A KINETIC STUDY OF CLASSICAL SOLID PHASE PEPTIDE SYNTHESIS REACTIONS

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REACTIONS

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PREFACE

A method for obtaining kinetic rate data of classical solid phase peptide synthesis reactions was developed. A small volume of the reactant solution was circulated through an ultraviolet spectrophotometer for continuous monitoring. The resulting absorbance curves were analyzed to give the desired kinetic information. Standard zero, first and second order analysis methods were used for this initial study.

Rate constants were determined over a range of reaction conditions for two amino acid derivatives with useful absorbance spectra. The procedure used should be applicable to as many as 9 additional amino acid derivatives commonly used in classical solid phase peptide synthesis.

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LIST OF ABBREVIATIONS

Ala	-	alanine
Asn	-	asparagine
Boc	-	tert-butyloxycarbonyl
Bpoc	-	2-(4-biphenylyl)propyl(2)oxycarbonyl
Bzl	-	benzyl
ClBzl	-	chlorobenzyl
DCC	-	dicyclohexylcarbodiimide
DCM	-	dichloromethane
DCU	-	dicyclohexylurea
DMF	-	dimethylformamide
EtOH	-	ethanol
Fmoc	-	9-fluorenylmethyloxycarbonyl
Glu	-	glutamic acid
Gly	-	glycine
Ile	-	isoleucine
Nps	-	2-nitrophenylsulfenyl
OBzl	-	benzyl ester
OEt	-	ethyl ester
OSu	-	N-hydroxysuccinimide ester
Pam	-	phenylacetamidomethyl
Phe	-	phenylalanine
Ser	-	serine
SPPS	-	solid phase peptide synthesis
TEA	-	triethyl amine
TFA	-	trifluoroacetic acid
Tos	-	4-toluenesulfonyl (tosyl)
Trp	-	tryptophan
Tyr	-	tyrosine

CHAPTER I

INTRODUCTION

The development of solid phase peptide synthesis (SPPS) by Merrifield[1] in 1963 greatly reduced the effort required to synthetically produce peptides. The major feature of this scheme is that the peptide is anchored to a solid support at its carboxyl end by an ester bond. The peptide remains attached to the resin support throughout the synthesis as each amyl residue is added in the desired sequence. The peptide is then cleaved from the resin by a strong acid such as HF. The biggest advantage of this method is that the intermediate purifications and the accompanying intermediate steps are replaced by simple rinsing of the resin.

The originally proposed procedure, now commonly called the classical method, contains several distinctive characteristics. First, the resin used is chloromethylated polystyrene crosslinked with 1-2% divinylbenzene. The chloromethylated sites provide the anchoring base and the low crosslinking percentage allows adequate swelling of the resin. The second feature of the classical method is that tert-butyloxycarbonyl (t-Boc or Boc) is used to protect the amine functionality while that amino acid is being added to the peptide. The final characteristic involves the method utilized to couple the amino acids to form the peptide bonds. Dicyclohexylcarbodiimide (DCC) is either used directly, or the amino acid symmetric anhydride, made

separately by reaction with DCC, is used in the classical method. Although variations and modifications of every facet of Merrifield's method have been attempted, it remains the most widely employed, and will be used for this study.

Because solid phase peptide synthesis is a repetitive addition of amino acids, the key to its' usefulness is the successful completion of each repetition. One failed addition, or, more importantly, successive partially incomplete additions results in both a low product yield and a mixture of similar peptides which are difficult to separate. To avoid these problems, many investigators allow the coupling step to continue substantially beyond the required time. Although this solution is acceptable on a lab-scale synthesis, a more efficient method is desirable for industrial use.

The purpose of this study is to provide information which will facilitate a more accurate prediction of the time required for the coupling step. Kinetic data and reaction rate constants can be obtained using several amino acids which have useful ultraviolet spectra. As the coupling reaction progresses, amino acid is attached to the anchored peptide and the solution concentration decreases. This drop is detected and recorded as the solution circulates through a flow cell in a U.V. spectrophotometer. The resulting absorbance curve can then be analyzed to determine the desired kinetic constants. This data can then be used as an aid for more efficient peptide production.

Four basic objectives will be addressed in this study. First, the experimental apparatus and design will be assembled and tested. Next, several experiments will be run to determine both the success of the reactions and the accuracy of this monitoring method. A list of

variables which may influence reaction kinetics and that can be studied will then be prepared. This will be accomplished by a literature review. Finally, initial kinetic data will be taken. This data will be used to qualitatively study some of the variables indicated as important by the literature review.

CHAPTER II

LITERATURE REVIEW

Solid Phase Peptide Chemistry

The classical method of solid phase peptide synthesis involves three basic chemical reactions, as illustrated in Fig. 1. The rinsed peptide-resin is first subjected to treatment with a 1:3 solution of trifluoroacetic acid : dichloromethane (DCM). This removes the t-Boc protecting group on the terminal amine. The next step is a treatment with 10% triethylamine in DCM. This base neutralizes the terminal end. The final step is coupling of the desired amyl residue. This is accomplished with either the DCC activated amino acid or with the preformed amino acid symmetric anhydride. Six solvent rinses with either DCM or dimethylformamide (DMF) are used between each of the above steps.

Several modified solid phase procedures have also been used and studied. Numerous protecting groups requiring milder deprotection conditions have been developed. These include Fmoc[2], Bpoc[3] and Nps[4]. Several coupling techniques attempting either to reduce reaction time or to improve coupling efficiency have also been tried. The use of anhydrides, activated esters of the amino acids[5] and various reagent additions to the coupling solution[6] are examples of such improvements. The resin support has also been altered. Polyacrylamides[7] and

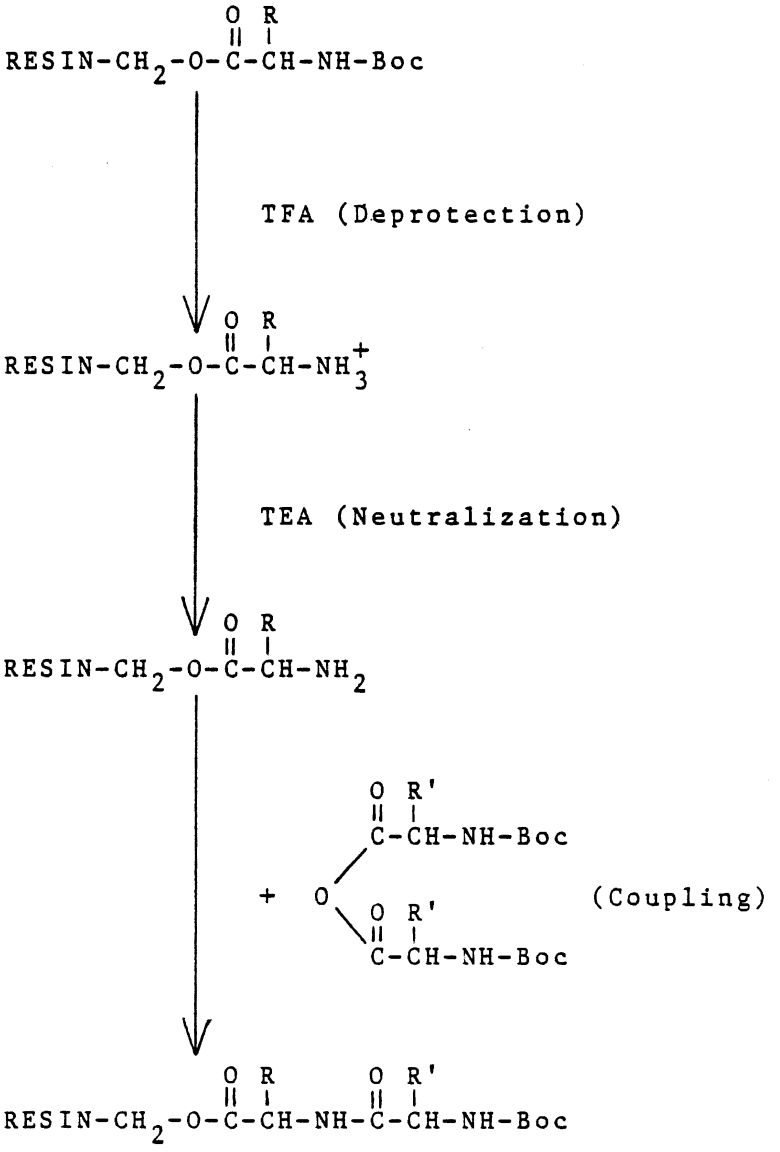

Figure 1. The Three Repetitive Steps of Solid Phase Peptide Synthesis

pellicular[8] resins are two variations developed in an attempt to eliminate any detrimental effects caused by the resin.

Reaction Monitoring Techniques

The need for nearly complete coupling has also led to various reaction monitoring techniques. Most methods involve a chemical test which indicates the presence of the deprotected amine functionality. Three disadvantages of these types of tests are the necessity of a peptide-resin sample, the time lag produced, and the small number of sites to be detected. However, these tests are still used extensively. The ninhydrin[9], quantitative ninhydrin[10] and a picrylsulfonic acid test[11] are three common indicating schemes. Ultraviolet absorbance of either a reactant or product in solution has been used as a nondestructive, continuous monitoring technique[12]. Other less common nondestructive methods include a hydrochloride procedure of Dorman[13] and the use of ^{36}Cl by Hancock[14].

Kinetic Variables

Numerous variables are known to influence the kinetics of SPPS, perhaps the most obvious of which is the reactants involved. Both the incoming amino acid and the anchored peptide are important factors. Competition studies have shown that isoleucine, valine and o-benzyl-threonine have substantially lower reactivities than other tested amino acids[15,16]. These same tests have also shown that the α-amine protecting group can also influence reactivity. Although amino acids protected by groups containing urethane structures all appeared to have similar reactivities, other sterically hindering protecting groups

decreased the reactivity[16]. Finally, the competition experiments showed that if dipeptides, instead of amino acids, are condensed on to the anchored peptide, the reactivities are lower. The three facts listed above all indicate that steric considerations of the incoming amino acid can be important in SPPS kinetics.

The role of the anchored peptide in reactivity and kinetics has also received increasing attention. Its influence is interrelated with the solvation properties of the solvent and resin. Several difficult synthesis problems, originally thought to be resin induced, have been shown to be due to solvation problems. Kent gives an interesting example of this in the synthesis of two tetrapeptides, Boc-Tyr(ClBzl)-Ile-Asn-Gly and Boc-Tyr(ClBzl)-Ile-Ala-Glu(OBzl)[17]. All variables were held constant except the two interior residues. The resulting rates were quite different though. 99% of the Ile-Ala-Glu(OBzl)-resin had reacted within 30 minutes while about 33% of the other resin remained unreacted. At 18 hours, nearly 100% coupling had been reached by the first resin while coupling had gone to only about 96% in the other. NMR spectra revealed intermolecular aggregation and decreased mobility, similar to precipitation, in the slower reacting peptide-resin. Using DMF, a better solvating solvent, completely eliminated these effects. The resin employed can also influence solvation. It has been shown that the peptide and resin exhibit mutual solvating effects[18]. Therefore, a better solvating resin, such as the Pam resins, could improve reaction rates.

Several other aspects of the importance of the anchored peptide on SPPS kinetics can also be considered. The aggregates described above occur most often between 4-18 residues[19], suggesting chain length

involvement. The secondary structure of the peptide chain may also influence kinetics, although peptide mobility has been shown to be similar inside the resin and in solution[17,20]. The reactivity of the terminal amino group on the peptide must also have a significant impact on reaction rates. The peptide bond is formed by a nucleophilic reaction of the terminal amine and hence pKa values are directly representative of reactivity. A study done on one sample peptide showed an increase of pKa over the first four residues which is equivalent to acylation rates increasing by a factor of 30. One additional experiment also showed that the reactivity of the terminal amino group changes throughout the course of a coupling reaction[21].

Other factors in SPPS have also been studied. Originally, resin induced problems were thought to be important. However, recent studies have often shown otherwise. Mass transfer into the resin beads has generally been accepted as not influencing rates[22]. High peptide and resin mobilities in most cases also indicates that internal diffusion is not rate limiting[17,20]. Internal volume and reactive space inside the resin actually increase with peptide chain length[18]. This fact has helped reduce concerns about both reaching a maximum chain length and having dramatic increases in steric hindrance. All of the results above are based on the use of a solvent which provides adequate swelling of the resin network. Finally, for several coupling schemes reaction rates tend to decrease with increasing solvent polarity[19,23].

Previous Experimental Work

Actual kinetic data on solid phase peptide synthesis is limited, and, available data often is presented for a different reaction

scheme. No kinetic data (rate constants or reaction half-times) were located for the classical SPPS method. For this reason, only relative and qualitative comparisons will be possible.

Several different types of data on SPPS kinetics are available. First, rate constants and half-times for other schemes are available. These include work on: non-crosslinked resins with active esters and DCC coupling[24], Nps protected active esters and Boc protected thiolesters[23], and Bpoc anhydrides[22]. Another source of qualitative information is the use of competitive reactions[15,16]. In these experiments an equimolar mixture of several different amino acid anhydrides is added to a peptide-resin. Analysis of the resulting peptides reveals relative reactivity between different amino acids. A final source of general kinetic information is overall reaction time used, combined with yield[25]. However, all of this data is inapplicable for improvements in the classical method of solid phase peptide synthesis.

CHAPTER III

EXPERIMENTAL APPARATUS

Several important considerations were involved in the development of the experimental apparatus. The major concern, and the biggest difference between this design and most other schemes, was the desire to have a continuous stream for monitoring. Besides having to handle this stream, an alternative to the standard mixing procedure was needed. Other factors which influenced the final design were the chemicals involved, the small volumes to be used and the general ease of operation. Figure 2 illustrates the final scheme used. A description of the experimental apparatus is given below.

