ism causes variations on certain carbon chemical shifts and line widths, especially at those carbons which are directly adjacent to the nitrogens which participate in this phenomenon. In the case of formycin (**4**) no carbon chemical-shift variations were observed which would account for any appreciable amine–imine tautomerism. Similarly, lactam–lactim tautomerism for those derivatives which have a 7-oxo function was not observed. Our data establish that the labile hydrogen in this portion of the pyrazolo[4,3-*d*]pyrimidine ring resides exclusively on the nitrogen (N6) adjacent to the oxo function. Therefore, the major tautomeric process observed in those derivatives studied is N(1)H ⇌ N(2)H. This prototropic process appears to be dependent on the substituent residing at C7, e.g., a hydrogen, amino, or oxo group. When a hydrogen atom is bonded to the C7 position of the pyrazolo[4,3-*d*]pyrimidine aglycon, the N(2)H tautomeric form was not detected. On the other hand, when an amino or oxo substituent is attached to this position, prototropic exchange between the N(1)H and N(2)H tautomers occurs. The data indicate that the less favorable (higher energy) N(2)H form is stabilized to a greater degree by the presence of an oxo group at this position as compared to an amino group. A possible explanation for the varying extent of tautomerism when the substituent at C7 is either a hydrogen, amino, or oxo group is their different electronic (resonance and/or inductive) influence on the pyrazolo[4,3-*d*]pyrimidine ring system.

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pH Dependence of Carbon-13 Nuclear Magnetic Resonance Shifts of Tetracycline. Microscopic Dissociation Constants

G. L. Asleson and C. W. Frank*

Contribution from the Department of Chemistry, University of Iowa, Iowa City, Iowa 52242. *Received November 14, 1975*

Abstract: Carbon signals of tetracycline hydrochloride were found to be very sensitive to pH in a 50/50 DMSO:water solvent system. The curves obtained by plotting the chemical shift of a signal vs. pH were related to the pK_a values which were determined to be 4.4, 8.1, and 9.8 in the mixed solvent system. These are compared to 3.3, 7.7, and 9.7 for tetracycline in aqueous solution. Microdissociation constants were determined and compared with values obtained by previous workers.

Proton NMR has been applied in the study of tetracyclines to elucidate stereochemical and structural features,[1-11] to determine microscopic dissociation constants,[12,13] to monitor the kinetics of epimerization at the 4 position,[14] and to study the binding sites of a number of metal ions.[15] Prior applications of ^{13}C NMR to tetracyclines have been the partial[16] and complete[17] assignment of ^{13}C NMR spectra, and studies concerning metal ion binding[18] and the effect of electrolytes on the metal binding sites of tetracycline.[19]

Previous discussions by Dias et al.[20] and Rigler et al.[12] indicated that it is possible that the microscopic dissociation constants of the tetracyclines are more reflective of the pro-

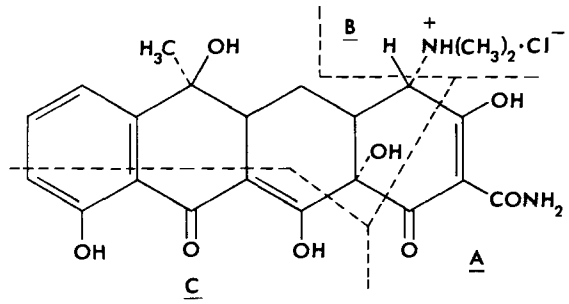

Figure 1. Sites of deprotonation for tetracycline hydrochloride.

cesses occurring within this molecule than are three macroscopic dissociation constants. Rigler et al.,[12] using a ^{1}H NMR technique, determined the twelve[12] microscopic pk_a's using indicator protons at the C-4 dimethylamino and C-8 positions. The proton NMR study on the pH dependence of tetracycline hydrochloride signals indicated the importance of the microscopic dissociation constants in understanding the chemical behavior of tetracycline, especially with regard to metal ion binding. Rigler et al.[12] assumed the shift of the dimethylamine protons to be exclusively a measure of the protonation at the B site and the shift of the C-8 proton to be a measure of the protonation at the C site. Extent of protonation at the A site was determined by difference.

