SOLID PHASE SYNTHESIS AND SELECTED NMR STUDIES OF PEPTIDES

A Thesis

Presented to

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Master of Science

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by

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To
Dr. Mythili Ramakrishnan

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ABSTRACT

Peptides corresponding to the sequences Pro-Tyr-Ser, Asp-Pro-Tyr-Arg, Asp-Pro-Tyr-Ser, and Glu-Tyr-Pro-Thr were synthesized by the standard solid phase method. Yields obtained were in the range of 20%. HF dried over CoF_3 was used to cleave the peptide from the resin. Co^{III} contamination during one synthesis of Asp-Pro-Tyr-Arg, warranted purification. Attempts to separate Co^{III} by EDTA lead to hydrolysis of the peptides, producing Tyr-Arg in very low yields.

The pure peptide Asp-Pro-Tyr-Arg was obtained later by cleaving the peptide from the resin with HF not dried over CoF_3.

All peptides synthesized were first deuterated and then studied by PMR at 100MHz. Peaks were assigned with the help of previously published data(1,2). Fully protonated peptide, Glu-Tyr-Pro-Thr in DMSO revealed an extremely complex PMR spectrum at variable temperatures, but does indicate some form of intramolecular hydrogen bonding. This could be due to the absence of any hindrance for the peptide to form a reverse turn and also due to 1 → 4 hydrogen bond stabilizing interaction.

Preliminary ^{1}H & ^{13}C spectra of the peptide Glu-Tyr-Pro-Thr indicated the possibility of two different conformational states for the peptides, but further work has to be done before any conclusive statements can be made. Such work is underway in our laboratory.

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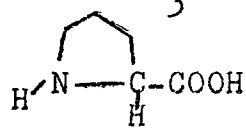
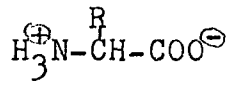
ABBREVIATIONS

Amino Acids

Abbreviation	Amino Acid	Structure of R
Asp or D	Aspartic acid	$-CH_2-COOH$
Tyr or Y	Tyrosine	$-CH_2-C_6H_4-OH$
Ser or S	Serine	$-CH_2-OH$
Arg or R	Arginine	$-CH_2-CH_2-CH_2-NH-C(=NH)-NH_2$
Glu or E	Glutamic acid	$-CH_2-CH_2-COOH$
Thr or T	Threonine	$-CHOH-CH_3$
Pro or P	Proline	

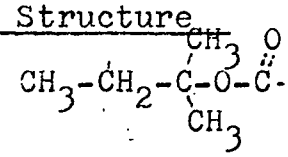
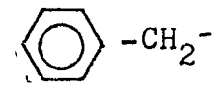
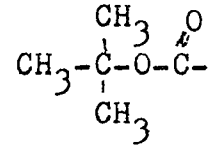
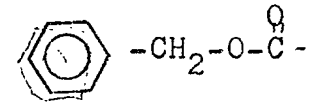
General Structure



Solvents and Reagents

Abbreviation	Solvent or Reagent
MeOH	Methanol
EtOH	Ethanol
DMF	N,N^1-Dimethylformamide
TFA	Trifluoroacetic acid
Ac_2O	Acetic anhydride
DCC	N,N^1-dicyclohexylcarbodiimide
Et_3N	Triethyl amine
t-BuOH	tert-Butanol

Protective Groups

Abbreviation	Protective Group	Structure
Aoc	tert-Amyloxycarbonyl	
Bzl	Benzyl	
Boc	tert-Butyloxycarbonyl	
OBzl	Benzyl ester	

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CHAPTER I

INTRODUCTION

INTRODUCTION

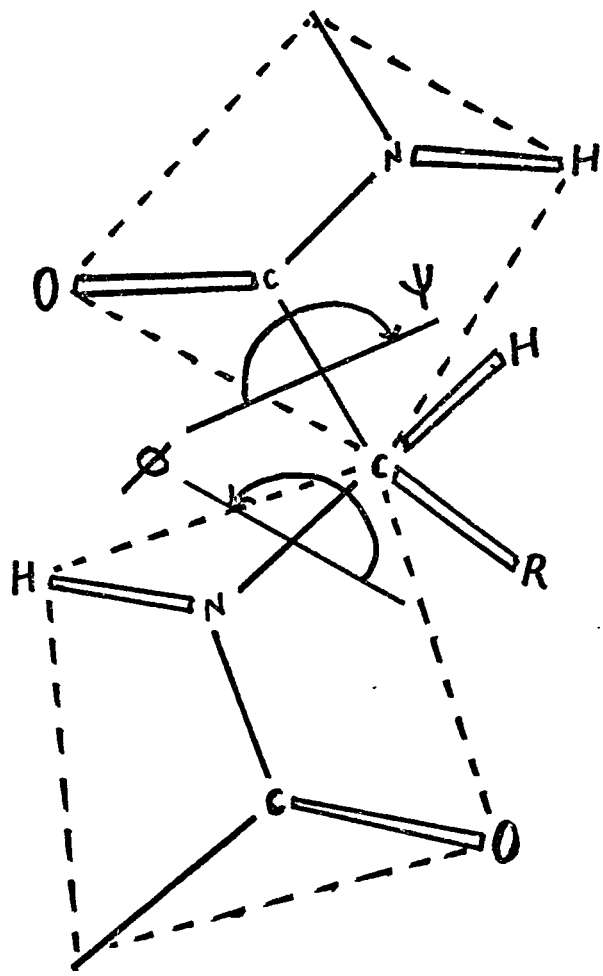
The reverse turn conformation in proteins is thought to play an important role in directing their folding. The reverse turn, first defined by Venkatachalam (4), consists of four amino acid residues, in a tight U conformation, with a hydrogen bond joining the >C=O of the first to >N-H of the fourth residue. This type of hairpinlike bend is also termed a β-turn (5).

The general conformation of a peptide molecule can be defined in terms of its ϕ and ψ angles (6). If the peptide bond is considered planar, then the polypeptide chain has only two degrees of freedom per residue: The first about C_α-N bond axis denoted by "ϕ", and that about C_α-C by "ψ".

Venkatachalam (4), has discussed the stereochemical criteria for polypeptides and proteins. He showed with computer assisted programming, that besides the 3_{10}-helix (3 residues with the total number of atoms comprising a hydrogen bonded unit being 10), two other non-helical structures exist, where intramolecular hydrogen bonding is possible. These two structures lead to chain reversal and have a $4 \rightarrow 1$ hydrogen bonding. The criteria for hydrogen bonding employed during computer programming was (a) the distance N.....O was required to be between 2.6 and 3.2 Å and (b) the angle NH-N....O was to be less than 30°. The type I differs from type II in only that the local conformation at C_2^α and C_3^α is different, with the C=O pointing

Figure 1

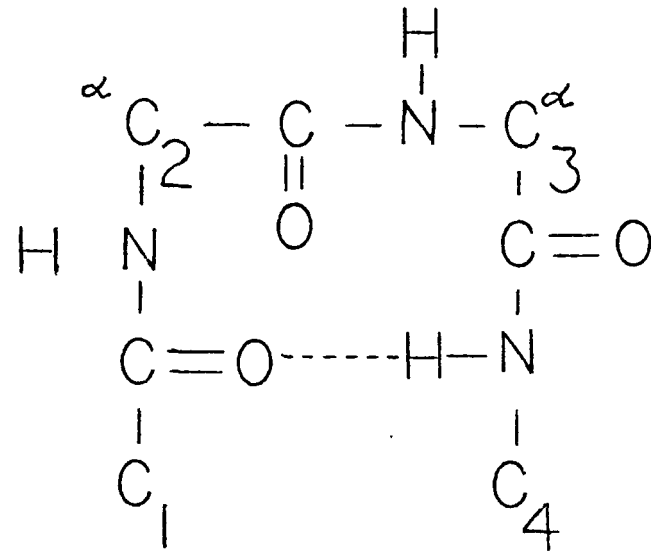
Diagrammatic representation of "ϕ" and "ψ" angles. All literature prior to 1970 follow this rule. The IUPAC-IUB Commission on Biochemical Nomenclature then introduced a shift in definition. The new (ϕ,ψ) values can be obtained from previous literature values by subtracting 180° from both angles.



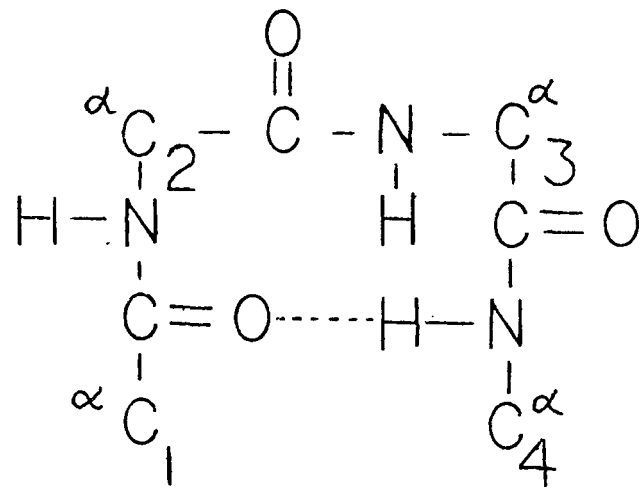
According to the new convention, in this diagram $\phi = -180$, $\psi = -180$

Figure 2

The two possible conformations of a tetrapeptide which lead to chain reversal and have 4 → 1 hydrogen bonding.



A



B

in one direction in I and in the opposite direction in II. Because of conformational restrictions only L-Gly can occur at position 3 in type II.

Figure 2 represents the two possible conformations of a tetrapeptide which lead to chain reversal and have 4 → 1 hydrogen bonding:

(A) Conformation of type I (B) Conformation of type II

Prediction of β-turns:

Chou and Fasman (3) developed a new predictive model for the secondary structure of proteins. Although uncertainty in the interpretation of x-ray data could lead to erroneous assignments of residues in β-turn conformations, such errors would likely cancel out if a large number of observations were utilized. X-ray data on 12 proteins were used and P_t values, based on this large statistical sampling, were computed. These values, representing an average, should be more accurate than those based on any individual protein. Table I lists the frequency of amino acids in the β-turns of 12 proteins.

The relative probability that a tetrapeptide will form a β-turn $P_t = f_i, f_{i+1}, f_{i+2}, f_{i+3}$...(eq. 1) where f_i, f_{i+1}, f_{i+2}, and f_{i+3} are respectively the frequency of

TABLE I

Frequency of Occurrence of Amino Acids
In the β turns of 12 Proteins.

Amino Acid	n[a]	(i)[b]	f_i[c]	(i+1)[b]	f_{i+1}[c]	(i+2)[b]	f_{i+2}[c]	(i+3)[b]	f_{i+3}[c]
Ala	204	10	0.049	10	0.049[d]	7	0.034	6	0.029
Arg	79	4	0.051	10	<u>0.127</u>	2	0.025	8	<u>0.101</u>
Asn	139	14	<u>0.101</u>	12	<u>0.086</u>	30	<u>0.216</u>	9	0.065
Asp	102	14	<u>0.137</u>	9	<u>0.088</u>	7	0.069	6	0.059
Cys	45	4	<u>0.089</u>	1	0.022	5	<u>0.111</u>	4	<u>0.089</u>
Gln	101	5	0.050	9	<u>0.089</u>	3	0.030	9	<u>0.089</u>
Glu	94	1	0.011	3	0.032	5	0.053	2	0.021
Gly	222	23	<u>0.104</u>	20	<u>0.090</u>	35	<u>0.158</u>	25	<u>0.113</u>
His	60	5	<u>0.083</u>	3	0.050	2	0.033	2	0.033
Ile	118	8	0.068	4	0.034	2	0.017	6	0.051
Leu	156	6	0.038	3	0.019	5	0.032	8	0.051
Lys	150	9	0.060	12	<u>0.080</u>	10	0.067	11	<u>0.073</u>
Met	28	2	0.070	2	0.070	1	0.036	2	0.070
Phe	64	2	0.031	3	0.047	4	0.063	4	0.063
Pro	81	6	<u>0.074</u>	22	<u>0.272</u>	1	0.012	5	0.062
Ser	201	20	<u>0.100</u>	19	<u>0.095</u>	19	<u>0.095</u>	21	<u>0.104</u>
Thr	162	10	0.062	15	<u>0.093</u>	9	0.056	11	0.068
Trp	44	2	0.045	0	0.000	2	0.045	9	<u>0.205</u>
Tyr	118	16	<u>0.136</u>	3	0.025	13	<u>0.110</u>	12	<u>0.102</u>
Val	175	4	0.023	5	0.029	2	0.011	5	0.029

[a] n = total occurence of each residue in the 12 proteins: carboxypeptidase A, α-chymotrypsin, ribonuclease S, myoglobin, lysozyme, subtilisin BPN', cytochrome b, cytochrome c, nuclease, elastase, papain, and thermolysin. [b] i, i+1, i+2, i+3 represent the total occurrence of each residue in the 1st, 2nd, 3rd, and 4th position of the β turn. [c] The frequency of occurrence is given by $f_i = i/n$, $f_{i+1} = (i+1)/n$, $f_{i+2} = (i+2)/n$, $f_{i+3} = (i+3)/n$. All f_is likely to occur in β turns are underlined.

occurrence for a certain residue at the first, second, third, and fourth position of a β-turn. For example, using eq.1 and Table I, P_t for the sequence Asp-Pro-Tyr-Ser is $(0.137)(0.272)(0.110)(0.104) = 4.26 \times 10^{-4}$. Preliminary investigation showed $P_t = 0.5 \times 10^{-4}$ to be a reasonable cut-off value in predicting the β-turns of the proteins studied.

In order to confirm the existence of β-turns in solution, tetrapeptides and their larger analogues have to be synthesized and their conformational analysis done under conditions not perturbing the molecule to a great extent, e.g. or, by NMR. Tetrapeptides selected for our study here primarily included two types viz. one which will be expected to form a β-bend in solution and the other which will not.

Asp-Pro-Tyr-Ser and Asp-Pro-Tyr-Arg were selected as examples for β-bend forms and the peptide Glu-Tyr-Pro-Thr was expected to be linear. These selections were based on the data given by Chou and Fasman (3). The P_t for Asp-Pro-Tyr-Ser is 4.26×10^{-4}, for Asp-Pro-Tyr-Arg is 4.14×10^{-4}, and for Glu-Tyr-Pro-Thr is 2.24×10^{-7}. For a tetrapeptide (i to i+4 residues) the sequence $Pro_{i+1}Tyr_{i+2}$ has a high probability of existence in a β-bend unit, whereas $Tyr_{i+1}Pro_{i+2}$ has very little probability. So these sequences were chosen, under the presumption that their conformational differences will probably show up in their NMR spectra. The N- and C- terminal residues were chosen on basis of their charged side chains for an indication

of the role of side chain charge-charge interactions in stabilizing reverse turns.

Peptide Synthesis:

Extensive coverage on the subject of peptide synthesis can be found in books published by Schroder and Lubke (7,8), Bodanszky and Ondetti (9), Bodanszky, Klausner, and Ondetti (10), and Law (11). In the classical method peptides are synthesized in the liquid phase. However, large peptides cannot be obtained quickly by this method. With the invention of solid phase methodology by R.B. Merrifield, a new era has dawned.(12,13,14,15). The advantages of this method are (a) simplicity in operation and (b) enormous savings in time. The serious drawbacks are (a) permanent chain termination and (b) incomplete reactions.

