is a reasonable expectation that significant amounts of monomers will form in the presence of 4 equiv of HMPA, particularly with THF as the solvent, and such species must be regarded as likely candidates for the reactive species. It will, however, be interesting to establish whether HMPA enhances or decreases the reactivity of lithium 3,5-dimethylphenolate in diethyl ether since there is no evidence for the formation of dissociated species in this system.

Acknowledgment. We gratefully acknowledge support for this work by grants (CHE-8801884 and CHE-9102732) from the National Science Foundation. We also thank Drs. Reich and Collum for supplying copies of their papers prior to publication.

Proton Chemical Shift Assignments in Citrate and Trimethyl Citrate in Chiral Media

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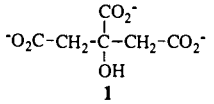
Abstract: The citrate ion gives rise to four different methylene proton chemical shifts in the presence of (*S*)-lactate and Pr^{3+}. Trimethyl citrate behaves similarly in the presence of (*S*)-2,2,2-trifluoro-1-(9-anthryl)ethanol. The four methylene shifts have been assigned in an absolute way by comparisons with those from the corresponding spectra of (2*R*,3*R*)-citrate-*2-d* and trimethyl (2*R*,3*R*)-citrate-*2-d*, respectively. The chemical shifts of the three methyl groups in trimethyl citrate in the presence of the anthryl shift reagent have also been assigned. Deuterium isotope effects on the proton chemical shifts of these molecules have been determined. In the absence of shift reagents these effects are mainly of the intrinsic type, but in their presence there are equilibrium perturbation contributions. The ^1H NMR line widths and chemical shifts in the citrate–lactate–$PrCl_3$ system depend strongly on the pH, spectrometer frequency, and temperature, with the best results obtained at room temperature, pH $\approx$ 3.8, and a spectrometer frequency of 200 MHz.

Introduction

Citric acid and its salts are of great importance in biochemistry and commerce. Citrate, which plays a key role in the metabolic pathway known as the Krebs, tricarboxylic acid, or citric acid cycle, has interesting stereochemical and symmetry features, despite its structural simplicity, lack of a stereogenic center, and achirality.[1-4] The inclusion of free citrate in the Krebs cycle was once considered to be impossible because experiments with isotopically labeled compounds seemed incompatible with the symmetry present in that ion.[5] However, in a classic paper in 1948, Ogston showed that the two $CH_2CO_2^-$ groups in citrate could be differentiated in theory by an enzyme, essentially because of the latter's chirality, and this molecular recognition was discussed in terms of a "three-point" complexation model.[6] Hirschmann has emphasized that this differentiation can be deduced from symmetry alone without the consideration of a specific complexation model.[7]

The two methylene groups in citrate (I) have different (diastereomeric) interactions with a chiral molecule such as the enzyme aconitase, which can selectively dehydrate citrate to aconitate with the stereospecific breaking of only one of the four C–H bonds in I. In principle, therefore, citric acid (or citrate) in any chiral



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medium should show four different CH chemical shifts in its ^1H NMR spectrum, but conditions under which these shifts are large enough to be resolved have not been reported, to our knowledge. This problem, which forms the subject of the present work, can be approached by means of chiral solvents or chiral shift reagents[8-13] or by converting citrate into an unsymmetrical derivative.[14]

Stereochemical Nomenclature. The ^1H NMR spectrum of the methylene groups in citric acid (or citrate) in D_2O, i.e., in an achiral medium, shows a single AB quartet and thus only two chemical shifts, in agreement with the symmetry of the molecule.[15]

(1) Stryer, L. *Biochemistry*, 2nd. ed.; Freeman: San Francisco, 1981; p 276.
(2) Glusker, J. P. *Acc. Chem. Res.* **1980**, *13*, 345.
(3) Emptage, M. H. *Metal Clusters in Proteins*; Que, L., Jr., Ed.; ACS Symposium Series 372; American Chemical Society: Washington, DC, 1988; Chapter 17.
(4) Bentley, R. In *Stereochemistry*; Tamm Ch., Ed.; Elsevier Biomedical Press: Amsterdam, 1982; pp 49-112.
(5) Wood, H. G.; Werkman, C. H.; Hemingway, A.; Nier, A. D. *J. Biol. Chem.* **1941**, *139*, 483.
(6) Ogston, A. G. *Nature* **1948**, *162*, 963.
(7) Hirschmann, H. *J. Biol. Chem.* **1960**, *235*, 2762.
(8) Weisman, G. R. In *Asymmetric Synthesis*; Morrison, J. D., Ed.; Academic Press: New York, 1983; Vol. 1, pp 153-171.
(9) Rinaldi, P. L. *Prog. Nucl. Magn. Reson. Spectrosc.* **1982**, *15*, 291.
(10) Pirkle, W. H.; Hoover, D. J. *Top. Stereochem.* **1982**, 263.
(11) Reuben, J. *Prog. Nucl. Magn. Reson. Spectrosc.* **1973**, *9*, 1.
(12) Fraser, R. R. In *Asymmetric Synthesis*; Morrison, J. D., Ed.; Academic Press: New York, 1983; Vol. 1, pp 173-196.
(13) Bertinini, I.; Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems*; Benjamin/Cummings; Menlo Park, CA, 1986.
(14) A monomethyl citric acid ester with the methyl attached to carbon 1 should show four methylene chemical shifts. However, this type of compound is chiral, and the determination of the position of a deuterium incorporated into the molecule would require a resolution step or an asymmetric synthesis of the ester. A more elegant method of observing four chemical shifts would be to convert the OH group attached to carbon 3 in citric acid (or, more conveniently, trimethyl citrate) into an ester function with a chiral acid, e.g., with optically active *O*-methylmandelic acid: Raban, M.; Mislow, K. *Tetrahedron Lett.* **1966**, 3961.
(15) A single AB quartet, $|\delta_A - \delta_B| = 0.16$ ppm, $|^2J| = 15$ Hz, is observed for tripotassium citrate in D_2O. The geminal coupling constants in the CH_2 groups of citric acid and related compounds are undoubtedly negative (Bovey, F. A.; Jelinski, L.; Mirau, P. A. *Nuclear Magnetic Resonance Spectroscopy*, 2nd ed.; Academic Press: New York, 1988; pp 191-195), and this is assumed to be so in the rest of the present paper. The two diastereotopic protons in either of the two $CH_2CO_2^-$ groups have different chemical shifts and are coupled to one another, whereas the two enantiotopic CH_2 groups are not differentiated in water, which is an achiral medium: Villafranca, J. J.; Mildvan, A. S. *J. Biol. Chem.* **1972**, *247*, 3454. These workers, who used a 100-MHz sweep NMR spectrometer with time averaging, were the first to observe the proton NMR spectrum of enzymatically prepared citrate-*2-d* and thereby showed that the proton that exchanges in D_2O in the presence of aconitase is one of the two lesser shielded protons that give rise to the AB quartet in citrate. They measured a deuterium isotope effect of −23 ppb on the CHD proton.

