A thesis entitled

"An Investigation into the Use of Cobalt Chelates in Peptide Synthesis"

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as per partial requirement for the degree of 'Master of Science' Massey University, Palmerston North.

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DEDICATION

This Thesis is dedicated to my parents for their encouragement throughout the last three years.

ABSTRACT

Preparative scale samples of $[Co(en)_2Cl_2]Cl$ using the method of Bailar (27) and $[Co(en)_2Co_3]Cl$ from $CoCl_6$ were produced. The carbonate complex was also prepared from $[Co(en)_2Cl_2]Cl$, using the method described by Buckingham (21). $[Co(en)_2CO_3]Cl$ was then converted to $[Co(en)_2Br_2]Br$. Alanato, Phenylalanato, N$^{\alpha}$ nitroarginato, Valinato, Prolinato and O-Benzylaspatato complexes were synthesised from $[Co(en)_2Br_2]Br$ using a modification of Meisenheimers method outlined by Dekkers (26). Isoleucinato, Phenylalaninato, N$^{\alpha}$ nitroarginato, O-benzyl-aspatato and O-benzyltyrosinato complexes were also prepared under non solution conditions described by Dekkers (26). The formation of glycinatobis(ethylene-diamine)cobalt(III) complex preparation by a modified Meisenheimer method, was used as model reactions to optimise the source of base, pH conditions, solvent conditions and reaction times. The use of methyltri-flouromethane sulfonic acid and triflouromethane sulfonic acid in methanol, to alkylate the amino-acido-bis(ethylene-diamine)cobalt(III) complex was demonstrated. The conditions required to optimise the yield of the condensation of these methylated amino acid cobalt(III) with amino acid esters or peptide esters, was established, and the conditions necessary for the rapid ion exchange separation of the products investigated. The most suitable of the methods for the removal of the peptide from the cobalt(III) complexes,

described by Dekkers, was established and Gel Filtration separation of the peptide products demonstrated. Problems with the instability of some peptide complexes, especially $Co(en)_2PhePheOC_6H_5$, were encountered and possible solutions tested. The synthesis using cobalt chelates of the amino acids of $PhePheOC_6H_5$, $ProPheOC_6H_5$, $AlaPheOC_6H_5$, $ProPhePheOC_6H_5$ and $AlaGlyGlyOC_2H_5$ had thus been attempted and the isolated products were submitted for amino analysis. The possible modification of some of the amino acids during the complex synthesis or isolation was indicated by the results of these amino acid analyses.

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The author wishes to acknowledge the assistance of Otago University Chemistry Section who performed the elemental analyses, and the Sequence Laboratory, Massey University who performed the amino acid analysis. The author particularly wishes to acknowledge the invaluable technical assistance offered by Dr D.K. Harding, Mr D. Knighton, Mr J. Battersby, Mr L. Meyers and the skillful typing of Mrs G. Shaw. The author would like to make special note of the help provided by Ms Helen Lallu, who spent many hours proof reading the original draft.

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INTRODUCTION

[0.0,0] A strategy for the formation of a known synthetic polypeptide sequence must require the construction of the amino acid chains of the correct sequence, retain the optical integrity of each asymmetrical centre and retain the chemical identity of the amino acid side chains. All the reactions leading to the production of the peptide or protein must be highly efficient, since a poor yield in an early step will adversely affect the possible yield of each subsequent step and that of the final product. The yield of each step compounds, thus putting a practical limit on the length of the polypeptide chain that a particular strategy can attain. Strategies designed to meet these aims presently in vogue, require blocking of the α amino group of the amino acid that is being added to the chain. This blocking action must render the α amino group unreactive during coupling conditions, but is such that the free α amino group can be regenerated after coupling, without damaging the protein or other longer term protecting groups. The side chains of the amino acids must remain unreactive during the synthesis. Protection for side chain groups which is stable under coupling conditions, but able to be removed at the last stage before final purification, is essential to any synthetic scheme. Identification and isolation of the product of each coupling reaction and at the end of the synthesis,

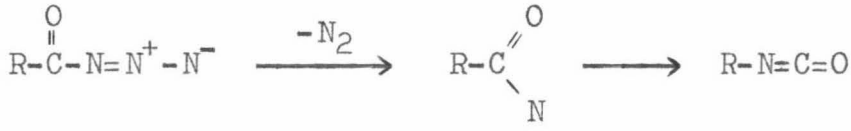
is an obvious requirement, but one that still limits the goals of protein synthesis. The interaction of these diverse requirements, in addition to the vigorous reaction conditions that are often needed to achieve the coupling of an amino acid to a peptide chain, are such that to date no general synthesis for all peptides and proteins exists.

Condensation of two amino acids to form a peptide is not a spontaneous process, except at elevated temperatures. Many activating schemes have been invoked, mostly involving modification of the carboxyl group to a more reactive form. With the model reaction, the formation of acetyl-L-leucine, Williams and Young (1) surveyed eight of the present most popular modification schemes, including Acid Azide, Cyanomethyl Ester, p-Nitrophenyl Ester, Dicyclohexylcarbodiimide, Tetramethylphosphite, Carbonic Mixed Anhydride, Phosphazo- and Phenyl-isonazolium methods. All reactions examined, except that of Acid Azide, resulted in the formation of some racemate during condensation. Using the Acid Azide method, however, racemisation occurred during the formation of some serine derivatives (2).

Not only do difficulties arise in achieving coupling of the amino acid units, but generating the active amino acid derivative efficiently can detract from the advantages of an activation scheme that is otherwise attractive.

Side reactions, during synthesis, have been reported for the Acid Azide scheme (3). These reactions are generally the result of the azide rearranging to the isocyanate.

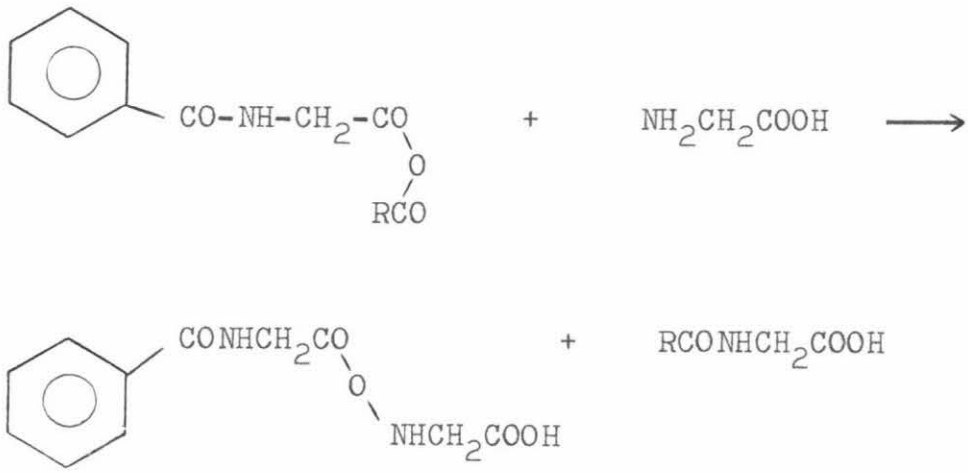
Fig. 0.0,1



The addition of the amino component can lead to urea formation,

Even an isolated crystaline azide rearranges slowly to the isocyanate at 0^{o}. Side reactions resulting in two acylation products may lead to complications using the Mixed Anhydride Method.

Fig. 0.0,2 Glycine Mixed Anhydride



Classical activation approaches by Fischer (5) using the α-halogen acyl derivative of the amino acid and subsequent modifications using tosylamino acid chlorides, are susceptible to fragmentation when exposed to alkali (6). The benzyloxycarbonyl acyl chloride derivatives form the Leuchs Anhydride on heating, or storage for long periods at room temperature (7). Production of the acyl chlorides using phosphorous oxychloride can result in side reactions which produce phosphorous containing peptide.

Preparation and handling difficulties with some of the activated ester derivatives of some amino acids has restricted the application of some amino acid activation schemes (2).

The sidechain protection system must remain unaffected by the amino acid carbonyl modification, activation, and condensation with a free amino group and removal of α-amino

protection reaction conditions. Thus some activation and side chain protection schemes are incompatible. Such incompatability can produce side reactions which may result in the untimely removal of the blocking group, degradation of the side-chain, or modification of the blocking function such that its eventual removal may be impossible. Benzyl mercaptan may be lost during formation of hydrazides of S benzyl cysteine. Azide activation in general, with the basic conditions of hydrazinolysis, causes some loss of N ε trifloroacetyl protection where steric hindrance of some trityl protecte amino acids may preclude formation of the active derivative. Under some conditions using dicyclohexyl-carbodiimde as a catalyst, Benzyloxycarbonyl protection of the α-amino group of lysine is inadequate (8). Use of triflouroacetic acid derivatives instead requires removal in basic conditions, which may damage some peptides. Removal of protecting groups using strong acid conditions with liquid HF, may cause deamination of asparagine and glutamine (8) and rearrangement of the peptide backbone.

The application of a chosen strategy may also generate some difficulties. Homogeneous synthetic approaches as typified by the work of Bodansky and Du Vigneaud (9), and Schwyser and Sieber (10) requires the purification and isolation of the product of each reaction, preferably in crystalline form, resulting in a great deal of work. The cumulative losses during purification can result in a very low yield of product. Heterogeneous synthesis,

the use of an insoluble resin support for the growing peptide chain, allows the SOLID PHASE APPROACH to enjoy considerable advantages over protein synthesis carried out in solution, such as facile removal of excess reagent, tremendous savings in time and avoidance of the problems of insolubility of large fragments of the peptide chain. However, reduced yield due to carboxyl catalysed intramolecular aminolysis of the peptide reaction site from the resin has been noted. This phenomenon occurs especially during the removal of t-butyoxycarbonyl protection of the amino terminal of the amino acid with acid conditions (11), (8). The cumulative reduction in the available free amino termini for further amino acid coupling becomes a significant factor in determining the final yield of product. Coupled with this are the differing degrees of reactivity of the resin sites. This is caused by the polymer-matrix interaction with the solvent. Swelling of the polymer is necessary to allow access to the reaction sites of the coupling amino acids and catalysts. A change in the nature of the polymer matrix caused by the changing character of the peptide as it is synthesised, has been indited for the formation of deletion and truncated sequences in Solid Phase Synthesis. Many of these sequences defy detection and separation with even the most sensitive of modern techniques (12) (13).

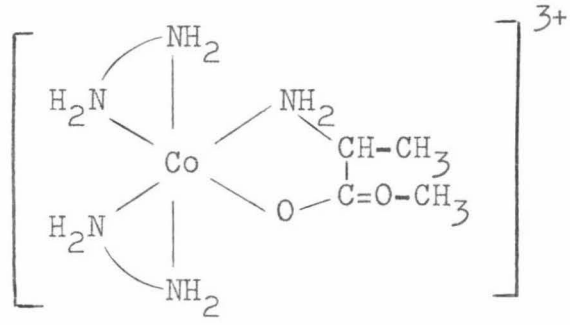
/~0.1,0_7 The use of cobalt(III) complexes of amino acids:

the condensation of some glycine alkyl esters complexed to cobalt(III) with ammonia or amines has been reported (14), (15) by Buckingham and coworkers. Further work by Buckingham and Dekkers (26) has demonstrated the preparation of eighteen amino acids complexed to cobalt(III). They have also demonstrated the preparation of alkyl ester derivatives of some of these complexes and the condensation of these derivatives with the free amino termini of amino acids, amino acid esters, or peptide chains. The product of such a condensation is a peptide chain, one amino acid unit longer, and complexed to a cobalt(III) ion by the N-terminal amino acid. Removal of the peptide chain from the complex using mildly acid reducing conditions, thus freeing the amino terminus of the peptide and allowing the possibility of another amino acid unit to be added as a cobalt complex, has achieved the stepwise preparation of tetrapeptide chains (26).

When chelated to the cobalt(III) ion, the amino acid was shown to be coordinated as a bidentate ligand using the amino nitrogen and a carbonyl oxygen. The other four ligand sites on the complex are occupied by amine nitrogen atoms. The ease of preparation of a large number of amino acid complexes with the ligand field around the central cobalt(III) ion completed by two ethylenediamine molecules was demonstrated by Dekkers and Buckingham (26). Ligand moieties other than ethylenediamine were considered

by these workers but difficulties in the preparation of these complexes forced the conclusion that they were not suited to a peptide synthetic scheme.

Fig. 0.1,1



The arrangement of Δ-Cis [Co(en)$_2$AlaOCH$_3$]$^{3+}$ ligand system.
en = ethylenediamine = H_2N—NH_2

The mechanism of the condensation reaction has been suggested by Buckingham et al (18) to be nucleophilic attack by the free amino nitrogen at the ester linkage, causing lysis of the ester. The carbonyl centre is made more vulnerable to such attack by the electron withdrawing ability of the metal ion.

Table 0.1,1

The Amino acidobis(ethylemediamine)cobalt(III)

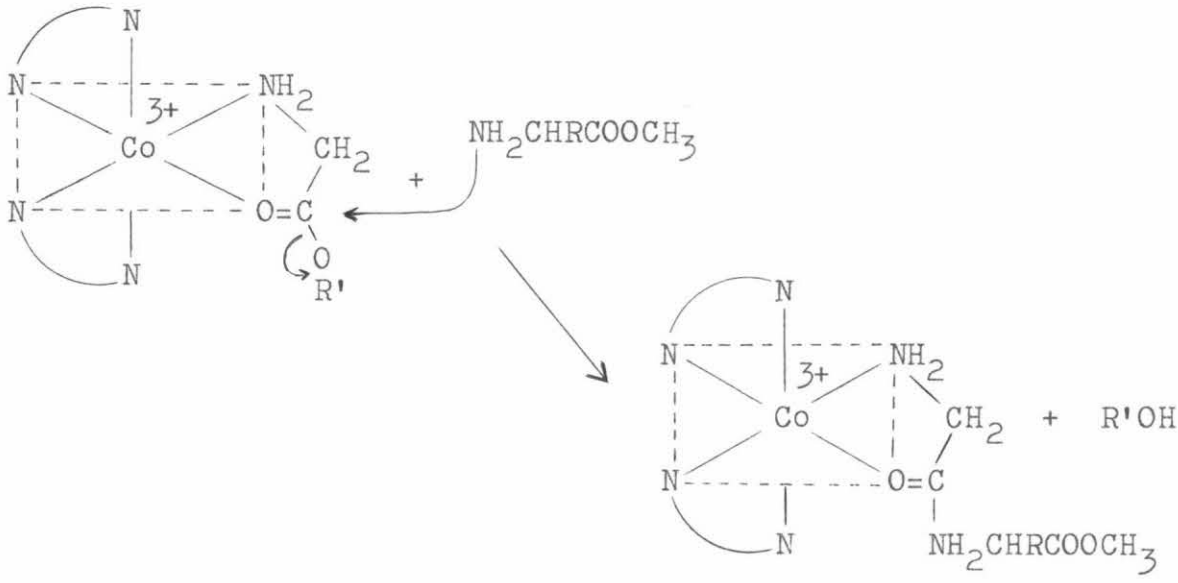
Complexes produced by D. Buckingham and J. Dekkers (26)

Complex[1]	λ MAX	ε MAX	λ MAX	ε MAX	% Yield
$[Co(en)_2GlyO]I_2$	487	97	347	107	80
$[Co(en)_2AlaO]I_2$	487	109	348	117	75
$[Co(en)_2ValO]I_2$	487	101	348	110	80
$[Co(en)_2LeuO]I_2$	486	109	347	130	75
$[Co(en)_2IleO]I_2$	486	110	347	132	78
$[Co(en)_2thrO]I_2$	486	102	347	110	70
$[Co(en)_2SerO]I_2$	486	118	345	135	68
$[Co(en)_2LysO]I_2$	487	101	348	120	76
$[Co(en)_2GlnO]I_2$	486	94	347	133	65
$[Co(en)_2AsnO]I_2$	486	103	347	133	50
$[Co(en)_2MetO]I_2$	486	102	346	134	70
$[Co(en)_2PheO]I_2$	487	103	346	128	82
$[Co(en)_2TyrO]I_2$	490	108	340	187	72
$[Co(en)_2TryO]I_2$	486	103	340	298	65
$[Co(en)_2ProO]I_2$	488	103	346	125	85

1. Abbreviation used for amino acids are listed in Appendix 3.3,0.

Fig. 0.1,2

Ester lysis, by an Amine, of a Cobalt Glycine Alkyl Ester Complex



N = nitrogens of amine chelates completing the ligand field, ethylenediamine in this study.

The mechanism described by Fig 0.1,2 requires the amino acid carbonyl group to be esterified. In this study the methyl function was selected, as with most studies cited in the literature, to avoid any steric hinderance from a bulkier group. Two methods of generating the methyl ester complexes are available (19) via (i) a monodentate amino acid ester complex of the form $cis[Co(en)_2X(NH_2CHRCOOR')]X_2$ where X = Cl^- or Br^-.

