are inconsistent with the hypothesis that carboxyl $n-\pi^*$ and/or $\pi-\pi^*$ transitions give rise to portions of the optical activity observed here. Rather, it is more likely that these spectral properties arise directly from the $\pi-\pi^*$ transitions of the chromophore common to all the derivatives studied, the indole residue of the tryptophan side chain. These conclusions apply with greater strength to the region of the ^1B_b band, due to the greater ability to resolve spectral components in this region. In the ^1B_a region contributions to the observed optical activity from amide $\pi-\pi^*$ transitions, as in the case of *N*-acetyl-L-alanine-*N'*-methylamide,[11] cannot be excluded on the basis of our present observations. Finally, it appears that in proteins the peptide chromophore will not couple with the tryptophan side-chain chromophore to generate new absorption or ellipticity bands.

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Nuclear Magnetic Resonance Study of the Interaction of Neodymium(III) with Amino Acids and Carboxylic Acids. An Aqueous Shift Reagent

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Abstract: Complexes of neodymium(III) with alanine, histidine, threonine, serine, acetate, butyrate, and 4-aminobutyrate have been studied in aqueous solution by proton magnetic resonance spectroscopy and potentiometric titrations. Stability constants for the various neodymium(III) complexes have been determined using an nmr shift method. The stability constants determined by the nmr method agree well with those determined by potentiometric titration.

Over the past few years there has been a great deal of interest in the use of various β-diketone complexes of lanthanide ions as shift reagents for use in the assignment of nmr spectra of complex organic molecules containing a donor atom.[3] These shift reagents have been used in noncoordinating organic solvents, and the size of the shifts produced generally obeys the $(3\cos^2\theta - 1)r^{-3}$ relation derived by McConnell and Robertson.[4] Armitage, *et al.*,[5] have recently discussed a procedure for evaluating the binding constants of complexes of amines and alcohols with one of the shift reagents, tris(dipivalomethanato)europium(III), whereas Kelsey[6] has carried out a similar study of complexes of organic acetates with tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium(III). Measurement of the induced shifts (δ) as a function of the Eu^{3+} complex concentration [EuL] at high substrate concentrations yields a plot of δ^{-1} *vs.* [EuL] which is linear. The binding constant is determined from the intercept and the chemical shift (ΔH_0) of the "bound" substrate is obtained from the slope. This method assumes only one substrate molecule binds one molecule of the Eu^{3+} shift reagent.

The shifts induced by the addition of lanthanide salts to aqueous solutions of amino acids have only recently been shown to obey the $(3\cos^2\theta - 1)r^{-3}$ relation.[7,8]

We have been interested for some time in using the lanthanide ions as spectroscopic probes of calcium ion binding sites in proteins.[7,9-11] Before an understanding of the aqueous protein systems can be realized, however, it is necessary to investigate model aqueous systems. We report here an nmr method of determining stability constants of amino acids and carboxylic acids in aqueous solution. We have compared the stability constants obtained with the nmr tech-

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nique with those obtained by the more conventional potentiometric method (pH titration) and have found excellent agreement between the two methods. It appears, therefore, that the nmr method may be applicable to more complex systems such as peptides and simple proteins.

Experimental Section

Materials. Samples of neodymium(III) oxide with a purity greater than 99.9% were provided by the Molybdenum Corp. of America and the American Potash and Chemical Corp. L-Histidine (Sigma), L-alanine, L-serine, L-threonine (Nutritional Biochemicals Corp.), *n*-butyric acid, 4-aminobutyric acid, and acetic acid (Eastman Organic Chemicals) were used without further purification. D_2O, DCl, and NaOD were purchased from Bio-Rad Laboratories and sodium 2,2-dimethyl-2-silapentanesulfonate (internal nmr standard) was purchased from Merck and Co. All other chemicals were Baker reagent grade. Distilled, deionized water was used in preparing all solutions.