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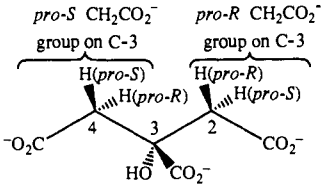
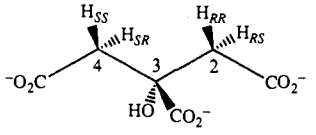
In Raban and Mislow's terminology,[16] the *two hydrogens* in either of the two CH_2 groups are diastereotopic to one another, since they are not related by any kind of symmetry[17] and thus cannot have exactly the same chemical shifts.[18] In contrast, the two $CH_2CO_2^-$ groups (or just the two CH_2 groups) in citrate (in isolation or in an achiral medium) are enantiotopic (rather than homotopic), since these groups are related by a plane of symmetry, but not by rotational symmetry. Enantiotopic and homotopic groups have the same chemical shifts, but only the former groups become diastereotopic when the molecule is placed in a chiral environment.

In Hanson's widely used terminology,[19] the central carbon of citrate is "prochiral", but as recently pointed out by Mislow and Siegel,[20] this term is better applied to citrate as a whole. In the latter's terminology, the central carbon in citrate is prostereogenic. Hanson's *pro-R–pro-S* nomenclature, however, is needed to describe fully the stereochemistry of molecules such as citric acid. For example, the citrate hydrogen that is attacked by aconitase is *pro-R* on the *pro-R* $CH_2CO_2^-$ group of citrate, as shown in Ia. We label this hydrogen as H_{RR}, where the first subscript refers to the $CH_2CO_2^-$ group and the second to the actual hydrogen on that group.

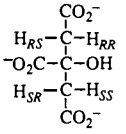


Ia

As shown in Ib and in the Fischer projection Ic, the four hydrogens in citrate can be labeled as H_{RR}, H_{SS}, H_{SR}, and H_{RS}. We

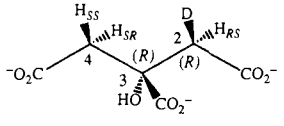


Ib

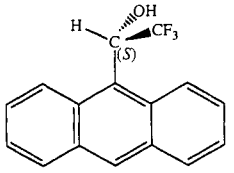


Ic

use the same designations for the hydrogens in the deuterated citric acid derivatives studied in the present work. In these compounds H_{RR} is replaced by a deuterium, as in I-*d*, so that H_{RR} does not appear in their ^1H spectra. The introduction of the single deuterium atom results in the formation of two stereogenic carbons (2 and 3 in I-*d*), both of which have the *R* configuration, as shown.



I-*d*



II

Pirkle first reported that the two enantiomers of a racemic compound dissolved in a (nonracemic) chiral solvent can give rise to distinct NMR spectra, at least under favorable circumstances.[21] Although *any* chiral solvent can in theory remove the degeneracy of the chemical shift of enantiotopic groups, the generation of an *experimentally observable* chemical shift difference between the resulting diastereotopic groups is generally not feasible in practice, unless special structural features are present in both the chiral molecule and the achiral (or racemic) substrate. The chiral fluorinated anthracene alcohol (II)[22] and various chiral paramagnetic lanthanide shift reagents, both aqueous and nonaqueous,[11-13,23,24] have been specially developed for this purpose and have proved useful, especially with racemic alcohols. However, these reagents are not general, and the successful observation of an induced diastereochemical shift difference is by no means easy or certain in any given case.

The alcohol II, other water-insoluble chiral molecules, and the paramagnetic lanthanide shift reagents derived from chiral β-diketones are best applied to compounds that are soluble in organic solvents, such as the trimethyl ester of citric acid (trimethyl citrate). Citrate salts and citric acid itself have solubility properties that are unsuitable for the application of these water-insoluble shift reagents. The aqueous chiral lanthanide reagents, originally developed by Reuben,[23,24] induce intramolecular diastereotopic chemical shift differences in salts of achiral carboxylic acids, such as glycolic acid[23,24] and oxydiacetic acid,[25] and should be applicable to citric acid. These reagents consist of D_2O solutions of lanthanide ions, such as europium (Eu^{3+}) or praseodymium (Pr^{3+}), complexed to readily available chiral ligands, such as lactate or mandelate, or to specially synthesized chiral ligands, such as the carboxymethyl ether of malic acid.[25]

Ion pairs derived from (chiral) alkaloid salts in a suitable solvent can induce diastereochemical shifts in racemic anions,[26] and alkaloids can therefore act as chiral shift reagents for acids as well as for other molecules to which the alkaloids can complex.[27] The interaction of citrate with a protein or enzyme in theory can lead to diastereomeric chemical shift differences. All of the above approaches have been investigated in the present work. Natural and synthetic chiral host molecules, which have been mostly applied to differentiating racemic compounds by NMR,[28] should be excellent shift reagents for achiral guests containing enantiotopic nuclei, but we are unaware of any host molecules for citrate or trimethyl citrate.

