Cyclodextrin-Catalyzed Hydrolyses of Acetanilides

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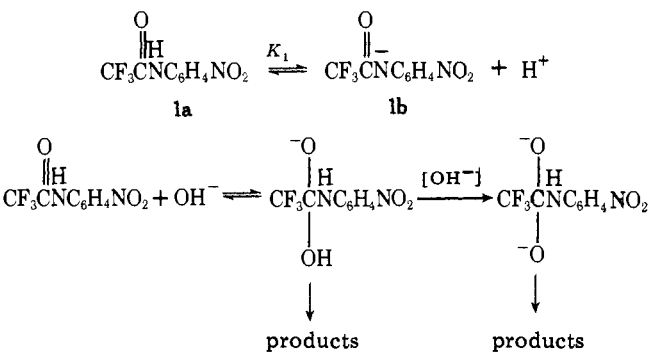
Abstract: The α-cyclodextrin (α-CD)-catalyzed hydrolyses of acetanilides were examined as models of hydrolytic enzymes. Hydrolysis of *p*-nitrotrifluoroacetanilide (**1**) catalyzed by α-CD was 16-fold faster than its alkaline hydrolysis at pH 6.0, 30 °C. Hydrolyses of trifluoroacetanilide (**3**) and *m*-nitrotrifluoroacetanilide (**4**) were also catalyzed by α-CD. However, cleavage of *p*-nitroacetanilide, a less activated substrate, was retarded by α-CD. The catalyses of hydrolyses of **1**, **3**, and **4** proceed via binding, acylation of α-CD, and deacylation of acyl α-CD. Like chymotrypsin reactions, the rate-determining step of hydrolyses of the amides is acylation. Thus, α-CD is a true catalyst here. The present findings indicate that cyclodextrins are excellent models of hydrolytic enzymes since with both catalysts, esters show rate-determining deacylation whereas amides exhibit rate-determining acylation.

Many studies have been done on the alkaline hydrolyses of anilides[1-10] and *N*-methylacetanilides.[11-15] One of the reasons for much interest in these reactions is that they can be a probe for the hydrolyses of amide compounds catalyzed by hydrolytic enzymes.

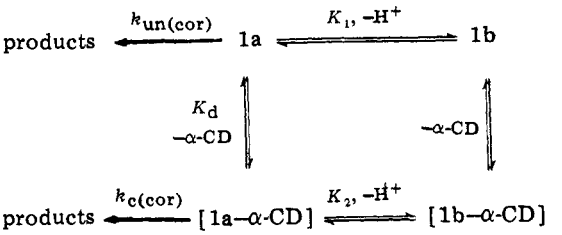
As recently reviewed by the present authors,[16] cyclodextrins, cyclic oligosaccharides, are good models for hydrolytic enzymes. Many investigations were carried out on the cleavage of phenyl esters.[17,18] However, there are few examples of the cyclodextrin-accelerated cleavage of amide bonds. Only the cleavages of the strained β-lactam ring of penicillins[19] and acylimidazoles[20] (both special cases) are known to be accelerated by cyclodextrins. Here, the α-cyclodextrin (α-CD)-catalyzed hydrolysis of *p*-nitrotrifluoroacetanilide (**1**) (an ordinary amide) is described. For comparison, the effects of α-CD on the hydrolyses of *p*-nitroacetanilide (**2**), trifluoroacetanilide (**3**), and *m*-nitrotrifluoroacetanilide (**4**) are also shown.

The alkaline hydrolysis of **1**, precisely studied by Pollack and Dumsha,[10] proceeds as shown in Scheme I; **1** is in equilibrium between the reactive un-ionized form (**1a**) and the unreactive anionic form (**1b**). Nucleophilic attack by hydroxide ion on **1a** forms a tetrahedral intermediate, which breaks down to products via two pathways. One is the pathway through the dianion. Another is general acid-catalyzed breakdown of the monoanionic form of the tetrahedral intermediate.