Nuclear magnetic resonance spectra were recorded on a JEOLCO PS-100 nuclear magnetic resonance spectrometer in the field internal mode at a probe temperature of ~22°. All spectra were run in D_2O with dimethylsilapentanesulfonate as an internal reference. The ligand–neodymium(III) samples were prepared as in the previous histidine work.[7] The ligand concentration was generally 0.05 *M* with the neodymium(III) concentration ranging from 0.05 to 0.10 *M*. The effect of changing ionic strength was checked by determining a neodymium(III) acetate stability constant with solutions in which the ionic strength was held constant at 0.20 by the addition of KCl. The shifts observed with these solutions were identical with those in which no KCl was added. We have found little influence of ionic strength upon the observed chemical shifts until the Nd^{3+} concentration reaches 0.25 *M*. All pH measurements were done with a Beckman zeromatic pH meter equipped with a Corning combination electrode. The pH values in Table I are actual meter readings and have not been corrected for a deuterium isotope effect (pD = meter reading + 0.4).

Table I. Stability Constants for the Reaction of Nd(III) with Amino Acids and Carboxylic Acids as Determined by an Nmr Method and a Potentiometric Titration Method

Ligand	pH[a]	ΔH_0, Hz[b]	Stability constants, l. mol^{-1} K_{nmr}	$K_{titration}$
Alanine	4.0	130	6.5	4.4
Histidine (monodentate)	4.0	122	1.8	
Threonine	4.0	100	7.6	
Serine	4.0	66	12.6	9.7
Histidine (bidentate)	7.0	257	123	230
Acetate	6.0	308	83	93
Butyrate	6.0	348	109	
4-Aminobutyrate	6.0	251	52	

[a] These are the pH values at which the stability constants were obtained by the nmr method. [b] The total isotropic shift values for the protons α to the carboxyl group as determined from the final plot of ΔH_{obsd}^{-1} *vs.* $[Nd^{3+}]^{-1}$.

Potentiometric Titrations. All measurements were performed using a Radiometer pH meter (Model TTT2) equipped with an autoburet (Model ABU 12b) and a titration assembly (Model TTA31). Approximately 10^{-4} mol of each ligand was added to two different 25-ml volumetric flasks. To one flask of each pair was added 0.40 ml of a 0.287 *M* $NdCl_3$ stock solution. A 0.116 *M* HCl solution (2.0 ml) was added to each volumetric flask to form the hydrochloride salt of each amino acid. The flasks were then brought to volume with 0.20 *M* KCl in order to maintain a constant ionic strength near 0.20.

Each solution was titrated three times using aliquots of 1.4, 1.7, and 2.0 ml. Each aliquot was diluted with 2.0 ml of the 0.20 *M* KCl solution resulting in amino acid concentrations of ~0.00165, 0.00183, and 0.00200 *M*. The solutions were titrated against ~0.10 *M* NaOH (standardized against Baker reagent grade potassium hydrogen phthalate) with pH readings taken at 0.010-ml increments. The concentration of neodymium(III) ion in the stock solutions was determined by titration with ethylenediaminetetraacetic acid (EDTA) using xylenol orange as an indicator in an acetate (pH 6) buffer.[12]

Analysis of Potentiometric Titration Data. The analysis of the titration data was carried out using an IBM 360/65 computer and the program SCOGS which was written by Perrin and Sayce.[13] The program was modified as follows: (a) all printed ouput could also be punched onto cards which could then be used in statistical analysis subroutines, and (b) the iteration procedure would cease whenever the change in the calculated stability constant was less than 5×10^{-5}.

As SCOGS minimizes the sum of the square of the residuals in volume of base added (*i.e.*, ΣR_i^2 is brought to a minimum where R equals calculated volume of base added minus actual volume of base added), three new subroutines were added to the program to aid in the interpretation of these residuals. One subroutine calculates the degree of randomness of the signs of the residuals assuming that, if a perfect fit is obtained between the experimental and the calculated pH *vs.* volume curves, the distribution of the signs of the residuals along the curve would be totally random. The other two subroutines together draw a graph of the residuals *vs.* pH on the computer line printer. From the graph, any nonrandom trends in the residuals are apparent and, therefore, the presence or absence of complex species becomes apparent.