Experimental Section

The shift reagent (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (II) (Aldrich Chemical Co.), which had the expected NMR spectrum, aconitase (citrate (isocitrate) hydrolyase, EC 4.2.1.3), and crystalline (*S*)-(+)-lactic acid ($CH_3CH(OH)CO_2H$); Sigma Chemical Co.) were used as received. NMR spectra were obtained on Bruker AM-500, AM-360, and MSL-300 and IBM AF-200 spectrometers operating at the indicated frequencies (MHz) for protons. Except for the trimethyl citrate–II solutions, which were measured in Wilmad thick-wall 5-mm tubes (2.4-mm i.d.) to minimize the amount (ca. 10 mg) of II needed, 5-mm thin-wall sample tubes were used. For NMR measurements in the presence of lanthanide ions, citric acid (1 mg or less), (*S*)-lactic acid (ca. 9 mg), and $PrCl_3$ or $EuCl_3$ (0.1 mL of a 0.06 M solution in D_2O) in 0.8 mL of D_2O were adjusted to the required uncorrected "pH" with a concentrated solution of Na_2CO_3 in D_2O. The summed free induction decays were processed by a Lorentzian–Gaussian (LG) line-narrowing function[29] with parameters dependent on the line widths of the citrate

(16) Mislow, K.; Raban, M. *Top. Stereochem.* **1967**, *1*, 1.
(17) We use symmetry on a time-averaged basis of the various conformations present in rapid equilibrium. In the molecules and ions considered here, a conformation of the quoted symmetry actually exists in the equilibrium mixture. This conformation may not have the lowest energy, but exchange between it and the other possible conformations is always fast on the NMR chemical shift time scale.
(18) A given proton on one CH_2 group, however, is enantiotopic to one and diastereotopic to the other proton in the second CH_2 group.
(19) Hanson, K. R. *J. Am. Chem. Soc.* **1966**, *88*, 2731.
(20) Mislow, K.; Siegel, J. *J. Am. Chem. Soc.* **1984**, *106*, 3319.
(21) Pirkle, W. H. *J. Am. Chem. Soc.* **1966**, *88*, 1837.
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(23) Reuben, J. *J. Am. Chem. Soc.* **1980**, *102*, 2232.
(24) Reuben, J.; Elgavich, G. *J. Magn. Reson.* **1980**, *39*, 421.
(25) Peters, J. A.; Vijverberg, C. A. M.; Kieboom, A. P. G.; van Bekkum, H. *Tetrahedron Lett.* **1983**, *24*, 3141.
(26) Anet, F. A. L.; Miura, S.; Siegel, J.; Mislow, K. *J. Am. Chem. Soc.* **1983**, *105*, 1419.
(27) Sweeting, L. M.; Anet, F. A. L. *Org. Magn. Reson.* **1984**, *22*, 539.
(28) Cram, D. J.; Cram, J. M. *Acc. Chem. Res.* **1978**, *8*, 11. Diederich, F. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 362 and references therein. Some chiral water-soluble cyclophanes induce diastereotopic shifts in racemates of mandelic and related aromatic acids but give negligible shifts with aliphatic molecules such as lactic acid: Takanashi, I.; Odashima, Koga, K. *Tetrahedron Lett.* **1984**, *25*, 973. Cyclodextrins induce diastereotopic shifts in pharmaceutically interesting racemic molecules containing aromatic rings: Casy, A. F.; Mercer, A. D. *Magn. Reson. Chem.* **1988**, *26*, 765. Greatbanks, D.; Pickford, R. *Magn. Reson. Chem.* **1987**, *25*, 208.
(29) Braoudakis, G.; Gerothanassis, I. P.; Lauterwein, J. *J. Magn. Reson.* **1983**, *52*, 288, and references therein.

resonances. For the lanthanide solutions the spectrometer parameters used were LB (exponential line broadening) = −10 Hz, GB = 0.15 (the maximum of the LG function occurs at the fraction GB of the acquisition time, AQ), and AQ = 0.4 s. The effect of the application of this LG function to the free induction decay is to replace a Lorentzian line ($\Delta\nu_{1/2}$ = 10 Hz) in the Fourier-transformed spectrum by a Gaussian line having a $\Delta\nu_{1/2}$ of $0.38[(-LB)/(GB)(AQ)]^{1/2}$, i.e., 5 Hz. For the trimethyl citrate soltuions, the parameters were LB = −0.3 to −1.0 Hz, GB = 0.25–0.6, and AQ = 3.3 s. Because of the low concentration of Pr^{3+}, the HOD peak was always sharp and its chemical shift was assumed to be the same as that in pure D_2O. The chemical shift scale was referenced to the HOD peak for the D_2O solutions (δ = 4.67 ppm at 25 °C with a temperature coefficient of −0.01 ppm/deg) and to internal tetramethylsilane (δ = 0) for the other solutions.