"Truncated sequences" are a result of permanent chain termination which could, for example, be caused by incomplete deprotection (if the protecting groups remain intact in subsequent cycles). A much more serious problem, however, is the formation of "failure sequences" or "deletion peptides". Deletion peptides could be formed as a consequence of incomplete coupling or of incomplete deprotection and cleavage of remaining protecting groups during the following cycle. With assumed 90% coupling yield and 10% formation of deletion peptides per cycle, for a tetrapeptide, the final product would contain 72.9% of the target sequence and 27.1% of a contaminant mixture consisting of fifteen different failure sequences. This leads to problems

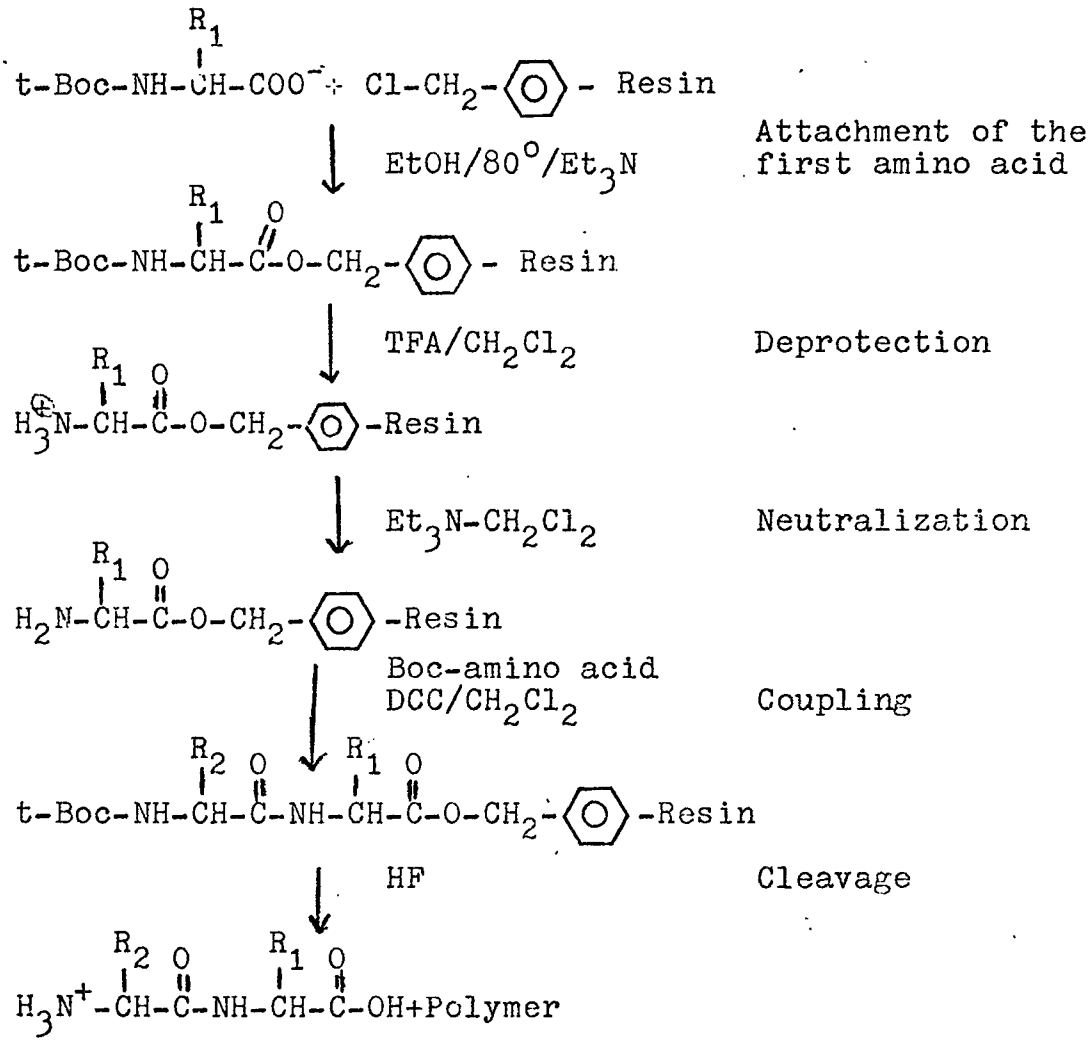
in purification of larger peptides which have an increased number of side-products.

Table II

Examples of achievements by Solid-Phase and by Solution Peptide Synthesis:

Solid-Phase Synthesis		Solution Synthesis	
Peptide and Reference	Size	Peptide and Reference	Size
Human Growth Hormone Li and Yamashiro (16)	188	Ribonuclease S. Protein Hirschmann et.al (21)	104
Ribonuclease A Gutte and Merrifield(17)	124	ACTH Porcine: Schwyzer & Sieber(22) Human: Bajusz et.al.(23)	39
Cytochrome C Sano and Kurihara(18)	104	Secretin Bodanzsky et.al.(24)	27
Bovine Basic Trypsin Inhibitor Noda et.al.(19) Izumiya et.al.(20)	58		

A standard experimental scheme for solid-phase peptide synthesis is shown below:



The solid support used is a cross-linked copolymer of styrene and divinyl benzene. It has the following properties: (a) complete insolubility in all solvents used, (b) chemical and physical stability, (c) it permits rapid diffusion of reactants into all reactive sites and easy removal of reactants, by-products, and side-products by washing and filtering and (d) possibility of substitution with a functional group for the attachment of the amino acid, by a stable chemical bond. It is estimated that for a 0.3 mmoles/gm substitution level, an average of 10^{12} reactive sites are available per single bend.

The first amino acid residue is attached to the chloromethylated resin through a benzyl ester linkage (25). This ester linkage requires drastic conditions for its cleavage, like HBr in presence of TFA or liquid hydrogen fluoride, this being one of its great disadvantages. Under these conditions the peptide is likely to be degraded.

The choice of a α-amino protecting groups is based upon the following conditions: (a) quantitative removal of the N_{α}-protecting group in a relatively short time and (b) complete stability of side-chain protecting groups. The most efficient method of removal of the Boc-group is treatment with TFA in CH_2Cl_2.

Side chain protection is employed in case of trifunctional amino acids. It is removed at the end of the synthesis along with the resin from the finished peptide-resin. A list of the protecting groups employed in the work

presented here, is shown in Table III.

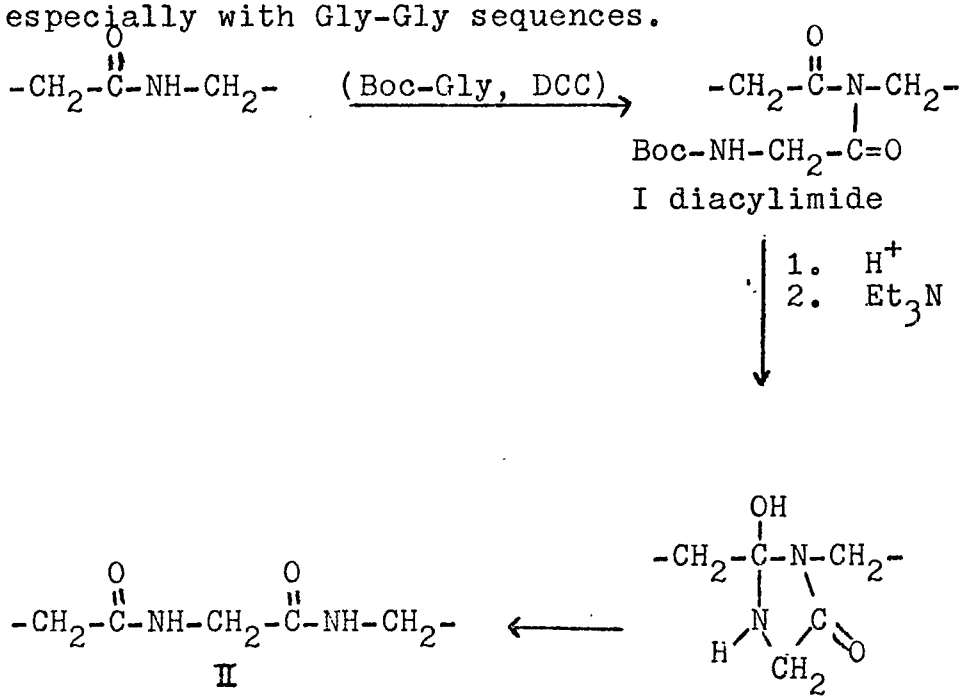
Table III

The most commonly used side-chain protection of functional amino acids and methods of cleavage.

Amino Acid	Side Chain Protection	Stabile	Cleaved
Arginine	Nitro(NO_2)	HCl, TFA HBr/TFA	H_2-Pd, HF
	p-toluenesulfonyl(Tos)	HCL, TFA HBr/TFA	Na-liq, NH_3-HF
Aspartic acid Glutamic acid	Benzyl ester(OBzl)	HCl, TFA	H_2-Pd, HF
Serine, Threonine Tyrosine	Benzyl ether(Bzl)	HCl, TFA	HBr/TFA, HF

The basic reaction in peptide synthesis is the formation of the amide bond. This step essentially consists of removing the elements of water. The carbodiimide method of Sheehan and Hess (26) is by far the most frequently used procedure for solid-phase synthesis. The use of DCC amounted to about 92% of all solid-phase synthesis, until November 1969. The other method, utilizes p-nitrophenyl ester and amounts to 6% of all synthesis, and was introduced by Bodanzsky (27,28). The DCC method has been found more useful, mainly because of its commercial availability and shorter reaction time. The majority of DCC couplings are believed to be complete within the first thirty minutes. Use of a large excess of Boc-amino acids and DCC relative to the substituted resin seems to improve the coupling yields. Too large an excess of acylating component and reagent can cause side reactions, such as diacylimide (I) formation (29) and amino acyl insertion (II)(30),

especially with Gly-Gly sequences.



Since 100% complete coupling cannot generally be taken for granted, "failure sequences" are bound to arise. Acylation with large excess of acetic anhydride in DMF in the presence of Et_3N was investigated by Merrifield (31), to block off irreversibly any remaining amino group. Ten fold excess of acetic anhydride and traces of Et_3N in CH_2Cl_2has been used here as the terminating agent.

Once the peptide has been synthesized on the polymer support, the resin has to be cleaved. HF has been found to be the most effective reagent for the cleavage (32,33). Ammonolysis can be used when a C-Terminal amide is desired(34). Transesterification is an alternative procedure, where the carboxyl end is obtained in the form of an alkyl ester. During HF treatment, HF is usually distilled from CoF_3 to eliminate traces of water. When tyrosine is present in the

peptide, an excess of anisole is added to act as a scavenger for nitronium ions, tert-butyl, and benzyl cations which would otherwise react with tyrosine. A special apparatus made of Kel-F is used to handle HF. The greatest advantage of HF is the simultaneous removal of all protecting groups from the peptide along with the resin. Yields obtained are relatively higher than other methods.

NMR Studies:

Reverse turns in peptides have been studied by NMR earlier (35,36,37). The conformation of Met_5-enkephalin, was discovered to be a β-turn with hydrogen bond between the amino proton of Met_5 and >C=O of Gly_2 by PMR. (38,39).

In a reverse turn conformation of a tetrapeptide unit, the intramolecularly hydrogen bonded proton on the nitrogen of the fourth amino acid residue is likely to be more shielded from the environment than other -NH protons. This fact was utilized by Gibbons et al. (39) to prove the β-bend conformation of enkephalin. PMR at variable temperatures in DMSO, revealed little change in the chemical shift of the methionine-NH proton as compared to the other -NH protons.

Urry et al. (35) studied the solvent titration (d_6-DMSO-D_2O) of the carbonyl carbon resonances in the protected linear pentapeptide $HCO\text{-}Val_i\text{-}Pro_{i+1}\text{-}Gly_{i+2}\text{-}Val_{i+3}\text{-}Gly_{i+4}\text{-}OCH_3$. Except for Val_i, the other carbonyl carbon resonances are seen to shift downfield as the volume percent of D_2O is increased to about 50%. This result can be inter-

preted to indicate that the carbonyl group of Val_i is shielded from the solvent, and is therefore involved in an intramolecular hydrogen bond.

It is apparent that the combination of ^{1}H and ^{13}C-NMR can lead to a quite detailed description of the hydrogen bonding pattern.

Kopple (37) studied derivatives of tetrapeptide sequences likely to form β-turns by PMR. Differential line broadening of N-H resonances by an added nitroxyl was used it indicate the presence of the sequestered N-H proton expected in β-turn conformations. The stable nitroxyl radical was generated by the addition of a low concentration of 3-oxyl-2,2,4,4-tetramethyl oxazolidine to the peptide solutions.

PMR of deuterated peptide samples are relatively less complex and aid in peak assignments. Protonation again, prior to introduction in DMSO, is required to view the N-H protons and their behavior at different temperatures, to study intramolecular hydrogen bonding.

CHAPTER II

EXPERIMENTAL

EXPERIMENTAL

Materials, Methods, and Instrumentation:

Amino acid derivatives were purchased from Peninsula Laboratories, San Carlos, California. Before using them, their purity was checked by TLC, m.p. and optical rotation, and the data was compared to those reported in the literature (39). Choromethylated polystyrene, (Merrifield resin) (40) 1% and 2% cross-linked with divinyl benzene (0.75 meq. of chlorine per gram: 200-400 mesh) were purchased from Schwarz-Mann Chemical, Orangeburg, New York; Pierce, Rockford, Illinois and Bio-Rad Laboratories, Richmond, California.

Analytical grade solvents and reagents were obtained and distilled prior to their use, as following: CH_2Cl_2 from $KHCO_3$, Et_3N from 2% α-naphthyl isocyanate or phenyl isocyanate, TFA from $CaSO_4$ and Ac_2O from anhydrous sodium acetate. Analytical grade 2-propanol was used with no further purification. Biogel P_2, cation and anion exchange resins were purchased from Bio-Rad Laboratory, Richmond, California.

Melting points were determined with a Thomas Capillary melting point apparatus and are uncorrected. The optical rotations were taken using a Perkin-Elmer Polarimeter. Amino acid analyses were performed on a Technicon Auto Analyzer. The samples were prepared for analyses by using 6N HCl at 110°, overnight, for acid hydrolysis. When analysis

of a peptide-resin sample was desired, 6N HCl and propionic acid, 1:1 by volume (41), or HCl:dioxane 1:1, was used under the same conditions as above. Fast hydrolyses, were performed at a temperature of 135° for two hours.

The hydrolyses were carried out under vacuum in a special hydrolysis tube. After the required time, the solution was evaporated to dryness and the hydrolysate was dissolved in pH 2.2 buffer, and used for analysis. (The resin was filtered out, in case of peptide-resin analysis.)

Thin layer chromatography was carried out on Analab and Analtech precoated silica gel plates in two solvents: (a) n-Butanol:acetic acid:water::4:1:1 and (b) n-Butanol: Pyridine:acetic acid:water::15:10:3:12. Detection was accomplished by chlorine starch-iodide spray (42) for peptides, ninhydrin spray (43) for amine functions, and the Sakaguchi test (44) for arginine.

The UV absorbance of the chromatographic fractions were determined on a LKB UVICORD II instrument at 280 nm.

PMR spectra were taken on a T-60 Varian instrument then on a Varian XL-100 spectrometer using data system from Nicolet Instrument Corporation. The solvents used were (a) 100% D_2O (Gold Label) and (b) 100% deuterated dimethyl sulfoxide (DMSO-d_6), both purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Temperature dependence studies were done using the latter instrument, which, incidentally is capable of giving ^{13}C spectra also. Peptide samples, run

using D_2O, were first lyophilized in D_2O, to replace the exchangeble protons with deuterium and those run in DMSO-d_6 were first lyophilized with H_2O, to protonate all possible positions in the molecule.

Solid Phase Synthesizer:

The solid phase syntheses were carried out in a Schwarz Bio Research peptide synthesizer. Silylation of the reaction vessel for synthesis was done with a 2% solution of trichloromethylsilane in toluene. The steps for an automated synthesis were programmed on a tape by means of a punching machine.

Operation:

The tape reader converts the punched tape into electrical information, which then activates the solenoid valves, releasing N_2 into the metering vessel. A motion sensor senses the number of motions of the reactor and after the required agitation the tape advances to the next function.

Programming the tape:

Using an 8-channel teletypewriter symbols corresponding to addresses are typed, and then subjected to run in the tape reader.

General Techniques Employed with the Solid Phase Synthesis:

(i) Attachment of the first amino acid residue to the resin:

Boc-amino acid (protected side group) and the chloromethylated resin were taken in equimolar ratio in sufficient quantity of absolute EtOH (10 ml/g) and 0.9 m of Et_3N were

added. The solution mixture was refluxed at 85° for two or three days. The resin was filtered and washed successively with EtOH, H_2O, MeOH, and CH_2Cl_2 three times with each solvent. The resin was then taken up in a separatory funnel, stirred, and suspended in CH_2Cl_2. After a clear line of demarcation appeared between the floating resin and the CH_2Cl_2, the bottom layer was discarded. Repetition of the procedure four or five times removed the fine particles which clog the reaction vessel during synthesis.

(ii) t-Boc-group cleavage:

We have employed two different concentrations of TFA/CH_2Cl_2 viz..25% and 50%. 50% TFA seems to be a better choice for the peptide that we prepared, because it yielded high purity of the peptide prior to ion-exchange purification.

(iii) Unreacted Chain Termination:

To avoid failure sequence formation acylation was employed to block off irreversibly any free amino group.. Ten fold excess of Ac_2O, in the required CH_2Cl_2 was taken and Et_3N added so as to make the molar ratio of Ac_2O:Et_3N::2.5:1.. The reaction time was usually ten minutes. Acetylation was performed at the end of every cycle.