Reactor

The reactor is probably the most important feature of the design. A continuous stream of the reacting solution is drawn from the bottom through a fine glass frit, which the resin cannot penetrate. The two side arms at the top of the reactor allow for return of the monitoring stream and addition of solvent and reagents. The third opening in the top of the reactor is used to supply nitrogen. Nitrogen is needed because gas bubbling, instead of the standard method of reactor inversion, is used for mixing. The total useable reactor volume is about 120 ml. Finally, the reactor is made of glass and is enclosed in a water jacket to provide temperature control. The reactor was made in

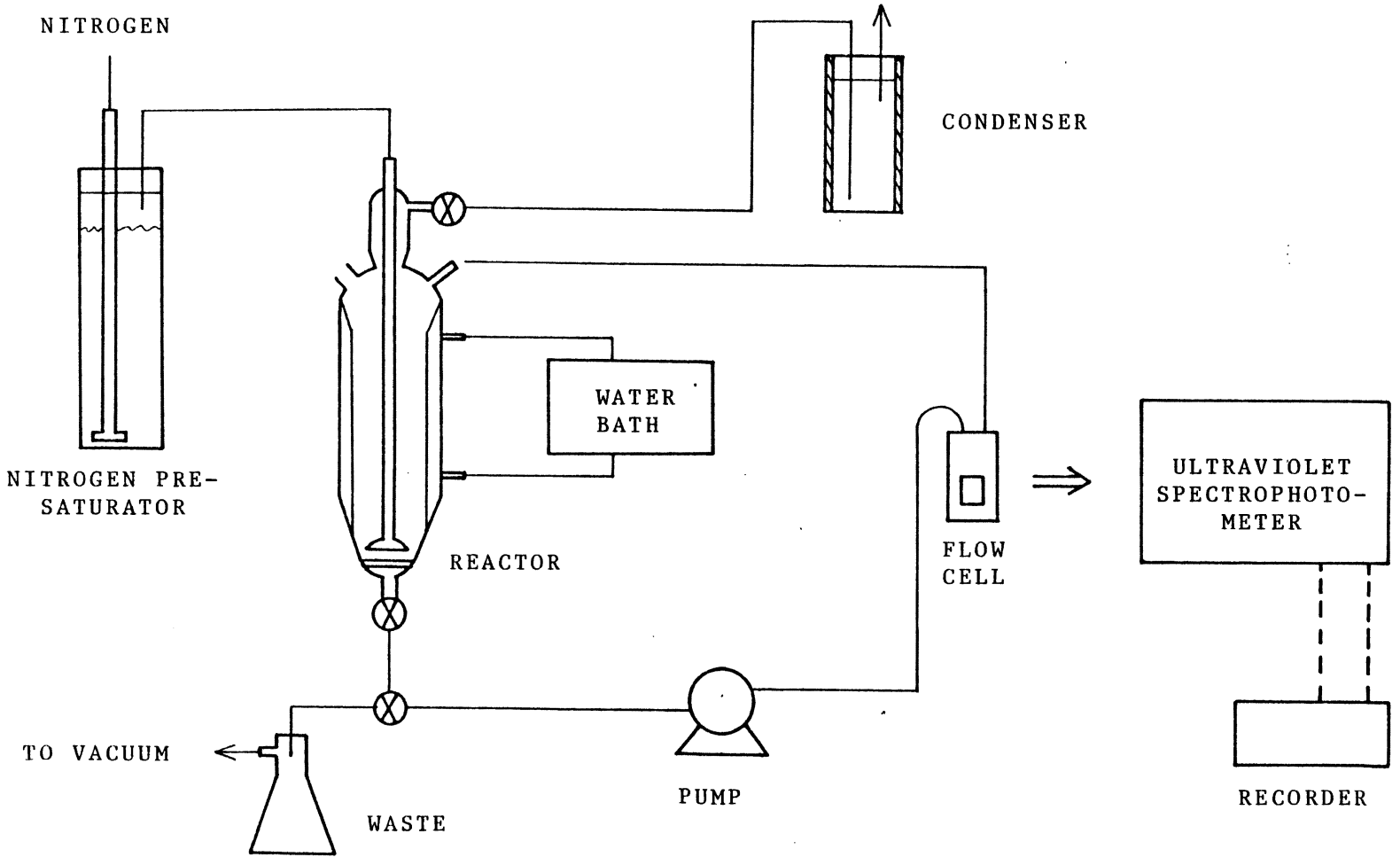

Figure 2. Final Equipment Design

the Oklahoma State University glass shop. A second, backup reactor with similar dimensions was also purchased. This allowed for more rapid changes of the resin and provided a margin of safety in case of reactor damage.

Monitoring Loop

The monitoring loop consists of a three-way stopcock, a piston pump, a flow cell and an ultraviolet spectrophotometer, all connected by about 4 ft of 1/8 and 1/16 inch FEP-teflon tubing. The total volume outside of the reactor is about 4 ml. The stopcock directs flow either to waste or to the circulating loop. A piston pump model RHSY1CKC, from Fluids Metering Inc. provides circulation. On the settings used, this pump gives flow rates between 10 and 25 ml/min and results in a lag time between reactor and detector of approximately 15 seconds. To achieve continuous ultraviolet monitoring, the solution is pumped through a quartz flow cell. The cell has a 10 mm light path and a capacity of 0.6 ml. An available Perkin-Elmer Lambda 3 UV/VIS spectrophotometer measures ultraviolet absorbance. This data is then recorded by a Perkin-Elmer 561 Recorder. This reactant solution then returns to the reactor.

Several design features are also involved in the resin mixing procedures. First, because of the high vapor pressure of DCM, the nitrogen is bubbled through a separate column of solvent to provide presaturation. The nitrogen is distributed by a fine gas distributor and then rises through approximately 8 inches of solvent in a large graduated cylinder. The saturated nitrogen then passes to the reactor

and is again bubbled by a gas distributor. A condenser is provided to remove solvent from the leaving nitrogen stream.

A few additional apparatus are included in the complete design. First, waste solvent and reagents are removed through the three-way stopcock under vacuum provided by a water aspirator. Temperature control is provided by a Haake D1 water bath. Finally, all of the equipment was placed in an enclosed hood, vented to outside the building.

Chemicals

Chemical supplies were obtained from several different sources. Boc-L-Proline (0.36 meq/gm) and Boc-N-tosyl-L-Arginine (0.45 meq/gm) polystyrene resins were from Bachem Inc. The Boc protected amino acids were all from Chemical Dynamics Corp. Indole was purchased from Eastman Kodak Co. and TFA and N,N-diisopropylethylamine was purchased from Sigma Chemicals. DCC and picrylsulfonic acid were obtained from Aldrich Co. All remaining chemicals were supplied by Fisher Scientific. The methylene chloride from Fisher was spectranalyzed® grade.

CHAPTER IV

EXPERIMENTAL PROCEDURE

Calibration Curves

Although most experimental work involves synthesis of peptides, some preliminary work is needed. This preliminary experimentation mainly consists of preparing calibration curves for both the amino acids and their symmetric anhydrides. A series of samples are made by diluting a solution of known amino acid or anhydride concentration. Ultraviolet absorbance of each sample is then taken at numerous wavelengths. By plotting absorbance vs. concentration at several wavelengths, calibration curves with the proper absorbance scale over a range of concentrations are obtained. Detailed description of anhydride preparation and spectrophotometer use are given in steps #4, 8 and 12 of the peptide synthesis procedure which follows.

Peptide Synthesis Procedure

Polystyrene resins with the first amyl residue already attached and analyzed are used exclusively in this study. Due to this fact, many common resin preparation steps are omitted. Stewart and Young provide detailed experimental procedures for these reactions and tests[26]. After a weighed sample of resin is placed in the reactor, the repetitive synthesis steps outlined by Stewart and Young and illustrated in Figure

STEP	REAGENT	VOLUME[a] (ml)	TIME[b] (min)
1	DCM	30	5
2	DCM (3 rinses)	30	2
3A	TFA/DCM	30	1.5
3B	TFA/DCM	30	30
5	DCM(6 rinses)	30	2
6	TEA/DCM (2 rinses)	30	1.5
7	DCM (6 rinses)	30	2
11-13	Symmetric Anhydride in DCM	15-30[c]	until u.v. absorbance is constant
16	DCM (3 rinses)	30	2

[a] Volume of each rinse.

[b] Time of each rinse.

[c] Varied to give desired concentration.

Figure 3. Solid Phase Peptide Synthesis Steps With Anhydride Coupling.

3 are followed. A detailed description of these reactions, based on a synthesis with two grams of resin, is given below.

1. The Boc-amyl-resin is allowed to swell in 30 mls DCM for 5 minutes.

2. Rinse the resin with 30 mls of DCM 3 times. Each wash is approximately 2 minutes in length. During these rinses and all following rinses and reactions, the resin is mixed occasionally by short, rapid bubbling by solvent saturated nitrogen.

3. Deprotection is accomplished using 60 mls of a 1:3 solution of trifluoroacetic acid:DCM which also contains a small, unmeasured amount of indole (less than 1 mg/ml). This reagent should be allowed to stand overnight before using. Half of the reagent is added to the resin for a 1.5 minute pre-treatment. Drain. Add the remaining solution and treat for 30 minutes. Step 4 can be started during this half hour.

4. The Boc-amino acid anhydride is prepared outside of the reactor in a small vial. Weigh out an amount of Boc-amino acid which gives a desired anhydride to resin sites mole ratio (typically between 1 and 3). Remember that two moles of amino acid are required to make one mole of anhydride. Dissolve the amino acid in 5-10 ml DCM. For insoluble amino acids, such as tryptophan, dissolve in the minimum volume of DMF and then add DCM to desired volume. The amino acid solution and a 1 M DCC in DCM solution are then cooled to 0°C. Enough DCC solution should be carefully prepared to complete an entire peptide synthesis. After being cooled, DCC solution is added to the amino acid solution such that the number of moles of DCC is half the moles of amino acid, i.e. 1:2 mole ratio DCC:amino acid. This solution is then kept at 0°C for 1-2 hours with occasional shaking.

5. Deprotection is followed by 6 DCM washes each of 30 ml. Additional washes should be added if the resin still retains purple coloring from the indole. The resin can be left overnight after this step. The final peptide-resin should be left deprotected.

6. The peptide is next neutralized by two treatments with a 1:9 solution of triethylamine:DCM. Each rinse is 30 mls and should be about 1.5 minutes in duration. The remaining steps should be carried out as quickly as possible.

7. Neutralization is followed by 6 DCM rinses, 30 mls each. During these rinses, preparations for following steps should be made. This includes starting the spectrophotometer (#8) and weighing filter paper.

8. With both channels completely empty, the ultraviolet spectrophotometer is turned on as instructed in the users' manual. This initiates a background light correction. When this is completed the flow cell and a clean, standard cell are inserted, with the flow cell in front. Scan to the wavelength to be used and then zero the absorbance reading. Rezero if necessary. Just before the coupling step (#12) is started add DCM to the rear reference cell. A small absorbance should result.

9. Turn on the chart recorder. Adjust the zero setting, chart paper speed and recording scale.

10. After the sixth rinse, dry the resin as much as possible. Use the pump for additional suction and for removing solvent from the tubing and stopcock.

11. To have resin in swelled state and to avoid preferential solvent absorption, add 5-10 ml DCM (accurately measured) to the resin.

12. To prepare the anhydride for coupling, the dicyclohexylurea precipitate must be removed. First, the anhydride is allowed to warm to room temperature, which is generally the reaction temperature. Anhydrides are very water sensitive and should be exposed to air as little as possible. Therefore, the following should be done only after all other preparations have been made. Quickly filter the anhydride solution through Whatman #54 filter paper into a graduated cylinder. Rinse vial with cooled DCM and pour through the filter. Rinse precipitate with additional DCM to bring concentration into calibration curve range. Measure the volume of the anhydride solution. Precipitate is weighed the next day after being dried.

13. As simultaneously as possible add anhydride to reactor, turn pump on and start recorder. Turn on nitrogen just enough to mix resin. Also stir by shaking reactor, if desired.

14. No change in u.v. absorbance for about 5 minutes is assumed to represent completion of reaction. At this point, the pump is reversed to clear the monitoring loop. Drain the reaction solution. Shut everything off. Rinse pump with ethanol.

15. Rinse resin with 30 mls of DCM 3 times. If DMF was used for solubility reasons, insert a rinse with 30 mls DMF before the DCM rinses. Finally, the resin can be left suspended in DCM.

16. Test for completeness of reaction, if desired. A modified version of Stahl, Walter and Smith's test gives good qualitative results. A 1% solution of picrylsulfonic acid is prepared in pure DMF. Approximately a 2 mg sample is placed in a very small test tube and two drops of the reagent and two drops of a 10% diisopropyl-ethylamine in chloroform solution is added. After 10 minutes at room

temperature, 1 ml of ethanol is added and the resin beads are viewed through a magnifier. All color should be associated with the beads. A negative test (little coupling) is indicated by a bright red color (approximately 0.5 mmol/g) to a faint yellow for almost complete coupling (0.001 mmol/g).

CHAPTER V

RESULTS

This initial study on the kinetics of classical solid phase peptide synthesis had several objectives. First, the experimental equipment was to be assembled and tested. Second, the entire method was to be examined for both success and accuracy. Next, a basic list of variables that might influence this systems' kinetics was needed. Finally, initial representative kinetic data was to be obtained and used to check variable influence qualitatively. The experimental results obtained for this approach are given below. Each specific goal is then addressed in detail in the Discussion chapter.

The first experimental results needed are the calibration curves for both the free amino acid and the anhydride of all of the amino acids to be used. Figure 4 presents the Boc-phenylalanine anhydride calibration curves at wavelengths of 274 and 276 nm. This data is a composite of three separate experiments. The Boc-phe, Boc-o-benzyl-tyr and Boc-o-benzyl-tyr anhydride curves, which are the results of two experiments each, are given in Appendix B. The data is fit by a system program called POLFIT on the Oklahoma State University IBM computer system. The calibration curve equations used throughout this study are listed in Table I. The choice of whether to use a first or second order polynomial is based on a significant improvement of the standard error for the estimation of the absorbance. Finally, data was also taken for

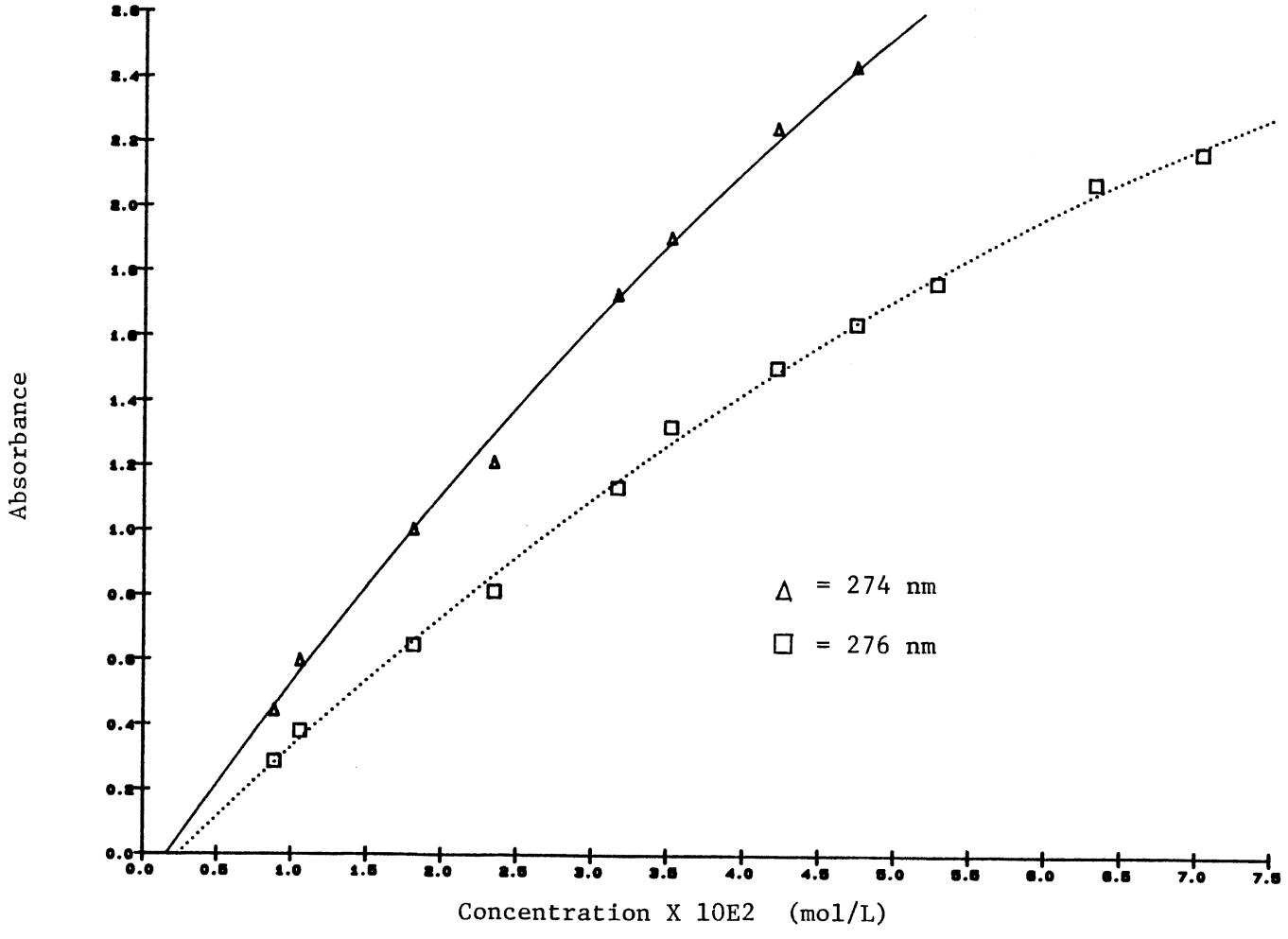

Figure 4. Boc-Phenylalanine Anhydride Calibration Curve

TABLE I

CALIBRATION CURVE EQUATIONS

Amino Acid	Wavelength	Absorbance =[a]	Standard Error of Absorbance Estimate
Phe Amino Acid	274	0.0068+9.7436 C	0.0440
	276	0.0062+5.8087 C	0.0[b]
Phe Anhydride	274	-0.1066+66.360 C-273.14 C^2	0.0444
	276	-0.1045+45.553 C-183.35 C^2	0.0324
Tyr Amino Acid	296	0.1160+27.803 C	0.0512
	300	0.0643+18.227 C	0.0311
Tyr Anhydride	296	0.1674+58.050 C	0.0335
	300	0.0688+38.453 C	0.0259

