Importance of Tautomers in the Chemical Behavior of Tetracyclines†

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Received April 28, 1998. Final revised manuscript received July 21, 1998.
Accepted for publication September 8, 1998.

Abstract □ We advance the concept that tautomerism is crucial for the understanding of the chemical behavior of tetracycline. Indeed, considering four deprotonations, there are 64 different possible tautomers to be considered for tetracycline. Our results indicate that tetracycline is a very adaptive molecule, capable of easily modifying itself through tautomerism in response to various chemical environments. Indeed, its situation in solution can be more accurately pictured as an equilibrium among a diversity of tautomeric species–an equilibrium that can be easily displaced depending on the various possible chemical perturbations, such as varying the pH or the dielectric constant of the solvent. Moreover, we also show that tetracycline could undergo four deprotonations and predict for it a fourth pK_a of 13 and refer to our experimental determination of this parameter, which yielded the value of 12. We conclude that tautomerism is essential to the comprehension of the chemical behavior of tetracycline as determined by the semiempirical method AM1 as well as by the self-consistent reaction field method, which estimates the effects of the solvent on the tautomers. All tautomers in their different conformations have been fully optimized for each of the possible degrees of protonation of this molecule. Thus, the relative stabilities of the different tautomeric species have been computed.

1. Introduction

Tetracycline (TC, Figure 1) and its derivatives are widely used antibiotics.[1] The chemical−structural properties of tetracycline have been extensively studied.[2] TC has different acid groups in its chemical structure and the possibility to adopt different conformations. The different proton-donating groups of this molecule offer several possibilities of metal ions substitution. The complexation with metal ions increases the stability of the various TC derivatives.[3] In some cases, metal ion complexation reduces the availability of TC in the blood plasma[4] or eliminates its biological activity.[5] It is known that TC forms complexes in different positions with calcium and magnesium ions that are available in the blood plasma.[6,7] Undoubtedly TC is a complex molecule and, despite all effort, its mechanism of action is still not well understood.

The three acid dissociation constants normally observed in potentiometric experiments and the appropriate assignment of the various acid groups of TC to the respective dissociation constants are important to understand the chemical behavior in blood plasma and the biological

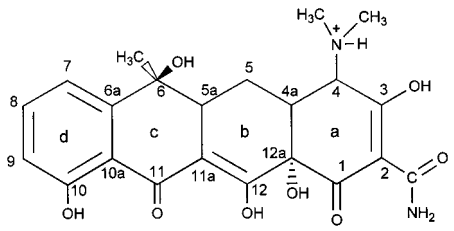

Figure 1—Chemical structure of TC.

activity of TC. The protonation scheme of TC has been the subject of controversy and intense study.[8−11] In 1956, Stephens et al.[8] proposed that pK_1 (= 3.30) is due to the protonation of the oxygen bonded to the C(3), pK_2 (= 7.68) is due to the protonation of the dimethylamino group, and pK_3 (= 9.69) is due to the protonation of the oxygen atoms bonded to the C(10) and C(12). Later, Leeson et al.[9] comparing the pK_as of different TC derivatives, suggested that pK_2 should be assigned to the protonation of the oxygen atoms bonded to the C(10) and C(12) and pK_3 to the protonation of the dimethylamino group. The effect of dielectric constant on the pK_a values of TC has been investigated by Garrett[12] studying TC in dimethylformamide−water mixtures. The observed decrease in pK_a values of uncharged acids with increasing dielectric constants supports the reversal in assignment of the 2nd and 3rd pK_as of TC. Rigler et al.,[10] using ^{1}H NMR, proposed a different protonation scheme. The chemical shifts of protons of the 4-dimethylamino group and the nonlabile protons of the phenolic group have been monitored as a function of pH. They assumed that the chemical shifts change depends exclusively on the respective protonation sites. Based on this assumption, they suggested that pK_2 and pK_3 have contributions from the dimethylamino and phenolic C(10) groups, respectively. The work of Asleson and Frank[11] using ^{13}C NMR supports the suggestion of Rigler et al.[10] and proposed that the protonation of the oxygen in C(12) is preferential with respect to the phenolic group C(10). They used the chemical shift of C(8) (see Figure 1) as a measure of protonation of the phenolic group C(10) and assumed that pK_1 is due to the tricarbonyl system of ring "a".

Another important aspect is the different conformations that this molecule can have depending on the medium. Mitcher et al.[13] have used circular dichroism (CD) to show that the conformation of TC in acid solution is different from the conformation it displays in neutral or basic solutions. Stezowski[14] showed that the hydrate crystals of TC are composed of zwitterionic structures. On the other hand, the anhydrous crystals of TC obtained by Stezowski and Jogun[15] have a different conformation. Stezowski et al.[16] studied the oxytetracycline in an ethanol/water solvent and showed evidence of the coexistence of zwitterionic and un-ionized forms of the free base in solution. They suggested that two conformations of TC are in equilibrium.

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10.1021/js980181r CCC: $18.00 Published on Web 12/11/1998

