bad output data is realized.

The specific objectives, by considering all the experimental conditions mentioned above, are as follow: As a first step, several experiments are performed under fixed operation conditions. After obtaining duplicated

results, the operation conditions are varied one at a time. Special care must be taken for every synthetic step. A failure at one step not only affects the data analysis for that reaction but also affects the subsequent series. This failure usually results in repeated experiments for the entire peptide. One synthesis step usually takes three hours. Working with two reactors at the same time reduces the average time to about two hours for each synthesis.

The second step is to analyze the experimental data. A kinetic model is proposed. The experimental data is compared with the model to determine the correctness. Whenever a kinetic model is proposed, an integral method is usually a good first try.

Finally, based on the knowledge of peptide synthesis chemistry and an adequate kinetic model, reaction mechanisms will be hypothesized. There may be several reaction mechanisms which can fit one kinetic model. The correct mechanism should be the one by which the experiment can be explained most reasonably.

CHAPTER IV

RESULTS

The major purpose of this study is to get kinetic data for selected hormones produced by SPPS. The output data are expressed by UV absorption behavior of amino acid symmetrical anhydrides. The concentration of symmetrical anhydride decreases as the reaction proceeds. This absorption curve is recorded by a chart recorder synchronized with the spectrophotometer. The absorption curve is then converted to concentration data. A prerequisite for this conversion is that the UV wavelength is chosen so that the concentration will change with absorption linearly. The effect of some factors on the kinetics is observed by changing operating conditions, such as temperature and concentration.

There are more than 400 individual attachments in this study for the eight peptides under consideration. Only a portion of the experimental data are presented in this chapter. The majority of data will be listed in appendixes.

Calibration Curves

The optimum UV absorption for most reactants in this study interferes with the solvent used, DCM. In order to

avoid this interference, the UV absorption for these reactants have to be at wavelengths higher than that of DCM. The absorption of DCM has a maximum at 233 nm and a tail through 262 nm. Beyond 262 nm, DCM is not detectable at all. Theoretically, the UV wavelength does not have to be at the maximum absorption point, as long as a linear relationship exists between absorption and concentration. But a deviation from the maximum point results in an increased sensitivity of absorption to measured component. As a result, the preparation of the measured components has to be more careful. Fortunately, the concentration information needed in this study comes from the difference of absorption data. Different solution concentration affects both the initial and end reading. However, their difference remains the same. The error caused by inaccurate reagent preparation is then canceled.

In order to test the linearity of absorption and concentration, standard calibration curves are necessary. Samples of symmetrical anhydride or amino acids in DCM with known concentration are prepared and measured spectrometricly.

Most of the calibration curves for both amino acids and their symmetrical anhydrides are listed in Appendix A. Some example curves are shown in Figures 3 to 5. There are several lines corresponding to different wavelengths in each figure. In practice, only one fixed wavelength for one amino symmetrical anhydride is used throughout the

Figure 3. Calibration Curves of Tyrosine

Figure 4. Calibration Curves of Tyrosine Symmetrical
Anhydride

Figure 5.   Calibration Curves of Proline Symmetrical
Anhydride

experiments in order to get comparable results. Figure 3 shows the calibration curves for Tyr. Figure 4 shows the curves for Tyr symmetrical anhydride. Figure 5 presents the absorption curves of Pro symmetrical anhydride, while the Pro itself is undetectable by UV in DCM. The absorptivities of amino acids and their symmetrical anhydrides are not a simply additive. For example, the spectrum of L-Tyrosine Symmetrical anhydride is obviously not simply the sum of 2 L-Tyrosine spectra, no matter what the wavelength. Both symmetrical anhydrides and their monomers exist in the experimental environment. The symmetrical anhydride comes from the excess mole ratio, while its monomer is released from the coupling reaction. As a result, the linearity is needed for both. The UV spectra for amino acid monomers, therefore, are measured whenever they are available. This non-additive behavior on UV spectra is also the explanation why a higher wavelength, where the solvent is undetectable, has to be chosen.

Time-Concentration Information

The time-concentration data come from the UV absorption spectra of symmetrical anhydride during the course of coupling reactions. An initial reading outside the reactor is used to overcome the time delay between the reactor and the flow cell. The absorption curve is extrapolated to this initial reading. Every attached amino symmetrical anhydride is fixed at one selected UV wavelength based on the

calibration curves. The wavelengths used in this study are listed in Table 1. The wavelength is also chosen so that the absorption curve will be well-located in a range of 0-1 or 0-2 cm^{-1} on chart paper.

TABLE 1

UV WAVELENGTHS USED IN THIS STUDY

Amino Acid	Gly	His	Ile	Leu	Pro	Ser	Lys	Phe	Tyr	Val
Wavelength (nm)	265	290	274	274	274	276	283	276	296	274

A typical absorption curve is shown in Figure 6. There are some small bumps on the curve due to air bubbles caused by pump-sucking and loop resistance. Pump speed can help to reduce the bubble size and frequency, and make the curve smooth. Usually, the time-lag is about 10 seconds. However, time is needed to dump the reagent into the reactor. The highest measured concentration occurs at about 15 seconds. The absorption curve gradually becomes horizontal, indicating a completed reaction. Now, if we consider the initial point as the start of reaction, corresponding to X_A = 0. (X_A is reacted mole fraction of reactive amino acid sites on the peptide fragments.) The horizontal line corresponds to X_A = 1. The concentration data is obtained from the equation of $C_A = C_{A0} (1 - X_A)$, where C_{A0} is the initial concentration of reactive sites on the resin, C_A is the concentration corresponding to X_A. The

Figure 6. UV Time-Absorption Curve of Valine in
[Val$_4$]-Angiotensin III, Position 6

total dropping of the absorption curve indicates the total concentration change, or the change of reacted mole fraction. The time from the initial reading to the first point of horizontal line is the total reaction time for the coupling reaction. Selected mole fraction vs. time curves for eight peptides at 78 ^{o}F are shown in Figures 7-14. The rest of the data at these conditions are listed in Appendix B. The properties of the previous amino acid and chain length may affect the reaction rate dramatically. These affects will be discussed in more detail in the next chapter.

Temperature Effect

All eight peptides are also synthesized at 58 ^{o}F. Some typical mole fraction vs. time curves for these peptides are shown in Figures 15-22. These curves can be compared with those at 78 ^{o}F (Figures 7-14). With decreased reacting temperature, the reaction rate is decreased. Other temperatures are tested for a selected peptide in order to obtain a quantitative conclusion about the temperature effect. These temperatures are 45 ^{o}F and 95 ^{o}F. The results are shown in Figure 23 and 24. Detailed data comparisons and discussions will be performed in the next chapter.

Concentration Effect

Concentration is primary factor affecting reaction

Figure 7. Reacted Mole Fraction of Amino Terminus
Versus Time (Bradykinin, 78 ^{o}F)

Figure 8. Reacted Mole Fraction of Amino Terminus
Versus Time (Tyr-Bradykinin, 78 $^\circ$F)

Figure 9. Reacted Mole Fraction of Amino Terminus
Versus Time (Tyr$_8$-Bradykinin, 78 $^\circ$F)

Figure 10. Reacted Mole Fraction of Amino Terminus Versus Time (Alpha-Neoendorphin, 78 $^{\circ}$F)

Figure 11. Reacted Mole Fraction of Amino Terminus Versus Time (Beta-Neoendorphin, 78 °F)

Figure 12. Reacted Mole Fraction of Amino Terminus Versus Time (Arg$_8$-Alpha-Neoendorphin, 78 $^\circ$F)

Figure 13. Reacted Mole Fraction of Amino Terminus
Versus Time (Angiotensin III, 78 $^\circ$F)

Figure 14. Reacted Mole Fraction of Amino Terminus Versus Time (Val$_4$-Angiotensin III, 78 °F)

Figure 15. Reacted Mole Fraction of Amino Terminus
Versus Time (Bradykinin, 58 °F)

Figure 16. Reacted Mole Fraction of Amino Terminus
Versus Time (Tyr-Bradykinin, 58 °F)

Figure 17. Reacted Mole Fraction of Amino Terminus
Versus Time (Tyr$_8$-Bradykinin, 58 $^{\circ}$F)

Figure 18. Reacted Mole Fraction of Amino Terminus
Versus Time (Alpha-Neoendorphin, 58 $^\circ$F)

Figure 19. Reacted Mole Fraction of Amino Terminus
Versus Time (Beta-Neoendorphin, 58 °F)

Figure 20. Reacted Mole Fraction of Amino Terminus
Versus Time (Arg_8-Alpha-Neoendorphin,
58 °F)

Figure 21. Reacted Mole Fraction of Amino Terminus Versus Time (Angiotensin III, 58 $^\circ$F)

Figure 22. Reacted Mole Fraction of Amino Terminus
Versus Time (Val$_4$-Angiotensin III,
58 ^{o}F)

Figure 23. Reacted Mole Fraction of Amino Terminus Versus Time (Angiotensin III, 45 °F)

Figure 24. Reacted Mole Fraction of Amino Terminus
Versus Time (Angiotensin III, 95 °F)

rate. A selected peptide, Bradykinin, is synthesized at several initial concentrations (Figures 25-27). This information can be used to test the effect of concentration on reaction rate. Also, it can be used to test the proposed reaction rate model, $-r_A = K\, f(C)$, where C represents the concentrations for all the reagents involved. In this model it is assumed that the reaction rate constant is a function of many factors.

Reaction Rate

A complete rate equation can be derived from concentration-time data. First of all, a reasonable mechanism should be hypothesized. Usually in a constant-volume system, the rate expression for the disappearance of reactant A will be of the form

$$-r_A = -dC_A\,/\,dt = f(K,\ C)$$

In this study, a more restricted case is considered, in which the disappearance rate of reactive sites is expressed as a multiplication of two terms:

$$-r_A = -dC_A\,/\,dt = K\,f(C)$$

With this hypothesized equation and the concentration-time experimental data, there are two methods which can be used to get kinetic information, the differential and integral methods. With the differential method, the slope of the concentration-time curve is needed. A slight uncertainty in the slope of the fitted curve will result in a large uncertainty in the evaluated slope. In these experiments,

Figure 25. Reacted Mole Fraction of Amino Terminus Versus Time (Bradykinin, 78 ^{o}F, Case 2)

Figure 26. Reacted Mole Fraction of Amino Terminus Versus Time (Bradykinin, 78 $^{\circ}$F, Case 3)

Figure 27. Reacted Mole Fraction of Amino Terminus
Versus Time (Bradykinin, 58 $^{\circ}$F,
Case 2)

the uncertainty comes not only from reading the slope, but also from the experimental accuracy. Most of the experiments reach completion very fast. As a result, the slope on the concentration-time curve is very steep, and not easy to read accurately. Considering this difficulty, the integral method was tried first. Several particular rate equations with different reaction orders are tested by comparing the predicted concentration versus time curves with experimental results. If there is no satisfactory agreement between the predicted model and experiment values, the rate equation and mechanism are rejected and another rate equation is tried. By this exclusive test, most data obtained from one percent DVB crosslinked resin show apparent second order behavior. The plot of $Ln[(M-X_A)/M(1-X_A)]$ vs. time gives a straight line to $X_A > 0.9$, where M is a ratio of initial concentration of loaded symmetrical anhydride and amino terminus. Figure 28-31 are typical examples for this trend. These data were obtained at 78 ^{O}F. The same trend is observed at other temperatures also (Figures 32-35).

Data for a few select amino acids are best fit by first order behavior at 78 ^{O}F. The straight line is obtained by plotting $-Ln(1 - X_A)$ vs. time, as shown in Figure 36 and 37.

The slope of the straight line based on second order rate model is $K(C_{BO} -C_{AO})$, where C_{BO} is the initial concentration of symmetrical anhydride. The apparent second order rate constant can therefore be calculated as

Figure 28. Second Order Plot (Bradykinin, 78 °F, Pro, Position 3)

Figure 29. Second Order Plot (Alpha-Neoendorphin, 78 $^\circ$F, Phe, Position 7)

Figure 30. Second Order Plot (Val$_4$-Angiotensin III, 78 °F, His, Position 3)

Figure 31. Second Order Plot $(Arg_8-Alpha-$
Neoendorphin, 78 ^{o}F, Lys,
Position 2)

Figure 32. Second Order Plot (Bradykinin, 58 ^{O}F, Pro, Position 3)

Figure 33. Second Order Plot (Arg_8-Alpha-Neoendorphin, 58 ^oF, Lys, Position 2)

Figure 34. Second Order Plot (Angiotensin III, 45 °F, Tyr, Position 5)

Figure 35. Second Order Plot (Angiotensin III, 95 $^{\circ}$F, Val, Position 6)

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Figure 36. First Order Plot (Bradykinin, 78 °F, Ser, Position 4)

Figure 37. First Order Plot (Arg$_8$-Alpha-
           Neoendorphin, 58 °F, Gly,
           Position 7)

$$K_s = Slope \; / \; (C_{B0} - C_{A0})$$

where, the subscript, s, stands for "second order." The slope of the straight line based on a first order rate model is the apparent rate constant directly. A symbol, K_f, is used for this first order rate constant.

Table 2 shows calculated reaction rate constants based on the result of least square analysis, either second order or first order, at 78 ^{O}F. Table 3 lists those rate constants at 58 ^{O}F. Table 4 lists the rate constants for Angiotensin III at four different temperatures. Table 5 shows the reaction rate constants of Bradykinin at different loading concentrations and different operating temperatures. The data shown in these tables are arithmetic averages for repeated experiments. Some clearly erroneous data are not included in these averages. These bad data mainly come from unskillful operation and occasionally from instrumental problems.