a. C is concentration of either the amino acid or anhydride. Concentration ranges between 4×10^{-3} - 7×10^{-2} M.
b. Only two data points used.

Boc-trp and Boc-o-benzyl-ser. However, this data was not used because of problems caused by tryptophan's insolubility and because only one serine run, near the end of the study, was done.

Reaction Success and Accuracy

The success and accuracy of this study is tested in two different ways. The first involves the analysis of each of the approximately 50 amino acid additions. Based on the concentration of anhydride in each experiment, the expected initial absorbance is determined using the appropriate calibration curve. Then, assuming 100% coupling, the expected final concentration and absorbance are calculated. The observed absorbances are then compared to these values to determine how accurate the initial point is and how well the final point represents 100% coupling. The results of this analysis are given in Table II. A listing of important procedures and variables, along with comments on every experiment are given in Appendix A.

The second general test of synthesis success is the measurement of overall weight gain of the resin. This represents the amount of amino acid incorporated as product and gives an approximation to the success of the synthesis. The results of this analysis are presented in Table III and the detailed calculations are given in Appendix C. The amino acid sequences of the four peptides weighed are given in Table IV.

Kinetic Data

The actual initial kinetic data desired to fulfill the fourth objective can be presented in several formats. Although it is not really kinetic data, the first method chosen is a compilation of times

TABLE II

ANALYSIS OF INITIAL AND FINAL ABSORBANCE READINGS

Peptide Number	Residue Number	Amino Acid	% Difference Between Expected and Measured Absorbances Initial	Final
1	5	Tyr	-31.9	-44.0
	6	Tyr	-20.8	-24.6
	7	Tyr	-18.8	-45.8
	8	Tyr	-17.6	-33.9
	9	Tyr	-15.5	-31.6
	10	Phe	1.8	-6.5
	11	Phe	0.6	9.1
	12	Phe	-10.9	-8.6
	13	Phe	3.1	68.7
	14	Phe	-10.7	-6.4
	15	Tyr	-10.5	-58.4
2	2	Phe	-71.7	-65.5
	3	Phe	-56.2	-38.9
	4	Phe	-0.4	-47.7
	5	Phe	-2.8	-34.6
	6	Phe	-0.9	-31.5
	7	Phe	-7.1	-44.2
	8	Phe	-33.6	12.1
	9	Phe	-27.7	-2.7
	10	Phe	-19.8	58.8
	11	Phe	2.3	36.8
	12	Phe	56.4	118.9
	13	Phe	-9.6	102.8
	14	Phe	15.1	97.5
3	2	Phe	9.2	-35.7
	3	Phe	6.1	-26.6
	4	Phe	7.3	-20.7
	5	Phe	8.6	-18.2
	6	Phe	34.3	34.8
	7	Phe	-9.4	6.1
	8	Tyr	-5.2	-3.2
	9	Phe	13.7	85.5
	10	Tyr	-6.6	-10.0
	11	Tyr	-0.7	-10.7
	12	Tyr	-6.0	-15.7
	13	Tyr	-2.2	-9.2
	14	Phe	18.6	209.3
	15	Tyr	-5.1	16.6
	16	Phe	5.7	419.8

TABLE II (Continued)

Peptide Number	Residue Number	Amino Acid	% Difference Between Expected and Measured Absorbances Initial	Final
4	2	Phe	4.6	12.7
	3	Phe	-1.3	14.9
	4	Tyr	-9.3	-8.5
	5	Tyr	-16.6	-5.3
	6	Phe	-43.9	23.1

TABLE III

TOTAL RESIN WEIGHT ANALYSIS

Peptide Number	Added Residues	Difference in Theoretical and Measured Added Weights (g)	Crude Yield of Total Peptide Mass (%)
1	16	-0.456	80.8
2	13	-0.122	91.2
3,fragment	5	+0.111	124.0
3,fragment	11	-0.300	65.2
3,total	16	-0.242	77.8
4	5	-0.114	86.7

TABLE IV

AMINO ACID SEQUENCES OF
THE PEPTIDES PRODUCED

Peptide	Sequence
1	Resin-Pro-$(Tyr(oBzl))_8$-Phe_5-Tyr(oBzl)-Trp_2
2	Resin-Pro-Phe_{13}
3,fragment 1	Resin-Pro-Phe_5
3,total	Resin-Pro-Phe_6-Tyr(oBzl)-Phe-$(Tyr(oBzl))_4$-Phe-Tyr(oBzl)-Phe-Ser(oBzl)
4	Resin-Arg(Tos)-Phe_2-$(Tyr(oBzl))_2$-Phe

TABLE V

TOTAL REACTION TIMES

Peptide	Residue Number	Mole Ratio Anhydride to Resin Sites	Initial Concentration (mM)	Time to Reaction End (min)
1	5	3.0	64.6	4
	7	2.5	57.6	15
	8	2.5	58.7	14
	9	2.0	51.0	30
	10	2.0	51.4	30
	11	2.0	51.3	30
	12	2.5	59.0	15
	13	1.0	39.2	70
	14	1.5	39.4	45
	15	1.0	35.2	80
	16	1.0	25.0	35
2	2	2.2	55.1	10
	4	1.2	29.0	5
	5	1.2	29.2	7
	6	1.2	29.2	7
	7	1.2	29.1	9
	8	1.0	25.4	7
	9	1.2	29.4	20
	10	1.2	29.2	30
	11	1.2	29.1	65
	12	1.2	29.1	60
	13	1.2	29.3	25
	14	1.2	29.1	50
3	2	1.2	29.2	9
	3	1.2	29.2	5
	4	1.2	29.2	9
	5	1.2	29.2	7
	6	1.2	29.3	15
	7	2.0	29.4	7
	8	1.0	24.9	20
	9	1.5	26.6	60
	10	1.2	29.3	60
	11	1.2	29.3	40
	12	1.2	29.3	40
	13	1.2	28.7	15
	15	0.6	14.4	55
	16	1.0	21.4	20
	17	1.0	21.3	25

TABLE V (Continued)

Peptide	Residue Number	Mole Ratio Anhydride to Resin Sites	Initial Concentration (mM)	Time to Reaction End (min)
4	2	1.5	44.9	5
	3	1.5	44.7	5
	4	1.5	44.0	3
	5	1.5	44.0	3

TABLE VI

APPARENT REACTION ORDERS OF SELECTED EXPERIMENT

Experiment Number	Peptide/Residue Number	Section of Reaction	Apparent Reaction Order
11	1/10	0-18%	4.2
		0-72%	1.6
		72-85%	15.1
13	1/11	0-35%	5.3
		0-65%	11.5
19	1/15	0-40%	0.7
		0-60%	1.4
25	2/4	0-84%	0.2
		84-98%	2.4
26	2/5	20-60%	-0.2
		45-90%	1.2
		75-97%	2.8
27	2/6	0-76%	0.1
		76-95%	3.4
28	2/7	0-50%	-2.2
		35-75%	-0.1
		50-95%	2.8
29	2/8	0-45%	0.8
		45-90%	2.8
30	2/9	0-50%	2.0
		50-83%	3.2
		0-83%	3.8
31	2/10	0-30%	2.8
		0-58%	6.8
32	2/11	0-40%	0.9
		20-76%	4.4
34	2/13	0.50%	0.1
		50-81%	11.4

TABLE VI (Continued)

Experiment Number	Peptide/Residue Number	Section of Reaction	Apparent Reaction Order
36	3/2	0-75%	-0.3
		25-75%	0.3
		75-97%	1.2
		0-97%	1.3
37	3/3	0-70%	0.0
		70-90%	1.8
39	3/5	0-70%	0.2
		72-97%	2.2
41	3/7	0-53%	1.2
		45-90%	11.0
42	3/8	0-50%	1.0
		35-83%	2.1
47	3/13	0-84%	1.5
		80-98%	2.4
		0-98%	2.1
59	4/5	0-50%	2.5
		80-93%	21.7

TABLE VII

EXPERIMENTS FIT BY ZERO ORDER
RATE EQUATION

Experiment Number	Peptide/Residue Number	Reaction Section	Best-Fit Order	K_o (mol/L·sec)x10^4
25	2/4	5-84%	0.15	4.5
26	2/5	20-60%	-0.20	5.8
27	2/6	0-75%	0.07	4.8
28	2/7	30-70%	-0.06	6.9
29	2/8	0-50%	0.79	5.2
34	2/13	5-50%	0.11	4.6
36	3/2	20-75%	0.34	12.
37	3/3	0-70%	0.01	20.
39	3/5	0-70%	0.25	20.

TABLE VIII

EXPERIMENTS FIT BY FIRST ORDER
RATE EQUATION

Experiment Number	Peptide/Residue Number	Reaction Section	Best-Fit Order	K_1 $(sec^{-1})x10^3$
11	1/10	0-72%	1.58	4.4
19	1/15	0-40%	0.74	77.
26	2/5	45-90%	1.20	35.
29	2/8	0-50%	0.79	37.
32	2/11	0-40%	0.86	10.
36	3/2	75-97%	1.25	15.
39	3/5	0-70%	0.90	95.
41	3/7	0-50%	1.18	18.
42	3/8	0-50%	1.05	29.
47	3/13	0-80%	1.50	27.

TABLE IX

EXPERIMENTS FIT BY SECOND ORDER
RATE EQUATION

Experiment Number	Peptide/Residue Number	Reaction Section	Best-Fit Order	K_2 (L/mol·sec)
11	1/10	0-72%	1.58	0.1
25	2/4	82-98%	2.37	16.
26	2/5	75-97%	2.79	3.5
29	2/8	50-92%	2.79	1.6
30	2/9	0-50%	1.97	1.2
31	2/10	0-30%	2.79	0.6
37	3/3	70-98%	1.79	3.5
39	3/5	70-97%	2.22	1.7
42	3/8	35-83%	2.14	1.8
47	3/13	0-99%	2.07	1.9
59	4/5	80-93%	2.49	0.6

to the reaction ends in Table V. Here, the leveling-off of u.v. absorbance is considered to be the reaction end. This does not necessarily represent 100% reaction however. In conjunction with the initial anhydride concentrations, this data indicates very general trends associated with chain length.

The second method of determining kinetic information is to find the reaction order and rate constants. This type of analysis was attempted with the 19 smoothest, most easily analyzed reaction curves, with limited success. By plotting ln(-dC/dt) vs. ln(Conc.) a line with slope equal to reaction order and a y-axis intercept equal to ln (rate constant) should result. This type of graph is particularly useful in determining shifts in reaction order. Table VI presents the results of this graphical procedure. The transition points between slopes were only rough estimates based on scattered data. However, a wide variance between apparent reaction orders is still obvious. The computer program used to calculate concentrations and concentration gradients is listed in Appendix D. This program utilizes three numerical techniques for determination of the slopes.

Although the variance of reaction orders prevents direct correlations with variables such as chain length, several representative rate constants were calculated. The values presented in Tables VII-IX are zero, first and second order rate constants for runs with best-fit orders near these. The constants were calculated using the following three standard plots. For zero order, a plot of concentration vs. time gives a line with slope equal to the negative of the rate constant. The first order constants are the slope of a graph of ln (concentration/ initial concentration) vs. time. Finally, the slope of a plot of

reciprocal concentration vs. time gives second order rate constants. Because the data was known not to be of exact reaction orders (Table VI), the graphs above were not plotted, but instead were simply fit by a least-squares program. However, correlation coefficients of the least-squares analysis did show relatively good fits, with values between 0.98 and 1.

CHAPTER VI

DISCUSSION

Discussion of experimental results, general trends and comments will be presented as they pertain to each of the study objectives given previously.

Experimental Technique Refinement

The first goal of this study was to design the experimental apparatus and to refine an experimental procedure suitable for obtaining ultraviolet monitoring. The final design, along with many of the considerations involved, is outlined in the Equipment chapter. Although the final experimental procedure is also given in the Procedure chapter, several comments are needed. For experiments 1-3 and several preliminary runs, DCC was added directly to the reactor. This resulted in a DCU precipitate which distorted absorbance readings. To remove precipitate before the solution was added to the reactor, the amino acid anhydride procedure was used for the remaining experiments, but refinements were still needed. First, the filtrate volume was measured instead of the volume of DCM used to dissolve the amino acid. This was implemented starting on experiment 6. Next, because more precipitate and lower initial absorbance readings than expected occurred, the precipitate was rinsed with DCM. This removed any residual amino acid or anhydride held by the precipitate. This rinsing began in experiment

10. Finally, starting with experiment 26, the anhydride solution was allowed to warm to room temperature before filtering. This resulted in the reactant solution being near the operating temperature.

Kinetic Variables

Another objective of this study was to develop a list of variables which might influence reaction kinetics. The results of the literature review are briefly summarized here. First, the amino acid to be attached plays a major role. Sterically hindered amino acids, such as Ile, Val and o-benzyl-Thr react significantly slower than the other amino acids. Second, the anchored peptide plays an important role. Reactivity of the terminal amine, steric considerations, chain length, and sequence-specific, low-reactivity aggregates are all possible influences of the anchored peptide. The synthesis scheme and the solvent used also affect the reaction rates. Finally, variables such as intraparticle diffusion, mass transfer into the resin beads and limited interior reactive volume have all been shown not to be reaction rate limiting.