The acid dissociation constants (β's) for each acid were determined. These values were subsequently used in determining the stability constants of each neodymium(III)–acid complex. Using the β's of the acids calculated by SCOGS compensates for any nonlinearity in the pH meter and also allows the calculation of β's for neodymium(III)–acid complexes under conditions identical with that for the free acids themselves (25°, μ = 0.20).

Results

Since a condition of rapid exchange exists between Nd^{3+} and its ligands in aqueous solution, only an average of the proton resonances of free and complexed ligand is observed by nmr. The observed isotropic shifts of any particular ligand proton (assuming only a single complex exists in solution) is therefore

$$\Delta H_{obsd} = \{[ML]/([ML] + [L])\}\Delta H_0 \qquad (1)$$

where ΔH_{obsd} is the observed difference between the proton resonance positions of the ligand in paramagnetic Nd^{3+} and diamagnetic La^{3+} solutions at the same pH. ΔH_0 is the maximum isotropic shift that would be observed if all the ligand molecules were complexed, and [ML] and [L] are the equilibrium concentrations of metal–ligand complex and free ligand, respectively. For a weak 1:1 complex governed by equilibrium 2,

$$Nd^{3+} + L \underset{}{\overset{K}{\rightleftharpoons}} Nd(L)^{3+} \qquad (2)$$

it is necessary to use eq 3 to calculate ΔH_0,[14] where K

$$\Delta H_{obsd} = \{K[Nd^{3+}]/(1 + K[Nd^{3+}])\}\Delta H_0 \qquad (3)$$

is the stability constant for the reaction of Nd^{3+} with ligand (L). Thus a plot of $(\Delta H_{obsd})^{-1}$ against $[Nd^{3+}]^{-1}$ should give an intercept of $(\Delta H_0)^{-1}$ and a slope of $(K\Delta H_0)^{-1}$. Early attempts were made to plot data at high metal ion concentrations where the initial Nd^{3+} concentration would approximate the free Nd^{3+} concentration. However, the effects of high ionic strength and the possibility of formation of 2:1 metal to ligand complexes with some of the potentially multidentate ligands made studies at high concentrations of Nd^{3+} ion ambiguous. We ultimately used data at low metal ion concentrations near a 1:1 metal to ligand ratio

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and used a computer program to calculate K. The program initially assumes $[Nd^{3+}] = [Nd^{3+}]_0$[15] and uses a least-squares subroutine to calculate a best value for K and ΔH_0. This value of K is then used to calculate new Nd^{3+} concentrations which are used in a second iteration. The iterations are repeated until $K_{n+1} - K_n \leq 0.1$. (K_n is K for the nth iteration.) At this point the calculated metal ion concentrations are equal to the equilibrium metal ion concentrations, $[Nd^{3+}]$, and from a plot of ΔH_{obsd}^{-1} *vs.* $[Nd^{3+}]^{-1}$ a maximum value of K is obtained. The stability constants as determined by the nmr shift experiments are compared with those determined by the potentiometric titration method in Table I.