Reaction Time

Reaction termination is indicated by the qualitative picrylsulfonic acid method. A material balance is performed after every peptide is synthesized to double check reaction completion. Since the reaction rate constants have been obtained, the reaction half-life can be determined either from the experimental output or theoretical calculation. The reaction time at any particular point can also be obtained in these ways, as long as the point is located in the range

TABLE 2

REACTION RATE CONSTANTS AT 78 °F

PEPTIDE	SEQUENCE								
1 (Arg)	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
	2.11	3.21	*0.051	3.49	*0.066	4.98	1.82	4.99	3.77
2 (Arg)	Tyr	Pro	Ser	Phe	Gly	Pro	Pro		
	2.34	5.27	*0.071	3.82	*0.085	4.00	1.55		
3 (Lys)	Pro	Tyr	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
	5.16	4.75	4.11	4.51	5.19	4.06	7.85	5.39	1.11
4 (Pro)	Tyr	Lys	Pro	Leu	Phe	Gly	Gly	Tyr	
	2.51	5.99	3.54	3.65	3.80	6.47	6.12	2.77	
5 (Arg)	Lys	Phe	Leu	Phe	Gly	Gly	Tyr		
	4.50	1.96	4.29	1.72	*0.052	*0.064	4.30		
6 (Phe)	Pro	His	Val	Tyr	Val				
	3.67	5.01	3.61	7.12	1.03				

Note: (1) Peptide 1 is [Tyr]-Bradykinin;
            Peptide 2 is [Tyr$_8$]-Bradykinin;
            Peptide 3 is Alpha-Neoendorphin;
            Peptide 4 is Beta-Neoendorphin;
            Peptide 5 is [Arg$_8$]-Alpha-Neoendorphin;
            Peptide 6 is [Val$_4$]-Angiotensin III.

       (2) *'s are for first order rate constant, s^{-1}.
            The others are second order rate constants,
            liter/mole sec.

       (3) The initial concentration of active site is
            0.0148 mole/liter.

TABLE 3

REACTION RATE CONSTANTS AT 58 °F

PEPTIDE	SEQUENCE								
1 (Arg)	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
	1.33	1.47	3.08	2.51	3.43	3.16	1.47	5.18	3.30
2 (Arg)	Tyr	Pro	Ser	Phe	Gly	Pro	Pro		
	1.88	2.28	3.36	3.59	5.15	2.41	1.37		
3 (Lys)	Pro	Tyr	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
	4.33	4.31	5.22	2.58	2.23	2.86	*0.043	3.20	2.19
4 (Pro)	Tyr	Lys	Pro	Leu	Phe	Gly	Gly	Tyr	
	2.22	3.57	2.67	2.51	2.49	4.59	4.55	2.51	
5 (Arg)	Lys	Phe	Leu	Phe	Gly	Gly	Tyr		
	2.05	1.92	1.71	1.54	3.28	*0.043	3.11		
6 (Phe)	Pro	His	Val	Tyr	Val				
	3.16	3.31	2.17	4.37	0.54				

Note: (1) Peptide 1 is [Tyr]-Bradykinin;
Peptide 2 is [Tyr_8]-Bradykinin;
Peptide 3 is Alpha-Neoendorphin;
Peptide 4 is Beta-Neoendorphin;
Peptide 5 is [Arg_8]-Alpha-Neoendorphin;
Peptide 6 is [Val_4]-Angiotensin III.

(2) *'s are for first order rate constant, s^{-1}.
The others are second order rate constants,
liter/mole sec.

(3) The initial concentration of active site is
0.0148 mole/liter.

TABLE 4

REACTION RATE CONSTANTS FOR
ANGIOTENSIN III

Temperature (^{o}F)	Phe-resin	Pro	His	Ile	Tyr	Val
45	--	2.61	4.30	1.41	3.64	0.61
58	--	3.02	2.93	2.64	5.60	0.76
78	--	3.67	5.18	2.72	5.85	0.90
95	--	4.34	5.37	4.16	6.09	1.30

Note: (1) The data are second order rate constants,
          liter/mole sec.

      (2) The initial concentration of active site is
          0.0148 mole/liter.

TABLE 5

REACTION RATE CONSTANT FOR BRADYKININ

Sequence	Conc.=0.0138	Conc.=0.0148		Conc.=0.0207	
(Arg-resin)	78 F	58 F	78 F	58 F	78 F
Phe	1.75	1.78	2.27	1.80	2.90
Pro	3.60	1.65	2.83	2.34	3.27
Ser	3.36	3.17	* 0.070	4.76	--
Phe	2.36	3.32	3.71	3.54	1.59
Gly	--	4.16	*0.093	--	--
Pro	4.53	3.65	6.12	3.50	3.33
Pro	2.49	1.33	1.98	1.67	2.32

Note: (1) *'s are for first order rate constant, s^{-1}.
The others are second order rate constants,
liter/mole sec.

(2) The unit of Conc. is mole/liter.

in which the reaction rate model is valid. The total
reaction time is read directly from experimental output
since the experiments show a deviation from the proposed
rate model when the reactions approach completion. The
reaction time for each individual amino acid attachment at
select moments are listed in Tables 6-14. All the data
shown are an average for several paralleled experiments.

TABLE 6

REACTION TIME FOR [TYR]-BRADYKININ

					Temperature = 58 °F					

Time					Sequence					
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
$t_{.5}$	--	29.1	26.5	12.6	15.5	11.3	12.3	26.4	7.5	11.8
$t_{.9}$	--	140	128	61	74	55	59	127	36	57
t_1	--	580	520	185	280	235	210	345	110	170

					Temperature = 78 °F					

Time					Sequence					
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
$t_{.5}$	--	18.4	11.8	13.7	11.1	10.4	7.8	21.3	7.8	10.3
$t_{.9}$	--	89	58	45	54	35	38	103	38	50
t_1	--	540	200	90	190	60	160	320	160	100

Note: (1) The units are in seconds.

(2) The subscript is the reacted mole fraction.

TABLE 7

REACTION TIME FOR [TYR$_8$]-BRADYKININ

Temperature = 58 ^{O}F							
Time				Sequence			
	Arg	Tyr	Pro	Ser	Phe	Gly	Pro	Pro
$t_{.5}$	--	20.7	17.0	11.6	10.8	7.5	16.1	28.3
$t_{.9}$	--	100	82	56	52	36	78	137
t_1	--	310	380	145	355	190	200	390

Temperature = 78 ^{O}F							
Time				Sequence			
	Arg	Tyr	Pro	Ser	Phe	Gly	Pro	Pro
$t_{.5}$	--	16.6	7.4	9.8	10.2	8.1	9.7	25.1
$t_{.9}$	--	80	36	32	49	27	47	121
t_1	--	370	200	80	310	70	120	350

Note: (1) The units are in seconds.

(2) The subscript is the reacted mole fraction.

TABLE 8

REACTION TIME FOR ALPHA-NEOENDORPHIN

Temperature = 58 °F									

Time					Sequence				
	Lys	Pro	Tyr	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
$t_{.5}$	--	9.0	9.0	7.4	15.1	17.4	13.6	16.3	12.2	17.8
$t_{.9}$	--	43	43	36	73	84	65	54	59	86
t_1	--	260	210	195	460	295	395	230	315	310

Temperature = 78 °F									

Time					Sequence				
	Lys	Pro	Tyr	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
$t_{.5}$	--	7.5	8.2	9.5	8.6	7.5	9.6	5.0	11.2	34.9
$t_{.9}$	--	36	39	46	42	36	46	24	54	168
t_1	--	160	230	180	240	250	245	90	170	420

Note: (1) The units are in seconds.

(2) The subscript is the reacted mole fraction.

TABLE 9

REACTION TIME FOR BETA-NEOENDORPHIN

Temperature = 58 °F								
Time				Sequence				
Pro	Tyr	Lys	Pro	Leu	Phe	Gly	Gly	Tyr
$t_{.5}$ --	17.5	10.9	14.6	15.5	15.6	8.5	8.5	15.5
$t_{.9}$ --	84	52	70	75	75	41	41	75
t_1 --	380	200	350	260	340	190	215	195

Temperature = 78 °F								
Time				Sequence				
Pro	Tyr	Lys	Pro	Leu	Phe	Gly	Gly	Tyr
$t_{.5}$ --	15.5	6.5	11.0	10.6	10.2	6.0	6.4	14.0
$t_{.9}$ --	75	31	53	51	49	29	31	68
t_1 --	520	120	320	180	210	50	65	495

Note: (1)   The units are in seconds.

(2)   The subscript is the reacted mole fraction.

TABLE 10

REACTION TIME FOR [ARG$_8$]-ALPHA-NEOENDORPHIN

Temperature = 58 $^\circ$F

Time	Sequence							
	Arg	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
$t_{.5}$	--	19.0	20.2	22.8	25.2	11.8	16.2	12.5
$t_{.9}$	--	91	98	110	121	57	54	60
t_1	--	255	420	385	500	210	190	160

Temperature = 78 $^\circ$F

Time	Sequence							
	Arg	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
$t_{.5}$	--	8.6	19.9	9.1	22.6	13.5	10.8	9.0
$t_{.9}$	--	42	96	44	109	45	36	44
t_1	--	120	360	250	415	60	50	240

Note: (1) The unit are in seconds.

(2) The subscript is the reacted mole fraction.

TABLE 11

REACTION TIME FOR [VAL$_4$]-ANGIOTENSIN III

Temperature = 58 ^{O}F					
Time			Sequence		
	Phe	Pro	His	Val	Tyr	Val
$t_{.5}$	--	12.3	11.8	17.9	8.9	72.4
$t_{.9}$	--	59	57	86	43	349
t_1	--	500	390	540	335	850

Temperature = 78 ^{O}F					
Time			Sequence		
	Phe	Pro	His	Val	Tyr	Val
$t_{.5}$	--	10.6	7.8	10.8	5.5	37.9
$t_{.9}$	--	51	37	52	26	183
t_1	--	275	300	385	450	730

Note: (1) The units are in seconds.

(2) The subscript is the reacted mole fraction.

TABLE 12

REACTION TIME FOR ANGIOTENSIN III

Temperature = 45 °F						
Time			Sequence			
	Phe	Pro	His	Ile	Tyr	Val
$t_{.5}$	--	14.9	9.0	27.6	10.7	63.8
$t_{.9}$	--	72	44	133	51	308
t_1	--	400	380	1080	300	960
Temperature = 58 °F						
$t_{.5}$	--	12.9	13.2	14.7	6.9	51.2
$t_{.9}$	--	62	64	71	33	247
t_1	--	350	300	810	215	740
Temperature = 78 °F						
$t_{.5}$	--	10.9	7.5	14.3	6.6	43.2
$t_{.9}$	--	51	36	69	32	208
t_1	--	230	260	500	390	840
Temperature = 95 °F						
$t_{.5}$	--	8.9	7.2	9.3	6.4	29.9
$t_{.9}$	--	43	35	45	31	144
t_1	--	200	150	270	90	420

Note: (1) The unit are in seconds.
      (2) The subscript is the reacted mole fraction.

TABLE 13

REACTION TIME FOR BRADYKININ (1)

Temperature = 58 ^{O}F

Time				Sequence				
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro
$t_{.5}$	--	21.9	23.6	12.3	11.7	9.3	10.7	29.2
$t_{.9}$	--	105	114	59	56	45	51	141
t_1	--	485	380	125	225	170	270	360

Temperature = 78 ^{O}F

Time				Sequence				
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro
$t_{.5}$	--	17.1	13.7	9.9	10.5	7.4	6.4	19.6
$t_{.9}$	--	83	66	33	50	25	31	94
t_1	--	450	220	70	200	70	120	250

Note: (1) The unit are in seconds.

(2) The subscript is the reacted mole fraction.

TABLE 14

REACTION TIME FOR BRADYKININ (2)

Temperature = 58 ^{O}F, Conc. = 0.0207 mole/l							
Time				Sequence			
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro
$t_{.5}$	--	15.4	11.9	5.8	7.9	--	8.0	16.7
$t_{.9}$	--	74	57.2	28	38	--	38	80
t_1	--	340	320	120	180	--	300	410

Temperature = 78 ^{O}F, Conc. = 0.0207 mole/l							
$t_{.5}$	--	9.6	8.5	--	17.4	--	8.3	12.0
$t_{.9}$	--	46	41	--	84	--	40	58
t_1	--	240	200	60	140	--	180	240

Temperature = 78 ^{O}F, Conc. = 0.0138 mole/l							
$t_{.5}$	--	23.8	11.6	12.4	17.7	--	9.2	16.8
$t_{.9}$	--	115	56	60	85	--	44	81
t_1	--	590	440	180	240	--	310	520

Note: (1) The units are in seconds.

(2) The subscript is the reacted mole fraction.

CHAPTER V

DISCUSSIONS

SPPS is a complex heterogeneous chemical reaction. So
far, most SPPS research has dealt with the underlying
chemistry, coupling efficiency and the functions of
different chemical reagents. Kinetics information is still
limited, and the study of the reaction mechanisms is almost
non-existent.

A kinetic study usually relies on a statistical
analysis of many data. This thesis shows that
reproducibility of this complex coupling is very sensitive
to operating conditions. A slight error in experimental
conditions can cause a severe deviation in the observed
reaction rate. The amount of data in this thesis provides a
chance to step into this challenging area - kinetic models
and reaction mechanisms of SPPS.

Experiment Facility and Operation

The major experiment is performed in a batch reactor.
A UV spectrophotometer is used to continuously monitor the
reaction. One problem of this design is the time delay
caused by circulation between the reactor and flow cell.
The volume of liquid in the loop was about 7% of total

liquid volume in experiments performed prior to this thesis. This volume is divided among the teflon tube and flow cell. In order to reduce this dead-volume, the tube length should be as short as possible and the flow cell volume should be small. In these experiments, the flow cell volume is 0.08 ml, a reduction from 0.6 ml previously. This change reduced the dead volume fraction to about 5% of total reacting volume and reduced the measurement error caused by the time-delay from the reactor to the detector.

The absorption represents the concentration of the reacting media at that moment. To assure an accurate solution concentration measurement, the paddle on the stirrer was increased to two pairs instead of the original one pair. Some resins, such as Phe-resin, are sticky and attach easily to the wall and the bottom of the reactor. Sometimes, even strong stirring cannot eliminate this adhesion. These experiments indicate that the adhesion can be removed by back-flushing the circulating pump. This back-flushing is particularly necessary before the coupling step. Without sufficient mixing, the absorption curve will be changed. Data obtained without good mixing cannot be used in the kinetic analysis.

Amino acid UV absorption relies on the detectable side-chain blocking group and/or the side-chain itself. The detectable groups include aromatics, imidazole and sulfur-containing functionalities. In this study, phenylalanine has a benzyl side-chain; serine, lysine and arginine have

benzyl side-chain protecting groups; tyrosine has both a benzyl side chain and protecting group; while histidine has imidazole and benzyl (Tosyl) protecting groups. These amino acids can be detected by UV directly. For those amino acids with no detectable side chain and/or side chain protecting groups, such as glycine, leucine, isoleucine, proline and valine, the UV absorption method fails. Fortunately, the reactant is the amino symmetrical anhydride rather than its monomer, and this study has shown that all the symmetrical anhydrides are detectable by UV and have a maximum absorption around 220 nm. This peptide-bond not only gives an extra benefit for detactability, but also makes the UV method reliable in this study.

