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A thesis entitled

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

submitted by Murray John Friar

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

April 1979

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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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,

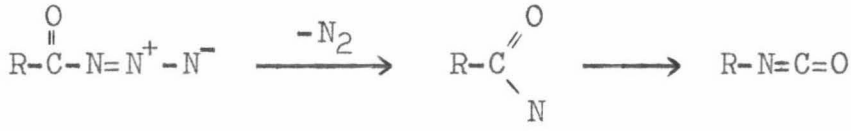
is an obvious requirement, but one that still limits the goals of protein synthesis. The interaction of these diverse requirements, in addition to the vigorous reaction conditions that are often needed to achieve the coupling of an amino acid to a peptide chain, are such that to date no general synthesis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

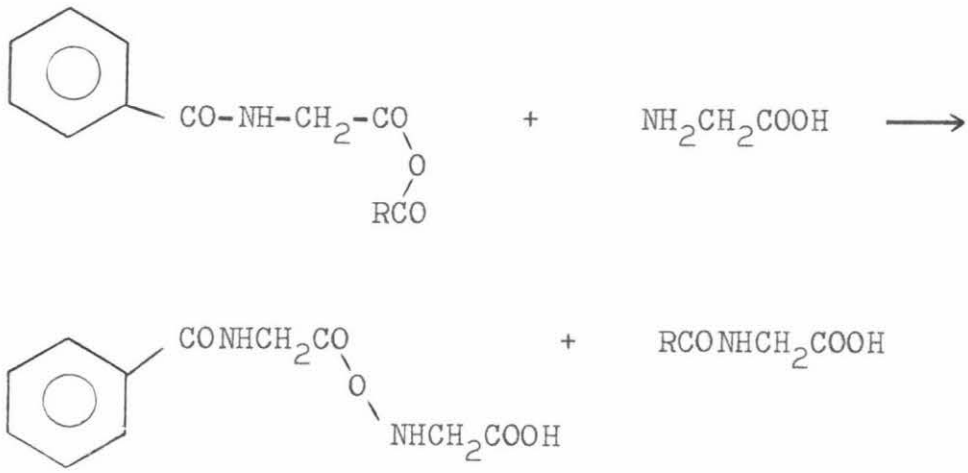
Fig. 0.0,1



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).

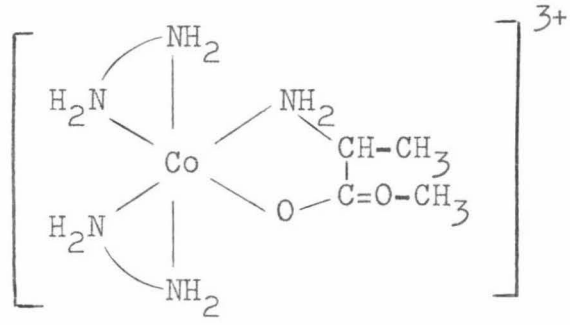
/~0.1,0_7 The use of cobalt(III) complexes of amino acids:

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)

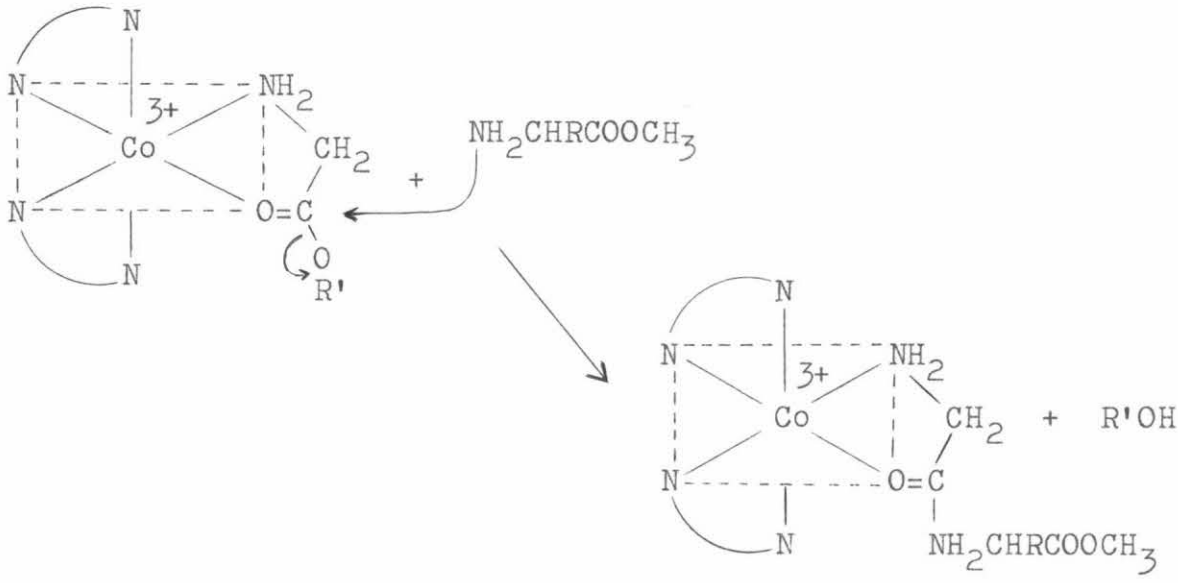
Complexes produced by D. Buckingham and J. Dekkers (26)

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)

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

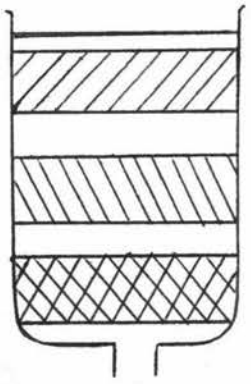
+ Unreacted $NH_2CHR'COOR''$ + (remaining Amino Acid Ester Complex)

(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.