CH-Acidity of Amino-acid Esters co-ordinated to Cobalt(III). Racemization during the Formation of Peptides

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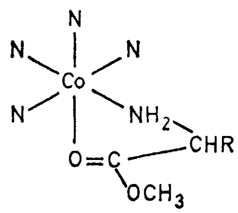
Several dipeptide complexes of general formula $[Co(tren)(NH_2CHRCONHCHR'COOCH_3)][CH_3C_6H_4SO_3\text{-}p]_3$ have been obtained by coupling a chelated ester complex with a free amino-acid ester. The rate of the reaction appears to be quite sensitive to steric hindrance. With chiral amino-acids, the product is a mixture of diastereomers; this result is consistent with a fast racemization coupled with a slight asymmetric induction. The half-life for proton exchange in the complex $[Co(en)_2(GlyOCH_3)][CH_3C_6H_4SO_3\text{-}p]_3$ under the conditions of peptide synthesis is 20 s. The hydrolysis of this ester in methanol (1 mol dm^{-3} in H_2O) is nevertheless faster than racemization.

SEVERAL years ago, a new method of peptide synthesis based on the activation of amino-acid esters by co-ordination to cobalt(III) complexes was proposed independently by two groups.[1,2] This new principle



(I)

appeared at the time to offer exciting new possibilities as a method of peptide synthesis. The cobalt(III) ion was shown to be both amino-protecting and carboxyl-activating; because the activation does not require a good leaving alcohol, the reactions could easily be run in hydroxylic solvents where a peptide would be reasonably soluble; it is likely that the synthesis of a peptide would require less side-chain protection than in other classical methods.

So far, however, no paper on the application of this system has appeared, among other reasons, because the synthesis of the crucial intermediate, the chelated ester (I) (N_4 = any amine or polyamine ligands) was easy only for the glycine derivative.

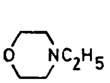
Now that chelated esters from optically active amino-acids are available,[3] several questions can be answered to appreciate the scope of this method of peptide synthesis: (i) can the coupling with a second amino-acid be effected while avoiding the racemization, (ii) is the reaction still reasonably fast when trying to couple two sterically hindered amino-acids, and (iii) if the answer to the first two questions is favourable, can the newly formed peptide complex be decomposed without at the same time hydrolyzing the peptide bond?

RESULTS

Steric Effects on the Rates of Condensation.—The dipeptides have been prepared according to the following sequence of reactions: synthesis (1) of the amino-acid complex (tren = 2,2′,2″-triaminotriethylamine), followed by exchange of the chloride counter ion for toluene-*p*-sulphonate, then 'one-pot' esterification (2) and condensation (3). Several reactions have been run with chelated amino-acid esters and free amino-acid esters of increasing bulkiness to determine the sensitivity of the reaction to steric hindrance. The percentage of cobalt complex transformed into a dipeptide was determined by chromatography of the reaction mixture (first diluted with an equal volume of water to hydrolyze the ester remaining at the end of the run) on an Amberlite IRC 50 column. The results are collected in

$$[Co(tren)Cl_2]Cl + NH_3^+CHRCOO^- \xrightarrow[4\,h/50\,°C]{pH\ 7{\cdot}5} [Co(tren)(NH_2CHRCOO)]Cl_2 \quad (1)$$

$$[Co(tren)(NH_2CHRCOO)][CH_3C_6H_4SO_3\text{-}p]_2 + [NH_3^+CHR'COOH][CH_3C_6H_4SO_3\text{-}p]$$
$$\xrightarrow[(MeO)_2SO]{CH_3C_6H_4SO_3H\text{-}p/MeOH} [Co(tren)(NH_2CHRCOOCH_3)][CH_3C_6H_4SO_3\text{-}p]_3 + [NH_3^+CHR'COOCH_3][CH_3C_6H_4SO_3\text{-}p] \quad (2)$$



$$\xrightarrow[pH\ ca.\ 7{\cdot}5]{} [Co(tren)(NH_2CHRCONHCHR'COOCH_3)][CH_3C_6H_4SO_3\text{-}p]_3 \quad (3)$$

Table 1. It appears that for the amino-acids other than glycine, the reaction time has to be increased to transform all the complex into a dipeptide; when the two amino-acids were bulky (Leu-Val) * only about 30% of the ester had reacted after 15 h at 50 °C. The chelated dipeptide esters were characterized by i.r. and ^{13}C n.m.r. spectroscopy and elemental analysis for the Ala-LeuOCH_3 complex used for the racemization studies.