Reaction Success, Accuracy and Reproducibility

The third goal of this study was to examine the general success of each reaction and then to determine approximate experimental accuracy and reproducibility. The success of reactions was tested by three techniques. These included a qualitative chemical test, a qualitative analysis of the absorbance curves and an overall mass balance for each peptide synthesis. Accuracy was calculated for several sections of the experimental procedure, however, an overall accuracy of the final rate

constants could not be determined. Finally, replicate runs to check reproducibility were basically sacrificed in this initial study so that qualitative analysis of the influences of several variables could begin.

The three techniques used to determine the approximate success of each reaction combined to show that, except for three or four experiments, the reactions generally proceeded as expected. The chemical test, a modified picrylsulfonic acid test, was used on about the first half of the experiments, with varied success. Although the resin samples changed color from red to yellow during reaction, the qualitative nature of determining colors greatly limited this method's usefulness. For research studies, a quantitative test, such as the quantitative ninhydrin procedure, should probably be used for completeness and thoroughness. The analysis of the absorbance curves to check reaction success involved comparing the total drop in absorbance to the approximate drop predicted by the calibration curves. The total drops were typically at least 80% of the expected values. The accuracy of the calibration curves prohibits using this method as a quantitative measure. Finally, the overall mass balances given in Table III also indicate that the reactions were occurring as expected. Crude peptide mass yields ranged from 65-91%, except for one short peptide that had an apparent yield of 124%.

Several comments and observations on coupling success and the reactions in general can be made. First, with the relatively low molar excesses of anhydride used, additions to longer peptides tended not to go to completion. This problem could be eliminated by using a higher molar excess or by using recoupling techniques. Second, several changes were noted at the fifth or sixth residue addition to proline-resin.

These differences included the final absorbances being significantly lower than expected for the first five or six residues, a small amount of resin sticking to the reactor wall for only the first few additions, and the fact that the first additions ended with a slow absorbance rise. Finally, possible explanations for low mass yield and their corresponding possible loss in yield are: a completely failed reaction (6-20%), successive partially incomplete failed reaction (depends on amount of incompletion), TFA chain cleavage or termination (as much as 1-2% per residue) and miscellaneous factors such as sampling or resin handling (1-3%).

Error and accuracy analyses were performed on several aspects of the experimental procedure. The sections studied included the calibration curves, the initial absorbance readings and the linearity of the plots used to determine rate constants. However, due to the lack of exact replicates and several inherent experimental uncertainties, the accuracy of the final rate constants could not be determined. One of the inherent experimental uncertainties was being unable to take separate initial absorbance readings of each run, because some solvent was added directly to the resin. The concentrated anhydride solution was quickly added and mixed with the solvent in the reactor to give the desired concentration.

The error and accuracy tests used are useful for checking several of the important analysis steps. As discussed above, the calibration curves resulted in a 2-10% error, depending on the magnitude of the absorbance. Errors of 3-7% applied to the ranges of absorbance which commonly occurred in this study. The wavelength repeatability of ±0.2nm, and, to a lesser extent, the photometric accuracy of ±0.005A and

photometric reproducibility of ±0.002A of the spectrophotometer used, could account for much of the calibration curve errors. The second test of accuracy was the comparison of expected and observed initial absorbances (Table II). This was the only means of determining how closely the concentration of the solution made was being represented. The initial absorbances fell within 11% of the expected values about 75% of the runs and showed some improvement as experimental techniques were refined. Finally, the rate constants given in Table VII-IX were all calculated from least squares analysis of standard kinetic plots. Although as few as three data points were used for some rate constant calculations, all of the correlation coefficients ranged from 0.98 to 1.0. The difficulties and uncertainties associated with analyzing these plots is discussed in the Kinetic Data section.

As previously mentioned, few replicate runs were conducted. Instead, conditions were changed to determine the effects of some variables qualitatively. Examples of this include producing only a few long peptides to test the role of chain length, using two different amino acids to compare reactivity, varying the molar excess of anhydride and using different resins. Experiments 38-41 were attempts to replicate experiments 25-28, but factors which could have influenced duplication still varied. Unfortunately, for example, experiment 24, which appeared to be a complete failure, was only the second experiment run by an assistant. Although this may not be a good excuse, doubt is raised about the true chain length and other possible influences important in replication. Comparisons between the rate constant data for experiments 26/39 and 28/41 have significant variation, but are inconclusive.

The overall results of meeting the third objective of this study are compiled in the Conclusion chapter.

Kinetic Data

Before kinetic data is presented, the influence of the reactor configuration should be discussed. Because the circulation loop is about 10% of the volume used, an initial test of the system dynamics was run. To accomplish this, a 20 ml solution of amino acid was added to 10 ml of DCM in the reactor. Every variable of this addition, except that no resin was used, was the same as those of the coupling reactions. The resulting curve contained a lag time of approximately 15 seconds, followed by a very rapid rise and leveling off which took about 5 seconds. These results provide two facts important for kinetic analysis. The first is that the method used for addition appears to give adequate mixing with the liquid already in the reactor. This is important because a homogeneous reaction solution is obviously desired. The second fact is that, for analysis purposes, the lag time plus about 4 seconds can be subtracted to give a good estimate of the starting time.

One final influence of the reactor configuration, and, in particular the circulation loop, was also noted. This was that although the volume and time of circulation were kept to a minimum, some discontinuity between reactor solution and returning solution concentrations occurred. Approximate mass balance showed that this effect could account for as much as a 5% jump in reactor concentration when the circulating stream first returns. In about 60% of the experiments a "bump" in the absorbance curve was noted (see Appendix A)

at the time that this effect would have been expected. Runs with low volumes, quicker reactions, slower pump speeds and lower molar excesses are affected most by this problem. This study neglected this phenomenon by smoothing the small discontinuity with an averaged curve.

Comments on kinetic data are also necessary before any analysis is done. Because this study was intended as a preliminary investigation, the specific results have restricted application. For many runs, several variables were altered simultaneously, which leads to difficulty in analysis. Another limitation of this study is that anhydride concentrations lower than commonly employed in industry were used. The reasons for using lower concentration were to slow the reactions and to amplify any mass transfer effects at low concentrations. Finally, it must be emphasized that some reactions, especially near the ends of syntheses, may not have gone to 100% coupling.

With the observations and precautions above, the kinetic data can be analyzed. The first, and perhaps most significant, results are the total reaction times presented in Table V. This method of comparison clearly shows that reactions do slow down significantly during the course of a peptide synthesis. Peptide #2 most readily illustrates this fact because only phenylalanine was used and anhydride concentrations were kept nearly constant. The first 6 measured additions to this peptide had u.v. absorbances that leveled off after 5-10 minutes. The next 6 additions lasted between 20-65 minutes. Peptide #3 showed a similar variation, despite the fact that both phenylalanine and tyrosine were used. However, insufficient repetitions of the same peptide prevented the determination of which variable or variables may be causing these increases in reaction times.

The apparent reaction order and rate constant data of Tables VI-IX can best be explained, and its significance determined, by examining sample data throughout the analysis procedure. Two experiments, numbers 27 and 39, are used as examples of better than average and average results, respectively. In addition, these experiments are also close to being replicates, with the only major difference being one extra attached residue for number 27. Tracings of the absorbance data, directly from the recorder, are given in Figures 5 and 6. They illustrate both the similarity of the two runs and the initial linearity, which represents nearly zero order reaction. The small discontinuities in the curves are caused by air bubbles drawn in by the pump from a small pocket of air which exists below the reactor frit.

The Kinetic data is obtained from the absorbance curves by the following procedure. First, absorbance readings are taken very carefully at 5 second intervals for the first two minutes of reaction. The time intervals are then increased to 10, 20 or 40 seconds when the curve begins to level off. This data is then entered in the computer program of Appendix D and the concentration at each point is calculated. The program also computes the slope or derivative of the concentration curve at each point using either a 3-point forward, 2-point central or 4-point central numerical technique. Finally, ln(-dC/dt) is plotted against ln(conc.) to give a graph with slope equal to the apparent reaction order (Figures 7 and 8).

Figures 7 and 8 illustrate the problem and limitations caused by the analysis methods used. First, the data contains significant scatter for several reasons. The biggest source of scatter is the non-ideality of the curves. Small bumps can result in large local changes in the

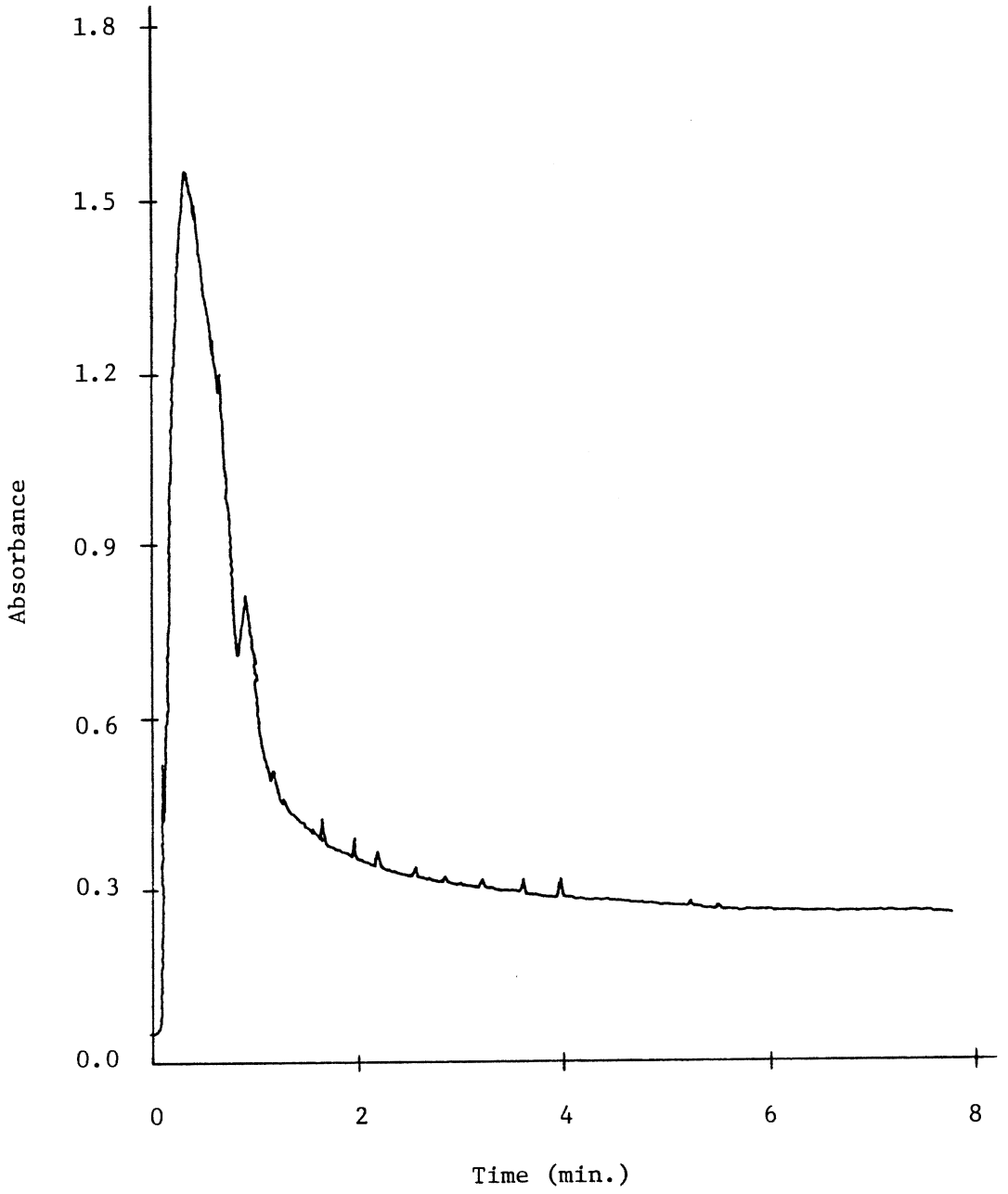

Figure 5. Absorbance Curve of Experiment 27

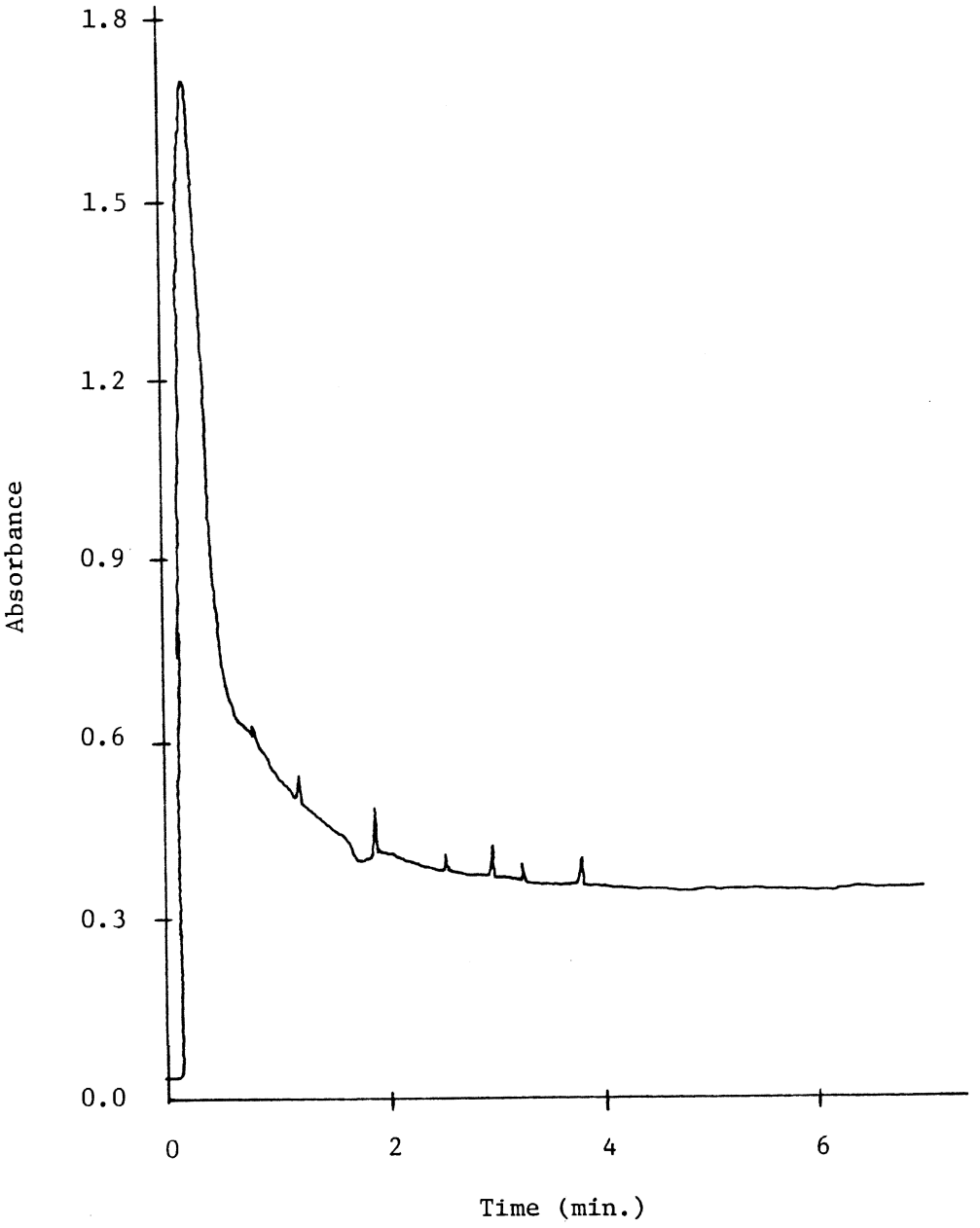

Figure 6. Absorbance Curve of Experiment 39.