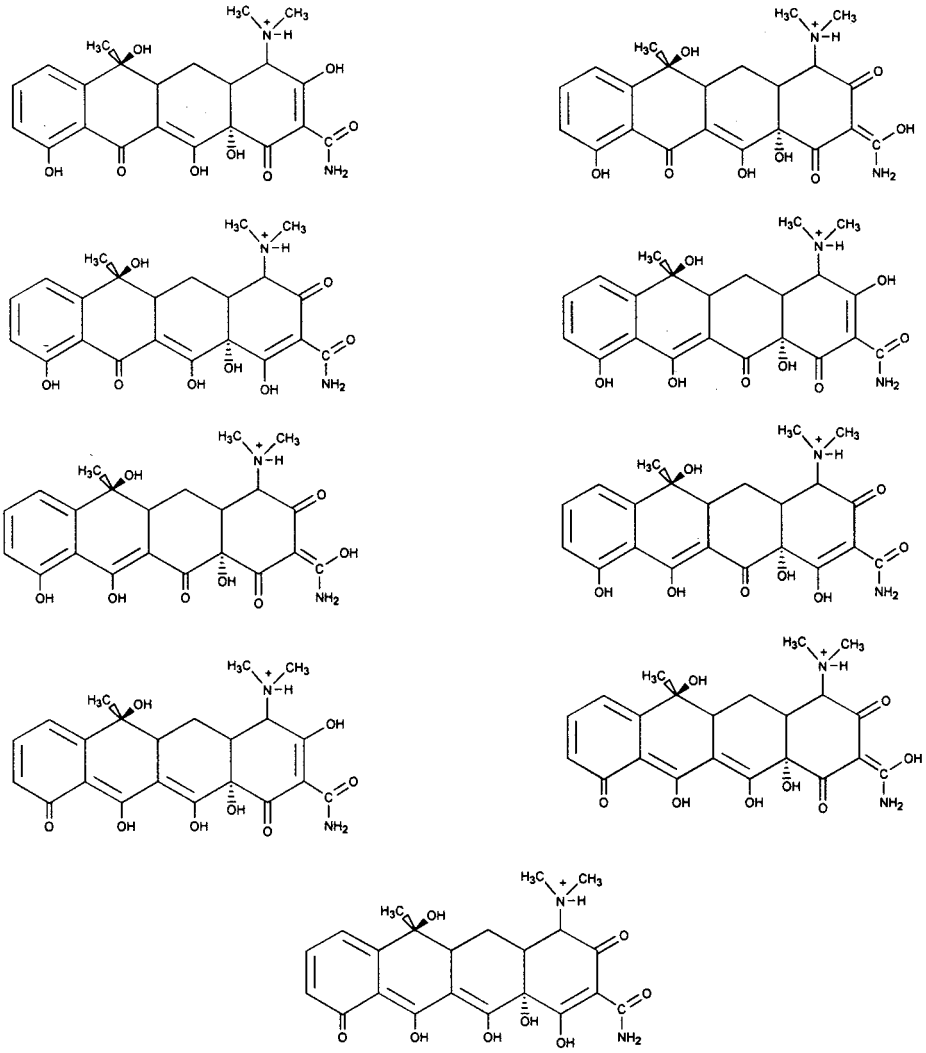


Figure 2—Possible tautomers of the completely protonated TC.

Metal ion coordination by TC has also received appreciable attention by researchers[6] due to the fact that some of the biological effects of TC and its derivatives arise from their interactions with metal ions.[17,18] TC has various possibilities for metal ion increasing the complexity of this system. Although some metallic complexes of TC in solid state have been isolated, most of them are studied in solutions. Complexes with Cr^{3+}, Nb^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Al^{3+}, VO^{2+}, and VO_2^{2+} have been studied[6,7,19–22] and, according to different reports, the metal ions are either bonded to the O(1) or O(3) and to the oxygen of the amide group, or bonded to the acidic groups of the rings b, c, and d. Recently, anhydrotetracycline, a decomposition product of TC, has also received attention because of its importance to the understanding of the side effects and the mechanism of action of this drug.[23]

Despite all studies reported concerning the chemical and structural properties of TC, there are still several aspects that remain not well understood. Figure 1 shows the tautomer normally used to describe the structure of TC. However, this molecule has several other possibilities of tautomerism (see Figure 2). It is evident that different tautomeric species are present in the medium contributing to the macroscopic chemical behavior of TC. The relative ratio of different tautomers depends on the difference of free energy between two tautomeric species.

To our knowledge, the tautomerism of TC and its consequences for the understanding of chemical properties has not received attention, although it is of clear interest. In this paper we advance the concept that different tautomers of TC may be involved in its protonation scheme, in its metal ion complexation, in its chemical behavior in blood plasma, in its biological activity, etc. Moreover, we also show theoretical and experimental evidences that TC can undergo four deprotonations.

Recently, quantum mechanical semiempirical methods have been applied successfully to perform a conformational analysis of anhydrotetracycline[24] and to investigate its near ultraviolet (UV) and visible (VIS) electronic spectra.[25]

In the present work we have studied all possible tautomers of TC in different conformations and degrees of deprotonation using the semiempirical method AM1. The solvent effects have been included using the self-consistent reaction field (SCRF) method. Potentiometric and spectrophotometric experiments are also being presented showing evidences that TC undergoes a 4th deprotonation.

2. Computational Aspects

The semiempirical AM1 method (Austin Model 1)[26] implemented in the program MOPAC 6.0[27] was used to study the tautomerism of TC in its different degrees of deprotonation. The geometries were fully optimized and vibrational analysis was performed. All frequencies were positive, ensuring that true minima in the potential energy surface had been found. Finally, the solvent effect was

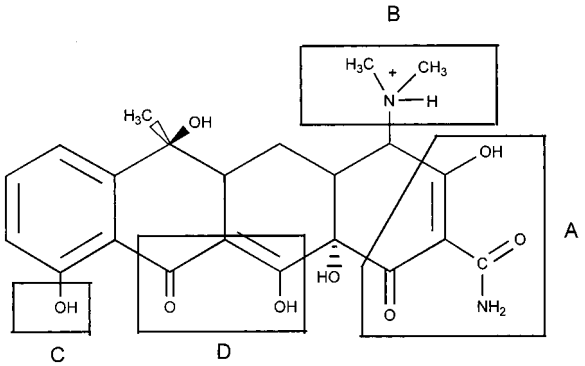


Figure 3—Possible sites of protonation of TC.

determined by the SCRF method, proposed by Tapia et al.,[28] merged with semiempirical methods of Zerner and co-workers.[29]

In this SCRF method, the solute lies inside a spherical cavity within a continuous dielectric medium representing the solvent. To determine the SCRF radius of the spherical cavity, rc, in which the tautomers are inserted, we used the method of Aguilar and Valle.[32] We chose this method because it is less arbitrary than most used to find the cavity radius. Indeed, it not only generates a single value per molecular structure but it is also based on a well-defined procedure for the adaptation of the molecular solute cavity size in a solvent that takes into account the charge distribution of the molecule.