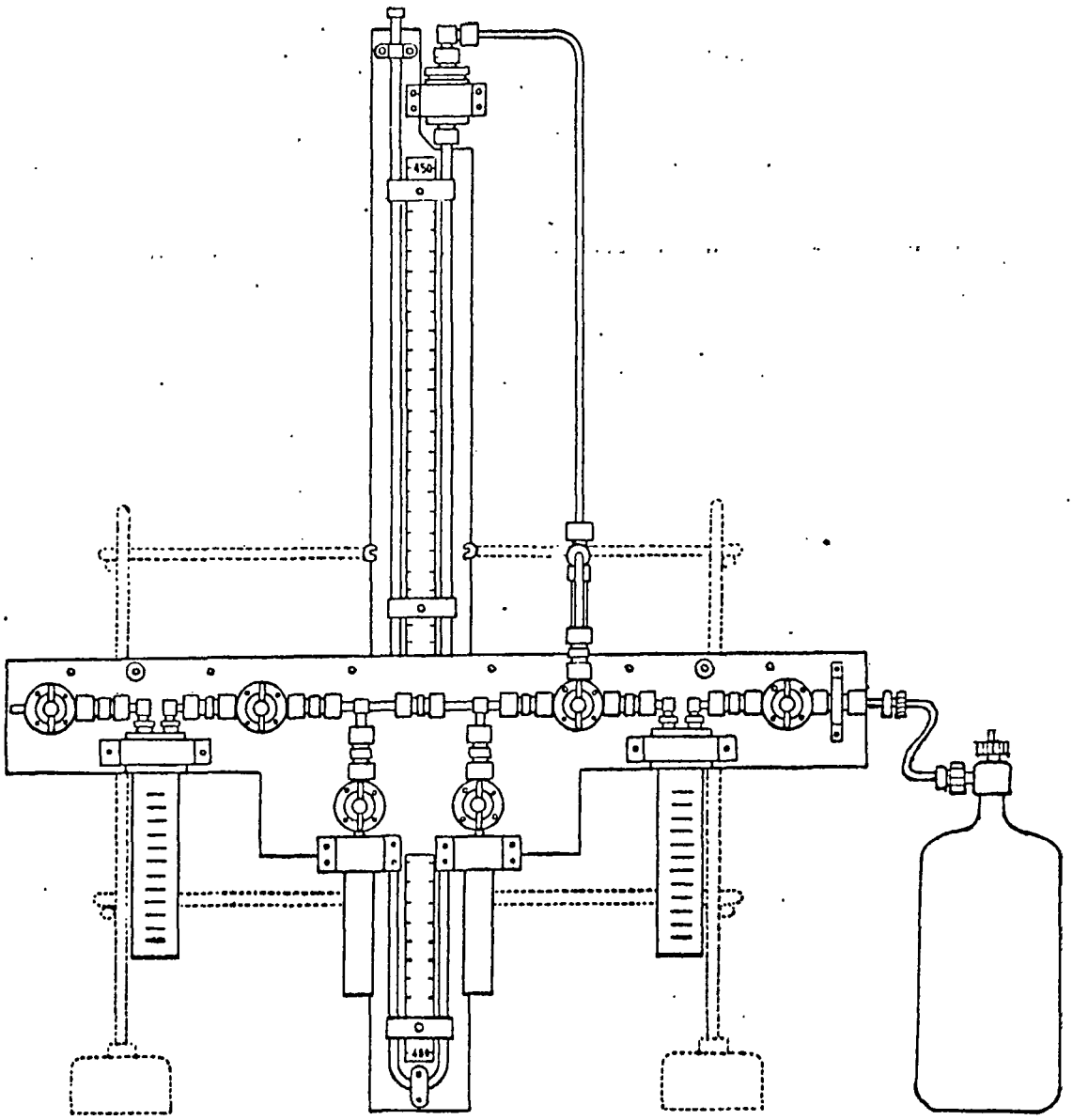
(iv) Cleaving the Resin from the Peptide:

(a) Cleavage using anhydrous HF

An HF reaction apparatus (Fig. 3), made primarily of diaflon (poly-trifluoromonochloroethylene), supplied by the Protein Research Foundation, Minoh, Osaka, Japan, was used which helped us

Figure 3

Diagram of HF Apparatus



to handle HF below 50°C safely.

Anhydrous liquid HF was first distilled into a reservoir maintained at a temperature of liquid N_2. This was done by evacuating the whole system and opening the HF tank. CoF_3 was usually placed in the reservoir to absorb traces of moisture.

The peptide-resin was placed in the reaction vessel and 50 fold excess of anisole was added. Then the desired amount of HF was distilled into the cooled reaction vessel. The reaction mixture was stirred for one hour at 0°C. After completion of the reaction the HF was stripped off *in vacuo*.

Any anisole left behind was extracted with an excess of ether. The product was then dried overnight under vacuum. The peptide was extracted with 1% HOAc and the resin filtered off. The HOAc extract was then subjected to purification procedures.

Synthesis of Z-Tyr-Otcp (46):

Z-Tyr (45) (4.73g 15mmoles) was added to 3.25g of trichlorophenol (16.5mmoles) in 50ml of EtOAc. The temperature was brought to -10°C in an acetone/ice bath and DCC (3.09g, 15mmoles) was added. After three hours at room temperature, the dicyclohexylurea formed, was removed by filtration.

To decompose the unreacted DCC, 10 drops of glacial acetic acid was added and the solution re-filtered. The unreacted Z-Tyr was removed by washing with 5% aq. $NaHCO_3$, eight times. The EtOAc layer was then dried over $MgSO_4$. The solution was filtered and evaporated to dryness. The

product was recystallized from EtOH. The weight of the dried crystalline product was 5.2g, corresponding to a yield of 52%; m.p.=145°, (Litt. value = 149° - 150°); $[\alpha]_D^{25}$= -29° (C 1 DMF) ; [Litt. value: -30° (C 1 DMF)]. TLC in $CHCl_3$: CH_3OH::9:1 showed one spot (R_f = 0.8.)

Synthesis of the Blocked Dipeptide Z-Tyr-Ser-OMe (47):

Ser-OMe·HCl (47) (1.4g, 9.0mmoles) m.p. 168° (Litt. value = 168°), $[\alpha]_D^{25}$ = +5.5 (Litt. value = +5.5) was taken in 10ml of EtOAc and $(C_3H_7)_2NC_2H_5$ (1.7ml, 9.0molles) was added and the mixture was stirred with cooling for 30 minutes. The precipitate of aminehydrochloride was removed by filtration. Next, Z-Tyr-Otcp (4.95g, 10mmoles) in 10ml EtOAc, was added to the above solution and the mixture cooled in an ice bath and stirred overnight.

The mixture was then washed seven times, with 150 ml. lots of 5% $KHCO_3$, to remove the trichlorophenol formed. NaCl was added during washing to effect separation of the aqueous and organic layer. The EtOAc layer was dried over $MgSO_4$ and then filtered. Evaporation and recrystallization under ether/ pet. ether:: 50/50 yielded 1g of the dipeptide corresponding to a yield of 26%, m.p. 130° [Litt. value:116°], $[\alpha]_D^{25}$ = +7.45 (C 2.2 DMF) [Litt. value = +13.5 (C 2.0 DMF)] (47). Amino acid analysis yielded Tyr and Ser exactly in the ratio of 1:1. TLC in EtOAc showed one spot, (R_f = 0.6).

Deblocking the Z-group (47) from Z-Tyr-Ser-OMe by Hydrogenolysis:

Z-Tyr-Ser-OMe (0.6g) was taken up in 20ml. CH_3OH and poured over reduced 10% palladium over charcoal (0.32g in

20ml CH_3OH), which was obtained by keeping it under H_2 at 50 psi for thirty minutes in a Parr apparatus. The reaction mixture was kept under H_2 at 50 psi overnight.

The TLC of the reaction mixture yielded several ninhydrin positive materials, relatively immobile in $CHCl_3$:CH_3OH:: 9:1 and EtOAc systems. Several repetitions of the above mentioned procedure did not yield a pure product. The Pd was filtered off and the solution was used as such for tripeptide synthesis.

Attempted Synthesis of the Tripeptide Z-Pro-Tyr-Ser-OMe:

Z-Pro-ONp (48) (0.60g, 1.7mmoles); m.p. 95° (Litt. value = 94° - 96°), $[\alpha]_D^{25}$ = -69.2° (C 2 DMF) (Litt. value = 68.2°,2,DMF) and the above deblocked peptide, 1.30mmoles (assuming 90% efficiency of deblocking), were mixed together in 20ml EtOAc and stirred overnight.

The reaction mixture was then washed eight times with 5% Na_2CO_3, to remove the para nitrophenol. The organic solvent was then evaporated and the product dissolved in a minimum quantity of MeOH:H_2O::80:20, and cooled. Crystals, which weighed 0.3g (0.6mmoles), (46% yield), were obtained. Amino acid analysis of the product yielded Pro-Tyr-Ser in the ratio 8:1:1.

Attempted Synthesis of the Dipeptide Z-Tyr-Arg-OMe:

Arg-OMe 2HCl (49) (0.4g, 15mmoles) m.p. = 193° (Litt. value - 196°), $[\alpha]_D^{25}$ = +17.28° (Litt. value = +21.7°, 2.5, MeOH) was taken up in 20ml DMF and Et_3N was added until the pH was 10. The solution was stirred for an additional half hour and the $Et_3N^+HCl^-$ formed was filtered off. Next, Z-Tyr-ONp (0.6g, 0.14mmoles) m.p. = 156° $[\alpha]_D^{25}$ = -13.3 (C 1 CH_3CN), was added and the reaction mixture was cooled and stirred overnight. TLC of the reaction mixture after 24 hours showed numerous spots ($CHCl_3$:CH_3OH::9:1).

The reaction mixture was then kept under 250ml of ether overnight at 0°, but no precipitate was obtained. The sides of the flask were scratched and the mixture kept again under 250ml ether, at 0°C for several days, but still no precipitate was obtained.

Synthesis of the Tripeptide Pro-Tyr-Ser (PYS) by Solid Phase Methodology:

(a) The synthesis was carried on a 1% cross-linked resin, with an aim of synthesizing the tetrapeptide, DPYS, and also its fragment, PYS. Since only the synthesis of the tripeptide was successful, experimental procedures for this peptide alone will be described.

Eight grams (i.e. 0.75 x 8 = 6.00mmoles of Cl) of 1% cross-linked resin and Boc-Ser (OBzl), 1.676g (6.00moles), were taken up in 40ml of absolute EtOH, and Et_3N, 0.756ml (5.4mmoles), was added. The mixture was then refluxed for forty hours at 85°C.

The product was worked up as described on page 18, in the experimental section. The dried product weighed 8.4g. Amino acid analysis performed on a 10mg sample of the material, indicated 0.22 mmoles/g substitution.

(b) The substituted resin was transferred to the reaction flask of a Schwarz/Mann Synthesizer and submitted to the program of Table V. The reaction vessel was silylated prior to introduction of the substituted resin. Boc-protected amino acids and DCC were added manually, prior to coupling. The protected amino acids Boc-Tyr (OBzl) and Boc-Pro were used in four fold excess, along with four fold excess of DCC in successive cycles. Each coupling was performed twice, at room temperature.

After incorporation of the three amino acids, corresponding to Pro-Tyr-Ser- resin, 4g of the material was removed from the reaction vessel and dried. A sample of 10mg of the material was hydrolyzed and subjected to amino acid analysis. The following result was obtained: Pro:Tyr:Ser::0.73:0.9:1.0.

(c) HF-Cleavage

HF, 40ml, was distilled over 2g of CoF_3 and dried overnight. The crude peptide-resin was taken in the reaction vessel along with anisole (28.00mmoles, $\sim$3ml) i.e. a 50 fold excess. It was then treated with HF at $0^{\circ}C$ for

Figure 4

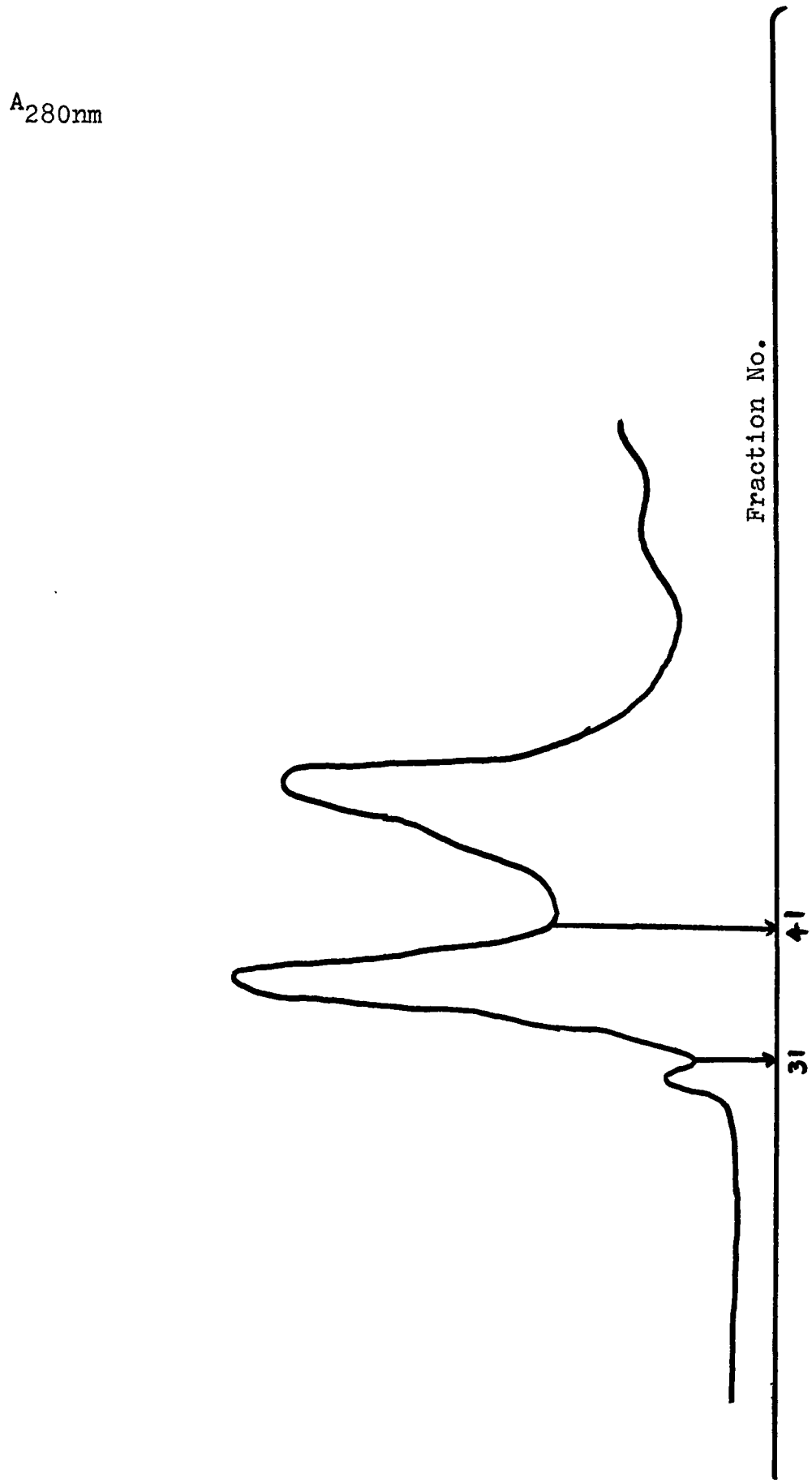
Elution diagram of the peptide, PYS on P_2 column:

Column = 2.8 x 40 cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 7.5ml


A_{280nm}
Fraction No.
41
31

one hour. The last traces of anisole were removed by washing with 250ml lots of pet-ether. The product weighed 4.00g. The peptide was extracted with 40ml of 0.1 AcOH and the resin was filtered off.

(d) Purification on P_2 BioGel Column:

The above solution was concentrated to 5ml by evaporation, loaded on a P_2 column and eluted with 0.1N AcOH; Seventy fractions, 7.5ml each were collected and the effluent was monitored by its absorbance at 280nm. (Fig. 4)

TLC of fractions 31 - 41 showed on spot with R_f = 0.21, in BAW:: 4:1:1. Amino acid analysis indicated Pro-Tyr-Ser in the ratio 1.00:0.80:1.00. UV absorbance at 280nm indicated the peptide content to be 100mg (0.27mmoles), corresponding to a 30% yield. Since the peptide obtained was sufficiently pure no further steps were taken for additional purification. The peptide, dissolved in 0.1N HOAc, was dried by evaporation, taken up in 35ml of water and lyophilized. Lyophilization was repeated four times, until all the HOAc was removed (monitored by the disappearance of PMR peak corresponding to $-\overset{O}{\overset{\|}{C}}-CH_3$ absorbance).

Synthesis of the Tetrapeptide Asp-Pro-Tyr-Ser by Solid Phase Methodology:

(a) The synthesis was carried out on 8g of 1% cross-linked Merrifield resin. Boc-Ser-OBzl (1.676g, 6mmoles) with was added to 8g of resin (6.00meq. of Cl), in 40ml of absolute EtOH. Et_3N (0.756ml, 5.4mmoles), was added and the mixture was refluxed forty hours. The product was worked up as mentioned in the experimental section, page 18.

The dried Boc-Ser(OBzl) resin weighed 8.5g. Amino acid analysis performed on a 10mg sample of the material indicated a substitution value of 0.21mmoles/g.

(b) The substituted resin was transferred to the reaction flask of a Schwarz/Mann synthesizer and submitted to the program of Table V. The side chain functional units used for the amino acids were β-Bzl for Asp, and OBzl for Tyr and Ser. Boc-amino acids and DCC were used in four fold excess. Double couplings were performed for all the amino acids.

After the incorporation of all the four amino acids to the resin, the Boc-group was cleaved by treatment with 50% TFA/CH_2Cl_2 for thirty minutes. The dried product weighed 8.7g. Amino acid analysis performed on a 10mg sample of the crude product indicated Asp:Pro:Tyr:Ser in the ratio 0.84:1.0:0.57:0.42.

(c) HF Cleavage

HF, 80ml, was distilled over CoF_3 (2g) and dried overnight. The crude peptide-resin was taken up in the reaction vessel of the HF apparatus and 9.67ml of anisole (50meq) was added. The mixture was treated with anhydrous HF for one hour at 0°C. The HF was stripped off and the product was washed six times with 250ml lots of pet-ether. The product weighed 5.8g. Amino acid analysis performed on a 10mg sample of the crude cleaved peptide-resin indicated 2.62μm, indicating 0.7g to be the upper limit of the target peptide that could be obtained.