TABLE 1

Experimental conditions and yields for the synthesis of the chelated dipeptides [a]

Complex	Reaction time/min	θ/°C	Yield (%)
$[Co(tren)(Gly\text{-}GlyOCH_3)]^{3+}$ [b]	10	r.t. [c]	99
$[Co(tren)(Gly\text{-}L\text{-}LeuOCH_3]^{3+}$	60	r.t.	99
$[Co(tren)\{Gly\text{-}L\text{-}Asp(OCH_3)_2\}]^{3+}$	90	r.t.	99
$[Co(tren)(Ala\text{-}L\text{-}LeuOCH_3)]^{3+}$	60	r.t.	95
	900	r.t.	99
$[Co(tren)(Ala\text{-}L\text{-}HisOCH_3)]^{3+}$	90	35	99
$[Co(tren)(Leu\text{-}L\text{-}AlaOCH_3)]^{3+}$	75	45	75
	900	r.t.	95
$[Co(tren)(Leu\text{-}L\text{-}ValOCH_3)]^{3+}$	900	50	30

[a] Yield determined by separation of the cobalt complexes in the reaction mixture after hydrolysis of the chelated esters on an ion-exchange column. [b] Product identical to that obtained by chelation of $[Co(tren)(Gly\text{-}GlyOCH_3)Cl]Cl_2$ with $Ag[p\text{-}O_3SC_6H_4CH_3]$. [c] r.t. = Room temperature.

Decomposition of the Complexes.—The dipeptide-ester complexes were first decomposed according to modifications of procedures developed for the decomposition of amino-acid complexes: reduction with $Na[BH_4]$ between pH 8 and 9 (ref. 4) and reaction with Na_2S [5] in acetate buffer. However, we have found that ' Jones reagent ' (prepared by reaction of an aqueous solution of $HgCl_2$ with 20—30 mesh granulated zinc) can perform the same task under cleaner conditions. The reduction takes place in acidic solution (0.1 mol dm^{-3} HCl) so that no racemization can occur during the decomposition of the complex. The dipeptide esters recovered are easily separated from the other constituents of the mixture by ion-exchange chromatography. They were characterized by i.r. and ^{13}C n.m.r. (Gly-GlyOCH_3 and Ala-LeuOCH_3) or shown to be identical with a material prepared by classical methods (Gly-GlyOCH_3). Because the dipeptides are extensively racemized, no further characterization was undertaken.

Racemization.—The extent of racemization during peptide synthesis was measured on the product of coupling of $[Co(tren)(AlaOCH_3)]^{3+}$ with L-leucine methyl ester. Two complementary peptide syntheses were run [reactions (4) and (5)]. The dipeptide complexes were isolated, characterized by i.r. and ^{13}C n.m.r. spectroscopy, and decomposed.

$$[Co(tren)(L\text{-}AlaOCH_3)]^{3+} + L\text{-}LeuOCH_3 \longrightarrow [Co(tren)(Ala\text{-}L\text{-}LeuOCH_3)]^{3+} \quad (4)$$

$$[Co(tren)(D\text{-}AlaOCH_3)]^{3+} + L\text{-}LeuOCH_3 \longrightarrow [Co(tren)(Ala\text{-}L\text{-}LeuOCH_3)]^{3+} \quad (5)$$

The dipeptides were then hydrolyzed in HCl (6 mol dm^{-3}). The total amino-acid content after hydrolysis was checked with the ninhydrin reagent and the D-amino-acid in the mixture determined by oxidation with a D-amino-acid oxidase. Because the second amino-acid (leucine) is very unlikely to racemize under the conditions of the experiment, the percentage of D-amino-acid obtained corresponds to the racemization of alanine. The dipeptide derived from $[Co(tren)(L\text{-}AlaOCH_3)]^{3+}$ contains 53% of D-alanine; the one derived from $[Co(tren)(D\text{-}AlaOCH_3)]^{3+}$, 55%.