Dias et al.[20] demonstrated mathematically the equivalence of the use of the macroscopic and microscopic dissociation constants, using the presumption that metal ions bind to a distinct chelation site.

Since it is generally agreed that the metal ion binding in tetracycline is dependent on the pH of the solution, it was necessary to study the pH dependence of tetracycline hydrochloride ^{13}C NMR chemical shifts. It was found that the low solubility of tetracycline in aqueous systems at midrange pH was not compatible with a ^{13}C NMR study. Of the several mixed solvent systems investigated, a 50/50 water:DMSO system was found to be the most similar to water in pH adjustment and allowed adequate solubility (0.1 M) at all pH values.

The dissociation constants for tetracycline hydrochloride in aqueous solution were reported as 3.3, 7.7, and 9.7, and the three pK_a values were assigned, respectively to the systems A, B, and C shown in Figure 1.[21] No effort was made to determine whether the C-10 hydroxyl or the C-12 hydroxyl was deprotonated. The relationships of macroscopic pK_a values to the microscopic pk_a values were discussed by Edsall and Wyman.[22] The pK_a values reported for the ^{1}H NMR study in 50/50 methanol:water were 4.4, 7.8, and 9.4 which were assigned to the C-3 hydroxyl proton, the dimethylammonium proton, and the C-10 hydroxyl proton, respectively.[12]

The macroscopic dissociation constants for tetracycline hydrochloride in the 50/50 water:DMSO system were determined by potentiometry to be 4.4, 8.1, and 9.8. These pK_a values agree well with those mentioned above for the methanol:water system and with those reported by Stoel (4.3, 7.7, and 9.7)[23] for a 50/50 water:DMF system.

Experimental Section

Potentiometric Titrations. The pK_a values for tetracycline hydrochloride in 50/50 water:dimethyl sulfoxide (DMSO) were determined from potentiometric data. Tetracycline hydrochloride (~1.4 mmol) was dissolved in 20 ml of the mixed solvent and titrated with 0.5 ml of 1 N sodium hydroxide which had been standardized with potassium hydrogen phthalate (KHP). An Agla micrometer syringe was used to dispense the titrant in 0.01-ml increments. The apparent pH values were monitored on a Heath EU-302A servo digital pH/volt meter using a Corning semimicro pH combination electrode (No. 476050) filled with 4 M KCl saturated with AgCl.

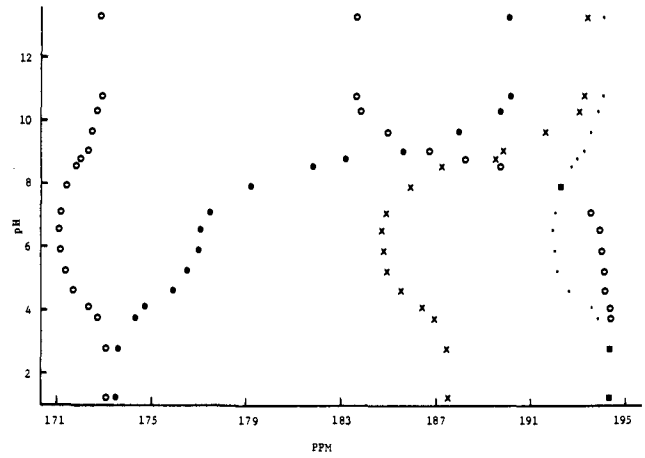

Figure 2. pH dependence of ^{13}C NMR signals for C-1, amide, C-3, C-11, and C-12 carbons: (•) C-1, (○) amide, (×) C-3, (□) C-11, (●) C-12.

The pK_a values were calculated by a computer program written by Jensen et al.[24] utilizing the method of Noyes of calculating pK_a values.[25]

Nuclear Magnetic Resonance Spectra. The ^{13}C NMR spectra were obtained on a Bruker HX-90-E high resolution Fourier transform spectrometer operating at 22.63 MHz. The accumulated interferograms were Fourier transformed by a Nicolet B-NC-12 computer. The samples used for the NMR titrations were 0.1 M in a 50/50 D_2O:d_6-DMSO solution with dioxane (67.4 ppm) as the internal standard. The desired pH was obtained by adding concentrated sodium hydroxide solution with a syringe. All samples were deoxygenated by bubbling with nitrogen for two reasons: broadening of signals because of paramagnetic oxygen is reduced and stability of tetracycline solution at high pH values is improved. All samples were spun in 10 mm tubes at a controlled temperature of 25 ± 1°. Deuterated solvents used for both ^{1}H and ^{13}C NMR were obtained from Stohler Isotopes, Bio-Rad Laboratories, and Aldrich Chemical Company.

