Journal of Chromatography, 313 (1984) 93–127
Chromatographic Reviews
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHREV. 187

REVERSED-PHASE HIGH-PERFORMANCE ION-PAIR CHROMATOGRAPHY OF COBALT(III) COORDINATION COMPLEXES

DAVID A. BUCKINGHAM
Department of Chemistry, University of Otago, P.O. Box 56, Dunedin (New Zealand)

CONTENTS

1. INTRODUCTION

The use of high-performance chromatography (HPC) for the separation and identification of charged metal coordination complexes is still in its infancy. Studies using inert supports such as silica and non-polar solvents (HPLC) has been largely reserved for uncharged coordination complexes[1] but some charged complexes have been investigated, notably those of cobalt(III). Thus Warner and Legg[2] extended their separations of Co(III) amino acid complexes to preparative HPLC on silica but with only moderate success. Earlier, Grassini-Strazza and Polcaro[3] had shown that *cis*-, and *trans*-$[Co(en)_2Cl_2]^+$ and *cis*- and *trans*-$[Co(en)_2(OCOC_6H_5)_2]^+$ could be resolved on a Micropak-silica column using aqueous ammonium acetate–alcohol eluents. However underivatized silica lacks the discriminatory power for most purposes.

Valenty and Behnken[4] were the first to use the reversed-phase high-performance ion-pair chromatography (RP-HPIPC) technique when they separated some monoester-monocarboxylate and dicarboxylate derivatives of $Ru(bipy)_3^{2+}$ using $CH_3SO_3^-$ or $CH_3(CH_2)_6OSO_3^-$ as ion-pair reagents in aqueous tetrahydrofuran. Since our own work began, Isied and co-workers[5,6] have used Radial Pak C_{18} cartridges (10 μm, Waters) to separate a variety of small peptides containing $Co(NH_3)_5^{3+}$ bound to the carboxyl terminus.

We and Searle[7] have for many years used ion-exchange chromatography (IEC) for the separation of isomeric charged cobalt(III) complexes. The popularity of this

0378-4355/84/$10.50

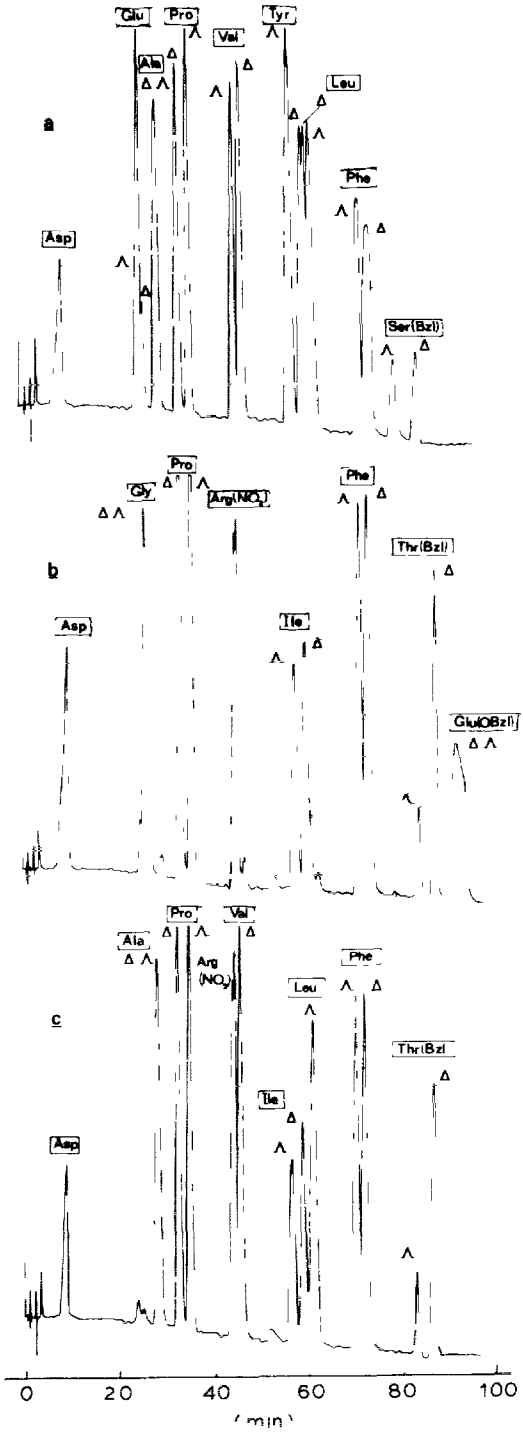


Fig 1 Elution profiles for mixtures of Δ,Λ-$[Co(en)_2AA]I_2$ complexes [AA = Asp, Glu, Gly, Ala, Pro, $Arg(NO_2)$, Val, Tyr, Ile, Leu, Phe Ser(Bzl), Thr(Bzl), Glu(OBzl)], (a) 2607 nmol, 50 μl, (b) 2347, 50, (c) 2424, 50, aqueous methanol gradient, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 cm^3 min^{-1}, a u f s, 0 01, λ = 480 nm

TABLE 1

COMPARISON OF CAPACITY FACTORS (k') FOR SOME $[Co(en)_2AA]A^{2+}$ COMPLEXES USING DIFFERENT PAIRING ANIONS

pH = 3 5, aqueous methanol eluent

$[Co(en)_2AA]^{2+}$ complex, AA =	*Eluent % methanol*	*Ion pairing anion*				
		10 mM	*25 mM*			
		HSA^-	*TSA^-*	*CSA^-*	*$As_2\text{-}tart_2^{2-}$*	*$Sb_2\text{-}tart_2^{2-}$*
ΔΛ Gly	2 85	–	1 36	3 43	0 19	0 28
ΔΛ Ala	2 85	3 03	1 42	3 63	0 22	0 30
Λ Pro	0 95	7 02	5 08	6 64	0 40	0 93
Δ Pro	0 95	5 29	3 70	4 83		0 44
Λ Val	2 85	15 44	7 22	13 91	0 56	0 86
Δ Val	2 85	19 07	9 41	15 38		
Λ Leu	2 85	–	34 00	–	0 76	1 95
Δ Leu	2 85		29 30			1 39
Λ Phe	14 25	20 45	8 39	22 12	2 28	6 79
Δ Phe	14 25		10 06		2 76	8 93

method is now almost universal and most geometric, optical, and configurational isomers can be separated using the appropriate eluent and Amberlite, Sephadex, or Dowex based ion-exchangers However, in most cases, the times required for separation are long (often many hours) and the separations often result in rather broad bands In our experience the transfer to high-pressure chromatography (HPIEC) has given only marginal improvement with recycling often being required to obtain a complete separation

However, the reversed-phase approach combined with ion pairing of the charged complexes by hydrophobic anions in solution makes the reversed-phase high-performance ion-pair chromatographic technique (RP-HPIPC) both fast and discriminating Our aim was to find and develop a rapid analytical method for identifying, and accurately estimating, the products of various spontaneous and induced reactions of cobalt(III) complexes This lecture describes some of these experiments Some of the results have been published[8,9] but many are new

2 SEPARATIONS OF $[Co(en)_2(AA)]^{+,2+}$ (AA = AMINO ACIDO) COMPLEXES

Fig 1 shows the usefulness of RP-HPIPC for the separation, and resolution of diastereoisomers, of several Δ,Λ-$[Co(en)_2(AA)]^{+,2+}$ complex ions Separations were achieved within 100 min of injection on a 30-cm C_{18} μBondapak column using a water–methanol gradient and 25 m*M* toluenesulfonate at pH 3 5 as the ion-pair reagent Detection was by λ = 480 nm absorption The chromatograms show sharp (*i e* narrow) bands, excellent resolution of diastereoisomeric pairs [*i e* $\Delta(S)$, $\Lambda(S)$], and complete separation of the different AA complexes Different ion-pair reagents give different results, *c f* Table 1, with the retention order increasing $As_2\text{-}tart_2^{2-}$ < $Sb_2\text{-}tart_2^{2-}$ < toluenesulfonate < camphorsulfonate < hexanesulfonate Inorganic ions such as SO_4^{2-} and HPO_4^{2-} give little or no retention despite their excellent

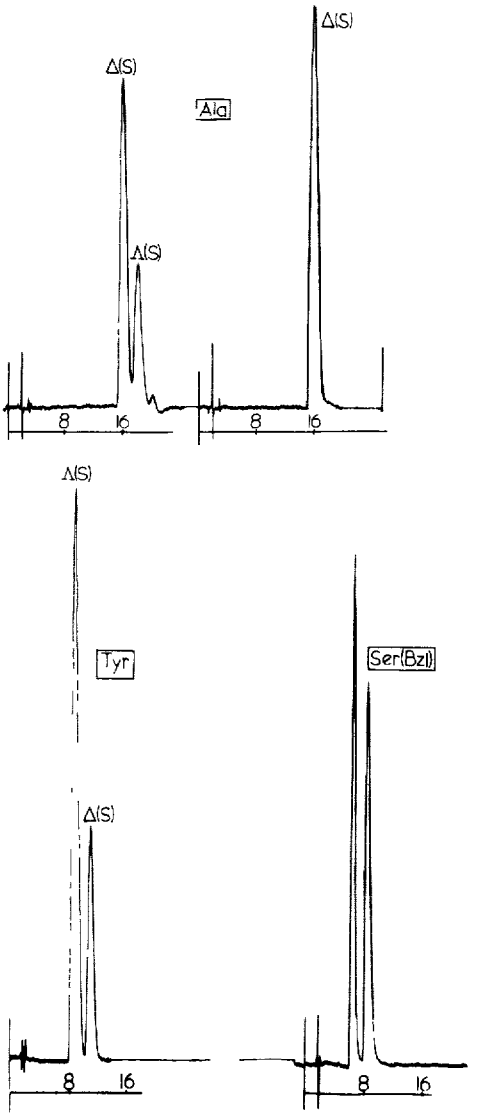


Fig 2 Separations of $\Delta(S)$-, $\Lambda(S)$-$[Co(en)_2Ala]I_2$ and $[Co(en)_2Tyr]I_2$ using 25 mM $(CH_3O)_2PO_2^-$, pH 3 4, aqueous methanol, λ = 480 nm

ion-pairing properties with cobalt(III) cationic complexes[7] The asymmetric anions Sb_2-$tart_2^{2-}$, As_2-$tart_2^{2-}$, and camphorsulfonate fail to resolve the $\Delta\Lambda$-$[Co(en)_2Gly)]^{2+}$ enantiomers and the $\Delta\Lambda$-$[Co(en)_2(Ala)]^{2+}$ diastereomers and in general give no better resolution of $\Delta\Lambda$-$[Co(en)_2(AA)]^{2+}$ diastereomers than do the achiral anions tosylate and hexanesulfonate Although the latter two ion-pair reagents fail to resolve Δ,Λ-$[Co(en)_2(Ala)]^{2+}$ it is resolved using 25 mM toluene phosphate at pH 3 5, Fig 2 The latter ion-pair reagent, $CH_3C_6H_5OPO_3H^-$, together with $CH_3OPO_3H^-$ and $(CH_3O)_2PO_2^-$ give excellent resolutions of diastereoisomeric

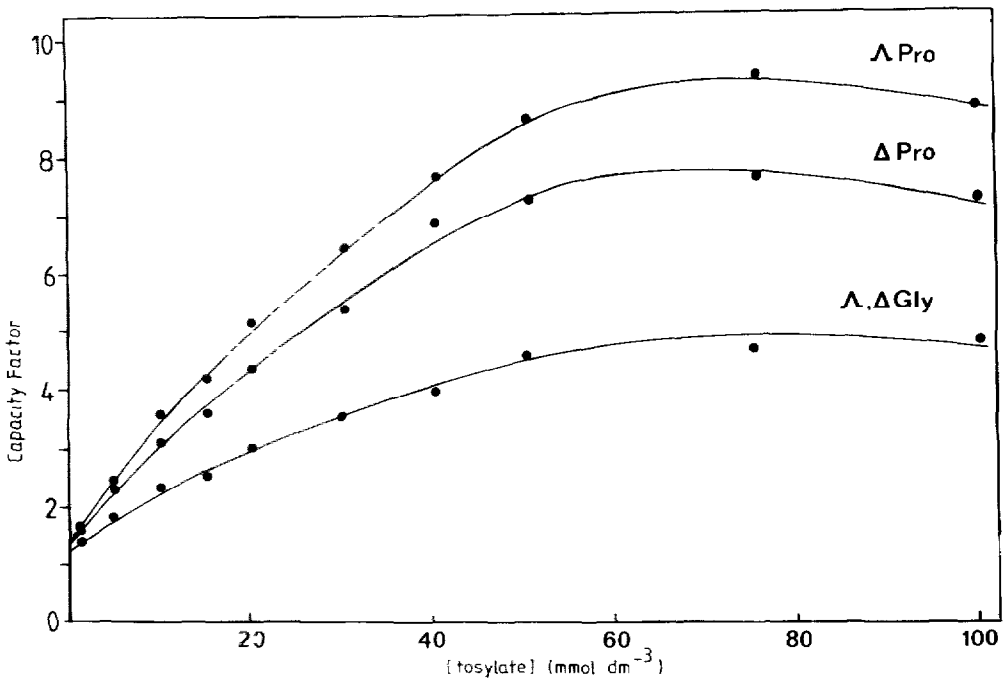


Fig 3 Variation of retention time (t_R) with toluenesulfonate concentration for $\Lambda(S)$-, $\Delta(S)$-$[Co(en)_2Pro]^{2+}$ and $\Delta\Lambda$-$[Co(en)_2Gly]^{2+}$ ions (2 5% aqueous methanol), λ = 480 nm

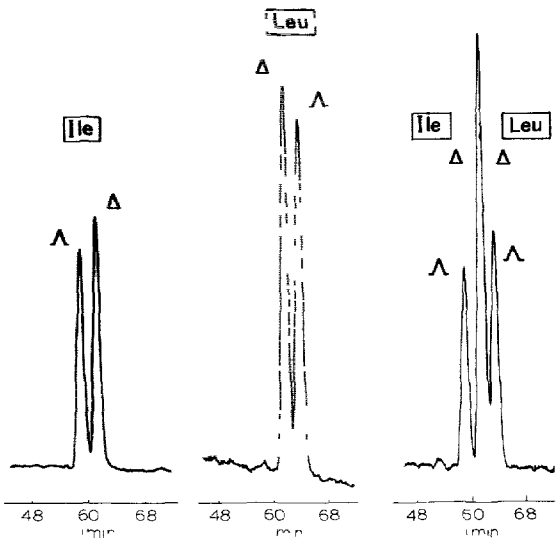


Fig 4 Elution profiles for Δ,Λ-$[Co(en)_2Ile]I_2$ and Δ,Λ-$[Co(en)_2Leu]I_2$ complexes, aqueous methanol gradient, 25 mM toluenesulfonate (pH 3 5), λ = 480 nm

systems and in this respect parallel the property of phosphate at pH ~ 6 8 in IEC However, much of our early work was with tosylate, $CH_3C_6H_5SO_3^-$, which is better than hexanesulfonate in this respect One advantage of tosylate is that it is inexpensive and easily recrystallised and although not transparent in the UV (190–250 nm) this property is often not required since cobalt(III) complexes can be easily detected in the visible (450–520 nm, extinction coefficients 50–200 M^{-1} cm^{-1})