Exchange of Citrate with D_2O in the Presence of Aconitase. A solution of aconitase (5 units, 175 mg) in a Tris buffer in D_2O (20 mL, 99.75% isotopic purity) was activated by incubation of 0 °C for 1 h with ferrous sulfate (0.5 mL of 0.001 M $FeSO_4$ in D_2O) and cysteine (1.1 mL of 0.05 M L-cysteine hydrochloride in D_2O) at pH 7.4. Citric acid (10 mg) in D_2O was added, the pH adjusted to about 7, and the solution monitored by NMR (the effect of pH near a value of 7–8 and of D_2O on the activity of aconitase is small[30]). Initially, a symmetrical AB quartet (δ = 2.39 and 2.51 ppm, ^2J = −15 Hz)[15] for the CH_2 groups of citrate was observed (if the aconitase concentration was too high, the spin–spin coupling was obscured). After 1 day at room temperature, the 200-MHz NMR spectrum showed the presence of a CHD proton signal as a broad singlet between the lines of the more shielded CH_2 doublet of citrate, whereas at 500 MHz the deuterium isotope effect (ca. −0.02 ppm)[15] is large enough to cause the broad singlet to overlap with the most shielded line of the AB quartet. Thus, it is easiest to monitor the incorporation of deuterium at spectrometer frequencies considerably less than 500 MHz. The solution was allowed to stand for 1–5 days in different experiments before being worked up.

Trimethyl (2*R*,3*R*)-Citrate-*2-d* (TMC-*2-d*). For conversion to the trimethyl ester, the solution containing the exchanged citrate was evaporated to dryness in a vacuum several times after the addition of methanol. A small drop of D_2SO_4 was then added to the residue in methanol, and the solution was refluxed for 15 h. The solution was evaporated to dryness in a vacuum and extracted with a small volume of pentane. Evaporation of the pentane extract gave a residue whose ^1H NMR spectrum was consistent with trimethyl citrate-*d* and contained no significant interfering impurities in the methylene proton region (at equilibrium, there is 88% citrate, 8% isocitrate, and 4% aconitate[4]). The deuterium incorporation was about 87%. Ordinary trimethyl citrate, mp 76 °C (lit.[31] mp 78.5–79 °C), was prepared by refluxing anhydrous citric acid in methanol saturated with HCl.

(2*R*,3*R*)-Citric Acid-*2-d*. The isolation of citric acid from the aconitase solution (in this case a small amount of anhydrous sodium acetate was used instead of a Tris buffer) was done by adjusting to pH 1 to precipitate most of the enzyme, which was removed by centrifugation, followed by readjusting to pH 7 and again removing the precipitate by centrifugation. Attempts to isolate the citrate as the insoluble calcium salt did not succeed because of extremely slow precipitation under these conditions, in contrast to pure citrate, which readily precipitates a crystalline calcium salt. Passage of the solution through a column (5 cm × 6 mm) of cation-exchange resin (Dowex 50X8-100 in the acid form in methanol) and evaporation of the collected acidic methanol elutate to dryness in a vacuum gave the desired partially deuterated citric acid (ca. 25% incorporation of deuterium), as shown by NMR.

Results and Discussion

Citric Acid and Citrate Ion in Chiral Media. The ^1H NMR spectra of citric acid or citrate in D_2O in the presence of proteins such as aconitase, lysozyme, ribonuclease, or egg white albumin show only a simple AB quartet, even at 500 MHz. A similar type of spectrum is obtained for the quinine salt of citric acid in $CDCl_3$/DMSO-d_6, and thus these systems fail to induce observable diastereomeric chemical shift differences in citrate.

The complexes formed by lanthanide ions and anionic ligands in aqueous solutions can be of various stoichiometries, but their NMR spectra are simple if chemical exchange between the various species is sufficiently fast on the chemical shift NMR time scale, as is generally,[23,24] but not necessarily always,[25] the case. If exchange is slow, the spectrum may be complicated but may still

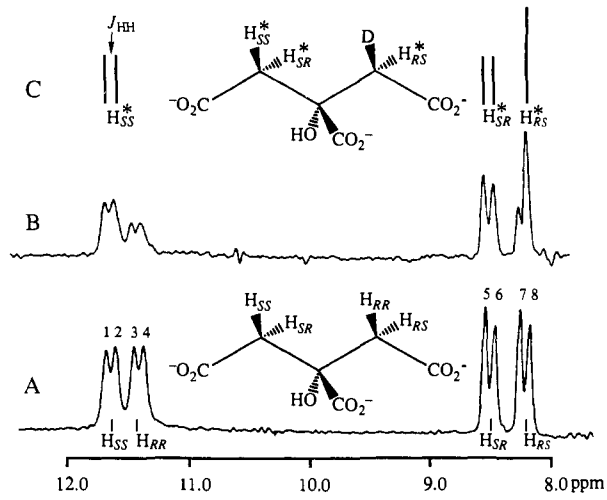

Figure 1. 200-MHz ^1H NMR spectra of the citrate methylene region in D_2O in the presence of (*S*)-lactate and $PrCl_3$ at pH 3.9 at 25 °C (resolution enhanced, see Experimental Section): (A) citrate, (B) 75% citrate/25% (2*R*,3*R*)-citrate-*2-d*, and (C) diagrammatic representation of the spectrum of (2*R*,3*R*)-citrate-*2-d*.

be interpretable. For intermediate rates of exchange, the resonances may be broad enough to obscure the chemical shift splittings. These considerations are important because citrate is expected to act as a multidentate ligand and therefore to have a much lower complexation–decomplexation rate on the lanthanide ion than a simpler ligand such as lactate. Fortunately, line broadening effects are manageable at pH below 6.0 for low concentrations of citrate in the presence of Pr^{3+} and (*S*)-lactate at 200 MHz and room temperature. Under other conditions, exchange effects can cause deleterious broadening as discussed further below. The citrate and lactate protons in the presence of Pr^{3+} are strongly deshielded from their normal chemical shifts, whereas they are more shielded in the presence of Eu^{3+}, in agreement with previous work on carboxylate complexes of these lanthanide ions.[23,24] We chose the Pr^{3+} system because it readily gives spectra where all the resonances are well separated from one another.