Reaction Rate Models

As mentioned in the last chapter, the general reaction rate model is supposed to be composed of a concentration-dependent and concentration-independent term. This hypothesis is generally correct for constant-volume systems. In order to test the correctness of the hypothesized model, some selected peptides were synthesized at several different initial loading concentrations with constant temperature. The reaction rate constants were calculated and listed in Table 15. From these data it can be seen that, with changing initial concentration, the reaction rate constants remain unchanged. Also, as reaction proceeded, the reaction rate constants remain unchanged over a broad range

TABLE 15

REACTION RATE CONSTANTS AT DIFFERENT INITIAL
CONCENTRATIONS FOR BRADYKININ

TEMP.	CONC.	SEQUENCE						
(^{O}F)	(mole/l)	Phe	Pro	Ser	Phe	Gly	Pro	Pro
	0.0138	1.75	3.60	3.36	2.36	--	4.53	2.48
78	0.0148	2.27	2.82	*0.070	3.71	*0.093	6.12	1.98
	0.0207	2.90	3.27	--	1.59	--	3.33	2.32
58	0.0148	1.78	1.65	3.17	3.32	4.16	3.65	1.33
	0.0207	1.80	2.34	4.76	3.54	--	3.50	1.67

Note: (1) *'s are for first order rate constant, s^{-1}.
The others are second order rate constant,
liter/mole sec.

of reacted mole fraction. This is indicated by a straight line passing through the origin at suitably chosen coordinate variables (Figure 38). The results in Table 15 and Figure 38 prove that the rate constant term is indeed concentration-independent.

Since SPPS is a heterogeneous reaction, the overall reaction may not be elementary. The overall reaction order and the concentration-independent rate constant can be obtained by the integral method as mentioned in the last chapter. Some simple integer orders were tried first. If these failed, then more complex models were tested. Using special-arranged groups as the coordinate variables, the reaction order and rate constant can then be obtained graphically. The method of plotting these data based on simple integer order models is classical. Levenspiel (1972, 1984) gave detailed explanations about these methods. For example, by integrating a first order reaction rate model and rearranging, the resulting expression will be $-Ln(1-X_A)=Kt$. If the first order hypothesis fits the experiment, a plot of $-Ln(1-X_A)$ vs. time should be a straight line passing through the origin. The slope of this straight line will be the first order rate constant. Zero, first, second and third orders were first tried in this study. Fortunately, second, and occasionally first, order models fit the data very well. In Chen's dissertation (1988), a shifting order model was obtained for resin with 2% crosslinked DVB. This phenomenon can not be duplicated

Figure 38. Reaction Rate Constant Plot for
Bradykinin at Different Loading
Concentrations

In this study with 1% crosslinked DVB resin.

Most data deviate from a first order rate model. However, Ser and Gly show first order behavior (Figure 36 and 37). This first order phenomenon occurs only with fast-reacting amino acids at higher temperature (78 ^{O}F). When the temperature is decreased to 58 ^{O}F, these same amino acids no longer obey first order and second order looks more reasonable. The second order behaviors of Ser and Gly at 58 ^{O}F are shown in Figure 39 and 40. It should be noticed that at lower temperature the reactions are slower. Generally speaking, the first order behavior occurs only for restricted cases and relies not only on the amino acid itself, but also on the operating conditions, such as temperature.

The calculated first order rate constants, as shown in Table 2, have a range of 0.04 to 0.09 s^{-1}. These numbers are different with those observed by Gut and Davidovich (1976), in which the first order rate constants had an order of magnitude of 10^{-4} s^{-1}. Their first order rate constants decreased during reaction which means that the reactions were not truly first order, and their rate constants are not concentration-independent. Therefore, their data may not be comparable with the first order data from these experiments.

The second order rate constants, as shown in Table 2, have a range of 0.5 to 8 liter/mole s. This order of magnitude is comparable with that obtained by Merrifield (1984), in which the rate constants for Bpoc-amino

Figure 39   Second Order Plot of Gly (Alpha-
Neoendorphin, 58 °F, Position 9)

Figure 40. Second Order Plot of Ser (Bradykinin, 58 $^\circ$F, Position 4)

symmetrical anhydrides to Val-resin were 0 6 to 6

liter/mole s, and for Ala-resin were 1 to 14 liter/mole s.

Merrifield's experiments were for the first attachment only.

The same order of magnitude for rate constants as

Merrifield's data confirms the observations of this study.

Second order rate constants were also obtained by Rudinger

and Buetzer (1975). Their data were lower than those in

these experiments by one order of magnitude.

All the reaction rate constants are obtained by least

square analysis with zero intercept The data fit a

straight line very well. When considering curve fitting,

one criterion is R^2, the coefficient of determination, or R,

the correlation coefficient For perfectly straight lines,

R^2 equals one. If R is less than 0 8 in absolute magnitude,

or R^2 less than 0 64, the linearity is doubtful. In this

analysis, all straight lines have R^2 greater than 0.9, with

most greater than 0 95

In plotting second or first order rate models, the

straight line is obeyed until X_A = 0 9   Some data are

linear for X_A greater than 0 95   Curvatures occurred beyond

that. These curvatures can be caused by the logarithm of X_A

as it approaches 1.0   Possibly, this phenomenon may

indicate some intra-particle diffusion   As the reaction

approaches completion, mass transfer resistance may

increase, leading to a reaction rate slowdown. That means

diffusion resistance dominates at the later stage of

reaction

Generally, desired mole fractions, such as 0 99, are close enough to reaction completion and tail-end problems are usually neglected   However, 99% completion may not be satisfactory for SPPS   A 99% completion at every attachment will lead to a yield of 82 wt % on a peptide of 20 amino acids. The reaction should approach 100% for SPPS to reduce separation and purification difficulties and production cost. Our experiments, shown in Tables 6-14, indicate that some reactions have longer tails as they approaching completion, resulting in longer total reaction time   For example, the second order rate constants for Lys and Leu in Arg_8-Alpha-Neoendorphin at 78 ^{o}F are similar, Lys has a value of 4 5 1/mole s and Leu has a value of 4.3 1/mole s (See Table 11). However, the total reaction time for Leu is more than double that for Lys, with 250 seconds and 120 seconds, respectively   This indicated that Leu has a longer tail-end than Lys. This may be caused by the intra-particle resistance of Beta-structure in leu, especially at the later stages of the reaction. A more detailed explanation about the tail-end phenomenon will require a further study of amino acid chemistry beyond the scope of this study. It should be emphasized that, from these observations, the tail-end analysis is indeed an interesting area. A higher concentration driving force may help to overcome intra-particle diffusion resistance   This gives high excess mole ratios an advantage   Promoting reaction with a higher mole ratio has been observed by Chen (1988)

All the results shown in Tables 2-14 are the arithmetic
mean of several paralleled experiments The rate constants
and the reaction half-life, as well as the total reaction
time, which are used to average values in Tables 2-14 are
all well-within a factor of two. For example, the averaged
second order rate constant for Phe at the second position in
Tyr-Bradykinin at 58 ^{O}F is 1 33 1/mole s. This value is an
average from three paralleled results, 1 42, 1 21 and 1.37.
Some typical plots which show error bars in these
experiments are shown in Figure 41 and 42.

The reactions discussed in this section, although they
show a simple reaction order, may be non-elementary because
of their heterogeneity. Some reaction mechanisms will be
studied in great detail in later sections. The "second" or
"first" order reaction rate constant is the "apparent"
reaction rate constant

Concentration Effect

The reaction rate is proportional to the reactant
loading concentration. Table 16 shows this effect on total
reaction time at different initial concentrations. The
reaction time increases with decreasing initial
concentration. The loading concentration is restricted by
many factors, such as reactor capacity, resin swolling
ability, solvent properties and economic considerations
Merrifield (1984) indicated that the actual coupling
condition was performed at an initial loading concentration

Figure 41. Error Bar Chart for Reacted Mole Fraction
(Phe in Tyr-Bradykinin, Position 2)

Figure 42. Error Range for Second Order Rate Constant
(Phe in Tyr-Bradykinin, Position 2)

TABLE 16

EFFECT OF CONCENTRATION ON TOTAL REACTION
TIME FOR BRADYKININ

TEMP.	CONC.	SEQUENCE						
(^{O}F)	(mole/l)	Phe	Pro	Ser	Phe	Gly	Pro	Pro
	0.0138	590	440	180	240	--	310	520
78	0.0148	450	220	70	200	70	120	250
	0.0207	240	200	60	140	--	180	240
58	0.0148	485	380	125	225	170	270	360
	0.0207	340	320	120	180	--	300	410

Note: (1) The units are in seconds.

Figure 43. Temperature Effect on Reaction Rate (Bradykinin, Phe, Position 2)

Figure 44. Temperature Effect on Reaction Rate
(Tyr-Bradykinin, Pro, Position 3)

Figure 45. Temperature Effect on Reaction Rate
(Tyr$_8$-Bradykinin, Tyr, Position 2)

Figure 46. Temperature Effect on Reaction Rate
(Alpha-Neoendorphin, Leu, Position 6)

Figure 47. Temperature Effect on Reaction Rate
(Beta-Neoendorphin, Lys, Position 3)

Figure 48. Temperature Effect on Reaction Rate
(Arg_8-Alpha-Neoendorphin, Tyr,
Position 8)

Figure 49. Temperature Effect on Reaction Rate
(Val$_4$-Angiotensin III, His,
Position 3)

TABLE 17

EFFECT OF TEMPERATURE ON REACTION HALF-LIFE

T(^{o}F)	Sequence									
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
78	--	18.4	11.8	13.7	11.1	10.4	7.8	21.3	7.8	10.3
58	--	29.1	26.5	12.6	15.5	11.3	12.3	26.4	7.5	11.8

	Arg	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
78	--	8.6	19.9	9.1	22.6	13.5	10.8	9.0
58	--	19.0	20.2	22.8	25.2	11.8	16.2	12.5

	Phe	Pro	His	Ile	Tyr	Val
95	--	8.9	7.2	9.3	6.4	29.9
78	--	10.6	7.5	14.3	6.6	43.2
58	--	12.9	13.2	14.7	6.9	51.2
45	--	14.9	9.0	27.6	10.7	63.8

Note: (1) The units are in seconds.

(2) Sequence 1 is [Tyr]-Bradykinin;
Sequence 2 is [Arg$_8$]-Alpha-Neoendorphin;
Sequence 3 is Angiotensin III.

TABLE 18

EFFECT OF TEMPERATURE ON TOTAL REACTION TIME

T(^{o}F)	Sequence									
	Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
78	--	545	200	90	195	60	160	320	160	100
58	--	580	520	185	280	235	210	345	110	170

T(^{o}F)	Arg	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
78	--	120	360	250	415	60	50	240
58	--	255	420	385	500	210	190	160

T(^{o}F)	Phe	Pro	His	Ile	Tyr	Val
95	--	200	150	270	90	420
78	--	230	260	500	390	840
58	--	350	300	810	215	740
45	--	400	380	1080	300	960

Note: (1) The units are in seconds.

(2) Sequence 1 is [Tyr]-Bradykinin;
    Sequence 2 is [Arg$_8$]-Alpha-Neoendorphin;
    Sequence 3 is Angiotensin III.

of 0.05 to 0.2 mole/liter. These experiments use lower
initial concentrations which should not affect the
concentration-independent term in the reaction rate model.
The initial concentration affects only total reaction rate
or reaction completing time.

Reaction Temperature

SPPS is generally operated at room temperature.
Although temperature can affect the reaction rate constant,
its range is limited by the properties of the solvent used
in SPPS. DCM, commonly used as a solvent in SPPS, has a
boiling point of 39.8 ^{o}C at atmosphere pressure. Exceeding
this temperature will bring a phase-change problem at
atmospheric pressure. However, the effect of temperature on
reaction rate is a major consideration in reaction kinetics.
Elevated temperature not only increases the reaction rate,
as observed by Chen (1988), but also increases the coupling
yield, as observed by Tam (1985). When increasing
temperature becomes critical, other solvents with higher
boiling points can be used in place of DCM. Tam (1985) used
1-methyl-2-Pyrrolidinone as a solvent and operated the
reaction at 50 ^{o}C. Of course, many other factors affect the
solvent choise, not just the boiling point.

Temperature data was very limited before this study.
From the results shown in Figures 43-49 and Table 17 and
18, reaction rate increases at higher temperature. The
reaction mechanism and rate model remain the same in most

cases as shown in Table 3. Table 19 lists some comparisons
between reaction rate constants at 78 ^{O}F and 58 ^{O}F.
Considering the micro-physical properties of peptides, we
may see that elevated temperature enhances the freedom of
peptide chain vibration, and the physical aggregation due to
hydrogen bonding may become weak at higher temperature. The
coupling reaction is then easier to perform at higher
temperature.

Although the enhancement of the reaction rate with
increasing temperature is evident, this increase is not very
large. This can be seen by calculating the apparent
activation energy for Angiotensin III. Based on Arrhenius's
law:

$$K = K_0 \, Exp(-E \, / \, RT)$$

where, K is the reaction rate constant, K_0 is the frequency
factor, E is the activation energy, T is temperature and R
is the gas constant. A plot of Ln(K) vs. 1/T based on four
selected temperatures for every single attachment in
Angiotensin III is linear, indicating that these reactions
obey Arrhenius's law (Figure 50). The slope of these
straight lines is -E/R and the intercepts are Ln(K_0). The
calculated results for E and K_0 in Angiotensin III are
listed in Table 20. From these data, the activation energy
in SPPS is on the order of 10° Kcal/mole, indicating that
SPPS are not very temperature-sensitive. But some reactions
still show relatively higher activation energy than others.
For example, the activation energy for Ile is almost nine

TABLE 19

EFFECT OF TEMPERATURE ON RATE CONSTANT

T(^{O}F)	Sequence							
	Arg	Tyr	Pro	Ser	Phe	Gly	Pro	Pro
78	--	2.34	5.27	*0.071	3.82	*0.085	4.00	1.55
58	--	1.88	2.28	3.36	3.59	5.15	2.41	1.37

	Lys	Pro	Tyr	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
78	--	5.16	4.75	4.11	4.51	5.19	4.06	7.85	5.39	1.11
58	--	4.33	4.31	5.22	2.58	2.23	2.86	*0.043	3.20	2.19

	Pro	Tyr	Lys	Pro	Leu	Phe	Gly	Gly	Tyr
78	--	2.51	5.99	3.54	3.65	3.80	6.47	6.12	2.77
58	--	2.22	3.57	2.67	2.51	2.49	4.59	4.55	2.51

	Phe	Pro	His	Val	Tyr	Val
78	--	3.67	5.01	3.61	7.12	1.03
58	--	3.16	3.31	2.17	4.37	0.54

Note: (1) *'s are for first order rate constants, s^{-1}.
The others are second order rate constants,
liter/mole sec.