The retention time varies with the concentration of the ion-pair reagent and some typical plots using tosyiate are given in Fig 3 Although higher concentrations

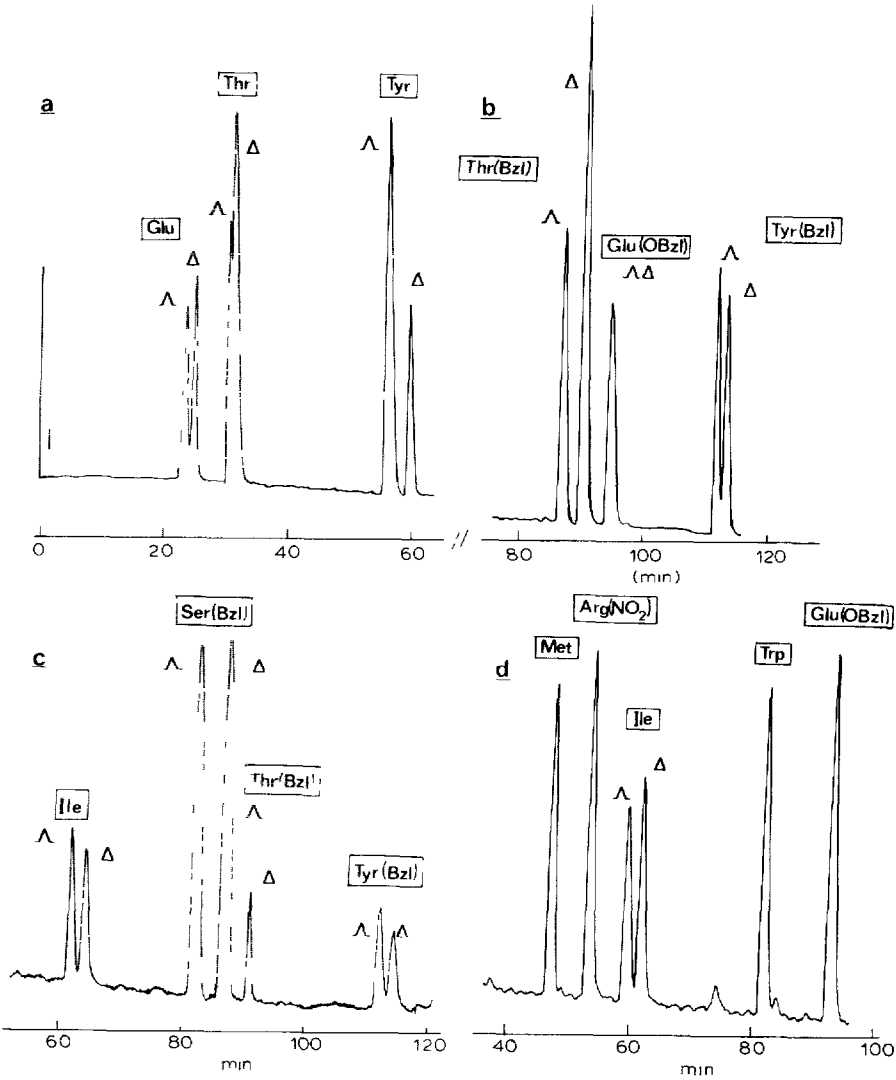


Fig 5 Elution profiles for mixtures of Δ,Λ-$[Co(en)_2AA]I_2$ [AA = Glu, Thr, Tyr, Thr(Bzl), Glu(OBzl), Tyr(Bzl), Ile, Ser(Bzl), Met, Arg(NO_2), Trp] (a) 1856 nmol (20 μl), (b) 2133 (5), (c) 480 (15), (d) 800 (25), aqueous methanol gradient, 25 mM toluenesulfonate (pH 3 5), flow-rate, 2 cm^3 min^{-1}, a u f s 0 02, 0 01, λ = 480 nm

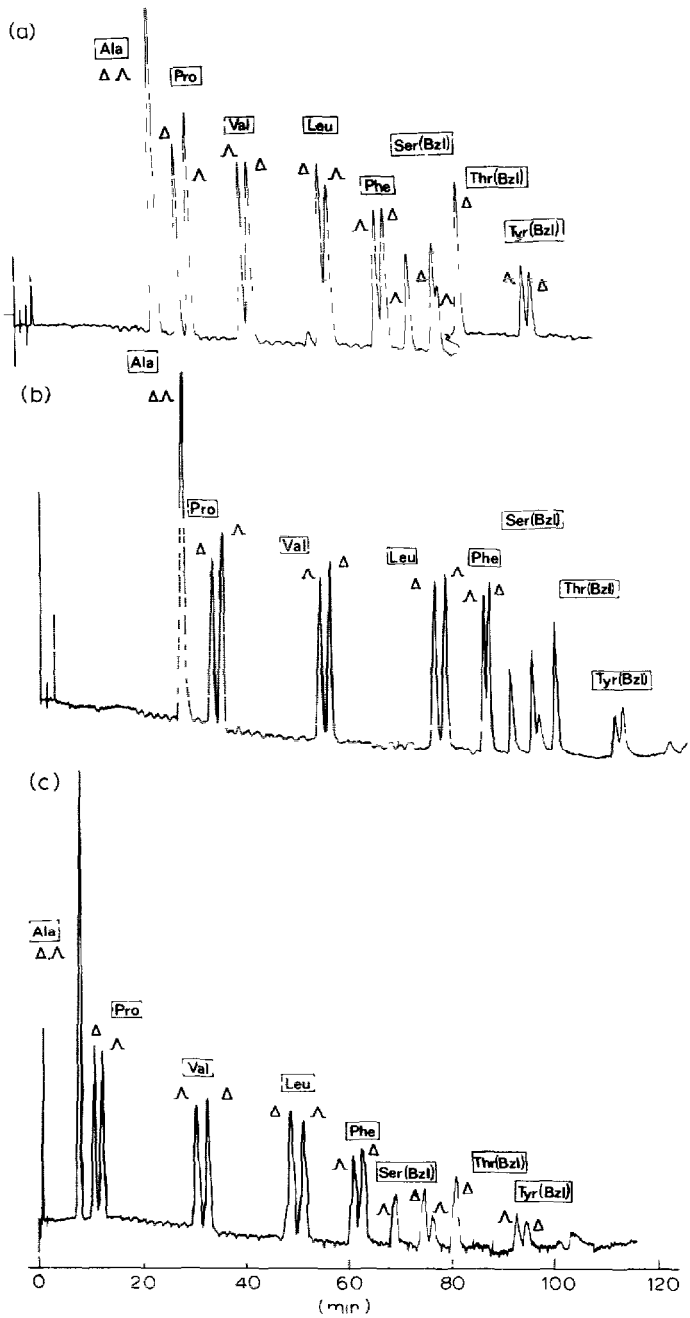


Fig 6 Elution profiles for Δ,Λ-[Co(en)$_2$AA] I_2 [AA = Ala, Pro, Val, Leu, Phe, Ser(Bzl), Thr(Bzl), Tyr(Bzl)], aqueous methanol gradient, 25 m*M* toluenesulfonate (pH 3 5) (a) 1000 nmol (20 μl), μBondapak C_{18}, 30-cm column, flow-rate, 2 0 cm^3 min^{-1}, (b) 1000 nmol (20 μl), C_{18} Radial Compression, 10-cm column, flow-rate 2 0 cm^3 min^{-} , 0 25% triethylamine (pH 3 5), (c) as in (b) but 3000 nmol (60 μl), flow-rate 8 0 cm^3 min^{-1}

increase retention and separation of the different 2+ complexes it also broadens the peaks and often does not give an improved resolution of the $\Delta(S)$, $\Lambda(S)$ diastereomers The optimum ion-pair concentration was found to be 20–30 mM Curved plots such as those given in the figure have been used to support a particular retention mechanism[10], but this aspect is not at all clear[11,12] Clearly, however, the hydrophobic and steric properties of the stationary phase are intimately tied up with the hydrophobic, steric, and ionic properties of the ion-pair reagent and cobalt(III) complex ion

The elution order Gly, Ala, Pro, Val, Ile, Leu, Phe clearly shows the importance of the hydrophobic property but other effects are more subtle Thus the $\Lambda(S)$ ion elutes before $\Delta(S)$ for all complexes except AA = Ala, Pro, Leu, and for AA = Pro, Glu the elution order for RP-HPIPC is the opposite to that found using IEC although it is the same for the others Obviously a complex combination of factors is operating and predictions are impossible Fig 4 shows elution profiles for the separate AA = Ile, Leu diastereoisomers, and in combination

Fig 5a shows the effect of benzylation of the side chain functionalities of Glu, Thr and Tyr, the complexes are retained more firmly Fig 5b shows a different combination including AA = Met, Arg(NO_2) and Ser(Bzl) These results extend the elution order to firstly the 1^+ ions Asp > Glu ions followed by the 2+ cations Gly > Ala > Thr > Pro > Val > Met > Arg(NO_2) > Tyr > Ile > Leu > Phe > Ser(Bzl) > Trp > Thr(Bzl) > Glu(OBzl) > Tyr(Bzl)

The use of a Waters radial compression module (RCC) allows larger loadings and faster flow-rates Fig 6a, b compares chromatograms for the same Δ,Λ[Co(en)$_2$(AA)]$^{2+}$ mixture using a 30-cm stainless-steel μBondapak C_{18} column and a 10-cm Waters RCC column with a water–methanol–tosylate eluent profile The only difference was that 0 25% triethylamine was added to the eluent used with the RCC before adjusting the pH to 3 5 The two chromatograms are similar but with somewhat longer retention times for the more hydrophobic ions using the RCC column Fig 6c gives the chromatogram for the same mixture but with three times the loading (3000 *vs* 1000 nmol) and at four times the flow-rate (8 0 cm^3 min^{-1} *vs* 2 0 cm^3 min^{-1}) Apart from the more rapid elution similar profiles obtain with no obvious diminution in resolution or discrimination

The loading factor was further examined Fig 7a shows the effect of increased loadings of Δ,Λ[Co(en)$_2$(Pro)]$^{2+}$ using the analytical μBondapak C_{18} column Good peak shape and resolution is maintained to 2500 nmol when peak splitting of the leading edge of the $\Delta(S)$ ion occurs Further loading results in further splitting followed by peak broadening at 5120 nmol Fig 7b gives results of the same experiments using the RCC Now good peak shape is maintained up to 25,600 nmol (14 mg) and some resolution still occurs at twice that loading Clearly the RC column can handle ten times as much sample and peak splitting (see below) has been eliminated This maximum loading also depends on the relative retention of the complex ion since some 6400 nmol of $\Delta\Lambda$-[Co(en)$_2$Ser(Bzl)]$^{2+}$ can be loaded on the μBondapak column before peak splitting occurs, Fig 7c

3 COMPARISONS WITH ION-EXCHANGE CHROMATOGRAPHY (IEC, IE-HPLC)

The $\Delta(S)$, $\Lambda(S)$ diastereoisomers of all the above [Co(en)$_2$(AA)]$^{+,2+}$ com-

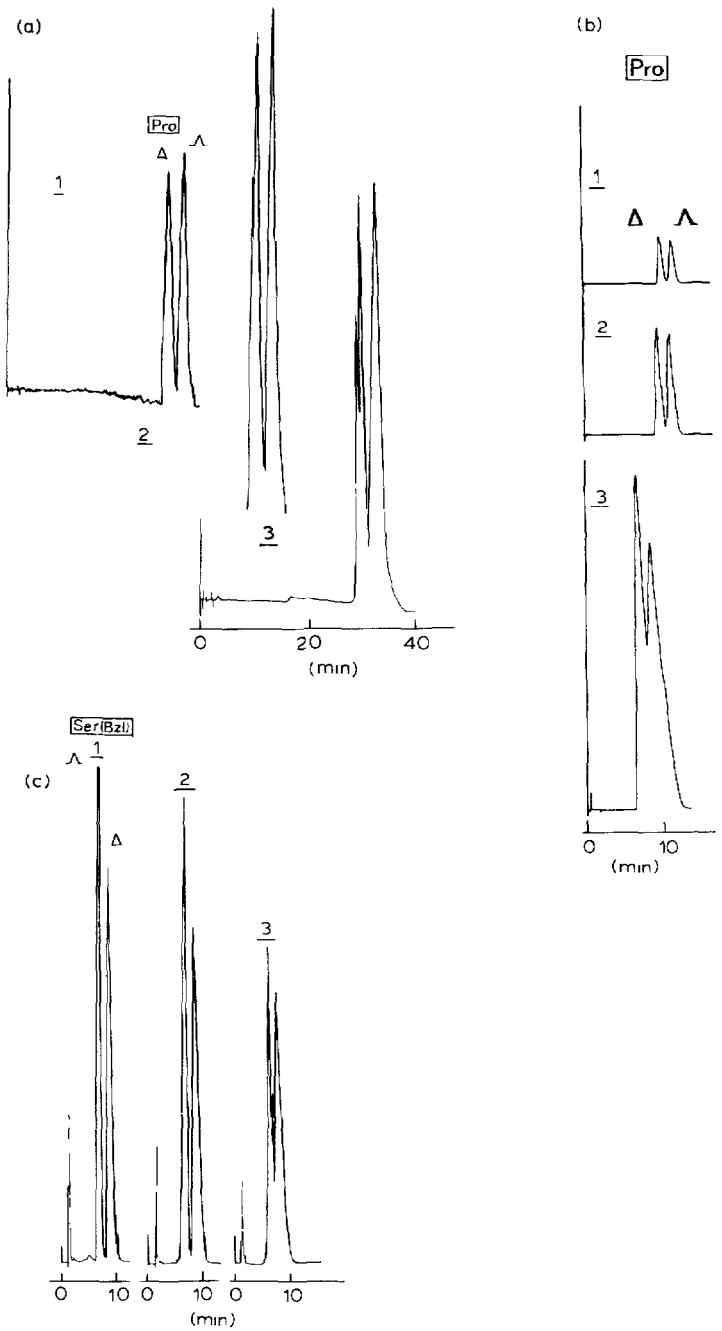


Fig 7 Effect of loading on μBondapak C_{18} 30-cm and C_{18} Radial Compression, 10-cm columns (a) Δ,Λ-$[Co(en)_2Pro]I_2$ 1, 320 nmol (5 μl), 2, 2560 (40), 3, 5120 (80) using μBondapak column, aqueous methanol gradient, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 0 cm^3 min^{-1} (b) Δ,Λ-$[Co(en)_2Pro]I_2$ 1, 2560 nmol (10 μl), 2, 25,600 (100), 3, 52,100 (200) using RCC, 11 4% aqueous methanol, 0 25% triethylamine (pH 3 5), 25 m*M* toluenesulfonate, flow-rate, 8 0 cm^3 min^{-1} (c) Δ,Λ-$[Co(en)_2Ser(Bzl)]I_2$ using μBondapak column 1, 3200 nmol (50 μl), 2, 6400 (100), 3, 12,800 (200), 38% aqueous methanol, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 0 cm^3 min^{-1}