The reaction scheme for the preparation of amino-acido

bis(ethylenediamine)cobalt(III) complex, from trans dibromobis(ethylenediamine)cobalt(III) bromide, via the monodentate ester is given in the reaction sequence [Co.1,1].

$$\underline{trans}\text{-}[Co(en)_2Br_2]Br + NH_2CHRCOOR' \rightarrow \underline{cis}\text{-}[Co(en)_2Br(NH_2CHRCOOR')]Br_2$$

[Co.1,1]

$$\underline{cis}\text{-}[Co(en)_2Br(NH_2CHRCOOR') + Ag^+ \rightarrow [Co(en)_2NH_2CHRCOOR']^{3+} + AgBr$$

via (ii) a bidentate complex of the form $[Co(en)_2NH_2CHRCOO]X_2$ where $X = I^-$ or CH_3COO^-, and alkylation using an alkylating agent.

The reaction scheme for the preparation of amino-acido-bis(ethylenediamine)cobalt(III) complex from trans dibromo-bis(ethylenediamine)cobalt(III)

$$\underline{trans}[Co(en)_2Br_2]Br + NH_2CHRCOOH \xrightarrow{XSNaI} [Co(en)_2NH_2CHRCOO]I_2$$

$$[Co(en)_2NH_2CHRCOO]^{2+} \xrightarrow[Agent]{Alkylation} [Co(en)_2NH_2CHRCOOR']^{3+}$$

Buckingham and Dekkers (26) concluded that the monodentate esters were difficult to isolate and the yields poor, except in the case of glycine.

The work of Buckingham et al has shown that it is possible to produce peptide chains using cobalt(III) chelates of the amino acid esters to induce the required chemical activity. The N-terminal of the amino acid remains complexed to the metal ion during the formation of the peptide linkages since under these conditions the amino terminus is no longer basic and thus reactive. Under coupling conditions no further protection of this site is required.

As well as providing N-terminal protection, the coloured cobalt complex may aid in separation of the product of the condensation reaction. See fig O.1,3.

Buckingham and Dekkers showed that within the accuracy of the uptake of oxygen measured at an oxygen electrode for a sample containing (R)-alanine treated with (R)-amino acid oxidase no racemisation could be detected for the complexing of alanine with cobalt(III), its methylation using PCl_3 in methanol, its condensation with glycine benzyl ester and the reduction of the cobalt complex (26). Although Keys and Legg showed that deuterium exchange takes place at the α carbon atoms of an aspatato-bis(ethylenediamine)cobalt(III) complex in a deuterium oxide solution at ph 9.5, over three days at 35° a slow rate of racemisation was observed (31) (less than 10%).

Fig 0.1,3

(i) Anhydrous reaction conditions

$[Co(en)_2NH_2CHRCOOCH_3]^{3+}$ + $NH_2CHR'COOR''$

(Excess Amino Acid Ester Complex)

$[Co(en)_2NH_2CHRCONHCHR'COOR'']^{3+}$ + $[Co(en)_2NH_2CHRCOOCH_3]^{3+}$

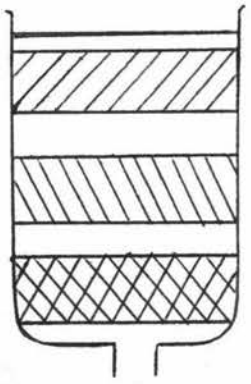
+ Unreacted $NH_2CHR'COOR''$ + (remaining Amino Acid Ester Complex)

(ii) Aqueous work up conditions pH 5.0 of reaction mixture hydrolysis of remaining Amino Acid Ester Complexes.

$[Co(en)_2NH_2CHRCONHCHR'COOR]^{3+}$ + $[Co(en)_2NH_2CHRCOO]^{2+}$ + CH_3OH

+ Unreacted $[NH_3CHR'COOR'']^{+}$

(iii) Ion Exchange Purification



Preparative Ion Exchange ph ≈ 5.0

—— $[Co(en)_2NH_2CHRCONHCHR'COOR'']^{3+}$

—— $[Co(en)_2NH_2CHRCOO]^{2+}$

—— Unreacted $[NH_3CHR'COOR'']^{+}$

The cobalt complex in the 2+ and 3+ bands are coloured orange-red and are easily detected.

The preliminary experiments reported in the literature thus indicate that the formation of amino acid cobalt(III) complexes and their condensation with other amino acids involves only a small loss, if any, of optical integrity at the optical centres. Using the cobalt(III) ion in peptide synthesis also has the additional advantage of providing α-amino protection of the amino acid which can be easily removed, and efficient purification of product by ion exchange chromatography.

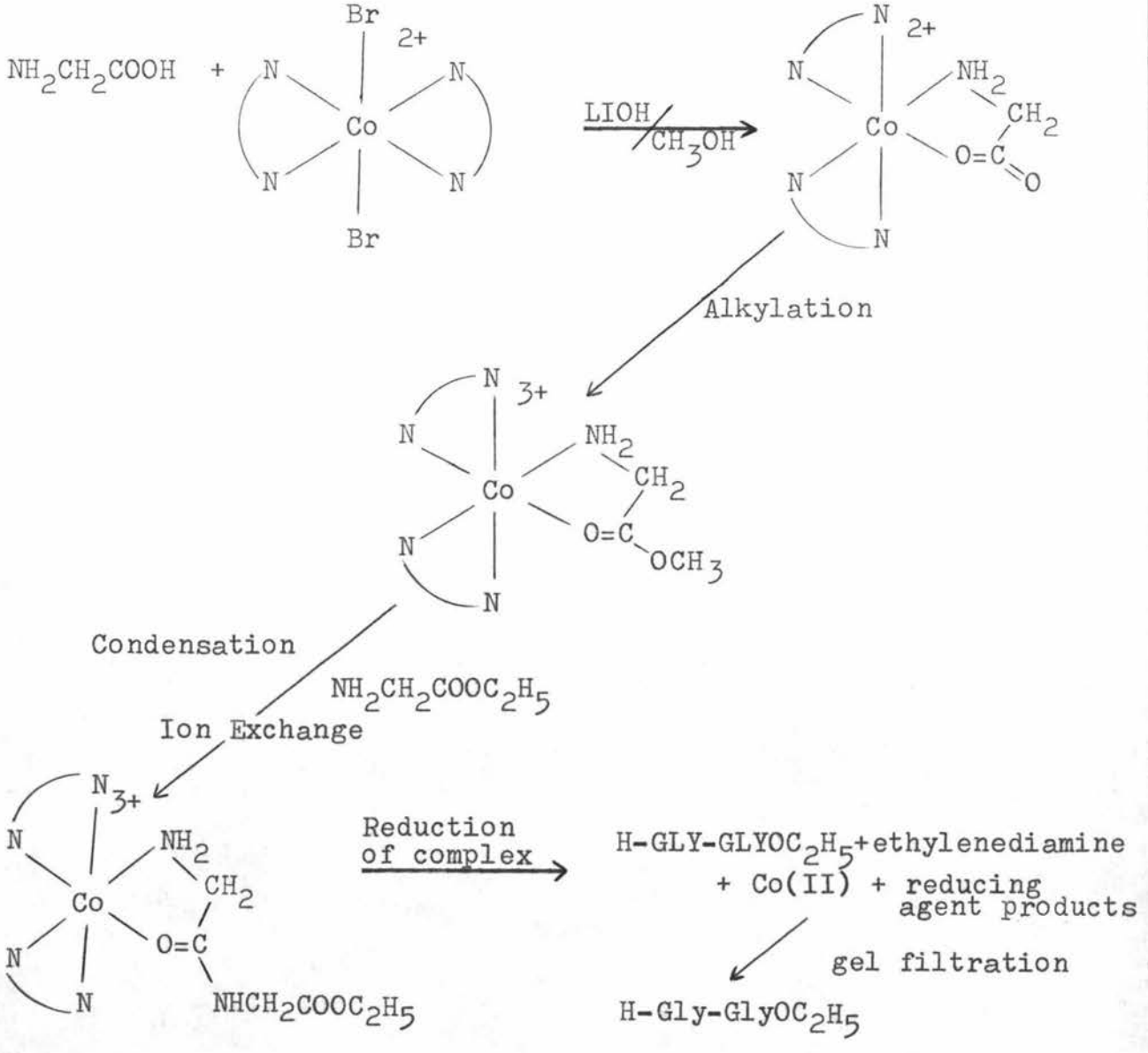
[0.2.0] The scope of this Thesis:

This study was designed to achieve the synthesis of the amino-acidobis(ethylenediamine)cobalt(III) complexes required for peptide synthesis on a preparative scale. The activation of these complexes by methylation by the methods described in the literature (26) require careful control of conditions to avoid unwanted side reactions and the isolation of the resulting product is often difficult. Reaction of the amino acid methyl ester complex in situ with amino acids does not occur with reproducable results and in some examples the yield is low (26), (24). Activation of the amino-acidobis(ethylenediamine)cobalt(III) complexes by two alternative methods and the condensation of these methyl ester complexes with high yield was demonstrated. Problems involved in the removal of the peptide from the complex and isolation and purification of

the complex were however observed. In addition the study was approached with the idea of investigating the potential of the small scale reactions so far demonstrated, to be developed in to an economical and attractive general approach to peptide synthesis.

The general synthetic approach using the amino acid cobalt(III) complexes is shown in the preparation of glycylglycine ethyl ester in Fig 0.2,1.

Fig. 0.2,1 The Synthesis of Glycylglycine ethyl ester.



CHAPTER (I)

PREPARATION OF THE COBALT AMINO ACID COMPLEXES

[1.0,0] Refer to appendix [3.1,0] for information regarding the preparation and purity of materials used in the trials described in the experimental reactions.

[1.1,0] Preparation of Dichlorobis(ethylenediamine) cobalt(III) Chloride (19)

A 10% aqueous ethylenediamine solution (600 ml) was added, with vigorous stirring, to a solution of cobalt chloride hexahydrate (160g, 0.4 mole) in 500 ml of water. A vigorous stream of air was drawn through the solution for 10-12 hours. Concentrated hydrochloric acid (325 ml) was added and the solution evaporated over a boiling water bath until it had been reduced to approximately one third of its original volume. The solution was then allowed to cool overnight and the bright green plates of trans-dibromobis(ethylenediamine)cobalt(III) chloride hydrochloride were filtered off. The crystals were washed with alcohol, then ether and dried at 110°C. This drove off the hydrochloride of crystallisation forming dull green crystals. Literature yield 78%.

Results

/1.1,1/ Table 1.0,1

Preparation of /$Co(en)_2Cl_2$/Cl

Trial	Scale[1]	Isolated Yield[2] grams	% Yield[3]
1	1	71	60
2	1	85	72
3	2	140	59
4	4	594	73

1. This method /1.1,0/ outlined the trial conditions, conducted on scale.
2. The weight of /$Co(en)_2Cl_2$/Cl isolated from each trial.
3. Based on the amount of cobalt chloride hexahydrate used in the trial.

The product of each trial was checked against an authenticated sample of /$Co(en)_2Cl_2$/Cl, by passing a small amount in aqueous solution down a SP-C25-120 Sephadex test column eluting with 0.1, 0.2 and 0.5 m HCl. (Refer appendix 3,2,0).

/1.2,0/ Preparation of Carbonatobis(ethylenediamine) cobalt(III) Chloride from Dichlorobis(ethylenediamine)cobalt(III) Chloride

Dichlorobis(ethylenediamine)cobalt(III) chloride (285.5g

1.0 mole) was dissolved in one litre of water, sodium carbonate (100g, 1.0 mole) was added and the solution heated under reflux for 60 minutes. The volume of the solution was reduced to 500 ml under reduced pressure and the product filtered off. Further product could be precipitated by adding 200 ml of methanol slowly, trituration and cooling overnight.

Results

[1,2,1] Table 1.2,1

Preparation of $[Co(en)_2CO_3]Cl$ from $[Co(en)_2Cl_2]Cl$

Trial	Scale[1]	Isolated Yield[2] grams	Yield[3] %
1	0.2	51.1	75
2	0.3	105.4	94
3	1	154	59
4	2	572	98

1. The method [1.2,0] outlined the trial conditions conducted on scale 1.
2. The weight of $[Co(en)_2CO_3]Cl$ isolated from each trial after recrystalisation.
3. Based on the amount dichlorobis(ethylenediamine) cobalt(III) chloride in the trial.

The $[Co(en)_2CO_3]Cl$ product was recrystalised by dissolving it in a minimum of hot water (approximately 5 ml/gram) and triturating the cooling solution. Further product could

be recovered from the mixture by adding methanol (approximately 10 ml methanol/100 ml of solution) slowly down the side of the flask, titration and cooling overnight. The purple red crystaline product was air dried, after being filtered off. The product was examined under a scanning microscope, if any contaminating crystals could be detected, the product was recrystalised again.

Yield of recrystalisation 70%.

[1.3,0_7 Preparation of Carbonatobis(ethylenediamine) cobalt(III) Chloride and Bromide (28)

A stream of carbon dioxide was bubbled through a mixture of 112 ml 98% ethylenediamine (112 ml 1.64 mole) and 160 ml of water, cooled in ice. The stream of carbon dioxide was maintained during the entire preparation. A solution of cobalt(II) chloride hexahydrate (195g, 0.82 mole) in 175 ml of water, or cobalt(II) bromide hexahydrate (268g, 0.82 mole) in 300 ml of water if preparing the bromide salt, at room temperature was added to the cold solution, which was continuously stirred. The addition of the cobalt(II) salt caused a violent evolution of carbon dioxide gas and the solution became red-violet in colour. (Sometimes the solution coagulated and became gel like.) The cooling and stirring were continued and the solution oxidised by the dropwise addition of 200 ml of 30% hydrogen peroxide for approximately 45 minutes. If a gel had formed, manual stirring was necessary during the addition of approximately 50 ml of hydrogen peroxide, until the solution again

appeared homogeneous. During the addition the temperature increased to about 35°C and the solution became a deeper red. The solution was heated to a temperature of 70-75°C and was kept at that temperature for 15 minutes, before being cooled in an ice bath to 20°C. Lithium hydroxide monohydrate (34.0g, 0.82 mole), finely powdered, was added to the reaction mixture under a vigorous stream of carbon dioxide, with thorough stirring and no cooling. The temperature of the reaction mixture rose to about 35°C. The solution was allowed to remain at room temperature, with constant stirring for thirty minutes, whereupon fine red crystals of $[Co(en)_2CO_3]Cl/Br$ began to form.

Isolation of the carbonatobis(ethylenediamine)cobalt(III) bromide product from the reaction mixture: the mixture was cooled in an ice bath and the resulting precipitate was filtered off, washed with 200 ml of 50% aqueous ethanol and two 200 ml portions of 95% ethanol and allowed to dry in air.

Literature yield, 71%.

Literature Elemental Analysis.

Calculated for $[Co(en)_2CO_3]Br$:

Co, 18.47; N, 17.56; C, 18.82; H, 5.06; Br, 25.05.

Found:

Co, 17.65; N, 15.27; C, 17.72; H, 5.10; Br, 26.82.

Isolation of the carbanatobis(ethylenediamine)cobalt(III) chloride product from the reaction mixture: Methanol, 500 ml, was added to the solution and the mixture cooled for two

hours in an ice bath to effect complete precipitation. The stream of carbon dioxide was maintained during the cooling. The precipitate was filtered off, washed with 200 ml of 50% v/v aqueous ethanol and air dried. Literature yield, 80%.

Literature Elemental Analysis.

Calculated for $[Co(en)_2CO_3]Cl$:

Co, 21.46; N, 20.40; C, 21.87; H, 5.87; Cl, 12.91.

Found:

Co, 21.59; N, 20.33; C, 21.61; H, 5.88; Cl, 12.99.

Results

[1.3,1] Table 1.3,1

Preparation of $[Co(en)_2CO_3]Cl/Br$ from $[CoCl_6].6H_2O/[CoBr_6].6H_2O$

Trial	Scale[1]	Counter Ion[2]	Isolated Yield grams[3]	Yield[4] %
1	1.0	Br	158	61
2	1.2	Br	173	67
3	1.0	Cl	179	66
4	1.0	Cl	183	68
5	1.0	Cl	191	71

1. The method [1.3,0] outlined the trial conditions conducted on scale 1.
2. The production of $[Co(en)_2CO_3]Br$ or $[Co(en)_2CO_3]Cl$ is the trial.

3. The weight of product isolated from each trial.
4. Based on the amount of cobalt chloride hexahydrate or cobalt bromide hexahydrate used in the trial.

The crystaline product was examined under a scanning microscope in an attempt to detect any gross contaminants.

Literature Elemental Analysis.

Calculated for $[Co(en)_2CO_3]Br$:

C, 18.82; H, 5.06; N, 17.56; Br, 25.05.

Found:

C, 17.7; H, 5.0; N, 15.7; Br, 26.5.

Calculated for $[Co(en)_2CO_3]Cl$:

C, 21.87; H, 5.87; N, 20.40; Cl, 12.91.

Found:

C, 21.5; H, 5.9; N, 20.2; Br, 13.0.

[1.4,0] Preparation of Carbonatobis(ethylenediamine) cobalt(III) Bromide from Carbonatobis(ethyl-enediamine)cobalt(III) Chloride

The carbonatobis(ethylenediamine)cobalt(III) chloride could be converted to the bromide salt by dissolving it in a minimum of cold water, then treating the solution with excess sodium bromide and cooling overnight.

Results

[1.4,1] The carbonatobis(ethylenediamine)cobalt(III) complex recovered as carbonatobis(ethylenediamine)cobalt(III) bromide was 63%.