(2) Sequence 1 is [Tyr$_8$]-Bradykinin;
Sequence 2 is Alpha-Neoendorphin;
Sequence 3 is Beta-Neoendorphin;
Sequence 4 is [Val$_4$]-Angiotensin III.

Figure 50. Activation Energy Plot (Angiotensin III)

TABLE 20

ACTIVATION ENERGY FOR ANGIOTENSIN III

Variable	Sequence					
	Phe	Pro	His	Ile	Tyr	Val
K_O	--	778.8	55.8	193000	21.1	18600
E (Kcal/mole)	--	3.18	1.42	6.65	0.76	4.48

times that of Tyr (6.65:0.76). This indicates that the reaction of Ile is more temperature-sensitive than that of Tyr. Increasing temperature may not be an effective way to improve the Tyr reaction rate, while this method will be better for Ile. Therefore, when dealing with the temperature effect, we may need to treat the reaction individually. The general conclusion is that running at highest convenient temperature is a good idea. There may be a trade off between reaction rate and efficiency. For those coupling reactions which have very low activation energy, some other ways of increasing reaction rate other than simply increasing temperature may be suggested. For those reactions with higher activation energy the increasing temperature may be more effective. Economic factors will play important roles in the decision on which procedure to choose. The activation energy for other model peptides in this study are shown in Table 21. The data in this table are based only on two temperatures. There are no published data for comparison.

Amino Acid Effect

Tables 6-14 show the reaction time data. These data indicate that the reaction time changes dramatically for different amino acids. Generally, glycine and serine are fast reacted (less than 100 seconds for completion at 78 ^{O}F). Valine and isoleucine are slowest (500 to 1000 seconds for completion at 78 ^{O}F). The others are intermediate.

TABLE 21

ACTIVATION ENERGY FOR SELECTED PEPTIDES

Bradykinin

	Phe	Pro	Ser	Phe	Gly	Pro	Pro
E Kcal/mole	3.74	8.31	--	1.71	--	7.97	6.14

[Tyr]-Bradykinin

	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Phe	Tyr
E Kcal/mole	7.08	12.0	--	5.03	--	7.03	3.33	--	2.05

[Tyr$_8$]-Bradykinin

	Tyr	Pro	Ser	Phe	Gly	Pro	Pro
E Kcal/mole	3.37	12.9	--	0.95	--	7.79	1.87

Alpha-Neoendorphin

	Por	Tyr	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
E Kcal/mole	2.70	1.50	--	8.61	13.0	5.35	--	8.05	--

Beta-Neoendorphin

	Tyr	Lys	Pro	Leu	Phe	Gly	Gly	Tyr
E Kcal/mole	1.86	7.97	4.34	5.75	6.48	5.27	4.54	1.53

TABLE 21 (Continued)

[Arg$_8$]-Alpha-Neoendorphin

	Lys	Phe	Leu	Phe	Gly	Gly	Tyr
E Kcal/mole	12.1	0.30	14.2	1.68	--	6.24	4.977

[Val$_4$]-Angiotensin III

	Pro	His	Val	Tyr	Val
E Kcal/mole	2.33	6.40	7.83	7.50	9.95

These reaction time differences can be explained chemically
from the structures of the attached amino acids. Now look
at the structures of some amino acids.

Valine - $-NH-CH-CO-$
                        |
                        CH
                       / \
                    3HC   CH3

Isoleucine - $-NH-CH-CO-$
                        |
                        CH
                       / \
                    3HC   CH2-CH3

It can be seen that Val and Ile are Beta-substituted amino
acids. This kind of side chain may bring extra steric
hinderence problems. The Beta-structure can increase
diffusion difficulty and cause a reaction slow-down. While
glycine has a chemical structure of

$$-NH-CH-CO-$$
$$|$$
$$H$$

and serine has a structure of

$$-NH-CH-CO-$$
$$|$$
$$CH_2OH$$

The side-chain for these two are small. We may expect the
smallest steric hindering for these amino acid reactions.

The secondary structure of proline and histidine may
also bring a diffusional problem and reduce reaction rate.
Proline has a structure of

```
        -NH-CH-CO-
            |    |
        2HC   CH2
             \  /
             CH2
```

Histidine has a structure of

```
        -NH-CH-CO-
             |
            CH2
             |
            C-NH
            ||    \CH
          HC-N/
```

Compared with Beta-substituted amino acids, the retardation effect of aliphatic residues is relatively low, even though these side-chains are big, such as those in lysine and leucine. The structure of lysine is

```
        -NH-CH-CO-
             |
           (CH2)4-NH2
```

The structure of leucine is

```
        -NH-CH-CO-
             |
            CH2
             |
            CH
          3HC/  \CH3
```

Phenylalanine and tyrosine have big aromatic residues also. They have a moderate effect on reaction rate, as does the secondary structure on Pro and His.

From this chemical structure analysis, it may be possible to estimate a qualitative reaction rate of an amino

acid coupling reaction. It should be noticed that the structures of side-chain protecting groups may also bring some diffusional problems to a reaction, and make the structural analysis more complicated. But in general, the chemical structures of amino acids indeed affect the reaction rates. This phenomenon was also observed by Merrifield (1984) and Gut and Davidovich (1976).

The data shown in Figure 51 can be used as a quantitative example of amino acid structure effects. The straight lines in the figure are used to obtain reaction rate constants. The slope of the straight line can be used to express the rate of reaction. A steeper slope means faster reaction. The data come from Tyr-Bradykinin, Bradykinin and Arg_8-Alpha-Neoendorphin, respectively. Three different amino acids are attached to the same peptide fragment and at the same position. They are all at the second position and attached to Arg-resin. The slopes for Tyr and Phe attachments are close. The calculated second order reaction rate constants for these two are 2.34 and 2.27 1/mole s, respectively, at 78 ^{o}F. While the slope of Lys is steeper with a second order rate constant of 4.50 1/mole s. These data show that the reaction rates of Tyr and Phe are similar, while the rate of Lys is faster. If we look at the structures of these amino acids, as shown above, we may see that both Phe and Tyr have an aromatic group on the side chain. Their chemical structures are quite similar. The Lys has an aliphatic group as a side-chain.

Figure 51. Amino Acid Effect on Reaction Rate
(Position 3, 78 °F)

The retardation effect of the aliphatic residue on the
reaction is relatively small compared with an aromatic
residue, as indicated above.

Another example is the comparison between the
attachment of Gly and Leu, as shown in Figure 52. The Gly
is in Tyr_8-Bradykinin. The Leu is in Alpha-Neoendorphin.
Both are at position 6, and both have Phe as the previous
amino terminus. But at 58 ^{O}F, the Gly has a calculated
second order rate constant of 5.150 l/mole s, while the Leu
has a second order rate constant of 2.231 l/mole s. Since
Gly has a smaller side chain (H), and the side chain of Leu
is a bigger aliphatic group, the higher reaction rate of Gly
is expected compared to that of Leu at otherwise the same
conditions.

Peptide Fragment Effect

The effect of chain length on reaction rate was
discussed by Chen (1988). With the chain length increasing,
the reaction rate of homooligopeptides decreases. This may
also be true for real peptide synthesis, but will be more
complex since the properties of the involved reactants have
to be considered. In this experiment, the reaction rate
sometimes decreases with increasing chain length. But in
some other cases this trend is not clear, or even contrary.
This uncertainty may also indicate the complexity of
chemical effects. The chemical effect comes from both amino
acid to be attached, as explained in a previous section, and

Figure 52. Amino Acid Effect on Reaction Rate
(Position 6, 58 °F)

the amino acid anchored to the peptide fragment. Reaction rate constant data in this study can be used to prove this peptide fragment effect, as shown in Figures 53-55. Figure 53 is the reactions of proline in Tyr-Bradykinin at position 3, 7 and 8, respectively. Figure 54 is the reaction of phenylalanine in Arg_8-Alpha-Neoendorphin at position 3 and 5, respectively. While Figure 55 is the reaction of glycine in Beta-Neoendorphin at position 7 and 8, respectively. From Figure 53, the highest reaction rate of proline is at position 7 rather than position 3, although the former has a longer chain length. This phenomenon can be explained by the structures of anchored amino acids. The anchored amino acid for position 3 proline is Phe, while that for position 7 proline is glycine. It has been explained structurally in a previous section that the glycine has a much faster reaction rate than Phe because of a smaller side-chain structure which may have lower diffusional difficulty for the next attachment. As a result, the next amino acid attached, proline here, may be synthesized easily. If this structure helps a lot, as the case discussed here, the observed chain length effect can be even offset. But the chain length effect still exists. The reaction of proline at position 8 has a lower rate than that at position 7. This chain length effect can be further proved from Figure 54. The Phe at position 3 is attached to Lys-peptide fragment, while the Phe at position 5 is attached to Leu-peptide fragment. It has been shown in the last section

Figure 53. Peptide Fragment Effect on Reaction Rate
(Tyr-Bradykinin, Pro)

Figure 54. Peptide Fragment Effect on Reaction Rate
(Arg_8-Alpha-Neoendorphin, Phe)

Figure 55. Peptide Fragment Effect on Reaction Rate
(Beta-Neoendorphin, Gly)

that the chemical structures of Lys and Leu are similar. Their blocking effect to the next reaction is similar, too. Therefore, the reaction rates for the phenylalanine adjacent to them are also similar. But the Phe at position 5 still has a lower rate than that at position 3. This indicates the chain-length effect. With the chain length increasing, the reaction rate decreases. Furthermore, compare the magnitude of reaction rate constants for Phe at these two positions. At position 3, the rate constant is 1.96 l/mole s. At position 5, the rate constant is 1.72 l/mole s. The decrease is small considering the chain length increased by 2 amino acids. This indicates that the properties of the anchored amino acid terminus may have a superior effect on the reaction rate to chain length.

The data shown in Figure 55 can also support the above explanation. The glycine at position 7 is attached to a Phe-peptide fragment, while that at position 8 is attached to a Gly-peptide fragment. Combining the effect of anchored amino terminus (slow-reacted Phe and fast-reacted Gly) and the effect of chain length (shorter chain-length with six amino acids and longer chain length with seven amino acids), the result is close reaction rate constants (4.59 l/mole s at position 7 and 4.55 l/mole s at position 8).

It should be mentioned that the peptides synthesized in this study are relatively short. The data which can be used for explaining the peptide fragment effects are still limited. With longer peptide chain and broader peptide

variety, the phenomenon and the explanation may both be more clear. By the way, the effect of peptide fragment on reaction rate was also noticed by Merrifield (1984). He mentioned that the amino methyl-resin, Ala-resin was more active than the Alpha-amine of an amino acid ester, Val-resin. But his data was very limited and only qualitative, without further explanation.

Polymer Support

The role of polymer support crosslinking has been proved to affect the coupling reaction (Periyasamy and Ford, 1985; Chen, 1988). In these experiments, the resins used have 1% crosslinked DVB since this lower percentage has been shown superior to 2% used in early SPPS (Merrifield, 1986). Generally, the reaction rate by using 1% DVB is higher than that using 2% DVB. While the comparative tests between this study and that of Chen (1988) indicate that the crosslinking percentage affects not only reaction rate but also the observed reaction rate expression. The experiments show that the coupling rate on 1% DVB resins is faster than those with 2% DVB crosslinking. Under the same operation conditions, the coupling time for Phe at some location could exceed 1500 seconds in Chen's experiment, with 2% DVB resin, while the time for Phe in this experiment never exceeded 500 seconds. Although this data comparison does not consider the effect of peptide fragment and other variables, the effect of resin crosslinking is still obvious from this time

difference. This is explained by considering the swelling
properties of different crosslinked resins. Based on Sarin
et al. (1984), the maximum swollen volume of resin with 1%
DVB is about four times that for 2% crosslinked resin. This
swelling was also observed in this study qualitatively. The
data clearly demonstrate that the resin with 1% DVB
crosslinking is superior to that with 2% crosslinking. An
interesting point from this study is that the first order or
second order behavior observed in 1% crosslinked resin
deviates significantly from 2% crosslinked resin. In Chen's
paper, a zero-first shifting order model was used to fit 2%
DVB attachments even though the reaction mechanism was not
clear in his study. This kinetic change can be explained
mechanistically by the addition of a mass transfer
resistance prior to the actual second order reaction. With
lower crosslinkage, this mass transfer resistance is
decreased and the second order or first order reaction model
is validated. Theoretically, at higher symmetrical
anhydride concentration in 2% DVB crosslinked resin, the
shifting order phenomenon should not be observed because of
higher concentration driving force, or chemical potential.
The higher the percentage crosslinking, the higher loading
concentration, also higher solution concentration, is
requested to prevent this shifting order phenomenon. More
data is needed to prove this explanation. However, the
loading concentration is restrained by reactor ability,
solvent property and economic considerations, as mentioned

in previous sections.

Reaction Mechanisms

The second, and occasionally first, order reaction rate
model has been proved in this experiment. The next step is
to explain the model mechanistically. In order to do so, we
need a stoichiometric equation for coupling reactions in SPPS.
This can be expressed as

$$B_2 + A = AB + B \tag{1}$$

where,

B_2 is amino symmetrical anhydride;

A is amino terminus on peptide fragment;

AB is coupling product; and

B is free amino acid monomer released from the

reaction.

From the discussions above, it can be assured that the
intra-particle diffusion cannot be ignored. The reaction
essentially is a liquid-solid two-phase heterogeneous
reaction.