TABLE V

Program Followed for the Synthesis of the Tetrapeptide, Asp-Pro-Tyr-Ser and the tripeptide, Pro-Tyr-Ser.

Step	Reagent	Minutes	No. of Operations	Vol of Reagent (ml) Per g of Resin
1	CH_2Cl_2	2	5	10
2	50% TFA/ CH_2Cl_2	5	1	10
3	50% TFA/ CH_2Cl_2	30	1	10
4	CH_2Cl_2	2	5	10
5	iPrOH	2	3	10
6	CH_2Cl_2	2	5	10
7	50% TFA/ CH_2Cl_2	5	1	10
8	50% TFA/ CH_2Cl_2	30	1	10
9	CH_2Cl_2	2	5	10
10	33% Et_3N/ CH_2Cl_2	2	1	10
11	33% Et_3N/ CH_2Cl_2	5	1	10
12	CH_2Cl_2	2	5	10
13	Boc-Amino acid(protected) + DCC(1:1 molar ratio) 4 fold excess	120		5
14	50% EtOH/ CH_2Cl_2	2	3	10
15	CH_2Cl_2	2	5	10
16	iPrOH	2	3	10
17	CH_2Cl_2	2	5	10
18	33% Et_3N/ CH_2Cl_2	2	1	10
19	33% Et_3N/ CH_2Cl_2	5	1	10
20	CH_2Cl_2	2	5	10
21	Boc-Amino acid(protected) +DCC(1:1 molar ratio) 4 fold excess	120		5
22	50% EtOH/ Ch_2Cl_2	2	3	10
23	CH_2Cl_2	2	5	10
24	iPrOH	2	3	10
25	CH_2Cl_2	2	5	10
26	Ac_2O/ CH_2Cl_2(10 fold Xs)	5	1	10
27	Ac_2O/ CH_2Cl_2(10 fold Xs)	10	1	10

The peptide was extracted with 36ml of 0.1N HOAc and the resin was filtered off. UV absorption of Tyr at 280nm indicated 1.772mmoles of Tyr content, corresponding to 0.819g of the peptide.

(d) Purification on BioGel P_2 Column:

Half of the above sample, (18ml), was taken and the volume reduced to 5ml by evaporation. It was loaded on a P_2 column, and eluted with 0.1N HOAc. Seventy fractions, 5.2ml each were collected. The effluent was monitored by its UV absorbance at 280nm (Fig. 5). Fraction number 41 was analyzed for amino acid composition and the following result was obtained: Asp:Pro:Tyr:Ser::0.96:1:0.83:0.73. TLC in BAW: 4:1:1 showed one spot with an R_f value of 0.39. Fractions 39 - 44 were pooled and the peptide content was estimated by UV absorption at 280nm. The amount of 0.15mmoles (85mg) corresponded to a yield of 17% from the substituted resin.

The acetic acid was evaporated and the peptide was lyophilized from 20ml water, five times.

(d') Purification of the other half of the crude peptide in acetic acid:

The other half (18ml), of 0·1N HOAc containing the peptide was evaporated to 5ml and loaded on a P_2 column and eluted with 0.1N HOAc. Seventy fractions, each measuring 5.2ml were collected and the effluent was monitored by it UV absorbance at 280nm (Fig. 6). Since it was known that the first peak contained the peptide (evidence from (d)), TLC in BAW;4:1:1 was run to determine the degree of purity. Fractions 41 - 45 showed presence of some fast moving impurity,

Figure 5

Elution diagram of the peptide DPYS on P_2 column (first half):

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 5.2ml

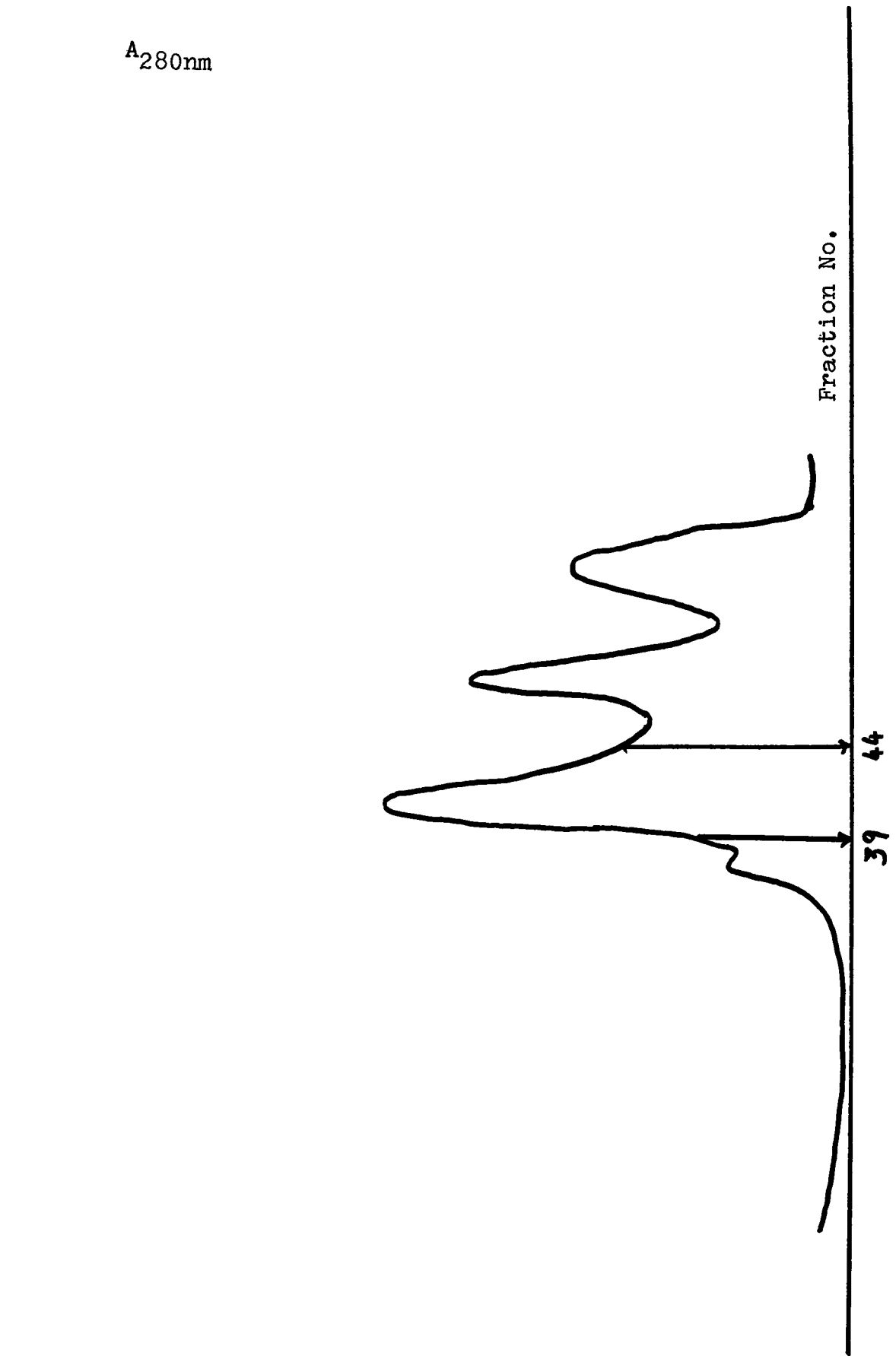

A_{280nm}
Fraction No.
44
39

Figure 6

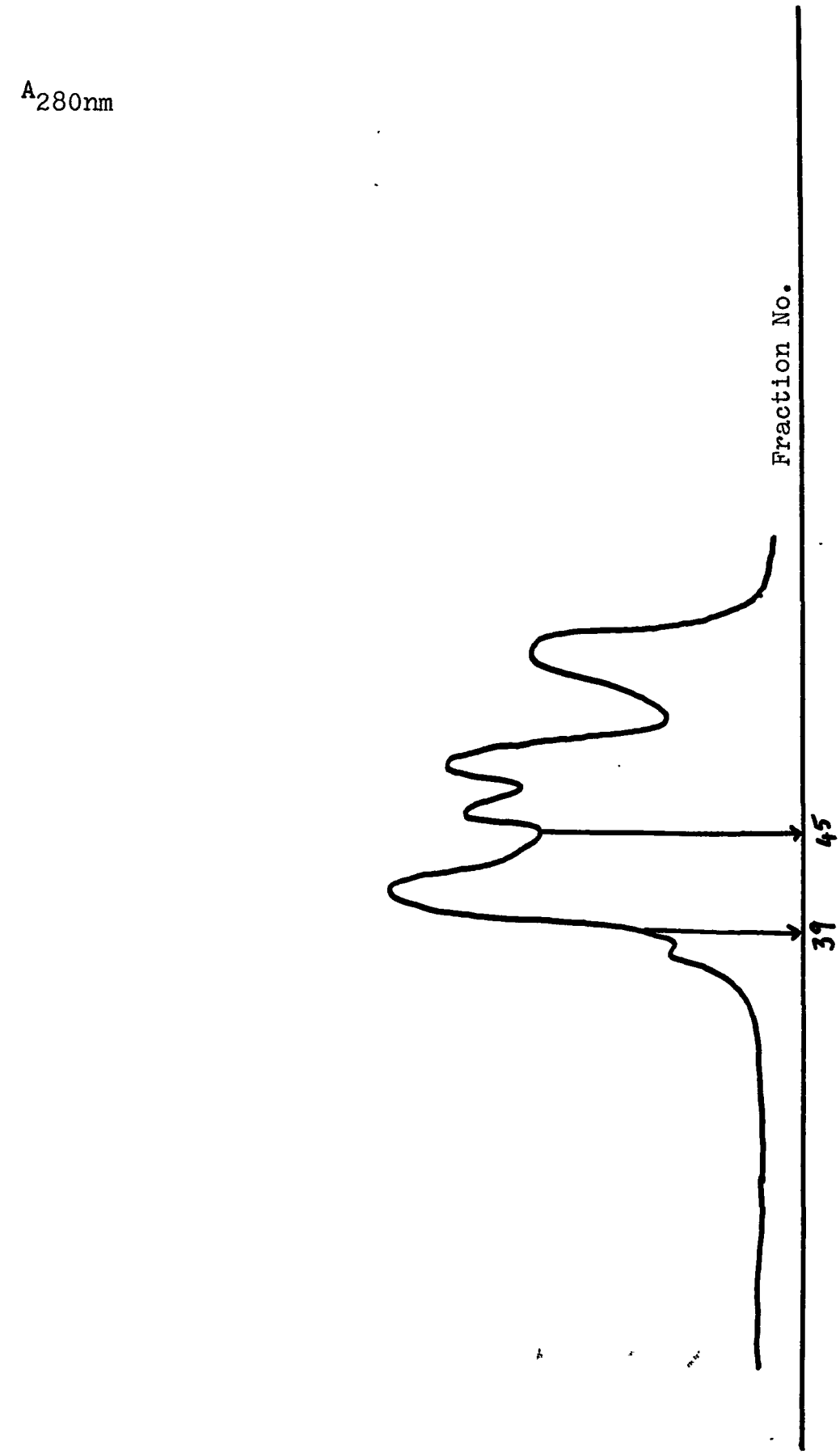
Elution diagram of peptide DPYS on P_2 column (second half)

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 5.2ml


A_{280nm}
Fraction No.
45
39

with R_f = 0.49.

(e) Ion-exchange purification on DEAE-BioGel A:

The sample to be purified (d') was taken up in 0.05M NH_4HCO_3 and subjected to ion-exchange chromatography on DEAE-BioGel A (50) (0.9 x 25cm) using a linear gradient of 0.05M to 0.2M NH_4HCO_3. One hundred and twenty fractions, 2ml each, were collected with a flow rate of 20ml/hour. No purification was achieved.

(f) DEAE-Sephadex:

Since separation from impurities could not be achieved by using DEAE-BioGel A, DEAE-Sephadex was tried forseparation under the same conditions.

The eluent containing the peptide from BioGel A, was diluted with water 0.05M in NH_4HCO_3. It was loaded on DEAE-Sephadex column (1.5 x 35cm) with 0.05M - 0.2M NH_4HCO_3 as a linear gradient and 120 fractions were collected, 2ml each, andthe eluent was monitored by its UV absorbance at 280nm.

TLC of all the peak fractions indicated absence of the target peptide.

The peptide was extracted using 6M Guanidine hydrochloride in 0.2M NH_4HCO_3, and a large peak was obtained in the UV spectrum. TLC (in BAW::4:1:1) indicated two components, one corresponding to Guanidine hydrochloride (R_f = 0.45) and the other due to the peptide DPYS (R_f= 0.39). The solution was evaporated to dryness and taken up in 10ml 0.1N HOAc and then passed through P_2BioGel A for

desalting.

Two purifications on P_2column, followed by lyophilization in water gave peptide weighing 40mg (0.08mmoles) corresponding to an 8% yield.

Synthesis of the Tetrapeptide, Asp-Pro-Tyr-Arg, by Solid Phase Methodology, resulting in cobalt contamination:

(a) The synthesis was carried out on 16g of 1% cross-linked Merrifield resin. Substitution of the resin with Aoc-Arg (Tos) was carried out in two different batches.

In the first batch, 8g of the resin (6.00mmoles of Cl) and Aoc-Arg (Tos), 2.667g (6.00mmoles) were taken up in 50ml of absolute EtOH. Et_3N, o.756ml (5.4mmoles) was added and the mixture refluxed for sixty hours at 85°C. The product was worked up as in page 18, and the dried product weighed 8.6g. Amino acid analysis performed on 10mg sample of the material, indicated a substitution value of 0.052mmoles/g.

Since the substitution came out to be so low, an additional 8.0g of the 1% cross-linked resin was refluxed with Aoc-Arg (Tos), 2.667g and 0.756ml of Et_3N in 50ml absolute EtOH for seventy two hours. After a work up as before, the dried product weighed 8.4g and amino acid analysis indicated 0.051mmoles/g substitution.

(b) The substituted resin, 17g, was transferred to the reaction flask of a Schwarz/Mann synthesizer and submitted to the program of Table V. The dried product weighed 15.9g. Amino acid analysis performed on 10mg sample of the material indicated the presence of all four amino acids, with Asp and Pro occuring in the same place.

Tyr and Arg were present in the ratio 0.86:1.0. This gave an upper limit of the peptide that could be obtained to be 0.48mmoles or 214mg.

(b) HF Cleavage:

HF, 120ml, was distilled over 2g of CoF_3 and dried overnight. The peptide-resin, which had 0.48mmoles of the peptide was taken up in the reaction flask with 2.5ml anisole, (i.e. 0.48 x 50 = 24.0mmoles).

The peptide-resin was treated with HF, for one hour at 0°C. Instead of the customary red coloration of the peptide-resin in contact with HF in presence of anisole, a dirty green color was observed. The HF was stripped off by evacuation for two hours, and any anisole left was extracted with ether. The product was dried and weighed. The material obtained weighed 16.0g.

The peptide was extracted with 81ml of 0.1N HOAc, which turned pink, and the resin (dirty green) was removed by filtration. UV absorbance at 280nm indicated 1.196mmoles of peptide present in the AcOH extract. Amino acid analysis, with 6N HCl (the solution turned blue) at 110°C overnight, indicated Tyr and Arg in the ratio 0.6:1.0. Asp and Pro appeared as an unsymmetrical peak.. The peptide content was thus estimated to be 0.015mmoles. The color change to blue, on acidification during hydrolysis indicated presence of cobalt.