[1.5,0] Preparation of trans Dibromobis(ethylenediamine)cobalt(III) Bromide (26),(21)

Hydrobromic acid 47% (200 ml), heated on a steam bath to approximately 70°C, was treated with carbonatobis-(ethylenediamine)cobalt(III) chloride (50g, 0.18 mole), or a molar equivalent of carbonatobis(ethylenediamine)-cobalt(III) bromide (58g, 0.18 mole), over two hours. Heating of the solution at 70°C was continued for four hours and after a further two hours at 20°C all the trans[Co(en)$_2$Br$_2$]Br was filtered off and washed with ethanol to remove traces of the soluble cis[Co(en)$_2$Br$_2$]Br.HBr and adhering HBr. Washing with ethanol was maintained until a sample of the product dissolved in water was neutral to litmus paper. The product was then washed with 80% aqueous ethanol and air dried.
Literature yield 94%

Results

[1.5,1] Table 1.5,1

Results from the Preparation of trans Dibromobis(ethylenediamine)cobalt(III) Bromide Trials

Trial	Scale[1]	Isolated Yield[2] grams	Yield[3] %
1	0.6	30	73
2	2.1	132	81
3	0.7	43	86
4	1.0	66	88

...continued

continued... Table 1.5,1

Trial	Scale[1]	Isolated Yield[2] grams	Yield[3] %
5	1.6	112	92
6	1	39	52
7	1	56	75

1. The method /⁻1.5,0_7 outlined the trial conditions on scale
2. The weight of /⁻$Co(en)_2Br_2$_7Br isolated from each trial.
3. Based on the amount of /⁻$Co(en)_2CO_3$_7.Br/Cl in the trial.

The product was viewed through a scanning microscope to detect any gross contamination. A sample of each trial was dissolved in water and compared chromatographically with a sample of /⁻$Co(en)_2Br_2$_7Br of known character under similar conditions.

/⁻1.6,0_7 Separation of the Amino-acidobis(ethylenediamine) cobalt(III) Complexes from Preparative Reaction Mixtures (20)

The reaction mixture was stripped under reduced pressure until all the non aqueous solvent had been removed. The resulting oil was taken up in water and loaded onto a Biorad Ag-50W-X2, 200-400 mesh, H^+ form, preparative column[1] (refer to appendix 3.2,0) and was eluted using four column volumes of 1.0 M HCl, followed by sufficient

[1] Complexes of phenylalanine were isolated using a SP-C25-120 Sephadex preparative column to avoid the stronger interactions of the phenylalanine side chain with the Ag-50W-X2 resin.

2.0 M HCl to completely separate the 2+ product band. Using a vacuum line and trap the resin carrying the coloured bands above that required were sucked off. The product band was similarly removed and treated with 3.0 M HCl, eluting off the cobalt amino acid complex. The eluant was then stripped under reduced pressure, water was added and the solution was stripped again. This was repeated until the odour of hydrogen chloride gas was removed from the stripped product. The resulting oil was dissolved in 100 ml of water, neutralised using 1.0 M sodium hydroxide, excess sodium iodide was added and the mixture cooled. The product that precipitated was filtered off. Further product was precipitated by reducing the solution volume to 50 ml and adding more sodium iodide. The isolated product was washed with acetone and dried under suction.

When possible a sample (approximately 0.01g) of the isolated product and a sample of the complex of known character were compared chromatographically.

[1.7.0] Preparation of Amino-acidobis(ethylenediamine) cobalt(III) Complex by a Modification of Meisenheimers Method (29)

Amino acid (0.01 mole) was dissolved in a minimumn of hot water and made up to 50 ml with methanol. trans-dibromobis-(ethylenediamine)cobalt(III) bromide (4.2 g, 0.01 mole) was added and the mixture treated with lithium hydroxide monohydrate (0.41g, 0.01 mole) in methanol 25 ml. The

solution was heated under reflux for 30 minutes, cooled, filtered and separated as in [1.6,0].

Results

[1.7,1] (S)-Alaninatobis(ethylenediamine)cobalt(III) Iodide (26)

Literature molar absorptivities,

ε_{487} 109 and ε_{348} 117, in water at 25°C.

Literature Yield, 75%.

Literature Elemental Analysis.

Calculated for $Co(C_7H_{22}N_5O_2)I_2.H_2O$:

C, 16.12; N, 11.51; H, 4.24; O, 8.91.

Found:

C, 16.2; N, 11.3; H, 4.3.

Trial Elemental Analysis:

Found:

C, 16.3; H, 4.5; N, 11.4; O, 8.8.

Trial Isolated Yield, 70%.

[1.7,2] (S)-Isoleucinatobis(ethylenediamine)cobalt(III) Iodide (26)

Literature molar absorptivities,

ε_{486} 110 and ε_{347} 130, in water at 25°C.

Literature Yield, 78%.

Literature Elemental Analysis.

Calculated for $Co(C_{10},H_{28},N_5,O_2)I_2$:

C, 21.33; H, 5.01; N, 12.44; O, 5.68.

Found:

C, 21.4; H, 5.0; N, 12.2.

Trial Elemental Analysis:

Found:

C, 22.2; H, 4.2; N, 11.8; O, 5.4.

Trial Isolated Yield, 65%.

[1.7,3] (S)-Phenylalaninatobis(ethylenediamine)-cobalt(III) Iodide (26)

Literature molar absorptivities,

ε_{487} 103 and ε_{346} 128 in water at 25°C

Literature Yield, 72%.

Literature Elemental Analysis.

Calculated for $Co(C_{13}H_{26}N_5O_2)I_2$:

C, 26.15; H, 4.39; N, 11.73; O, 5.36.

Found:

C, 26.6; H, 4.3; N, 11.8.

Trial Elemental Analysis.

Found:

C, 26.2; H, 4.2; N, 11.8; O, 5.4.

Trial Isolated Yield, 70%.

[1.7,4] (S)-Prolinatobis(ethylenediamine)cobalt(III) Iodide (26)

Literature molar absorptivities

ε_{488} 103 and ε_{346} 125, in water at 25°C.

Literature Yield, 85%.

Literature Elemental Analysis.

Calculated for $Co(C_9H_{24}N_5O_2)I_2$:

C, 19.36; H, 4.42; N, 12.80; O, 5.85.

Found:

C, 19.1; H, 4.6; N, 12.4.

Trial Elemental Analysis.

C, 19.0; H, 4.4; N, 13.0; O, 5.7.

Trial Isolated Yield, 85%.

[1.7,5] (S)-Valinatobis(ethylenediamine)cobalt(III) Iodide (26)

Literature molar absorptivities,

ε_{487} 101 and ε_{348} 110, in water at 25°C.

Literature Yield, 80%.

Literature Elemental Analysis.

Calculated for $Co(C_9H_{26}N_5O_2)I_2$:

C, 19.69; H, 4.77; N, 12.75; O, 5.83.

Found:

C, 19.6; H, 4.8; N, 10.7.

Trial Elemental Analysis.

Found:

C, 19.9; H, 5.2; N, 12.8; O, 6.5.

Trial Isolated Yield, 55%.

The reaction conditions were optimised using the simple acid preparations above. This was followed by a series of trials complexing amino acids that have side chains that require protection from reaction conditions to maintain their integrity.

[1.7,6] Nα Nitroarginatobis(ethylenediamine)cobalt(III) Acetate

The method [1.7,0] was adopted, except for the isolation of the product from the reaction solution. After heating under reflux for 60 minutes the organic solvent was removed under reduced pressure. The remaining solution was sorbed on a SP-C25-120 Sephadex, acetate form, preparative column (refer to appendix 3.2,0). The column was eluted by two column volumes of 0.1 M pyridinium acetate, followed by successive elutions of 0.2 M, 0.3 M, 0.5 M and 1.0 M pyridinium acetate, each of two column volumes. When the separation had been sufficiently developed the resin overlaying the 2+ product band was removed using a vacuum line and trap. The resin carrying the product was removed by the same method and the cobalt amino acid complex stripped off using 3.0 M pyridinium acetate. The resulting solution was reduced in volume under reduced pressure and then freeze dried.

No satisfactory elemental analysis could be obtained for the glassy orange product. It did however, show the chromatographic behaviour characteristic of the 2+ amino-acidobis(ethylenediamine)cobalt(III) complex.
Trial Yield,40%.

[1.7,7] O-Benzylaspatartobis(ethylenediamine)cobalt(III) Iodide

The standard method [1.7,0] for the preparation of amino acid complexes was adopted except that the amino acid was dissolved in a minimum of hot water and an equivalent amount of methanol was added, rather than the final volume being made up to 50 ml.

Trial Elemental Analysis.

Calculated for $Co(C_5H_{22}N_5O_4)I_2$:

C, 27.50; H, 4.30; N, 10.74.

Found:

C, 27.4; H, 4.4; N, 10.5.

The product showed the chromatographic behaviour of the 2+ amino-acidobis(ethylenediamine)cobalt(III) complex. Trial Yield, 15%.

[1.8,0] The Preparation of O-Benzyltyrosinatobis-(ethylenediamine)cobalt(III) Iodide

O-benzyltyrosine (7.0g) was dissolved in 250 ml of dimethylsulfoxide, (1.2g, 0.02 mole) trans-dibromobis-(ethylenediamine)cobalt(III) bromide was added, followed by 1.2g lithium hydroxide monohydrate. The mixture was heated under reflux for 30 minutes and the resulting product stripped under reduced pressure. The product was a dark oil, which was taken up in 50 ml of water. A purple residue was filtered off. Adding methanol to the aqueous product solution induced the precipitation of fine orange crystals, which were filtered off. This orange product was sorbed on a SP-C25-120 Sephadex

preparative column, (refer to appendix 3.2,0) and the separation developed by eluting with 0.2 M HCl. The resin carrying the 2+ orange colour band was separated off using a vacuum line and trap and the colour eluted from the resin with 1.0 M HCl. The solvent was removed under reduced pressure, the product thus produced was taken up in 50 ml of water and the solution stripped again. This procedure was repeated until no smell of hydrogen chloride could be detected. The produce was taken up in 50 ml of water, the solution neutralised using 1 M NaOH and the product precipitated by addition of excess sodium iodide and cooling. The precipitate was filtered off, washed with acetone and air dried.

Results.

/1.8,1/ Trial Elemental Analysis.

Calculated for $Co(H_{32}C_{20}N_5O_3)I_2$:

C, 34.16; H, 4.59; N, 9.96.

Found:

C, 35.0; H, 4.7; N, 10.2

Trial Yield, 30%.

The product was found to be chromatographically clean of any detectable cobalt compound contamination and showed the chromatographic character of amino-acidobis(ethylenediamine)cobalt(III) complexes.

/1.9,0/ Variation in the Yield of the Preparation of Glycinato-bis(ethylenediamine)cobalt(III) Complex with Time of Heating under Reflux

trans-Dibromobis(ethylenediamine)cobalt(III) bromide (1.59g, 4.0 M mole) was stirred in 10 ml of methanol. To this was added (0.29g, 4.0 M mole) glycine dissolved in 10 ml of water. The mixture was treated with 0.13g of lithium hydroxide monohydrate and heated under reflux for 180 minutes. Samples (2 ml) were removed at time intervals of 15, 30, 45, 60, 90, 120, 150 and 180 minutes. The samples were placed on SP-C25-120 Sephadex test columns, H^+ form, eluted with 50 ml of water, followed by 50 ml portions or 0.1 M, 0.2 M and 0.3 M HCl respectively. The eluant containing the orange 2+ bands were separated, stripped under reduced pressure, taken up in 5 ml of water and the amount of product determined spectrophotometrically, λ_{MAX} 487 ε_{MAX} 97. This determination was used to calculate the ratio of 2+ cobalt complex product isolated over the total cobalt expected in a 2 ml sample, expressed as a percentage. This percentage was called the "Potential Yield".

Results

[1.9,1] Table 1.9,1

Variation in the Yield of Glycinatobis(ethylenediamine)cobalt(III) Complex with Heating Time

Time of Heating Under Reflux (Minutes)	Potential Yield[1] %
15	81
30	83
45	82
60	73

...continued

continued... Table 1.9,1

Time of Heating Under Reflux (Minutes)	Potential Yield[1] %
90	69
120	62
150	55
180	57

1. The number of moles of the amino-acidobis(ethylenediamine)cobalt(III) complex product, as a percentage of the total number of moles of cobalt in the trial.

/~1.10,0_7 Variation in the Yield, of the Preparation of Alaninatobis(ethylenediamine)cobalt(III) Complex Reaction, with Time of Heating Under Reflux

(S)-Alanine (9.0g, 0.1 mole) was added to a hot suspension of dibromobis(ethylediamine)cobalt(III) bromide (38.09, 0.1 mole), in 50% aqueous methanol (200 ml) and treated with lithium hydroxide monohydrate (4.2g, 0.1 mole). The mixture was heated under reflux for 180 minutes, 2 ml samples having been removed at 10, 15, 30, 60, 120 and 180 minutes of heating time. The samples were treated the same as those in trial /~1.9,0_7.

Results

/1.10,1/ Table /1.10,1/

Variation in the Yield of
Alaninatobis(ethylenediamine)cobalt(III)
Complex Preparation Reaction, with Heating Time

Time of Heating Under Reflux (Minutes)	Potential Yield[1] %
10	80
15	85
30	83
45	86
60	73
90	67
120	62
150	57
180	55

1. The number of moles of amino-acidobis(ethylenediamine)-cobalt(III) complex product, as a percentage of the total number of mole of cobalt in the trial.

/1.11,0/ Variation in the Yield in the Preparation of Glycinatobis(ethylenediamine)cobalt(III) Complex with Variations in the Methanol Water Solvent Ratio

Four trials were conducted using glycine (0.29g, 4.0 m mole) and dibromobis(ethylenediamine)cobalt(III) bromide (1.59g, 4.0 m Mole)

slurried in 20 ml of solvent. The solvents were (i) 60%, (ii) 70%, (iii) 80% and (iv) 100% Methanol/Water respectively. The slurrys were treated with 0.15g lithium hydroxide monohydrate and heated under reflux for 30 minutes. The product solutions were cooled, filtered, and a 2 ml sample of each analysed as described in [1.9,0].

Results

[1.11,1] Table [1.11,1]

The Variation of Glycinatobis(ethylenediamine)cobalt(III) Complex Yield, with Variation of the Methanol/Water Ratio of the Preparative Mixture

Solvent (% Methanol)	Potential Yield[1] %
50	81
60	78
70	85
80	88
100	85

1. The number of moles of glycinatobis(ethylenediamine) cobalt(III) complex, expressed as a percentage of the total number of mole of cobalt in the trial.

/1.12,0/ Preparation of Glycinatobis(ethylenediamine) cobalt(III) Complex, using Dichlorobis(ethylenediamine)cobalt(III) Chloride as the Starting Material

Glycine (0.2g, 2.7 m Mole) was dissolved in 10 M of hot water and added to a slurry of dichlorobis(ethylenediamine) cobalt(III) lithium hydroxide monohydrate (0.13g, 3.0 m Mole) and heated under reflux for 30 minutes. A 2 ml sample was removed and analysed as described in trial /1.9,0/. The reaction mixture was heated for a further 30 minutes under reflux and another 2 ml sample taken and analysed as above. The product was isolated from the solution as described in method /1.6,0/.

Results

/1.12,1/

(1) Potential Yield after 30 minutes heating of the reaction mixture - 76%.
(2) Potential Yield after 60 minutes heating of the reaction mixture - 78%.
Yield of Isolated Product, 68%.

/1.13,0/ Preparation of Alaninatobis(ethylenediamine) cobalt(III) Iodide using Dimethylsulfoxide as a Solvent

Alanine (1.78g, 20 m Mole) was dissolved in 100 ml of

dimethylsulfoxide, dibromobis(ethylenediamine)cobalt(III) bromide (8.4g, 20 m Mole) was added and the mixture was treated with (0.71g, 20 m Mole) lithium hydroxide monohydrate. The suspension was heated under reflux for 30 minutes. The resulting solution was stripped under reduced pressure, dissolved up in 10 ml of water, which was filtered and sorbed on a SP-C25-120, H^+ form, Sephadex preparative column (refer to appendix 3.2,0). The system was eluted with two column volumes of water, then with a gradient solvent ranging from 0.1 M to 1.0 M HCl. When the product band had been eluted and separated, a 10 ml sample of its solution was stripped under reduced pressure, taken up in 5 ml of water and the amount of product alaninatobis(ethylenediamine)cobalt(III) complex determined spectrophotometrically. The solution containing the bulk of the product was reduced to 50 ml volume, treated with excess sodium iodide and cooled. Further reduction in the solvent volume and cooling isolated more product. The orange product crystals were filtered off, washed with acetone, and air dried.

Results

[1.13,1]

Potential Yield of product - 68%

Isolated Yield of product - 58%

The product showed identical chromatographic behaviour to that of a characterised sample of alaninatobis(ethylenediamine)cobalt(III) iodide.