TABLE 2

ELUTION ORDER OF THE $[Co(en)_2AA]^{2+}$ DIASTEREOISOMERS USING CONVENTIONAL ION-EXCHANGE METHODS

AA	*Column support*	*Ion pairing reagent*	*Elution order* Λ,Λ	Δ,Λ	*Ref* **
Ala	Dowex 50W-X 2	NaCl	X		a
Pro	Dowex 50W-X 2	NaCl	X		a
Cys(Me)	Dowex 50W-X 2	NaCl	X		a
homo-Ser	Dowex 50W-X 2	NaCl	X		a
Glu	Dowex 50W-X 2	NaCl	X		a,b,c,d
Asn	Dowex 50W-X 2	NaCl	X		a,c
Asp	Dowex 50W-X 2	NaCl	X		a,c,d
Cys	Sephadex SP-C25	$Sb_2tart_2^{2-}$	X		e
Gly	Sephadex SP-C25	$Sb_2tart_2^{2-}$	X		f
Phe	Dowex 50W-X 2	Sb-*d*-$tart_2^{2-}$, $tart_2^{2-}$	X		g,h,ı
Thr	Dowex 50W-X 2	HCl	X		j
Leu	Dowex 50W-X 2	NaCl		X	a
Glu	Dowex 50W-X 2	NaCl (pH 7 0)		X	a,c
Ala*	Sephadex SP-C25	$H_2PO_4^-$, HPO_4^{2-}	X		k
Val*	Sephadex SP-C25	$H_2PO_4^-$, HPO_4^{2-}	X		k
Phe*	Sephadex SP-C25	$H_2PO_4^-$, HPO_4^{2-}	X		k

* Tetraamine ligands (N_4) = 3(S),8(3)-$(CH_3)_2$-trien, 2(S),10(S)-$(CH_3)_2$-2,3,2-tet, 3(S),9(2)-$(CH_3)_2$-2,3,2-tet, SS-pyht

** (a) W E Keyes and J I Legg, *J Amer Chem Soc*, 98 (1976) 4970, (b) D A Buckingham, J Dekkers, A M Sargeson and L G Marzilli, *Inorg Chem*, 12 (1973) 1207, (c) W E Keyes, R E Caputo, R D Willett and J I Legg, *J Amer Chem Soc*, 98 (1976) 6939, (d) J I Legg and J Steele, *Inorg Chem*, 10 (1971) 2177, (e) H Nakazawa, S Yamazaki and H Yoneda, *36th Annual Meeting of the Chemical Society of Japan, Osaka, 1975*, (f) H Yoneda, S Yamazaki and K Maruyama, *26th Symposium on Coordination Chemistry in Japan, Sapporo, 1976*, (g) M M Deva, *M Sc Thesis*, University of Otago, Dunedin, New Zealand, 1982, (h) T Taura, H Tamada and H Yoneda, *Inorg Chem*, 17 (1978) 3127, (i) C R Clark, unpublished work, (j) J C Dabrowiak and D W Cooke, *Inorg Chem*, 14 (1975) 1305, (k) M Yamaguchi, S Yamamatsu, T Furusawa, S Yano, M Saburi and S Yoshikawa, *Inorg Chem*, 19 (1980) 2010

plexes have been previously separated in our laboratory or in the laboratories of others (*cf* Table 2), using IEC on Dowex or Sephadex cation-exchange resins A mixture of $[Co(en)_2(AA)]^{2+}$ ions has also been separated on cellulose using a water saturated *n*-butanol–acetic acid–pyridine eluent[13] These separations often require long columns and elution times in excess of 24 h Broad bands are usually obtained with the long elution times and sometimes the separations are incomplete However, for large scale preparative work the IEC method is far less expensive, and is therefore the method of choice

An IE-HPLC study using silica gel bonded with sulfonic acid groups (Whatman Partisil-10-SCX column) showed reasonably sharp peaks for the AA = Gly, Ala and Pro complexes (Fig 8), but the separations of the $\Delta(S)$ isomers, $\Lambda(S)$ were poor Varying the eluent [$H_2PO_4^-$ – HPO_4^{2-} (pH 6 8 and 4 5), Cl^-, ClO_4^-, bromocamphorsulfonate$^-$, Sb_2-$tart_2^{2-}$] confirmed that this system was inferior to RP-HPIPC Another IE stationary phase may be more suitable

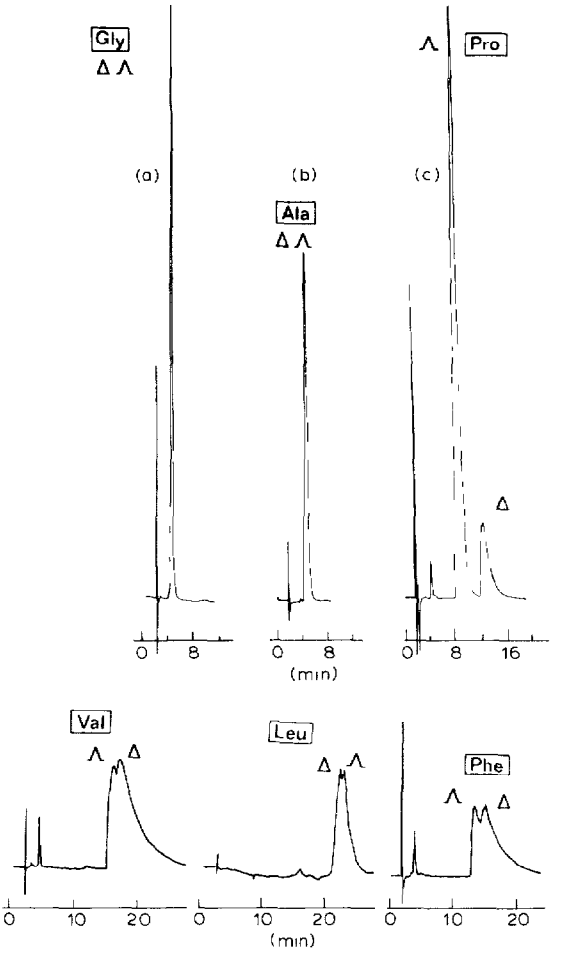
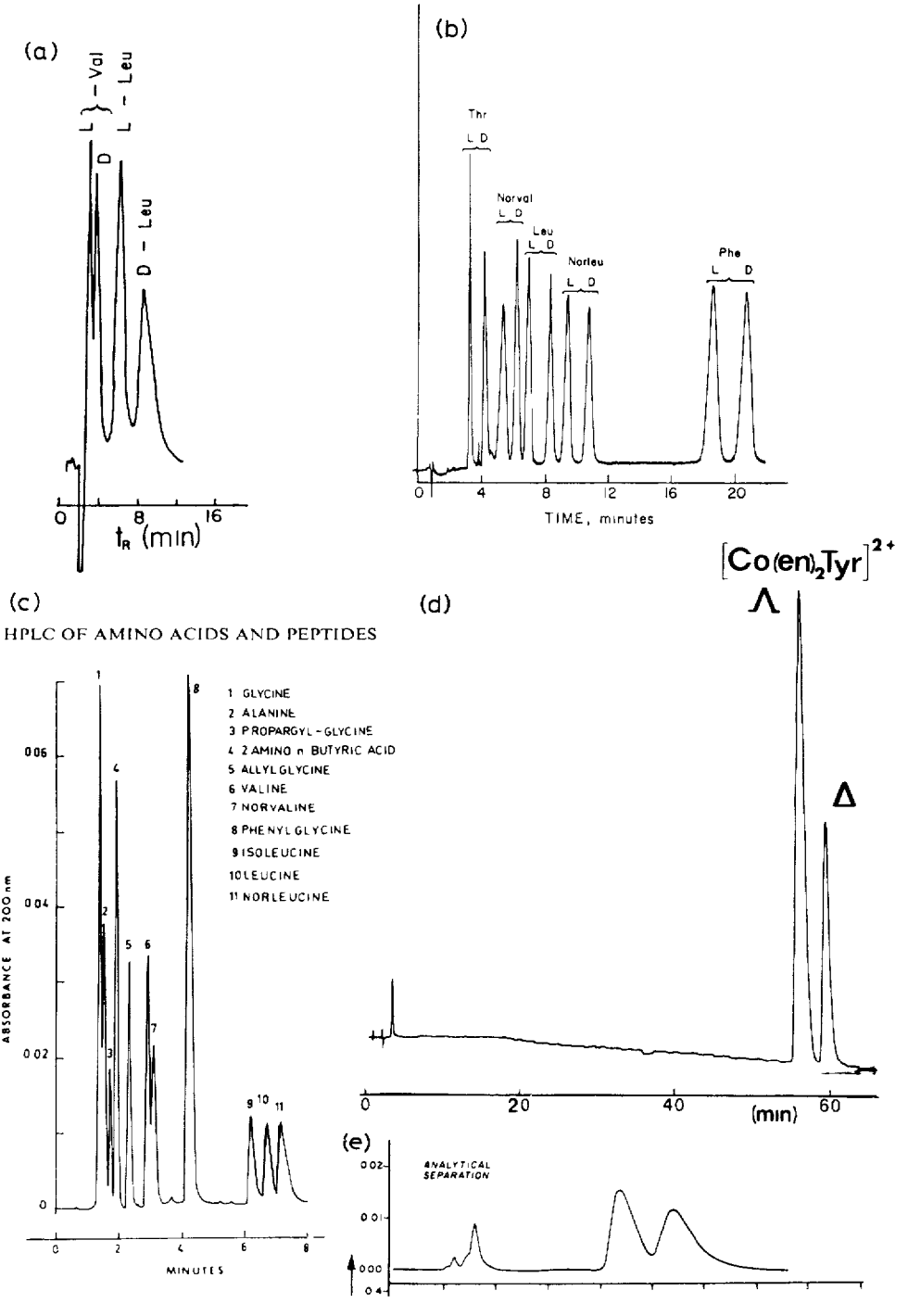


Fig 8 Elution profiles for various Δ,Λ-$[Co(en)_2AA]I_2$ (AA = Gly, Ala, Pro, Val, Leu, Phe) using high-performance ion-exchange chromatography (Whatman Partisil-10-SCX 30-cm column), λ = 480 nm (a) 160 nm (10 μl), 1 0 *M* phosphate buffer (pH 6 45), flow-rate, 1 5 cm^3 min^{-1}, a u f s , 0 02 (b) 400 (25), 1 0 *M* (6 45), 1 5, 0 01 (c) 400 (25), 1 0 *M* (6 45), 1 5, 0 02 (d) 400 (25), 0 2 *M* (4 50), 2 0, 0 02 (e) 160 (16), 0–0 2 *M* Sb_2-$tart_2^{2-}$ gradient (pH 4 20), 1 5, 0 02 (f) 400 (25), 1 0 *M* phosphate buffer (6 45), 1 5, 0 05

4 COMPARISONS WITH HPLC AND RP-HPLC

Fig 9 gives some examples of previous separations of amino acids or their derivatives using RP packings on silica Molnar and Horváth[14] observed that amino acids elute in order of increasing hydrophobicity of the side chain (Fig 9c), and Grushka and co-workers[15,16] found that by adding a Cu(II) or Zn(II) complex of Asp-Phe-OCH_3 or analogous asymmetric ligands that the *R*- and *S*-amino acids could be resolved (Fig 9a) Likewise, a Cu(II) proline complex in the mobile phase has been used to separate *R*- and *S*-amino acids using ion-exchange methods[17] Davankov and Semechkin[18] and Lefebure *et al*[19] have used ligand-exchange chromatography with resolution via a Cu(II) amino acid complex grafted to the stationary

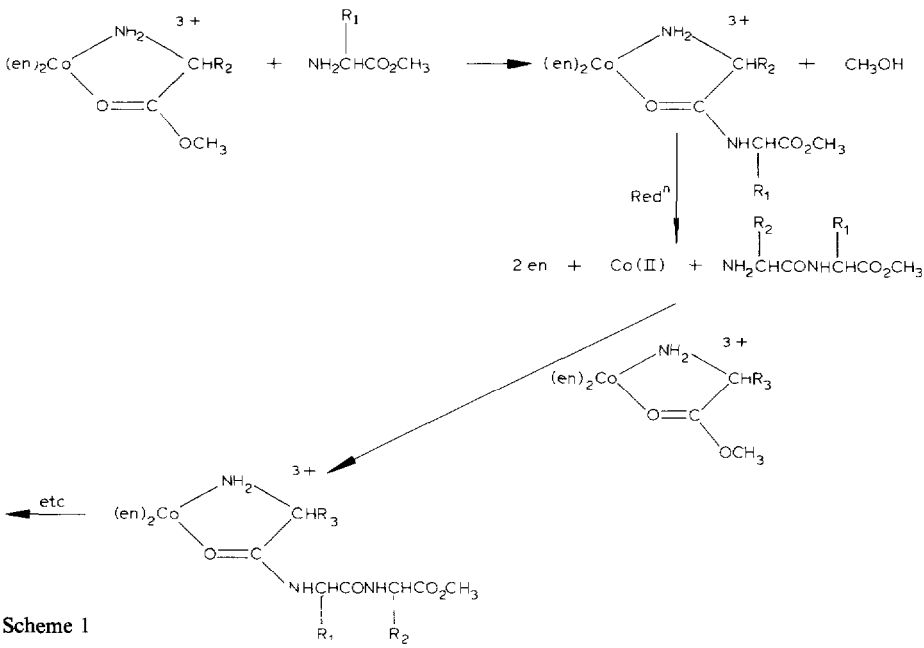

(a)
L } - Val
L - Leu
D
D - Leu
0
8
16
t_R (min)
(b)
Thr
L D
Norval
L D
Leu
L D
Norleu
L D
Phe
L D
0
4
8
12
16
20
TIME, minutes
(c)
HPLC OF AMINO ACIDS AND PEPTIDES
1 GLYCINE
2 ALANINE
3 PROPARGYL - GLYCINE
4 2 AMINO n BUTYRIC ACID
5 ALLYLGLYCINE
6 VALINE
7 NORVALINE
8 PHENYLGLYCINE
9 ISOLEUCINE
10 LEUCINE
11 NORLEUCINE
ABSORBANCE AT 200 nm
0.06
0.04
0.02
0
MINUTES
(d)
$[Co(en)_2Tyr]^{2+}$
Λ
Δ
0
20
40
60
(min)
(e)
ANALYTICAL SEPARATION
0.02
0.01
0.00
0.4

phase, Cram and his group[20] have used chiral crown ethers bound to the stationary phase Le Page *et al* [21] have modified the mobile phase by adding the Zn(II) complex of *R*-2-alkyl-4-octyldiethylenetriamine to resolve the dansylated *R*- and *S*-amino acids (Fig 9b)

They[21] appear to have obtained the best results with complete separation and resolution and good peak shape within 20 min of injection Our results are comparable, especially when toluenephosphate is used as ion-pair reagent On a preparative scale cobalt(III) can be easily removed by reduction to the labile Co(II) oxidation state (using zinc amalgam, or H_2S)