These results are confirmed by a close inspection of the ^{13}C n.m.r. spectra (data given in the Experimental section) where it is seen that several peaks are split; from the ratios of intensities, the percentage of the diastereomers in the mixture is obtained. The results are collected in Table 2, together with data on the Ala-His, Leu-Ala, and Leu-Val complexes. It is clear that extensive racemization has taken place in each case.

Further experiments have been conducted to determine whether some racemization takes place during the formation of the amino-acid complex (step 1) and/or its esterification (step 2). The complex directly derived from the coupling of L-alanine with $[Co(tren)Cl_2]Cl$, or recovered after a cycle of esterification and hydrolysis of the ester, was decomposed, the amino-acid recovered, and the percentage of D-amino-acid measured as above. Within the limits of experimental error, no racemization was detected after the first step; however, after esterification and hydrolysis, the sample contained 2% of D-alanine.

TABLE 2

Percentage of predominant diastereomer as determined from ^{13}C n.m.r. measurements

Complex	Origin	% Predominant diastereomer [a]	Ratio of diastereomers
$[Co(tren)(Ala\text{-}L\text{-}LeuOCH_3)]^{3+}$	$[Co(tren)(L\text{-}AlaO)]^{2+}$	52.6 ± 2.4 [b]	1.11
$[Co(tren)(Ala\text{-}L\text{-}LeuOCH_3)]^{3+}$	$[Co(tren)(D\text{-}AlaO)]^{2+}$	53.3 ± 1.5 [b]	1.14
$[Co(tren)(Ala\text{-}L\text{-}HisOCH_3)]^{3+}$	$[Co(tren)(L\text{-}AlaO)]^{2+}$	53.8 ± 2.4	1.16
$[Co(tren)(Leu\text{-}L\text{-}AlaOCH_3)]^{3+}$	$[Co(tren)(L\text{-}LeuO)]^{2+}$	53.6 [c]	1.15
$[Co(tren)(Leu\text{-}L\text{-}ValOCH_3)]^{3+}$	$[Co(tren)(L\text{-}LeuO)]^{2+}$	58.0 ± 2.0	1.38

[a] Mean measured from the ratios of intensities of the two parts of the split peaks (both Co and both C; see Experimental section). [b] D-Ala-L-LeuOCH_3 is the predominant isomer. [c] Measured only on one split peak because the separation in the others was too small.

Finally, the rate of exchange of the CH_2 protons of glycine has been measured directly for the complex $[Co(en)_2(GlyOCH_3)]^{3+}$. The esterification was run in CH_3OD, then the pH was brought to 9.8 † with *N*-ethylmorpholine (final concentration 3 mol dm^{-3}); after a few seconds (between 6 and 30) the exchange reaction was stopped simply by addition of water. This method is effective because it is known from the previous experiments that the hydrolysis is faster than the proton exchange.