Figure 7

Elution diagram of the Co (III) contaminated peptide DPYR on P_2 Bio Gel column:

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 5.1ml

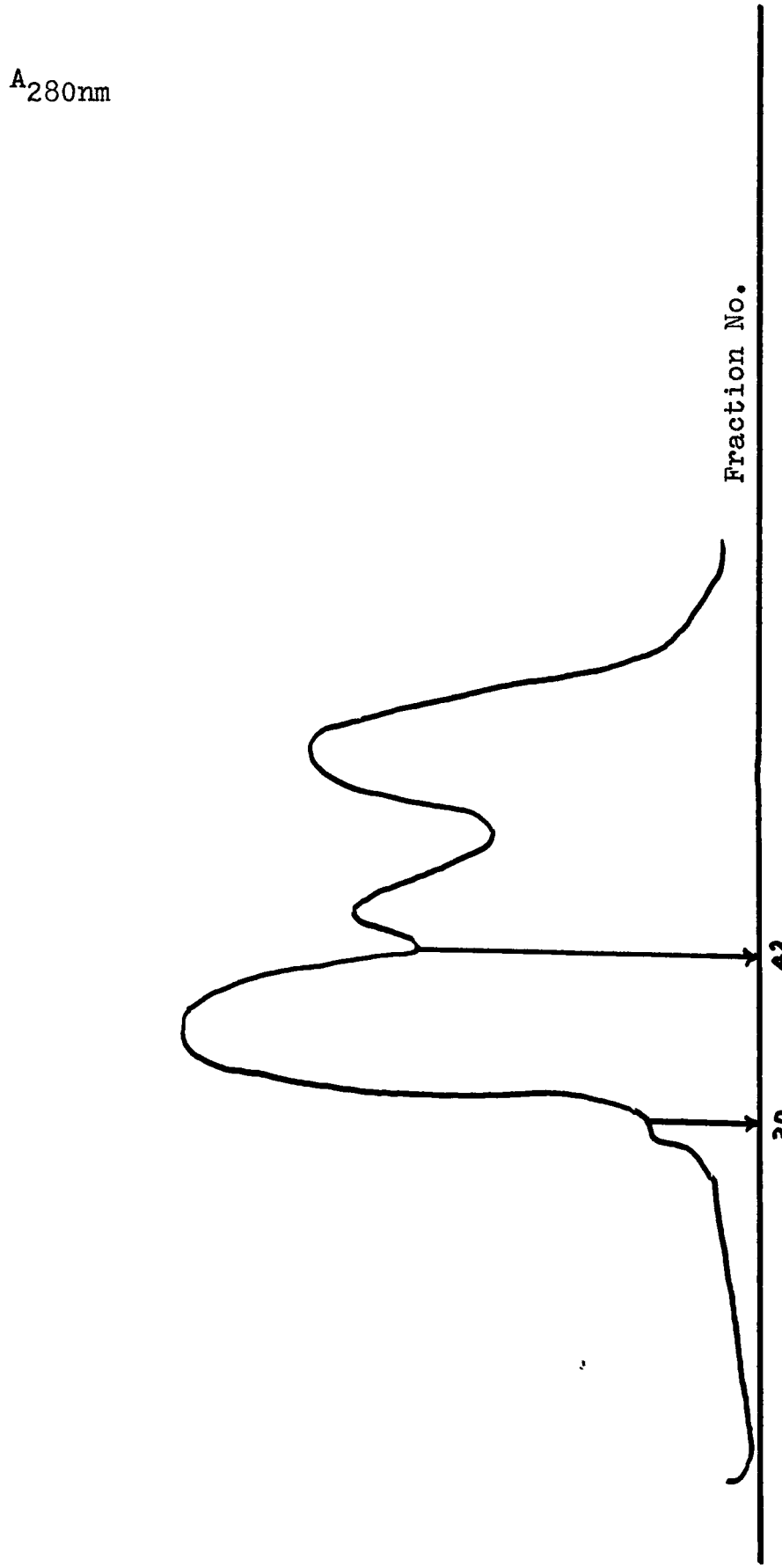

A_{280nm}
Fraction No.
42
30

Figure 8

Elution diagram of the peptide DPYR (of #31) for the separation of Co (III) ions, after treatment with EDTA:

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 5.1ml

A_{280nm}

Figure 9

Elution diagram of the Co (III) contaminated peptide DPYR (the whole lot) for the separation of Co (III) ions, after treatment with ten fold excess of EDTA:

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 5.5ml

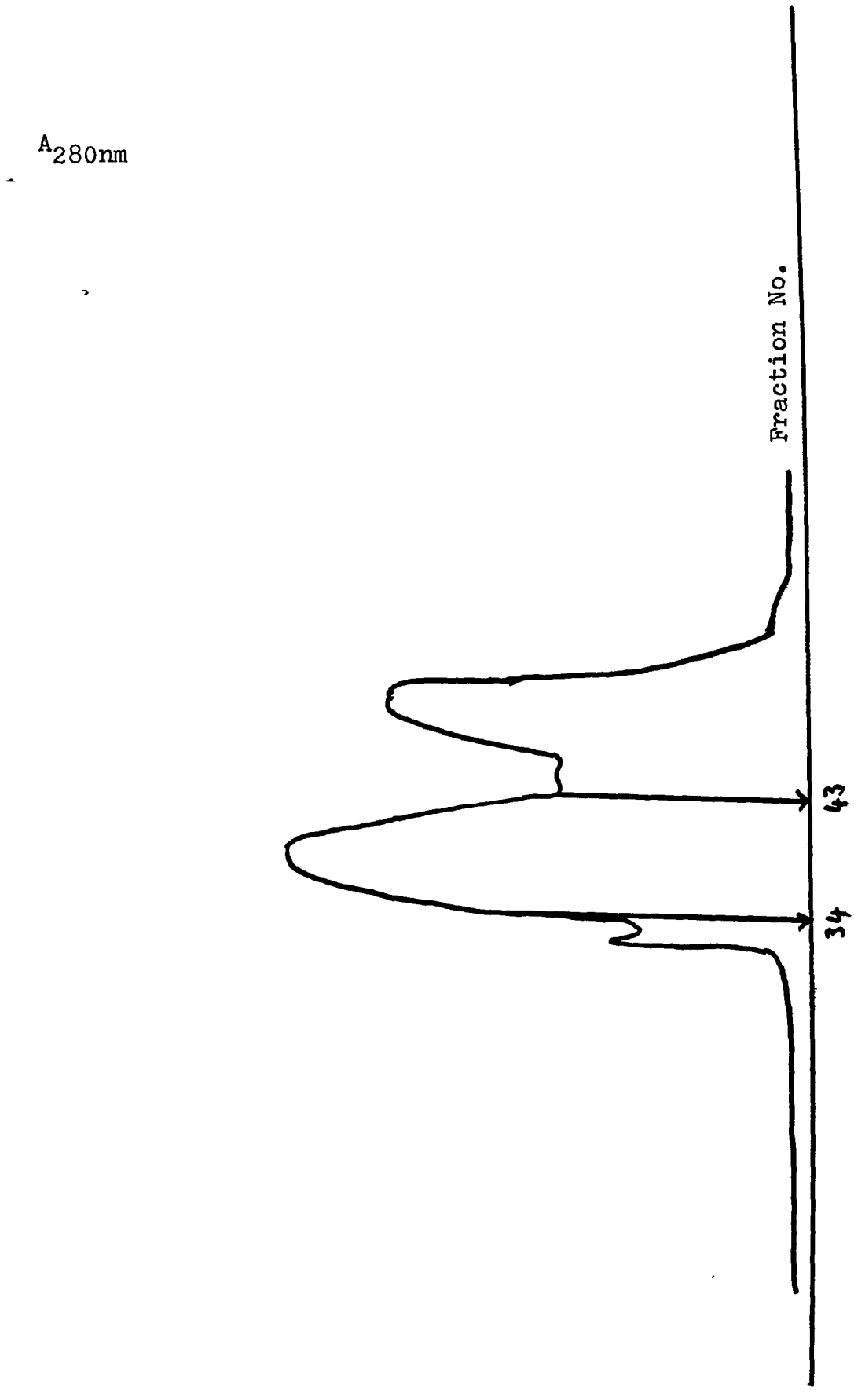

A_{280nm}
Fraction No.
43
34

Figure 10

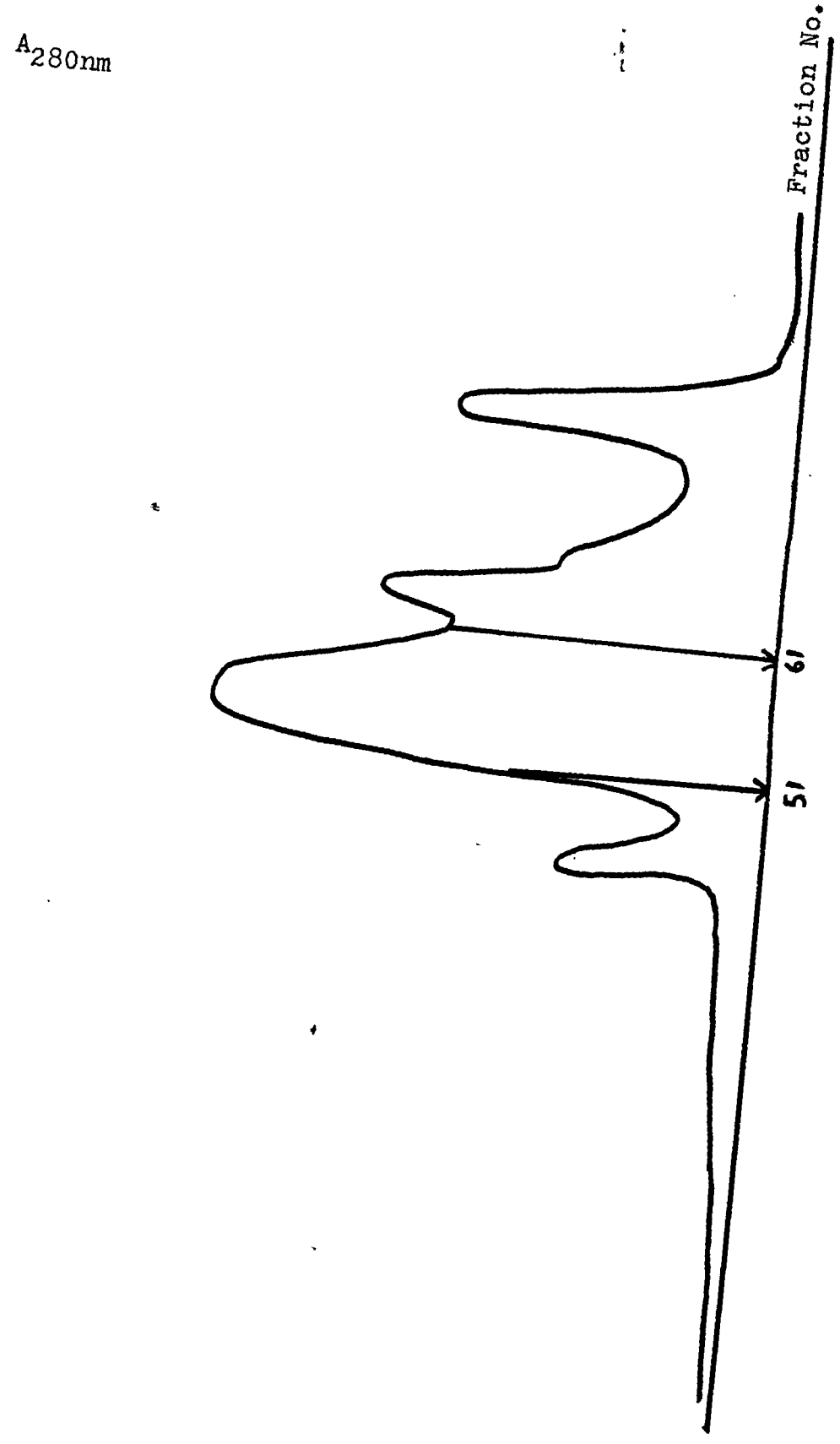
Elution diagram of the peptide DPYR for the separation of Co (III) ions, after treatment with EDTA (second purification):

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 3.6ml


A_{280nm}
Fraction No.
61
51

Purification on BioGel P_2 Column:

The 81ml sample from above was evaporated to 5ml and passed through a P_2column. Seventy fractions, 5.1ml each were collected and the effluent was monitored by its absorbance at 280nm (Figure 7). Amino acid analysis of fraction #56 showed very low Tyr concentration. Fractions 28 - 42, which were pink in color, comprised the first peak. UV absorption at 280nm revealed fraction 31 to have 0.0056mmoles of Tyr. With an idea of separating the peptide from Co^{III} ions, 5.6ml of 10mmoles EDTA solution was added to #31 on an experimental basis and the solution was stirred for two hours. The solution was loaded on a P_2column and eluted with 0.1N HOAc (Figure 8). Seventy fractions, 5.1ml each, were collected. Fractions 32 → 36 were pink. UV absorption at 280nm was negative. Fractions 36 → 46 did not exhibit any coloration. Tyr absorption was positive, (however the peptide elution had considerably slowed down since the volume of eluent required to elute the sample was 204.0ml as compared to 175ml during P_2 purification earlier).

Since separation seemed to have been achieved, the whole Co^{III} contaminated peptide sample was treated with 70ml (0.7mmoles) of 10mM solution of EDTA and the mixture was stirred for two hours. The solution was evaporated to dryness and taken up in 10ml of 0.1N HOAc and passed through P_2 column (Figure 9). Seventy fractions were collected, 5.5ml each and the effluent was monitored by its UV absorbance at 280nm. Fractions 30 → 33 had majority of the pink color. Fractions 34 → 43 however had some Co^{III} in them. Amino acid analysis

of fraction #50 revealed the presence of only Tyr in low concentration.

Fractions 34 → 43 were pooled (and also the fraction number 31, purified (Fig. 8), and treated with 70ml of 10mM EDTA. The solution was then stirred for two hours. The volume was reduced to 10ml by evaporation and passed through P_2 column and the effluent was monitored by its UV absorbance at 280nm (Fig. 10). Ninety fractions were collected, measuring 3.6ml each, with 0.1N HOAc as eluent. Fractions 40 → 45 showed absence of Tyr. Fractions 50 to 60 were pooled and the Tyr content of 0.05mmoles was estimated by it UV absorbance at 280nm. Amino acid analysis revealed only Tyr and Arg in the ratio 0.8:1.0. The material was lyophilized from H_2O four times and 28.1mg of material was obtained, corresponding to a yield of nearly 7%. TLC in BAW::4:1:1 gave only one spot with an R_f value of 0.26. NMR after lyophilization in D_2O, showed only Tyr and Arg peaks. The product exists as an acetate salt because of the presence of Arg side chain.

Synthesis of the Tetrapeptide Asp-Pro-Tyr-Arg by the Solid Phase Methodology:

(a) The synthesis was carried out on 8g of 2% chloromethylated resin (i e. 1.5 x 8 = 12mmoles of Cl). Aoc-Arg (Tôs), 5.33g (12mmoles) was added to the resin, in 70ml absolute EtOH. Et_3N, 1.6ml (10.8mmoles) was then added and the mixture was refluxed for sixty hours at 85°C.

The product was worked up as described on page 18, and vacuum dried overnight. The dried product weighed 9.4g.

Amino acid analysis performed on a 19mg sample of the material, yielded a substitution value of 0.189mmoles/g.

(b) The substituted resin was transferred to the reaction flask of a Schwarz-Mann Synthesizer and submitted to the program of Table V. The product was dried and it weighed 12.4g. A sample of 10mg of the material was hydrolyzed and subjected to amino acid analysis. The following result was obtained, Asp:Pro:Tyr::0.86:0.91:1.0. UV absorption at 280nm indicated 2.4mmoles of peptide in 12.4g of peptide-resin. The amino acid analysis was performed on a long column, so it was inconvenient to wait for the Arg peak to appear. However the presence of Arg could be assumed and the resin was cleaved by HF treatment.

(c) HF Cleavage

HF, 80ml, was distilled in a reservoir without any CoF_3 in it. The remaining peptide-resin, weighing 12.4g was mixed with 13ml anisole (120mmoles) and treated with 80ml of HF for one hour at $0^{\circ}C$. The peptide was extracted with 32ml of 0.1N HOAc and the resin filtered off. A 100µl sample of this solution was subjected to hydrolysis for amino acid analysis. The following result was obtained: Asp:Pro:Tyr::0.97:0.98:1.0. This value indicated 0.41mmoles of the peptide in the solution. The UV absorption at 280nm gave a concentration of 0.97mmoles peptide content.

(d) Purification on P_2 Column:

The HOAc solution containing the peptide was concentrated to 5ml by evaporation and loaded on a P_2 column, then eluted with 0.1N HOAc. Seventy fractions each measuring 5.3ml were collected and the effluent was monitored by its UV

Figure 11

Elution diagram of the peptide DPYR on P_2 column (after HF cleavage of the resin):

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml/hr

Vol. of each fraction = 5.3ml

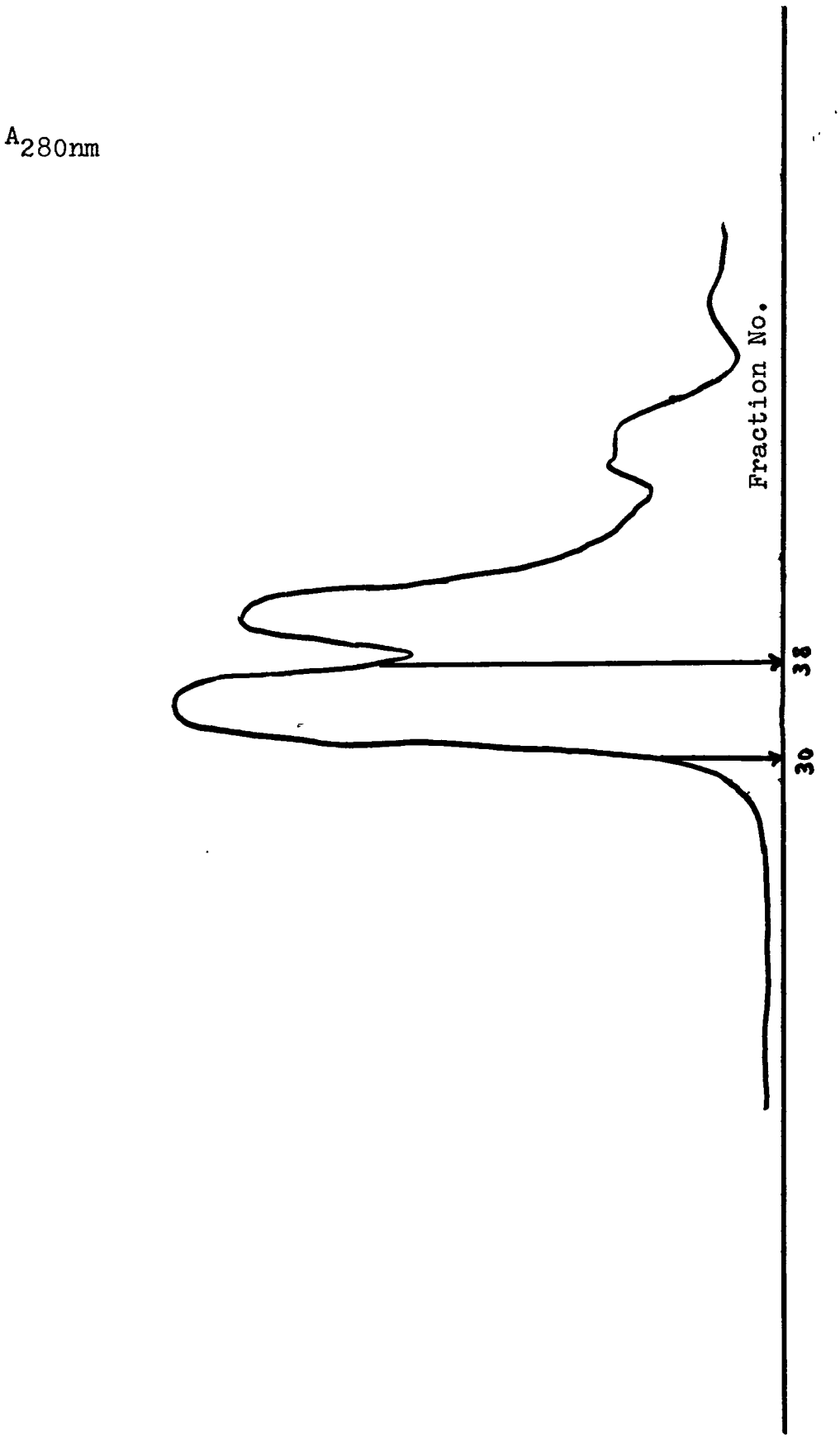

A_{280nm}
Fraction No.
38
30

Figure 12

Apparatus used for ion-exchange column having Dowex 50-X8 used for the purification of the peptide DPYR.