5 SYNTHESIS OF DIPEPTIDES AND THEIR RP-HPIPC

For several years we have been evaluating an alternative approach to synthesising small peptides using a cobalt(III) centre to activate the formation of the



Scheme 1

Fig 9 Comparisons with previous HPLC, RP-HPLC of amino acids (a) Enantiomeric separation of *R,S*-valine and *R,S*-leucine[15,16] (RP-column, flow-rate, 2 0 cm^3 min^{-1}, 34°C, 1 0 mmol dm^{-3} *R*-aspartylcyclohexylamide–Cu(II), λ = 230 nm) (b) Separation of *R,S*-Dns-amino acids[21] (Hypersil C_8 packing, flow-rate, 2 0 cm^3 min^{-1}, 30°C, 0 65 mmol dm^{-3} L-2-isopropyl-dien–Zn(II), 0 17 mol dm^{-3} ammonium acetate to pH 9 0 with aqueous ammonia, acetonitrile–water (35 65), λ = 254 nm) Solutes Thr = threonine, Norval = norvaline, Leu = leucine, Norleu = norleucine- Phe = phenylalanine (c) Chromatogram of non-polar amino acids[14] (LiChrosorb RP-18 packing, flow-rate, 2 0 cm^3 min^{-1}, 70°C, 0 5 mol dm^{-3} $HClO_4$, pH 0 2, ν = 210 nm) (d) Elution profile (gradient 2) of Δ,Λ-$[Co(en)_2Tyr]^{2+}$ ions (μBondapak C_{18} packing, flow-rate, 2 0 cm^3 min^{-1}, 25°C, 25 mmol dm^{-3} TSA^-, pH 3 5, λ = 480 nm, chartspeed, 0 25 cm min^{-1}), total Co, 512 nmol (8 μl) (e) Elution profile (isocratic) of Δ,Λ-$[Co(en)_2Tyr]^{2+}$ ions[2] (Silica packing, flow-rate, 6 0 cm^3 min^{-1}, 2 0 mol dm^3 isopropyl alcohol, pH 9 0- triethylammonium bicarbonate buffer (70 30), λ = 510 nm) 2 mg of mixture (20 μl)

amide bond[22] Essentially this method is an active ester method with development of the peptide from the C-terminal end in a stepwise manner (Scheme 1)
As well as monitoring the formation of the amide bond RP-HPIPC is proving very useful in evaluating mutarotation at the α-carbon centre (As well as activating the carbonyl centre towards nucleophilic attack the cobalt(III) centre also activates the α-carbon centre in the 5-membered chelate towards proton exchange and hence to the possibility of mutarotation) Fig 10 gives an example of monitoring the reaction

$$\Delta,\Lambda\text{-}[Co(en)_2(Phe\text{-}OCH_3)]^{3+} + Phe\text{-}OCH_3 \xrightarrow[25°C]{DMSO} \Delta\Lambda\text{-}[Co(en)_2(Phe\text{-}Phe\text{-}OCH_3)]^{3+} + CH_3OH \quad (1)$$

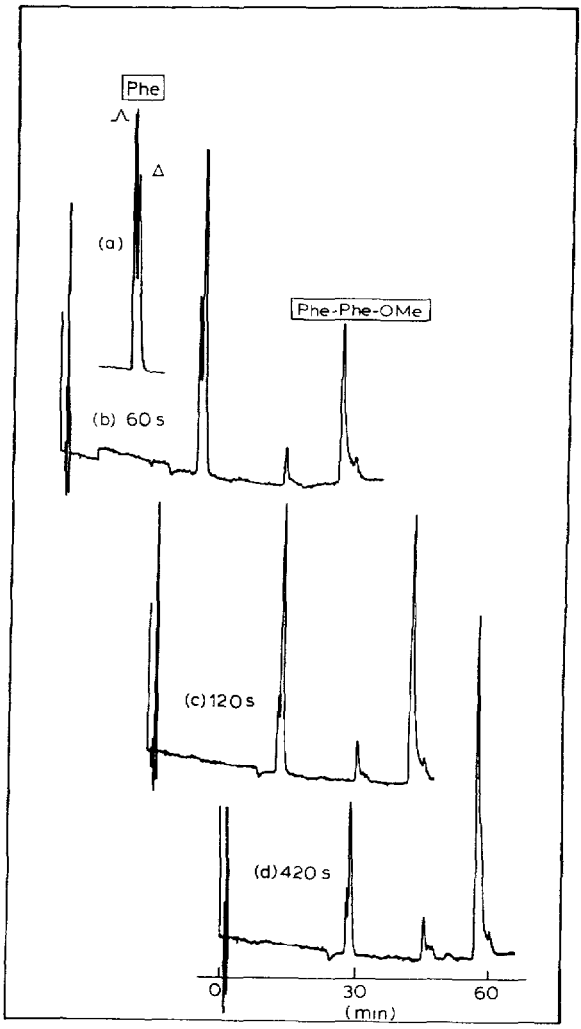


Fig 10 Elution profiles for the products of reacting Δ,Λ-$[Co(en)_2Phe\text{-}OCH_3](CF_3SO_3)_3$ with Phe-OCH_3 after (b) 60 sec, (c) 120, (d) 420, (a) inset, Δ,Λ-$[Co(en)_2Ala](CF_3SO_3)_2$, aqueous methanol, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 0 cm^3 min^{-1}, λ = 480 nm, a u f s , 0 01

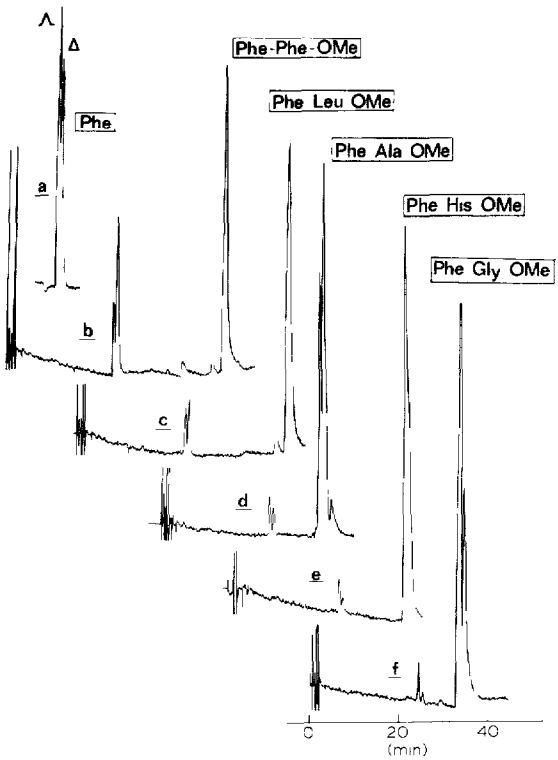


Fig 11 Elution profiles for the products of reacting Δ,Λ-$[Co(en)_2Phe\text{-}OCH_3](CF_3SO_3)_3$ with AA-OCH_3, AA = Phe (b), Leu (c) Ala (d), His (e), Gly (f) after 420 sec reaction time Inset (a) Δ,Λ-$[Co(en)_2Phe](CF_3SO_3)_2$, aqueous methanol, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 0 cm^3 min^{-1}, λ = 480 nm, a u f s , 0 02)

Starting with approximately equal amounts of the reactant $\Delta(S)$, $\Lambda(S)$ diastereomers subsequent chromatograms show the growth of the product dipeptide complex with a reaction half-time of *ca* 2 min Although using toluenesulfonic acid as ion-pair reagent no resolution of diastereoisomeric products is observed an indication that the $\Lambda(S)$ reactant reacts faster than the $\Delta(S)$ reactant is seen from the more rapid disappearance of the absorption due to the former This asymmetric process has been confirmed by both rate studies on the separate diastereomeric reactants and by recovering the product from preparative experiments, which is largely Λ-$[Co(en)_2(Phe\text{-}Phe\text{-}OCH_3)^{3+}$ Fig 11 gives elution profiles for reacting the same active ester with Gly-OCH_3 His-OCH_3, Ala-OCH_3, Leu-OCH_3 and Phe-OCH_3 In some cases differentiation of diastereoisomeric products is seen but the resolution is not good with this ion-pair reagent

Fig 12 gives another sequence of chromatograms for the reaction

$$\Delta,\Lambda\text{-}[Co(en)_2(Ala\text{-}OCH_3)]^{3+} + AA\text{-}OCH_3 \xrightarrow{DMSO} \Delta,\Lambda\text{-}[Co(en)_2(Ala\text{-}AA\text{-}OCH_3)]^{3+} + CH_3OH \quad (2)$$

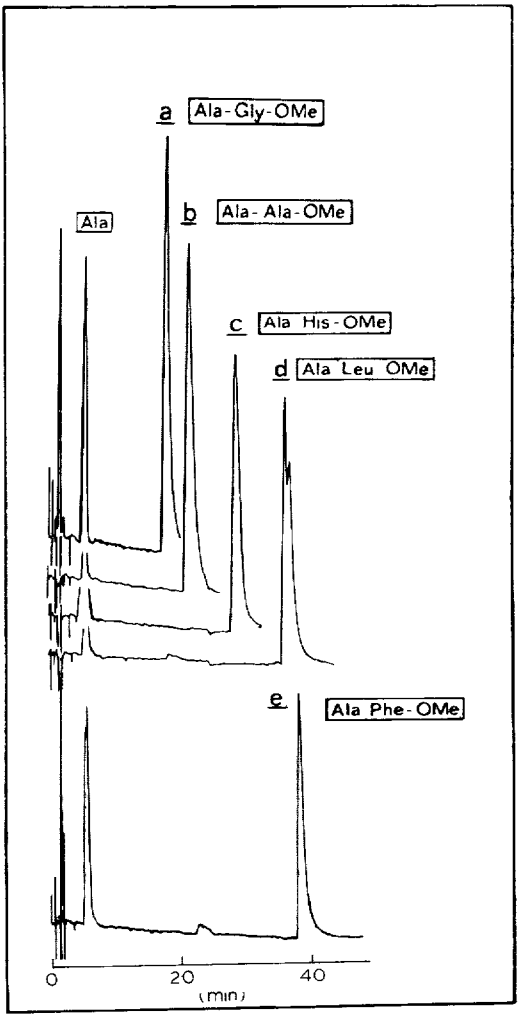


Fig 12 Elution profiles for the products of reacting Δ,Λ-$[Co(en)_2Ala\text{-}OCH_3](CF_3SO_3)_3$ with AA-OCH_3, AA = Gly (a), Ala (b), His (c), Leu (d), Phe (e) after 420 sec reaction time, aqueous methanol, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 0 cm^3 min^{-1}, λ = 480 nm, a u f s , 0 02

but again the diastereoisomers are not resolved From these experiments it is clear that retention on the chromatographic column is largely controlled by AA rather than by Ala, the more hydrophobic amino acids give rise to longer retention times, AA = Gly < Ala < His < Leu < Phe This is a general characteristic of such dipeptide complexes with the C-terminal amino acid playing a dominant role An unusual reversal for the dipeptide complexes of Ala-Phe-OCH_3, Gly-Phe-OCH_3 and Ala-Gly-OCH_3, Gly-Gly-OCH_3 was found, with the alanine systems eluting before the glycine dipeptides, for the 2+ ions $[Co(en)_2(Gly)]^{2+}$ and $[Co(en)_2(Ala)]^{2+}$ the former elutes before the latter

The question of mutarotation at the α-carbon centre is an important one since any synthetic procedure must reduce this possibility to the bare minimum, preferably

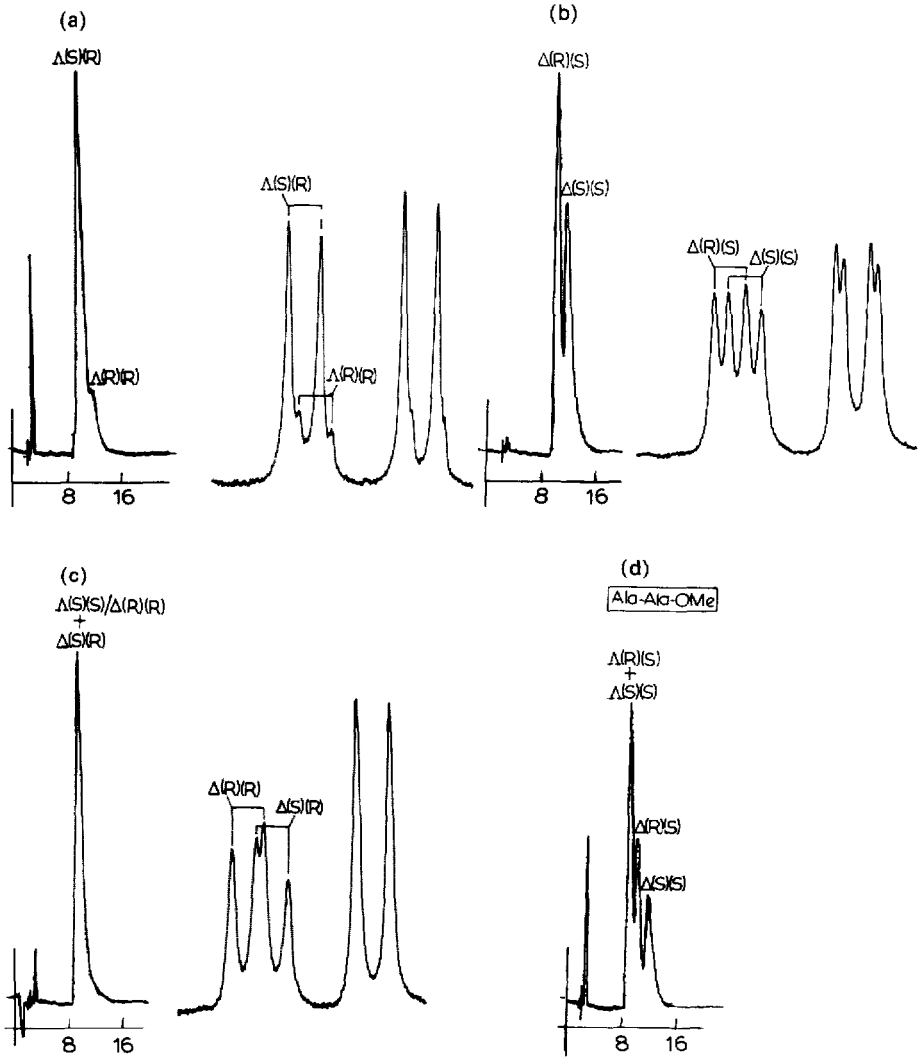


Fig 13 Elution profile and ^{1}H NMR (α-CH_3 region, $\delta \approx 1.5$ ppm) for products, after 2 min of coupling (a) Λ-[Co(en)$_2$-(*S*)-Ala-OCH_3]$(CF_3SO_3)_3$ with (*R*)-Ala-OCH_3, (b) Δ-[Co(en)$_2$-(*S*)-Ala-OCH_3]$(CF_3SO_3)_3$ with (*S*)-Ala-OCH_3, (c) Δ-[Co(en)$_2$-(*S*)-Ala-OCH_3]$(CF_3SO_3)_3$ with (*R*)-Ala-OCH_3, (d) elution profile only for products after 2 min of coupling Δ,Λ-[Co(en)$_2$(*S*)-Ala-OCH_3]$(CF_3SO_3)_3$ with (*R*)-Ala-OCH_3, aqueous methanol, 37 m*M* dimethyl phosphate (pH 3 25), λ = 480 nm, a u f s , 0 02, flow-rate, 2 0 cm^3 min^{-1}

to zero (Clearly mutarotation could occur in either the $[Co(en)_2(AA\text{-}OCH_3)]^{3+}$ reactant or in the $[Co(en)_2(AA\text{-}AA'\text{-}OCH_3)]^{3+}$ product) We are currently looking at this problem using tritium-labelling techniques but RP-HPIPC is also proving to be most useful Using $(CH_3O)_2PO_2^-$ as ion-pair reagent at pH 3 4 most of the diastereoisomeric possibilities can be separated Fig 13a gives the chromatogram and ^{1}H NMR spectrum of the chelate ester ring alanine methyl protons for the dipeptide product of the reaction

$$\Lambda\text{-}\{Co(en)_2[(S)Ala\text{-}OCH_3]\}^{3+} + (R)Ala\text{-}OCH_3 \rightarrow$$
$$\Lambda\text{-}\{Co(en)_2[Ala\text{-}(R)Ala\text{-}OCH_3]\}^{3+} + CH_3OH$$