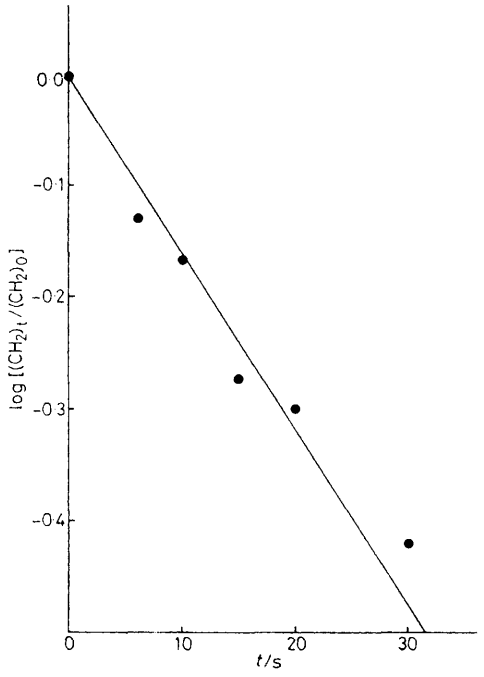
The percentage of non-exchanged protons was determined by ^1H n.m.r. by relative integration of the CH_2 signal of glycine and CH_2 signal of the ethylenediamines. The point at zero time was obtained by running the reaction for 20 s in CH_3OH. The results are presented as a plot of $\log[(CH_2)_t/(CH_2)_0]$ as a function of time (Figure). Under

* See footnote on the title page of the preceding paper.

† pH 9.8 and 11.4 in pure methanol correspond to approximately 7.8 and 9.8 respectively in 50% MeOH–H_2O (see ref. 12).

the conditions of the experiment, the half-life for exchange was 19 s.

For comparison, the rate of exchange of the CH_2 protons in the complex $[Co(en)_2(GlyO)][CH_3C_6H_4SO_3\text{-}p]_2$ in a NMe_3–$(CH_3C_6H_4SO_3\text{-}p)NMe_3^+$ buffer at pH 11.4 * was measured by monitoring the disappearance of the CH_2 signal in CD_3OD at 25 °C; the half-life was 253 h.



Exchange of the methylene protons (in methanol, 3 mol dm^{-3} *N*-ethylmorpholine buffer at pH 9.8) for $[Co(en)_2(GlyOCH_3)][CH_3C_6H_4SO_3\text{-}p]_3$ at 25 °C. Half-life for exchange = 19 s

DISCUSSION

A definite answer can be given to the questions raised in the introduction: although it appears that the release of the dipeptide unit from the complex can be performed easily without significant hydrolysis, the scope of this method of activation for the synthesis of peptides is very limited for two reasons. First, the rate of the reaction appears to be even more sensitive to steric hindrance than in conventional methods of peptide synthesis. Second, the racemization is complete despite the fact that the pH of the reaction mixture was kept rather low by using *N*-ethylmorpholine instead of triethylamine to maintain the basic conditions necessary for coupling. This second observation renders the method totally unsuitable except in special cases where the first amino-acid could be protected against racemization by anchoring the side chain itself to the cobalt centre. This could be possible for cysteine and histidine derivatives.

For the racemization study, we have worked with complexed derivatives of $[Co(tren)Cl_2]Cl$, because the Co(tren) moiety contains a plane of symmetry. Had we worked with complexes in which the amino-acid is attached to $Co(en)_2$ or Co(trien) units, the interpretation of the results would have been less straightforward because the asymmetric cobalt centre would be likely to interact with the asymmetric carbon on the amino-acid (aa). In fact, epimerization of chelated amino-acids leads to equilibrium mixtures $\Delta[Co(en)_2(\text{D-aa})]/\Delta[Co(en)_2(\text{L-aa})] = 1.0 \pm 0.2$ and 1.7 ± 0.14 for alanine and valine respectively.[6] When a chiral polyamine ligand is used, even larger isomer ratios can be obtained.[7] From this work, there appears to be a slight interaction between the two asymmetric centres of the dipeptide; it remains weak, however, as the diastereomer ratios remain below 1.5. This ratio can in fact be either the thermodynamic equilibrium if the first amino-acid is able to epimerize after the dipeptide is formed or a kinetically controlled ratio if it results from a combination of a fast epimerization of the ester and a slight preference of the L-free ester for the D-complex, the epimerization in the dipeptide being very slow. The experimental observations reported do not allow the question to be settled, but it is clear that the epimerization of the ester is certainly fast enough compared to the rate of coupling for the second mechanism to play a role.