In the coupling reaction of SPPS, it is reasonable to
assume that the reactant is first diffused to the reaction
site. Some active molecule is formed as an intermediate
after the diffusion. Then chemical reaction follows. At
the same time, the active molecule may reversely diffuse
back to the bulk phase. This mechanism can be expressed as
follows:

$$B_2 + A \overset{K_1}{\underset{K_2}{\rightleftharpoons}} B_2A^* \overset{K_3}{\dashrightarrow} AB + B \qquad (2)$$

This series of reactions can be split into several elementary steps. The first step is

$$B_2 + A \overset{K_1}{\dashrightarrow} B_2A^* \qquad (3)$$

The second step is

$$B_2A^* \overset{K_2}{\dashrightarrow} B_2 + A \qquad (4)$$

The third step is

$$B_2A^* \overset{K_3}{\dashrightarrow} AB + B \qquad (5)$$

The Equation 3 can be considered as the formation of energized molecule by diffusion. The Equation 4 means that the intermediate returns to stable form from the active site by reverse diffusion. The Equation 5 is that the active molecule simultaneously decomposes into product which is a peptide with one more anchored amino acid. The free amino acid is also released in this step. In these equations,

K_1 is the diffusion rate in positive direction;

K_2 is the diffusion rate in reverse direction; and

K_3 is the chemical reaction rate constant.

Based on mass balance for individual components, we may write the expression for the rate of disappearance of active amino acid sites on the peptide fragment, A. Since this component is involved in Equation 3 and 4, its overall rate of change is the sum of the individual rates. Notice that the component A is consumed in Equation 3 while it is formed in Equation 4. Thus, if we use a positive sign for formation

and a negative sign for consumption, the rate of change for

component A is

$$r_A = -K_1[B_2][A] + K_2[B_2A^*]$$ (6)

where,

r is rate of change;

subscript A stands for component A; and

[ ] means concentration of a component.

Since the concentration of intermediate B_2A^* is not

measurable, the above rate expression cannot be tested in

its present form. This intermediate should be replaced by

the components that can be measured, such as B_2 and A. In

order to do so, we need to consider the elementary reactions

which involve B_2A^*. It has been found that the intermediate

relates to all three reactions. It is formed from Equation

3; consumed from Equation 4 and 5. Therefore, the overall

rate of change for this intermediate is

$$r_{B_2A^*} = K_1[B_2][A] - K_2[B_2A^*] - K_3[B_2A^*]$$ (7)

Because the rate of change for the intermediate is

always extremely fast, we may consider a steady state

condition, that is, the rate of change for the intermediate

B_2A^* is assumed to be zero. Then the equation is simplified

to

$$0 = K_1[B_2][A] - K_2[B_2A^*] - K_3[B_2A^*]$$ (8)

From this steady-state approximation we get

$$[B_2A^*] = \frac{K_1[B_2][A]}{K_2 + K_3}$$ (9)

The Equation 9 is substituted into Equation 6. The result

is

$$r_A = -K_1[B_2][A] + K_1K_2 \frac{[B_2][A]}{K_2 + K_3} \qquad (10)$$

Equation 10 can be further simplified to give

$$r_A = - \frac{K_1 K_3}{K_2 + K_3} [B_2][A] \qquad (11)$$

If we let the coefficient group in this equation as one

constant, we will have

$$\frac{K_1 K_3}{K_2 + K_3} = K_S \qquad (12)$$

We can see that K_S is nothing but the apparent second order

rate constant observed in this experiment study. In

searching for a reaction rate model consistent with observed

kinetics, it is found that the final form of Equation 12:

$$r_A = -K_S[B_2][A] \qquad (13)$$

is identical with the observed overall second order rate

model. The mechanism proposed thus matches with kinetic

model. Keep in mind that the apparent second order rate

constant K_S in Equation 13 is a function of many factors,

such as temperature, amino acid attached, anchored peptide

terminus, chain length, resin type, etc. These factors

affect the rate of both diffusion and chemical reaction.

Both terms are proved to contribute to the overall rate of

change. The detailed discussion about the function of each

factor has been performed in previous sections.

As a special case, if the concentration of A changes

with time fast, as in some reaction cases, the reactant B_2

may be treated as a constant, while the A will dominate the rate of change. In this situation, Equation 6 may become

$$r_A = - K_1'[A] + K_2[B_2A^*] \tag{14}$$

where K_1' is a combination constant of forward diffusion rate and the relatively slow-changed concentration of B_2. Equation 7 will become

$$r_{B_2A^*} = K_1'[A] - K_2[B_2A^*] - K_3[B_2A^*] \tag{15}$$

Using the same steady-state treatment as before, we will get from Equation 15 that

$$K_1'[A] - K_2[B_2A^*] - K_3[B_2A^*] = 0 \tag{16}$$

Therefore,

$$[B_2A^*] = \frac{K_1'[A]}{K_2 + K_3} \tag{17}$$

By substituting Equation 17 into Equation 14, we end up with

$$r_A = -K_1'[A] + \frac{K_2K_1'[A]}{K_2 + K_3} \tag{18}$$

If, once again, let

$$\frac{K_1'K_3}{K_2 + K_3} = K_f \tag{19}$$

Equation 18 then becomes

$$r_A = -K_f[A] \tag{20}$$

Equation 20 is just the observed first order rate model. K_f is the apparent pseudo first-order rate constant. Again, K_f is a function of those factors mentioned above.

Finally, considering coupling as a constant-volume reaction, we can express the rate change of component A as

$$r_A = \frac{dC_A}{dt} \tag{21}$$

Then the second order rate model, Equation 13, has a conventional form of

$$\frac{dC_A}{dt} = -K_s[B_2][A] \tag{22}$$

While the first order rate model, Equation 20, has a final form of

$$\frac{dC_A}{dt} = -K_f[A] \tag{23}$$

The minus sign here means that the rate of change for component A is negative, or "consumed."

It should be indicated that other mechanisms may also fit the observed kinetic models. A reasonable mechanism should be one by which the experimental phenomenon can be explained chemically.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

A kinetic study has been performed for the production
of Bradykinins, Angiotensins and Endorphins by solid phase
synthesis on polystyrene resin with 1% DVB crosslinking.
Reactor liquid concentrations were continually monitored
using a UV detector and a flow cell. The objectives of this
study were to experimentally determine the factors which may
affect the reaction rate, to derive suitable reaction
models, and to propose mechanisms for these models. Based
on the statistical results of more than four hundred
individual attachments, some conclusions are:

1. Reaction rates show second order behavior primarily
with apparent reaction rate constants between 0.5 and 8
liter/mole s. Some data for fast reactions show first order
behavior with apparent rate constants between 0.04 and 0.09
s^{-1}. Deviating from these rate models occurs only at the
late stages of reaction.

2. The models are overall results for several
individual steps. The reaction is essentially
heterogeneous, and non-elementary. The overall reaction
includes diffusion in forward and reverse directions and
chemical reaction. This mechanism can explain the

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phenomenon of both second and first order observations.

3. Reaction rate is affected by the chemical structure of the amino symmetrical anhydride and the anchored amino terminus, chain length of the peptide fragment, and reactor mixing.

4. Increasing operating temperature promotes reaction. The apparent activation energy ranges from 0.5 to 10 Kcal/mole. This low range indicates that the reaction is not very temperature sensitive. For those reactions which show low temperature sensitivity, increasing temperature may show only a small increase in reaction rate.

5. Increasing initial loading on the resin promotes reaction.

6. As the reaction approaches completion, the mass transfer resistance becomes dominant and causes the reaction to slowdown.

7. Although changing resin crosslinked DVB was not tested in this study, a comparison with previous OSU work shows that the DVB crosslinkage affects the observed reaction rate and the observed reaction mechanism. With increasing DVB percentage, mass transfer resistance becomes significant. Higher symmetrical anhydride concentrations, which produces higher concentration driving force, should reduce this resistance.

8. The UV monitoring technique works for all reactants due to the symmetrical anhydride bond in addition to detectable groups on side-chains or their protecting groups.

This is an extra benefit of using this coupling method.

These experiments improve our understand of SPPS reaction kinetics and guide large-scale reactor design and production. However, since SPPS is a complex process, much additional work needed is on reaction kinetics. Some research directions can also be identified from this study; these are summarized with the following recommendations.

1. The tail-end kinetics should be studied. Although the kinetic models fit the data over a broad range, there are deviations observed when the reaction approaches completion. This tail-end phenomenon may suggest an increased mass transfer resistance. The goal is to find better models to fit these data where mass transfer resistance may be critical. Diffusional models which include particle diffusions and chemical reaction may prove to be helpful.

2. For large-scale production, continuous operation may be attractive. Experimentation on continuous systems should be performed.

3. The peptides synthesized in these experiments are relatively short and chain length effects and steric hindering may be more severe with increased chain length. Therefore, studying longer peptide chains may be of interest.

4. Data over broader operational ranges should be obtained. For example, the initial loading concentration promotes reaction rate, as does increasing temperature.

However, these advantages are restrained by solvent properties. With regard to DCM temperature restrictions, some other solvents may be evaluated.

Since mass transfer resistance is increased with increased DVB crosslinking, this observation is still qualitative. Experimentation with varying DVB crosslinkage can help to quantify diffusional problems.

5. The scaling factors in SPPS should be determined experimentally.

6. The monitoring technique used in this study can only test overall yield of the coupling reaction. And the percentage reacted for an individual reaction is calculated from a global average of total synthesized product. Since the peptide synthesis requires high purity, other instrumentational methods may be considered to monitor desired product yield and quantify reaction completion.

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APPENDIX A

CALIBRATION CURVES

Phe
UV=274, 276, 278

◇ UV=274
+ UV=276
□ UV=278

His
UV=290, 292, 294

◇ UV=290
+ UV=292
□ UV=294

Lys
UV=281, 283, 285

◇ UV=281
+ UV=283
□ UV=285

Gly. Sym.
UV=265, 270 274

His. Sym.
UV=290, 292, 294

Ile, Sym.
UV=272, 274, 276

Leu, Sym.
UV=272, 274, 276

Lys. Sym.
UV=281, 283, 285

◇ UV=281
+ UV=283
□ UV=285

Absorption

Conc (mole/l)

Phe. Sym.
UV=274, 276, 278

◇ UV=274
+ UV=276
□ UV=278

Absorption

Conc (mole/l)

Ser, Sym.
UV=274, 276, 278

Val. Sym
UV=272, 274, 276

APPENDIX B

EXPERIMENTAL DATA AT 78 ^{O}F

Part of the experimental data are presented here. The operational conditions for these data are:

Temperature: 78 ^{O}F

Initial concentration of amino terminus:

0.01482 mole/l

Excess mole ratio, M: 1.5 : 1

Resin: 1 % DVB

The data are listed in Table 22. Y2 is for obtaining second order rate constant, $Y2=Ln[(M-X_A)/(1-X_A)/M]$. Y1 is for obtaining first order rate constant, $Y1=-Ln(1-X_A)$. The X_A represents the amino terminus mole fraction reacted on peptide fragment. n is amino acid position excluding first one.

TABLE 22

Experimental Data at 78 ^{O}F

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
Bradykinin, n=1, Phe			
0	0.000	0.000	
20	0.667	0.512	
30	0.745	0.681	
40	0.818	0.916	
50	0.854	1.085	
60	0.873	1.190	
150	0.960	2.197	
450	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=2, Pro			
0	0.000	0.000	
20	0.665	0.508	
30	0.760	0.721	
40	0.820	0.922	
50	0.871	1.180	
60	0.897	1.361	
130	0.979	2.823	
200	1.000	-----	
n=3, Ser			
0	0.000		0.000
20	0.847		1.879
30	0.875		2.079
40	0.944		2.890
50	0.979		3.871
60	1.000		-----
n=4, Phe			
0	0.000	0.000	
20	0.704	0.585	
30	0.790	0.814	
40	0.869	1.167	
50	0.924	1.618	
100	0.988	3.356	
130	1.000	-----	
n=5, Gly			
0	0.000		0.000
20	0.907		2.380
30	0.963		3.296
40	0.993		4.905
50	1.000		-----
n=6, Pro			
0	0.000	0.000	
20	0.817	0.913	
30	0.909	1.463	
40	0.942	1.857	
50	0.963	2.261	
60	0.975	2.643	
100	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=7, Pro			
0	0.000	0.000	
20	0.529	0.318	
30	0.624	0.440	
40	0.733	0.651	
60	0.838	1.003	
80	0.886	1.276	
130	0.962	2.242	
190	1.000	-----	
n=1, Phe (Duplicated)			
0	0.000	0.000	
20	0.509	0.297	
40	0.710	0.597	
60	0.790	0.812	
100	0.885	1.271	
160	0.942	1.866	
400	1.000	-----	
n=2, Pro			
0	0.000	0.000	
20	0.638	0.462	
40	0.800	0.847	
60	0.874	1.196	
100	0.948	1.950	
130	0.971	2.512	
300	1.000	-----	
n=3, Ser			
0	0.000		0.000
20	0.726		1.293
30	0.860		1.964
40	0.915		2.461
60	0.976		3.714
80	1.000		-----
n=4, Phe			
0	0.000	0.000	
20	0.697	0.569	
30	0.773	0.758	
40	0.827	0.952	
60	0.887	1.289	
140	0.965	2.331	
300	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=5, Gly			
0	0.000		0.000
20	0.813		1.679
30	0.896		2.259
40	0.951		3.026
50	0.970		3.512
70	1.000		-----
n=7, Pro			
0	0.000	0.000	
30	0.550	0.341	
40	0.673	0.521	
60	0.784	0.792	
100	0.883	1.258	
130	0.936	1.776	
250	1.000	-----	
Tyr-Bradykinin, n=1, Phe			
0	0.000	0.000	
30	0.723	0.625	
40	0.783	0.790	
60	0.866	1.151	
80	0.901	1.394	
220	0.986	3.172	
510	1.000	-----	
n=2, Pro			
0	0.000	0.000	
30	0.700	0.575	
40	0.798	0.839	
50	0.867	1.153	
80	0.944	1.897	
200	1.000	-----	
n=3, Ser			
0	0.000		0.000
35	0.834		1.797
45	0.904		2.342
55	0.939		2.800
65	0.961		3.253
120	1.000		-----