Results and Discussion

The ^{13}C NMR signals are divided into two general groups: (1) those signals which shift with the first pK_a (4.4), and (2) those signals which shift with the remaining two pK_a's (8.1, 9.8).

Carbon Signal Shifts pH 1 to 6. The carbon signals that have an appreciable shift (>1.0 ppm) in the pH 1–6 range are the C-1, C-2, amide, C-3, C-4, dimethylamine, C-4a, C-5, C-12, and C-12a. The fact that all of these carbons are in the A and B rings supports the previous assignments of the first pK_a to the tricarbonyl system.

The pH dependence of the C-1 signal is presented in Figure 2. The signal was shifted upfield 2.3 ppm in the pH 1–6 range. Similar behavior was noted for the C-3 and amide carbon signals which were shifted upfield 2.7 and 1.9 ppm, respectively (Figure 2). These three oxygen-bonded carbons make up the tricarbonyl system of the A ring. It is possible for the acidic hydrogen of this system to be associated with any of the three oxygens. Thus, it is reasonable that the C-1, C-3, and amide carbon signals were affected similarly by dissociation of the first acidic proton.

The C-2 carbon signal was shifted downfield 5.6 ppm in this pH range as shown in Figure 3. The fact that approximately 75% of its total shift occurred in this pH range gave additional evidence for the assignment of the first pK_a to the tricarbonyl system. The C-4 carbon signal was shifted 1.6 ppm for this pH range or 25% of its total shift as shown in Figure 4. Its shift over this pH range is attributed to its proximity to the tricarbonyl system.

The dimethylamine and the C-4a carbon signals were shifted upfield 1.0 and 1.3 ppm, respectively, while the C-5 carbon signal was shifted 1.4 ppm downfield. The effect of pH on these signals is shown in Figures 5 and 6. These three signals are

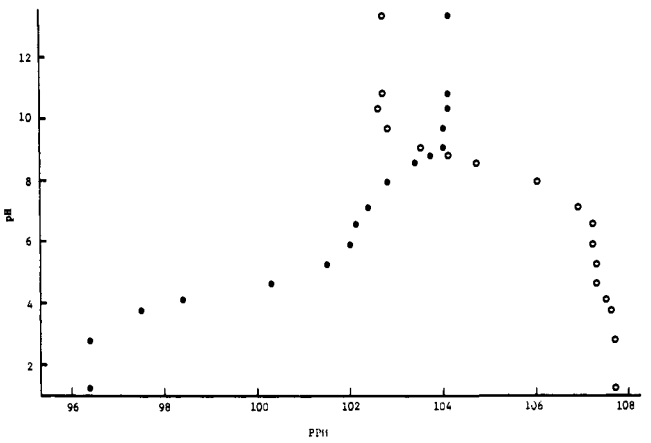

Figure 3. pH dependence of ^{13}C NMR for C-2 and C-11a carbons: (●) C-2, (○) C-11a.

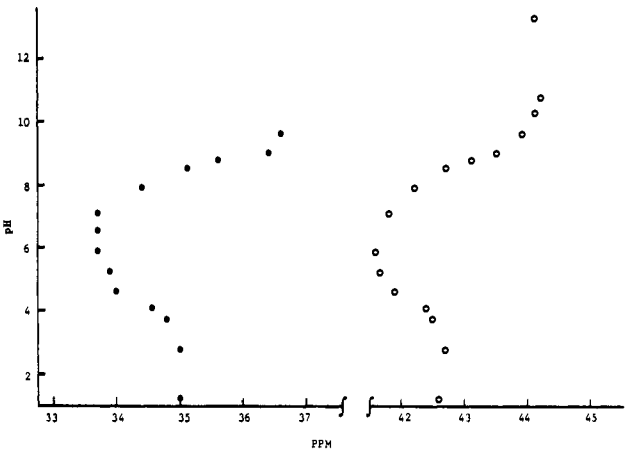

Figure 5. pH dependence of ^{13}C NMR signals for dimethylamine and C-4a carbons: (○) dimethylamine, (●) C-4a.