Scheme I



Scheme II



Experimental Section

Materials. **1**, **3**, and **4** were synthesized from trifluoroacetic anhydride and the corresponding anilines: mp **1**, 150 °C (lit.[21] 147 °C); **3**, 87 °C (lit.[21] 86.5 °C); **4**, 87 °C (lit.[21] 88 °C). **2** and α-CD were purified by recrystallization from water. All water used in the kinetic studies was doubly distilled.

Kinetics. The cleavages of **1** and **2** were followed at 400 nm on a Cary Model 14 PM spectrophotometer equipped with a thermostated cell compartment, while the cleavages of **3** and **4** were followed at 265 nm. The reaction was initiated by the addition of 15 μL of stock solution of the substrate in acetonitrile to 3 mL of thermoequilibrated buffer, followed by thorough mixing of the solution. The initial concentration of the substrate was 10^{-4} M, and the concentration of α-CD was larger than 50 times that of the substrate. All the reactions followed first-order kinetics. Infinite absorbance values were obtained after at least 8 half-lives. For the deuterium oxide experiments, pD was determined using the equation pD = pH meter reading + 0.4.[22] Phosphate, Tris-HCl, and carbonate buffers, respectively, were used in the pH ranges of 6.0–8.0, 8.0–9.0, and 9.0–11.5. The ionic strength was kept at 0.2 M by use of KCl.

The cleavage of **1** in the presence of α-CD proceeds as shown in Scheme II, where [**1a**–α-CD] and [**1b**–α-CD] represent the inclusion complexes of un-ionized **1** (**1a**) and anionic **1** (**1b**) with α-CD; K_d is the dissociation constant of [**1a**–α-CD] to free **1a** and free α-CD; K_2 is the equilibrium constant for release of a proton of **1a** in [**1a**–α-CD], converting it to [**1b**–α-CD]. $k_{un(cor)}$ and $k_{c(cor)}$ are the rate constants, which are corrected for ionization of **1** by dividing the values by the percent of active form of **1**. The anionic form (**1b**) is unreactive.[4,6,8-10] Thus, the observed rate constant, k_{obsd}, is represented by eq 1, under the condition that $[\alpha\text{-CD}]_0 \gg [\mathbf{1}]_0$:

$$k_{obsd} = \{k_{un(cor)}[\mathbf{1a}] + k_{c(cor)}[\mathbf{1a}\text{–}\alpha\text{-CD}]\}/[\mathbf{1}]_0 = \left(k_{un(cor)}\frac{K_d}{[\alpha\text{-CD}]_0} + k_{c(cor)}\right) \times \frac{1}{\left(1 + \frac{K_d}{[\alpha\text{-CD}]_0} + \frac{K_1K_d}{[\alpha\text{-CD}]_0[H^+]} + \frac{K_2}{[H^+]}\right)} \quad (1)$$

where the subscript 0 refers to the initial concentration. The value of pK_1 was determined to be 8.2 in ref 10.

(1) When $[H^+] \gg K_1$, K_2, the rate constants $k_{c(cor)}$ and $k_{un(cor)}$ were almost equal to k_c and k_{un}, the uncorrected rate constants, since almost all of **1** is in the active form. Equation 2 was obtained from eq 1, and the values of k_c and K_d were obtained by plotting $1/(k_{obsd} - k_{un})$ vs. $1/[\alpha\text{-CD}]_0$ as in eq 2.

$$\frac{1}{k_{obsd} - k_{un}} = \frac{K_d}{k_c - k_{un}}\frac{1}{[\alpha\text{-CD}]_0} + \frac{1}{k_c - k_{un}} \quad (2)$$

(2) When $[H^+] \ll K_1$, K_2, eq 1 can be simplified to

$$\frac{1}{k_{obsd} - k_{un}} = \frac{K_1K_d}{K_2(k_c - k_{un})}\frac{1}{[\alpha\text{-CD}]_0} + \frac{1}{k_c - k_{un}} \quad (3)$$