(2 min reaction time in DMSO) Optical integrity is obtained at cobalt and at the C-terminal amino acid function in all such couplings and the only question concerns retention in the chelate ring amino acid It is clear that the major product has the required $\Lambda(S)$ (R) configuration but some (*ca* 5%) mutarotation to $\Lambda(R)$ (R) has occurred A similar, but not identical, result is obtained on coupling (*S*)-Ala-OCH_3 to the same $\Lambda(S)$ active ester complex However for the reaction

$$\Delta\text{-}\{Co(en)_2[(S)Ala\text{-}OCH_3]\}^{3+} + (S)Ala\text{-}OCH_3 \rightarrow$$
$$\Delta\text{-}\{Co(en)_2[Ala\text{-}(S)Ala\text{-}OCH_3]\}^{3+} + CH_3OH$$

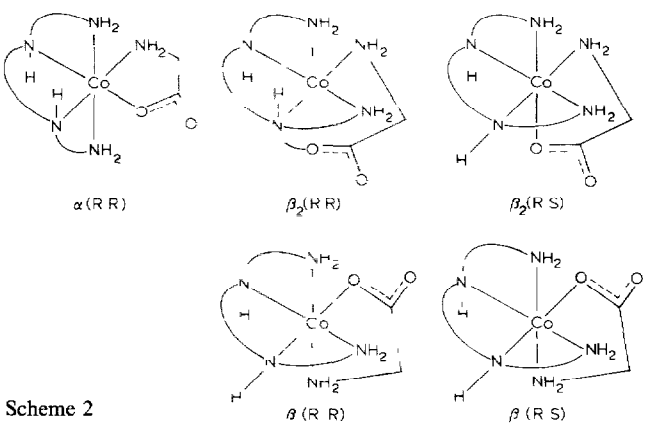
the result is somewhat different Both the chromatogram and the ^1H NMR spectrum now show two species to be present, (Fig 13b), and identification with known compounds establish these as the $\Delta(S)$ (S) and Δ (R) (S) isomers Clearly considerable mutarotation has occurred during synthesis and other experiments establish that this occurs in the active ester rather than in the dipeptide product Fig 13c gives results for the

$$\Delta\text{-}\{Co(en)_2[(S)Ala\text{-}OCH_3]\}^{3+} + (R)Ala\text{-}OCH_3 \rightarrow$$
$$\Delta\text{-}\{Co(en)_2[Ala\text{-}(R)Ala\text{-}OCH_3]\}^{3+} + CH_3OH$$

coupling but now RP-HPIPC does not distinguish the $\Delta(S)$ (R) and $\Delta(R)$ (R) products although the 100 MHz ^1H NMR does Thus three out of the four possible diastereoisomers $\Lambda(S)$ (R), $\Lambda(R)$ (R), Λ (R) (S), Λ (S) (S) can be distinguished in this system by the chromatographic method, Fig 13d shows a mixture of these

6 IDENTIFICATION OF CONFIGURATIONAL ISOMERS, THE $[Co(trien)Gly]^{2+}$ AND $[Co(trien)Ala]^{2+}$ IONS

This section deals with the RP-HPIPC identification of geometric and optical isomers resulting from the arrangement of the tetradentate linear chelating ligand triethylenetetramine about Co(III) Ten diastereomers of $[Co(trien)Gly]^{2+}$ are possible and all have been identified previously[23,24] The five Λ-isomers are depicted in Scheme 2



Scheme 2

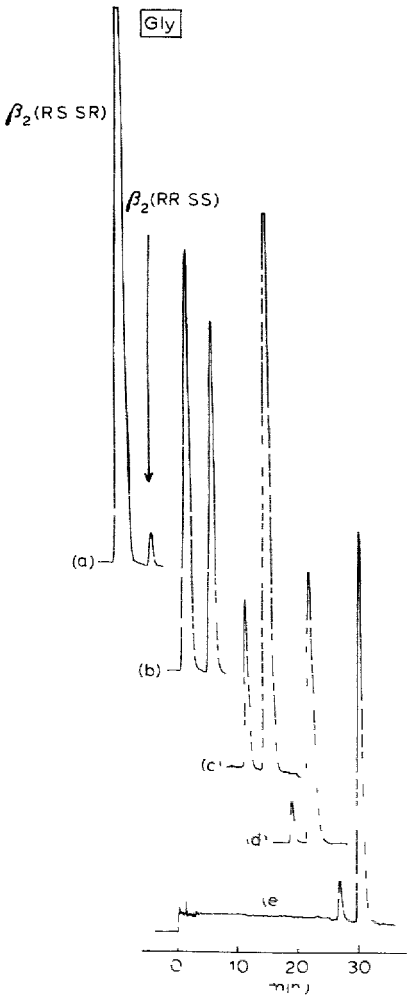


Fig 14 Elution profiles following mutarotation in β_2-(*RS,SR*)-[Co(trien)Gly]$^{2+}$ in water Aqueous methanol, 25 m*M* toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1}, λ = 480 nm (a) 512 nmol (8 μl) after 1 h, (b) 23 h, (c) 96 h, a u f s , 0 02, (d) 512 (8) after 8 days, a u f s , 0 05, (e) 192 (6) after 9 days, a u f s , 0 05

There are similar mirror image forms The $\beta_2(R,R)$, $\beta_2(R,S)$ and $\beta_1(R,R)$, $\beta_1(R,S)$ pairs are known to mutarotate to equilibrium distributions of *ca* 9 1 and *ca* 1 1 respectively under alkaline conditions (this is slow in neutral aqueous solution) since proton exchange is a necessary precursor to inversion at the "planar" secondary N centre Fig 14 gives chromatograms of aliquots of an aqueous solution of racemic $\beta_2(RS,SR)$-[Co(trien)-Gly]I_2 taken at different times Clearly the growth of the more retained species occurs at the expense of the initial complex and the half-time of this process, *ca* 100 h, is entirely consistent with the $\beta_2(RS,SR) \rightleftharpoons \beta_2(RR,SS)$ equilibrium with a final product distribution of *ca* 1 9 after 9 days (*i e* $k_{obs} \approx 4 \ 10^{-6}$ sec^{-1}) This is in agreement with the earlier work[23,24] Fig 15 gives the chromatogram of a sample of $\beta_2(RS,SR)$-[Co(trien)Gly]$^{2+}$ following equilibration with sodium hydroxide for a

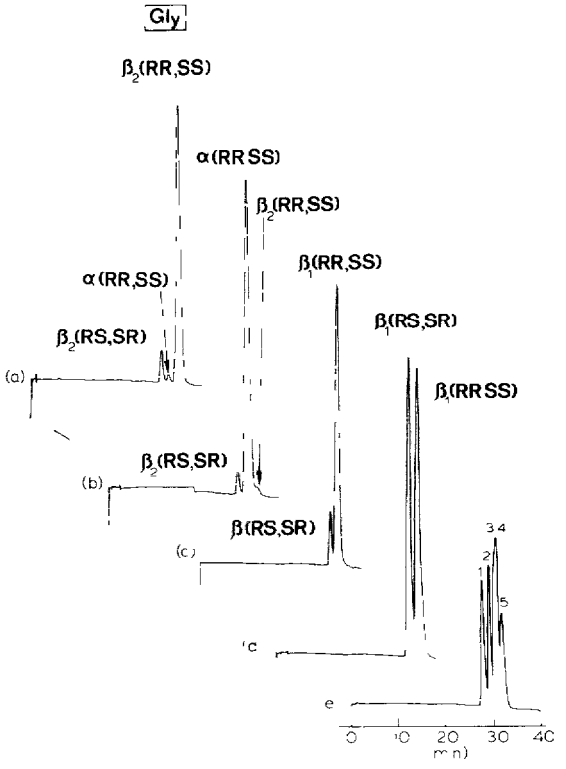


Fig 15 Elution profiles for various [Co(trien)Gly]I_2 isomers Aqueous methanol, 25 m*M* toluenesulfonate (pH 3 5), λ = 480 nm, a u f s , 0 02, flow-rate, 2 0 cm^3 min^{-1} (a) 288 nmol (6 μl), (b) 384 (6), (c) 380 (2), (d) 600 (6), (e) 288 (6) containing a mixture of the five isomers (1) $\beta_2(RS,SR)$, (2) $\beta_1(RS,SR)$, (3) $\alpha(RR,SS)$, (4) $\beta_1(RR,SS)$, (5) $\beta_2(RR,SS)$

few seconds (and then neutralised) It duplicates the distribution given in Fig 14e but also contains a small $\alpha(RR,SS)$ impurity Likewise, the chromatograms of supposedly "pure" $\alpha(RR,SS)$ and $\beta_1(RR,SS)$ isomers are shown to be slightly impure with the $\beta_1(RS,SR)$ contaminant being confirmed by mutarotation of the $\beta_1(RR,SS)$ sample at pH *ca* 13 for 2 min and adjustment back to pH *ca* 6 (Fig 15d) Finally, Fig 15e shows a mixture of all five isomers with the elution order $\beta_2(RS,SR) > \beta_1(RS,SR) > \alpha(RR,SS) \approx \beta_1(RR,SS) > \beta_2(RR,SS)$ Although the $\beta_2(RS,SR)/\beta_2(RR,SS)$ and $\beta_1(RS,SR)/\beta_1(RR,SS)$ pairs of isomers had previously been separated by ion-exchange chromatography[23,24] this took over 24 h to achieve and a separation of the complete mixture was never attempted Fig 16 gives the chromatogram of a preparative sample of [Co(trien)(Ala)]I_2 Clearly two isomers are present with the suggestion of a shoulder on the following edge of the slower species Mutarotation of this sample at pH *ca* 13 followed by neutralisation to pH *ca* 6 gives the second chromatogram and it resembles that of Fig 15e except that the $\alpha(RR,SS)$ adsorption appears to be absent The $\beta_2(RS,SR)$, $\beta_1(RS,SR)$, $\beta_1(RR,SS)$ and $\beta_2(RR,SS)$ ions appear to be present (in that order) but the relative stabilities of the $\beta_2(RS,SR)$, $\beta_2(RR,SS)$ ions seem to be reversed compared to the AA = Gly system Obviously further experiments are necessary to confirm this

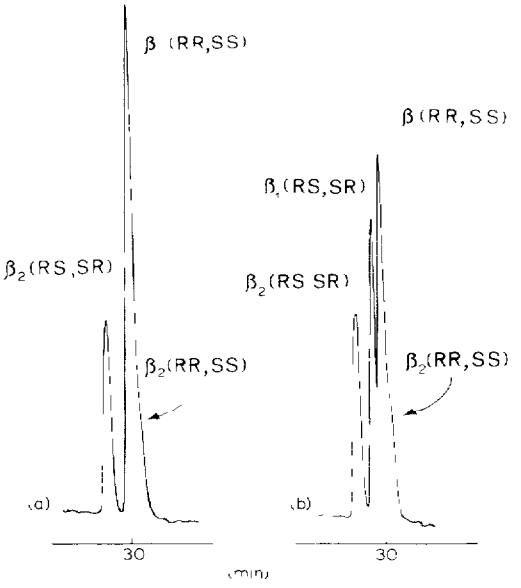
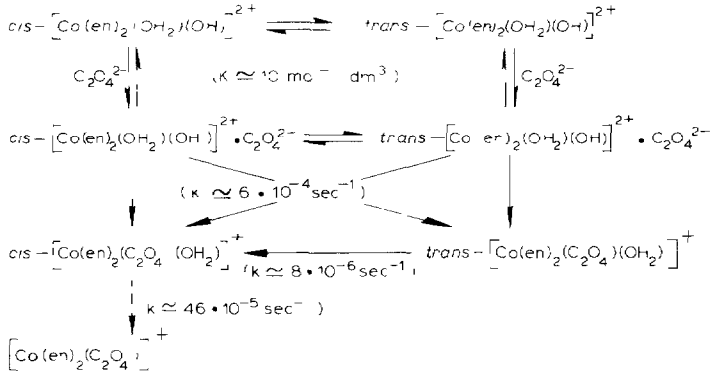


Fig 16 Elution profile for [Co(trien)Ala]I_2 from preparative mixture (a) 300 nmol (6 μl) in water, (b) 300 (6) after mutarotation at pH 13 and neutralisation Aqueous methanol, 25 m*M* toluenesulfonate (pH 3 5), flow-rate, 2 0 cm^3 min^{-1}, a u f s , 0 02, λ = 480 nm

7 THE ANATION OF $[Co(en)_2(OH_2)(OH)]^{2+}$ BY $C_2O_4^{2-}$

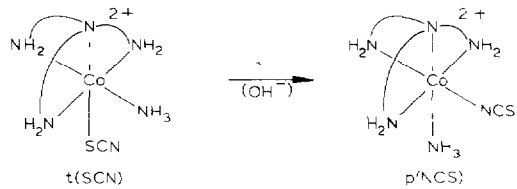
This example illustrates the ability of RP-HPIPC to detect and measure the concentrations of all species in a relatively complicated reaction sequence (Scheme 3) At pH *ca* 7 the anation occurs via the *cis* and *trans* hydroxo-aqua ions with little or no involvement of the diaqua and dihydroxo species Ion-pairing with $C_2O_4^{2-}$ is substantial, and is complete at $[C_2O_4^{2-}] > 0\,3$ mol dm^{-3} for $[Co]_T \approx 10^{-3}$ mol dm^{-3} Anation occurs via the ion-paired species with both *cis*- and *trans*-$[Co(en)_2(C_2O_4(OH_2))]^+$ intermediates being formed Isolation of the *trans* intermediate allowed its chemistry to be studied independently[25], it isomerises to the *cis*-monodentate which finally chelates via dual paths, displacement of water at the metal centre and attack by coordinated water at the carboxyl function Apart from the ^{18}O-tracer work all these processes can be followed chromatographically by quenching the reaction at appropriate times Fig 17 shows an example of this under the conditions $[Co]_T = 8\ 10^{-3}$ mol dm^{-3}, $[C_2O_4]_T = 0\,3$ mol dm^{-3}, 25 0°C and I = 1 0 mol dm^{-3} The amounts of *cis*- and *trans*-$[Co(en)_2$-$C_2O_4(OH_2)]^+$, and $[Co(en)_2C_2O_4]^+$ were monitored by quenching to pH *ca* 3 and freezing samples in liquid nitrogen until injection The decay of the reactant ions and the growth of the two monodentate oxalato intermediates can be easily seen as can the slower growth of the final $[Co(en)_2C_2O_4]^+$ product At higher pH values these reactions are reversed with $[Co(en)_2C_2O_4]^+$ and *cis*- and *trans*-$[Co(en)_2(C_2O_4)(OH)]$ giving rise to *cis*-$[Co(en)_2(OH)_2]^+$ and little or no *trans*-$[Co(en)_2(OH)_2]^+$ Clearly such processes are difficult, if not impossible, to quantify in the absence of a method which clearly distinguishes between alternative ionic species