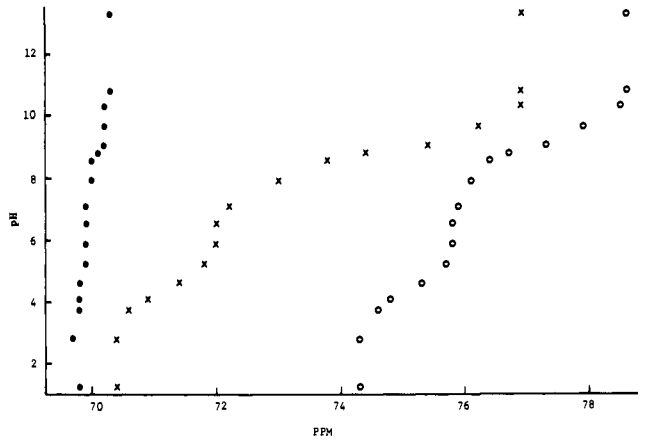

Figure 4. pH dependence of ^{13}C NMR signals for C-4, C-6, and C-12a carbons: (×) C-4, (●) C-6, (○) C-12a.

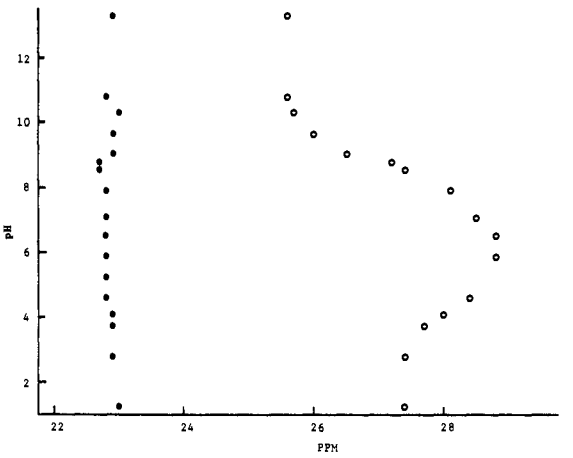

Figure 6. pH dependence of ^{13}C NMR signals for C-5 and C-6 methyl carbons: (○) C-5, (●) C-6 methyl.

shifted as a result of deprotonation in the nearby tricarbonyl system.

The pH dependence of the C-12 and C-12a carbons is shown in Figures 2 and 4. For the pH 1-6 range, the C-12 and C-12a signals were shifted 3.5 and 1.5 ppm downfield, respectively. Although the shift of 3.5 ppm for the C-12 signal is quite large, it is only 21% of the total shift observed for the signal. These shifts could be a combination of proximity to the tricarbonyl system and partial deprotonation of the C-12 hydroxyl group.

Ten carbon signals assigned to carbon atoms in the A and B rings were observed to have appreciable shifts in the pH 1-6 region. The only exceptions were the C-5a[26] and C-11a carbons which bridge the B and C rings. The largest shifts were observed for the four carbon signals of the tricarbonyl system. The shifts observed for the other carbon signals can be attributed to a proximity to the tricarbonyl system and/or a partial dissociation of the other acidic portions of the molecule.

Carbon Signal Shifts pH 6 to 13. The second and third pK_a values for tetracycline hydrochloride were determined to be 8.1 and 9.8. Just as a titration curve of tetracycline hydrochloride fails to give two distinct breaks for the last two deprotonations, an analysis of the shifts of carbon signals in the pH 6-13 range cannot easily distinguish the effects of deprotonation of the last two acidic sites. The carbon signals that were observed to have an appreciable shift (>1.0 ppm) in this pH range were the C-1, C-2, amide, C-3, C-4, dimethylamine, C-4a, C-5, C-6a, C-7, C-8, C-9, C-10a, C-11, C-11a, C-12, and C-12a. The C-1, C-2, amide, and C-3 carbon signals were shifted downfield 1.9, 2.1, 1.6, and 8.3 ppm, respectively, as shown in Figures 2 and 3. For the first three signals, the shift in the pH 6-13 range was less than that observed for the pH 1-6 range which might be expected for carbons of the tricarbonyl system. However, the large shift observed for the C-3 signal is somewhat unexpected and is attributed to its proximity to the dimethylamine group.