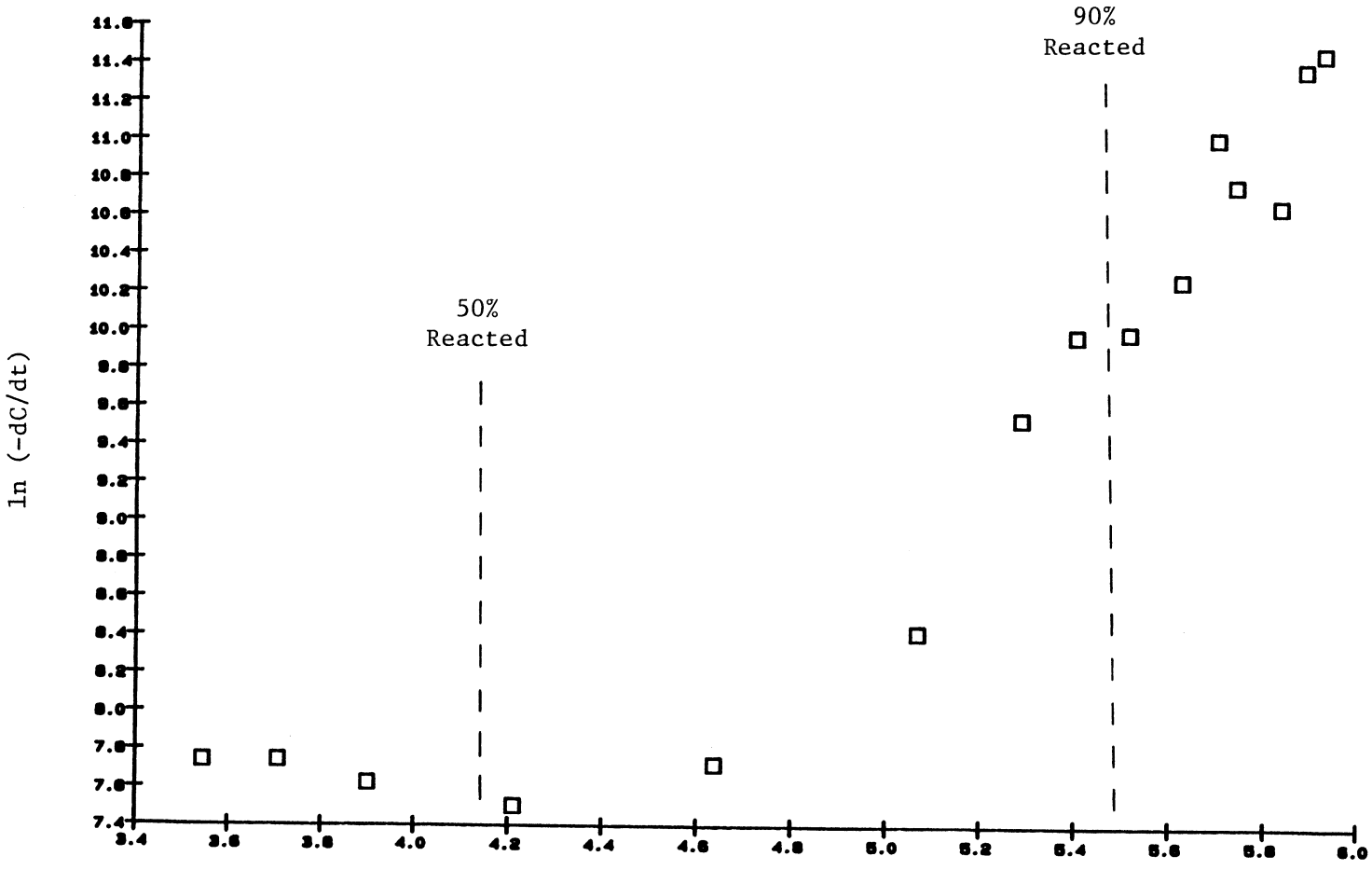

Figure 7. Apparent Order Plot for Experiment 27

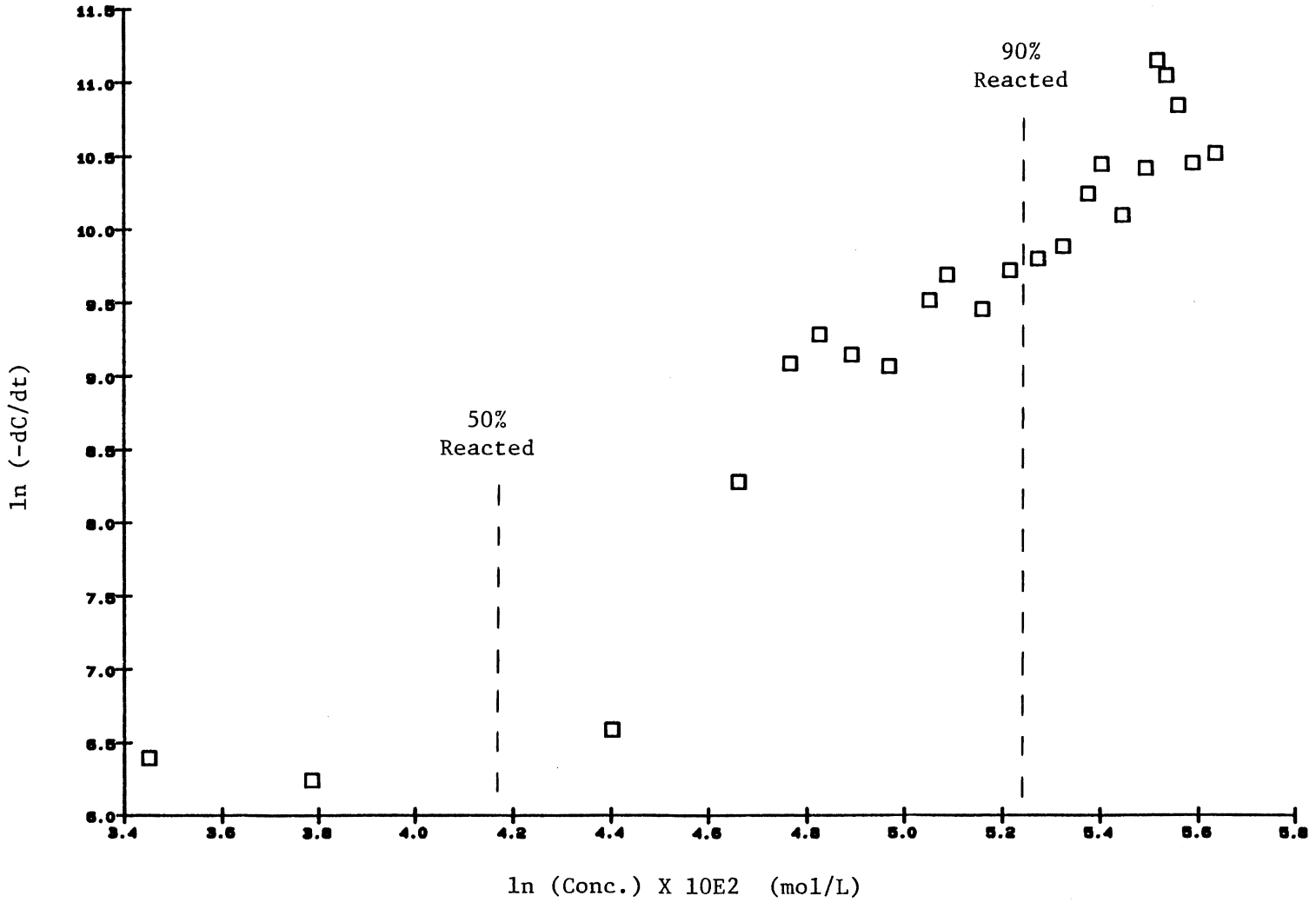

Figure 8. Apparent Order Plot for Experiment 39

slope, which this procedure readily detects. Another source of scatter is that, at the chart recorder speeds used, five second intervals are approximately the lower limit of measurement. Less than a sixteenth of an inch on the chart recorder represents five seconds. Using a faster speed would reduce this problem, but would amplify discontinuities or bumps. This time interval limitation, combined with the rapid reaction rates, also causes another problem. This is that relatively few data points can be obtained for the initial sections of reactions. Experiment 39, for example, was 75% completed in 20 seconds, which gives only three data points to base the zero order analysis on. Zero order analysis was still used though, because the absorbance curve and the relatively limited data both suggested its applicability. Another source of uncertainty is added when shifts in slope are estimated. For this initial study, the changes in slope were generally based only on inspection of the graphs, but, occasionally points were included if correlation coefficients improved. Finally, by noting that the scatter of data with a slope near zero can cause negative slopes, the negative apparent reaction orders can be explained.

Despite the uncertainties listed above, several generalizations can be made based on the data of Table VI. First, the zero and first order reactions tended to occur in the early parts of the reaction, while second order reactions occurred in the latter stages with lower concentrations. However, the initial sections of couplings to longer chains were also fit best by second order analysis. Another generalization can be recognized by noting the trend of the cut-off points between orders. This trend is that as chain length increases, the jump to higher orders occurs earlier in the reaction. An attempt to

correlate the order shifts with solution concentration was generally unsuccessful.

After the various best-fit orders of Table VI were found, a method for comparing the rate constants was needed. To accomplish this, all of the reaction sections with orders near 0, 1 and 2 were analyzed with the assumption that the order was actually the nearest integer. Although this assumption adds some inherent error depending on how nearly the integer represents the true order, this method provides the best comparison possible. Intermediate orders were fit by both adjacent orders. Tables VII, VIII and IX give these calculated rate constants. However, even these tabulations are difficult to analyze because different sections of the reactions are represented.

Again, several broad generalizations can be made from the rate constant data. First, based on relatively few tyrosine runs, Boc-o-benzyl-tyrosine and Boc-phenylanine anhydrides appear to have similar reactivity. Second, the zero order constants may be relatively constant for a particular resin. This may indicate that a maximum reaction rate, controlled by another system variable, is occurring. Also, examination of Table IX indicates that the second order rate constants may decrease with chain length. Finally, because of the scatter and lack of replicates, these initial rate constants and generalizations should be viewed only as initial data.

No direct comparisons of rate constants with literature values is possible. However, two comparisons with rate constants from systems similar enough to be applicable and significant can be made. Because the other systems are not indentical, these comparisons can only be used to give order-of-magnitude similarity. First, Merrifield[22] presents

second order constants for additions of Bpoc protected anhydrides to val-resin. The results he obtained ranges from 3.4-7.2 L/mol • sec. The closest comparable reaction from this study was the last 30% of experiment 37, which was the addition of Boc-phenylalanine to phe-phe-resin. The rate constant calculated was 3.5 L/mol • sec. The second system is indirect data for Boc-alanine addition to val-resin, which I calculated from time and yield data presented by Esko and Karlsson[25]. A first order plot of the first 90% of their reaction gave a rate constant of 6.4×10^{-3} sec^{-1}. The best reaction for comparison with this is experiment 36, which is also a second residue addition. The first order rate constant for this experiment was $15. \times 10^{-3}$ sec^{-1}, which is about an order of magnitude greater. This is expected though, because Esko and Karlsson's data was for DCC activated coupling, which is considerably slower than anhydride coupling. Overall, despite the differences in schemes, the limited results of this study compare favorably with published results.

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Although the chemistry of solid phase peptide synthesis has been widely studied, relatively little data on the reaction kinetics is available. Kinetic data on the classical method of SPPS, the most commonly used scheme, is even more limited, bordering on non-existent. This study used the ultraviolet absorbance properties of two amino acids to provide monitoring of the progress of the reactions. This data was then used to calculate representative rate constants. The important findings of this study are listed below.

1. The experimental procedure, combined with the calibration curves, do provide a relatively accurate means of monitoring the reactions to obtain kinetic data. However, occasionally a very poor run did occur.

2. Total reaction times increased with increasing chain length. This could be especially important in commercial production of small peptides.

3. The reaction orders varied widely and apparently shifted during the course of a reaction. This shift was usually from either zero or first order to second order to apparent higher orders.

4. Second order rate constants fit best at lower concentrations, while zero and first order rates applied best for the initial sections

of couplings to short peptides.

5. Boc-o-benzyl-tyrosine anhydride appears to have similar, or slightly higher, reactivity than Boc-phenylalanine anhydride.

6. Several variables which may influence SPPS kinetics have been compiled. These include: a.) solvent, b.) amino acid used, c.) reactivity of terminal amine, d.) aggregate formation (secondary peptide structure), e.) chain length.

7. No direct rate constant comparison could be found with published data. However, data for other amino acids and for differently blocked anhydrides did compare favorably.

8. Crude total added peptide mass showed good yield when compared to the theoretical maximum.

Recommendations

Recommendations for further possible research using the techniques employed in this study are listed below.

1. The monitoring procedure developed for the three amino acids which contain a natural benzyl ring can be extended to several other Boc-amino acids. This can be accomplished by the proper choice of side-chain protecting groups. Commonly used amino acid derivatives which are monitorable are: a.) Boc-o-benzyl-serine, b.) Boc-o-benzyl-threonine, c.) Boc-N-tosyl-arginine, d.) Boc-tosyl-lysine, e.) Boc-methoxybenzyl-L-cysteine, and f.) Boc-benzyloxymethyl-histidine or Boc-im-benzyl-L-histidine. Boc-xanthyl asparagine and glutamine may also have limited possibilities. Finally, the benzyl-esters of aspartic acid and glutamic acid could be used if another method of removing DCU could be found.

2. To provide more useful results, several model, commercial peptides should be produced a number of times. This would allow direct comparisons between syntheses.

3. A higher mole ratio of 1.5:1 of anhydride to resin sites should be used extensively. This ratio is closer to that used in industry. Higher mole ratios would also tend to force more reactions to nearer completion.

4. The monitoring loop volume and lag time should be reduced further. This would improve the accuracy of the absorbance curves and would help decrease the "bump" in absorbance caused by the initial return of solution from the monitoring loop. To further reduce this effect, larger volume should be used.

5. For experimental completeness, the coupling reactions should always be tested chemically for $99\%^{+}$ coupling. The quantitative ninhydrin test should be used.