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=4, Phe			
0	0.000	0.000	
20	0.683	0.541	
40	0.860	1.115	
60	0.921	1.583	
110	0.979	2.811	
270	1.000	-----	
n=5, Gly			
0	0.000		0.000
25	0.851		1.906
30	0.905		2.358
40	0.986		4.304
50	1.000		-----
n=6, Pro			
0	0.000	0.000	
30	0.876	1.212	
40	0.914	1.527	
50	0.945	1.900	
90	0.974	2.600	
210	1.000	-----	
n=7, Pro			
0	0.000	0.000	
30	0.578	0.376	
50	0.696	0.567	
80	0.797	0.838	
120	0.865	1.142	
190	0.932	1.723	
430	1.000	-----	
Tyr_8-Bradykinin, n=1, Tyr			
0	0.000	0.000	
20	0.570	0.365	
40	0.769	0.746	
60	0.850	1.058	
80	0.891	1.312	
150	0.961	2.231	
410	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=2, Pro			
0	0.000	0.000	
20	0.764	0.732	
30	0.829	0.961	
50	0.941	1.843	
80	0.973	2.582	
200	1.000	-----	
n=3, Ser			
0	0.000		0.000
20	0.650		1.051
25	0.741		1.352
30	0.818		1.705
40	0.944		2.883
50	1.000		-----
n=4, Phe			
0	0.000	0.000	
20	0.752	0.697	
30	0.847	1.044	
40	0.902	1.402	
60	0.936	1.765	
80	0.966	2.356	
310	1.000	-----	
n=5, Gly			
0	0.000		0.000
25	0.853		1.920
30	0.913		2.446
35	0.960		3.219
50	0.993		5.011
60	1.000		-----
n=6, Pro			
0	0.000	0.000	
20	0.706	0.589	
30	0.797	0.837	
40	0.881	1.241	
60	0.940	1.833	
80	0.964	2.300	
270	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=7, Pro			
0	0.000	0.000	
30	0.544	0.335	
40	0.680	0.535	
60	0.765	0.735	
100	0.851	1.064	
200	0.943	1.875	
400	1.000	-----	
n=1, Tyr (Duplicated)			
0	0.000	0.000	
20	0.770	0.748	
30	0.826	0.950	
50	0.879	1.227	
100	0.938	1.793	
310	1.000	-----	
n=2, Pro			
0	0.000	0.000	
20	0.847	1.045	
30	0.907	1.443	
40	0.940	1.832	
60	0.969	2.447	
200	1.000	-----	
n=3, Ser			
0	0.000		0.000
20	0.807		1.646
30	0.922		2.547
40	0.970		3.503
50	0.988		4.419
60	1.000		-----
n=4, Phe			
0	0.000	0.000	
20	0.681	0.538	
40	0.865	1.144	
60	0.921	1.588	
80	0.949	1.978	
120	0.984	3.083	
300	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
Alpha-Neoendorphin, n=1, Pro			
0	0.000	0.000	
20	0.721	0.621	
30	0.839	1.009	
40	0.906	1.441	
50	0.930	1.689	
100	0.978	2.773	
240	1.000	-----	
n=2, Tyr			
0	0.000	0.000	
30	0.844	1.032	
40	0.896	1.355	
50	0.935	1.758	
60	0.958	2.162	
110	0.990	3.489	
200	1.000	-----	
n=3, Lys			
0	0.000	0.000	
30	0.826	0.948	
40	0.866	1.152	
50	0.914	1.517	
60	0.942	1.858	
140	0.972	2.546	
260	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.814	0.900	
40	0.897	1.362	
60	0.947	1.944	
150	0.972	2.544	
260	1.000	-----	
n=5, Leu			
0	0.000	0.000	
20	0.809	0.882	
30	0.880	1.238	
40	0.917	1.549	
50	0.939	1.815	
100	0.980	2.970	
180	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=6, Phe			
0	0.000	0.000	
30	0.850	1.060	
50	0.910	1.473	
80	0.958	2.151	
100	0.974	2.586	
230	1.000	-----	
n=7, Gly			
0	0.000	0.000	
40	0.967	2.380	
50	0.980	2.865	
120	1.000	-----	
n=8, Gly			
0	0.000	0.000	
30	0.820	0.923	
50	0.886	1.278	
60	0.919	1.564	
80	0.949	1.974	
140	0.976	2.677	
290	1.000	-----	
n=9, Tyr			
0	0.000	0.000	
20	0.255	0.108	
40	0.516	0.304	
80	0.707	0.591	
120	0.803	1.626	
200	0.904	1.423	
350	0.957	2.140	
500	1.000	-----	
n=1, Pro (Duplicated)			
0	0.000	0.000	
20	0.817	0.910	
30	0.880	1.240	
40	0.932	1.718	
60	0.970	2.470	
150	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=6, Phe			
0	0.000	0.000	
20	0.730	0.642	
30	0.834	0.983	
50	0.928	1.667	
60	0.942	1.856	
130	0.983	3.031	
260	1.000	-----	
n=8, Gly			
0	0.000	0.000	
30	0.918	1.557	
40	0.961	2.216	
50	0.977	2.724	
120	1.000	-----	
n=9, Tyr			
0	0.000	0.000	
40	0.643	0.471	
60	0.738	0.662	
90	0.811	0.889	
140	0.874	1.198	
200	0.934	1.738	
680	1.000	-----	
Bata-Neoendorphin, n=1, Tyr			
0	0.000	0.000	
20	0.628	0.447	
40	0.790	0.813	
60	0.841	1.015	
200	0.937	1.785	
570	1.000	-----	
n=2, Lys			
0	0.000	0.000	
20	0.806	0.870	
30	0.892	1.321	
40	0.967	2.383	
80	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=3, Pro			
0	0.000	0.000	
20	0.644	0.472	
40	0.863	1.131	
60	0.912	1.495	
100	0.955	2.089	
300	1.000	-----	
n=4, Leu			
0	0.000	0.000	
20	0.679	0.533	
40	0.818	0.916	
60	0.903	1.312	
70	0.918	1.556	
150	0.961	2.211	
235	1.000	-----	
n=5, Phe			
0	0.000	0.000	
20	0.689	0.552	
40	0.841	1.018	
60	0.913	1.503	
80	0.948	1.953	
300	1.000	-----	
n=6, Gly			
0	0.000	0.000	
20	0.839	1.009	
30	0.905	1.430	
40	0.971	2.492	
50	1.000	-----	
n=7, Gly			
0	0.000	0.000	
20	0.823	0.936	
30	0.896	1.352	
40	0.938	1.792	
70	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=8, Tyr			
0	0.000	0.000	
20	0.557	0.350	
30	0.715	0.609	
50	0.846	1.038	
60	1.000	-----	
n=3, Pro (Duplicated)			
0	0.000	0.000	
20	0.698	0.571	
40	0.873	1.188	
50	0.899	1.375	
70	0.936	1.775	
300	1.000	-----	
n=4, Leu			
0	0.000	0.000	
20	0.727	0.635	
40	0.893	1.328	
60	0.934	1.745	
150	1.000	-----	
n=5, Phe			
0	0.000	0.000	
20	0.658	0.495	
40	0.862	1.123	
80	0.951	2.009	
150	0.991	3.638	
230	1.000	-----	
n=8, Tyr			
0	0.000	0.000	
20	0.441	0.233	
40	0.750	0.693	
60	0.888	1.294	
80	0.924	1.624	
330	0.967	2.380	
560	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=3, Pro (Duplicated)			
0	0.000	0.000	
20	0.699	0.573	
40	0.865	1.140	
70	0.930	1.689	
180	0.983	3.025	
360	1.000	-----	
n=4, Leu			
0	0.000	0.000	
20	0.666	0.509	
40	0.869	1.164	
60	0.922	1.598	
80	0.951	2.011	
150	1.000	-----	
n=5, Phe			
0	0.000	0.000	
20	0.721	0.621	
40	0.913	1.501	
60	0.951	2.013	
120	1.000	-----	
[Arg_8]-Alpha-Neoendorphin, n=1, Lys			
0	0.000	0.000	
25	0.773	0.758	
40	0.899	1.378	
60	0.949	1.983	
160	1.000	-----	
n=2, Phe			
0	0.000	0.000	
30	0.674	0.524	
40	0.739	0.664	
70	0.823	0.936	
130	0.904	1.422	
240	1.000	-----	
n=3, Leu			
0	0.000	0.000	
25	0.810	0.883	
40	0.893	1.464	
80	0.941	1.843	
250	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=4, Phe			
0	0.000	0.000	
20	0.522	0.311	
40	0.729	0.641	
80	0.868	1.159	
200	0.974	2.617	
300	1.000	-----	
n=5, Gly			
0	0.000		0.000
30	0.754		1.404
50	0.947		2.944
70	0.965		3.350
190	1.000		-----
n=6, Gly			
0	0.000		0.000
20	0.745		1.366
30	0.847		1.877
40	0.949		2.976
50	1.000		-----
n=7, Tyr			
0	0.000	0.000	
30	0.808	0.878	
40	0.895	1.344	
50	0.992	3.807	
60	1.000	-----	
n=4, Phe (Duplicated)			
0	0.000	0.000	
20	0.477	0.266	
40	0.671	0.518	
60	0.777	0.771	
100	0.860	1.117	
300	0.971	2.493	
530	1.000	-----	
[Val$_4$]-Angiotensin III, n=1, Pro			
0	0.000	0.000	
20	0.618	0.431	
30	0.786	0.799	
50	0.901	1.397	
90	0.954	2.064	
300	1.000	-----	

TABLE 22 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=2, His			
0	0.000	0.000	
20	0.713	0.602	
30	0.869	1.164	
50	0.942	1.857	
180	1.000	-----	
n=3, Val			
0	0.000	0.000	
20	0.697	0.569	
30	0.807	0.873	
50	0.899	1.377	
120	0.963	2.276	
270	1.000	-----	
n=4, Tyr			
0	0.000	0.000	
20	0.794	9.825	
30	0.922	1.594	
50	0.975	2.642	
120	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.380	0.186	
40	0.581	0.380	
60	0.659	0.497	
120	0.795	0.830	
300	0.938	1.801	
660	1.000	-----	
Angiotensin III, n=1, Pro			
0	0.000	0.000	
20	0.719	0.616	
40	0.903	1.408	
60	0.946	1.921	
120	0.987	3.271	
210	1.000	-----	
n=2, His			
0	0.000	0.000	
20	0.741	0.669	
40	0.919	1.564	
60	0.935	1.759	
270	1.000	-----	

TABLE 22 (CONTINUED)

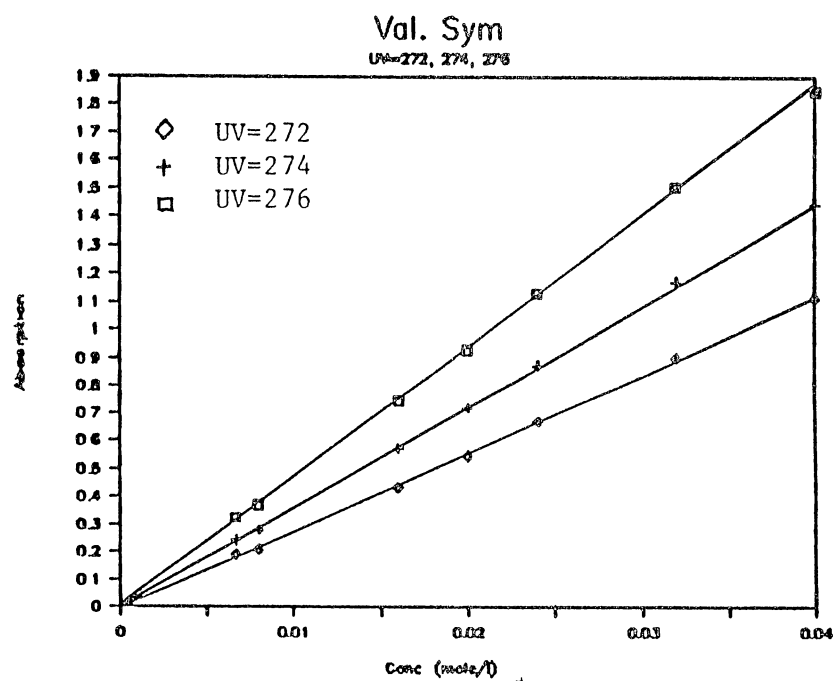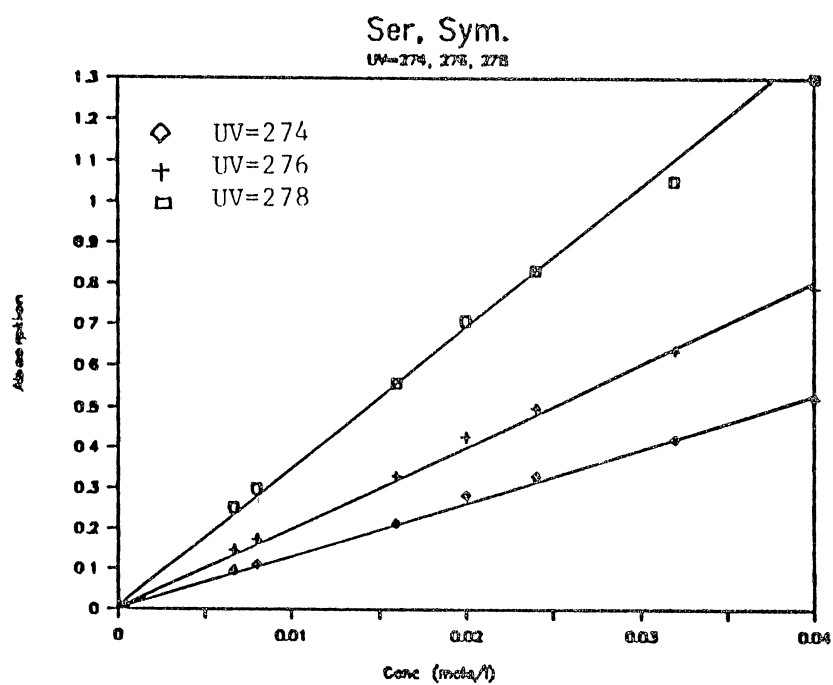| Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=3, Ile			
0	0.000	0.000	
20	0.611	0.421	
40	0.812	0.893	
60	0.858	1.103	
120	0.924	1.618	
480	1.000	-----	
n=4, Tyr			
0	0.000	0.000	
20	0.730	0.641	
30	0.885	1.273	
40	0.943	1.868	
90	0.984	3.045	
150	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.332	0.153	
40	0.543	0.333	
90	0.708	0.593	
120	0.763	0.730	
360	0.943	1.876	
720	1.000	-----	

APPENDIX C

EXPERIMENTAL DATA AT 58 ^{O}F

Part of the experimental data are presented here. The operational conditions for these data are:

    Temperature: 58 ^{O}F

    Initial concentration of amino terminus:

                              0.01482 mole/l

    Excess mole ratio: 1.5 : 1

    Resin: 1 % DVB

The data are listed in Table 23. Y2 is for obtaining second order rate constant, $Y2=Ln[(M-X_A)/(1-X_A)/M]$. Y1 is for obtaining first order rate constant, $Y1=-Ln(1-X_A)$. The X_A represents the amino terminus mole fraction reacted on peptide fragment. n is amino acid position excluding first one.