The C-4 signal in Figure 4 was noted to shift downfield 4.9 ppm (75% of the total shift). The C-4a, dimethylamine, and C-5 signals all were observed to have approximately 70% of their shift in this pH range as shown in Figures 5 and 6. The C-4a and dimethylamine signals were shifted downfield 2.9 and 2.4 ppm, respectively, while the C-5 signal was shifted upfield 3.2 ppm.

The C-6a, C-7, C-8, C-10, C-9, and C-10a carbon signals shown in Figures 7 and 8 have no appreciable shifts in the pH 1-6 range since they are well removed from the tricarbonyl system. The C-6a, C-7, C-8, C-9, and C-10 signals were shifted upfield 2.0, 1.0, 4.5, 2.1, and 0.4 ppm, respectively, in the pH 6-13 range, and the C-10a signal was shifted downfield 2.8 ppm.

The pH dependences of the C-11, C-11a, C-12, and C-12a carbon signals are shown in Figures 2, 3, and 4. In the pH 6-13 range the C-11 and C-11a signals were shifted upfield 10.4 and 4.5 ppm, respectively, while the C-12 and C-12a signals were shifted downfield 13.0 and 2.8 ppm, respectively. Since the C-12 signal was observed to have a very large shift in this pH range, it seems reasonable to assign the deprotonation to the

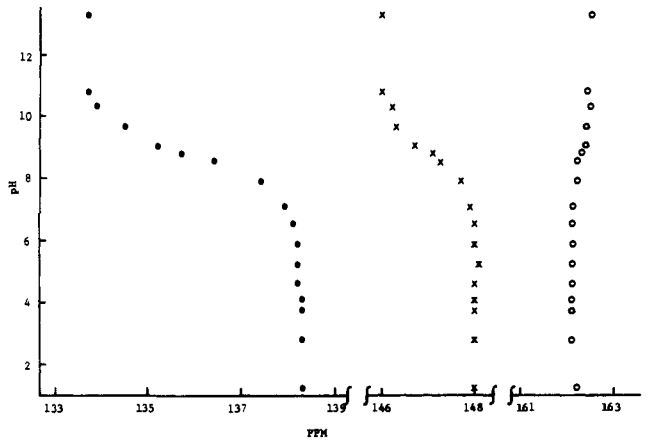

Figure 7. pH dependence of ^{13}C NMR signals for C-6a, C-8, and C-10 carbons: (×) C-6a, (●) C-8, (○) C-10.

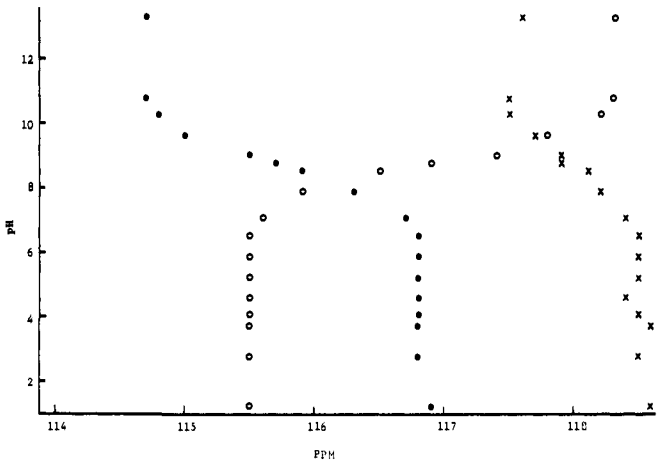

Figure 8. pH dependence of ^{13}C NMR signals for C-7, C-9, and C-10a carbons: (×) C-7, (●) C-9, (○) C-10a.