The spectrum of a citrate–(*S*)-lactate–Pr^{3+} solution with (*S*)-lactate in large excess (Figure 1A) shows four CH_2 chemical shifts for citrate, as two separate AX quartets (i.e., four doublets) with $^2J_{AX}$ = −15 Hz. The chemical shift differences between geminal protons have become very large (ca. 3.5 ppm) as compared to those for citrate or citric acid in water, and the systems are therefore labeled AX instead of AB. Decoupling of the most shielded proton doublet (lines 7 and 8 in Figure 1A) changes the next to most deshielded proton doublet (lines 3 and 4) into a singlet. Furthermore, as the concentration of (*S*)-lactate is lowered, the resonances of the two most shielded proton doublets gradually merge to become a single doublet (^2J = −15 Hz), and the same thing happens to the resonances of the two least shielded protons. The enantiotopic and diastereotopic relationships of the four CH_2 protons in citrate under these conditions, in terms of chemical shift assignments, are clearly defined by the above evidence. The absolute chemical shifts in these solutions are sensitive to the concentrations of the components and to the pH, but the *order* of the chemical shifts remains unchanged. A large excess of lactate is required in order to resolve the resonances of the two most deshielded protons in citrate.

In order to make an absolute assignment of the citrate chemical shifts, a sample of citrate-*2-d* was prepared by exchanging one of the four methylene protons in citrate with a deuteron by means of aconitase in a D_2O solution.[15] The partially exchanged sample contained about 25% monodeuterated citrate in ordinary citrate. Figure 1B shows the NMR spectrum of this sample in the presence of Pr^{3+} and (*S*)-lactate. Two citrate proton resonances are clearly affected by the deuteration: The next to most deshielded proton doublet (lines 3 and 4 in Figure 1A) has a reduced intensity, while the most shielded proton doublet (lines 7 and 8 in Figure 1A) gives

(30) Thomson, J. F.; Nance, S. L.; Bush, K. J.; Szczepanik, P. A. *Arch. Biochem. Biophys.* **1966**, *117*, 65.
(31) Hunäus, P. *Ber.* **1876**, *9*, 1749.

Table I. Proton Chemical Shift Assignments in Citrate and Citrate-*2-d*

solution	chemical shift[a] (δ, ppm)			
	H_{RR}	H_{SS}	H_{RS}	H_{SR}
citrate–(*S*)-lactate–$PrCl_3$[b]	11.98	12.15	8.42	8.60
citrate-*2-d*–(*S*)-lactate–$PrCl_3$[b]		12.15	8.38	8.60[c]

[a] Unless otherwise indicated, the resonances are doublets with ^2J = −15 Hz. [b] In D_2O at pH 3.9 and 25 °C; see Figure 1. [c] Singlet.

Table II. Temperature Dependence of the Chemical Shifts in the Citrate–(*S*)-Lactate–$PrCl_3$ System

T (°C)	NMR freq (MHz)	line width lactate CH (Hz)	chemical shift (δ, ppm)[a]		
			CH (lactate)	$(\delta_{SR} + \delta_{RS})/2$	$(\delta_{RR} + \delta_{SS})/2$
5	200	65	6.40	8.32[b]	12.42[c]
25	200	20	6.50[d]	8.53[e]	12.06[e]
25	500	70	6.5	8.53[f]	12.06[f]
50	500	40	6.65	8.70[c]	11.68[c]
70	200	*g*	6.65	8.96[f]	11.48[c]

[a] The same sample (pH 3.8) was used in all measurements, and the spectra were referenced to the temperature-corrected HOD peak (see Experimental Section). [b] Resolved chemical shifts ($\Delta\delta$ = 0.3 ppm), but unresolved ^2J splittings. [c] Unresolved chemical shifts and *J* splittings. [d] Lactate methyl, δ = 2.97 ppm, ^3J = 7 Hz. [e] Resolved chemical shifts and ^2J splittings (Table I), doublets, $^2J \approx -15$ Hz. [f] Resolved chemical shifts, but unresolved ^2J splittings. [g] Barely resolved 1:3:3:1 quartet.

rise to a superposed doublet and singlet.[32] The latter signal arises from a CHD group and shows an isotope effect (shielding) of ca. −40 ppb compared to the chemical shift of the corresponding proton in undeuterated citrate, which gives rise to the doublet. This chemical shift isotope effect is about twice as large as that in citrate-*2-d* in D_2O[15] or in trimethyl citrate-*2-d* (see below) and presumably has a contribution of about −20 ppb from an equilibrium isotope effect.[33-35] The sign convention[35] (more shielded is negative) for isotope effects on chemical shifts used here is consistent with that for substituent effects, but is opposite to that in Hansen's review.[33]

The absolute configuration of citrate-*2-d* obtained by enzymatic exchange is known to be 2*R*,3*R*,[1-4,36-38] and thus the chemical shifts of citrate-*2-d* under the above conditions can be assigned as shown in Figure 1C and Table I.

Exchange Effects in the Citrate–(*S*)-Lactate–Pr^{3+} System. Both the citrate and lactate line widths and chemical shifts in solutions containing Pr^{3+} depend on concentration, pH, temperature, and the spectrometer frequency (Table II). A pH less than about 3 results in sharp lines but small shifts, presumably because of protonation of the ligands and decomplexation, and a pH greater than about 6 results in the formation of a precipitate. The concentrations used by previous workers with the lactate–Pr^{3+} shift reagents[21,22] are too high for use with citrate and also lead to precipitation. The solutions used for the data in Table II have a large mole ratio (16:1) of lactate to Pr^{3+}, so that much of the lactate is probably uncomplexed. Lower lactate concentrations give smaller chemical shift differences for the four citrate CH_2 protons and also lead to overlap of the CH lactate resonance with the resonances of the two most shielded citrate protons.