Table I. Values of k_c, k_{OH}, and K_d for the Cleavages of Acetanilides

Substrate	Temp, °C	pH (pD)	k_c (or k_{obsd}), 10^{-5} s^{-1}	k_{OH} (or k_{OD}), 10^{-5} s^{-1}	k_c/k_{OH} (or k_c/k_{OD})	K_d,[d] 10^{-2} M
1	30	6.0	11 ± 1	0.62 ± 0.03	16	6.2 ± 0.5
	30[a]	6.0[a]	2.6 ± 0.2[a]	0.13 ± 0.01[a]	20[a]	6.1 ± 0.5[a]
	30	6.9	49 ± 5	3.0 ± 0.2	16	6.1 ± 0.5
	70	6.0	430 ± 30	46 ± 2	9.3	14 ± 1
	70	6.9	1100 ± 80	110 ± 5	10	13 ± 1
2	70	12.3	130 ± 4[b]	165 ± 10		
	70	12.3	95 ± 6[c]	165 ± 10		
3	30	9.0	4.9 ± 0.4[b]	2.7 ± 0.2		
	70	9.0	41 ± 3[b]	28 ± 2		
	70	9.0	59 ± 4[c]	28 ± 2		
4	30	9.0	4.3 ± 0.3[b]	1.7 ± 0.2		
	70	9.0	72 ± 5[b]	29 ± 2		
	70	9.0	95 ± 6[c]	29 ± 2		

[a] In D_2O. [b] Values obtained by extrapolating k_{obsd} in the presence of 0.03 M α-CD to zero buffer concentration. [c] Values of k_{obsd} extrapolated to zero buffer concentration in the presence of 0.06 M α-CD. [d] Kinetically determined.

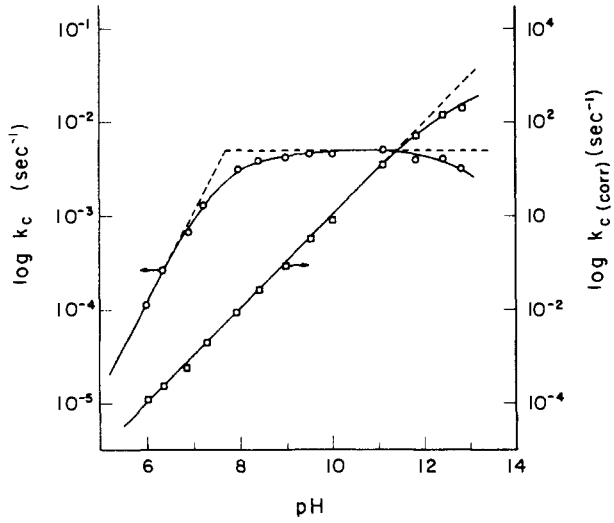


Figure 1. pH–k_c and pH–$k_{c(cor)}$ profiles in the α-CD-catalyzed cleavage of **1** at 30 °C.

In this case, the value of k_c was determined by plotting $1/(k_{obsd} - k_{un})$ vs. $1/[\alpha\text{-CD}]_0$.

Determination of the Dissociation Constants of Inclusion Complexes by Absorption Spectroscopy. The dissociation constants of the inclusion complexes between α-CD and **1** were determined at 30 °C, I = 0.1 M, pH 6.0, 9.5, and 13.0, by spectrophotometric methods. $[C]_0[S]_0/\Delta Abs$ was plotted vs. $([C]_0 + [S]_0)$ according to the method of Benesi and Hildebrand.[23] The slope and the intercept of the straight line gave $1/\Delta\epsilon$ and $K_d/\Delta\epsilon$, respectively. Here, Δ Abs is the observed change in absorbance on addition of α-CD to an aqueous solution of **1**, and $\Delta\epsilon$ is the difference between the molar absorption coefficient of the α-CD–**1** complex and that of **1**. Since appreciable hydrolysis occurred at pH 9.5 and 13.0, the absorbancies were extrapolated to the time of mixing.