The AM1/SCRF method is a reasonably good one to study this highly versatile and adaptive character of TC. This can be verified by examining the studies by Zerner et al.[29–31] on the tautomeric equilibria of the 5-nitroimidazole and 4-nitroimidazole, on the tautomeric equilibria of five-membered rings with two heteroatoms, and on 2-, 3-, and 4-substituted pyridines in aqueous solution.

3. Results and Discussion

3.1. Conformations and Protonation Sites of TC—The acid/base properties of TC are difficult to analyze because of its several protonation sites. Its deprotonation scheme has been subject of discussion and controversy.[8–11] Figure 3 shows the totally protonated TC and its four sites of protonation: $A^0B^+C^0D°$. According to this notation (and hereafter), A, B, C and D are protonation sites of TC. The superscripts −1 and 0 represent the ionic charge of the respective site. Therefore, superscripts + and − mean that this site has either received or lost one proton.

We have analyzed the two conformations that have been reported from X-ray experiments, the extended and folded conformations, by optimizing the zwitterionic form of TC, $A^{-1}B^+C^0D^0$ structure. The conformation we have obtained is similar to the one observed by Stezowski[14] when he analyzed hydrated crystals of TC; that is, the extended conformation. The difference between the experimental and calculated bond distances is <0.02 Å, which is in agreement with the expected accuracy of the calculated bond distances calculated by the AM1 method.[27] The dihedral angles are compared with the experimental data in Table 1. The differences for some dihedral angles can be explained if one takes into account that TC has been calculated in the gas phase without intermolecular interactions and the experimental geometry has been obtained from crystals.

The folded conformation of oxytetracycline has been observed in anhydrous crystals[14] in its nonionic form, $A^0B^0C^0D^0$ structure (according to the notation adopted in the present work). Because oxytetracycline and tetracycline are very similar,[14] the oxytetracycline is a suitable reference to compare with the calculated values of TC. The bond distances are <0.02 Å different from the available experimental data. The dihedral angles shown in Table 2 are in reasonable agreement with the experimental data.

Table 1—Calculated and Experimentally Determined Dihedral Angles of Extended Tetracycline

formula	angle, ° AM1[a]	angle, ° TC[b]	formula	angle, ° AM1[a]	angle, ° TC[b]
$C_{12}C_{12a}C_1C_2$	–147.2	–169.5	$C_{11}C_{11a}C_{5a}C_6$	46.0	41.6
$C_{12a}C_1C_2C_3$	4.5	5.4	$C_5C_{5a}C_6C_{6a}$	173.3	181.0
$C_1C_2C_3C_4$	–11.6	34.1	$C_{5a}C_6C_{6a}C_7$	–140.9	–146.6
$C_2C_3C_4C_{4a}$	36.6	–30.2	$C_6C_{6a}C_{10a}C_{11}$	–1.1	–3.2
$C_3C_4C_{4a}C_5$	65.6	110.5	$C_8C_9C_{10}C_{10a}$	–0.3	–1.0
$C_4C_{4a}C_{12a}C_1$	48.0	49.4	$C_9C_{10}C_{10a}C_{11}$	–178.9	–179.3
$C_{11}C_{11a}C_{12}C_{12a}$	174.4	179.7	$C_{10}C_{10a}C_{6a}C_7$	1.2	–0.3
$C_{11a}C_{12}C_{12a}C_1$	106.1	102.3	$C_6C_{6a}C_7C_8$	179.5	183.1
$C_{12}C_{12a}C_{4a}C_5$	48.6	47.7	$C_{6a}C_7C_8C_9$	0.6	–0.1
$C_4C_{4a}C_5C_{5a}$	172.7	173.3	$C_7C_8C_9C_{10}$	0.2	0.6
$C_{4a}C_5C_{5a}C_6$	166.7	171.5	C_1C_2C(amide)O(amide)	116.9	8.6
$C_5C_{5a}C_{1a}C_{12}$	–9.0	–17.6	$C_2C_3C_4N$(amine)	–97.4	–163.3
$C_{10}C_{10a}C_{11}C_{11a}$	161.1	167.1	$C_3C_4N_4C$(amine A)	37.5	73.2
$C_{10a}C_{11}C_{11a}C_{12}$	174.7	177.8	$C_3C_4N_4C$(amine B)	172.4	201.0

[a] Calculated by the AM1 method. [b] From crystallographic data of the zwitterionic structure (Ref 14).

Table 2—Dihedral Angles of the Folded Tetracycline AM1: Calculated Geometry. OTC: Crystallographic Data of Oxytetracycline