A second-order rate constant for the methoxide-ion catalyzed exchange of the methylene protons in $[Co(en)_2(GlyO)][CH_3C_6H_4SO_3\text{-}p]_2$ can be calculated from the pH and the ionic product of methanol: $K_{MeOH} = 10^{-15.9}$ mol dm^{-3} (ref. 8), $k_{calc.} = 2.2 \times 10^{-2}$ dm^3 mol^{-1} s^{-1}. This value is similar to the rate constant reported by Buckingham *et al.*[6] for the exchange of the methine protons in $[Co(en)_2(ValO)]^{2+}$ and $[Co(en)_2(AlaO)]^{2+}$ complexes in water [(2.4—5.4) $\times 10^{-2}$ at 34.3 °C]. After correction for the difference in the pH of the measurements, the rate of the exchange in the ester is 1.9×10^6 times larger. The comparison, however, should not be taken too literally, because due to experimental limitations, the data on the ester had to be obtained at high buffer concentration and the basic form of the buffer can catalyze the deprotonation.

A few experiments have been run to try to take advantage of the increased acidity of the methylene after esterification. We have tried to condense aldehydes or benzyl bromide with the ester complex under slightly basic conditions. At this stage, however, this system does not appear to offer any advantage over the more simple method, condensation of the non-esterified amino-acid complex under basic conditions,[9] presumably because the chelated ester is more sensitive than the acid to side-reactions like disproportionation.

EXPERIMENTAL

Formation of the Dipeptides.—The amino-acid complexes were obtained as described in the previous paper.[3] The esterification and the formation of the peptide were run as a 'one-pot' reaction. The reaction was conducted in a 25-cm^3 two-necked flask bearing a rubber septum and a condenser connected to a solid-NaOH trap, followed by a sulphuric-acid trap. The reagents were introduced: $[Co(tren)(aa)][CH_3C_6H_4SO_3\text{-}p]_2\cdot H_2O$ (10^{-3} mol), the second amino-acid

* pH 9.8 and 11.4 in pure methanol correspond to approximately 7.8 and 9.8 respectively in 50% MeOH–H_2O (see ref. 12).