Scheme 3

8 THE BASE HYDROLYSIS OF THE *t*-$[Co(tren)(NH_3)SCN]^{2+}$ ION

The question initially here was, does this ion undergo a base induced isomerisation process where the metal bound thiocyanate undergoes both isomerisation to metal bound isothiocyanate and concomitant stereochemical change (*viz* Scheme 4) We had previously suggested such a process for *trans*-$[Co(en)_2\text{-}NH_3(SCN)]^{2+}$ (ref 26), but only *ca* 0 5% *cis*-$[Co(en)_2NH_3(NCS)]^{2+}$ was accredited to the intramolecular path against a backdrop of 8% *cis*- and *trans*-isothiocyanate product Also, the analysis procedure used previously is now considered uncertain We have recently prepared the above *t*(SCN) complex, and other studies[27] had shown that base catalysed loss of an acido group from this position resulted in 80–90% stereochemical change to *p*(OH) It was hoped that substantial *t*(SCN) → *p*(NCS) isomerisation might also occur (*t* signifies *trans* to tertiary N centre, *p* signifies *trans* to primary N center)



Scheme 4

Base hydrolysis of *t*-$[Co(tren)(NH_3)(SCN)]^{2+}$ (Scheme 5) follows the expected second-order rate law, rate = k_{OH} [CoSCN][OH^-] with k_{OH} = 3 1 10^{-2} mol^{-1} dm^3 sec^{-1} at 25 0°C, I = 1 0 mol dm^{-3} Approximately three-quarters of the immediate product is $[Co(tren)(NH_3)OH]^{2+}$ of which *ca* 85% has the *p*-configuration and *ca* 15% is *t*(OH) The remaining products proved difficult to determine by conventional ion-exchange chromatography but RP-HPIPC came to the rescue Fig 18 shows chromatograms of the 1+ and 2+ products (*i e* 30% of the total) resulting from the hydrolysis of a 3 mg sample of *t*-$[Co(tren)(NH_3)SCN]Br_2$ in 0 4 cm^3 of 0 25 mol dm^{-3} sodium hydroxide at various times Firstly, no *p*(NCS) is formed, but *ca* 5% isomerisation to *t*(NCS) occurs (possibly via a tight ion-pair) However considerable hydrolysis of the *p*-NH_3 ligand occurs and the chromatograms show that both the

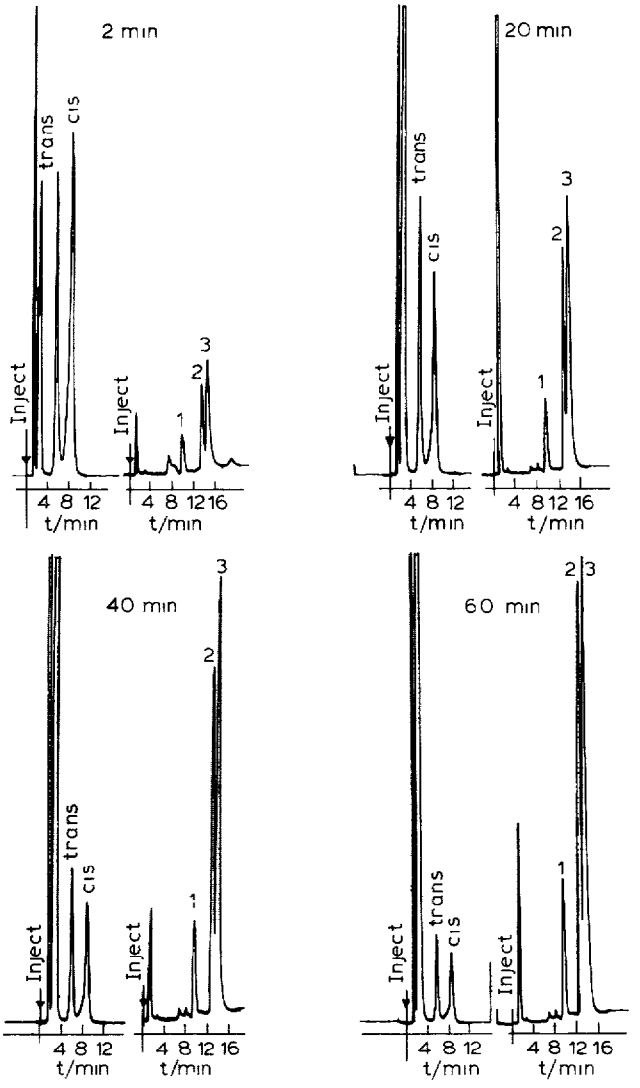
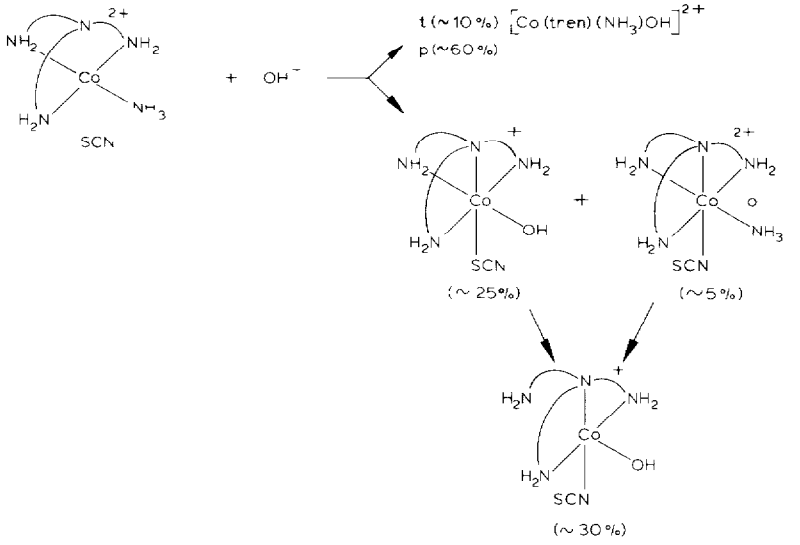


Fig 17 Samples (10 μl) of a reaction mixture consisting initially of $[Co(en)_2(OH_2)(OH)](ClO_4)_2$ (8 10^{-3} mol dm^{-3}), pH = 7 40 (0 1 *M* HEPES), 25 0°C, $[C_2O_4^{2-}]$ = 0 3 mol dm^{-3} The *trans*-, *cis*-$[Co(en)_2(C_2O_4)(OH_2)]^+$ (1, 2) and $[Co(en)_2C_2O_4]^+$ (3) ions were eluted with a 0–13 5% methanol gradient, 25 m*M* hexanesulfonate (pH 3 4), the *trans*- and *cis*-$[Co(en)_2(OH_2)]^{3+}$ ions were eluted with 40 5% methanol, 25 m*M* hexanesulfonate (pH 3 4)

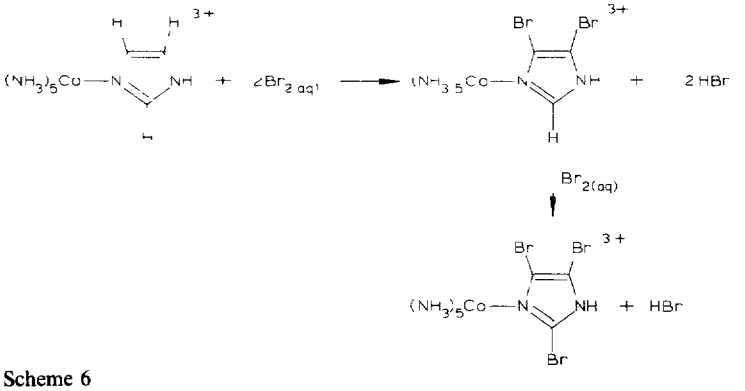
t(SCN)p(OH) and t(NCS)p(NH_3) products subsequently hydrolyse to t(NCS)p(OH) The surprising result here is that t(SCN)p(OH) appears to isomerise totally to t(NCS)p(OH) under the alkaline conditions and we are presently investigating this process



Scheme 5

9 BROMINATION OF COORDINATED IMIDAZOLE

Fig 19 shows chromatograms following the bromination of $[(NH_3)_5CoIm](ClO_4)_3$ in aqueous Br_2 solutions (Scheme 6) The difficulty here was that the bromo products [especially $(NH_3)_5Co(Br_3Im)^{3+}$] are light sensitive and ion-exchange chromatography (on Sephadex) must be done in the complete absence of light to have any chance of success RP-HPIPC is both fast and quantitative and with extinction coefficients at hand for each species relative yields can be evaluated The obvious result from these chromatograms is that no mono-bromo complex can be detected, and the stoichiometry requires only one H-atom substitution per Br_2 molecule Also the product/reactant distribution requires the relative bromination rates to be monobromo ≫ dibromo > H Clearly RP-HPIPC shows considerable promise in the area of reactions of coordinated ligands



Scheme 6

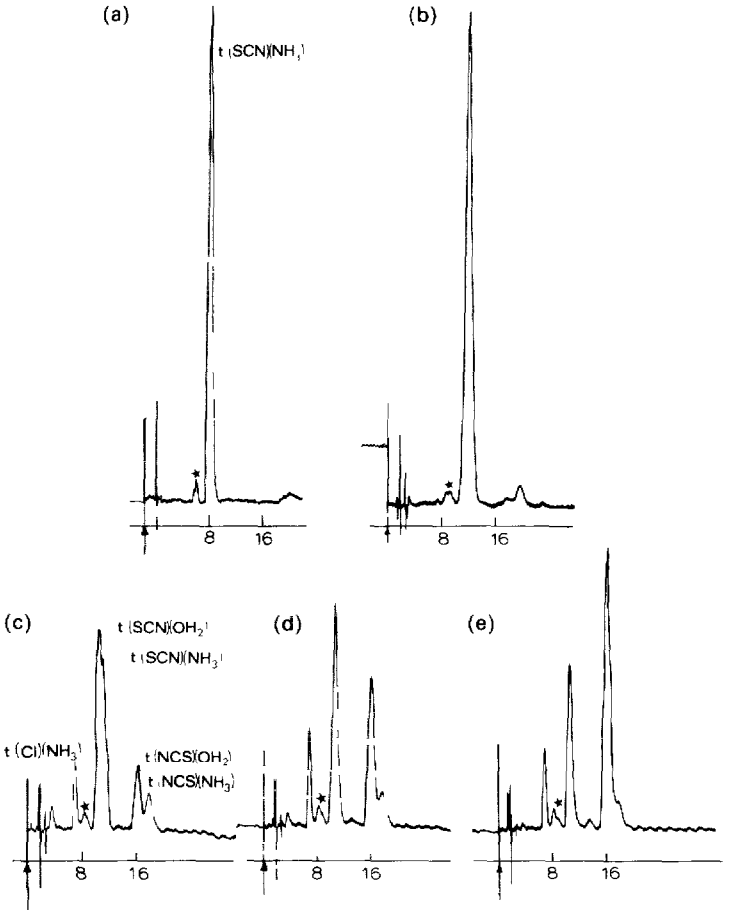


Fig 18 Samples (40 μl) following hydrolysis of 3 12 mg *t*-[Co(tren)(NH_3)SCN]Br_2 in 0 4 cm^3 0 25 *M* sodium hydroxide at 25 0°C for (a) immediate quench, (b) $t_{\frac{1}{2}}$ (45 sec), (c) $2t_{\frac{1}{2}}$ (3 min), (d) $5t_{\frac{1}{2}}$ (7 5 min), (e) $10t_{\frac{1}{2}}$ (15 min) Aliquots were quenched into 0 5 *M* hydrochloric acid (5% methanol, 25 m*M* toluenesulfonate (pH 3 5) flow-rate, 2 cm^3 min^{-1}, a u f s , 0 02, λ = 500 nm)

10 MECHANISM OF AQUATION OF ACIDO COMPLEXES

There still remains considerable debate as to the details of substitution at a Co(III) centre The reactions are largely dissociative and the central question concerns whether a 5-coordinate intermediate exists, and if so for how long Much of the evidence along these lines concerns the ability to generate a common set of products from related, but different, reactants A common set of products suggests an intermediate which exists long enough to come to equilibrium with its immediate environment, or possibly the bulk environment One difficulty here has been the inability to measure ion-pair constants for the reactant complexes and the products resulting from these ion-pairs under conditions of low ionic strength so that the results can be placed on a more firm theoretical footing To do this requires low ($< 10^{-2}$ mol dm^{-3}) substrate concentrations and low ($< 10^{-1}$ mol dm^{-3}) anion concen-

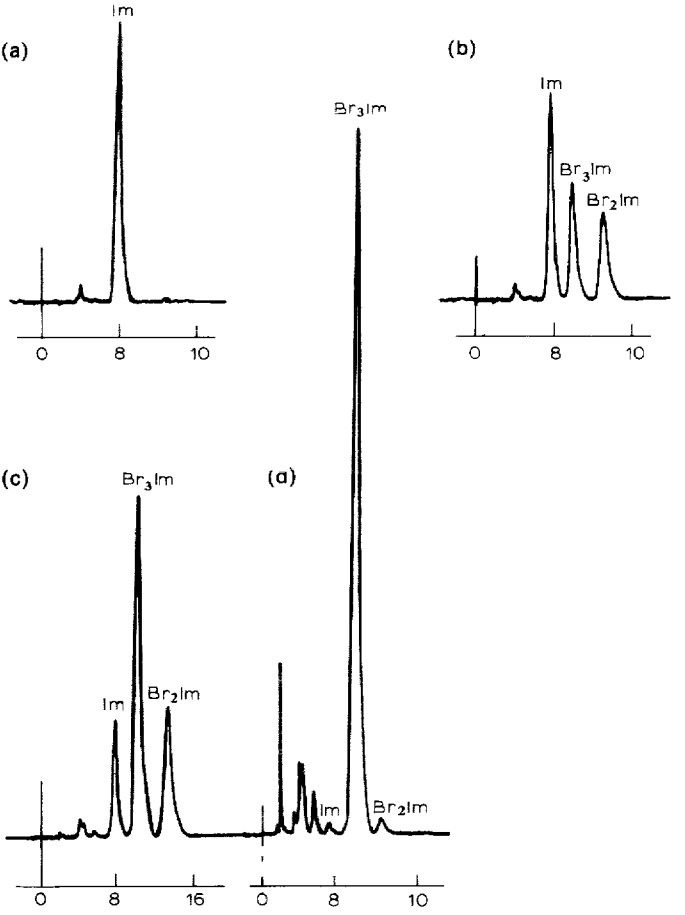


Fig 19 Samples (10 μl) of $[Co(NH_3)_5(imidazole)](ClO_4)_3$ (2 5 10^{-2} mol dm^{-3}) following addition of varying amounts of aqueous Br_2 (0 224 mol dm^{-3}) (a) 0 mol equiv , (b) 1 0 mol equiv , (c) 2 3 mol equiv , (d) 5 0 mol equiv (λ = 250 nm, a u f s = 0 5, 42% methanol, 25 m*M* hexanesulfonate (pH 3 4), flow-rate 2 cm^3 min^{-1})

trations and this means accurately determining different products in the *ca* 10^{-5} mol dm^{-3} range