TABLE 23

Experimental Data at 58 ^{O}F

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
Bradykinin, n=1, Phe			
0	0.000	0.000	
20	0.540	0.332	
40	0.701	0.578	
60	0.781	0.782	
130	0.888	1.296	
300	0.966	2.344	
550	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=2, Pro			
0	0.000	0.000	
30	0.595	0.399	
50	0.763	0.729	
80	0.853	1.078	
130	0.927	1.650	
250	0.979	2.807	
400	1.000	-----	
n=3, Ser			
0	0.000	0.000	
20	0.599	0.404	
40	0.841	1.014	
60	0.894	1.336	
100	0.966	2.354	
150	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.689	0.552	
40	0.862	1.129	
60	0.923	1.614	
100	0.972	2.537	
160	1.000	-----	
n=5, Gly			
0	0.000	0.000	
20	0.704	0.583	
40	0.869	1.169	
60	0.910	1.483	
130	0.975	2.621	
200	1.000	-----	
n=6, Pro			
0	0.000	0.000	
20	0.668	0.513	
40	0.858	1.103	
60	0.906	1.441	
160	0.982	2.947	
290	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=7, Pro			
0	0.000	0.000	
20	0.397	0.198	
40	0.595	0.398	
80	0.734	0.653	
160	0.848	1.051	
250	0.916	1.530	
600	1.000	-----	
n=1, Phe (Duplicated)			
0	0.000	0.000	
20	0.522	0.310	
40	0.753	0.701	
80	0.907	1.443	
150	0.978	2.762	
230	1.000	-----	
n=2, Pro			
0	0.000	0.000	
20	0.535	0.324	
40	0.679	0.534	
80	0.837	0.999	
130	0.916	1.536	
200	0.970	2.459	
340	1.000	-----	
n=4, Phe			
0	0.000	0.000	
35	0.814	0.898	
50	0.896	1.355	
80	0.943	1.880	
130	0.983	3.038	
200	1.000	-----	
n=5, Gly			
0	0.000	0.000	
20	0.710	0.596	
40	0.898	1.367	
60	0.956	2.102	
140	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=6, Pro			
0	0.000	0.000	
20	0.606	0.414	
40	0.869	1.165	
60	0.931	1.707	
120	0.975	2.639	
240	1.000	-----	
n=7, Pro			
0	0.000	0.000	
20	0.379	0.185	
40	0.684	0.542	
60	0.774	0.762	
120	0.921	1.585	
240	1.000	-----	
n=1, Phe (Duplicated)			
0	0.000	0.000	
20	0.421	0.217	
40	0.680	0.536	
60	0.767	0.740	
120	0.877	1.220	
240	0.969	2.428	
420	1.000	-----	
n=2, Pro			
0	0.000	0.000	
20	0.470	0.259	
40	0.713	0.603	
60	0.783	0.790	
120	0.900	1.386	
240	0.972	2.543	
390	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.562	0.356	
40	0.824	0.940	
60	0.899	1.378	
120	0.969	2.433	
240	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1

Tyr-Bradykinin, n=1, Phe

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
0	0.000	0.000	
20	0.493	0.281	
40	0.678	0.531	
60	0.745	0.681	
100	0.821	0.929	
200	0.903	1.416	
400	0.961	2.220	
760	1.000	-----	

n=2, Pro

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
0	0.000	0.000	
20	0.525	0.314	
40	0.714	0.606	
80	0.846	1.041	
130	0.912	1.495	
200	0.960	2.207	
440	1.000	-----	

n=3, Ser

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
0	0.000	0.000	
20	0.689	0.553	
40	0.850	1.060	
60	0.896	1.357	
150	0.979	2.818	
230	1.000	-----	

n=4, Phe

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
0	0.000	0.000	
30	0.611	0.422	
60	0.827	0.953	
100	0.919	1.569	
160	0.974	2.599	
280	1.000	-----	

n=5, Gly

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
0	0.000	0.000	
20	0.618	0.431	
40	0.865	1.141	
60	0.917	1.551	
80	0.959	2.170	
160	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=6, Pro			
0	0.000	0.000	
20	0.552	0.344	
40	0.840	1.012	
60	0.913	1.502	
90	0.951	2.003	
230	1.000	-----	
n=7, Pro			
0	0.000	0.000	
20	0.384	0.189	
40	0.687	0.549	
60	0.758	0.716	
120	0.872	1.185	
180	0.929	1.678	
280	1.000	-----	
n=8, Phe			
0	0.000	0.000	
20	0.771	0.752	
40	0.916	1.537	
60	0.952	2.021	
150	1.000	-----	
n=9, Tyr			
0	0.000	0.000	
20	0.622	0.437	
40	0.794	0.826	
60	0.880	1.238	
100	0.944	1.887	
200	1.000	-----	
n=1, Phe (Duplicated)			
0	0.000	0.000	
20	0.395	0.197	
40	0.660	0.499	
60	0.749	0.690	
130	0.868	1.158	
200	0.927	1.654	
400	0.977	2.726	
540	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=2, Pro			
0	0.000	0.000	
20	0.393	0.195	
40	0.655	0.490	
60	0.754	0.704	
120	0.885	1.271	
240	0.965	2.328	
420	1.000	-----	
n=3, Ser			
0	0.000	0.000	
20	0.557	0.350	
40	0.807	0.874	
60	0.880	1.238	
100	0.964	2.283	
150	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.497	0.285	
40	0.805	0.865	
60	0.890	1.310	
90	0.944	1.888	
120	0.973	2.575	
210	1.000	-----	
n=5, Gly			
0	0.000	0.000	
20	0.566	0.361	
40	0.846	1.040	
60	0.907	1.445	
120	0.968	2.398	
220	1.000	-----	
n=9, Tyr			
0	0.000	0.000	
20	0.535	0.324	
40	0.893	1.330	
60	0.940	1.821	
90	0.972	2.535	
150	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=3, Ser (Duplicated)			
0	0.000	0.000	
20	0.543	0.333	
40	0.819	0.920	
60	0.889	1.303	
90	0.950	1.988	
120	0.975	2.634	
170	1.000	-----	
Tyr$_8$-Bradykinin, n=1, Tyr			
0	0.000	0.000	
15	0.417	0.214	
35	0.667	0.511	
55	0.760	0.721	
85	0.833	0.981	
105	0.880	1.237	
205	0.953	2.055	
390	1.000	-----	
n=2, Pro			
0	0.000	0.000	
25	0.698	0.572	
45	0.791	0.817	
65	0.854	1.080	
105	0.916	1.538	
165	0.958	2.157	
405	1.000	-----	
n=3, Ser			
0	0.000	0.000	
15	0.550	0.342	
35	0.820	0.924	
55	0.904	1.427	
105	0.973	2.585	
165	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.558	0.351	
40	0.849	1.057	
60	0.927	1.657	
120	0.985	3.126	
210	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=5, Gly			
0	0.000	0.000	
20	0.704	0.583	
40	0.920	1.578	
60	0.963	2.269	
90	0.983	3.004	
180	1.000	-----	
n=6, Pro			
0	0.000	0.000	
15	0.464	0.253	
35	0.760	0.720	
55	0.842	1.021	
85	0.901	1.397	
135	0.957	2.136	
325	1.000	-----	
n=7, Pro			
0	0.000	0.000	
25	0.550	0.342	
45	0.687	0.550	
65	0.756	0.709	
105	0.825	0.943	
165	0.887	1.283	
255	0.945	1.906	
455	1.000	-----	
n=1, Tyr (Duplicated)			
0	0.000	0.000	
15	0.448	0.240	
35	0.724	0.629	
55	0.821	0.926	
85	0.900	1.386	
165	0.983	2.996	
205	1.000	-----	
n=2, Pro			
0	0.000	0.000	
20	0.508	0.296	
40	0.775	0.766	
60	0.856	1.094	
90	0.919	1.565	
180	0.982	2.942	
300	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=7, Pro			
0	0.000	0.000	
20	0.104	0.204	
40	0.702	0.580	
60	0.770	0.750	
120	0.881	1.213	
180	0.945	1.901	
300	1.000	------	
n=1, Tyr (Duplicated)			
0	0.000	0.000	
20	0.335	0.155	
40	0.641	0.467	
60	0.757	0.713	
120	0.913	1.500	
240	0.981	2.881	
330	1.000	------	
Alpha-Neoendorphin, n=1, Pro			
0	0.000	0.000	
20	0.702	0.560	
40	0.882	1.253	
60	0.934	1.743	
60	0.960	2.191	
200	1.000	------	
n=2, Tyr			
0	0.000	0.000	
20	0.642	0.469	
40	0.889	1.299	
60	0.946	1.917	
120	0.983	2.993	
280	1.000	------	
n=3, Lys			
0	0.000	0.000	
20	0.518	0.306	
40	0.790	0.812	
60	0.899	1.374	
90	0.971	2.485	
180	1.000	------	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=4, Phe			
0	0.000	0.000	
20	0.594	0.397	
40	0.728	0.638	
60	0.886	1.280	
120	0.944	1.888	
420	1.000	-----	
n=5, Leu			
0	0.000	0.000	
20	0.505	0.293	
40	0.733	0.650	
60	0.841	1.018	
120	0.959	2.177	
300	1.000	-----	
n=6, Phe			
0	0.000	0.000	
25	0.725	0.631	
45	0.849	1.055	
65	0.888	1.294	
105	0.929	1.679	
375	1.000	-----	
n=7, Gly			
0	0.000		0.000
30	0.680		1.139
50	0.888		2.185
60	0.925		2.590
120	0.978		3.794
220	1.000		-----
n=8, Gly			
0	0.000	0.000	
20	0.734	0.653	
40	0.844	1.030	
60	0.906	1.440	
100	0.938	1.792	
290	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=9, Tyr			
0	0.000	0.000	
20	0.524	0.313	
40	0.752	0.699	
60	0.821	0.926	
120	0.937	1.789	
240	0.994	4.080	
300	1.000	-----	
n=1, Pro (Duplicated)			
0	0.000	0.000	
20	0.733	0.649	
40	0.917	1.546	
60	0.952	2.024	
120	0.981	2.904	
240	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.501	0.288	
40	0.735	0.655	
60	0.891	1.313	
120	0.960	2.194	
380	1.000	-----	
n=6, Phe			
0	0.000	0.000	
20	0.574	0.371	
40	0.797	0.836	
60	0.876	1.213	
120	0.945	1.910	
320	1.000	-----	
n=7, Gly			
0	0.000		0.000
20	0.548		0.794
40	0.819		1.710
60	0.921		2.537
120	0.972		3.567
240	1.000		-----

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=8, Gly			
0	0.000	0.000	
20	0.705	0.587	
40	0.872	1.182	
60	0.912	1.492	
120	0.962	2.250	
290	1.000	-----	
n=9, Tyr			
0	0.000	0.000	
20	0.526	0.315	
40	0.779	0.776	
60	0.839	1.007	
120	0.943	1.867	
290	1.000	-----	
n=8, Gly (Duplicated)			
0	0.000	0.000	
20	0.571	0.367	
40	0.779	0.778	
60	0.858	1.104	
180	0.956	2.106	
360	1.000	-----	
Beta-Neoendorphin, n=1, Tyr			
0	0.000	0.000	
20	0.625	0.442	
40	0.797	0.837	
60	0.868	1.160	
100	0.939	1.809	
200	0.991	3.584	
250	1.000	-----	
n=2, Lys			
0	0.000	0.000	
20	0.703	0.582	
40	0.901	1.396	
60	0.956	2.110	
80	0.985	3.106	
140	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=3, Pro			
0	0.000	0.000	
20	0.756	0.709	
40	0.883	1.256	
60	0.926	1.641	
100	0.971	2.489	
200	1.000	-----	
n=4, Leu			
0	0.000	0.000	
20	0.813	0.895	
40	0.931	0.701	
60	0.967	2.367	
130	1.000	-----	
n=5, Phe			
0	0.000	0.000	
20	0.680	0.535	
40	0.815	0.902	
60	0.865	1.142	
100	0.921	1.585	
200	0.981	2.887	
370	1.000	-----	
n=6, Gly			
0	0.000	0.000	
20	0.811	0.889	
40	0.937	1.786	
50	0.959	2.177	
80	0.984	3.085	
120	1.000	-----	
n=7, Gly			
0	0.000	0.000	
20	0.774	0.760	
40	0.903	1.414	
60	0.947	1.932	
120	0.990	3.509	
200	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=8, Tyr			
0	0.000	0.000	
20	0.646	0.475	
40	0.802	0.853	
60	0.858	1.103	
90	0.914	1.516	
120	0.952	2.025	
240	1.000	-----	
n=1, Tyr (Duplicated)			
0	0.000	0.000	
50	0.765	0.734	
80	0.807	0.873	
200	0.899	1.374	
400	0.965	2.331	
600	1.000	-----	
n=2, Lys			
0	0.000	0.000	
20	0.729	0.639	
40	0.843	1.026	
60	0.883	1.255	
100	0.940	1.826	
240	1.000	-----	
n=3, Pro			
0	0.000	0.000	
20	0.683	0.541	
40	0.813	0.894	
60	0.861	1.119	
100	0.924	1.619	
200	0.978	2.750	
310	1.000	-----	
n=4, Leu			
0	0.000	0.000	
20	0.637	0.461	
40	0.799	0.845	
60	0.846	1.041	
130	0.931	1.708	
280	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=5, Phe			
0	0.000	0.000	
20	0.625	0.442	
40	0.769	0.747	
60	0.830	0.965	
90	0.885	1.268	
180	0.952	2.037	
420	1.000	-----	
n=6, Gly			
0	0.000	0.000	
20	0.761	0.725	
40	0.873	1.188	
60	0.922	1.593	
100	0.964	2.297	
200	1.000	-----	
n=6, Gly			
0	0.000	0.000	
20	0.747	0.684	
40	0.884	1.265	
60	0.925	1.626	
120	0.978	2.780	
240	1.000	-----	
[Arg$_8$]-Alpha-Neoendorphin, n=1, Lys			
0	0.000	0.000	
20	0.444	0.236	
40	0.698	0.571	
60	0.791	0.815	
120	0.930	1.695	
240	0.988	3.378	
300	1.000	-----	
n=2, Phe			
0	0.000	0.000	
20	0.493	0.281	
40	0.733	0.651	
60	0.828	0.958	
120	0.938	1.804	
240	0.980	2.861	
380	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1