(1.5×10^{-3} mol), toluene-*p*-sulphonic acid (6×10^{-3} mol), methanol (3.5 g), and dimethyl sulphite (3.5 g, 3×10^{-2} mol). After 3 h of esterification at 70 °C, *N*-ethylmorpholine (2.5 cm^3) previously dried and distilled on $Li[AlH_4]$ was added. At the end of the reaction, the mixture was diluted (1 : 1) with water and charged on an Amberlite IRC 50 column (Na form). It was eluted first with a solution 0.4 mol dm^{-3} in NaCl. After the elution of the excess of the second amino-acid, the *N*-ethylmorpholine and the doubly charged complex $[Co(tren)(aa)]^{2+}$, the column was washed with water and the chelated dipeptide removed with 0.1 mol dm^{-3} HCl. The complexed dipeptide solution was evaporated to dryness. The complex was taken up in methanol, the NaCl filtered out, and evaporated twice to dryness. The i.r. spectra of all the complexed dipeptides show peaks at 1 740 cm^{-1} (non-co-ordinated ester), 1 630 cm^{-1} (co-ordinated amide), 1 575 cm^{-1} (NH_2 bending). The complexes were further characterized by their ^{13}C n.m.r. spectra (shifts in p.p.m. from 4,4-dimethyl-4-silapentanesulphonate). $[Co(tren)(Gly\text{-}LeuOCH_3)]Cl_3$: CH_2(tren), 61.3, 58.3, 44.6, 44.1; CO(Gly), 181.1; CH_2(Gly), 47.6; CO(Leu), 172.2; CH^{α}, 52.5; $CH^{\beta}{}_2$, 38.4; CH^{γ}, 23.4; $CH^{\delta}{}^{+}{}^{\delta'}{}_3$, 21.0 and 20.1; OCH_3, 52.8. $[Co(tren)\{Gly\text{-L-}Asp(OCH_3)_2\}]Cl_3$: CH_2(tren), 61.5, 58.4, 44.8, 44.4; CO(Gly), 181.3; CH_2(Gly), 48.0; CO(Asp), 172.8 and 171.5; CH^{α}, 50.0; $CH^{\beta}{}_2$, 34.3; OCH_3, 52.9 and 52.0. $[Co(tren)(Ala\text{-L-}LeuOCH_3)]Cl_3$: CH_2(tren), 61.5, 58.4, 45.1, 44.5; CO(Ala) (split), 183.5 and 182.8; CH, 55.9; CH_3 (split), 18.4 and 18.1; CO(Leu) (split), 172.2 and 172.0; CH^{α}, 53.3; $CH^{\beta}{}_2$ (split), 38.8 and 38.1; CH^{γ} (split), 23.6 and 23.4; $CH^{\delta}{}^{+}{}^{\delta'}{}_3$, 21.3 and 20.8; OCH_3, 52.9 (Found: C, 35.1; H, 7.45; N, 15.45. $C_{16}H_{40}Cl_3CoN_6O_6{\cdot}H_2O$ requires C, 35.2; H, 7.4; N, 15.4). $[Co(tren)(Ala\text{-L-}HisOCH_3)]Cl_3$: CH_2(tren), 61.5, 58.4, 44.8, 44.5; CO(Ala) (split), 184.1 and 183.7; CH, 56.1; CH_3 (split), 18.3 and 18.0; CO(His) (split), 170.6 and 170.4; CH, 53.5; CH_2 (split), 24.8 and 24.6; ring, 132.9, 126.6, and 117.0; OCH_3, 53.3. $[Co(tren)(Leu\text{-L-}AlaOCH_3)]Cl_3$: CH_2(tren), 61.2, 58.3, 44.7, 44.3; CO(Leu) (split), 183.6 and 183.1; CH^{α}, 58.1; $CH^{\beta}{}_2$ (split), 40.4 and 39.9; CH^{γ}, 23.2; $CH^{\delta}{}^{+}{}^{\delta'}$, 21.9 and 19.1; CO(Ala) (split), 173.8 and 173.6; CH, 55.6; CH_3 (split), 18.6 and 18.3; OCH_3, 52.6. $[Co(tren)(Leu\text{-L-}ValOCH_3)]Cl_3$: CH_2(tren), 61.4, 58.3, 44.9, 44.2; CO(Leu) (split), 184.4 and 183.8; CH^{α}, 58.0; $CH^{\beta}{}_2$ (split), 40.6 and 40.1; CH^{γ}, 23.4; $CH^{\delta}{}^{+}{}^{\delta'}{}_3$, 21.8 and 18.6; CO(Val) (split), 171.6 and 171.1; CH^{α}, 48.2; CH^{β} (split), 29.7 and 28.8; $CH^{\delta}{}^{+}{}^{\delta'}{}_3$, 17.4 and 17.2; OCH_3, 52.4.

Decomposition of the Complexes.—(*a*) *Preparation of the reductor* (*Jones reagent*). Mercury(II) chloride (2.7 g) dissolved in water (100 cm^3) was added to granular zinc (32.5 g, 30 mesh). The mixture was stirred for 3 min and rinsed with water.

(*b*) *Reduction of the complexes and recovery of the dipeptides.* A portion of complex (10^{-3} mol) dissolved in HCl (15 cm^3, 0.1 mol dm^{-3}) was added on the reductor and stirred for 10 min. The solution turns rapidly from orange to rose; it was then charged on a Dowex 50W × 8 column (20 × 1.5 cm). After washing with water, the dipeptide was eluted with NaCl (0.1 mol dm^{-3})–HCl (0.01 mol dm^{-3}). The Ala-L-$LeuOCH_3{\cdot}HCl$ dipeptide was characterized by ^{13}C n.m.r.: CO(Ala), 173.8; CH (split), 48.2 and 48.0; CH_3, 15.8; CO (L-Leu), 170.0; CH^{α} (split), 50.7 and 50.6; $CH^{\beta}{}_2$, 38.2; CH^{γ}, 23.5; $CH^{\delta}{}^{+}{}^{\delta'}{}_3$, 21.2 and 9.6; OCH_3, 52.1.