RP-HPIPC allows such experiments to be carried out, with rapid separation of the various products and their accurate estimation Fig 20b shows the separation of the $(NH_3)_5CoNCS^{2+}$ and $(NH_3)_5CoSCN^{2+}$ ions resulting from SCN^- competition in the base hydrolysis of $(NH_3)_5CoONO_2^{2+}$, and Fig 20a a similar separation of the $(NH_3)_5CoONO^{2+}$ and $(NH_3)_5CoNO_2^{2+}$ isomers Fig 20c shows a separation of a $(NH_3)_5CoX^{2+}$ (X = NCS^-, SCN^-, Cl^-, Br^-) mixture, the latter separations have never been achieved by ion-exchange chromatography Fig 21 shows a chromatogram at 300 nm for the aquation of $(NH_3)_5CoOSO_2CF_3^{2+}$ in the presence of very low levels of N_3^- ($\leqslant$0 1 mol dm^{-3}) At these levels entry of N_3^- accounts for $<1\%$ of the product and it is clear that good sensitivity is achieved Clearly RP-HPIPC will allow the above studies to be carried out

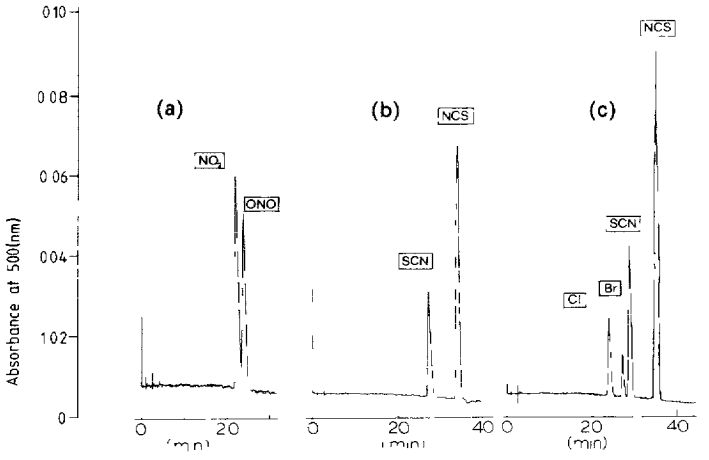


Fig 20 Chromatograms of isomeric $[Co(NH_3)_5X]^{2+}$ complex ions (a) X = NO_2^-, ONO^- (480 nmol, 15 μl), (b) X = SCN^-, NCS^- (320, 10) and (c) X = Cl^-, Br^-, SCN^- NCS^- (426, 20) Aqueous methanol gradient, 25 mM toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1}, λ = 480 nm

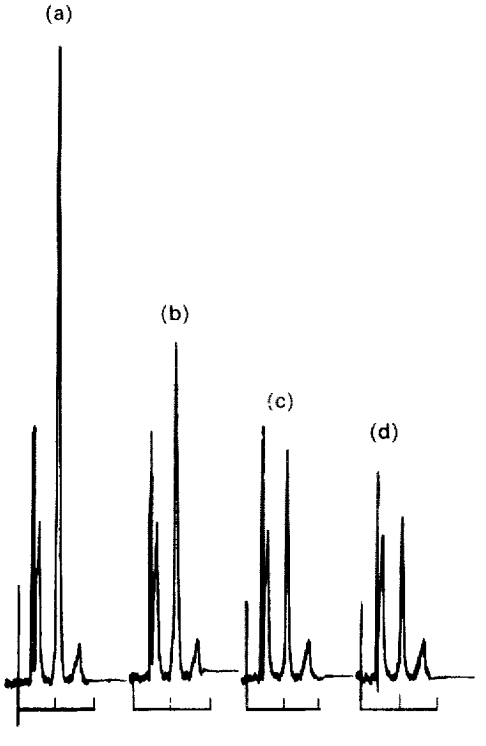


Fig 21 Samples (50 μl)following hydrolysis of $[Co(NH_3)_5OSO_2CF_3](CF_3SO_3)_2$ (6 26 10^{-3} mol dm^{-3}) in aqueous solutions of NaN_3 at pH = 10 7, I = 0 1 ($NaOSO_2CF_3$) (a) 0 10 mol dm^{-3} N_3^-, (b) 0 05 mol dm^{-3} N_3^-, (c) 0 033 mol dm^{-3} N_3^-, (d) 0 025 mol dm^{-3} N_3^- (λ = 300 nm, a u f s = 0 05, 42% methanol, 25 mM hexanesulfonate (pH 3 4), flow-rate, 2 cm^3 min^{-1})

11 A CONCENTRATION-DEPENDENT PEAK SPLITTING EFFECT

During the course of these studies a concentration-dependent splitting of peaks for single complex ions was observed We mention this here because we believe it to be a potential problem in any reversed-phase ion-pair chromatography experiment dealing with cationic complexes, the only way to be certain that separate peaks mean different species is to run them again separately after they have been separated to ensure that they have different k' values

Fig 22 shows the clear separation of optically pure Δ-$[Co(en)_2(AA)]^{2+}$ ions (AA = Gly, Pro, Val, Leu, Phe) On increasing the sample loading at constant ion-pair concentration (5 mM tosylate) the AA = Gly, Pro, peaks each split into two, with the earlier major component preceding, by progressively shorter retention times (t_R abnormal), the minor component which has the expected retention time (t_R normal) The splitting, Δt = t_R (normal) − t_R (abnormal), appears to be related to the total amount (moles) of complex loaded and not to an excess of any one species

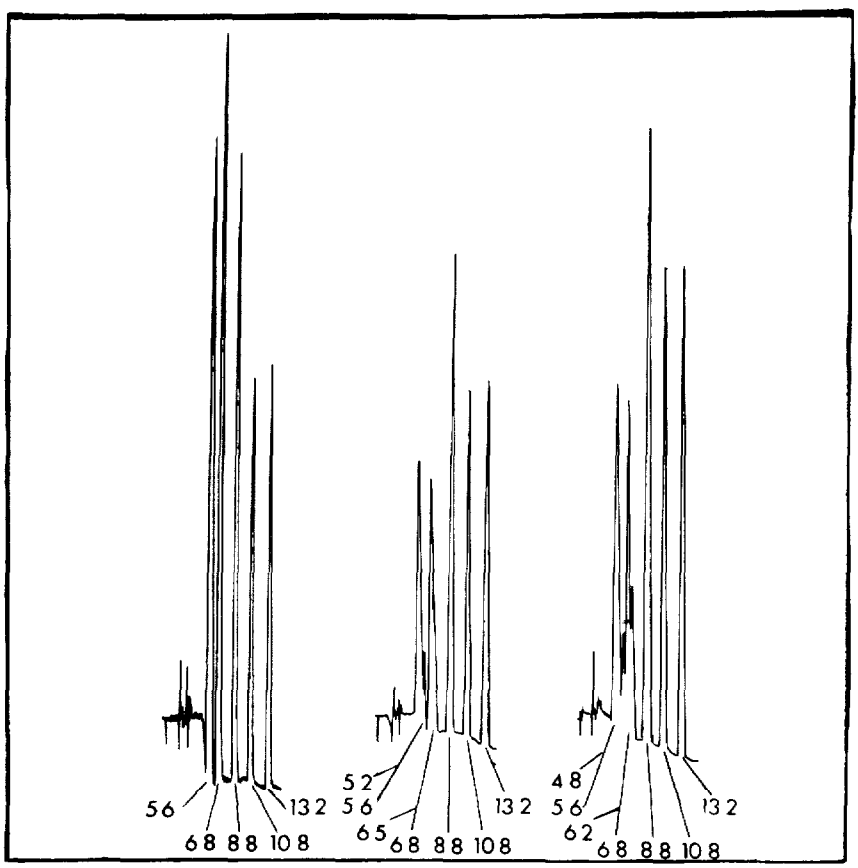


Fig 22 The effect of overloading a μBondapak C_{18}, 30-cm column with Δ-$[Co(en)_2AA]I_2$ complexes (AA = Gly, Pro, Val, Leu, Phe, in order of elution) 0–100% aqueous methanol gradient, 5 mM toluenesulfonate, flow-rate, 2 5 cm^3 min^{-1} (a) 367 nmol (10 μl), (b) 734 (20), (c) 1101 (30)

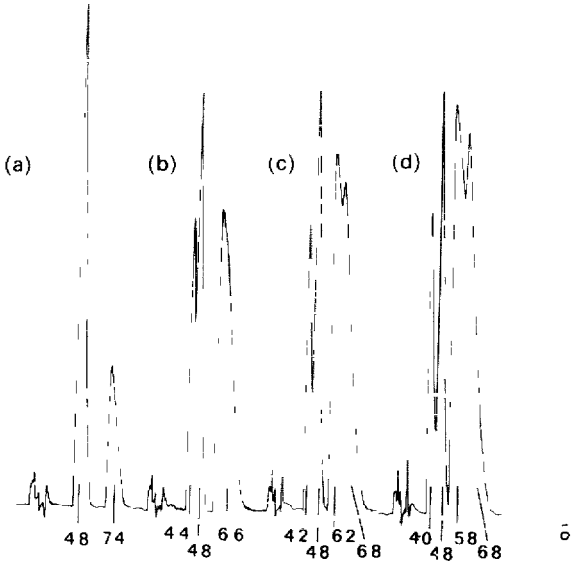


Fig 23 Elution of Λ-[Co(en)$_2$Gly]I$_2$, 438 nmol (30 μl) and Δ-[Co(en)$_2$Pro]I$_2$, x nmol (y, μl) (a) x = 270 (y = 5), (b) 1080 (20), (c) 1620 (30), (d) 2160 (40) 3 5% aqueous methanol, 5 mM toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1} λ = 480 nm

Thus, Fig 23 shows that increasing amounts of AA = Pro splits a constant loading of AA = Gly before the loading becomes sufficient to split itself This sharp separation is clearly distinguished from normal overloading shown in Fig 24 for AA = Gly by itself, here the broad asymmetric peak preceding a sharp spike shows overloading as it is often encountered when the buffer capacity of the ion-pair reagent is inadequate[28] Indeed, with the two complexes AA = Gly, Val, it is possible to

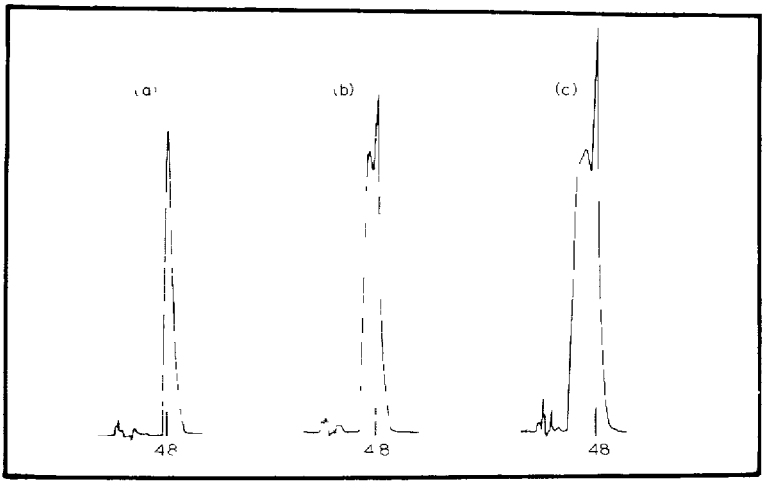


Fig 24 Overloading as it is normally observed with a one-component mixture, Λ-[Co(en)$_2$Gly]I$_2$ (a) 591 μmol (30 μl), (b) 1182 (60), (c) 1970 (100) 2 5% aqueous methanol, 5 mM toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1}

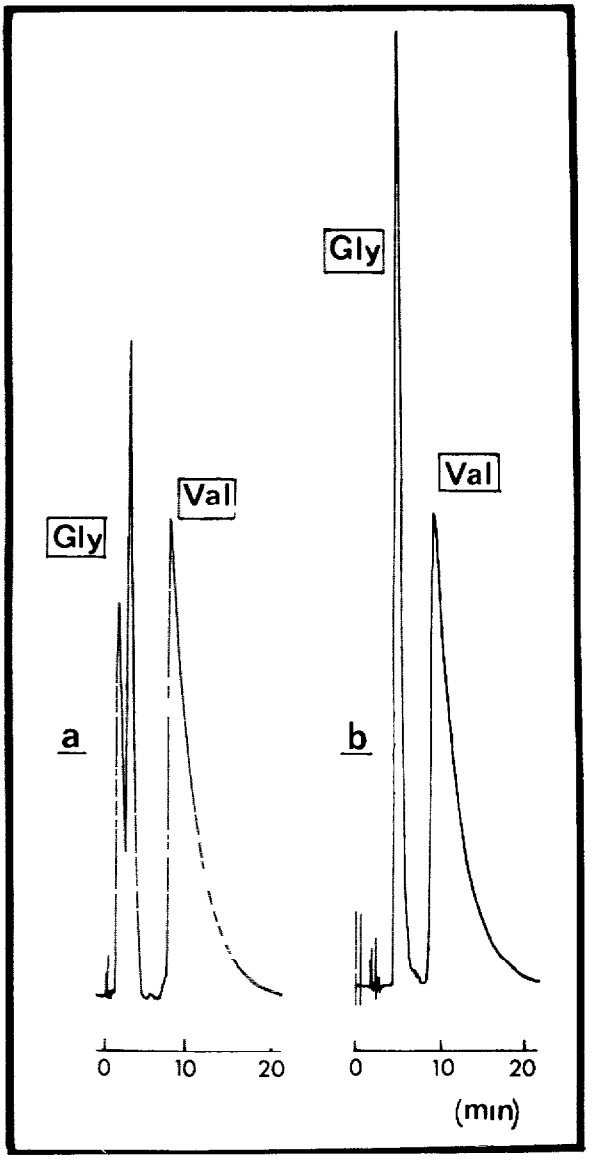


Fig 25 Overloading following injection of Δ-$[Co(en)_2Val]I_2$, 3605 nmol (35 μl) followed by Λ-$[Co(en)_2Gly]I_2$, 1090 (10), after (a) 10 sec, (b) 30 sec 2 5% aqueous methanol, 10 m*M* toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1}

split the AA = Gly peak into two by initially loading AA = Val followed after 10 sec by AA = Gly (Fig 25a) If the delay is 30 sec the AA = Gly complex still overtakes the AA = Val species on the column but now it is not split into two (Fig 25b) This experiment shows that the effect is not due to loading difficulties or that it need occur at the loading zone of the stationary phase, but is a property of the two complexes as they pass down the column However, it is an overloading phe-