n=3, Leu

0	0.000	0.000	
20	0.396	0.198	
40	0.629	0.447	
60	0.746	0.684	
120	0.871	1.181	
240	0.929	1.674	
300	0.975	2.639	
450	1.000	-----	

n=4, Phe

0	0.000	0.000	
20	0.403	0.203	
40	0.652	0.485	
60	0.751	0.697	
120	0.875	1.203	
300	0.947	1.933	
600	1.000	-----	

n=5, Gly

0	0.000	0.000	
20	0.594	0.397	
40	0.831	0.970	
60	0.891	1.315	
120	0.966	2.353	
260	1.000	-----	

n=6, Gly

0	0.000		0.000
20	0.454		0.604
40	0.794		1.579
60	0.907		2.377
90	0.959		3.188
180	1.000		-----

n=7, Tyr

0	0.000	0.000	
20	0.549	0.340	
40	0.878	1.224	
60	0.933	1.729	
90	0.951	2.015	
150	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=1, Lys (Duplicated)			
0	0.000	0.000	
20	0.469	0.258	
40	0.755	0.706	
60	0.853	1.076	
90	0.918	1.557	
160	1.000	-----	
n=2, Phe			
0	0.000	0.000	
20	0.484	0.272	
40	0.721	0.621	
60	0.787	0.802	
120	0.883	1.255	
240	0.957	2.138	
400	1.000	-----	
n=3, Leu			
0	0.000	0.000	
20	0.405	0.204	
40	0.686	0.547	
90	0.868	1.159	
120	0.913	1.506	
240	0.979	2.821	
340	1.000	-----	
n=4, Phe			
0	0.000	0.000	
20	0.462	0.251	
40	0.711	0.599	
60	0.794	0.827	
120	0.886	1.276	
180	0.950	1.994	
300	0.977	1.994	
450	1.000	-----	
n=5, Gly			
0	0.000	0.000	
20	0.685	0.545	
40	0.863	1.131	
60	0.909	1.462	
90	0.950	1.988	
150	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=6, Gly			
0	0.000		0.000
20	0.467		0.629
40	0.803		1.623
60	0.901		2.316
120	0.967		3.414
210	1.000		-----
n=7, Tyr			
0	0.000	0.000	
20	0.444	0.236	
40	0.765	0.736	
60	0.858	1.103	
90	0.932	1.718	
180	1.000	-----	
n=1, Lys (Duplicated)			
0	0.000	0.000	
20	0.449	0.240	
40	0.720	0.618	
60	0.804	0.861	
120	0.925	1.634	
180	0.972	2.530	
300	1.000	-----	
n=2, Phe			
0	0.000	0.000	
20	0.565	0.360	
40	0.744	0.678	
60	0.802	0.856	
120	0.888	1.295	
240	0.953	2.058	
480	1.000	-----	
n=3, Leu			
0	0.000	0.000	
20	0.530	0.319	
40	0.755	0.705	
60	0.818	0.916	
90	0.882	1.249	
180	0.958	2.143	
360	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=4, Phe			
0	0.000	0.000	
20	0.438	0.231	
40	0.702	0.580	
60	0.790	0.812	
120	0.891	1.312	
240	0.956	2.114	
440	1.000	-----	
n=6, Gly			
0	0.000		0.000
20	0.568		0.839
40	0.893		2.240
60	0.941		2.827
90	0.970		3.520
180	1.000		-----
[Val$_4$]-Angiotensin III, n=1, Pro			
0	0.000	0.000	
20	0.643	0.471	
40	0.844	1.029	
60	0.894	1.336	
150	0.954	2.067	
400	0.984	3.066	
600	1.000	-----	
n=2, His			
0	0.000	0.000	
20	0.675	0.526	
40	0.852	1.069	
60	0.896	1.356	
100	0.916	1.537	
200	0.967	2.377	
430	1.000	-----	
n=3, Val			
0	0.000	0.000	
20	0.595	0.399	
40	0.756	0.708	
60	0.804	0.862	
160	0.907	1.445	
300	0.957	2.132	
530	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=4, Tyr			
0	0.000	0.000	
20	0.742	0.672	
40	0.893	1.329	
50	0.915	1.522	
100	0.964	2.303	
250	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.470	0.259	
40	0.532	0.321	
80	0.616	0.429	
250	0.832	0.975	
400	0.911	1.485	
500	0.947	1.944	
670	1.000	-----	
n=1, Pro (Duplicated)			
0	0.000	0.000	
20	0.660	0.500	
40	0.840	1.009	
60	0.901	1.397	
80	0.932	1.718	
160	0.984	3.041	
250	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.305	0.136	
60	0.602	0.408	
120	0.728	0.638	
360	0.903	1.411	
480	0.940	1.825	
600	0.963	2.272	
870	1.000	-----	
Angiotensin III, n=1, Pro			
0	0.000	0.000	
20	0.650	0.482	
50	0.830	0.968	
80	0.896	1.351	
100	0.920	1.571	
200	0.972	2.522	
400	1.000	-----	

TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=2, His			
0	0.000	0.000	
20	0.664	0.506	
40	0.820	0.922	
60	0.881	1.245	
240	0.961	2.222	
480	1.000	-----	
n=3, Ile			
0	0.000	0.000	
30	0.737	0.660	
90	0.944	2.887	
200	0.992	3.750	
350	1.000	-----	
n=4, Tyr			
0	0.000	0.000	
20	0.816	0.909	
30	0.873	1.191	
60	0.915	1.526	
160	0.980	2.864	
260	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.281	0.122	
60	0.611	0.421	
140	0.788	0.807	
200	0.849	1.053	
300	0.900	1.388	
500	0.964	2.291	
700	1.000	-----	
n=1, Pro (Duplicated)			
0	0.000	0.000	
20	0.710	0.596	
40	0.871	1.179	
60	0.914	1.513	
120	0.959	2.178	
300	1.000	-----	

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TABLE 23 (CONTINUED)

Time (Sec.)	Mole Fraction Reacted	Y2	Y1
n=4, Tyr			
0	0.000	0.000	
20	0.833	0.981	
40	0.933	1.735	
50	0.963	2.278	
120	0.983	3.029	
180	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.361	0.173	
40	0.565	0.360	
90	0.750	0.693	
180	0.850	1.061	
240	0.889	1.299	
600	0.974	2.604	
810	1.000	-----	

APPENDIX D

EXPERIMENTAL DATA AT OTHER CONDITIONS

Part of the experiment results with different operational conditions other than those presented in Appendix B and Appendix C are listed here in Table 24.

TABLE 24

Experimental Results at Other Conditions

Time (sec)	Reacted Mole Fraction	Y2	Y2
Angiotensin III, Temperature = 45 ^{O}F			
n=1, Pro			
0	0.000	0.000	
20	0.571	0.368	
40	0.806	0.868	
60	0.869	1.164	
90	0.926	1.640	
180	0.981	2.900	
300	1.000	-----	
n=2, His			
0	0.000	0.000	
20	0.709	0.594	
40	0.924	1.624	
60	0.945	1.909	
180	1.000	-----	

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TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2
n=3, Ile			
0	0.000	0.000	
20	0.399	0.200	
40	0.674	0.524	
60	0.733	0.649	
90	0.788	0.805	
180	0.862	1.128	
600	0.961	2.214	
1080	1.000	-----	
n=4, Tyr			
0	0.000	0.000	
20	0.666	0.509	
40	0.863	1.130	
60	0.916	1.538	
120	0.970	2.471	
300	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.305	0.137	
40	0.528	0.317	
60	0.605	0.413	
120	0.735	0.654	
240	0.821	0.927	
360	0.894	1.338	
600	0.960	2.192	
960	1.000	-----	

Angiotensin III, Temperature = 95 $^{\circ}$F

n=1, Pro			
0	0.000	0.000	
20	0.676	0.527	
40	0.898	1.368	
50	0.920	1.576	
120	0.964	2.307	
250	1.000	-----	

TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2
n=2, His			
0	0.000	0.000	
20	0.738	0.662	
40	0.925	1.633	
60	0.959	2.184	
150	1.000	-----	
n=3, Ile			
0	0.000	0.000	
20	0.638	0.463	
40	0.888	1.295	
60	0.929	1.680	
120	0.975	2.646	
270	1.000	-----	
n=4, Tyr			
0	0.000	0.000	
20	0.743	0.675	
40	0.941	1.839	
60	0.980	2.864	
90	1.000	-----	
n=5, Val			
0	0.000	0.000	
20	0.327	0.150	
40	0.606	0.414	
60	0.707	0.591	
120	0.862	1.127	
300	0.978	2.745	
420	1.000	-----	

Bradykinin, Temp. = 78 ^{O}F, C_{B0} = 0.0138 mole/l

n=1, Phe			
0	0.000	0.000	
20	0.473	0.261	
40	0.691	0.557	
60	0.779	0.777	
100	0.857	1.099	
260	0.961	2.222	
590	1.000	-----	

TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2
n=2, Pro			
0	0.000	0.000	
20	0.683	0.541	
40	0.835	0.987	
60	0.915	1.525	
80	0.946	1.917	
180	0.983	2.987	
440	1.000	-----	
n=3, Ser			
0	0.000	0.000	
20	0.623	0.439	
40	0.813	0.897	
60	0.901	1.398	
80	0.944	1.885	
100	0.975	2.653	
180	1.000	-----	
n=4, Phe			
0	0.000	0.000	
40	0.760	0.721	
60	0.851	1.064	
100	0.920	1.576	
160	0.973	2.578	
240	1.000	-----	
n=6, Pro			
0	0.000	0.000	
30	0.849	1.058	
50	0.926	1.645	
80	0.966	2.346	
150	0.989	3.401	
310	1.000	-----	
n=7, Pro			
0	0.000	0.000	
30	0.728	0.638	
50	0.816	0.907	
80	0.880	1.238	
150	0.944	1.897	
300	0.982	2.979	
520	1.000	-----	

TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2

Bradykinin, Temp. = 78 ^{O}F, C_{BO} = 0.0207 mole/l

n=1, Phe

0	0.000	0.000	
30	0.845	1.033	
50	0.906	1.438	
80	0.953	2.049	
140	0.985	3.161	
240	1.000	-----	

n=2, Pro

0	0.000	0.000	
30	0.854	1.082	
60	0.956	2.111	
100	0.987	3.289	
200	1.000	-----	

n=4, Phe

0	0.000	0.000	
30	0.677	0.530	
50	0.812	0.893	
80	0.876	1.212	
160	0.940	1.832	
330	0.983	3.005	
460	1.000	-----	

n=6, Pro

0	0.000	0.000	
40	0.890	1.308	
70	0.950	1.988	
200	0.978	2.762	
600	1.000	-----	

n=7, Pro

0	0.000	0.000	
30	0.631	0.452	
60	0.810	0.886	
100	0.889	1.300	
130	0.919	1.570	
250	0.971	2.511	
580	1.000	-----	

TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2
Bradykinin (Duplicated)			
n=1, Phe			
0	0.000	0.000	
40	0.901	1.392	
60	0.949	1.976	
80	0.969	2.445	
160	0.998	4.929	
360	1.000	-----	
n=2, Pro			
0	0.000	0.000	
20	0.604	0.411	
40	0.787	0.803	
60	0.875	1.203	
100	0.932	1.726	
250	0.984	3.090	
485	1.000	-----	
n=4, Phe			
0	0.000	0.000	
40	0.766	0.738	
60	0.843	1.025	
80	0.874	1.199	
150	0.927	1.657	
350	0.985	3.122	
480	1.000	-----	
n=6, Pro			
0	0.000	0.000	
40	0.931	1.708	
60	0.962	2.241	
130	0.984	3.114	
300	1.000	-----	
n=7, Pro			
0	0.000	0.000	
30	0.636	0.460	
50	0.766	0.738	
100	0.904	1.416	
160	0.963	2.267	
250	0.991	3.600	
370	1.000	-----	

TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2

Bradykinin, Temp. = 58 ^{O}F, C_{B0} = 0.0207 mole/l

n=1, Phe

0	0.000	0.000
20	0.636	0.459
40	0.819	0.918
80	0.892	1.325
200	0.969	2.427
350	0.992	3.808
510	1.000	-----

n=2, Pro

0	0.000	0.000
30	0.839	1.005
50	0.910	1.472
80	0.947	1.934
150	0.985	3.128
210	1.000	-----

n=3, Ser

0	0.000	0.000
30	0.942	1.862
60	0.978	2.776
160	1.000	-----

n=4, Phe

0	0.000	0.000
20	0.780	0.782
40	0.915	1.520
50	0.932	1.720
100	0.976	2.684
190	1.000	-----

n=6, Pro

0	0.000	0.000
40	0.927	1.652
60	0.950	1.991
150	0.987	3.234
300	1.000	-----

TABLE 24 (CONTINUED)

Time (sec)	Reacted Mole Fraction	Y2	Y2
n=7, Pro			
0	0.000	0.000	
20	0.597	0.401	
40	0.770	0.750	
60	0.827	0.952	
130	0.926	1.646	
250	0.985	3.103	
360	1.000	-----	
Bradykinin (Duplicated)			
n=2, Pro			
0	0.000	0.000	
20	0.667	0.512	
40	0.832	0.975	
60	0.892	1.326	
140	0.962	2.238	
300	1.000	-----	

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