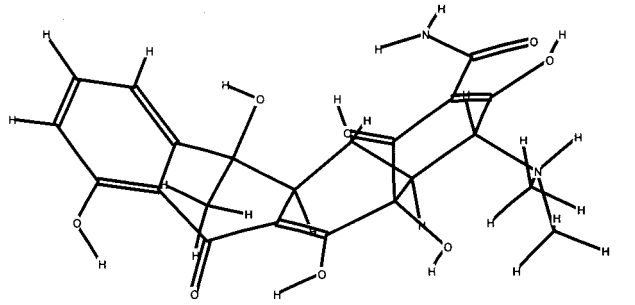
angle	AM1[a]	OTC[b]	angle	AM1[a]	OTC[b]
$C_{12}C_{12a}C_1C_2$	–79.0	–72.7	$C_{11}C_{11a}C_{5a}C_6$	26.3	35.3
$C_{12a}C_1C_2C_3$	–11.5	–16.4	$C_5C_{5a}C_6C_{6a}$	–178.6	–171.8
$C_1C_2C_3C_4$	–3.3	–9.1	$C_{5a}C_6C_{6a}C_7$	–143.7	–159.9
$C_2C_3C_4C_{4a}$	–13.6	2.0	$C_6C_{6a}C_{10a}C_{11}$	–1.9	–6.1
$C_3C_4C_{4a}C_5$	167.1	153.8	$C_8C_9C_{10}C_{10a}$	–1.5	2.2
$C_4C_{4a}C_{12a}C_1$	–58.8	–53.2	$C_9C_{10}C_{10a}C_{11}$	–178.2	–175.7
$C_{11}C_{11a}C_{12}C_{12a}$	179.6	183.0	$C_{10}C_{10a}C_{6a}C_7$	–0.1	–0.4
$C_{11a}C_{12}C_{12a}C_1$	164.0	152.8	$C_6C_{6a}C_7C_8$	–179.7	–170.0
$C_{12}C_{12a}C_{4a}C_5$	–59.3	–58.3	$C_{6a}C_7C_8C_9$	1.2	–0.7
$C_4C_{4a}C_5C_{5a}$	–89.0	–79.0	$C_7C_8C_9C_{10}$	0.1	–3.2
$C_{4a}C_5C_{5a}C_6$	138.5	123.0	C_1C_2C(amide)O(amide)	160.9	182.7
$C_5C_{5a}C_{1a}C_{12}$	–31.6	–22.8	$C_2C_3C_4N$(amine)	–142.2	–122.7
$C_{10}C_{10a}C_{11}C_{11a}$	156.0	180.3	$C_3C_4N_4C$(amine A)	–133.1	–84.1
$C_{10a}C_{11}C_{11a}C_{12}$	–164.6	–186.1	$C_3C_4N_4C$(amine B)	86.2	44.8

[a] Calculated geometry. [b] Crystallographic data of oxytetracycline.

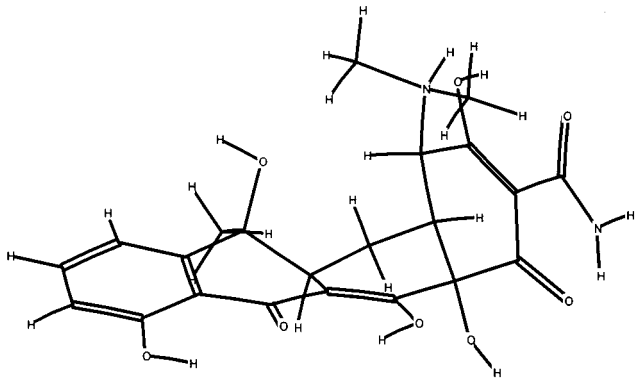
The two conformations shown in Figure 4 have been used to generate all the tautomers studied in their different degrees of protonation.

3.2. Tautomerism and the Chemical Behavior of TC in Solution—It is important to analyze all possible tautomers of this molecule in their different degrees of protonation and conformation to understand the role of tautomerism in the chemical behavior of TC. We have optimized the structures of all 64 tautomers and calculated their heats of formation ($\Delta H_f°$). The use of heat of formation instead of the more correct Gibbs free energy is because it is difficult to evaluate the entropy factor for molecules in solution. Tests with totally protonated species in the gas phase showed that the entropy contribution to the relative stability is of about 0.1 kcal mol^{-1} and therefore much lower than the relative accuracy of AM1. This result has also been pointed out by Santos et al.[24] in their study of anhydrotetracycline, in which they used a thermodynamic cycle to justify the use of the entropy calculated in the gas phase as an estimate of the entropy contribution to the Gibbs free energy of species in solution.

There are different tautomers in equilibrium in each degree of protonation of TC. They have similar stabilities



extended conformation



folded conformation

Figure 4—Two conformations of TC obtained from AM1 calculations.

and, therefore, they are present in considerable amount in the medium. The average error of the calculated heat of formation using AM1 is about 7 kcal mol^{-1} compared with experimental values.[26] This error should be much smaller when the relative stability of species is analyzed. Previous works showed that the relative stability of species calculated by the AM1 method is correctly predicted even for challenging systems.[29–31] However, the error bars of the calculated differences of enthalpies of different species are still too large to allow estimation of equilibrium constants.

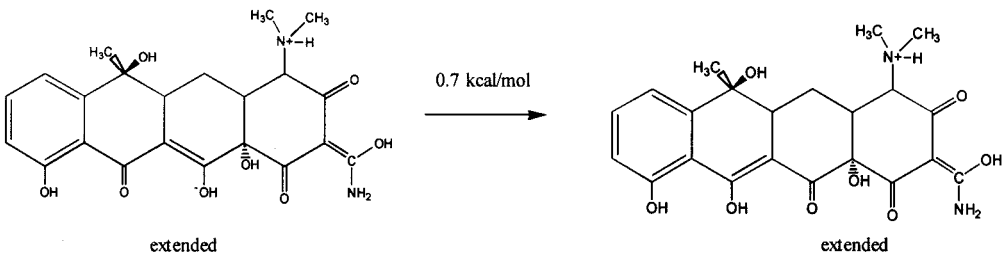
It is important to observe that the interaction of those species with the solvent has been described by the SCRF method, which does not include specific interactions with the solvent. In the gas phase, the favored species are different, due to the lack of interaction with the solvent i.e. (with the dielectric constant of the medium). Therefore, one species that is favored in aqueous solution may not be favored in ethanol solution, due to the difference in the dielectric constant. These two species can have different conformations and different protonated sites. Stezowski,[16] for example, has shown that two species (zwitterionic and nonionized) are in equilibrium in ethanol/water mixtures. One could also argue that specific interactions (which are not taken into account in our model) of the tautomers with the solvent may also alter with a change of the medium. Thus, these specific interactions would modify the relative stabilities of different species. The chemical behavior of TC can therefore change depending on the conditions of the medium (solvent, ionic force, concentration, etc.) because different tautomers have different chemical properties.