Results

α-CD accelerated the cleavage of **1**. At pH 6.0, 6.4, and 6.9, where most of **1** is in the reactive form, the values of k_c and K_d were determined from fair linear relationship between $1/(k_{obsd} - k_{un})$ and $1/[\alpha\text{-CD}]_0$ (eq 2). Table I lists these values as well as the first-order rate constant due to alkaline hydrolysis, k_{OH}. k_{OH} was determined by extrapolating k_{un} to zero buffer concentration.

The k_c values for **1** are 16- and 10-fold larger than the values of k_{OH} at 30 and 70 °C, respectively. Both K_d and k_c/k_{OH} at pH 6.0 are identical with those at pH 6.9.

The D_2O solvent isotope effect for the α-CD-accelerated cleavage of **1** was examined at pH (pD) 6.0. At this pH (pD), almost all of **1** is in the reactive form (**1a**) considering its pK_a (8.2).[10] The k_c value in D_2O, obtained by plotting $1/(k_{obsd} - k_{un})$ vs. $1/[\alpha\text{-CD}]_0$ using eq 2, was 4.2 ± 0.5-fold smaller than that in H_2O. The K_d in D_2O, however, is identical with that in H_2O within experimental error.

The cleavages of **3** and **4** were also accelerated by 0.03 M α-CD by a factor of 1.5–3. However, the cleavage of **2**, which is a less activated substrate, was retarded by α-CD.

Plotting the logarithm of k_c vs. pH for the cleavage of **1** exhibited a straight line with a slope of 1.0 below pH 7.0, as shown in Figure 1. On the other hand, the value of k_c in the pH region 9.5–11.2, obtained from a plot of $1/(k_{obsd} - k_{un})$ vs. $1/[\alpha\text{-CD}]_0$ in eq 3, hardly depended on the pH. The coordinate of the intersection of these two straight lines is 7.7, which corresponds to the pK_2 value. Importantly, when pK_2 was taken as 7.7, the K_d value is 0.062 ± 0.004 M independently of pH in the pH region 9.5–11.2, which is equal to the value below pH 7.0. Furthermore, all the values of k_c in the pH range 7.0–9.5, determined from eq 1 using pK_2 = 7.7 and K_d = 0.062 M, fit the theoretical lines (Figure 1). The theoretical line was calculated by use of

$$k_c = k_{c(cor)} \frac{[H^+]}{K_2 + [H^+]} \quad (4)$$

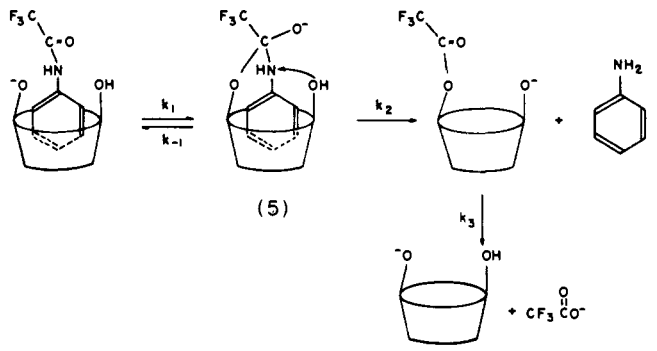
Below pH 11.2, the proportion of catalytically active α-CD, containing the anionic form of the secondary hydroxyl groups, increases directly with pH, since their pK_a is 12.1.[18] Figure 1 also shows the pH – $k_{c(cor)}$ profile, which is a straight line with a slope of 1.0 below pH 11.2.