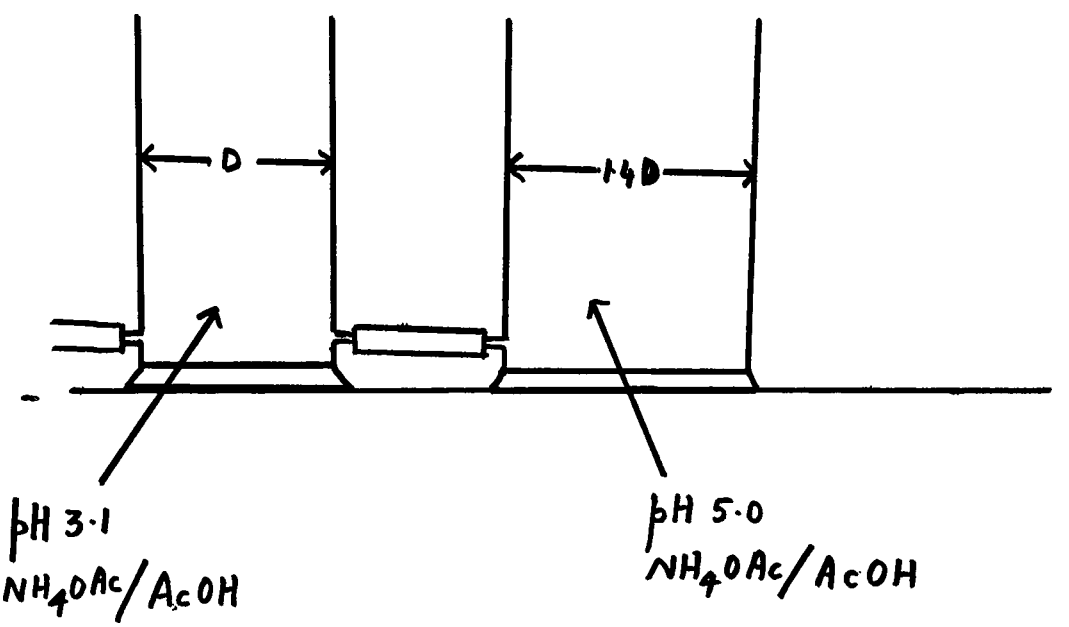

D
1.4D
pH 3.1
NH4OAc/AcOH
pH 5.0
NH4OAc/AcOH

Figure 13

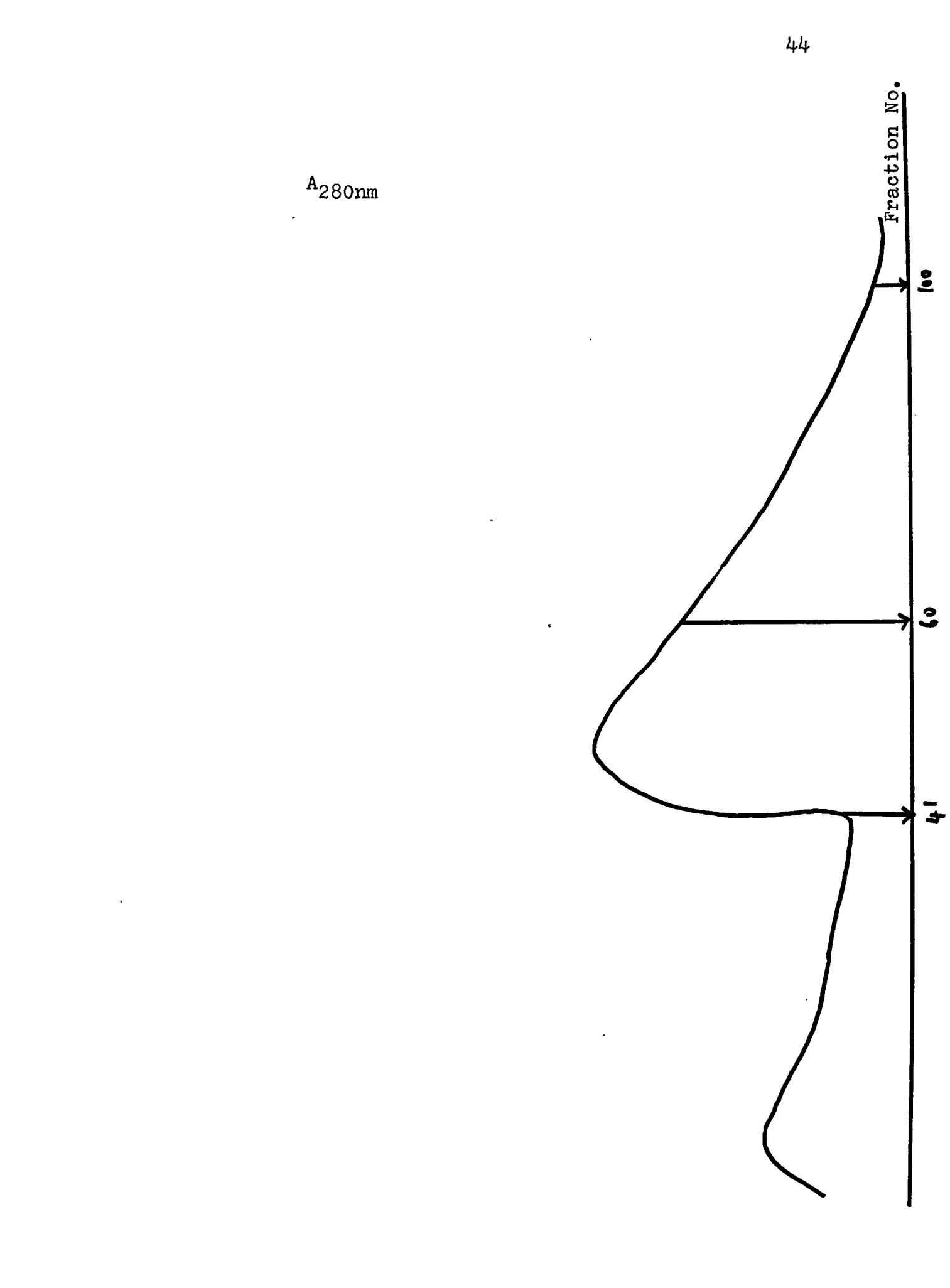
Elution diagram for the purification of the peptide (DPYR) by ion-exchange (Dowex 50-X8):

Column = 1.75 x 35cm

Eluent = pH 3.1 - 5.00
$NH_4OAc/AcOH$ buffer

Flow rate = 20ml/hr

Vol. of each fraction = 2.0ml


A_{280nm}
Fraction No.
100
60
41

absorbance at 280nm (Fig.11). TLC of fractions 30 - 37 in BAW::4:1:1 indicated the presence of a major, slow moving product. Three other relatively dim, but fast moving products were observed. Amino acid analysis performed on 0.15ml sample of fraction #34 gave the following result: Asp:Pro:Tyr:: 0.98:0.96:1.0.

(e) Purification on ion-exchange column having Dowex 50-X8 (strongly acidic ion-ex change with sulfonate groups):

Since TLC of P_2 fractions indicated three other dim spots besides the one we wanted, 30 - 38 were pooled and subjected to ion-exchange purification.

Preparation of a Dowex 50-X8 column (3.5 x 35cm) (50):

Fifty grams of the resin was washed with 500ml water, followed by 500ml in NaOH, 500ml water, 300ml 3N HCl, 500ml H_2O and finally with 300ml of pH 3.1 buffer (NH_4OAc/HOAc, 1M in acetate). The resin was then suspended in pH3.1 buffer, so that the ratio of the supernatant buffer to the settled resin was 2:1. The column was loaded with the resin and equilibrated with pH 3.1 buffer. The sample to be purified was evaporated to dryness and taken up in 5ml of pH 3.1 NH_4OAc/AcOH buffer. It was loaded in the column and 110 fractions, each measuring 2ml were collected and the effluent was monitored by its UV absorbance at 280nm (Fig. 13). TLC of the various fractions were performed. Fractions 3 - 12 carried the fast moving impurities. Fraction #30 was blank, and #41 - 60 showed one spot corresponding to the desired peptide, (R_f=0.2). Fraction #100 showed one spot which was relatively fast moving (R_f=0.4).

Fractions 41 through 60 were pooled, evaporated to dryness and taken up in 7ml of 0.1N HOAc. It was then loaded on a P_2 column and desalted. The single peak fraction was pooled and peptide content was estimated, to be 90mg. Amino acid analysis performed on a 2ml sample of the solution on a long column did not work well, probably due to interference of NH_4OAc during hydrolysis.

The sample was passed through P_2 column again and desalted once more. The single peak fraction was pooled and lyophilized three times with water and three times with D_2O. The product weighed 90mg (0.16mmoles). This corresponded to a yield of 9% from the substituted resin. TLC of the product showed one spot with R_f = 0.2 in BAW::4:1:1 system. Amino acid analysis revealed Asp:Pro:Tyr in the ratio 0.91:0.87:1.0.

Synthesis of the Tetrapeptide Glu-Tyr-Pro-Thr (EYPT) by the Solid Phase Technique:

(a) Preparation of Boc-Thr (Bzl)-resin:

Sixteen grams of 1% cross-linked Merrifield resin (Biobeads SX-1, chloromethylated, Bio-Rad), of actual capacity (0.7 x 16) i.e. 11.2meq of Cl/g was added to Boc-Thr (Bzl) 3.47g (11.2mmoles), in 90 ml.EtOH. Et_3N, 1.4 ml (10.08mmoles) was added and the mixture was refluxed for forty hours at 90°C. The product was then worked up as described on page 18 and dried overnight. Amino acid analysis performed on 20mg sample of the material, after hydrolysis in 1:1 HCl:propionic acid at 110° overnight, gave a Thr substitution value of 0.27mmoles/g.

(b) Only 8g (2.16mmoles of substituted Thr) of the substituted resin was used for synthesis. This was introduced into a silylated reaction vessel of a Schwarz/Mann synthesizer and submitted to the program of Table V.

The dried material weighed 10.0g. A 10mg sample of the material was hydrolyzed in 1:1 HCl:propionic acid, and subjected to amino acid analysis. The following ratio of amino acids were obtained Glu:Tyr:Pro:Thr::1.0:0.77:0.95:0.75.

(c) HF Cleavage:

The dried peptide-resin obtained from above was taken up in a reaction vessel of an HF apparatus, along with 9ml of anisole (50 fold in excess of Tyr content) and treated with 90ml of HF, for one hour at $0^{o}C$. The product was washed with ether and then dried overnight under vacuum.

The peptide was extracted with 65ml of 0.1N HOAc and the resin was filtered.

(d) Purification on P_2 BioGel Column:

The 65ml solution from above was reduced to 10ml by evaporation and loaded on a P_2 column, and eluted with 0.1N HOAc. Seventy fractions each measuring 5.2ml were collected and the effluent was monitored by it UV absorbance at 280nm (Fig. 14). TLC of various fractions indicated fractions #30 to 51, to contain the majority of the peptide (R_f value of 0.32) along with other impurities both slower and faster moving. Tyr content by UV absorption gave a value of 1.15mmoles. Two different ion-exchange

Figure 14

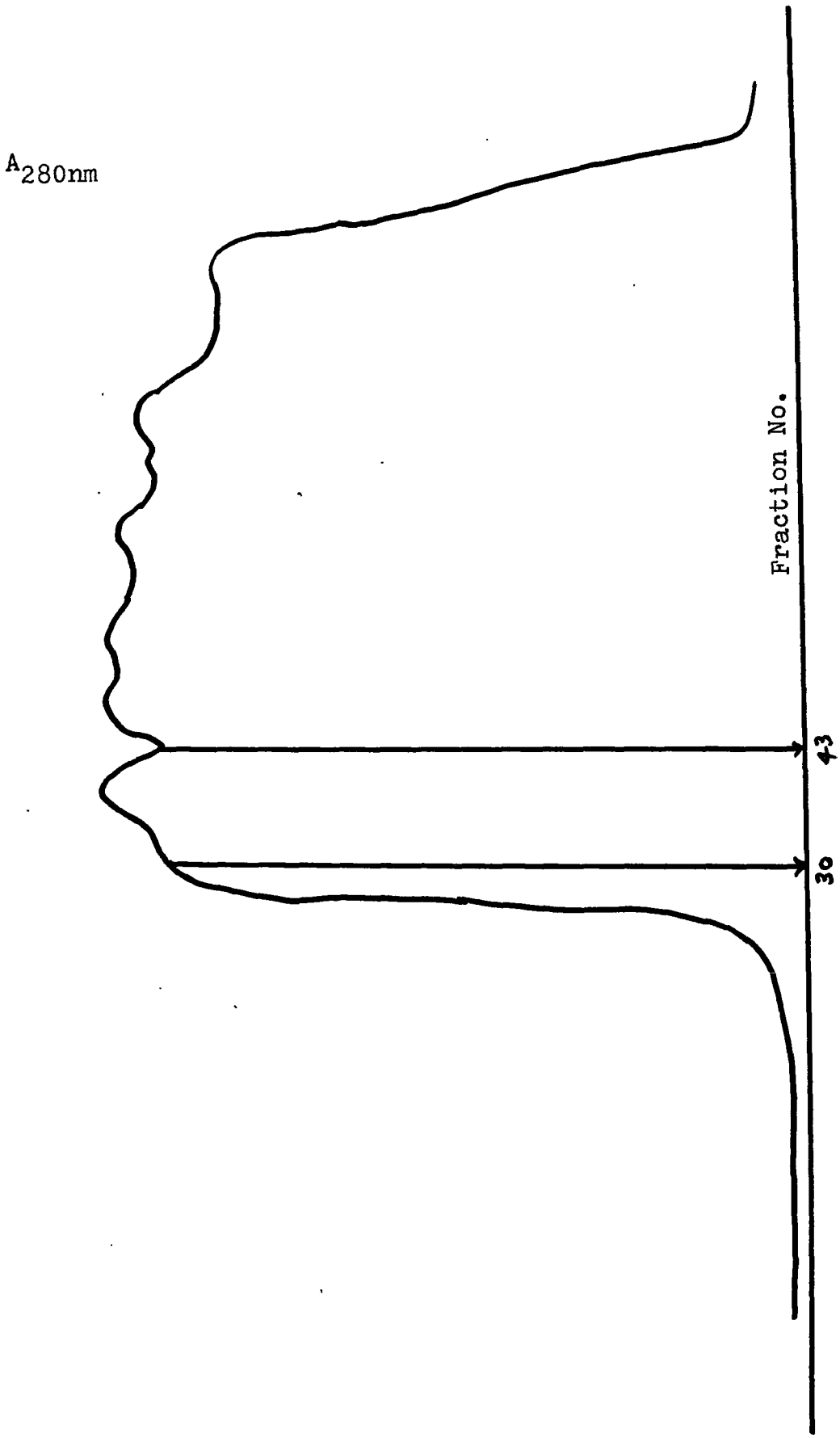
Elution diagram for purification of the peptide EYPT on P_2 Bio Gel column:

Column = 2.8 x 40cm

Eluent = 0.1N HOAc

Flow rate = 60ml

Vol. of each fraction = 5.2ml


A_{280nm}
Fraction No.
43
30

purifications were carried out, one for fractions 30 to 43 and the other for fractions 43 to 51.