/1.14,0/ Preparation of Alaninatobis(ethylenediamine) cobalt(III) Complex using a Variety of Sources of Base

Four trials were conducted, in each transdibromobis(ethylenediamine)cobalt(III) bromide (4.15 g, 0.01 mole) was added to 50 ml of methanol, alanine (0.89g, 0.01 mole), dissolved in a minimum of hot water (approximately 10 ml), was added and the solution made up to 70 ml with methanol. The mixtures were then treated with 0.1 M equivalent of base and heated under reflux for 30 minutes. From the resulting product solutions a 2 ml sample was taken and tested as described in /1.8,0/.

Results

/1.14,1/ Table 1.13,1

The Preparation of $/Co(en)_2AlaO/^{2+}$ Using Different Sources of Bases

Trial	Base Used[1]	Potential Yield[2] %
1	$LiOH.H_2O$	85
2	NaOH	0
3	KOH	0
4	Na_2CO_3	70

1. Base used in each preparation.
2. The percentage yield determined to be in each product

solution, based on the amount of trans-dibromobis-(ethylenediamine)cobalt(III) bromide in the trial.

/1.15,0/ Preparation of Alaninatobis(ethylenediamine) cobalt(III) Iodide Using Sodium Hydroxide as the Source of Base

trans-Dibromobis(ethylenediamine)cobalt(III) bromide (4.15, 0.01 mole) and alanine (0.9g, 0.01 mole) was slurried in 70 ml of 60% aqueous methanol and treated with 1 M sodium hydroxide solution until the pH was 8.0. This mixture was then heated under reflux for 30 minutes. A 2 ml sample of the resulting solution was analysed as described in /1.8,0/ and the product extracted as described in /1.6,0/.

Results

/1.15,1/

Potential Yield - 62%

Isolated Yield - 55%

The isolated product showed identical chromatographic behaviour to that of an authentic sample of characterised alaninatobis(ethylenediamine)cobalt(III) iodide sample under the same conditions.

/1.16,0/ Preparation of Aminoacidobis(ethylenediamine) cobalt(III) Iodide complexes in Non Solution Conditions (26)

A slurry of trans-dibromobis(ethylenediamine)cobalt(III)

bromide (4.15g, 0.01 mole) and the amino acid (0.01 mole) were slurried in 50 ml of 50% aqueous methanol at 50°C and treated with lithium hydroxide monohydrate (0.41g, 0.01 mole) in methanol (25 ml) over 15 minutes with stirring. The mixture was slowly evaporated to near dryness. Water, 25 ml, was added, the mixture heated to 70°C for 15 minutes, cooled and filtered. The product was isolated as in [1.6,0]. Where possible the amino-acidobis(ethylenediamine)cobalt(III) complex prepared was compared with an authenticated sample of the product complex, chromatographically.

Results

[1.16,1] (S)-Isoleucinatobis(ethylenediamine)cobalt(III) Iodide

Trial Yield, 60%

Trial Elemental Analysis.

Calculated for $Co(C_{10}H_{28}N_5O_2)I_2$:

C, 21.33; H, 5.01; N, 12.84; O, 5.68.

Found:

C, 21.3; H, 5.0; N, 12.5; O, 5.7.

[1.16,2] (S)-Phenyladaninatobis(ethylenediamine)cobalt(III) Iodide

Trial Yield, 60%

Trial Elemental Analysis.

Calculated for $Co(C_{13}H_{26}N_5O_2)I_2$:

C, 26.15; H, 4.39; N, 11.73; O, 5.36.

Found:

C, 26.2; H, 4.3; N, 11.5; O, 5.3

/1.17,0/ Nε — Nitroaginatobis(ethylenediamine)cobalt(III) Acetate Preparation in Solid Conditions

The method /1.16,0/ was adopted, except that the product was isolated by sorbing the preparative solution on CM-C25-120 Sephadex column (refer appendix /3.2,0/). The column was developed by eluting with 0.1 M pyridinium acetate and the 2+ product band eluted with 0.3 M pyridinium acetate. The product solution was reduced in volume under reduced pressure and then freeze dried.

Results

/1.17,1/ Trial Yield, 40%.

/1.18,0/ O-Benzylaspatatobis(ethylenediamine)cobalt(III) Iodide

The method /1.16,0/ was adopted with the product being isolated as described in method /1.6,0/

Results

/1.18,1/ Trial Yield, 20%.

Trial Elemental Analysis.

Calculated for $Co(C_{15}H_{28}N_5O_4)I_2$:

C, 27.50; H, 4.30; N, 10.74; O, 9.77.

Found:

C, 28.0; H, 4.7; N, 10.5.

/1.19,0/ O-Benzyltyrosinatobis(ethylenediamine)cobalt(III) Iodide Preparation, using Non Solution Conditions

The method [1.16,0] was adopted. However, when the reaction mixture was filtered approximately 1.3g of O-benzyltyrosine was recovered. This was slurried with a further (4.2g, 0.01m mole) trans-dibromobis(ethylenediamine)cobalt(III) bromide in 50 ml of 50% aqueous methanol at 50°C and treated with lithium hydroxide monohydrate (0.41g, 0.01m mole). The preparation method [1.16,0] was repeated using this mixture. Product solution was isolated from both the first and second preparations separately using a Ag-50W-X2 BioRad resin preparative Ion Exchange column described in [1.6,0].

Results

[1.19,1] The first section of the preparation showed an Isolated Yield of 15%.

The second section of the preparation showed an Isolated Yield of 25%.

In each section a sample of the isolated product was chromatographically compared to a sample of the complex of established character. The product of this trial showed identical character to that of the known sample.

[1.20,0] Preparation of an Amino Acid Methyl Ester Cobalt(III) Complex using Triflouromethane Sulfonic Acid in Methanol, as the Alkylating Agent

Anhydrous aminoacidobis(ethylenediamine)cobalt(III) iodide (10 m Mole) (refer to appendix [3.1,0]), was slurried in 50 ml of anhydrous methanol, in a dry reaction vessel. Triflouromethanesulfonic acid (10 ml) was added slowly. The mixture was stirred gently, forming a red solution about 10 minutes after the addition of the alkylating agent. Stirring was maintained for 30 minutes after the formation of the solution and the product was then isolated. The isolation was effected by increasing the stirring rate and rapidly adding 600 ml of anhydrous diethyl ether (refer to appendix [3.1,0]). The product precipitated as an oil, the diethylether was decanted off. The final precipitation was achieved by the addition of 400 ml of anhydrous ether, accompanied by rapid stirring. The product was filtered off and placed under vacuum over P_2O_5.
A sample of the product was tested as described in [1.20,1].

[1.20,1] Testing of the "Peptide Formation Potential" of an Amino Acid Methyl Ester Cobalt(III) Complex

A 1M glycine methyl ester hydrochloride standard solution in anhydrous methanol (refer [3.1,0]) was prepared.
A 1ml aliquot of the solution containing the amino acid methyl ester cobalt(III) complex to be tested, or approximately 0.01g solid amino acid methyl ester complex dissolved in 1 ml of anhydrous methanol, was treated with 1 ml of the glycine methyl ester hydrochloride standard solution. A 1ml volume of 1M triethylamine solution in anhydrous methanol was added to the test solution, and the

reaction quenched after 10 seconds using 1 drop of glacial acetic acid.

Fig. [1.20,1]

$$NH_2\text{-}CH_2\text{-}COO\text{-}CH_2CH_3.HCl \xrightarrow{\text{Triethyl amine}} NH_2\text{-}CH_2\text{-}COO\text{-}CH_2CH_3 +$$

$$[Co(en)_2AAOCH_3]^{3+} \longrightarrow [Co(en)_2AAONH\text{-}CH_2COO\text{-}CH_2CH_3]^{3+}$$

A 1ml sample of the solution to be tested, or 1ml of anhydrous methanol solution of approximately 0.01g of the solid to be tested, was treated with 1ml of anhydrous methanol saturated with ammonia and after 10 seconds the reaction was quenched using 1 drop of glacial acetic acid.

Fig. [1.20,2]

$$NH_3 + [CO(en)_2NH_2\text{-}CHR\text{-}COOCH_3]^{3+} \xrightarrow{NH_3} [Co(en)_2NH_2CHR\text{-}COONH_2]^{3+} + CH_3OH$$

The resulting test solutions were analysed by stripping off the methanol under reduced pressure, taking up the resulting oil in 5ml of water separating the cobalt complexes in the solution using a SP-C25-120 Sephadex, H^+ form, test column (refer to appendix [3.2,0]) eluting with 0.2M HCl. The 3+ product band was eluted, stripped under reduced

pressure, taken up in 5ml of water, and determined spectrophotometrically at λ_{max} 490, ε 100. The number of moles of 3+ product, thus determined, expressed as a percentage of the theoretically possible yield was defined to be the "Peptide Formation Potential" of the ester complex tested.

/~1.21,0_7 Preparation of (S)-Proline-Methyl-Esterbis-(ethylenediamine)cobalt(III) Triflouromethane Sulfonate *

The method /~1.20,0_7 was adopted on the scale described.

Results

/~1.21,1_7 Testing the isolated product using (I) Glycine Methyl Ester method gave a Peptide Formation Potential of 65%.

Testing the isolated product using (II) Ammoniated Methanol method gave a Peptide Formation Potential of 100%.

Isolated Yield, 80%.

/~1.22,0_7 Preparation of (S)-Phenylalanine-Methyl-Esterbis(ethylenediamine)cobalt(III) Triflouromethane Sulfonate *(22)

The method /~1.20,0_7 was adopted on the scale described.

* See page 51

Results

/1.22,0/ Testing the isolated product using (I) Glycine Methyl Ester method gave a Peptide Formation Potential of 45%.

Testing the isolated product using (III) Ammoniated Methanol method gave a Peptide Formation Potential of 95%.

Isolated Yield, 80%.

/1.23,0/ Preparation of Amino Acid Methyl Ester-bis-(ethylenediamine)cobalt(III) Triflouromethane Sulfonate,* using Methyl Trifloromethane Sulfonic Acid as an Alkylating Agent

Anhydrous amino-acidobis(ethylenediamine)cobalt(III) iodide (10 m Mole) was slurried in 20 ml anhydrous trimethylphosphate (refer to appendix /3.1,0/). Methyl triflouromethane sulfonic acid (7.5g, 50m Mole) was added with stirring under anhydrous conditions. The reaction was strongly exothermic and the temperature rose to approximately 50°C. Stirring was maintained until all the amino acid complex had dissolved and was then continued for an additional 30 minutes. A 1 ml sample of the reaction solution was tested using the "Ammoniated Methanol" method (refer to appendix /1.20,1/). The product was then isolated by slowly pouring the reaction mixture into 800 ml of rapidly stirred anhydrous diethyl ether. The product precipitated as an oil. The diethyl ether was

* See page 51

decanted off and the product oil dissolved in 20 ml of anhydrous methanol. The product was once again precipitated from this solution by adding the solution to 800 ml of rapidly stirred anhydrous diethyl ether. The precipitation and redissolving procedure was repeated until the product precipitated as fine pink crystals. These crystals were filtered off and placed over P_2O_5, at 60°C, under oil pump vacuum.

Results

[1.23,1] (S)-Proline Methyl Ester Complex bis(ethylenediamine)cobalt(III) Triflouromethane Sulfonate *

A number of trials were conducted on a variety of scales. The "Ammoniated Methanol" test method (refer to [1.20,1]) was applied to the product solution and the result of this test reported as "Potential Yield". The product was isolated from the reaction solution and reported as "Isolated Yield". The isolated product was tested using the "Ammoniated Methanol" method (refer to [1.20,1]) and the result of this test reported as "Peptide Formation Potential".

* See page 51.

Table [1.23,1]

The Alkylation of Prolinatobis(ethylenediamine) cobalt(III) Iodide using Methyl Triflouromethane Sulfonic Acid as the Alkylating Agent

Trial	Scale[1]	Potential Yield[2] %	Isolated Yield[3] %	Peptide Formation Potential[4] %
1	0.5	100	85	100
2	1.0	95	70	100
3	1.57	80	75	80
4	2.1	100	90	100

1. All trials were carried out with materials in the proportions shown in method [1.23,0], the method was, however, scaled by the factors given.
2. The number of mole of $[Co(en)_2AAOCH_3]^{3+}$ determined to be in the reaction solution before isolation.
3. The number of mole of $[Co(en)_2AAOCH_3]^{3+}$ isolated.
4. The percentage of the isolated product that was determined to be active $[Co(en)_2AAOCH_3]^{3+}$ by reaction with ammoniated methanol.

[1.23,2] (S)-Phenylalamine Methyl Ester Complex bis(ethylenediamine)cobalt(III) Triflouromethane Sulfonate *

A number of trials were conducted on a range of scale and the results reported under "Potential Yield","Isolated

* See page 51.

Product Yield" and "Peptide Formation Potential".

Table [1.23,2]

The Alkylation of (S)-Phenylalaninatobis(ethylenediamine) cobalt(III) Iodide, using Methyl Triflouromethane Sulfonic Acid as the Alkylating Agent

Trial	Scale[1]	Potential Yield[2] %	Isolated Product Yield[3] %	Peptide Formation Potential[4] %
1	0.2	100	80	75
2	0.5	50	40	50
3	1.0	95	85	100
4	1.4	100	95	90
5	2.0	100	90	100

1. The method [1.23,0] outlined the trial conditions conducted on scale 1.
2. The number of mole of $[Co(en)_2PheOCH_3](F_3CSO_3)_3$ determined to be in the reaction mixture before isolation, expressed as a percentage of the maximum theoretically possible, based on the amount of phenylaninatobis(ethylenediamine)cobalt(III) iodide used in the trial.
3. Based on the amount of phenylalaninatobis(ethylenediamine) cobalt(III) iodide used in the trial.
4. The percentage of the isolated product determined to be active $[Co(en)_2AAOCH_3]^{3+}$.

[1.23,3] (S)-Alanine Methyl Ester Complex bis(ethylenediamine)cobalt(III) Triflouromethane Sulfonate *

A number of trials were conducted, on a range of scale and the results are reported under "Potential Yield", Isolated Product Yield" and "Peptide Formation Potential".

Table [1.23,3]

The Alkylation of (S)-Alaninatobis(ethylenediamine)-Cobalt(III) Iodide, using Methyl Triflouromethane Sulfonic Acid as the Alkylating Agent

Trial	Scale[1]	Potential Yield[2] %	Isolated Product Yield[3] %	Peptide Formation Potential[4] %
1	1.0	100	80	100
2	0.75	100	85	100

1. The method [1.23,0] outlined the trial conditions conducted on scale 1.
2. The number of mole of $[Co(en)_2AlaOCH_3](F_3CSO_3)$ determined to be in the reaction mixture before isolation, expressed as a percentage of the maximum theoretically possible, based on the amount of alaninatobis(ethylenediamine)cobalt(III) iodide used in the trial.

* See page 51.

3. Based on the amount of alaninatobis(ethylenediamine) cobalt(III) iodide used in the trial.

4. The percentage of isolated product determined to be active $[Co(en)_2AAOCH_3]^{3+}$ by reaction with ammoniated methanol.

* No elemental analysis were attempted in this study. The elemental composition and confirmation of the triflouromethane sulfonate as the counter ion of these complexes was reported by Buckingham and Clark (25).

DISCUSSION AND CONCLUSION

[1.24,0] A number of compounds are cited in the literature as having two ethylenediamine molecules complexed to a central cobalt(III) ion, but also carrying other ligand species that can be displaced by an amino acid thus forming the amino acidobis(ethylenediamine)cobalt(III) complex (21), (22), (26), (27), (28).
Methods [1.1,0], [$Co(en)_2Cl_2$]Cl; [1.2,0], [$Co(en)_2CO_3$]Cl; [1.3,0], [$Co(en)_2CO_3$]ClBr; [1.4,0], [$Co(en)_2CO_3$]Br; [1.5,0], t-[$Co(en)_2Br_2$]Br, demonstrated the preparation of these starting compounds and in some cases, [1.2,0] [1.4,0], the conversion of one to another.

Method [1.1,0] detailed the preparation of dichlorobis-(ethylenediamine)cobalt(III) chloride. The preparation scheme was scaled up such that reactions yielding approximately 600g (refer to Table 1.0,1 trial 4) of product and recording an acceptable 70% Percentage Yield, were demonstrated. The product was, in general, suitable for further study without recourse to recrystalisation.

Carbonatobis(ethylenediamine)cobalt(III) complex was prepared by two different methods, [1.2,0] from [$Co(en)_2Cl_2$]Cl, [1.3,0] directly and with two different counter ions, bromide and chloride.

The results contained in table [1.2,1] indicate that the

preparation of the carbonatobis(ethylenediamine)cobalt(III) complex, can be conducted on a large scale with very high yield. The achievement of this yield, however, required careful isolation of the [Co(en)$_2$CO$_3$]Cl from the produce solution. Lack of such care resulted in the percentage yield 59% reported for trial 3 (table 1.2,1). The product of this trial preparation usually required recrystalisation, thus the overall isolation of pure product was, by the method [1.2,0], 70%. The preparation of the carbonatobis(ethylenediamine)cobalt(III) complex by the direct method [1.3,0] gave an average crude yield of 67%. If care was taken with the isolation of the product from solution, further study could be conducted using this material without recrystalisation. Considering the two approaches to the synthesis of carbonato-bis(ethylenediamine)cobalt(III) complex, the losses involved in the necessary preparation of dichlorobis-(ethylenediamine)cobalt(III) chloride (refer to table 1.1,1) for method [1.2,0], make this method less attractive than the direct synthesis method [1.3,0]. The table 1.3,1 shows no evidence to suggest any preference toward the synthesis of the chloride or bromide product, other than the initial cost of the starting materials. Cobalt chloride hexahydrate, which is cheaper, may be favoured over the Bromide.