Nucleophilic attack by a secondary hydroxyl ion of α-CD on **1** was indicated by the gradual decrease of slope above pH 11.2 in the pH–$k_{c(cor)}$ profile, which corresponds to a gradual decrease of k_c above pH 11.2. This can be ascribed largely to the ionization of the secondary hydroxyl groups of α-CD in this pH region. A similar phenomenon was observed in the α-CD-accelerated cleavage of phenyl esters.[18]

The constancy of the K_d, determined kinetically, irrespective of pH indicated that the [**1a**–α-CD] complex is the only reactive species. This was confirmed by the determination of the K_d by spectrophotometric methods. Table II shows the values of K_d at pH 6.0, 9.5, and 13.0. The K_d for the complex between **1a** and α-CD (6.6×10^{-2} M) is identical with the kinetically determined K_d (6.2×10^{-2} M) within experimental error. The K_d for the complex of anionic **1** (**1b**) with α-CD is about fourfold smaller than that for the complex of neutral **1** (**1a**) with α-CD. However, anionic **1** (**1b**) did not show any complex formation with anionic α-CD, which is probably due to electrostatic repulsion between the two negative charges.

The regeneration of α-CD was observed in the α-CD-cata-

Scheme III

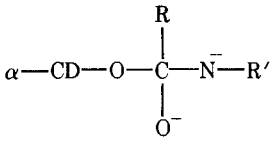


lyzed hydrolysis of **1**, when the initial concentration of **1** was larger than that of α-CD by a factor of 3–4. For example, k_{obsd} remained 2.5 times that in the absence of α-CD up to at least 100% conversion with respect to α-CD, under the conditions that $[\mathbf{1}]_0 = 0.04$ M, $[\alpha\text{-CD}]_0 = 0.01$ M, and pH 7.0, 30 °C in 30% (v/v) acetonitrile–H_2O solution. This shows that the concentration of α-CD remains almost constant during the course of the reaction.

Discussion

From the above results, the mechanism of α-CD-catalyzed hydrolysis of **1** (after complexation) is proposed as in Scheme III. Nucleophilic attack by a secondary hydroxyl ion at the carbonyl carbon atom of **1** included in the cavity of α-CD results in the tetrahedral intermediate (**5**). The breakdown of **5** to the acyl α-CD and aniline proceeds through general acid catalysis without formation of a dianionic intermediate. Formation of a dianionic intermediate in the α-CD-catalyzed hydrolysis is energetically unfavorable, although a dianionic intermediate has been observed in alkaline hydrolysis. In alkaline hydrolysis, this is possible because there are two OH groups in the tetrahedral intermediate, whereas in the α-CD-catalyzed hydrolysis there is only one OH group and one oxygen atom attached to the cyclodextrin.

The dianionic intermediate of the form



would be unfavorable since two proton transfers would be necessary to form an amine. Furthermore, if this intermediate were largely involved, a region with a slope larger than unity would be observed in the pH–$k_{c(cor)}$ profile. This is not the case.

Probably, in the breakdown of **5**, the un-ionized secondary hydroxyl group(s) functions as intracomplex general acid catalyst either directly or via water molecules.[5,10] The ratio of $k_{c(cor)}(H_2O)/k_{c(cor)}(D_2O)$, 4.2, is considerably larger than that for the β-cyclodextrin-accelerated cleavage of *trans*-cinnamoylimidazole, 3.4,[20] and that for the α-CD-accelerated cleavage of *m-tert*-butylphenyl acetate, 3.2.[24] This can be associated with general acid catalysis in the breakdown of **5** (the rate constant, k_2), though this is not definitely proved in light of complexity of the D_2O effect noted in alkaline hydrolysis.[13,14]

No general acid catalyses were observed in the cyclodextrin-accelerated cleavages of acylimidazoles and phenyl esters, since they have good leaving groups. The difference of k_c for the cleavages of these compounds in H_2O and in D_2O is due to differences of the pK_a of the hydroxyl groups of cyclodextrins in H_2O and in D_2O.