(e) Ion-exchange purification on DEAE-Sephadex for fractions 30→ 43 from above:

A DEAE-Sephadex ion-exchange column was set up (50) with 0.05M - 0.2M NH_4HCO_3 as a linear gradient. Fractions 30 to 43 pooled from above were evaporated to dryness and taken up in 0.05M NH_4HCO_3, the inital buffer. This was loaded on the ion-exchange column and 160 fractions were collected, each measuring 2ml (Figure 15). Fractions 110 to 160 were pooled and lyophilized in H_2O, 8 times and in D_2O 4 times. The material obtained weighed 100mg.

(e') Ion-exchange purification on DEAE-Sephadex of fractions 44 → 51 from (d):

The ion-exchange column of (e) was equilibrated with 0.5M NH_4HCO_3. Fractions 44 - 51 were evaporated to dryness, taken up in 5ml of 0.05M NH_4HCO_3 and loaded to the column. One hundred and sixty fractions, each measuring 2ml were collected and the effluent was monitored by its UV absorbance at 280nm.(Fig. 16). TLC of various fractions were taken and this showed that fraction 80 onwards contained the peptide. Fractions 80 - 160 were pooled and evaporated to dryness. The material was lyophilized 8 times with H_2O and 5 times in D_2O. This was mixed with the material purified from (e) and a total of 0.76mmoles was obtained, corresponding to a yield of 35% from the substituted resin. Amino acid analysis performed on a 10mg sample of the material indicated Glu:Tyr: Pro:Thr in the ratio ::0.8:0.9:1.0:0.95.

Figure 15

Elution diagram for the purification of the peptide EYPT (fractions 30 - 43 from P_2) by ion-exchange on DEAE-Sephadex:

Column = 1.5 x 35cm

Eluent = 0.05M - 0.2M NH_4HCO_3

Flow rate = 20ml/hr

Vol. of each fraction = 2.0ml

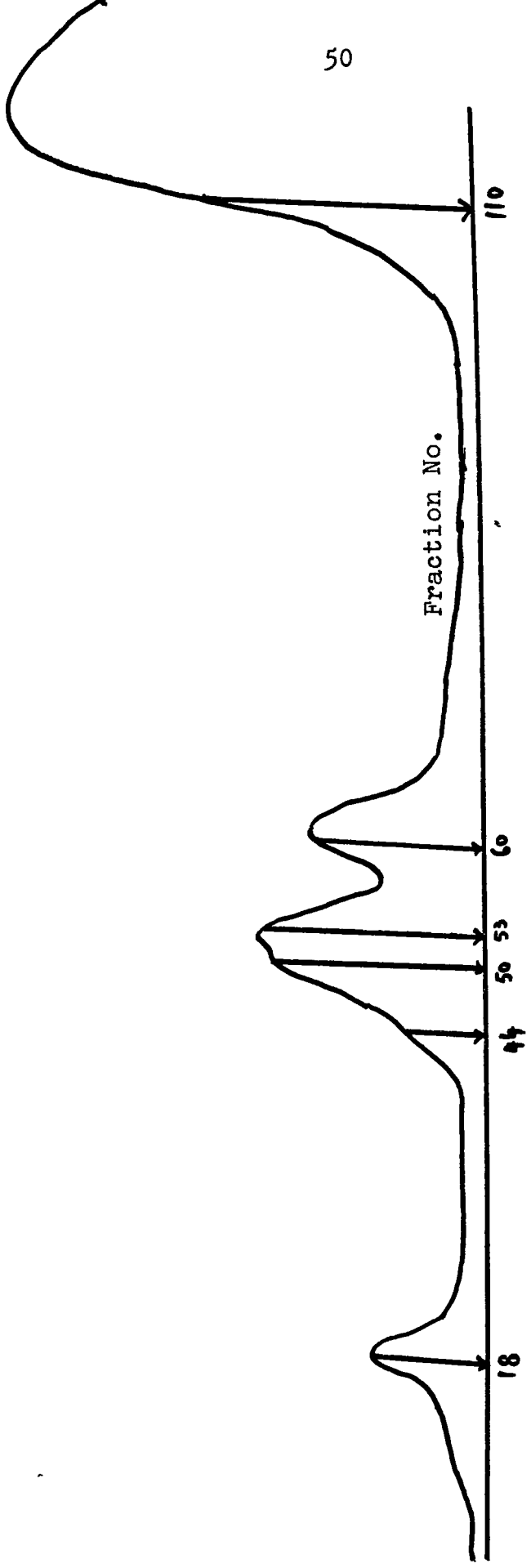

A_{280nm}
Fraction No.
110
60
53
50
44
18

Figure 16

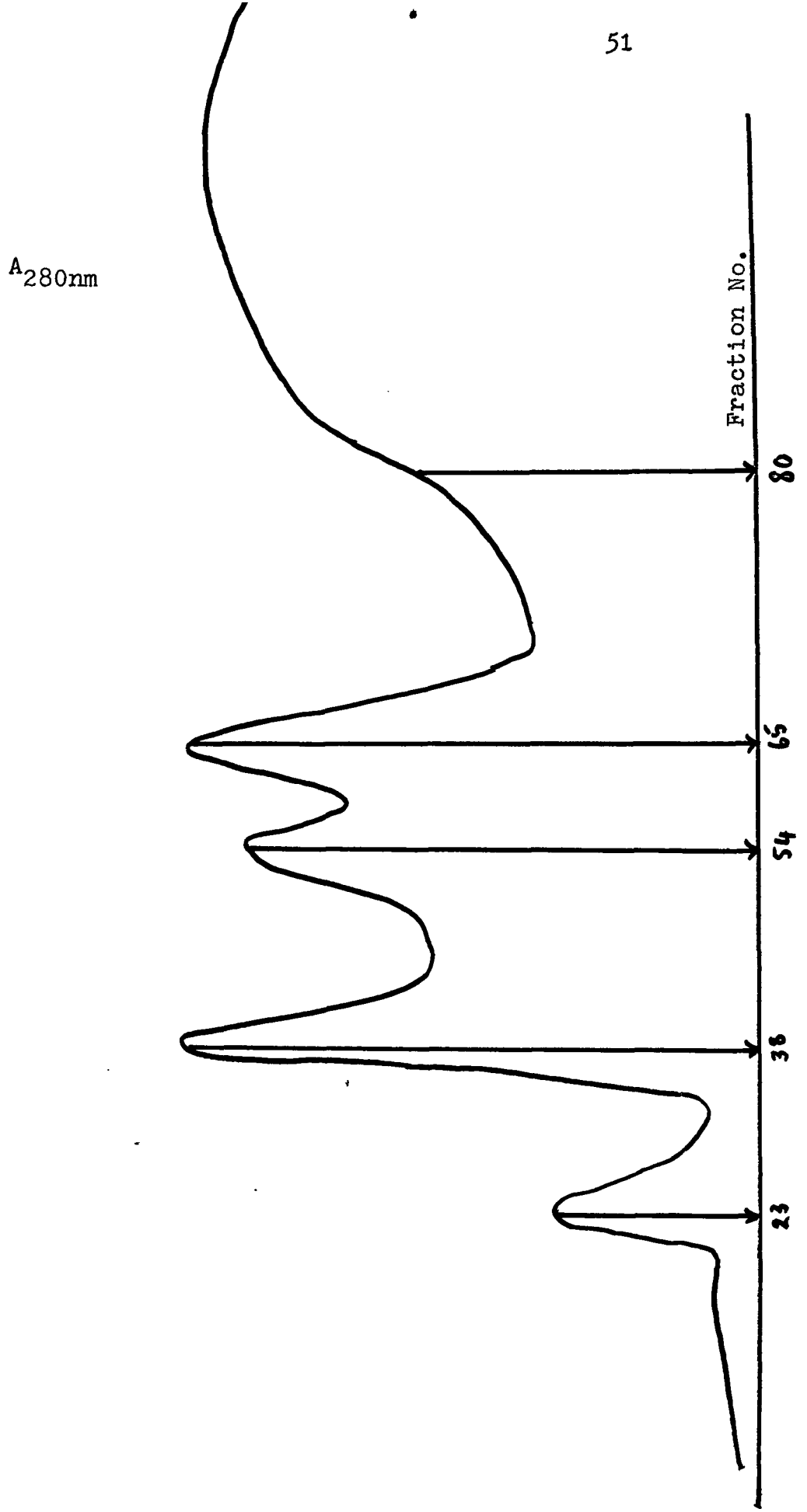
Elution diagram for the purification of the peptide EYPT (fractions 44 - 51 from (d)) by ion-exchange on DEAE-Sephadex:

Column = 1.5 x 35cm

Eluent = 0.05M - 0.2M NH_4HCO_3

Flow rate = 20ml/hr

Vol. of each fraction = 2.0ml


A_{280nm}
Fraction No.
80
65
54
38
23

NMR Studies

All the peptides synthesized were studied for their nuclear magnetic properties. Proton magnetic resonance studies in 100% D_2O were done initially for peak assignments. The sophistication of our instrument allowed us to utilize $1 \rightarrow 3$mg of the material, and the signal to noise ratio was improved by storing the data in a computer of average transients.

The samples were lyophilized in 100% D_2O, three to four times to deuterate all exchangeable protons. This procedure considerably reduced the complexity of the PMR Spectrum. No internal references were used even though DSS (2, 2-dimethyl-2-silapentane-5-sulfonate) could have served as one, in D_2O.

The other solvent used was 100% deuterated DMSO. The samples were lyophilized in H_2O prior to introduction in DMSO. The PMR spectrum of this sample allowed us to view the -NH protons. The spectrum was more complex than the deuterated sample due to the presence of additional hydrogen atoms on the amide and the side chain nitrogen atoms. However the low field portion between 6.00 to 9.00ppm where the -NH protons appeared, was relatively less complex, which allowed us to study the change in chemical shifts of these protons, under the influence of variable temperatures.

CHAPTER III

DISCUSSION

DISCUSSION

Synthesis:

In contrast to the time consuming classical synthesis, solid phase technique greatly reduces time consumption. However yields obtained are very low. This is because of the formation of "truncated" and "failure" sequences, discussed earlier in the experimental section. Also the HF treatment, to cleave the peptide form the resin, probably degrades the target sequence. However due to milligram quantity requirement of the peptide for NMR work, solid phase methodology was found to be the most convenient. In the syntheses undertaken here, not much difficultly was encountered, except during purification.

Purification:

(a) Pro-Tyr-Ser

Solid phase synthesis of this peptide on 1% cross-linked Merrifield resin was followed by HF treatment, and purification on P_2 Bio Gel column. TLC in BAW::4:1:1 indicated only one spot and the ratio of amino acids were in good agreement by amino acid analysis. So further purification was not essential.

(b) Asp-Pro-Tyr-Ser

Here also, as in (a) the peptide was obtained as a single spot by TLC in BAW::4:1:1 and showed good amino acid analysis, after P_2 Bio Gel purification. Further purification was not undertaken. Purification of small peptides

as compared to larger ones, should not be much of a problem, because impurities accumulated during the course of the synthesis are fewer in number. Also failure sequence peptides of nearly equal molecular weight will be fewer in number.

(c) Asp-Pro-Tyr-Arg (II)

The fraction obtained from the P_2 Bio Gel column was not very pure and had to be subjected to ion-exchange purification on Dowex 50-X8. Pure fraction came out last and gave only one spot on TLC.

(d) Glu-Tyr-Pro-Thr

TLC of the first fraction obtained from the P_2 column, in BAW::4:1:1 indicated numerous spots and had to be definitely purified. Ion-exchange column carrying DEAE-Sephadex was used, and pure Glu-Tyr-Pro-Thr was obtained as a last fraction with 0.20M NH_4HCO_3 as eluent. The sample had to be lyophilized to remove the bicarbonate.

Cobalt contamination during Asp-Pro-Tyr-Arg synthesis:

Co^{III} contamination to the peptide-resin during resin cleavage with HF, was followed by an attempt to remove the metal. The amino acid analysis of the final product (slightly pink) showed Tyr and Arg in the ratio 0.8:1.0.

Buckingham and Collman (52,53) have earlier studied the hydrolysis of N-terminal peptide bonds by β-hydroxy-aquotriethylene tetramine cobalt (III) ion (β-[Co(triene) OH $(H_2O)]^{2+}$). β-[Co(trien) OH $(H_2O)]^{2+}$ was found to react with peptides to form complex ions, β-[Co(triene)(amino

acid anion)] $^{2+}$, in which the N-terminal amino acid residue resulting from the hydrolysis of the amide becomes coordinated to the metal. The pH value in their study was 7.5.

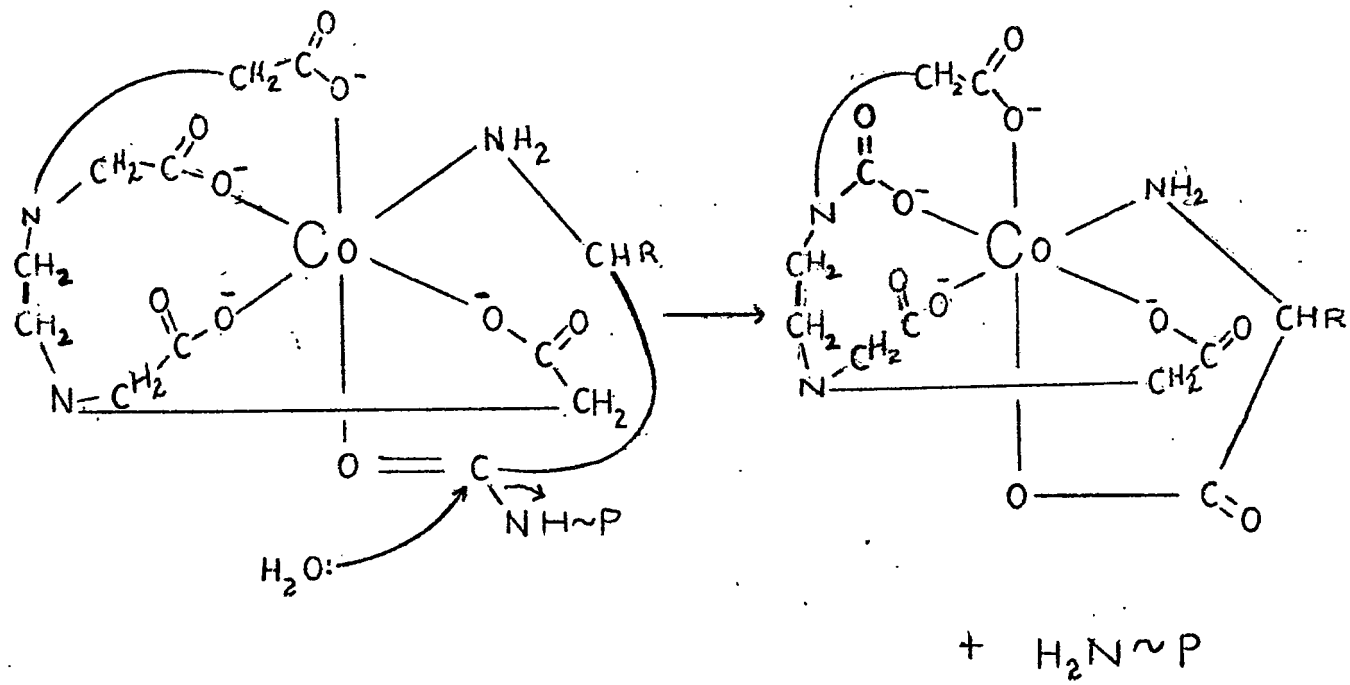
$$\beta\text{-}[Co(trien)\,OH\,(H_2O)]^{2+} + H_2NCHRCNHR' \rightarrow \beta\text{-}[Co(trien)H_2NCHRCO_2]^{2+} + H_2NR'$$

A possible explanation for the hydrolysis observed during Asp-Pro-Tyr-Arg synthesis is the following reaction:

$$[Co(EDTA)(H_2O)_2]^{-1} + H_2N\text{-}CHR\text{-}C(=O)\text{-}NH\sim P \rightarrow [Co(EDTA)(H_2N\text{-}CHR\text{-}C(=O)\text{-}O^-)]^{-1} + H_2N\sim P$$

A plausible mechanism, analogous to the one described by Buckingham and Collman (52,53) is:

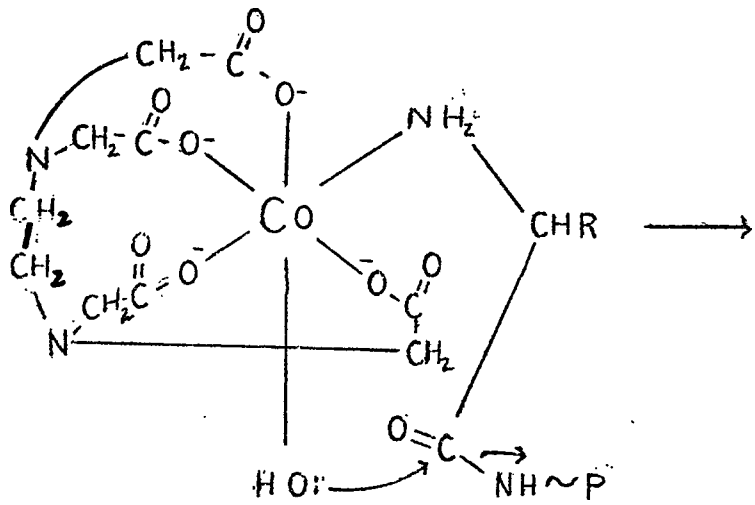
$$[Co(EDTA)(H_2O)_2]^{-1} + H_2N\text{-}\underset{R}{CH}\text{-}C(=O)\text{-}NH\sim P$$