C-12 hydroxyl rather than the C-10 hydroxyl as reported by Rigler et al.[12] Deprotonation of the C-10 hydroxyl would be expected to manifest itself by a shift of greater than 0.4 ppm because the hydroxyl bearing carbon in phenol has been reported to shift 11 ppm over pH 1–13.[26]

The carbon signals that were observed to have very small shifts over the entire pH range were the C-6, C-6 methyl, and C-10. This was not unexpected for the C-6 and C-6 methyl which are somewhat isolated from all three of the acidic centers.

Microscopic Dissociation Constants. The microscopic dissociation constants were calculated in the following manner. Since a distinct break was noted for the first pK_a, it was assumed that at the pH equal to pK_1, eq 1 is valid

$$[A^0B^+C^0] = [A^-B^+C^0] = 0.5T \quad (1)$$

where T is the total concentration of tetracycline. A similar assumption was made so that at a pH equal to pK_3, eq 2 is also valid.

$$[A^-B^0C^-] = [A^-B^0C^0] + [A^-B^+C^-] = 0.5T \quad (2)$$

The microscopic equilibria for such a scheme are shown in Figure 9. This is in contrast to the classic microscopic equilibrium scheme of Rigler et al.,[12] who assumed that the first deprotonation could occur at any of the three acidic sites as shown by the microscopic equilibria in Figure 10.

As examples the microconstants k_1 and k_{123} were calculated from the following equations,[26] defining pH = pK_1 in eq 3 and pH = pK_3 in eq 3a.

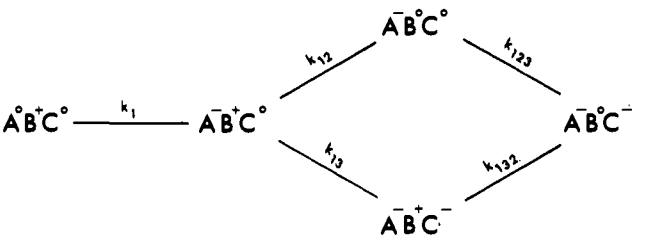

Figure 9. Microscopic equilibria for tetracycline hydrochloride assuming first deprotonation exclusively from tricarbonyl system (A site).

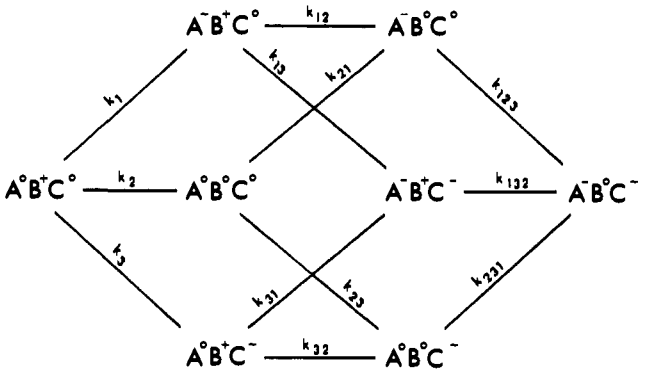

Figure 10. Microscopic equilibria for tetracycline hydrochloride.

Table I. Percent Protonation at Various Values of n^a

					Site		
n	pH	A	B	C	A^b	B^b	C^b
3	3.5	100	100	100	100	100	100
2.5	4.4	50	100	100			
2	6.1	0	100	100	19	90	91
1.5	8.1	0	74	76			
1	9.1	0	66	34	13	16	51
0.5	9.8	0	36	14			
0		0	0	0	0	0	0

[a] n = equivalents of dissociable hydrogen remaining. [b] Rigler et al.

$$k_1 = \frac{[H^+][A^-B^+C^0]}{[A^0B^+C^0]} = \frac{K_1[A^-B^+C^0]}{0.5T} \quad (3)$$

$$k_{123} = \frac{[H^+][A^-B^0C^-]}{[A^-B^0C^0]} = \frac{K_3(0.5T)}{[A^-B^0C^0]} \quad (3a)$$