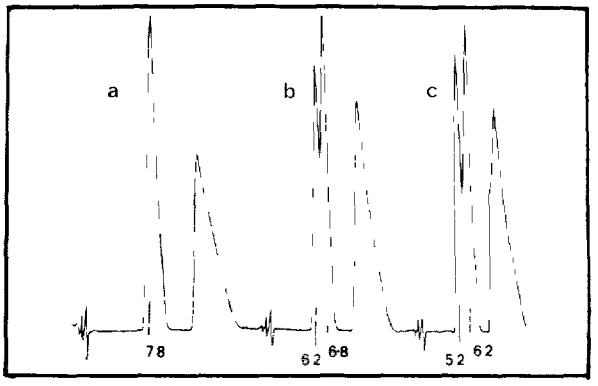


Fig 26 The effect of decreasing ion-pair reagent on peak splitting Λ-$[Co(en)_2Gly]I_2$ (1640 nmol), Δ-$[Co(en)_2Pro]Cl_2$ (1616 nmol), 80 μl (a) 30 mM, (b) 20 mM, (c) 15 mM toluenesulfonate 2·5% aqueous methanol, flow-rate, 2·0 cm^3 min^{-1}

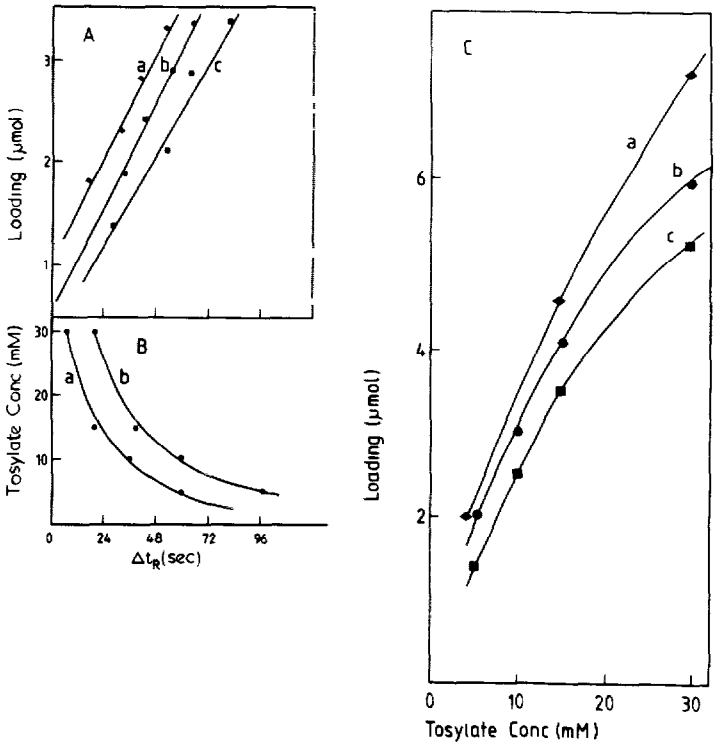


Fig 27 (A) Relationship between Δt_R (sec) and loading (μmol) using 10 mM toluenesulfonate (B) Relationship between Δt_R and ion-pair concentration (mM) with (a) 2000 nmol, and (b) 3000 nmol total loadings (C) Relationship between loading and [ion pair] for a constant Δt_R = 60 sec Data are for equal amounts of $[Co(en)_2AA]^{2+}$ [AA = Gly, Pro (●), AA = Ala, Val (■) and $[Co(NH_3)_5X]^{2+}$ (X = $OCOCH_3^-$, $OCOC_2H_5^-$) (◆)]

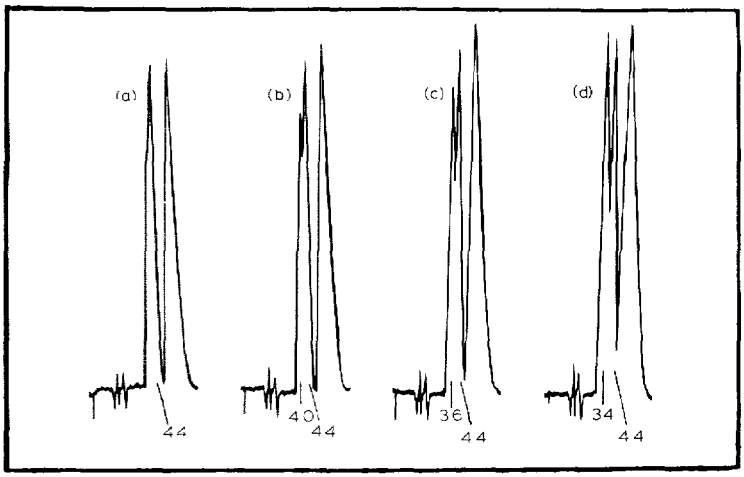


Fig 28 Peak splitting for a mixture of $[Co(NH_3)_5OCOCH_3](ClO_4)_2$ and $[Co(NH_3)_5OCOC_2H_5](ClO_4)_2$ complexes (a) 759 nmol (30 μl), (b) 886 (35), (c) 1012 (40), (d) 1265 (50) 2 5% aqueous methanol, 5 m*M* toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1}

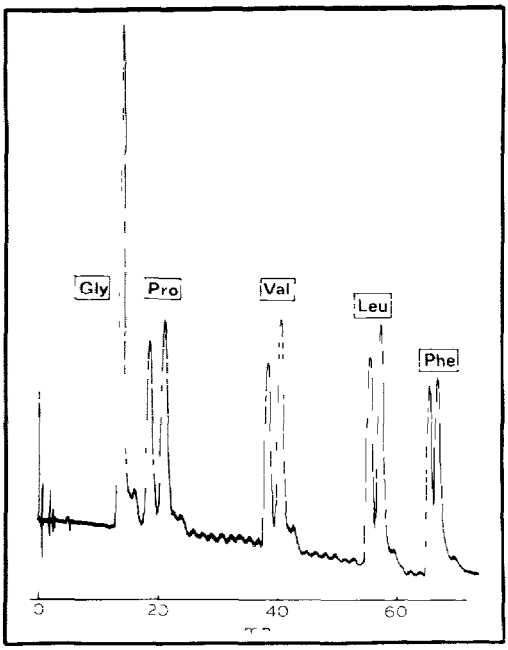


Fig 29 The effect of 0 25% $(C_2H_5)_3NH^+$ (pH 3 5) in the mobile phase is shown as a "shadow" following the peaks for a Δ,Λ-$[Co(en)_2AA]I_2$ mixture (810 nmol), aqueous methanol gradient, 25 m*M* toluenesulfonate, flow-rate, 2 0 cm^3 min^{-1}, λ = 480 nm

nomenon since it only occurs under conditions of high complex concentration in the matrix of the stationary phase (When R C columns are used, where the sample is distributed over a larger surface area at the loading zone, the effect has not been observed even with high loadings) Splitting can also be observed if lower ion-pair concentrations are used Thus decreasing the tosylate concentration from 30 to 15 mM for a fixed loading of AA = Gly (1640 nmol) Pro (1616 nmol) results in the splitting of the AA = Gly peak and a slight broadening of the AA = Pro peak (Fig 26)

The splitting Δt_R increase linearly with sample loading (n in nmoles) but decreases non-linearly with ion-pair concentration (Fig 27), and there is a non-linear correlation between n and [ion pair] for a given Δt_R (60 sec) Similar correlations hold for different combinations of complexes From such results we devised the working rule that for a methanol–water (2 5 97 5) mobile phase containing [ion pair] <30 mM peak splitting can be prevented for 2+ ions (*i e* $\Delta t_R < 12$ sec) if n/[ion pair] < 100 This useful guide however changes for different solvent–ion pair combinations

Fig 28 shows that this phenomenon is not restricted to chiral complexes with increasing amounts of $[Co(NH_3)_5X]^{2+}$ (X = $CH_3CO_2^-$, $C_2H_5CO_2^-$) giving rise to splitting of the X = $CH_3CO_2^-$ peak Also the presence of both NH_4^+ or $(C_2H_5)_3NH^+$ in the mobile phase can result in broadening or "shadow" peaks (Fig 29), and care needs to be exercised with R C columns in this respect since we found the presence of $(C_2H_5)_3NH^+$ to be beneficial in other respects with low coating packings

Δt_R was found to be independent of the concentration of the injected sample, whether it was introduced as the I^-, Cl^-, $CH_3CO_2^-$ or ClO_4^- salt, and of the ratios of the various complexes making up the sample This last point is demonstrated by Fig 30 where the AA = Val Λ-Gly ratio changes from 0 25 to 0 04 but Δt_R remains

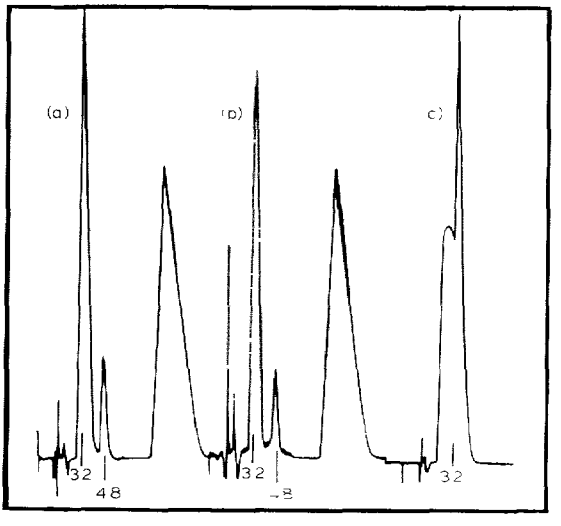


Fig 30 The effect of different ratios in a binary mixture of Λ-$[Co(en)_2Gly]I_2$ (x, nmol) and Λ-$[Co(en)_2Val]I_2$ (y, nmol), (a) x = 511, y = 2065 (b) 102, 2479, (c) 2410 2 5% aqueous methanol, 5 mM toluenesulfonate, flow-rate 2 0 cm^3 min^{-1}

the same at 1 6 min The complete separation of the two Λ-Gly peaks in this experiment allowed them to be recovered and analysed Following desalting on Sephadex SP-C25, optical rotations gave $[M]_{589}$ = (1 45 ± 0 03) × 10^3, (1 48 ± 0 03) × 10^3 deg M^{-1} cm^{-1} which is to be compared with that for Λ-$[Co(en)_2(Gly)]^{2+}$ of (1 45 ± 0 03) × 10^3 deg M^{-1} cm^{-1} Fig 30c shows the chromatogram with the same total loading of AA = Λ-Gly by itself and comparison with the others shows the clear difference between the two types of overloading phenomena

12 SUMMARY

These experiments show that there is some sort of interaction between two (or more) complex ions which influence their distribution ratios with the stationary phase and with the ion-pair reagent This is in addition to the ion-pair depletion effect noted previously by others at high sample concentrations These interactions lead to species of different counterion stoichiometry, or geometry, which have sufficient lifetime on the matrix of the stationary phase to allow their separation as discrete entities This would require abnormally slow rates for the establishment of cation–ion pair–stationary phase equilibria,

13 ACKNOWLEDGEMENTS

My colleague Dr C R Clark and our students Drs R F Tasker and W S Webley, and Messrs M M Deva, G S Binney, G M Miskelly, W Fairbrother and Miss M Gaudin have all contributed to the development work described in this article

REFERENCES

1 B R Willford and H Veening, *J Chromatogr*, 251 (1982) 61
2 B D Warner and J I Legg, *Inorg Chem*, 20 (1981) 1625
3 G Grassini-Strazza and C M Polcaro, *J Chromatogr*, 147 (1978) 516
4 S J Valenty and P E Behnken, *Anal Chem*, 50 (1978) 834
5 S S Isied J Lyon, A Vassilian and G Worosila, *J Liq Chromatogr*, 5 (1982) 537
6 S S Isied, A Vassilian and J M Lyon, *J Am Chem Soc*, 104 (1982) 3910
7 G H Searle, *Aust J Chem*, 30 (1977) 2625
8 D A Buckingham, C R Clark, R F Tasker and M T W Hearn, *J Liq Chromatogr*, 4 (1981) 689
9 D A Buckingham, C R Clark, M M Deva and R F Tasker, *J Chromatogr*, 262 (1983) 219
10 M T W Hearn, B Grego and W S Hancock, *J Chromatogr*, 185 (1979) 429
11 Cs Horváth, W Melander, I Molnar and P Molnar, *Anal Chem*, 49 (1977) 2295
12 P T Kissinger, *Anal Chem*, 49 (1977) 883
13 D A Buckingham and J P Collman, *Inorg Chem*, 6 (1967) 1803
14 I Molnar and Cs Horvath, *J Chromatogr*, 142 (1977) 623
15 C Gilan, R Lesham, Y Tapuhi and E Grushka, *J Am Chem Soc*, 101 (1979) 7612
16 C Gilan, R Lesham and E Grushka, *Anal Chem*, 52 (1980) 1206
17 P E Hare and E Gil-Av, *Science*, 204 (1979) 1226
18 V A Davankov and A V Semechkin, *J Chromatogr*, 141 (1977) 313
19 B Lefebure, R Audebert and C Quivoron, *J Liq Chromatogr*, 1 (1978) 761
20 G Dotsevi, J Sogah and D J Cram, *J Am Chem Soc*, 98 (1976) 3038
21 J N Le Page, W Lindner, G Davies, D E Seitz and B L Karger, *Anal Chem*, 51 (1979) 433
22 D A Buckingham, C R Clark, R Tasker, D Knighton, D Harding and W S Hancock, *J Am Chem Soc*, 103 (1981) 7023

23 D A Buckingham, M Dwyer, G J Gainsford, V Janson Ho, L G Marzilli, W T Robinson, A M Sargeson and K R Turnbull, *Inorg Chem*, 14 (1975) 1739
24 D A Buckingham, P J Cresswell, R J Dellaca, M Dwyer, G J Gainsford, L G Marzilli, I E Maxwell, W T Robinson, A M Sargeson and K R Turnbull, *J Am Chem Soc*, 96 (1974) 1713
25 D A Buckingham, G M Miskelly, C R Clark and J Simpson, *Inorg Chem*, 22 (1983) 3237
26 D A Buckingham, I I Creaser, W Marty and A M Sargeson, *Inorg Chem*, 11 (1972) 2738
27 D A Buckingham, C R Clark and W S Webley, *Aust J Chem*, 33 (1980) 263
28 E Tomlinson, T M Jefferies and C M Riley, *J Chromatogr*, 159 (1978) 315