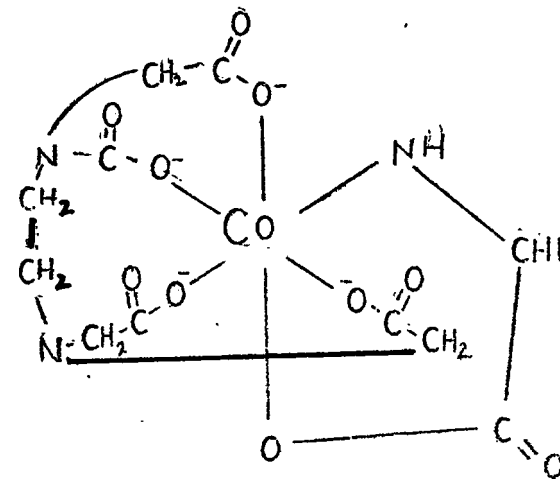


$$+\ H_2N\sim P$$

or $[Co(EDTA)(H_2O)_2]^{-1}$ + $H_2N-\underset{R}{CH}-\overset{O}{C}-NH\sim P$

↓ pH 6.7





+ $H_2N\sim P$

NMR Studies:

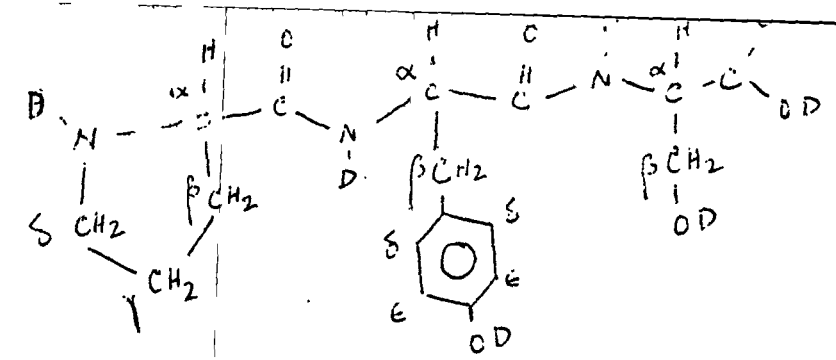
PMR studies in D_2o (100%) were done primarily for peak assignments. Sophistication of the instrument allowed us to utilize 1-3mg of the material and the signal to noise ratio was improved by storing the data in a computer of average transients.

The samples were lyophilized in 100% D_2O, three to four times to deuterate all exchangeable protons. This procedure considerably reduced the complexity of the PMR spectrum.. No internal reference was used.

Water had to be completely excluded during PMR studies of deuterated peptides. A huge water peak otherwise not only hid other peaks but also interfered with data recording. The chemical shifts of various amino acid residues, are not significantly dependent of their position in a peptide. This makes sequencing by NMR very difficult, however recognition of patterns is possible. Once a sequence is known, peaks can be assigned with the help of previously published data. Any controversy could be verified by performing double resonance experiments. Even though we have had difficulty assigning certain peaks, double resonance experiments have not been performed so far. But we hope to do them once the method of operation is known.

Figure 17

100MHz PMR spectrum of PYS in D_2O.



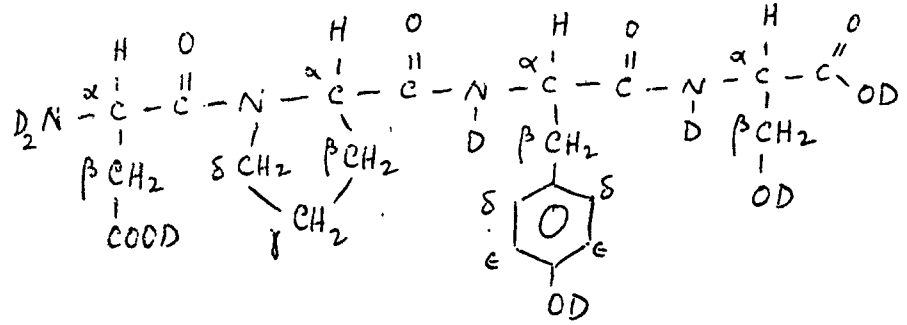
HDO
δ-Tyr
ε-Tyr
α-Tyr
α-Pro, α-Ser
β-Ser
δ-Pro
β-Tyr
β-Pro
γ-Pro
7
6
5
4
3
2
1
0
ppm

Pro-Tyr-Ser

The peptide is expected to be linear. The spectrum obtained is relatively simple. The broad peak seen at 4.75ppm is due to water. The spectrum of Tyrosine is very clear and indicates an AA'BB' system. There is some controversy over the assignment of α-Ser and α-Tyr protons but we hope to clear it once we are able to perform double resonance experiments.

Figure 18

60MHz PMR spectrum of DPYS in D_2O.



δ-Tyr
ε-Tyr
Side band
HOD
β-Ser
Side band
δ-Pro
β-Asp
β-Tyr
γ-Pro, β-Pro
8
7
6
5
4
3
2
1
0
ppm

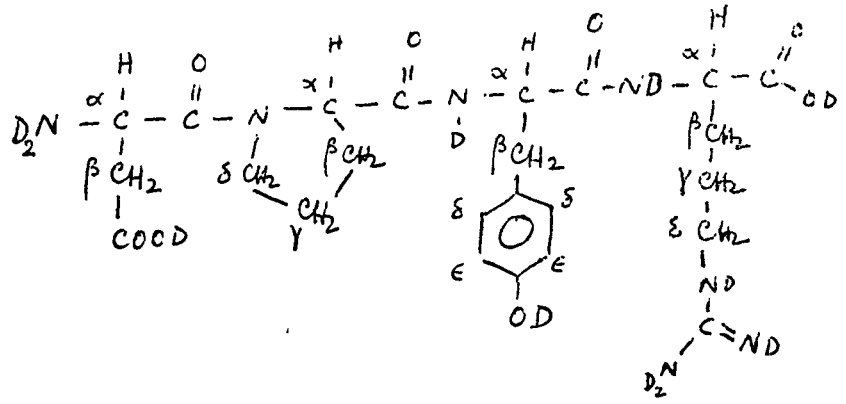
Asp-Pro-Tyr-Ser

This spectrum is more complex than Pro-Tyr-Ser. The Tyr spectrum shows splitting patterns, indicative of some hindered rotation.

Figure 19

100MHz PMR spectrum of DPYR in D_2O.

62



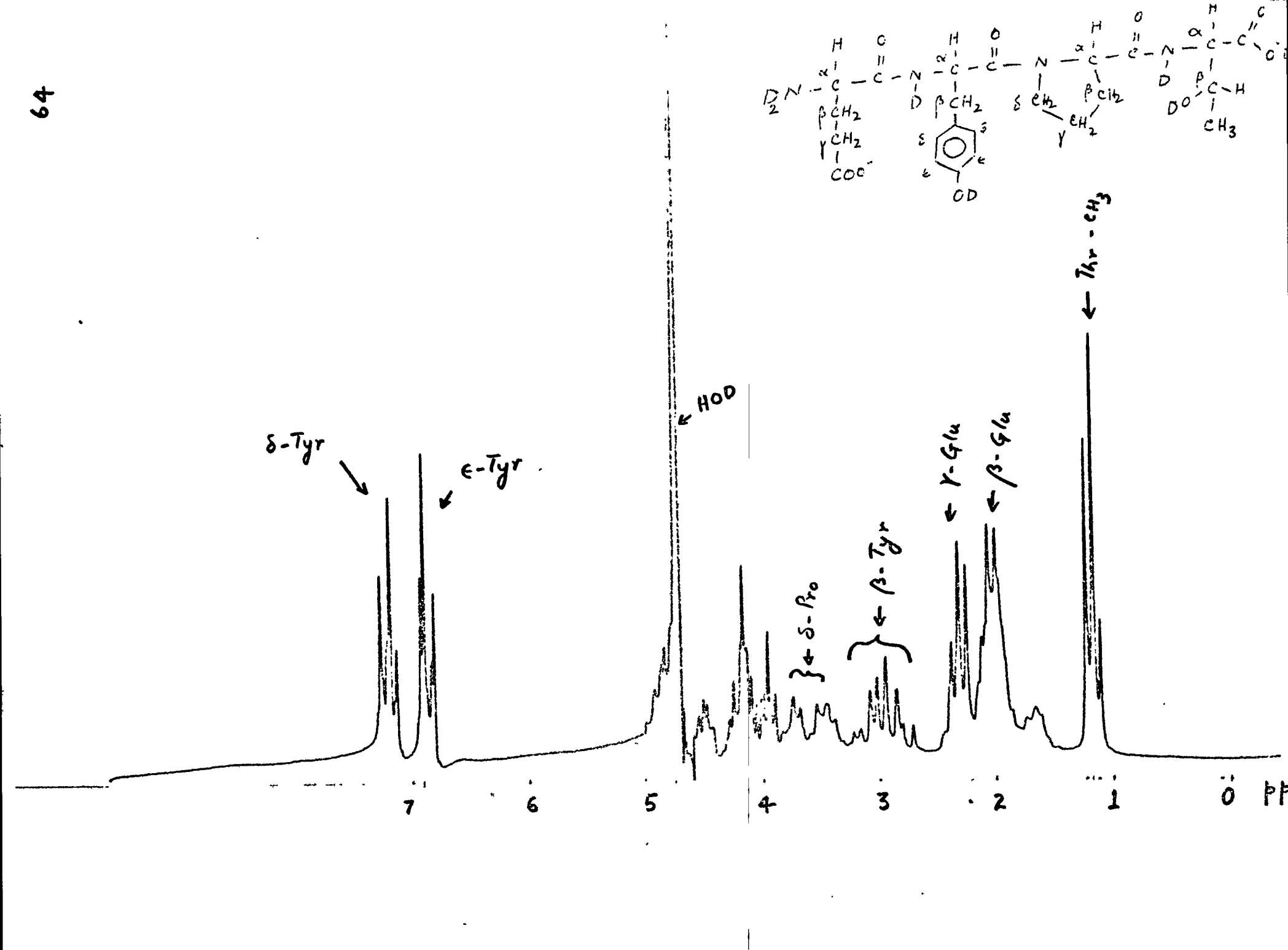
Asp-Pro-Tyr-Arg

The spectrum of this molecule is very complex. The only region with some degree of clarity is the Tyr region. The splitting pattern is indicative of AA'BB' system, however there seems to be a small degree of hindered rotation of the aromatic ring. According to Chou and Fasman's rule, we would expect this peptide to exist in the form of β-bend. The extreme complexity of even the deuterated peptide, makes it more difficult to study it in 100% d_6-DMSO in the fully protonated state. This kind of peptide can be studied more easily by ^{13}C-NMR.

The large singlet seen close to 2.00ppm is due to the CH_3- of acetate moeity. The peptide exists as an acetate salt, when lyophilized from CH_3COOH.

Figure 20

100MHz PMR spectrum of EYPT in D_2O.


δ-Tyr
ε-Tyr
HOD
δ-Pro
β-Tyr
γ-Glu
β-Glu
Thr-CH3
7
6
5
4
3
2
1
0
PPM

Glu-Tyr-Pro-Thr

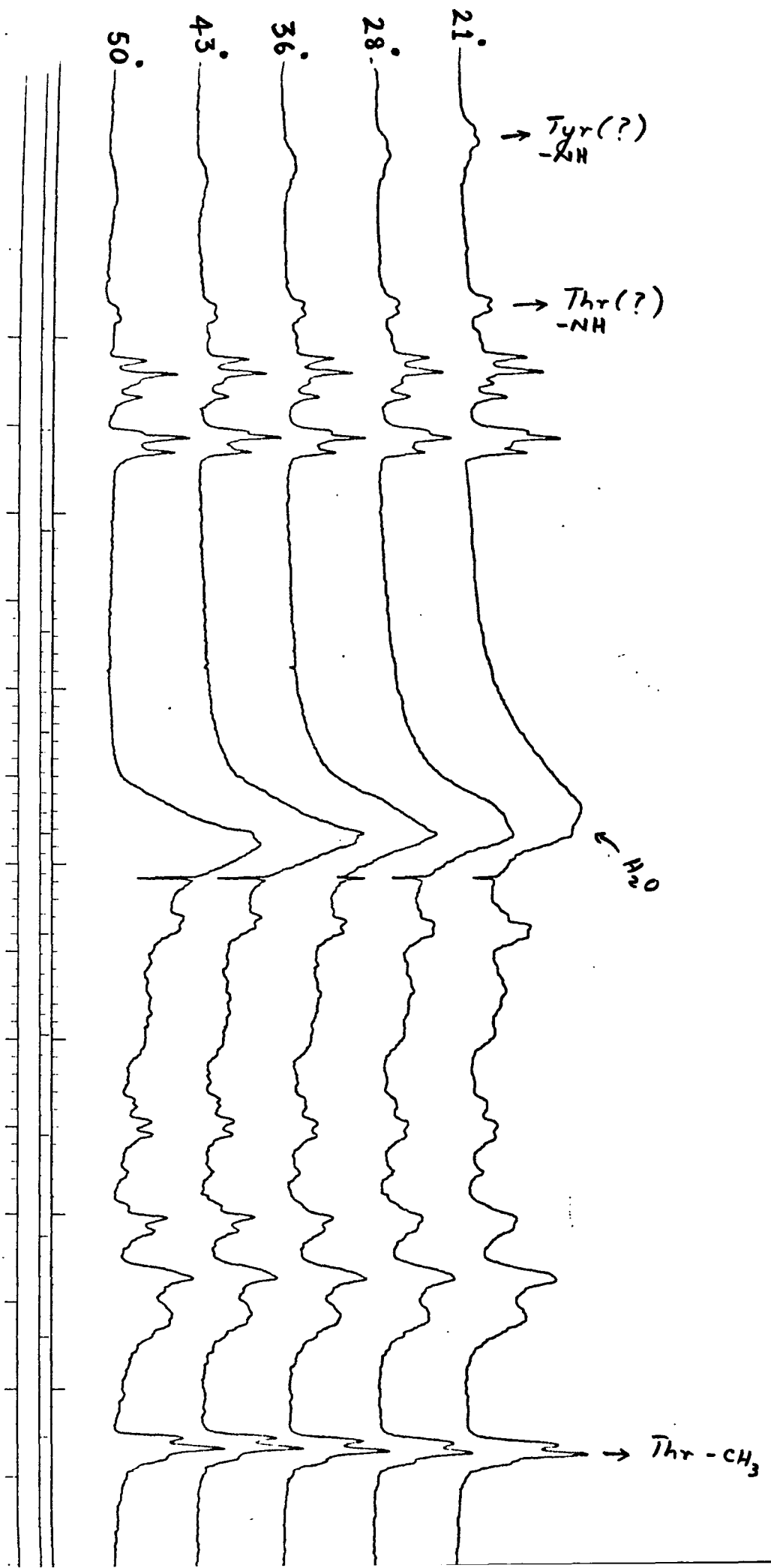
The spectrum of Glu-Tyr-Pro-Thr is the most interesting. We observe the threonine methyl as an apparent triplet. A pure conformer would be expected to give a doublet for the methyl group. One possible explanation would be the existence of diastereomers giving rise to two overlapping doublets. Another possibility is magnetically non-equivalent methyl protons due to hindered rotation of this group. This seem possible once the peptide is envisioned as existing in a β-turn conformation. However this statement cannot be made categorically.

Interestingly enough, the Tyr spectrum confirms to a great degree of restricted rotation. The spectrum is not of a simple AA'BB' system, but is more indicative of a conformationally prefered state.

A third possible explanation is the presence of cis- and trans- isomers of the proline moeity, which would then alter the magnetic environment of all protons, thus giving rise to spectra of two isomers overlapped one over the other.

Figure 21

100MHz PMR spectra of EYPT in DMSO at different temperatures.


50°
43°
36°
28°
21°
→ Tyr(?) -αNH
→ Thr(?) -NH
← H2O
→ Thr - CH3

Temperature Study

Temperature study done on Glu-Tyr-Pro-Thr, which would not be expected to form β-bend, according to Chou and Fasman's data (3), indicates some form of intramolecular hydrogen bonding involving the Thr -NH proton. As the temperature is raised the chemical shift of Tyr -NH proton significantly changes as compared to very little change of Thr -NH proton. This would mean the Tyr -NH is more exposed to the environment and that the Thr -NH is in a caged surrounding. The Tyr protons can be seen, but the rest of the signals are broad and non-interpretable. The Thr CH_3- appears at the extreme right (high field).

Also as the temperature is increased we observe a change in the relative peak heights of the multiplets. This is evident in case of Tyr protons and the Thr -CH_3 protons. This might be arising due to conformational differences rather than configurational differences.

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