Conformations, protonation sites and metal ion complexation sites of TC and its derivatives have been extensively studied in different solvents.[6–11,13–16,19–22] However, the difficulties and disagreements among researchers with respect to the interpretation of their experimental results and the assignment of metal ion complexation sites of TC, as a whole, constitute circumstancial evidence of the influence of the tautomers.

Now, we turn to the discussion of the tautomers in each one of the degrees of protonation of TC.

3.3. Degrees of Protonation of TC—*Totally Protonated Species*—The extended conformation is favored with respect to the folded one by about 2.8 kcal mol^{-1}. However, the most stable tautomer is the one with the proton of the site A on the amide group instead of the oxygen bonded to the C(3). It is important to notice that Stezowski[33] has already observed this type of tautomerism in solid state for a tetracycline derivative. Crystals of α-6-deoxyoxytetracycline have the amide oxygen of the site A protonated. The second most stable tautomer is 0.7 kcal mol^{-1} less stable than the first one. It is extended and has one proton on the oxygen bonded to the C(11) instead of the oxygen of the C(12). Figure 5 shows these two tautomers. Other tautomers lie at least 2.8 kcal mol^{-1} higher in energy than the most stable tautomer.

1st Deprotonation—Species resulting from the 1st deprotonation can be either in a neutral or zwitterionic form, in which case a positive charge is located on the amine group of the C(4) and a negative one on the site A (Figure 3). It has been experimentally shown that, in aqueous solution, TC attains the zwitterionic form. However, in the gas phase (or in a nonpolar solvent), it is expected that the neutral form of TC is favored. In solution, the separation of charge is stabilized by the polarization of the solvent around the TC. This stabilization depends on the value of the dielectric constant of the medium, this being the basic idea of the SCRF method used. However, the SCRF method has not been able to sufficiently stabilize the zwitterionic tautomers in order to invert its relative stability with respect to the neutral species. The results show that, despite the major stabilization of the zwitterionic species (because they have the largest dipole moments) with respect to the neutral ones, they are still not favored. It is important to observe that the SCRF method does not consider the specific interactions such as those arising from intermolecular hydrogen bonding and dipole–dipole interactions. These facts are important and should be kept in mind, because, the neutral species of TC are present in hydrophobic media like those found in biological systems.[16] Figure 6 shows the 10 most stable species. Folded conformation is favored, but the species still have the proton on the amide group.



extended extended

Figure 5—Most stable completely protonated species according to AM1/SCRF calculations. The indicated value is $\Delta(\Delta H_f; r_c = 5.5$ Å).