The pair H_{RR}–H_{SS} in citrate becomes more shielded as the temperature is increased, but the H_{RS}–H_{SR} pair and the lactate CH unexpectedly show the opposite behavior. This means that the diastereochemical shift differences between the protons within the CH_2 groups, which are of the order of 3 ppm and are therefore much larger than those for uncomplexed citrate in D_2O ($\Delta\delta \approx$ 0.1 ppm), decrease with increasing temperature (Table II). Also, the diastereochemical shift difference between the protons within each CH_2 group in citrate as a lanthanide complex decreases as the temperature is increased. However, the mean citrate CH_2 chemical shift remains essentially constant (δ = 10.3 ± 0.1). A simple equilibrium between uncomplexed and complexed ligands that is not strongly one-sided should shift toward the uncomplexed state at higher temperatures, and all the chemical shifts should strongly decrease. This clearly does not happen, and it appears that the excess of lactate and the strong complexing ability of citrate maintain the Pr^{3+} nearly fully complexed at all temperatures. The relatively small observed chemical shift changes are possibly the result of a (rapid) conformational equilibrium within the complex.

Relaxation effects in paramagnetic systems can be complicated, and the following interpretations of line broadening, although reasonable, should be regarded as tentative.[13,39] The line widths of citrate and lactate in the presence of $PrCl_3$ show changes with both temperature and spectrometer frequency (Table II). The resonance of the lactate CH is narrow at high temperatures, and very broad at low temperatures. The line width of this resonance at a given temperature is much larger at 500 MHz than at 200 MHz, indicating that the broadening arises from a dynamic NMR (exchange) effect between complexed and uncomplexed lactate. Intermediate line widths are observed at 300 and 360 MHz. The citrate CH_2 lines are also much broader at 500 MHz than at 200 MHz, but unlike the lactate CH signal they broaden at both lower *and* higher temperatures. Thus, the best resolved citrate spectrum is obtained at 200 MHz near room temperature. It has previously been observed that exchange broadening in 1-nonanol in the presence of β-diketone complexes of paramagnetic lanthanide ions is much larger at 400 MHz than at 100 MHz.[40]

The citrate resonances broaden below room temperatures, and this can be ascribed to a lowered rate of molecular tumbling of the fairly large complex. The increased broadening of the citrate resonances observed at 70 °C as compared to room temperature may be the result of a very small amount of free (or partially free) citrate at higher temperatures. The low citrate and high lactate concentrations should favor a small dissociation of the citrate ligand, with the equilibrium being highly temperature dependent because of an expected large positive entropy favoring free citrate. This may not give much broadening at room temperature because of either a very low concentration of uncomplexed citrate or a very small exchange rate. At higher temperatures the free citrate concentration still may be too low to make much of a difference to the observed chemical shifts, but it might be large enough to give some broadening.[41,42] This explanation requires an appreciable exchange rate between bound and unbound citrate at these higher temperatures. If the exchange rate is large enough to give averaged signals, then the greater broadening of the H_{RR} and H_{SS} than of the H_{SR} and H_{SR} resonances becomes understandable. Under these conditions the broadening should be larger for the

(32) The geminal coupling constant (J_{HD} = −2.3 Hz) is not resolved because the quadrupolar relaxation of the deuteron is expected to be fast and because the proton line widths are too large.

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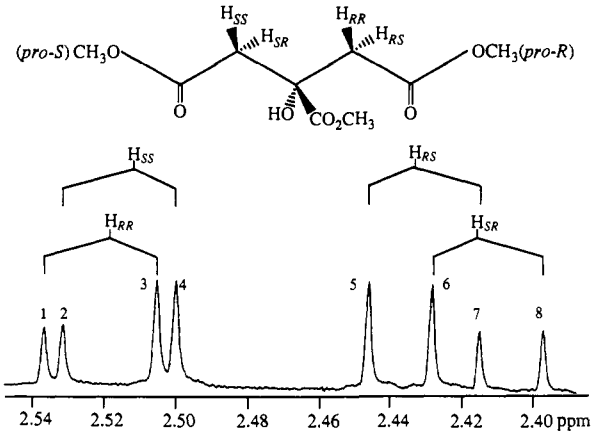

Figure 2. 500-MHz ^{1}H NMR spectra of the methylene region in trimethyl citrate (TMC) with Pirkle's chiral alcohol (II) in CCl_4/C_6D_6 (ca. 10:1) showing two AB quartets. The diagrammatically represented splittings arise from $^2J_{AB}$. The ester methyl groups give singlets at δ = 3.351 (*pro-R* methyl), 3.357 (*pro-S* methyl), and 3.520 ppm (central methyl) under these conditions.

protons that are most shifted,[41] i.e., H_{RR} and H_{SS}, as observed.

Trimethyl Citrate (TMC) in Chiral Media. The hydroxyl group in TMC should facilitate complexation to a chiral lanthanide shift reagent. Unfortunately, even with resolution enhancement at 500 MHz, TMC shows only a single AB quartet in the presence of the Eu^{3+} tris(chelate) derived from 3-[(trifluoromethyl)-hydroxymethylene]-(+)-camphor. However, the two $CH_2CO_2CH_3$ branches in TMC show separate methyl resonances in the presence of this shift reagent. Attempts to induce splittings in the AB quartet of TMC with various resolved chiral compounds, such as valine methyl ester, methyl *p*-tolyl sulfoxide, *tert*-butyl *p*-tolyl sulfoxide, and dihydrobenzothiophene 1-oxide derivatives,[27] were all unsuccessful.