Determination of the Racemization.—The dipeptide Ala-L-$LeuOCH_3$ derived from $[Co(tren)(L\text{-}AlaO)][CH_3C_6H_4SO_3\text{-}p]_2$ and $[Co(tren)(D\text{-}AlaO)][CH_3C_6H_4SO_3\text{-}p]_2$ were hydrolyzed in 6 mol dm^{-3} HCl (reflux, 7 h). After evaporation to dryness, the amino-acids were taken up in water, neutralized and the solutions brought to 100 cm^3. The total amino-acid concentration in the sample was checked by an analysis with ninhydrin according to the method of Moore.[10] The percentage of D-amino-acid in the sample was measured by stereospecific oxidation with a D-amino-acid oxidase according to Walsh *et al.*:[11] in a test tube were incubated (at 30 °C), 1 cm^3 of sample solution, pyrophosphate buffer (0.975 cm^3, 0.05 mol dm^{-3}, pH 8.5), D-amino-acid oxidase (20 μl, Boehringer lot No. 1426117, crystalline suspension in 3.2 mol dm^{-3} ammonium sulphate, 15 units per mg of protein), and catalase (5 μl, Boehringer lot No. 15005168, crystalline suspension in water, 65 000 units per mg of protein). After 90 min, 250 μl of that solution were added to 2.5 cm^3 of a solution of thiosemicarbazide solution in acetate buffer (0.91 g in 100 cm^3 of 0.1 mol dm^{-3} acetate at pH 5.4). After 30 min at 30 °C, the absorbance at 285 nm was read against a blank. The D-amino-acid content was determined by comparison with a calibration curve obtained on 8 samples of known composition of DL-alanine, $\Delta\varepsilon = (1.86 \pm 0.02) \times 10^4$ dm^3 mol^{-1} cm^{-1}.

Determination of the Rate of Exchange of the Methylene Protons.—(*a*) *In* $[Co(en)_2(GlyOCH_3)][CH_3C_6H_4SO_3\text{-}p]_3$. The complex (0.16 mmol) was dissolved with dimethyl sulphite (0.41 g, 3.8 mmol) and $CH_3C_6H_4SO_3H{\cdot}H_2O$ (0.18 g, 0.95 mmol) in 4 cm^3 of CH_3OD. After 2 h of refluxing, the mixture was cooled down to 25 °C. Then, *N*-ethylmorpholine (1.5 cm^3, 11.7 mmol) was added with stirring. The reaction was stopped by injection of water. The complex was precipitated with ether, the precipitate being washed with thf, CH_2Cl_2, and methanol. It was recovered in a minimum of water and purified by passing on a Sephadex G_{10} column. After evaporation to dryness, it was taken up in D_2O and analyzed by ^1H n.m.r. spectroscopy. The pH of the reaction mixture was measured with a Radiometer GK 2401C electrode, left to equilibrate in dry methanol for more than 2 d. The Radiometer pHM 64 pH-meter was calibrated with salicylate and oxalate buffers as described in ref. 12.

(*b*) In $[Co(en)_2(GlyO)][CH_3C_6H_4SO_3\text{-}p]_2$. The complex (0.03 g, 0.064 mmol) was dissolved in 2 cm^3 of CD_3OD. Toluene-*p*-sulphonic acid (0.018 g, 0.095 mmol) and trimethylamine (0.012 g, 0.19 mmol) were added on cooling. The tube was sealed and kept at 25 °C. The ^1H n.m.r. spectrum was recorded every day for 15 d. The pH of the reaction mixture was measured at the end of the experiment.

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