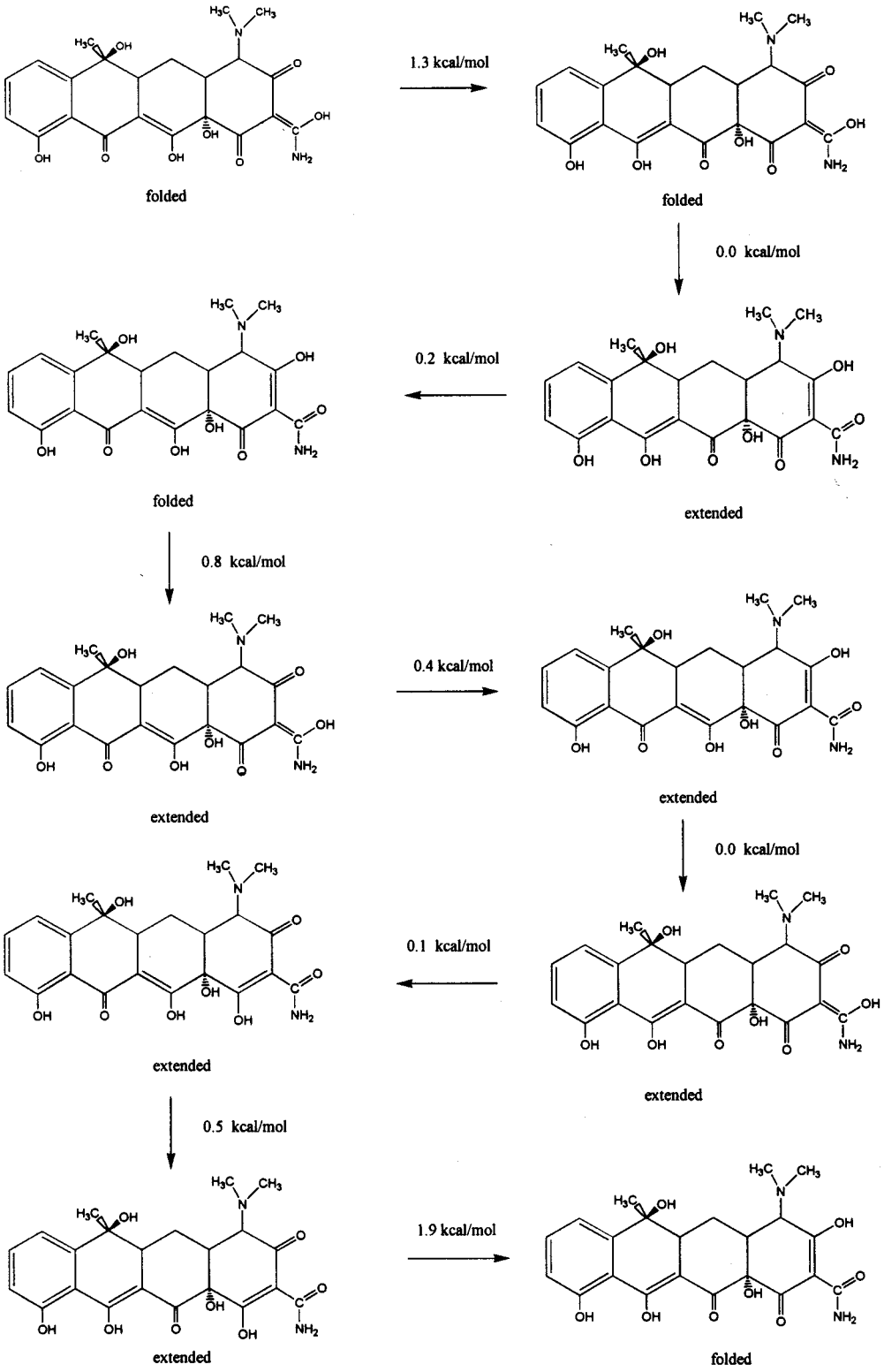


Figure 6—Most stable tautomers that arise from the 1st deprotonation according to AM1/SCRF calculations. The indicated values are $\Delta(\Delta H_f; r_c = 5.5$ Å).

2nd Deprotonation—It is accepted that the 2nd deprotonation lead to the formation of species of the form $A^{-1}B^0C^0D^0$ (i.e., species that have lost protons from sites A and B). Our results are in good agreement with these assumptions because the most stable tautomers present sites A and B deprotonated. The different tautomers arise from different conformations and from the exchange of protons between the oxygen bonded to C(11) and C(12). Figure 7 shows the most stable species.

3rd Deprotonation—The 3rd deprotonation occurs in the C and D sites. In fact, it is not possible to distinguish between these two sites. The most stable species have a proton bonded to one of the oxygens of either C(10), C(11), or C(12). Figure 8 shows the three most stable species. The most stable conformation is a folded one.

4th Deprotonation of TC—Even though only three acidic protons have been observed in potentiometric experiments with TC,[8–11] the analysis of its structure shows the pos-

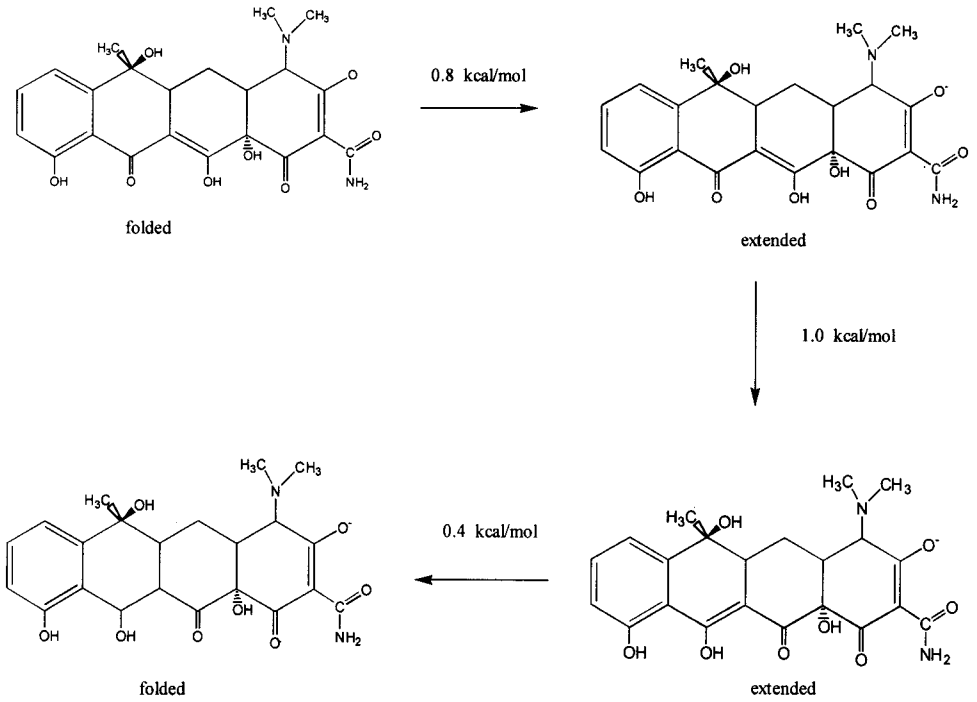


Figure 7—Most stable tautomers that arise from the 2nd deprotonation according to AM1/SCRF calculations. The indicated values are $\Delta(\Delta H_f;\ r_c = 5.5\ \text{Å})$.