The cleavages of **3** and **4** accelerated by α-CD probably proceed in the same way as **1**. Retardation of cleavage of **2** by α-CD is in accord with Scheme III, since alkaline cleavage of **2** proceeds only through a dianionic intermediate without concurrent breakdown of the monoanionic tetrahedral intermediate by general acid catalysis.[2,6] However, an alternative explanation might be possible for the retardation of cleavage of **2** by α-CD. That is, the rate of the α-CD reaction might be larger than the rate through the monoanionic intermediate in alkaline hydrolysis. Here, retardation by α-CD can be still observed, since the alkaline hydrolysis of **2** at pH 12.3 proceeds predominantly through a dianionic intermediate.[6]

Table II. Values of K_d for the Inclusion Complexes between **1** and α-CD[a]

pH	Ionic states of species[b]		K_d, 10^{-2} M
	1	α-CD	
6.0	Neutral (**1a**)	Neutral	6.6 ± 0.5
9.5	Anionic (**1b**)	Neutral	1.7 ± 0.2
13.0	Anionic (**1b**)	Anionic	*c*

[a] Determined by spectrophotometric method, 30 °C, $I = 0.1$ M. [b] At the pH's indicated, the designated species exist to the extent of 90% or greater. [c] There was no measurable change in absorption spectrum on addition of α-CD to an aqueous solution of **1**.

In contrast to acceleration by α-CD in the cleavages of **1**, **3**, and **4**, neither methanol nor ethanol exhibited important effects in these reactions. This supports the predominant role of inclusion complex formation in these catalyses.

Another mechanism such as general base catalysis by alkoxide ion, which does not involve formation of acyl α-CD, is unlikely. Only the nucleophilic reaction was observed in the cyclodextrin-accelerated cleavage of phenyl esters, whereas no general base catalysis was observed in these reactions.[17,18]

An alkaline hydrolysis mechanism assisted by the hydroxyl group(s) of α-CD, which does not involve formation of **5**, can be ruled out by the same reason.

In the α-CD-catalyzed hydrolysis of **1**, α-CD is used as a true catalyst. The result of the experiment, where a substrate concentration larger than the α-CD concentration was employed, showed that the concentration of α-CD remains the same as the initial concentration during the process. This means that α-CD is indeed regenerated by the rapid hydrolysis of the acyl α-CD. Thus, the rate-determining step of the α-CD-catalyzed hydrolysis of **1** is the *acylation* step. In the cyclodextrin-accelerated hydrolyses of phenyl esters, however, the deacylation step is rate determining. These kinetic results parallel those of chymotrypsin reactions exactly.

pK_2, the dissociation constant for **1** in the α-CD complex, is smaller than pK_1, the dissociation constant for **1** in aqueous solution, by 0.5 pH unit. This is consistent with the absorption spectroscopy showing that the dissociation constant of the [**1b**–α-CD] complex, K_d', is about fourfold smaller than that of the [**1a**–α-CD] complex, K_d, since the relation $K_1K_d = K_2K_d'$ should hold. Furthermore, a previous titration study showed that the pK_a of *p*-nitrophenol is decreased by 0.94 pH unit on addition of 0.02 M α-CD to the aqueous solution.[25]

In conclusion, α-CD catalyzes the hydrolyses of certain anilides. The reactions proceed via binding, acylation of α-CD, and deacylation of acyl α-CD. Thus, the process involves esterification of the catalyst by the amide, followed by the hydrolysis of the esterified catalyst, which is identical with serine protease catalyzed hydrolyses of amide compounds. α-CD is a true catalyst of hydrolysis, since α-CD is used repeatedly. Like chymotrypsin reactions, the rate-determining step of amide hydrolysis is acylation whereas the rate-determining step of ester hydrolysis is deacylation. Thus α-CD is a true catalyst in amide hydrolysis whereas it is not in ester hydrolysis. The

present findings indicate that cyclodextrins are excellent models of hydrolytic enzymes.