6. A longer peptide, with up to 25-30 residues should be made. This could be used to check the role of chain length beyond the commonly used region. Several of the influences of chain length may eventually level off with longer peptides.

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APPENDIX A

DESCRIPTION OF EXPERIMENTAL RESULTS

A total of four peptides were prepared during this study. Specific experimental operating conditions and comments on each amino acid addition are given below. The time to reaction end data given for most runs is an approximate time of when the u.v. absorbance leveled off. This was interpreted as the reaction end, but does not necessarily represent 100% reaction.

Experiment 1

Peptide Number: 1

Amino Acid: Tyr Chain Length: 1 residue

Attached Peptide: Resin-Pro

Mole Ratio Anhydride to Resin Sites: 2.5 : 1*

Temperature: ?

Comments: The mole ratio given above is actually the amino acid:resin sites ratio. This run did not use preformed anhydride, but instead used amino acid and DCC together in the reactor. A DCU precipitate resulted in off-scale absorbance readings. This run helped pick a practical wavelength.

Experiment 2

Peptide Number: 1

Amino Acid: Tyr Chain Length: 2 residues

Attached Peptide: Resin-Pro-Tyr

Mole Ratio Anhydride to Resin Sites: 1.5 : 1*

Temperature: 80.0°F

Comments: Again, amino acid and DCC were both placed in the reactor, however, they were quickly mixed outside of the reactor. This solution was to be filtered, but precipitate did not form until inside of the reactor. Although the absorbance curve was smooth and on scale, the precipitate was again suspected of interference.

Experiment 3

Peptide Number: 1

Amino Acid: Tyr Chain Length: 3 residues

Attached Peptide: Resin-Pro-Tyr-Tyr

Mole Ratio Anhydride to Resin Sites: 2.5 : 1*

Temperature: 82°F

Comments: This was the last run before using symmetrical anhydrides. Initially only amino acid was to be used to test reaction without DCC. After about 25 minutes of little change, DCC was added and precipitate caused readings off scale.

Experiment 4

Peptide Number: 1

Amino Acid: Tyr Chain Length: 4 residues

Attached Peptide: Resin-Pro-Tyr-Tyr-Tyr

Mole Ratio Anhydride to Resin Sites: 3.0 : 1

Approximate Time to U.V. Minimum: 200 sec

Temperature: ?

Comments: For this first anhydride run the precipitate was not rinsed and the filtrate volume was not measured. This may explain why the

readings were substantially lower than expected. Beyond the 200 seconds at which the absorbance had leveled off, a slow but steady rise occurred until the reaction was stopped.

Experiment 5

Boc-o-benzyl-Tyr symmetric anhydride calibration curve.

Comments: The precipitate was not rinsed and the final filtrate volume was not measured. For this reason this run was not used.

Experiment 6

Peptide Number: 1

Amino Acid: Tyr Chain Length: 5 residues

Attached Peptide: Resin-Pro-$(Tyr)_4$

Mole Ratio Anhydride to Resin Sites: 2.5 : 1

Approximate Time to U.V. Minimum: ?

Temperature: 82°F

Comments: The filtrate volume was measured this time but the precipitate was not rinsed. For the first time DCM was added to the resin. This was done in hopes of eliminating any preferential absorption of solvent and to start resin in a completely swelled state. The reaction time could not be determined because a large, wide hump occurred between about 240-480 seconds. This run was the only time any phenomena like this happened. A very slight rise in absorbance occurred after this hump. Absorbance values were still difficult to determine because the appropriate wavelength had not been found.

Experiment 7

Peptide Number: 1

Amino Acid: Tyr Chain Length: 6 residues

Attached Peptide: Resin-Pro-$(Tyr)_5$

Mole Ratio Anhydride to Resin Sites: 2.5 : 1

Approximate Time to Reaction End: 15 minutes

Temperature: ?

Comments: Several things were done differently. First, because of vacuum trouble, the resin was left in the deprotected state overnight in a 50/50 solution of EtOH/DCM. This also caused the anhydride to be left overnight at 0°C. This could have caused some anhydride dissociation. The precipitate was still not rinsed. The absorbance curve did not slowly rise this time.

Experiment 8

Peptide Number: 1

Amino Acid: Tyr Chain Length: 7 residues

Attached Peptide: Resin-Pro-$(Tyr)_6$

Mole Ratio Anhydride to Resin Sites: 2.5 : 1

Approximate Time to Reaction End: 14 minutes

Temperature: ?

Comments: Again the precipitate was not rinsed. This still is causing all readings to be low. For this run and all remaining runs on this peptide, the U.V. readings either remained leveled off or dropped very slightly at the end.

Experiment 9

Comments: This was a run to study the precipitate some. Precipitate from a previous run was rinsed with about 9 ml DCM in hopes of removing any trapped or attached amino acid. U.V. absorbances showed that a significant amount of amino acid was being lost.

Experiment 10

Peptide Number: 1

Amino Acid: Tyr Chain Length: 8 residues

Attached Peptide: Resin-Pro-$(Tyr)_7$

Mole Ratio Anhydride to Resin Sites: 2.0 : 1

Approximate Time to Reaction End: 30 minutes

Temperature: 79.0°F

Comments: For the first time the precipitate was rinsed. Also, adding DCM to the resin has become standard procedure. The amount of DCC added on this run may have been slightly incorrect. This is because only a little DCC solution was left and bubbles were drawn into the pipet at 0.1 ml before the proper amount. In this run the absorbances were closer to expected. However, with comparison to later runs, chain length may be causing some problems.

Experiment 11

Peptide Number: 1

Amino Acid: Phe Chain Length: 9 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$

Mole Ratio Anhydride to Resin Sites: 2.0 : 1

Approximate Time to Reaction End: 30 minutes

Temperature: 79.0°F

Comments: This was the first run with Phe anhydride which resulted in using an inappropriate wavelength. This run was close to the expected absorbances for both the maximum and minimum absorbances. A rather large depression in the absorbance curve occurred at approximately the 70 second mark and lasted about 30 seconds. This type of bump appeared in about half of the remaining runs.

Experiment 12

Boc-phenylalanine Symmetric Anhydride Calibration Curve.

Experiment 13

Peptide Number: 1

Amino Acid: Phe Chain Length: 10 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-Phe

Mole Ratio Anhydride to Resin Sites: 2.0 : 1

Approximate Time to Reaction End: 30 minutes

Temperature: 80.0°F

Comments: Again a bump occurred but this time at the beginning of the curve. Its' duration was about 20 seconds. This caused some doubt in the initial absorbance reading.

Experiment 14

Boc-phenylalanine Symmetric Anhydride Calibration Curve.

Experiment 15

Peptide Number: 1

Amino Acid: Phe Chain Length: 11 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-Phe-Phe

Mole Ratio Anhydride to Resin Sites: 2.5 : 1

Approximate Time to Reaction End: 15 minutes

Temperature: ?

Comments: Bump occurred at about 20 seconds and lasted about 10 seconds.

Experiment 16

Peptide Number: 1

Amino Acid: Phe Chain Length: 12 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-$(Phe)_3$

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 70 minutes

Temperature: 78.0°F

Comments: This was the first low mole ratio run. As can be seen, the reaction time was increased significantly. Both terminal absorbance peaks are close to expected. The bump occurred at about 30 seconds and lasted 20 seconds.

Experiment 17

Peptide Number: 1

Amino Acid: Phe Chain Length: 13 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-$(Phe)_4$

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: 45 minutes

Temperature: 77.5°F

Comments: Both terminal absorbances were a little low. Bump appeared at 40 seconds and lasted 15 seconds.

Experiment 18

Boc-o-benzyl-Tyrosine Symmetric Anhydride Calibration Curve.

Experiment 19

Peptide Number: 1

Amino Acid: Tyr Chain Length: 14 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-$(Phe)_5$

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 80 minutes

Temperature: 78.5°F

Comments: Again low 1:1 mole ratio caused large increases in reaction time. Small bump occurred at about 30 seconds with 10 second duration. Both terminal absorbances were again lower than expected.

Experiment 20

Peptide Number: 1

Amino Acid: Trp Chain Length: 15 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-$(Phe)_5$-Tyr

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 30 minutes*

Temperature: 78.5°F

Comments: The incorporation of tryptophan was not accomplished successfully in this study. Several problems resulted with tryptophan. First, this amino acid is not soluble in DCM.

Therefore, 2 mls of DMF were added to the coupling solution. The anhydride was also slow to form. It took approximately 1 hr for precipitate to appear compared to about 2 minutes for the other amino acids used. The precipitate may also have been partially soluble in the DMF. Also, a small amount of precipitate formed during reaction and while the calibration curves were being prepared. Finally, the DMF may have had slight absorbance at the wavelength used. No bump appeared during this run. Also, the anhydride was added to dry resin.

Experiment 21

Boc-tryptophan Symmetric Anhydride Calibration Curve.

Comments: As noted above, the precipitate continued to form during the calibration readings.

Experiment 22

Peptide Number: 1

Amino Acid: Trp Chain Length: 16 residues

Attached Peptide: Resin-Pro-$(Tyr)_8$-$(Phe)_5$-Tyr-Trp

Mole Ratio Anhydride to Resin Sites: 2.0 : 1

Approximate Time to Reaction End: ?

Temperature: 78.0°F

Comments: This was the last run using Trp. The anhydride was added to wetted resin. A large bump occurred at about 5 seconds and leveled off at its end. It is unclear whether any significant amount of Trp was attached.

Experiment 23

Peptide Number: 2

Amino Acid: Phe Chain Length: 1 residue

Attached Peptide: Resin-Pro

Mole Ratio Anhydride to Resin Sites: 2.2 : 1

Approximate Time to Reaction End: 10 minutes*

Temperature: ?

Comments: This run produced a curve which had only a slight decrease in absorbance. The success of coupling is questionable. No bump occurred. Readings were much lower than expected.

Experiment 24

Peptide Number: 2

Amino Acid: Phe Chain Length: 2 residues

Attached Peptide: Resin-Pro-Phe

Mole Ratio Anhydride to Resin Sites: 2.3 : 1

Approximate Time to Reaction End: ?*

Temperature: 78.0°F

Comments: This run gave an absorbance curve which rose smoothly and leveled off, giving no sign of reaction. This occurred one other time in run 60. No explanation for this was found. Readings were much lower than expected.

Experiment 25

Peptide Number: 2

Amino Acid: Phe Chain Length: 3 residues

Attached Peptide: Resin-Pro-Phe-Phe

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 5 minutes

Temperature: 76.5°F

Comments: This run had initial readings consistent with the calibration curve. The minimum was lower than expected. The absorbance was rising slightly when the reaction was stopped. A slight bump appeared at 40 seconds and lasted about 10 seconds.

Experiment 26

Peptide Number: 2

Amino Acid: Phe Chain Length: 4 residues

Attached Peptide: Resin-Pro-$(Phe)_3$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 7 minutes

Temperature: 77.5°F

Comments: Similar to experiment 25. Initial peak close but minimum was lower than expected. Finished with rising absorbance. Small bump at 40 seconds.

Experiment 27

Peptide Number: 2

Amino Acid: Phe Chain Length: 5 residues

Attached Peptide: Resin-Pro-$(Phe)_4$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 7 minutes

Temperature: 78.0°F

Comments: Similar to the preceding two experiments. One difference was

that precipitate was allowed to warm to room temperature before filtering. This was hoped to cause more uniform temperature. Absorbance was rising slightly when reaction was stopped.

Experiment 28

Peptide Number: 2

Amino Acid: Phe Chain Length: 6 residues

Attached Peptide: Resin-Pro-$(Phe)_5$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 9 minutes

Temperature: ?

Comments: No bump. The absorbance was rising very slightly when reaction was stopped. The anhydride was again allowed to warm before filtering.

Experiment 29

Peptide Number: 2

Amino Acid: Phe Chain Length: 7 residues

Attached Peptide: Resin-Pro-$(Phe)_6$

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 7 minutes

Temperature: ?

Comments: This run gave a smooth curve with no bump. However, the initial reading is considerably off. The final reading is close. It is possible that the first few seconds of this run may have been missed due to pump not being on.

Experiment 30

Peptide Number: 2

Amino Acid: Phe Chain Length: 8 residues

Attached Peptide: Resin-Pro-$(Phe)_7$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 10 minutes

Temperature: ?

Comments: This is similar to the previous experiment. No bump occurred, the initial reading was substantially low and the final reading was close. Also note that allowing anhydride to warm to room temperature before filtering has become standard.

Experiment 31

Peptide Number: 2

Amino Acid: Phe Chain Length: 9 residues

Attached Peptide: Resin-Pro-$(Phe)_8$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 30 minutes

Temperature: 76.5°F

Comments: Again the initial point is substantially low. No bump.

Experiment 32

Peptide Number: 2

Amino Acid: Phe Chain Length: 10 residues

Attached Peptide: Resin-Pro-$(Phe)_9$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 65 minutes

Comments: Initial and final readings were close to expected values. No bump occurred.

Experiment 33

Peptide Number: 2

Amino Acid: Phe Chain Length: 11 residues

Attached Peptide: Resin-Pro-$(Phe)_{10}$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 60 minutes

Temperature: 77.5°F

Comments: The initial reading was much higher than expected. The reason for this is unknown. A slight bump occurred at 50 seconds.

Experiment 34

Peptide Number: 2

Amino Acid: Phe Chain Length: 12 residues

Attached Peptide: Resin-Pro-$(Phe)_{11}$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 25 minutes

Temperature: ?

Comments: The initial reading was substantially lower than expected. No bump occurred.

Experiment 35

Peptide Number: 2

Amino Acid: Phe Chain Length: 13 residues

Attached Peptide: Resin-Pro-$(Phe)_{12}$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 50 minutes

Temperature: 77.5°F

Comments: Initial absorbance was too high. Bump occurred at 50 seconds.

Experiment 36

Peptide Number: 3

Amino Acid: Phe Chain Length: 1 residue

Attached Peptide: Resin-Pro

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 9 minutes

Temperature: 76.0°F

Comments: No bump occurred. Some resin "clumps" were noted several rinses before coupling. Small clumps may have remained during the coupling step, however it was hard to see them. Such clumps may represent added mass transfer resistance. Absorbance remained level at end.

Experiment 37

Peptide Number: 3

Amino Acid: Phe Chain Length: 2 residues

Attached Peptide: Resin-Pro-Phe

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 5 minutes

Temperature: 76.5°F

Comments: Initial peak was close to expected but minimum was lower than expected. The absorbance began rising significantly after minimum had been reached for a while. No bump occurred.

Experiment 38

Peptide Number: 3

Amino Acid: Phe Chain Length: 3 residues

Attached Peptide: Resin-Pro-Phe-Phe

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 9 minutes

Temperature: 76.5°F

Comments: Similar to previous two runs. Absorbance was rising when reaction was stopped. A slight bump appeared.

Experiment 39

Peptide Number: 3

Amino Acid: Phe Chain Length: 4 residues

Attached Peptide: Resin-Pro-$(Phe)_3$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 7 minutes

Temperature: 76.5°F

Comments: Final absorbance was steady. No bump.