Discussion

The stability constants determined by the two methods are in generally good agreement. The only pair which do not compare favorably are the values obtained for histidine binding to neodymium(III) in a bidentate manner (pH 7.0). The nmr experiment assumes only one type of metal–ligand complex is contributing to the observed shift at a given pH. This is apparently a good assumption at pH 4.0 since the stability constants determined for Nd^{3+} complexed with alanine, histidine, serine, and threonine agree very well with the values reported for glycine and alanine.[16–18] At pH 4.0 only the carboxyl groups will be significantly deprotonated and therefore only the monodentate complexes should contribute to the observed shifts. This method appears to be sensitive enough to measure a smaller stability constant for the Nd^{3+}–histidine complex than the Nd^{3+}–alanine complex at pH 4. This smaller stability constant probably reflects the additional repulsion between the protonated imidazole group in histidine and the highly charged Nd^{3+} ion. The increase in K from alanine to serine may also reflect the attraction or association between the OH group and the metal ion. As the pH of these solutions is increased, binding of other deprotonated groups to Nd^{3+} becomes possible. As shown previously,[7] beginning at pH 6.0, Nd^{3+} will bind to histidine in a bidentate manner *via* the carboxyl group and the imidazole nitrogen. Thus the stability constant measured by the nmr method at pH 7.0 is considerably larger than at pH 4.0. At pH 7.0, however, there is still a small amount of the metal bound to histidine *via* the carboxyl group only. Since the shifts are an average over all complexes in solution, the shift due to this small amount of monodentate complex is included in the calculation. Thus, the K calculated by nmr is a lower limit of the actual K as determined by the titration procedure. Increasing the pH above 7.0 to eliminate contribution of the monodentate complex results in precipitation of hydroxy species. An additional error in the nmr calculation is the assumption that no $Nd(OH)^{2+}$ species is present. The potentiometric method indicates that a significant amount of the hydroxy species is present at pH 7. The stability constant of 230 for bidentate

(15) $[Nd^{3+}]_0$ is the total concentration of metal ion, $[Nd^{3+}] + [NdL^{3+}]$, present.
(16) A. Aziz and S. J. Lyle, *J. Inorg. Nucl. Chem.*, 33, 3407 (1971).
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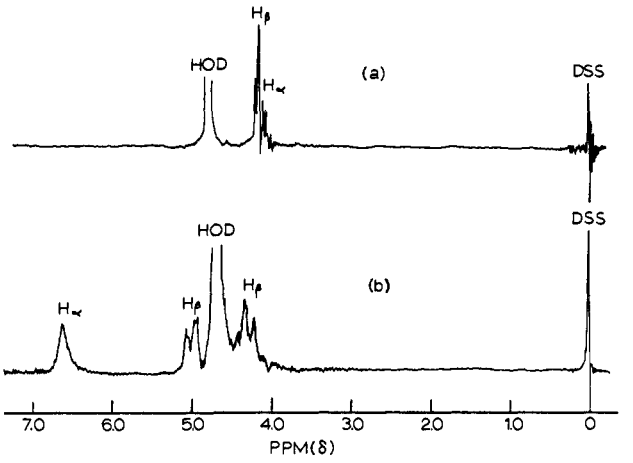

Figure 1. Nmr spectra (100 MHz) observed for a solution of (a) 0.05 M $NdCl_3$ and 0.05 M serine at pH 4.0 (the α and β protons have been shifted ~15 Hz from their diamagnetic positions) and (b) 0.10 M $NdCl_3$ and 0.10 M serine at pH 6.5.

neodymium(III)–histidine coordination obtained by potentiometric titration agrees very well with an earlier value reported by Jones and Williams,[19] even though their calculations included no $Nd(OH)^{2+}$ species.

The nmr spectra of the neodymium(III)–serine and neodymium(III)–threonine solutions change substantially as the pH is increased to 6.5. The spectra of 1:1 neodymium(III)–serine complexes at pH 4.0 and 6.5 are shown in Figure 1. The pH 4.0 spectrum is identical with that of serine alone except that both the α- and β-proton peaks have been shifted downfield by ~15 Hz. At pH 6.5, however, all of the peaks have broadened and have been shifted downfield even further (Figure 1). The α-proton shift is now much larger than the β-proton shift and the β protons have become magnetically nonequivalent. From a spin decoupling experiment it is clear that each nonequivalent β proton is splitting the other β proton into a doublet, thus resulting in the two broad doublets one in each side of the HOD peak. The α-proton peak has lost all of its fine structure, probably due to chemical-exchange spin decoupling,[20] and shifts downfield very rapidly as the Nd^{3+} ion concentration is increased. These observations make it tempting to conclude that serine is binding the Nd^{3+} ion in a tridentate manner at this pH. This type of complex would explain the large shift observed in the α proton and the magnetic nonequivalence of the β protons. However, calculation of a stability constant at this pH gives a K of 18. This value does not seem nearly large enough for tridentate coordination of serine to Nd^{3+} but may reflect the small concentration of the tridentate complex expected at this low pH. Also, we have no evidence of Nd^{3+} binding to the α-amino group of any other amino acid over the pH ranges we have studied (up to pH 7.2). Thus, the serine and threonine complexes with Nd^{3+} will require further investigation to elucidate their structures in solution.