Method [1.5,0], cobalt(III) bromide, was demonstrated on

a preparative scale, while maintaining an acceptable percentage yield. This yield, however, seemed dependent on the quality of the $[Co(en)_2CO_3]^+$ used. Trial 6 (Table 1.5,1) was conducted using crude $[Co(en)_2CO_3]Cl$ product from method [1.2,0]. Viewing this carbonato complex through a scanning microscope showed dark contaminating crystals. The product isolated from trial 6 (table 1.5,1) was similarly contaminated, the dark crystals being visible to the naked eye. Preparative applications of method [1.5,0] have been reported using 125 g of $[Co(en)_2CO_3]Br$ starting material and recording satisfactory $[Co(en)_2Br_2]Br$ product yields of 60%, (29).

Preparation of amino acidobis(ethylenediamine)cobalt(III) complexes have been produced, on a preparative scale, by two general solution methods, [1.7,0] and in a solid mull [1.16,0], adapted from the work of Buckingham and Dekkers (26). The general efficiency of either method compared to the other, can be judged by comparing [1.7,2] and [1.16,1], [1.7,3] and [1.16,2], [1.7,6] and [1.17,1], [1.7,7] and [1.18,0] and [1.8,1] and [1.19,1]. The trial yields for the preparations of the isoleucinato complexes by method [1.7,0] and [1.16,0] gave 65% and 60% respectively. For phenyalalinato, trial yields for each method were 70% [1.7,0] and 60% [1.16,0]. Considering the amount of work required for solid mull method, [1.16,0], heating the reaction

mixture under reflux as described in method [1.7,0] was more attractive. For amino acid derivatives that are not particularly soluble in methanol/water mixtures, such as o-benzyltyrosine, the solution method [1.16,0] may show better yields. However, if the methanol solvent method [1.7,0] is modified as described in trial [1.8,0] to dimethylsulfoxide, preparation of the amino-acidobis(ethylenediamine)cobalt(III) complex in solution remains more attractive.

Alaninatobis(ethylenediamine)cobalt(III) complex was prepared in an aqueous methanol solvent ([1.7,1], [1.10,0]) and in dimethylsulfoxide solvent conditions. A comparison of these trials and the variation of the preparative solvent in [1.11,1] suggests that aminoacido-(ethylenediamine)cobalt(III) complexes can be synthesised on a preparative scale by adapting the solvent conditions to the soluble characteristics of the amino acid, to achieve the maximum yield of product. The literature also reports 60-70% reaction for the preparation of alanine and proline in non aqueous solvents (26), (21). The results [1.11,1] on trials altering the solvent conditions, along with Buckingham and Dekkers reported yields (26) using aqueous solution conditions for the preparation of alaninato, valinato, leucinato, isoleucinato, serinato and phenylalaninato complexes, suggest that the maximum yield

of amino acid complex product is achieved by keeping the amount of water in the solvent to a minimum.

Trials [1.9,0] [1.10,0] (21) on varying reaction heating times confirmed that the yield of aminoacidobis-(ethylenediamine)cobalt(III) complex by the solution method [1.17,0] generally maximised with 30 minutes heating under reflux. Trials [1.14,0] and [1.15,0] using different bases in the solution method indicate that good preparative yields can be maintained, using cheaper sources of base than lithium hydroxide monohydrate to adjust the reaction conditions to pH 8.0. The use of dichlorobis(ethylenediamine)cobalt(III) chloride as the starting complex in the solution method [1.7,0] was in trial [1.12,0] compared with the potential yield results for the similar preparation using dibromobis-(ethylenediamine) bromide in trial [1.9,0]. This showed similar results for the glycinato complex. These results agree with observations reported in the literature (21) for proline. Having demonstrated and gained experience in preparing the amino-acidobis(ethylenediamine)cobalt(III) complexes efficiently and on a large scale, attention was then turned to the alkylating agent reported by Buckingham and Dekkers (26) which was PCl_3 in methanol under anhydrous conditions. Further study of this scheme by Reynolds (30) showed that large excesses of the alkylating reagent were required, coupling yields of an amino acid with the

alkylated complex in the alkylating solution were poor and separation of the products difficult.

Efficient preparation and isolation of alkylated amino acid complexes was achieved in the study using two methods, and the potential usefulness of the products tested, using glycine methyl ester and ammoniated methanol procedure. The two test procedures consistently showed glycine methyl ester predicting a lower "Peptide Formation Potential" than ammoniated methanol. This led to the hypothesis that the acidic conditions that may exist in the solution of the "Glycine Methyl Ester Method", may lead to protonation of the glycine ester, thus rendering the protonated molecule unavailable for condensation. Excessively basic conditions (greater than ph 6.0 when 1 ml of the methanol solution is dissolved in 1 ml of water) can lead to the formation of a brown product that irreversably binds to the Sephadex SP test column. Ammoniated methanol was thus adopted as a more realistic indication of the "Peptide Formation Potential". Comparison of the trial [1.21.0] the preparation of $[Co(en)_2ProOCH_3]^{3+}$ Table 1.23,1 and trial [1.22,0] the preparation of $[Co(en)_2PheOCH_3]^{3+}$ Table [1.23,2] showed acceptable amounts of product isolated, having a high "Peptide Formation Potential". High isolation yields resulted when the product precipitated in large lumps. If however, the precipitate

formed a very fine suspension, product was lost when the diethyl ether was decanted off. The use of triflouromethane sulfonic acid as the alkylating agent was more inclined to produce such a fine precipitate, thus methyl-triflouromethane sulfonic acid was adopted as the standard methylation technique. Anhydrous conditions were essential to either scheme and failure to exclude water at all stages resulted in hydrolysis of the product. Freshly prepared methyl triflouromethane sulfonic acid also gave higher isolated product yields. The low yield of trial 2 table 1.23,2 occurred when the bottle of methyl triflouromethane sulfonic acid had been opened then resealed repeatedly. This study demonstrated, however, that alkylation using triflouromethane sulfonic acid or methyl triflouromethane sulfonic acid, could prepare on a large scale, with little inconvenience, the alkylated amino acid cobalt(III) complexes required for synthetic peptide production.

CHAPTER (II)

Peptide Formation Using Amino Acid Cobalt(III) Chelates

/2.1,0/ Selection of a Resin for the Ion Exchange Separation of the Condensation Reaction Products

The synthetic scheme outlined in the "Discussion and Conclusion" section /1.24,0/ of Chapter 1 included an Ion Exchange separation of the coupling reaction products. Four cationic exchange resins were investigated in an attempt to complete the separation as efficiently as possible.

The resins examined were (i) SP-C25-120 Sephadex, (ii) CM-C25-120 Sephadex, (iii) Whatman CM32 Carbonymethyl Cellulose and CM Sepharose CL-6B. Each resin was swollen according to the recommendations of the manufacturer and precycled as required. The resin was then converted into the pyridinium form by treatment with 1.0 M pyridinium acetate, followed by equilibration in 0.05 M pyridinium acetate buffer pH 5.1. That each resin had reached equilibrium with the eluting 0.05 M pyridinium acetate buffer, was determined by comparing pH and conductivity readings for the eluant and effluent solutions. The swollen and equilibrated resins were packed in glass chromatography columns of 12 MM diameter which had a No.2 scintered glass plug to retain the resin, thus forming a bed 12x100 MM in depth.

A 0.1 g phenylalanine methyl ester cobalt(III) complex sample was dissolved in 10 ml of anhydrous methanol and treated with 10 ml of ammoniated methanol and the reaction quenched after ten seconds by the addition of approximately 1 ml of glacial acetic acid. The methanol was stripped off under reduced pressure and the reaction products formed an oil. This oil was dissolved in 5 ml of water, 0.1 g $[Co(en)_2PheO]I_2$ and 0.1 g $[Co(en)_3]I_3$ were added to the solution and the solution diluted to 20 ml. This solution was then divided into four parts and each part sorbed onto one of the cation exchange resins under examination. Each column was eluted by a buffer gradient.

The gradient was generated by having a chamber connected to the top of the column, with the flow of its contents controlled by a tap, initially containing 100 ml of 0.05 M pyridinium acetate. The fluid volume was maintained in this chamber by 1.0 M pyridinium acetate drawn from an adjacent reservoir and the contents of the chamber were mixed with a mechanical stirrer. The development of the separation could be followed by the naked eye and the time from the start of elution, flow rate of the solvent and the conductivity of the effluent was noted as each band of colour separated.

/2.1,1/ Results Table 2.1,1

The Separation of a Simulated Condensation Reaction Solution using a Variety of Ion Exchange Resins

Resin	Time[1] (min)	Flow Rate[2] (ml/min)	Band[3]	Concentration[4] (M)
SP-C25-120 Sephadex	14	3.5	Purple	0.09
	30	2.9	Yellow	0.15
	40	2.3	Orange 1/2	0.35
CM-C25-120 Sephadex	10	3.0	Purple	0.07
	15	2.7	Orange 2	0.10
	24	2.5	Orange 1/ Yellow	0.18
CM Cellulose	8	3.2	Purple	0.07
	12	3.0	Orange 2	0.10
	17	3.0	Orange 1/ Yellow	0.20
*CL6B CM Sepharose	20	1.4	Purple	0.10

1. Measured from the start of the elution of the column.
2. Determined in ml/minute of effluent from the column measured over 20 seconds.
3. The colour of the band isolated. The orange band isolated near the top of the column was labelled orange band 1 that below it orange band 2.
4. Concentration of the pyridinium acetate in the effluent. This concentration was determined by reading the concen-

tration from the conductivity of the effluent, on a straight line plot of the conductivity of 0.05, 0.1, 0.2, 0.3 and 1.0 M pyridinium acetate.

* The low flow rate of this column may have been due to blocking of the glass scinter. This trial was abandoned.

NB 1. From the descriptions offered by Buckingham and Dekkers (26) and the experience gained with the trials described in chapter 1 the band colours were identified as:

Yellow	$[Co(en)_3]^{3+}$
Orange 1	$[Co(en)_2Phe\text{-}NH_2]^{3+}$
Orange 2	$[Co(en)_2PheO]^{2+}$
Purple	$[Coen_2(PheO)_2]^{+}$

NB 2. From a similar trial using $[Co(en)_2AlaO]^{2+}$ complex the band labelled orange 2 when chromatographed on SP-C25-120 Sephadex resin eluted with 0.2 M pyridinium acetate formed an orange foreshadow. Resin carrying this foreshadow was separated out using a vacuum line and trap. Cobalt complex from the main band and this foreshadow was isolated and an element analysis carried out.

Elemental Analysis.

Calculated for $Co(H_{14},N_5,C_9,O_2)I_2$:

C, 19.76; H, 4.42; N, 12.80

Found:

Foreshadow	C, 19.8;	H, 5.0;	N, 12.8
Main Band	C, 20.1;	H, 4.5;	N, 12.7

[2.2,0] Preparation of Phenylalanine Benzyl Ester p-Toluenesulfonate: A Modification of the Method of Zervas, Winitz and Greenstein (31)

Phenylalanine (48.5g, 0.025 mole) and p-Toluenesulfonic acid monohydrate (48.5, 0.25 mole) were added to 100 ml of benzyl alcohol and 50 ml of benzene. The mixture was then heated under reflux with the liberated water being removed with the aid of a Dean and Stark apparatus. After approximately 9 ml of water had been collected the Dean and Stark receiver was drained and filled with 3Å molecular sieves that had been dried at 110°C. Reflux was continued overnight. The mixture was permitted to cool and 250 ml of benzene was added to the reaction flask. Approximately 300 ml of anhydrous diethyl ether was added slowly until precipitation was induced and the mixture was left sitting at room temperature for 60 minutes. The waxy precipitate was washed with diethyl ether, after being filtered from the mother liquor. Further product could be precipitated from the mother liquor by the addition of a further 100 ml of diethyl ether and cooling overnight at 4°C.

The crude product, isolated from the reaction solution, was purified by dissolving it in a minimum of hot benzene (approximately 100 ml), cooling slowly and trituration.

/2.2,1/ Results

Trial Yield, 80%.

Product Melting Point 168-171°C.

Literature Melting Point 170°C.

/2.3,0/ The Condensation of an Amino Acid Methyl Ester Cobalt(III) Complex with Phenylalanine Benzyl Ester: A General Method

The phenylalanine benzyl ester was generated by adding a solution of triethylamine (0.2g, 2.0 m Mole) in 10 ml of methanol, to (0.9g, 2.0 m Mole) phenylalanine benzyl p-toluenesulfonate. The amino acid methyl ester cobalt(III) complex (1.0 m Mole) was then added to this solution and the mixture stirred for 30 minutes. The methanol was then stripped of the resulting solution under reduced pressure and the product taken up in 20 ml of 0.05 M pyridinium acetate. This solution was filtered and the product sorbed onto a CM 32 Carboxymethyl Cellulose pyridinium form Peptide Complex Separation Column (refer to appendix 3.2,0). The column was eluted with 50 ml of 0.05 M pyridinium acetate followed by enough 0.1 M pyridinium acetate to separate the product band labelled orange 1 in section /2.1,0/ from the other bands. This product band was then eluted using 0.2 M pyridinium acetate and its resulting solution was reduced in volume to approximately 20 ml then freeze dried.

Testing the condensation reaction solution: a 1 ml sample of the condensation reaction solution was taken before proceeding with the product isolation, stripped under reduced pressure, taken up in 5 ml of water and sorbed on a SP-C25-120 Sephadex test column (refer to appendix [3.2,0] and the chromatogram developed using 0.1 M HCL. All the cobalt colour, except that identified as belonging to the dipeptide product complex was pooled, stripped and taken up in 10 ml of water and the amount of cobalt determined spectrophotometrically (ε_{MAX}.104 λ487 NM). The amount of dipeptide product complex was similarly stripped and determined separately. Since the phenylalanine benzyl ester was in excess in the reaction solution, all the amino acid complex should theoretically have condensed and be separated as the dipeptide complex product. The percentage of the dipeptide complex product to the total amount of cobalt complexes in the test solution gave an indication of the success of the condensation and was defined as the "Indicated Coupling Yield".

[2.3,1] Results Table 2.3,1

The Condensation of an Amino Acid Methyl Ester Complex with Phenylalanine Benzyl Ester

Trial	Amino Acid Ester Complex Used[1]	Scale[2]	Indicated Coupling Yield[3] %	Isolated Yield[4] %
1	$[Co(en)_2PheOCH_3]^{3+}$	2.0	90	40
2	$[Co(en)_2PheOCH_3]^{3+}$	1.0	60	50
3	$[Co(en)_2PheOCH_3]^{3+}$	1.5	100	30
4	$[Co(en)_2PheOCH_3]^{3+}$	2.5	80	4
5	$[Co(en)_2PheOCH_3]^{3+}$	2.5	90	0
6	$[Co(en)_2PheOCH_3]^{3+}$	0.5	trace	0
7	$[Co(en)_2PheOCH_3]^{3+}$	4.0	30	10
8	$[Co(en)_2ProOCH_3]^{3+}$	3.0	10	10
9	$[Co(en)_2ProOCH_3]^{3+}$	1.7	80	80
10	$[Co(en)_2AlaOCH_3]^{3+}$	1.0	80	80

1. Was reacted with excess phenylalanine benzyl ester.
2. The trials were conducted with the materials in the proportions given in the general method [2.3,0] but on a greater or lesser scale.
3. The amount of dipeptide product determined to be in the product solution immediately after reaction based on the initial amount of amino acid methyl ester complex.

4. The amount of dipeptide isolated from the product solution based on the initial amount of amino acid ester complex used.

[2.4,0] The Condensation of Phenylalanine Methyl Ester Complex with Phenylalanine Benzyl Ester, using Dimethylsulfoxide as a Solvent

Phenylalanine benzyl ester p-toluenesulfonate (0.9 g, 2.0 m Mole), was treated with a triethylamine (0.2 g, 2.0 m Mole) and the mixture dissolved in 50 ml of anhydrous dimethylsulfonide (refer to appendix [3.1,0]). Phenylalanine methyl ester cobalt(III) complex (0.8 g 1.0 m Mole) was added and the resulting solution stirred for 30 minutes. The solution was stripped under reduced pressure and the dipeptide product isolated as described in the general method [2.3,0] using a CM-32 Cellulose pyridinium form Peptide Complex Separation Column.
A 1 ml sample of the product solution was also tested after being stripped under reduced pressure as described in general method [2.3,0], the dipeptide product being determined spectrophotometrically and the "Indicated Coupling Yield" calculated.

/⁻2.4,1_7 Results. Table 2.4,1

The Coupling of /⁻$Co(en)_2PheOCH_3$_7^{3+} with $PheOC_6H_5$ using Anhydrous Dimethylsulfonide as a Solvent

Trial	Scale[1]	Indicated Coupling[2] Yield %	Isolated[3] Yield %
1	1	100	50
2	1	50	10

1. Trials were conducted with the materials in the proportions of the description /⁻2.4,0_7 but may be on a different scale.
2. The amount of dipeptide product determined to be in the product solution immediately after reaction based on the amount of cobalt complex used.
3. Based on the amount of cobalt complex used.