Experiment 40

Peptide Number: 3

Amino Acid: Phe Chain Length: 5 residues

Attached Peptide: Resin-Pro-$(Phe)_4$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 15 minutes

Temperature: 77.0°F

Comments: The initial reading was substantially higher than expected. This was followed by a rapid drop and a bump at about 30 seconds. The absorbance was rising at the end.

Experiment 41

Peptide Number: 3

Amino Acid: Phe Chain Length: 6 residues

Attached Peptide: Resin-Pro-$(Phe)_5$

Mole Ratio Anhydride to Resin Sites: 2.0 : 1

Approximate Time to Reaction End: 7 minutes

Temperature: 76.0°F

Comments: This run had several major differences. First, after experiment 40 the resin was deprotected, dried and weighed. Although the weight was higher than expected, the resin was split into two equal halfs to insure the proper number of resin sites. Then one half was returned to the reactor. Comparing this run with the rest indicates that freshly charged resin and a cleaned reactor may influence the results. This is discussed in the Discussion chapter. The absorbance was rising at the end of the run. No bump occurred.

Experiment 42

Peptide Number: 3

Amino Acid: Tyr Chain Length: 7 residues

Attached Peptide: Resin-Pro-$(Phe)_6$

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 20 minutes

Temperature: ?

Comments: The low mole ratio increased reaction time. This run, along with most of the remaining runs on this peptide were done in significantly lower volumes (about half). This would be expected to magnify errors due to inaccurate measurements or possible evaporation. A bump occurred at about 30 seconds.

Experiment 43

Peptide Number: 3

Amino Acid: Phe Chain Length: 8 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: 60 minutes

Temperature: 78.0°F

Comments: The volume was not quite as low as last run. The final reading was much higher than expected. One major item was different. The resin was left deprotected in DCM for about 4 hours. Also, the final rinse before coupling was pumped through monitoring system for cleaning purposes.

Experiment 44

Peptide Number: 3

Amino Acid: Tyr Chain Length: 9 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 60 minutes

Temperature: 77.5°F

Comments: A large bump occurred at about 20 seconds. Low volume was used. The reactor was shaken by hand with no N_2 bubbling. This is due to the low volume. The final rinse was pumped through the flow system.

Experiment 45

Peptide Number: 3

Amino Acid: Tyr Chain Length: 10 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-Tyr

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 40 minutes

Temperature: 78.5°F

Comments: Again low volume and hand shaking of reactor were used. Also, a large bump at about 20 seconds occurred again. The final rinse was pumped through system for cleaning.

Experiment 46

Peptide Number: 3

Amino Acid: Tyr Chain Length: 11 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-Tyr-Tyr

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 40 minutes

Temperature: 78.0°F

Comments: Again a large bump occurred at 20 seconds. A small volume

and hand shaking were used again. The final rinse was again pumped through the system.

Experiment 47

Peptide Number: 3

Amino Acid: Tyr Chain Length: 12 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-$(Tyr)_3$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: 13 minutes*

Temperature: ?

Comments: The time to reaction end is questionable. The chart recorder shows 13 minutes but the recorder may have been stopped near the end of the reaction. No bump occurred in this run. Small volume and nitrogen bubbling were used.

Experiment 48

Peptide Number: 3

Amino Acid: Phe Chain Length: 13 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-$(Tyr)_4$

Mole Ratio Anhydride to Resin Sites: 1.2 : 1

Approximate Time to Reaction End: ?*

Temperature: 78.0°F

Comments: The initial reading was higher than expected and the final reading was much higher than expected. The absorbance curve dropped rapidly to an absorbance considerably lower than the final value. The curve leveled off in about 2 minutes and then began to rise

slowly. Success of this run is questionable. Some DCU may also have been seen on the reactor frit.

Experiment 49

Peptide Number: 3

Amino Acid: Tyr Chain Length: 14 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-$(Tyr)_4$-Phe

Mole Ratio Anhydride to Resin Sites: 0.6 : 1

Approximate Time to Reaction End: 55 minutes

Temperature: 77.5°F

Comments: By accident, only half the desired amino acid was used. This resulted in all the anhydride reacting with some sites remaining. A bump occurred at about 20 seconds.

Experiment 50

Boc-o-benzyl-serine Calibration Curve

Comments: For some reason the U.V. only read absorbances to about 1.5. This must be redone.

Experiment 51

Boc-o-benzyl-serine Anhydride Calibration Curve

Comments: Again the U.V. read to only about 1.36. This must be redone.

Experiment 52

Peptide Number: 3

Amino Acid: Phe Chain Length: 15 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-$(Tyr)_4$-Phe-Tyr

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 20 minutes

Temperature: 78.0°F

Comments: The end absorbance is way off from the expected value. The initial value is close. A small bump occurred. Recall that the last run may not have coupled all the sites. This would mean that these sites were open for nearly two weeks. Side reactions may have occurred.

Experiment 53

Peptide Number: 3

Amino Acid: Ser Chain Length: 16 residues

Attached Peptide: Resin-Pro-$(Phe)_6$-Tyr-Phe-$(Tyr)_4$-Phe-Tyr-Phe

Mole Ratio Anhydride to Resin Sites: 1.0 : 1

Approximate Time to Reaction End: 25 minutes

Temperature: 78.0°F

Comments: The pump speed was set lower to reduce pulsing action that was drawing in air. This resulted in a smooth curve with no bump. However, initial readings may have been missed. Also, a slightly bigger volume was used.

Experiment 54

System Analysis

Comments: An attempt to measure exact dead time and volume was made. This did not work well however. The pump had to be run backwards and air was drawn in. Also, after the first test, some DCM remained in the tubing.

Experiment 55

Peptide Number: 4

Amino Acid: Phe Chain Length: 1 residue

Attached Peptide: Resin-Arg

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: 5 minutes

Temperature: 77.5°F

Comments: A large bump in the reaction curve appeared at about 20 seconds. Approximately 98% of the U.V. absorbance drop occurred within the first two minutes. At this point the absorbance dropped slightly below the leveled-off value. After about 10 minutes, the absorbance started to rise slowly. Note that a new resin is being used.

Experiment 56

Boc-o-benzyl-serine Anhydride Calibration Curve

Experiment 57

Peptide Number: 4

Amino Acid: Phe Chain Length: 2 residues

Attached Peptide: Resin-Arg-Phe

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: 5 minutes

Temperature: 77.0°F

Comments: No bump occurred, however, again at about two minutes a rapid drop followed by a rapid rise occurred. The initial and final absorbance readings of this bump were approximately equal, resulting

in no overall concentration change. The absorbance began to rise slowly at the end of the experiment. Several things were changed with this peptide run. First, the reactor stopcock leaked and could not be replaced for several days. Solution did not leak out, however, air was drawn in. To repair this the stopcock ends were taped with Teflon tape in an open position. Also the pump was used to drain solvents instead of the vacuum line. This reduced the experimental time greatly. One problem with this was that some DCM added to the resin before the coupling step went through the stopcock. Although the volume of this was only about 0.5 ml it could have caused inaccurate initial readings. This same procedure was used for the next three runs also.

Experiment 58

Peptide Number: 4

Amino Acid: Tyr Chain Length: 3 residues

Attached Peptide: Resin-Arg-Phe-Phe

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: 3 minutes

Temperature: 76.0°F

Comments: No bump occurred, however a large drop at about 30 seconds occurred. Again, this went well below the final level. Also, the absorbance rose slowly near the end. Comments from Experiment 57 apply.

Experiment 59

Peptide Number: 4

Amino Acid: Tyr Chain Length: 4 residues

Attached Peptide: Resin-Arg-Phe-Phe-Tyr

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: 3 minutes

Temperature: 71.0°F

Comments: Note the low temperature. This curve was similar to experiment 57. No bump appeared, but at about 1.5 minutes a very large drop followed by a matching rinse occurred.

Experiment 60

Peptide Number: 4

Amino Acid: Phe Chain Length: 5 residues

Attached Peptide: Resin-Arg-Phe-Phe-Tyr-Tyr

Mole Ratio Anhydride to Resin Sites: 1.5 : 1

Approximate Time to Reaction End: ?*

Temperature: 76.5°F

Comments: This run was similar to run 24. Absorbance rose smoothly and leveled off, showing no sign of reaction. Two experimental differences were that only 5 mls of DCM were added prior to coupling and the TEA was left at least 10 minutes longer than desired.