Pirkle's chiral anthracene alcohol (II), which is available commercially in both enantiomeric forms, does induce splittings in the AB quartet of TMC. In $CDCl_3$, II causes a small splitting in the more shielded CH_2 proton doublet of TMC. With benzene-d_6 as the solvent, the splitting is larger (0.014 ppm) but there is no observable splittings in the other (less shielded) doublet. When a solution of TMC in CCl_4/benzene-d_6 (ca. 10:1) is saturated with II, two resolved AB quartets are obtained (Figure 2) with splittings of 0.0184 and 0.0053 ppm. The assignments shown in Figure 2 are based on decoupling data and on correspondence with analogous spectra of TMC-*d* presented below.

Decoupling experiments of the type described earlier on citrate in the chiral aqueous media are not possible in the TMC-II system because the induced shifts are smaller (in absolute terms) than the geminal coupling constant ($^2J_{AB}$ = −15.6 Hz). Irradiation by a weak radio-frequency magnetic field on a single transition in the spectrum of Figure 2, i.e., in a "tickling experiment",[43,44] allows the eight CH_2 lines to be grouped into two separate AB quartets. Experiments were carried out on the lines (5–8) of the two most shielded protons, because these resonances are fairly well separated. In each case, one of the four lines (1–4) of the two most deshielded protons splits into a sharp doublet (regressive transition), while the other affected line splits into a broad doublet (progressive transition). For example, irradiation of line 8 splits line 4 into a sharp doublet and line 2 into a broad doublet. These 1-D experiments are much faster than 2-D COSY measurements which could give the same information. This is because the spectrum is simple, and the critical information depends on distinguishing lines that are 2.5 Hz apart, necessitating good digital resolution which is time consuming in 2-D spectroscopy.

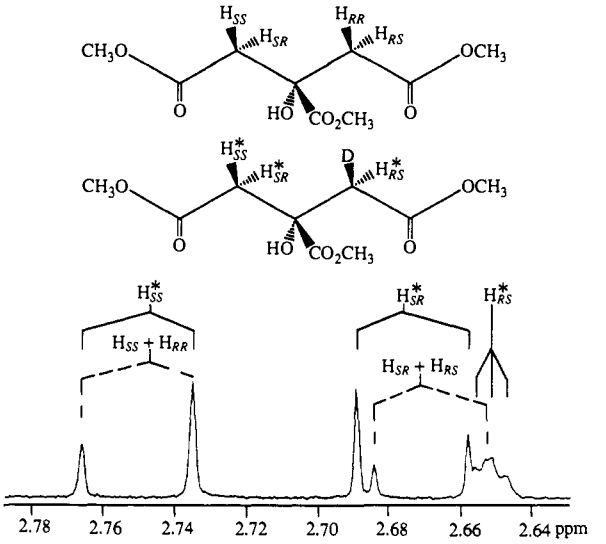

Figure 3. 500-MHz ^{1}H NMR spectra of the methylene region in trimethyl citrate-*2-d* (TMC-*2-d* containing 13% TMC as an isotopic impurity) in CCl_4/C_6D_6 (ca. 10:1). The peaks labeled with an asterisk are those of the deuterated compound; those without an asterisk arise from TMC. The 1:1:1 triplet given by H^*_{RS} arises from coupling (^2J) to the deuteron.

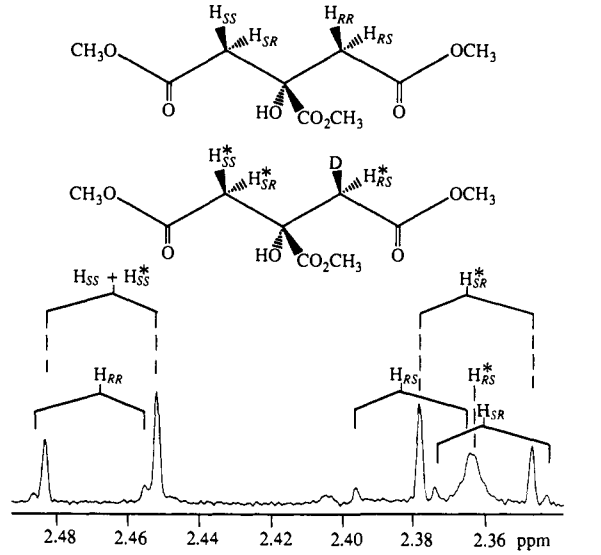

Figure 4. 500-MHz ^{1}H NMR spectra of the methylene region in trimethyl citrate-*2-d* (TMC-*2-d* containing 13% TMC as an isotopic impurity) with Pirkle's chiral alcohol (II) in CCl_4/C_6D_6 (ca. 10:1). The peaks labeled with an asterisk are those of the deuterated compound; those without an asterisk arise from TMC.

The TMC-II solution gives two closely spaced signals from the terminal methyls and to a less shielded and well separated signal from the central methyl group. The chemical shifts of the former methyls can be assigned by decoupling these protons selectively from the CH_2 AB quartets to which they have an unresolved long-range (^5J) coupling.[45] Irradiation of the more shielded methyl causes the lesser shielded CH_2 quartet to sharpen but leaves the other quartet unchanged, and vice versa for the more shielded methyl. This, together with evidence presented below leads to the assignments given in Figure 2.

Figure 3 shows a 500-MHz ^{1}H NMR spectrum of TMC-*2-d*, which contains ca. 13% TMC as an isotopic impurity. The TMC-*2-d* was made by esterification of (2*R*,3*R*)-citric acid-*2-d*.