/⁻2.5,0_7 The Generation of an Amino Acid Methyl Ester Complex and its Condensation with an Amino Acid Benzyl Ester In situ

A 10 ml anhydrous methanol solution of the amino acid benzyl ester (2.0 m Mole) was generated, if necessary by adding 1 molar equivalent of triethylamine in 10 ml of anhydrous methanol to the p-toluenesulfonate salt of the amino acid benzyl ester. Anhydrous amino-acidobis(ethylenediamine)-

cobalt(III) iodide (1.0 m Mole) was slurried in 40 ml of anhydrous trimethylphosphate (refer to appendix [3.1,0]). Methyl triflouromethane sulfonic acid (2 ml) was added and the mixture gently stirred. The stirring was maintained for 30 minutes after the amino acid complex had gone into solution. A 1 ml sample of this solution was then tested using ammoniated methanol as described in general method [1.20,17] and the "Peptide Formation Potential" determined. If the test indicated less than satisfactory generation of the amino acid methyl ester complex, another 1.0 ml of methyltriflouromethane sulfonic acid was added and a further 1 ml sample tested as above. When all the amino acid complex had been methylated as indicated by the tests, the product was precipitated as an oil by pouring the reaction solution into 200 ml of rapidly stirred anhydrous diethyl ether. The amino acid methyl ester complex product oil was then dissolved in 40 ml of anhydrous methanol, after the diethyl ether had been decanted off. This solution of the amino acid methyl ester was then treated with the amino acid benzyl ester solution initially generated. This mixture was stirred gently for 10 minutes, then a 1 ml sample was taken, stripped under reduced pressure and the "Indicated Coupling Yield" determined spectrophotometrically as described in the general method [2.3,0]. The dipeptide complex product was then isolated from the main reaction solution by stripping of the solvent under reduced pressure, separating out the complex using Ion Exchange, then freeze-drying the resulting solution as described in the general method [2.3,0].

/2.5,1/ Results Table 2.5,1

The In situ Generation and Condensation of an Amino Acid Methyl Ester Cobalt(III) Complex with Amino Acid Benzyl Ester

Trial	Scale[1]	Amino Acid Cobalt(III) Complex[2]	Amino Acid Benzyl Ester[3]	Indicated Coupling Yield[4] %	Isolated Yield[5] %
1	1	Phe	$PheOC_6H_5$	100	10
2	10	Phe	$PheOC_6H_5$	60	10
3	4	Ala	$PheOC_6H_5$	80	60
4	1	Ala	$GlyOC_6H_5$	40	40

1. The trials were conducted with the materials in the proportions described in /2.5,0/ but may be on a greater or smaller scale.
2. The amino acid complex used to generate the amino acid methyl ester complex using methyltriflouromethane sulfonic acid.
3. The amino acid benzyl ester generated to condense with the cobalt(III) complex.
4. The amount of dipeptide complex product determined to be in the product solution immediately after reaction.
5. Based on the amount of cobalt complex used.

From the results of coupling trials quoted so far, it can be noted that the Indicated Yield for the coupling of a

particular amino acid methyl ester to a particular amino acid benzyl ester can vary greatly for a number of similar trials. As suggested in the Discussion and Conclusion section of chapter 1 [1.24,07] the difference in the "Peptide Formation Potential" predicted for the same amino acid methyl ester product by the "Glycine Methyl Ester" and the "Ammoniated Methanol" test procedure may have been due to the protonation of the amino acid ester under the conditions of the "Glycine Methyl Ester" test. An aqueous solution of 0.1 g of each of the amino acid methyl ester prepared as described in Chapter 1, in 1 ml of water showed a variation in the pH from <1 to approximately 3.5. It had also been noticed that condensation of an amino acid methyl ester complex with an amino acid benzyl ester, glycine methyl ester, or ammonia, under potentially basic conditions can lead to the formation of dark brown cobalt polymer that will irreversably bind to a cationic exchange resin (33).

[2.6,0] <u>The Condensation of Alanine Methyl Ester Cobalt(III) Complex with Phenylalanine Benzyl Ester, Using Triethylamine to avoid Protonation of the Phenylalanine Benzyl Ester</u>.

Phenylalanine Benzyl Ester p-toluenesulfonic acid (9.0 g, 0.02 Mole) was treated with an anhydrous methanol solution (2.0 ml) of triethylamine (2.0 g, 0.02 Mole).

Anhydrous alanatobis(ethylenediamine)cobalt(III) iodide (5.2 g, 0.01 Mole), was slurried in 40 ml of anhydrous trimethylphosphate, treated with methyltriflouromethane sulfonic acid (8 ml) and the mixture gently stirred. The stirring was maintained for 30 minutes after the amino acid complex had dissolved. A 1 ml sample labelled sample 0, was removed and the "Peptide Formation Potential' of the solution determined by treatment with ammoniated methanol as described in [1.20,1]. The amino acid methyl ester complex was isolated by pouring the reaction solution into 800 ml of rapidly stirred anhydrous diethyl ether. The ether was decanted off the product oil which had formed, and the oil was dissolved in 40 ml of anhydrous methanol. This amino acid methyl ester complex solution was added to the phenylalanine benzyl ester solution prepared initially and the mixture gently stirred for 30 minutes. Sample 1: The Indicated Coupling Yield was determined spectrophotometrically after preliminary ion exchange separation of a 1 ml sample of the reaction mixture as described in the general method [2.3,0]. A 1 ml sample of the solution was taken, diluted with 1 ml of water and the pH determined. The main reaction mixture was placed in a beaker and mixed using a magnetic stirrer. A pH meter was used to get a rough indication of the hydrogen ion concentration. Triethylamine was added until the pH meter reading was approximately 3.0.

Sampling 2: A 1 ml sample of this mixture was removed, dissolved in 1 ml of water and the pH determined. Another 1 ml sample of the mixture was taken and the Indicated Coupling Yield determined as described above. Triethylamine 2 x 1 ml was added. Sampling 3 and 4: After the addition of each 1 ml lot, 2 x 1 ml samples were taken. One was diluted with 1 ml of water and the pH determined and the other used to calculate the Indicated Coupling Yield.

The dipeptide complex product was isolated from the reaction mixture by ion exchange using a CM-Cellulose, pyridinium form column, as described in general method [2.3,0] and the resulting solution freeze dried.

[2.6,1] Results

Sample O: "Peptide Formation Potential" of the $[Co(en)_2AlaOCH_3]^{3+}$ = 80%.

Table 2.6,1

The Variation of the Dipeptide Complex Product in the Preparation Solution of $[Co(en)_2AlaOGlyOC_6H_5]^{3+}$ with the addition of Triethylamine

Sampling	pH[1]	Indicated Coupling Yield[2] %
1	1.1	10
2	4.6	60
3	6.1	75
4	7.5	70

1. The pH measurement of a 1 ml sample of the reaction mixture diluted with 1 ml of water.
2. The dipeptide complex product yield based on the amount of cobalt complex in the reaction mixture.

[2.7,0] A Modification of the General Method for the Generation and Condensation of an Amino Acid Methyl Ester Cobalt(III) Complex with an Amino Acid Benzyl Ester, or Dipeptide Ester, using Triethylamine to avoid Protonation of the Amino Acid/Peptide Benzyl Ester

A 10 ml anhydrous methanol solution was produced containing 1.0 m Mole of the amino acid benzyl ester or dipeptide benzyl ester that was to form the carbonyl terminal amino

acid of the peptide. If the required amino acid benzyl ester was phenylalanine benzyl ester this was generated by treating phenylalanine benzyl ester p-toluenesulfonate; (0.45 g, 1.0 m Mole), with a 10 ml anhydrous methanol solution of triethylamine (0.1 g, 1.0 m Mole).

Amino-acidobis(ethylenediamine)cobalt(III) iodide (anhydrous) (2.0 m Mole) was slurried in 4.0 ml of anhydrous trimethylphosphate and treated with methyltriflouromethane sulfonic acid (2.0 ml) and the mixture gently stirred for 30 minutes after all the complex had dissolved. A 1 ml sample, Sample 0, of this solution was removed and tested using ammoniated methanol and the "Peptide Formation Potential" determined as described in [1.20,1]. The amino acid methyl ester complex was extracted from the reaction solution by pouring the solution into 100 ml of rapidly stirred anhydrous diethyl ether. The product formed an oil, the ether was decanted and the oil was dissolved in 10 ml of anhydrous methanol. A 1 ml sample, Sample 1, was removed and treated as described below. The remaining solution was added to the amino acid/dipeptide benzyl ester solution prepared initially, and this main reaction mixture was stirred for approximately 30 minutes.

Glycine ethyl ester, (0.14 g, 1.0 m Mole) was dissolved in (4.0 ml) anhydrous methanol. A 1 ml sample of this solution was added to the Sample 1 of the amino acid methyl ester complex solution described above. This mixture was diluted

with 2 ml of water and enough triethylamine was added to give a pH reading of 6.0. From this test a volume of triethylamine was calculated that would give a similar pH reading from the main reaction mixture, if it were to be diluted with an equal volume of water. This calculated volume of triethylamine was added to the main reaction mixture and the reacting species stirred gently for 10 minutes. A 1 ml sample, Sample 2, was removed and the "Indicated Coupling Yield" was determined as described in trial [2.3,0]. The Indicated Coupling Yield calculation was based on the amount of peptide ester or amino acid ester in the trial.

[2.7,1] Results Table 2.7,1

'Peptide Formation Potential' of Sample 1 = 90%.

Peptide Complex Preparation Using Triethylamine to Avoid Protonation of the Coupling Amino Acid/Peptide Benzyl Ester

Trial	Scale[1]	Amino Acid[2] Complex Used	Amino Acid/ Peptide[3] Ester Used	Indicated Coupling[4] Yield %	Isolated[5] Yield %
1	10	$[Co(en)_2PheO]^{2+}$	$PheOC_6H_5$	100	45
2	5	$[Co(en)_2PheO]^{2+}$	$PheOC_6H_5$	100	50
3	1	$[Co(en)_2ProO]$	$PheOC_6H_5$	95	90
4	2	$[Co(en)_2ProO]$	$GlyOC_6H_5$	90	90
5	5	$[Co(en)_2AlaO]$	$PheOC_6H_5$	100	90
6	1	$[Co(en)_2ProO]$	$PhePheOC_6H_5$	80	75
7	10	$[Co(en)_2ProO]$	$GlyGlyOC_2H_5$	100	100
8	1	$[Co(en)_2AlaO]$	$GlyGlyOC_2H_5$	100	100

1. The trials were conducted with the materials in the proportions described in general method [2.7,0] but on a greater or lesser scale.
2. Amino-acidobis(ethylenediamine)cobalt(III) complex used.
3. Peptide Ester that was coupled to the complex.
4. Yield of peptide complex product determined to be in the reaction solution, based on the amount of peptide/ amino acid ester used;- calculated from the test on Sample 2.
5. Based on the amount of peptide/amino acid ester used.

[2.8,0] Removal of the Peptide from a Peptide Cobalt(III) Complex using Vanadous Sulphate Reduction

15 g of zinc metal was shaken for a short time in 1.0 M HCl. When the metal was clean a mercuric chloride solution (1.0 g in 10 ml of water) was added and the zinc shaken until the granules were covered with mercury, 50 ml of mercury was then added and the mixture left over night. The vandous sulphate solution was generated by adding approximately 10 ml of the zinc mercury amalgam produced above to 10 ml of a 0.5 M vanadyl sulphate solution in 0.1 M HCl. The amalgam and the vanadyl sulphate were shaken until the bright blue colour of the vanadyl sulphate changed to an imperial purple.

Peptide Cobalt(III) complex (1.0 m Mole) was treated with a ten molar excess of the freshly prepared vanadous sulphate

solution produced above. The mixture was shaken for 10 minutes, then filtered through Watman No 1 filterpaper. The resulting solution was stripped under reduced pressure, taken up in distilled water and loaded onto a preparative P_2 Gel Filtration Column. (Refer to appendix [3.2,0]). The column was eluted with distilled water and the column effluent collected by a fraction collector. A 1 ml sample from each tube was spotted onto blotting paper and the paper sprayed with a 2% solution of ninhydrin. The blue colour that developed on the spots with peptide, as well as the uv monitoring at 254 NM and 280 NM of the column effluent, permitted the tubes carrying the peptide to be identified. The liquid in these tubes was pooled and freeze dried.

[2.8,1] Results Table 2.8,1

The Separation of the Peptide from the Products of the Peptide Complex Reduction

Trial	Complex[1]	Amount m Mole[2]	Recovered Peptide[3] grams	Isolated Yield[4] %
1	$[Co(en)_2PhePheOC_6H_5]^{3+}$	12.0	1.35	28
2	$[Co(en)_2ProPheOC_6H_5]^{3+}$	5.0	-	-
3	$[Co(en)_2ProGlyGlyOC_2H_5]$	6.0	1.74	97
4	$[Co(en)_2ProPhePheOC_6H_5]$	5.0	2.45	98
5	$[Co(en)_2AlaPheOC_6H_5]$	2.0	-	-

1. The peptide complex reduced using freshly prepared vanydale sulphate solution and the peptide isolated using P_2 gel filtration.
2. Number of mole of complex in trial.
3. Number of mole of peptide material recovered.
4. Based on amount of complex used.

In the case of trials 2 and 5 the P_2 gel filtration column did not completely separate the blue coloured reduction products from the peptide material, the isolated peptide product was coloured faintly blue. In all other cases except trial 3 the low recovery of peptide could be due to some of the product being collected in tubes containing the vanadium ion reduction products and thus masking the peptide during the scanning of the fraction collector tubes. The P_2 Gel Filtration column became contaminated with a blue colour after two separations. This colour appeared to be irreversably bound.

[2.9,0] Reduction of Prolinylglycylglycine Ethyl Ester Cobalt(III) Complex using Sodium-Borohydride

Prolinylglycylglycine ethyl ester cobalt(III) complex, (0.6g 1.0 m Mole) was dissolved in 2 ml 0.1 m HCL and

treated slowly with sodium borohydride (0.5 g, 10 m Mole). Acetic acid was added to maintain the acid conditions, the mixture was shaken vigorously for 1 minute and left to stand at room temperature for 30 minutes. The mixture was then filtered through a Millipore, pore size 0.5 /um filter, and loaded onto a test P_2 Gel Filtration column and eluted with distilled water. Fractions of the column effluent were collected (2 ml) and a sample of each fraction spotted onto blotting paper and the spots developed by spraying with a 2% ninhydrin solution.

Results

/~2.9,1_7 The column became contaminated by blue/black reduction products. The peptide was only partially separated from the reduction products.

/~2.9,2_7 Reduction of Peptide Ester Complexes using 2% Zinc Amalgam and the Isolation of the Peptide Ester

The peptide ester complex (1.0 m Mole) was dissolved in 2 ml 0.2 m HCL and treated with an equal volume of 2% zinc amalgam. The mixtures were shaken together vigorously for approximately 10 minutes then left standing for 24 hours. The aqueous solution was separated from the amalgam by filtration. The solution was then loaded onto a

preparative P_2 Gel Filtration column and the separation of the products proceeded as described in [2.8,0] by elution of the column with distilled water.

Results

[2.9,3] Table 2.9,1

The Isolation of the Peptide from a Peptide Cobalt(III) Complex using 2% Zinc Amalgam

Trial	Complex[1]	Amount m Mole[2]	Weight of Peptide grams	Isolated Yield[3] %
1	$[Co(en)_2ProPheOC_6H_5]$	0.5	0.10	58
2	$[Co(en)_2ProGlyOC_6H_5]$	1.0	0.19	72
3	$[Co(en)_2ProGlyGlyOC_2H_5]$	5.0	1.42	95
4	$[Co(en)_2AlaGlyGlyOC_2H_5]$	2.0	0.46	98

1. Peptide cobalt(III) complex reduced in the trial.
2. Amount of complex used in the trial. The proportions of materials used in the trials are given in section [2.9,2].
3. Based on the amount of complex used in the trial.

[2.10,0] Results presented in table 2.3,1 trials 1, 2, 3, 4, 5 and table 2.4,1 trials 1 and 2 show a very much reduced "Isolated Yield" of product when compared to the

yields expected from the 'Indicated Coupling Yield'. It was noticed in some cases, especially those involving the condensation of $[Co(en)_2PheOCH_3]^{3+}$ with an amino acid ester, that when the product solution was separated by Ion Exchange chromotography the peptide complex product band would gradually smear and in some cases elute under the conditions that were used to mobilise 2+ and lesser positively charged ions on the column. The degree to which this occurred varied with the peptide product involved. An attempt was made to purify a sample of $[Co(en)_2PheOPheOC_6H_5](CH_3COO)_3$ by precipitating the dipeptide complex from a small amount of warm water as the iodide salt, by adding excess sodium iodide. It had been shown previously by chromatography that the sample carried 60% of the cobalt as a 3+ charged species, the remainder being 2+. Immediately after the addition of the sodium iodide, an attempt was made to induce precipitation by sorbing a small amount of the solution onto an H+ form SP-C25-120 Sephadex test column (refer to appendix [3.2,0]) and eluting with 0.1 M HCL. Only one coloured band could be detected on the column and this showed the chromatographic behaviour of a cobalt complex carrying a 2+ charge.