Substitution of eq 4 and 4a into eq 3 and 3a yields the microdissociation constant. In these equations (eq 4 and 4a) $f_{2.5A}$ is the percent protonation at the A site when 2.5 equiv of dissociable hydrogen remain and $f_{0.5c}$ is the percent protonation at the C site when 0.5 equiv remains.

$$[A^-B^+C^0] = (1 - f_{2.5A})T \quad (4)$$

$$[A^-B^0C^0] = (f_{0.5c})T \quad (4a)$$

The values for the percent protonation are presented in Table I. Two assumptions were made in the determination of these values: (1) that pK_1 is caused totally by deprotonation of the tricarbonyl system, and (2) the pH dependence for the C-8 carbon is a good probe to measure the degree of protonation at the C site since this carbon is well removed from either the A or B site.[12] The percent protonation at the B site was determined by difference. The microscopic constant k_{132} was calculated analogous to k_{123}. The two remaining microcon-

Table II. Microscopic Dissociation Constants for Tetracycline Hydrochloride

Calcd	Lit. data[a]	Calcd	Lit. data[a]
$pk_1 = 4.40$	$pk_1 = 4.49$	$pk_{123} = 9.25$	$pk_{123} = 9.11$
$pk_{12} = 8.65$	$pk_{12} = 8.00$	$pk_{132} = 9.66$	$pk_{132} = 8.60$
$pk_{13} = 8.24$	$pk_{13} = 8.51$		

[a] Rigler et al.

stants k_{12} and k_{13} were calculated using the relationship $K_1K_2K_3 = k_1k_{12}k_{123} = k_1k_{13}k_{132}$.

The microscopic dissociation constants determined in this manner are compared in Table II to those reported by Rigler et al.[12] The differences arise for several reasons. First, Rigler et al.[12] did not make an assumption that the A site is totally deprotonated at pH 6, even though they observed a distinct break in this region. Their protonation data also included a reprotonation at the A site at higher pH values which is difficult to justify even when considering the zwitterionic character of the A ring. The other differences arise because Rigler et al.[12] believe that the B site is deprotonated prior to the C site while the ^{13}C data indicates that initially the two sites are nearly equivalent and later deprotonation at the C site is favored (see Table I).

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Charge Distributions and Chemical Effects. 11. On the Charge Dependence of ^{13}C Chemical Shifts in Adamantane and Related Six-Membered Polycyclic Molecules

G. Kean, D. Gravel, and S. Fliszár*

Contribution from the Department of Chemistry, University of Montreal, Montreal, Quebec H3C 3VI, Canada. *Received December 12, 1975*

Abstract: The analysis of ab initio charge distributions in adamantane and selected polycycloalkanes constructed from chair and/or boat cyclohexane units indicates that no effect beyond what is included in the relationship $\delta_C = -237.1q_C + 242.64$ ppm from TMS between ^{13}C chemical shifts and C net charges (as determined for acyclic alkanes and cyclohexanes) contributes to any significant extent to the shielding of the carbon atoms. The optimized ab initio charges satisfying this δ_C-q_C relationship reflect, hence, all effects which in usual empirical calculations are itemized as $\alpha, \beta, \gamma, \ldots$ contributions.

Recent studies[1] have indicated that in acyclic alkanes the carbon-13 nuclear magnetic resonance shifts, δ_C, are linearly related to the carbon net charges, q_C, i.e.,

$$\delta(^{13}C) = -237.1q_C(\text{rel}) + 242.64 \text{ ppm} \quad (1)$$

from TMS, with a standard error of ±0.3 ppm. The choice of tetramethylsilane as a standard of reference is, in itself, arbitrary and represents purely a matter of practical convenience. In fact, choosing ethane as the reference compound for defining chemical shifts, eq 1 can be written as follows

$$\delta(^{13}C) = -237.1(q_C - 1) \text{ ppm} \quad (2)$$

from ethane, which in certain cases turns out to be a more practical form than (1). Of course, in these equations q_C is to be expressed in the system of relative units defined by setting the C net charge of ethane equal to 1 arbitrary unit, along the lines described in ref 1-3. The merit of eq 2 is to express clearly that the only empirical parameter arising from the correlation between chemical shifts and carbon net charges is the slope, −237.1 ppm/relative charge unit.