The stability constants of Nd^{3+} complexes of three carboxylic acids were determined at pH 6.0 (Table I). The constant determined for the acetate complex of Nd^{3+} by the nmr method is in good agreement with

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that determined by the potentiometric method and with literature values.[21-23] As in the histidine case, the decrease in the value of K from butyrate to 4-aminobutyrate probably reflects the additional positive charge on 4-aminobutyrate at pH 6.0.

The total isotropic shift of a complex, ΔH_0, is defined as that shift which would result if the equilibrium was shifted entirely toward the metal–ligand complex so that only complex species exist in solution. The ΔH_0 values determined by the nmr method for the α proton of each system are reported in Table I. Assuming a pseudocontact mechanism, these shifts are proportional to a $(3\cos^2\theta - 1)/r^3$ angle and distance factor.[5] For monodentate coordination, the angle factor should be nearly the same for the α proton of each ligand and the total isotropic shift should depend only upon the cube of the metal–proton distance, r. The ΔH_0 values found for the three carboxylic acids indicate a decrease in ΔH_0 with decreasing K. We might expect the neodymium(III)–α proton distance in the 4-aminobutyrate complex would be longer than the neodymium(III)–α proton distance in the butyrate complex merely on electrostatic arguments because of the additional protonated amino group of the former ligand. This is reflected in the smaller ΔH_0 value of the α proton found for the 4-aminobutyrate complex. For complexes which have more than one observable nmr proton peak, such as butyrate and 4-aminobutyrate, we have calculated the stability constants using the α-, β-, and γ-proton shifts. In all cases, the same stability constant was obtained. The ΔH_0 values, however, decrease from α to β to γ as the metal–proton distance becomes larger. The ΔH_0 values for the α, β, and γ protons of butyrate are 348, 176, and 96 Hz, respectively, while the analogous values for 4-aminobutyrate are 251, 132, and 84 Hz, respectively.

The ΔH_0 values for the α and β protons of serine and threonine at pH 4 are equal (ΔH_0 (serine) = 66 Hz, ΔH_0 (threonine) = 100 Hz), whereas the ΔH_0 values for the α and β protons for alanine at pH 4 are 130 and 84 Hz, respectively, and the ΔH_0 values for histidine at pH 4 for the α and β protons are 122 and 62 Hz, respectively. Since both serine and threonine have a hydroxyl group which is capable of coordinating to the metal ion, it is reasonable to assume that the equal values of the α and β shifts arise from the formation of a bidentate complex with the metal ion. Since the angle and distance parameters of such a complex are uncertain due in part to the unknown position of the molecular axis, a calculation of the expected shifts of the α and β protons is not practical. However, coordination of the hydroxyl group must bend the carbon chain of the molecule closer on the average to the metal ion (compared to monodentate coordination) making the distance of the α and β protons similar, and hence the isotropic shifts similar.

This study has shown that the nmr technique is a rapid method for determining stability constants of Nd^{3+} complexes in aqueous solution. The results obtained by the nmr method agree well with the potentiometric determinations. In contrast to the potentiometric method, the nmr technique has the distinct advantage of allowing conclusions to be made concerning the mode of metal ion coordination to ligands. A disadvantage of the method is that the experimental nmr spectrum at any particular pH is an average spectrum of all species present in solution. However, a complete pH study together with the use of a program such as SCOGS would allow a determination of stability constants of several complex species in solution.

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