APPENDIX B

CALIBRATION CURVES

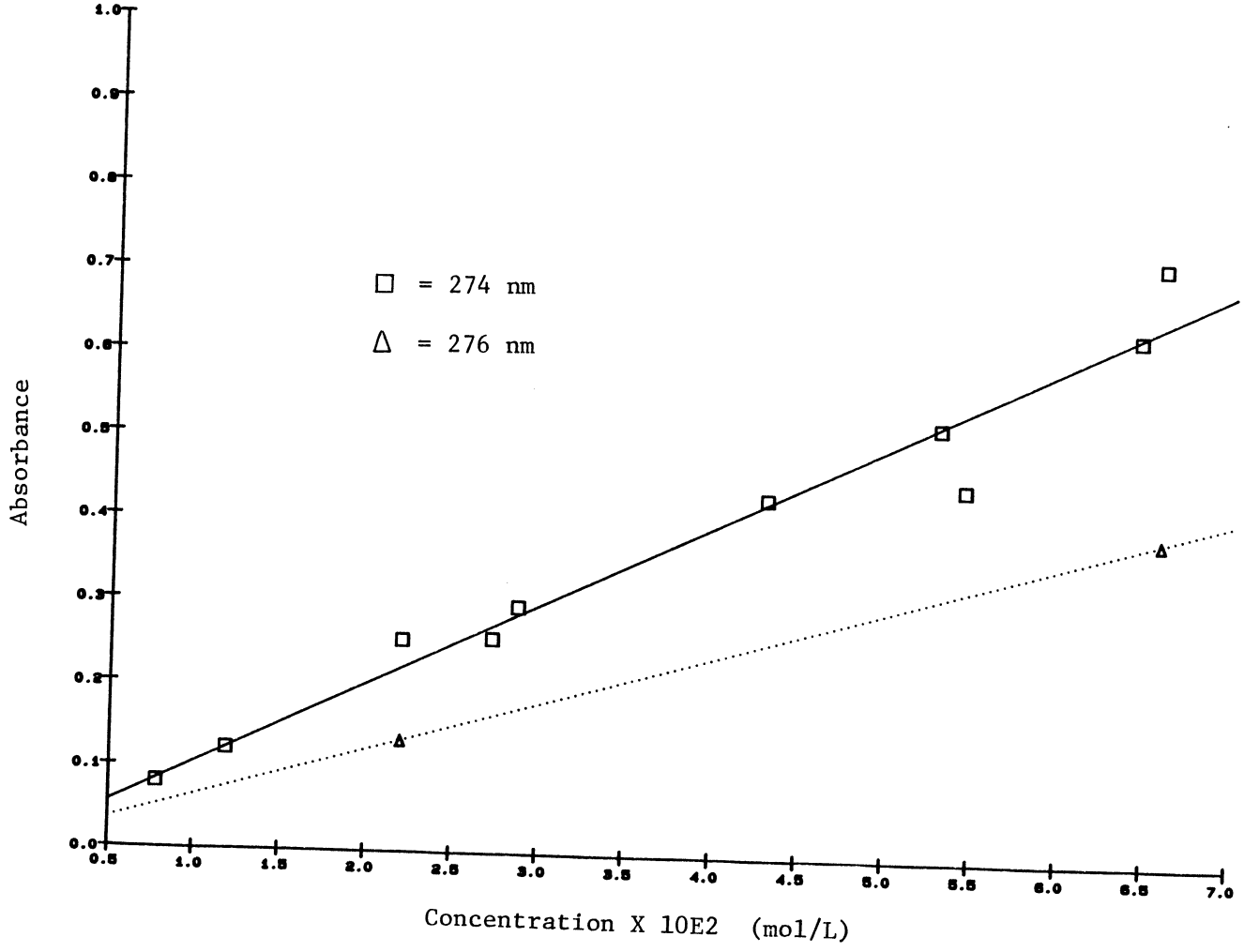

Figure 9. Boc-Phenylalanine Calibration Curve

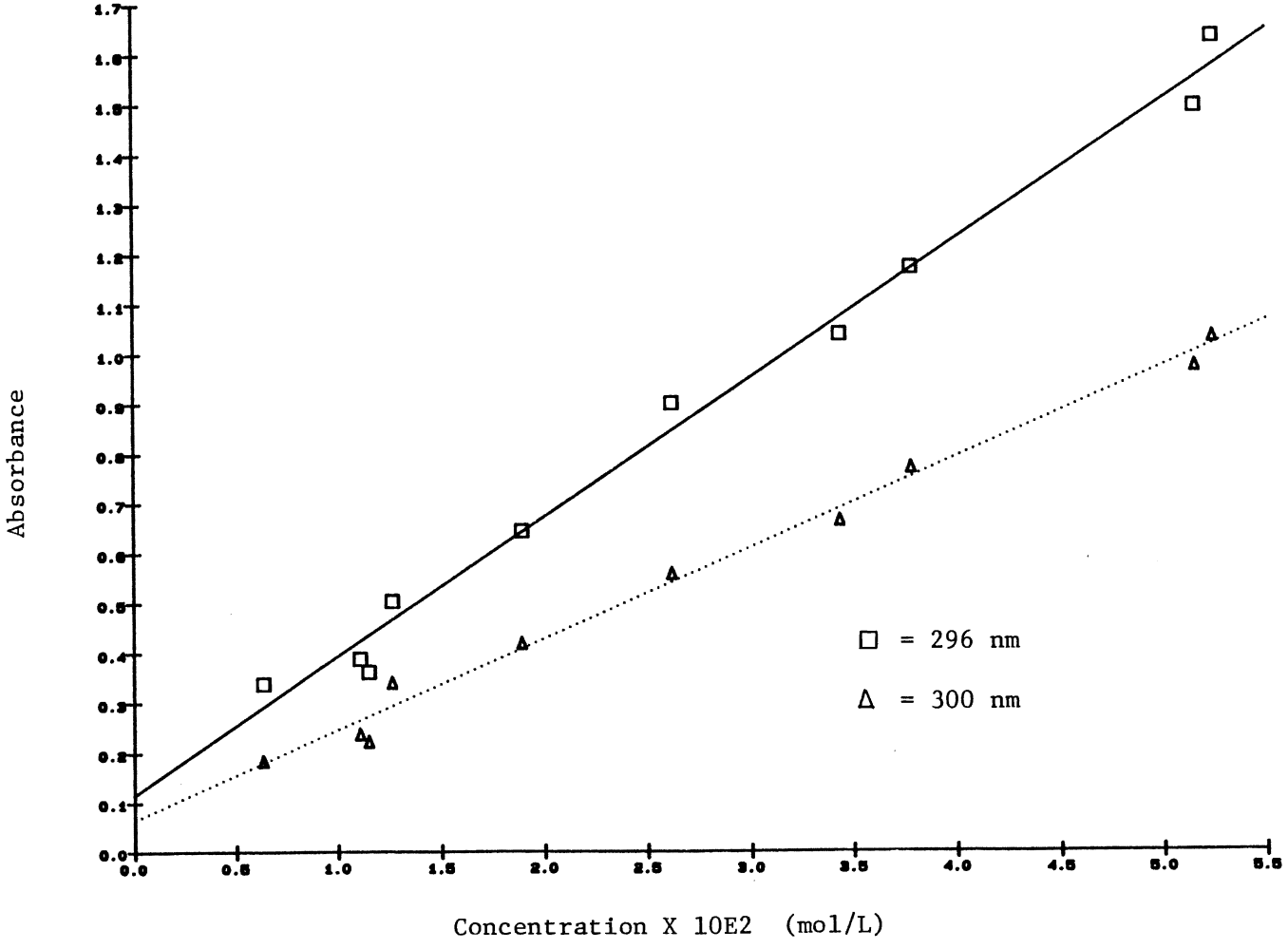

Figure 10. Boc-o-benzyl-Tyrosine Calibration Curve

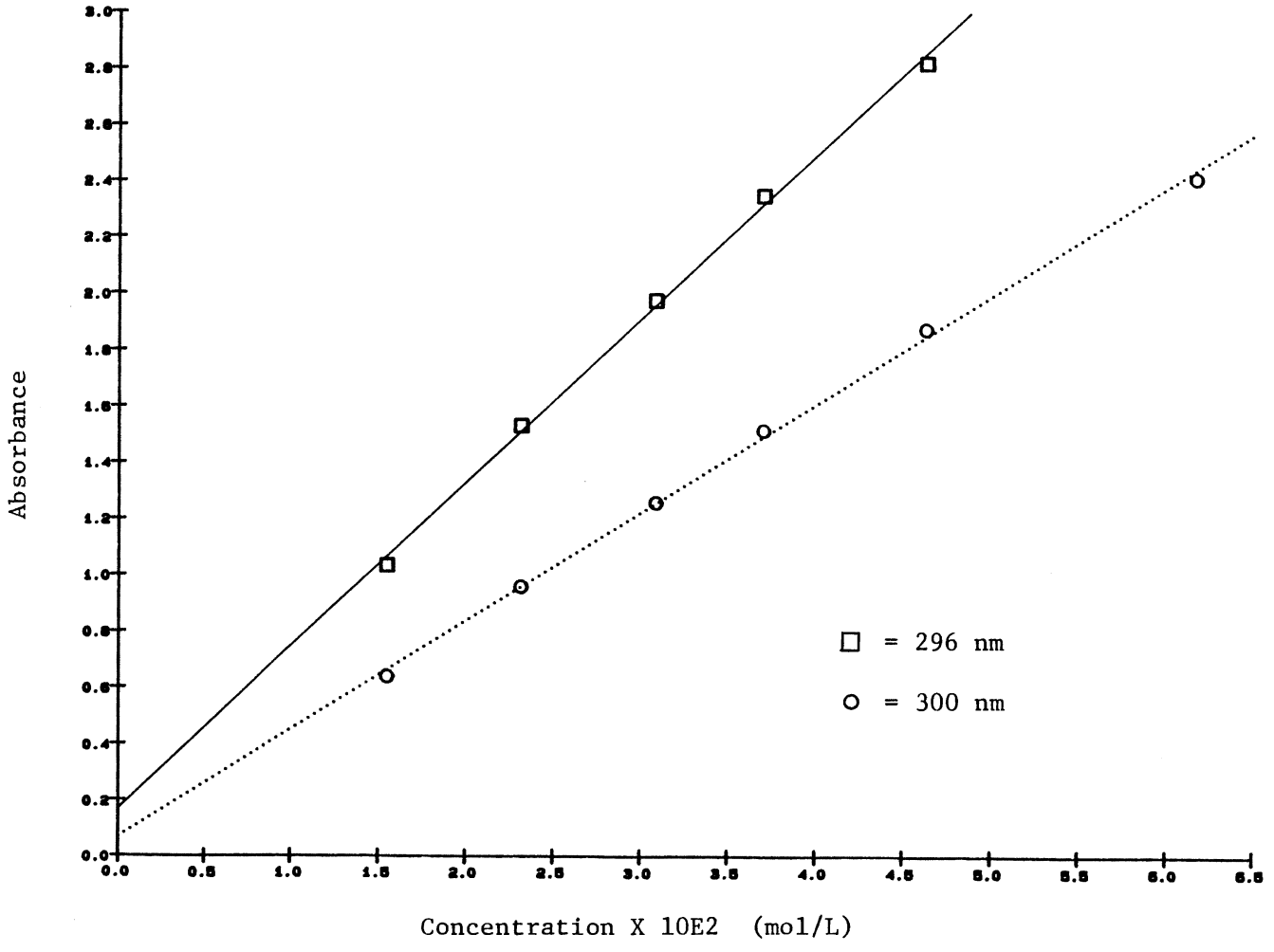

Figure 11. Boc-o-benzyl-Tyrosine Anhydride Calibration Curve

APPENDIX C

CALCULATIONS FOR RESIN WEIGHT ANALYSIS

PEPTIDE #1

Experiments 1-23

Theoretical

Initial Weight		2.00011 g
One Boc group lost (left deprotected)		
= 100 g/mol * 0.00072 mol		- 0.07200 g
Tyrosine Added (9 residues)		
= (371.44-100-17) * 0.00072 mol * 9		+ 1.64877 g
Phenylalanine Added (5 residues)		
= (265.31 - 117) * 0.00072 * 5		+ 0.53392 g
Tryptophan Added (2 residues)		
= (304.35-117) * 0.00072 * 2		+ 0.26978 g
Expected Weight	=	4.38058 g
Measured Weight	=	3.92455 g
Difference		-0.56604 g
Expected Added Peptide Weight	=	2.38047 g
Measured Added Peptide Weight	=	1.92444 g
Crude Yield	=	80.8%

PEPTIDE #2

Experiments 24-35

Initial Weight of Resin		1.99980 g
Phenylaline Added (13 residues)		
= (265.31-117) * (0.00072) * (13)		+ 1.38818 g
Expected Weight	=	3.38798 g
Measured Weight	=	3.26576 g
Difference	=	- 0.12222 g
Expected Added Peptide Weight	=	1.38818 g
Measured Added Peptide Weight	=	1.26596 g
Crude Yield	=	91.2%

PEPTIDE #3, FRAGMENT 1

Experiments 36-40

Initial Weight of Resin		2.00004 g
Phenylalanine Added (5 residues)		
= (265.31 - 117) * 0.00072 * 5		0.53392 g
Final Deprotection		
= 100 g/mol * 0.00072		- 0.07200 g
Expected Weight	=	2.46196 g
Measured Weight	=	2.57275 g
Difference	=	+ 0.11079 g
Expected Added Peptide Weight	=	0.46192 g
Measured Added Peptide Weight	=	0.57271 g
Crude Yield	=	124.0%

PEPTIDE #3, FRAGMENT 2

Experiments 41-53

Initial Weight of Resin		1.28895 g
Phenylalanine Added (4 residues)		
= (265.31 - 117) * 0.00036 * 4		+ 0.21357 g
Tyrosine Added (6 residues)		
= (371.44-117) * 0.00036 mol * 6		+ 0.54959 g
Serine Added (1 residue)		
= (295.26-117) * 0.00036 * 1		+ 0.06417 g
Left Protected		
= 100 * 0.00036		+ 0.03600 g
Expected Weight	=	2.15228 g
Measured Weight	=	1.85185 g
Difference	=	- 0.30043 g
Expected Added Peptide Weight	=	0.86333 g
Measured Added Peptide Weight	=	0.56290 g
Crude Yield	=	65.2%

PEPTIDE #3, TOTAL

Experiments 36-53

Initial Weight of Resin		1.00002 g
Added Amino Acids		1.09429 g
Left protected		+ 0.00000 g
Expected Weight	=	2.09431 g
Measured Weight	=	1.85185 g
Difference	=	- 0.24246 g
Expected Added Peptide Weight	=	1.09429 g
Measured Added Peptide Weight	=	0.85183 g
Crude Yield	=	77.8%

PEPTIDE #4

Experiments 55-60

Initial Weight of Resin		2.00005 g
Phenylalanine Added (3 residues) = (265.31 - 117) * 0.0009 * 3		+ 0.40044 g
Tyrosine Added (2 residues) = (371.44-100-17) * 0.0009 mol * 2		+ 0.45799 g
Left Protected		- 0.00000 g
Expected Weight	=	2.85843 g
Measured Weight	=	2.74395 g
Difference	=	- 0.11453 g
Expected Added Peptide Weight	=	0.85843 g
Measured Added Peptide Weight	=	0.74390 g
Crude Yield	=	86.7%

APPENDIX D

COMPUTER PROGRAM FOR CONCENTRATION AND GRADIENT ANALYSIS

```
$JOB                                                                    00000010
C  ********************************************************************* 00000020
C                                                                       00000030
C       PROGRAM FOR ANALYSIS OF CONCENTRATION AND                       00000040
C                 CONCENTRATION GRADIENT                                00000050
C                                                                       00000060
C                       WRITTEN BY                                      00000070
C                                                                       00000080
C                    WILLIAM DIETRICH                                   00000090
C                                                                       00000100
C                       FALL, 1986                                      00000110
C                                                                       00000120
C  ********************************************************************* 00000130
C                                                                       00000140
C                                                                       00000150
C     THIS PROGRAM CONVERTS THE ABSORBANCE CURVES INTO CONCENTRATION     00000160
C     CURVES.  THEN THREE DIFFERENT NUMERICAL TECHNIQUES ARE USED TO     00000170
C     FIND DERIVATIVES OF THE CONCENTRATION CURVES.  THESE METHODS       00000180
C     INCLUDE A 2-POINT, A CENTRAL 3-POINT AND A CENTRAL 5-POINT         00000190
C     SCHEME.                                                           00000200
C                                                                       00000210
C                                                                       00000220
C     NOMENCLATURE:                                                     00000230
C                                                                       00000240
C       ABB    -  ARRAY OF ABSORBANCE READINGS                          00000250
C       AMAX   -  HIGHEST ABSORBANCE READING                            00000260
C       AMIN   -  LOWEST ABSORBANCE READING                             00000270
C       C      -  ARRAY OF CONCENTRATIONS                               00000280
C       CMAX   -  HIGHEST CONC. CALCULATED                              00000290
C       CMIN   -  LOWEST CONC. CALCULATED                               00000300
C       DCDT   -  ARRAY OF SLOPES                                       00000310
C       H      -  TIME STEP SIZE                                        00000320
C       N      -  NUMBER OF ABSORBANCE READINGS                         00000330
C       PERC   -  PERCENTAGE OF REACTION COMPLETED                      00000340
C       X      -  LN (CONC.)                                            00000350
C       Y      -  LN (SLOPE)                                            00000360
C                                                                       00000370
C                                                                       00000371
      DIMENSION ABB(50),DCDT(50),C(50),PERC(50)                         00000380
      READ,N,H,AMIN,AMAX                                                00000390
      C(1)=0.12149-SQRT(4287.2-1092.4*AMAX)/546.2                       00000400
      PRINT,'              CONC                  DC/DT                P00000410
     DERC                  LN CONC                     LN DC/DT'        00000420
      CMAX=C(1)                                                         00000430
      ABB(1)=AMAX                                                       00000440
C                                                                       00000441
C        THE FOLLOWING LOOP READS IN ABSORBANCE VALUES AND CALCULATES   00000442
C        THE CORRESPONDING CONCENTRATIONS.  A DIFFERENT SET OF          00000443
C        EQUATIONS IS USED FOR EACH BB, CC AND C, DEPENDING ON THE      00000444
C        AMINO ACID.  THE LISTED EQUATIONS ARE FOR PHENYLALANINE AT     00000445
C        A WAVELENGTH OF 274.                                           00000446
C                                                                       00000447
      DO 10 I=2,N                                                       00000450
      READ,ABB(I)                                                       00000460
      BB=56.616-546.2*CMAX                                              00000470
      CC=ABB(I)+0.09983-66.36*CMAX+273.1*CMAX**2                        00000480
      C(I)=0.10365-SQRT(BB**2-1092.4*CC)/546.2                          00000490
      IF(C(I).LT.0.0) STOP                                              00000500
   10 CONTINUE                                                          00000510
      CMIN=C(N)                                                         00000520
      L=N-1                                                             00000530
C                                                                       00000531
C        THE FOLLOWING LOOP CALCULATES THE SLOPE OF THE CONCENTRATION   00000532
C        CURVE, CALCULATES APPROPRIATE NATURAL LOGS AND PRINTS RESULTS. 00000533
C                                                                       00000534
      DO 110 I=1,L                                                      00000540
      IF(I.NE.1) GO TO 20                                               00000550
C                                                                       00000551
C        THIS SECTION USES A 3-POINT FORWARD FORMULA TO CALCULATE THE   00000552
C        SLOPE AT THE FIRST POINT.                                      00000553
C                                                                       00000554
      DCDT(I)=((-1.0)*C(3)+4.0*C(2)-3.0*C(1))/(2.0*H)                   00000560
```

```
      GO TO 100                                                          00000570
   20 IF(I.NE.2) GO TO 30                                                00000580
C                                                                        00000581
C        THIS SECTION USES A 2-POINT CENTRAL FORMULA TO CALCULATE THE    00000582
C        SLOPE AT THE SECOND POINT                                       00000583
C                                                                        00000584
      DCDT(I)=(C(3)-C(1))/(2.0*H)                                        00000590
      GO TO 100                                                          00000600
   30 M=N-1                                                              00000610
      IF(I.NE.M) GO TO 40                                                00000620
C                                                                        00000621
C        THIS SECTION USES A 2-POINT CENTRAL FORMULA TO CALCULATE THE    00000622
C        SLOPE OF THE SECOND TO LAST POINT                               00000623
C                                                                        00000624
      DCDT(I)=(C(N)-C(I-1))/(2.0*H)                                      00000630
      GO TO 100                                                          00000640
   40 IF(I.NE.N) GO TO 50                                                00000650
C                                                                        00000651
C        THIS SECTION USES A 3-POINT BACKWARD FORMULA TO CALCULATE THE   00000652
C        SLOPE OF THE LAST DATA POINT                                    00000653
C                                                                        00000654
      DCDT(I)=(C(N-2)*(-1.0)+4.0*C(N-1)-3.0*C(N))/(2.0*H)                00000660
      GO TO 100                                                          00000670
C                                                                        00000671
C        THIS SECTION USES A 5-POINT CENTRAL FORMULA TO CALCULATE THE    00000672
C        SLOPE OF THE INTERIOR DATA POINTS                               00000673
C                                                                        00000674
   50 DCDT(I)=(8.0*(C(I+1)-C(I-1))+C(I-2)-C(I+2))/(12.0*H)               00000680
  100 Y=ALOG(ABS(DCDT(I)))                                               00000690
      X=ALOG(C(I))                                                       00000700
      PERC(I)=(CMAX-C(I))/(CMAX-CMIN)                                    00000710
      PRINT,C(I),DCDT(I),PERC(I),X,Y                                     00000720
  110 CONTINUE                                                           00000730
      STOP                                                               00000740
      END                                                                00000750
$ENTRY                                                                   00000760
$IBMSYS                                                                  00000770
```

VITA

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Master of Science

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