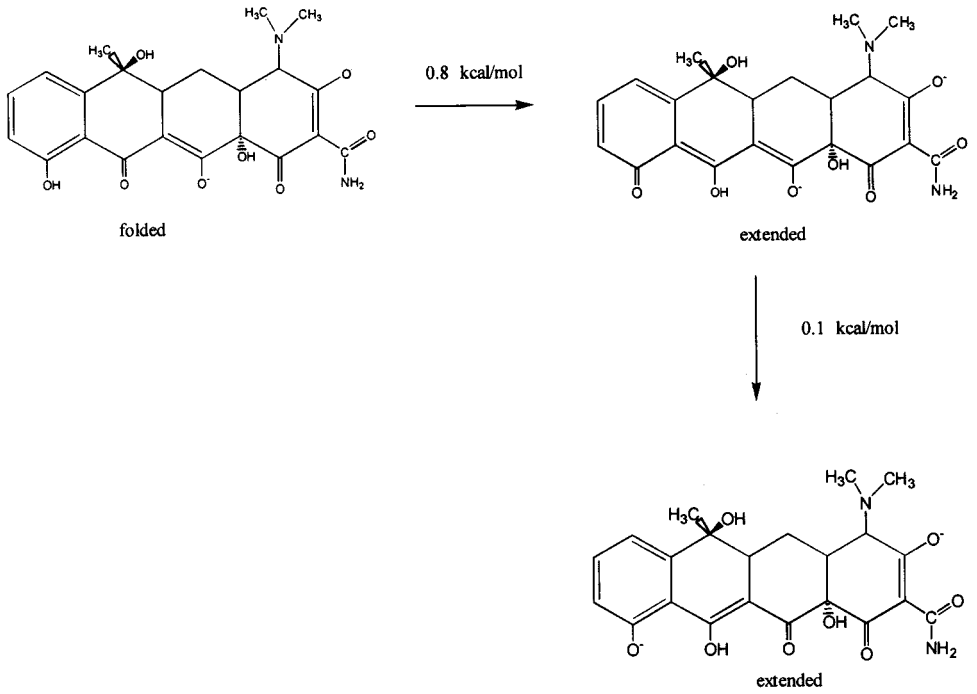


Figure 8—Most stable tautomers that arise from the 3rd deprotonation according to AM1/SCRF calculations. The indicated values are $\Delta(\Delta H_f;\ r_c = 5.5\ \text{Å})$.

sibility of a 4th deprotonation. Therefore, we have also analyzed the completely deprotonated species, aiming to contribute to the understanding of this so far unresolved problem. Our results indicate that the completely deprotonated species is a folded one and the extended conformation lies 4.4 kcal mol^{-1} higher in energy than the extended one.

Tetracycline methiodide possesses the equivalent of the TC acidic site B blocked by a methyl group. Therefore, Rigler et al.[10] had already suggested that the existence of three pK_as of tetracycline methiodide, the third equaling 10.67, should be construed as an evidence that TC itself should have a fourth acid group, which to date has seemingly not been accessible to potentiometric experiments.

One could suggest using the calculated heat of formations of the species to fit the known pK_a and to use this to estimate, by extrapolation, the rth dissociation constant of TC. This procedure is reasonable because a direct absolute calculation of pK_as would require computation of the proton solvation energy, which is very difficult to be evaluated theoretically with high accuracy.[34] Furthermore, the AM1 calculated values of Gibbs free energy[27] are in error of only about 7 kcal mol^{-1}, which is an additional argument in favor of the fit.

$$\Delta = A - B \ln K_n \qquad (1)$$

where

Table 3—Estimate of pK_4 According to Equations 1 and 2

n	Δ (kcal/mol)	-ln(K)[a]	pK_a[a]
4	–104.0	7.6 (8.2)	3.3 (3.6)
3	–87.1	17.5 (15.8)	7.6 (6.9)
2	–70.9	22.1 (23.2)	9.6 (10.1)
1	–54.0	(30.9)	(13.4)

[a] Estimated values in parentheses.

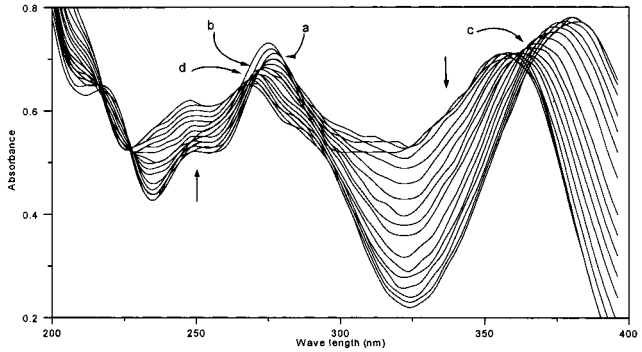


Figure 9—Spectra of a 0.5 mmol L-1 aqueous TC solution in the pH (–log(H^+)) range from 3.6 to 11.5 (as indicated by the arrows). Small letters correspond to isosbestic points. See text for details.

$$\Delta = \Delta H_{H_{n-1}L} - \Delta H_{H_nL} \qquad (2)$$

with n = 1, 2, 3, and 4 and H4L being the completely protonated TC. The values of A and B can be determined with the three experimental acid dissociation constants (3.3, 7.68, and 9.69) by least-squares fit. Accordingly, the estimated values of A and B are −121.8 and 2.19 kcal mol^{-1}, respectively, with correlation factor (**R**) of 0.98. Table 3 shows the experimental and estimated values of the pK_as using eq 1, together with the estimation of the pK_4 of about 13. Although these results are not conclusive, they are evidence that the discussions concerning a 4th deprotonation are meaningful.

If the possibility of a 4th deprotonation is accepted, then the value of pK_3 should be smaller. The determination of the dissociation constants from the experimental data neglecting the 4th deprotonation probably led to an overestimation of the pK_3 to compensate the lack of the 4th deprotonation.

3.4. Spectrochemical Evidence of the 4th deprotonation of TC—At the biological pH (about 7.4), the 4th deprotonation does not interfere in acid/base equilibria and can be neglected. However, in multiligand/multimetal systems, like blood plasma, considering the 4th dissociation constant of TC (hereafter the totally protonated tetracycline species is represented as H_4L^+) may have important consequences for the understanding of its chemical behavior. Therefore, we have carried out simultaneous potentiometric and spectrophotometric experiments (see *Appendix* for details of the experiments), searching for experimental evidences of the 4th deprotonation.