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Table III. Proton Chemical Shift Assignments in Trimethyl Citrate (TMC) and Trimethyl Citrate-2-d (TMC-2-d)

solution	chemical shift[a] (δ, ppm)			
	H_{RR}	H_{SS}	H_{RS}	H_{SR}
TMC[b]	2.747	2.747	2.672	2.672
TMC-2-d[b]		2.747	2.651[c]	2.675
TMC-II[d]	2.459	2.454	2.372	2.347
TMC-2-d-II[d]		2.454	2.35[e]	2.351

[a] Unless otherwise indicated, the resonances are doublets with ^2J = −15.6 Hz. [b] Corresponds to the spectrum shown in Figure 3. [c] Broad 1:1:1 triplet, with outer lines broader than the center line because of quadrupolar relaxation of the deuteron; $^2J_{HD}$ = −2.3 ± 0.2 Hz. [d] Corresponds to the spectrum shown in Figure 5. [e] Broad singlet whose position is not well defined because of peak overlap (cf. Figure 4).

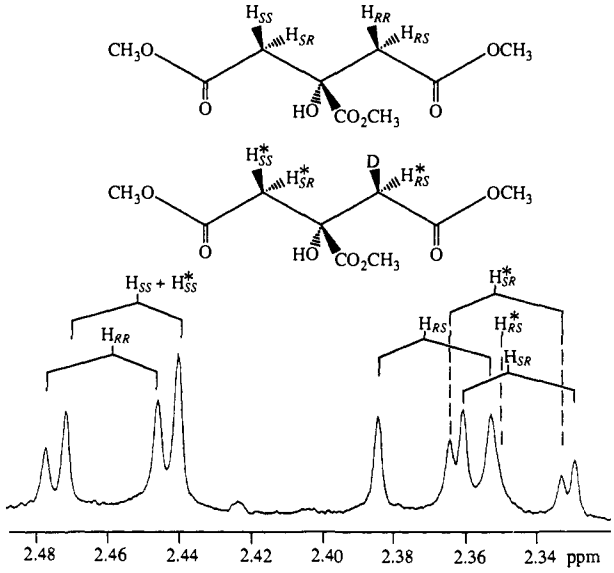

Figure 5. 500-MHz ^{1}H NMR spectra of the methylene region in a mixture of trimethyl citrate (TMC) and trimethyl citrate-2-d with Pirkle's chiral alcohol (II) in CCl_4/C_6D_6 (ca. 10:1). The peaks labeled with an asterisk are those of the deuterated compound; those without an asterisk arise from TMC.

The deuterium isotope effect on the chemical shift of the CHD proton (H_{RS}) in TMC-2-d is −23 ppb (Table III), and this is a typical value for a two-bond deuterium isotope effect.[33] A small four-bond *positive* isotope effect (4 ppb) is observed for H_{SR}• while H_{SS}• shows no observable isotope effect (<2 ppb).

Figure 4 shows a 500 MHz ^{1}H NMR spectrum of TMC-2-d in the presence of II. The concentrations of the substrate and of II are significantly less than those for the corresponding undeuterated sample, and thus the absolute chemical shifts are not meaningful. The 13% TMC that is an isotopic impurity provides an internal reference for the resonances of TMC-2-d. Figure 5 shows the spectrum of TMC-2-d to which has been added a substantial amount of TMC in order to make sure that the weak peaks observed in Figure 4 indeed arise from TMC. Although the absolute chemical shifts are different from those in both Figures 2 and 4, there is no difficulty in recognizing the peaks of TMC and TMC-2-d in Figure 5. The chemical shifts of H_{RS} (part of a CH_2 group) and H_{SR} (also part of a CH_2 group) in TMC-II differ by 25 ppb (Figure 5; Table III) because of the effect of the chiral shift reagent II. The corresponding resonances of TMC-2-d (indicated by asterisks in Figures 4 and 5) have nearly the same chemical shifts because H_{SR}• is now part of a CHD group and there is a compensating isotope effect of ca. −23 ppb.

Simple citrate salts in the crystalline state are known to exist with carbons 1–5 in the anti–anti or anti–gauche conformations,[2] and thus TMC probably exists in solution as a conformational mixture whose equilibrium constant could be perturbed by deuterium substitution. However, there is no evidence for a significant equilibrium contribution to the isotope effect,[33,34] unlike the situation with the aqueous lanthanide shift reagent described above.

Conclusions. In appropriate chiral media, the citrate ion and trimethyl citrate each give rise to four CH_2 proton chemical shifts with known absolute configurational assignments. An easy NMR determination of the absolute configurations at the two different stereogenic centers of any of the four possible monodeuterated citric acid or trimethyl citrate isotopomers (deuterated at a methylene group) is now possible[46] and may be useful in mechanistic studies related to aconitase.[3,47] Related tritiated citric acid species,[4] with sufficient concentrations of tritium for NMR observation, should be differentiated clearly by means of ^{3}H NMR with the same shift reagents, and absolute assignments become possible if the NMR of a randomly tritiated citrate sample is also measured for comparison.

Acknowledgment. The NMR spectrometers were purchased through funds provided in part by the National Science Foundation and the National Institutes of Health. We thank Debbie Crans for discussion on water-soluble lanthanide shift reagents.

Registry No. $PrCl_3$, 10361-79-2; D_2, 7782-39-0; citrate ion, 126-44-3; trimethyl citrate, 1587-20-8; (2*R*,3*R*)-citrate-2-d, 137623-72-4; trimethyl (2*R*,3*R*)-citrate-2-d, 137540-87-5; (*S*)-lactate, 72-08-2; (*S*)-2,2,2-trifluoro-1-(9-anthryl)ethanol, 60646-30-2; (2*R*,3*R*)-citric acid-2-d, 137623-73-5.

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