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Interaction of Cis Platinum(II) Compounds with Poly(L-glutamate). A Doubly Anchored Spin-Label and a Doubly Anchored Chromophore-Label[1]

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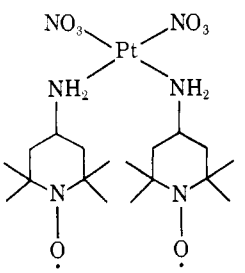
Abstract: The free-radical 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy (ATMPO) yields *cis*-Pt(ATMPO)$_2$(NO$_3$)$_2$, which is used to label poly(L-glutamate) [(Glu)$_n$], poly(L-aspartate) [(Asp)$_n$], and poly(L-lysine) [(Lys)$_n$]. Labeling occurs by displacement of nitrate by polymer side chains. Electron spin resonance (ESR) spectra of oriented films of labeled (Glu)$_n$ are strongly anisotropic; several arguments suggest that the major cause is g anisotropy. Spectra of solutions, in several solvents, of labeled (Glu)$_n$ are also anisotropic and monitor the helix–coil transition and polymer aggregation. Since monofunctional, side-chain labels show only isotropic motions, Pt must be bifunctionally anchored to adjacent carboxylates, requiring the label to follow backbone segmental motions. With shorter side chains [(Asp)$_n$], adjacent double anchoring is impossible; with longer side chains [(Lys)$_n$], flexibility reduces coupling to backbone motion; in each, therefore, spectra are isotropic. Chromophoric compounds, particularly [*cis*-Pt(bpy)(H$_2$O)$_2$][NO$_3$]$_2$, are similarly used. Bifunctional attachment is evidenced by the absence of base-induced ultraviolet (UV) spectral shifts (characteristic of attachment of OH$^-$ to Pt) shown by label alone, and by similarity of the spectra of labeled polymer and labeled oxalate. Induced circular dichroism (CD) appears for α helix in the region of the chromophore π–π^* bands; transition to random coil drastically reduces this CD. With extensively labeled polymer, differences in the course of the helix–coil transition as monitored by CD in the backbone region with that monitored in the chromophore region show that the label stabilizes its attached helical residue. A study of Corey–Pauling–Koltun (CPK) models and extant theories suggests that the induced CD arises by coupling of the carboxylate π–π^* and the bound chromophore $^1B_1$ electric transition moments.

Spin-labels have been widely used in a variety of systems. The resulting ESR spectra presumably contain information on the mobility of the host macromolecule. However, since the attachment of the label is usually monofunctional, the spectra often also strongly reflect the more or less independent motion of the spin-label itself. In many cases, it is impossible to sort out the various motional contributions to the spectrum. For example, an ordinary monofunctional spin-label attached to a side chain of poly(L-glutamate) [which we sumbolize (Glu)$_n$] gives an unrevealing, rather mobile, spin spectrum.[3] Hitherto, only in one very special case, in which a monofunctional label was rather *rigidly* attached to the carboxyl end group of poly(benzyl-L-glutamate),[4] has it been found that the ESR spectrum of a synthetic polypeptide is sensitive to the anisotropic motion that should be characteristic of the macromolecule itself.

We describe here an attempt to eliminate, or at least decrease, the complications ascribable to independent motion of the spin-label by employing a side-chain spin-label that is anchored to the macromolecule at *two* points and is thus required to follow more closely the segmental motion of its host. We therefore synthesized, and used as a label for (Glu)$_n$, the biradical platinum *cis*-(4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy) dinitrate [called *cis*-Pt(ATMPO)$_2$(NO$_3$)$_2$]:



We were hopeful that the carboxylate side chains on the (Glu)$_n$ polymer would be favorably suited to displace the nitrates, resulting in a bifunctionally labeled macromolecule.