These observations suggested that some of the peptide cobalt(III) complexes were unstable and that the

degradation of the peptide complex varied with the chemical environment of the complex.

[2.11,0] The Degradation of Peptide Cobalt(III) Complexes under a variety of Hydrogen Ion Concentration Conditions

The amino acid methyl ester cobalt(III) complex (1.0 m Mole) was dissolved in 5 ml of anhydrous methanol and this solution was treated with one mole equivalent of the amino acid methyl ester, or peptide alkyl ester and stirred gently for 30 minutes. The resulting mixture was stripped under reduced pressure and the product oil dissolved in 15 ml of water. This was then split into three equal parts and each solution was then accurately made to 10 ml with a different pH buffer. A 2 ml sample was then removed from each of the buffered solutions, stripped under reduced pressure and sorbed onto a SP-C25-120 Sephadex H+ form test column. The column was eluted with 50 ml of 0.1 m HCL followed by 50 ml of 0.2 m HCL. The effluent containing the 3+ peptide product was separated and the rest of the effluent was discarded. The effluent solution containing 3+ ion was stripped under reduced pressure to approximately 5 ml, then made up accurately to 10 ml with distilled water. The amount of peptide product complex was determined spectrophotometrically and its percentage of the total cobalt in the 2 ml sample calculated. A 2 ml sample was removed from the buffered solutions and similarly tested every 48 hours.

Results

$[2.11,1]$ $[Co(en)_2PheOPheOC_6H_5]^{3+}$. Table 2.11,1

Degradation of $[Co(en)_2PhePheOC_6H_5]^{3+}$

Time[1] Hours	Percentage Peptide Complex[2] Buffer[3] pH 6.5	pH 5.1	pH 2.1
0	62	62	62
48	35	56	56
96	26	49	49
144	13	37/	45
192	-	32	38

$[2.11,2]$ $[Co(en)_2ProPheOC_6H_5]^{3+}$. Table 2.11,2

Degradation of $[Co(en)_2ProPheOC_6H_5]^{3+}$

Time[1] Hours	Percentage Peptide Complex[2] Buffer[3] pH 6.5	pH 5.1	pH 2.1
0	88	88	88
48	79	87	88
96	71	84	85
144	62	79	82
192	57	78	80

[2.11,3] $[Co(en)_2AlaPheOC_6H_5]^{3+}$. Table 2.11,3

Degradation of $[Co(en)_2AlaPheOC_6H_5]^{3+}$

Time[1] Hours	Percentage Peptide Complex[2] Buffer[3] pH 6.5	pH 5.1	pH 2.1
0	75	75	75
48	73	75	75
96	68	72	75
144	65	72	75
192	68	79	75

[2.11,4] $[Co(en)_2ProPhePheOC_6H_5]^{3+}$. Table 2.11,4

Degradation of $[Co(en)_2ProPhePheOC_6H_5]^{3+}$

Time[1] Hours	Percentage Peptide Complex[2] Buffer[3] pH 6.5	pH 5.1	pH 2.1
0	57	57	57
48	52	56	57
96	47	56	51
144	44	53	52
192	42	50	49

$\lfloor$2.11,5$\rfloor$ $\lfloor$Co(en)$_2$ProGlyGlyOC$_2$H$_5\rfloor^{3+}$. Table 2.11,5

Degradation of $\lfloor$Co(en)$_2$ProGlyGlyOC$_6$H$_5\rfloor^{3+}$

Time[1] Hours	Percentage Peptide Complex[2] Buffer[3]		
	pH 6.5	pH 5.1	pH 2.1
0	92	92	92
48	92	92	92
96	91	90	92
144	90	90	92
192	88	90	92

1. Elapsed from first sample tested time 0 hours.
2. Percentage of the total amount of cobalt ion in the sample in the form of peptide complex product.
3. pH at which the sample had been maintained.

/~2.12,0_7 Preparation of Phenyalanylphenylalanine Benzyl Ester Cobalt(III) Complex and its Immediate Reduction to Avoid Auto Hydrolysis of the Peptide

Phenalamine methyl ester Triflouromethane sulfonate; (0.80g, 1.0 m Mole), was dissolved in 10 ml of anhydrous methanol, treated with a 10 ml anhydrous methanol solution of phenylalanine benzyl ester p-toluenesulfonate (0.45g, 1.0 m Mole) and one molar equivalent of triethylamine. The mixture was stirred gently for 10 minutes, then a calculated volume of triethylamine was added. The volume of triethylamine was such that if an equal volume of water was added to the reaction mixture the pH of the mixture would be approximately 6.0. A 1 ml sample, Sample 1, of the reaction mixture was removed five minutes after addition of the triethylamine. This sample was diluted to 5 ml with water sorbed onto a SP-C25-120 Sephadex test column, eluted with 0.2 M HCl and the amount of peptide complex product determined spectrophotometrically. This was expressed as a percentage of the total amount of cobalt in the sample, thus giving the 'Indicated Coupling Yield'. The main reaction mixture was treated with 5 ml of 1.0 M HCl and stripped to dryness under reduced pressure then taken up in 20 ml of 0.2 M HCl and shaken vigorously with 20 ml of 2% Zn/Hg amalgam. The product solution was then filtered off and

and loaded onto a preparative P_2 Gel Filtration Column and the peptide product eluted with distilled water, detected, and the product solution freeze dried as described in general method [2.8,0].

[2.12,1] Results

The Indicated Coupling Yield of Sample 1 was 100%. Freeze-drying the main solution and making allowance for the amount of the solution that was removed for testing, 92% of the phenyalanine complex initially added to the reaction mixture could be accounted for as the dipeptide benzyl ester product isolated (0.37g, 0.92 m Mole).

[2.13,0] Amino Acid Analysis of Some of the Peptide Esters Prepared Via Cobalt Chelates of the Amino Acids

A sample of the peptide ester (0.01g) was prepared for amino acid analysis by acid hydrolysis. The hydrolysis was achieved by heating the sample with 3.0 ml of 6N HCl, to 110°C, in a sealed glass tube for six hours. The seal was then broken and the acid evaporated.

Results

[2.13,1] $PhePheOC_6H_5$

No amino acids detected.

[2.13,2] $AlaPheOC_6H_5$

No Phenylalanine detected.

[2.13,3] $ProPheOC_6H_5$

No Phenylalanine detected.

[2.13,4] $ProPhePheOC_6H_5$

Pro 1.0 Phe 10.69 Ratio of products detected with respect to proline.

[2.13,5] $ProGlyGlyOC_2H_5$

Pro 1.0 Gly 12.24 Ratio of products detected with respect to proline.

[2.13,6] $AlaGlyGlyOC_2H_5$

Ala 1.0 Gly 1.79 Ratio of products detected with respect to alanine.

[2.13,7] $ProGlyGlyOC_2H_5$

Pro 1.0 Gly 12.13 Ratio of products detected with respect to proline.

[2,14.0] DISCUSSION AND CONCLUSION

One of the attractive features, as pointed out in the introduction to this study, in using cobalt chelates of the amino acids for polypeptide synthesis, was the possibility of using Ion Exchange techniques to purify and isolate the product from the condensation reaction mixture. A number of Cation Exchange Resins were reviewed in trial [2,1.0]. The initial flow rate of the CM Sepharose CL-6B resin was obviously inferior to the other resins under study, and on this basis this cation exchange system was dropped from any further consideration. The remaining three systems all demonstrated that they could separate the different cobalt III complexes from the mixture that was tested. The SP-C25-120 resin also may have given partial separation of optical isomers of the amino acid complexes $[Co(en)_2AlaO]^{2+}$. The carboxymethyl based resins performed the separation using a much milder change in the concentration of the eluting buffer, than the sulfopropyl based system. The Sephadex resin bed, whether CM or SP, tended to shrink during the development of the chromatogram, thus reducing the flow rate. Thus on the basis of these trials Whatman Carboxymethyl CM 32 Cellulose Cationic Exchange resin was used to separate most of the condensation reaction mixtures.

Many of the peptides prepared had phenylalanine benzyl ester as the carbonyl terminal amino acid. The

phenylalanine benzyl ester was prepared by the esterification, by benzyl alcohol, of phenylalanine. Other carbonyl terminus amino acid esters were obtained from commercial sources (refer to appendix [3.1,0]).

The results presented in table 2.3,1 show the condensation trials carried out with a amino acid emthyl ester cobalt complex and phenylalanine benzyl ester, in methanol under anhydrous conditions. From the 'Indicated Coupling Yield' data presented in this table it can be seen that the amount of dipeptide complex produced varied greatly from trial to trial even when the apparent conditions of each trial were the same. It can be noted that in the case of trial 3, producing phenylalanylphenylalanine benzyl ester complex, the coupling appeared to be carried out to completion within the detection limits of the test. For trial 6 however, virtually no condensation to form the same product took place. For the trials 1-7 in table 2.3,1 in most cases less than 50% of the dipeptide product formed could be isolated. The product appeared to be degrading during the time that the product was sorbed onto the Ion Exchange Resin and its elution. This degradation process was often visible to the naked eye as a gradual smearing of the dipeptide product band as the separation of the condensation reaction mixture proceeded.
Trials 8, 9 and 10 suggested that the dipeptide complexes of prolinylphenylalanine benzyl ester and alanylphenylalanine benzyl ester are much more stable

under the conditions which the Ion Exchange Separation was conducted.

Using dimethylsulfoxide as the solvent for the condensation of the phenylalanine methyl ester cobalt(III) complex, the phenylalanine benzyl ester produced the dipeptide with the same variability in reaction yield as was noted for the same reaction in methanol solvent. The dipeptide prepared in the dimethylsulfoxide also showed the susceptability to degrade during its isolation from the reaction mixture, as has already been noted above and can be seen by comparing the 'Indicated Coupling Yield' and the 'Isolated Yield' columns in table 2.4,1.

That the condensation could be carried out without first isolating the amino acid methyl ester complex in the crystalline form is demonstrated in the trials results presented in table 2.5,1. A great variation in the product yield could be noted from these results also, and the tendency for the dipeptide complex products not containing phenylalanine to be isolated in greater yeilds than those containing phenylalanine can also be observed in these results.

With the losses of product noted in Chapter 1 section [1.23,0] for the isolation of the amino acid methyl ester complexes, the <u>in situ</u> generation and condensation of the amino acid methyl ester complexes must result in an enhancement in the overall efficiency of the scheme.

This technique was thus adopted as a standard approach.

As has been explained in the Results Section, the condensation yields had been variable. Although, as in trials 1, 1 and 3 of tables 2.4,1, 2.4,1 and 2.3,1 respectively the very high coupling yields, as required for a practical peptide synthetic scheme, were demonstrated, these results could not be reliably repeated. The treatment of the reaction mixture, formed by the condensation of phenylalanine methyl ester complex and phenylalanine benzyl ester, with triethylamine was conducted in trial [2.6,0] and the amount of dipeptide product monitored at points during the addition. From the 'Indicated Coupling Yields' presented in table 2.6,1 and calculated from the samplings 1-4, taken after each addition of triethylamine, the best coupling yield was observed in a mixture that, when an equal volume of water was added, had a pH of approximately 6. The 'General Method' outlined in section [2.3,0] was altered to include the addition of triethylamine, such that the pH conditions discussed above were realised. The results presented in table 2.7,1 show a higher uniformity of coupling yields for a range of condensations, benefiting from the moderation of conditions by triethylamine addition. Trials 1 and 2, table 2.7,1, involving the condensation of phenylalanine methyl ester complex and phenylalanine benzyl ester, indicate that repeatable condensation yields of a very high value can be achieved. Trial 5, in the same table, suggests that the condensation

yield for alanine methyl ester complex and phenylalanine benzyl ester may be increased with the coupling conditions being regulated using triethylamine. This can be seen by comparing the results of trial 5 table 2.7,1, 100%, and condensation in unregulated conditions shown by the results of trial 3 table 2.5,1, 80%, and trial 10 table 2.3,1, 80%. As already noted in the discussion prior to section [2.6,0], the addition of excessive amounts of triethylamine can lead to the formation of a polymeric cobalt ion complex, or to a decrease in the yield of condensation product as indicated by the results of section 2.6,0.

Buckingham and Dekkers (26) have suggested that the peptide can be isolated by destroying the complex, using a variety of reducing agents. The reducing agents investigated by Buckingham and Dekkers and their comments on their use are reproduced below.

Table 2.14,1

Reducing Agents Investigated by Buckingham and Dekkers (26) for the Removal of Peptide from Co(III) Complex[1]

Reagent	Comment
Chromous Chloride	Oxygen free conditions required Rapid Reaction

continued ...

Table 2.14,1 continued...

Reagent	Comment
Lithium Aluminium Hydride	Reduction complete in 5 minutes
Sodium Borhydride	Reduction complete in 5 minutes
Lithium tri-(t-butony)-Alaminium Hydride	Reduction complete in 5 minutes
Sodium Thiosulphate	Very slow reduction Ca. 20 hours
Sodium Cyanide	Rapid reduction. Handling problems
Ammonium Sulphide	Reduction complete after 30 minutes at 40°
Hydrogen Sulphide	Reduction complete after 30 minutes at 40°
Vanadous Sulphate	Reduction complete in 5 minutes
Zn/HCl	Reduction complete after 18 hours

1. Complex 0.05 M, reducing reagent 50% excess.

Buckingham and Dekkers adopted $NaBH_4$ or VSO_4 as a standard reducing agent for this purpose. The peptide and ethylenediamine products from the reduction were then separated from other contaminants by passing the reduction mixture down a Na^+ form cation exchange resin. Co(II) and V(IV) ions were sorbed onto the resin and the peptide and ethylenediamine were eluted at the solvent point. The peptide and ethylenediamine were then separated by thin layer chromatography. While this scheme is satisfactory

for a small scale synthesis, this study investigated the use of Gel Filtration techniques to accomplish the required separation in one step.

Reduction of the peptide cobalt(III) complexes with vanadous sulphate solutions resulted in a deep green solution. This solution when passed down a Gel Filtration column yielded the free peptide if the column was long enough. Using a column of insufficient length resulted in the peptide being contaminated with a blue colour as in trials 2 and 5 reported in table 2.8,1. Using this Gel Filtration Resin and the column described in section [2.8,0] and appendix [3.2,0], a dipeptide such as that in trial 1, in table 2.8,1 $PhePheOC_6H_5$ followed very closely behind the coloured band containing the metal ions Co(II) and V(IV). A much greater separation was achieved when a tripeptide such as that in trials 3 and 4 was involved, that is $ProGlyGlyOC_2H_5$ and $ProPhePheC_6H_5$ respectively. This technique should become even more successful in separating the peptide from the other products of the reduction reaction as the size of the peptide involved increases. The metal ions produced by the reduction reaction, however, tended to bind irreversably to the Gel Filtration Resin and gradually caused the resin's performance to deteriorate. In an attempt

to avoid this deterioration by contamination of the P_2 resin, sodium borohydride was tried as the reducing agent. Prolinylglycylglycine ethyl ester complex, from which the peptide had been successfully separated from the other products of the reduction, was thus reduced as reported in table 2.8,1 trial 3. Using sodium borohydride, however, resulted in an incomplete isolation of this peptide from other contaminants. Further studies by Buckingham (22) also suggested that reduction of the complex using vanadous sulphate or tithium aluminium borohydride may cause racemisation of the peptide.

The reduction of the peptide complex without introduction of contaminants that may be difficult to remove, or that would reduce the performance of a Gel Filtration system, were avoided by using 2% zinc mercury amalgam. The results of such reductions and peptide separations are reported in table 2.9,1. Trial 1 reported in this table involved the isolation of prolinylphenylalanine benzyl ester, a peptide that had not been reduced using vanadous sulphate.

Of the techniques used to reduce the peptide cobalt(III) complexes, zinc amalgam appeared the most compatable with subsequent isolation of the peptide by Gel Filtration. For the tripeptide isolations using

Gel Filtration, good recovery of the peptide as required in a Peptide Synthetic Scheme was achieved. Efficient recovery of dipeptides may, however, pose a problem.

For some peptide complex products, such as $[Co(en)_2PhePheOC_6H_5]^{3+}$, $[Co(en)_2ProPheOC_6H_5]^{3+}$, $[Co(en)_2AlaPheOC_6H_5]^{3+}$ and $[Co(en)_2ProPhePheOC_6H_5]^{3+}$ the 'Isolated Yield' was significantly smaller than one would expect from the predictions of the 'Indicated Coupling Yield'. As has been noted [2.10,0], peptide product was seen to degrade from a 3+ charged species to a 2+ charged species, on an ion exchange resin, when being eluted with pH 5.1 pyridinium acetate buffer over a period of 10 hours. It has also already been noted that when $[Co(en)_2PhePheOC_6H_5]^{3+}$ complex sample, that had been determined to be one hundred percent 3+ species, was dissolved in a little hot water and precipitation was attempted to be induced by the addition of sodium iodide, only 2+ species could be detected. On other occassions, such as during the stability trials [2,11,0], the decrease in the amount of $[Co(en)_2PhePheOC_6H_5]^{3+}$ table 2.3,1, table 2.4,1, table 2.5,1, table 2.7,1: $[Co(en)_2ProPheOC_6H_5]^{3+}$ table 2.3,1, table 2.7,1: $[Co(en)_2AlaPheOC_6H_5]^{3+}$ table 2.5,1, table 2.7,1: $[Co(en)_2ProPheOC_6H_5]^{3+}$

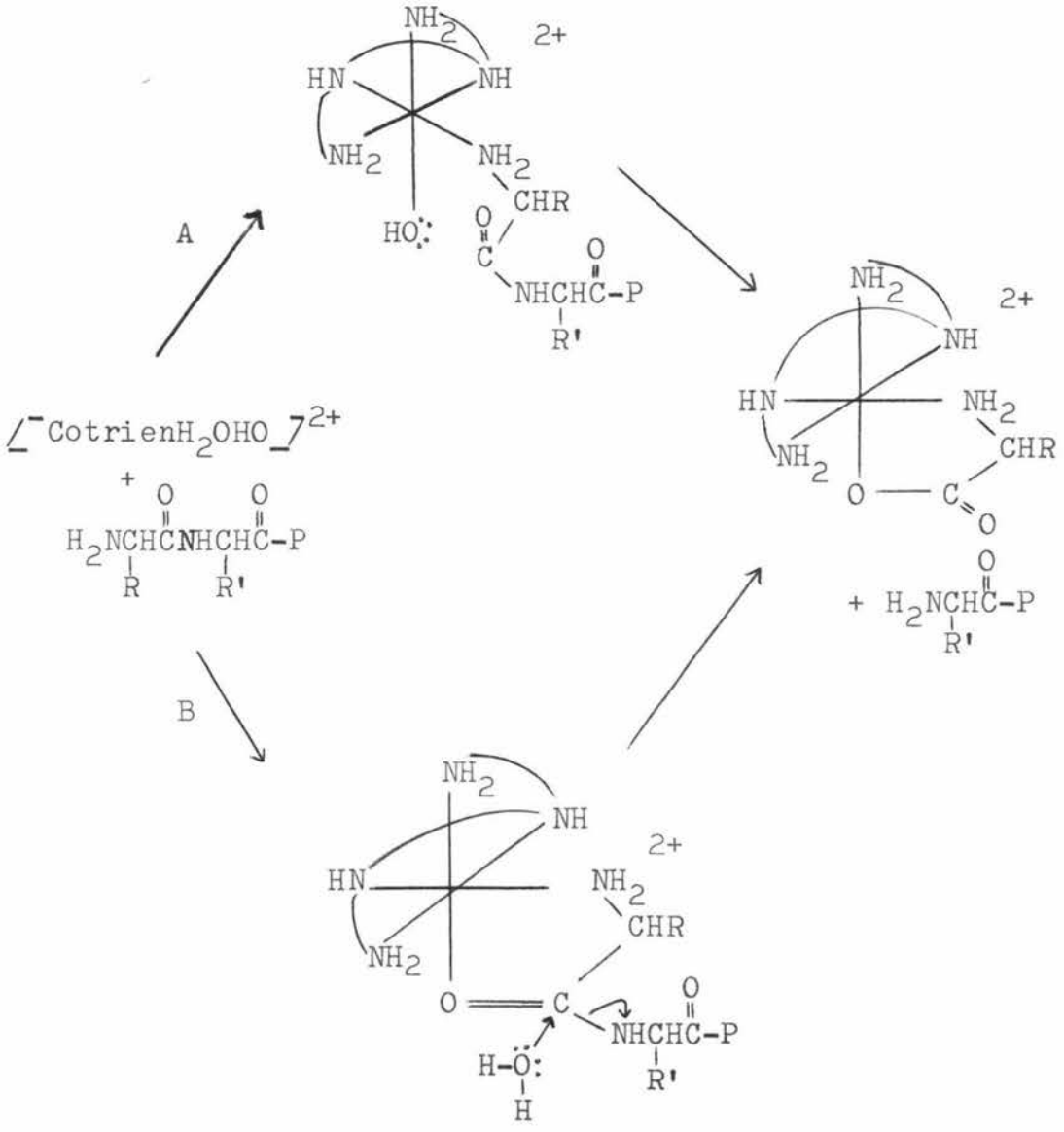
table 2.7,1, 3+ peptide complex species was accompanied by an increase in the $Co(en)_3^{3+}$ species. From the stability trials, [2.11,0] and the results presented in tables 2.11,1 - 5, the trial conducted at pH 6.5 for all peptide complex products showed the greatest loss of product. The peptide complex products maintained at pH 2.1 were generally shown to be the more stable, and in some cases, such as for $[Co(en)_2ProGlyGlyOC_6H_5]^{3+}$ table 2.11,5, no deterioration of the peptide complex could be detected.

By comparing the results for the trials conducted at pH 6.5 for the dipeptide complexes $[Co(en)_2PhePheOC_6H_5]^{3+}$ table 2.11,1 and the other dipeptide trials at pH 6.5, some complexes are far more easily degraded than others, $[Co(en)_2ProPheOC_6H_5]^{3+}$ being particularly susceptable. Comparing $[Co(en)_2ProPheOC_6H_5]^{3+}$ results table 2.11,2 and $[Co(en)_2ProPhePheOC_6H_5]^{3+}$ table 2.11,4 suggests that the tripeptide is significantly more stable than the dipeptide having the same n-terminal and n-terminal adjacent amino acid sequence. The lack of stability of peptide complexes containing phenylalanine is again noted from the results tabled for $[Co(en)_2ProPhePheOC_6H_5]^{3+}$ table 2.11,4 and $[Co(en)_2ProGlyGlyOC_2H_5]^{3+}$ table 2.11,5.

Buckingham et al (34) have shown that the n-terminal amino acid of a peptide chain may be selectively removed

from the chain or an amino acid ester hydrolysed by first forming the complex with $[^{-}CotrienH_2OHO]^{2+}$. Two possible mechanisms were proposed:

Fig. 2.14,1



It was noted in this reference that "glycine ester and glycinamide bonds are rapidly hydrolyzed at room temperature over the pH range 6.5-7.5 in concentrated aqueous or non-aqueous solutions," and also "using

glycinamide as the substrate, hydrolysis rates (at 40°C) were measured in buffered solutions for a series of pH values. The crude pH profile passed through a maximum at pH 6.4-6.6. The similarity between the glycine ester or glycinamide complex formed as the intermediate in mechanism "A" fig. 2,15,1 and the peptide complexes prepared in this study is noted, and also the apparent pH dependence of their degradation rate. Similar behaviour is also recorded for the hydroxoaquobis-(ethylenediamine)cobalt(III) ion by Buckingham and Collman (16).

It would, however, be unwise to suggest at this stage, that hydrolysis of the peptide complexes at the N-terminal peptide bond was occurring. The gradual change of the 3+ peptide complex products to 2+ species, during the ion exchange separation of the reaction production of $Co(en)_3^{3+}$ observed during the stability trials, section [2.11,0], cannot be explained by simple peptide bond hydrolysis. It was not the intention of this study to identify the mechanism by which some peptide complexes were being degraded. This phenomenon could, however, restrict the yields attainable for certain particularly sensitive amino acid sequences when complexed to cobalt(III) ions.

The most sensitive sequence identified in the stability trials, section [2.11,0] was [$Co(en)_2PhePheOC_6H_5$].

It may be possible to prepare these unstable sequences, without severe loss of product, by immediately removing the peptide from the complex following its synthesis. That significantly higher yields could be obtained using this approach was demonstrated in section 2.12,0 with the synthesis of [Co(en)$_2$PhePheOC$_6$H$_5$], its immediate reduction using zinc mercury amalgam without first isolating the peptide complex, then isolation of the dipeptide.

Samples of the peptides prepared were subjected to Amino Acid Analysis, section [1.13,0]. The results of these analysis show that only in one case [12.13,6] were the expected ratios even approached. The ratios reported for [2.13,4] ProPhePheOC$_6$H$_5$, [2.13,5] ProGlyGlyOC$_2$H$_5$, [2.13,7] ProGlyGlyOC$_2$H$_5$ show a greatly reduced amount of the N-terminal amino acid than expected. Since the conditions for hydrolysis of the peptides is not expected to produce any degradation of the amino acids, the digestion or alteration of the amino acid ratios must have occurred at some stage during the preparation, isolation, or purification of the peptide. Once again phenylalanine seems particularly affected, no trace of the amino acid being found in PhePheOC$_6$H$_5$ [section 2.13,1] or AlaPheOC$_6$H$_5$ [2.13,2].

This study has produced, on a reasonable scale, the

condensation product of a cobalt amino acid complex and an amino acid ester or peptide ester. This condensation product had demonstrated the 3+ ion exchange behaviour of the peptide complex product that was expected. The product complex was reduced and a product giving a positive test to ninhydrin, but not ethylenediamine, had been isolated. At some stage during the process, however, some of the amino acids were modified to such an extent that they were rendered unrecognisable to an amino acid analyser. Such modification of the amino acids cannot be tolerated in a peptide synthesis scheme.

[2.15,0] SUMMARY

This study has demonstrated the production of amino-acidobis(ethylenediamine)cobalt(III) complex, with acceptable efficiency and with due regard to using cheap starting materials on a scale necessary to support a preparative scheme for peptide synthesis. Experience was gained in the chemical characteristics of amino-acidobis(ethylenediame)cobalt(III) complexes, recognition, optomisation of the preparative conditions, and isolation of these complexes for further processing. Problems with the solubility of some amino acids in some solvents were encountered. It was demonstrated that the efficiency and scale of the preparative reactions could be maintained by tailoring the solvent conditions to suit the solubility characteristics of the amino acids being complexed to the cobalt(III) ion ligand system.

Two methods of methylating the amino acid carbonyl group in the amino-acidobis(ethylenediamine)cobalt(III) complexes were investigated. These methods were shown to efficiently produce the active methylated complexes which, if the acid condition of the product was monitored, could easily and with very high yield condense with an amino acid or peptide ester. Conditions necessary to achieve a reliably high coupling yield were investigated and the products of the coupling

reaction monitored. Problems with the stability and integrity of these products were identified. Problems with the isolation of the peptide from the cobalt(III) complex were encountered and in some cases possible solutions demonstrated.

One major feature to be noted from this study was the instability and loss of integrity of some amino acids, or sequences of amino acids, under reaction conditions. Such phenomenon had not been reported in the literature surveyed. Future workers in this area will have to identify other amino acids or sequences which may require special treatment. Conditions will have to be found with these sensitive amino acid sequences to carry out the required reactions while avoiding damage to the growing peptide or its amino acid units.

The closer monitoring of pH conditions during condensation and purification, the use of new Ion Exchange Resins such as Reconstituted Viscose or Sulphated Viscose which have the potential for very rapid separation of the reaction products; conditions which will allow the removal of the peptide from the complex without modifying the amino acid chain, and the possible tagging onto the carbonyl terminal of the peptide chain of a group which will allow the easy detection of the peptide produce; these techniques may allow the use of cobalt(III) chelates of amino acids to be used as a general synthetic technique for the preparation of

polypeptides. To this end, amino acid side chain protective modification schemes may have to be developed that are suited to the conditions encountered using this approach. Just as some amino acids have proven to be more sensitive to some reaction conditions than others, so the possibility for racemisation at the asymetric centres still exists and will have to be investigated. Some amino acids or amino acid sequences may prove to be susceptable to such optical rearrangement. Problems encountered with other techniques may still affect the use of cobalt(III) chelates - sequence dependent solubility problems, detection and truncated product formation and their detection etc. Cobalt(III) chelates have demonstrated a reasonable flexibility of application. The features outlined in the introduction that make them attractive as a synthetic peptide preparative scheme still command attention, and their adaption to a highly automated approach such as Solid Phase Synthesis is not beyond the realms of reasonable possibility.

[3.1,0] APPENDIX 1

Materials: Grade and Preparation.

(i) Analar grade reagents and solvents were used in all trials detailed in this thesis, without further purification unless otherwise stated.

(ii) Solid materials prepared in trials detailed in this thesis that were required to be anhydrous and anhydrous solid reagents prepared from the analar grade commercially available material, were placed over P_2O_5 under an oil pump vacuum and heated at 60°C for 24 hours.

(iii) Anhydrous liquids required for the trials detailed in this thesis were prepared from the commercially available analar grade material, and treated immediately prior to use.

Diethyl Ether: Dried by placing over calcium chloride pellets for four days, decanting off, and treated with calcium hydride for two days, then placed over dried molecular sievies 3Å for storage in capped winchesters.

Dimethylsulfoxide: Redistilled under reduced pressure, then placed over dried molecular sieves 3Å for storage in capped winchesters.

Methanol: Redistilled, treated with calcium hydride, redistilled from the calcium hydride and stored over dried molecular sieves 3Å in oven dried bottles sealed by a rubber sceptum.

Triethylamine: Stored over dried molecular sieves 3Å, in an oven dried reagent bottle, sealed with a rubber sceptum.
Trimethylphosphate: Stored over dried molecular sieves 3Å, in an oven dried reagent bottle, sealed with a rubber sceptum.
Methyltriflouromethane Sulphonic Acid: Was stored in the flasks it was shipped in, in a dessicator over blue silica gel crystals at 4°C. The cap was sealed around the bottom with paraffin wax.

(iv) Dried Reagent Vessels: Were placed in an oven at 100°C for 30 minutes. The reaction glassware was assembled hot and allowed to cool with atmospheric moisture being excluded by plugging drying tubes with glass wool and packing blue silica crystals in all unstoppered inlets.

(v) Dried Molecular Sieves 3Å: were prepared by heating B.D.H 3Å Molecular Sieves in an oven to 110°C for 48 hours.

(vi) Dispensing Anhydrous Liquids from Containers Sealed by Rubber Sceptums: This was achieved using a hypodermic syringe filled to the volume of liquid that was required with dry nitrogen gas. The reagent bottle was inverted, the rubber sceptum pierced by the hypodermic syringe and the dry nitrogen gas introduced into the reagent bottle. The required amount of liquid was forced out of the reagent bottle into the syringe by the increased pressure within the bottle.

(vii) Dispensing Methyltriflouromethane Sulfonic Acid: The desicator containing the methyltriflouromethane sulfonic acid flask was allowed to come to room temperature, the paraffin wax seal on the flask broken and the liquid removed using a glass bodied hypodermic syringe. The seal was replaced and the flask returned to dry cool conditions as soon as possible.

(viii) Handling Anhydrous Solids: This was done, as far as possible, under the dry nitrogen atmosphere in a Glove Bag.

(ix) Preparing "Ammoniated Methanol": Anhydrous methanol (200 ml) was saturated with ammonia gas, supplied from commercial sources, under pressure, through a scintered glass gas bubbler.

[3.2,0] APPENDIX 2 - Equipment

(i) 'Test Column': A glass column 12 mm I.P. x 95 mm, a No.1 scintered glass disc set in the bottom end, and the effluent flow controlled by a tap. The column was fitted at the top end with a standard "Quickfit" female adaptor. An eluting buffer reservoir could be mounted with an air-tight seal above the column, the buffer being gravity fed to the column.

(ii) 'Amino-acidobis(ethylenediamine)cobalt(III) Complex Preparative Column': A glass column 70 mm I.D. x 50 mm, a No.3 scintered glass disc set in the bottom end and the effluent flow controlled by a tap. The column was fitted at the top end with a standard "Quickfit" female adaptor. An eluting buffer reservoir could be mounted with an air-tight seal above the column, the buffer being gravity fed to the column.

(iii) 'Peptide Complex Preparative Column': A Pharmacia Solvent column 26 mm x 700 mm. The column effluent passed through two LKB UVFCORD II UV dectectors at wavelengths λ_{254} and 280 mm and a conductivity meter. The data from these instruments was recorded on a LKB 6 CHANNEL point recorder linked in pairs. The effluent was collected by a LKB ULTRORAL 7000 Fraction Collector. The complete system was maintained at $6^{o}C$.

(iv) 'Peptide Isolate Gel Filtration Preparative Column': A 20 mm x 700 mm Pharmacia column with the same support apparatus as described for the Peptide Complex Preparative Column.

(v) An eluting buffer gradient could be applied to the "Preparative Column" using a Pharmacia 9 M gradient mixer.

(vi) Scanning UV Visible spectra were run on a Unicam SP800 Ultraviolet Scanning Spectrophotometer

(vii) Solution volumes were reduced using Buchi Rotavapor-R Rotaryevaporator.

(viii) Solutions were freeze dried on a FTS Systems Inc Freeze Dryer.

(ix) Amino Acid Analysis was carried out on a Bechman 120C Amino Acid Analyser.

[3.3,0] APPENDIX 3

Abbreviations used for Amino Acids are those proposed by B IUPAC (37)

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine	Asn	Methionine	Met
Asparatic Acid	Asp	Phenylalanine	Phe
Cysteine	Cys	Proline	Pro
Glutamic Acid	Glu	3 Hydroxyproline	3-Hyp
Glutamine	Gln	Serine	Ser
Glycine	Gly	Threonine	Thr
Histidine	His	Tryptophan	Trp
Isolecicine	Ile	Tyrosine	Tyr
		Valine	Val

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