A series of spectra of a 0.5 mmol·L^{-1} TC aqueous solution in the pH range 3.5−11.5 is shown in Figure 9. Close inspection of these spectra reveal the existence of several isosbestic points, which prevail in limited pH ranges. Considering only the data >250 nm, four isosbestic points can be noticed. Below pH 5, a first one "a" can be seen at 280 nm and it can be related to the H_4L^+/H_3L equilibrium. In the pH interval from 5.5 to 7.5, two isosbestic points are seen at 272 "b" and 360 "c" nm, respectively, and they can be related to the H_3L/H_2L^- equilibrium. A fourth isosbestic point can be seen at 260 nm "d", limited to the pH range 9−10.5. This point can be related to the H_2L^-/HL^{2-} equilibrium. Its disappearance above pH 10.5 is clear evidence of a 4th deprotonation. Another piece of evidence is obtained when the absorbance at 380 nm is plotted against pH, as shown in Figure 10, together with a species distribution diagram. The absorbance at this wavelength increases up to pH 10 while the first three deprotonations occur, and absorbance starts to decrease when the 4th deprotonation starts to take place. A rapid acidification restored the spectrum of H_4L^+, ruling out the hypothesis of ligand degradation associated with the spectral modifications observed in high pH values. As mentioned before, the low concentration of L^{3-} in the pH range studied could explain the absence of the fourth dissociation constant in the literature.

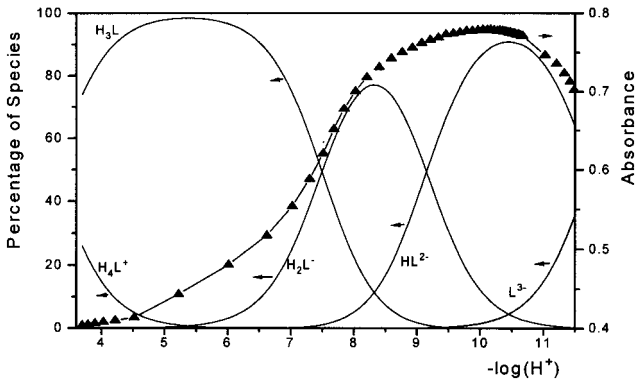


Figure 10—Distribution of TC species and the absorbance of TC in aqueous solution measured at 380 nm as functions of –log(H^+).

Table 4—Deprotonation Constants of Tetracycline at 25 °C (I = 0.1 mol/L, $NaClO_4$)

	measurements	
pK_n	potentiometric	spectrophotometric
1	3.24	3.36
2	7.49	7.33
3	9.15	8.97
4	11.75	11.81

Table 4 shows the estimated dissociation constants for TC obtained from potentiometric and spectrophotometric measurements. The different measurements have led to estimates of the dissociation constants that are in a good agreement with each other. With regard to the potentiometric data, we have observed that if the 4th deprotonation is neglected, pK_3 is estimated to be 10.7, one logarithmic unit larger than the value reported in the literature, which is 9.7. In addition, the statistical parameters C^2 and S, calculated by SUPERQUAD, decrease from 81.5 to 5.6 and from 12.24 to 0.86, respectively, when the 4th deprotonation is taken into account.

3.5. Importance of Tautomers in the Analysis of the Experimental Data—The tautomers of TC seem to have an important and so far unsuspected role in the actual behavior of TC, which explains a certain level of confusion present in the various interpretations of experimental results that appear in the scientific papers dealing with TC.

For example, the protonation scheme of TC and its derivatives has been a subject of discussions and disagreements between researchers about the interpretation of experimental results. Stezowski et al.[16] studied the behavior of free TC in ethanol/water solution using circular dichroism. According to them, two species are in equilibrium and are represented by (i) a zwitterionic species with extended conformation, which predominates in aqueous solution, and (ii) a neutral one with folded conformation,

which predominates in ethanol. It is important to note that our results are in agreement with this observation. Depending on the ethanol/water proportion, not only the conformation can be changed but also the tautomeric form of TC. However, our results do not support that the 1st deprotonation is due to site A. According to our results, the heat of solution of the zwitterionic species has to be >30 kcal mol^{-1} to invert the relative stability of the neutral form. This value is much larger than the expected error bar of the calculations performed in the present work. On the other hand, the solvent effects calculated by the SCRF method do not take into account the specific interactions (hydrogen bonding, dipole−dipole, etc.), and this could perhaps explain the discrepancies between calculated and the current interpretation of experimental results concerning the 1st deprotonation. The dimeric form of TC also has to be taken into account because it has been shown by Bogardus and Blackwood[35] that such species are also formed as a result of intermolecular hydrogen bonding of phenolic diketone system.

Stephens et al.[8] have eliminated some protonation sites of TC through reactions of etherification. The product dimethiloxytetracycline has the methoxy groups of the C(12) and C(1) and/or C(3) blocked for protonation. By means of potentiometric and UV spectra they determined two pK_as (7.5 and 9.4) and they concluded that the pK_2 = 7.5 should be assigned to site B. Therefore, the pK_1 = 3.3 of TC could not be assigned to site B because the electronegative inductive effect of the methoxy group could not increase the pK_1 from 3.3 to 7.5. It is necessary to consider that in the case of dimethiloxytetracycline, the pK_2 = 7.5 is related to a deprotonation of a positively charged species (because the site A is blocked by a methoxy) to a neutral one. In the case of TC, the situation is different. Supposing that pK_a = 7.68 is the deprotonation of site B, it would be related to a deprotonation of a neutral species to a negatively charged one. Thus, the two models are not so similar as to warrant straightforward conclusions.

Leeson et al.[9] have also compared values of three molecules derived from TC, however, these molecules have different total charges. Furthermore, different TC derivatives have different tautomers and conformations. Consequently, the chemical properties of the molecule can be completely changed. So, comparison between different molecules derived from TC, all probably also presenting a large degree of tautomerism, must be done with caution.

Rigler et al.[10] attributed the chemical shift of the dimethylamonium group exclusively to the protonation state of site B and the chemical shifts of the protons bonded to the phenolic ring to the protonation state of this group. They concluded that pK_2 and pK_3 should have contributions from sites B and C or D. According to Martin,[2] their supposition is not valid and the conclusions consequently not reasonable. For example, Martin mentioned the fact that after addition of 1 equivalent of base to the TC, the site A is 19% protonated, and after addition of 2 equivalents, 33% of site A remains protonated. It is known that chemical shifts are modified by the tautomeric forms and that has to be taken into account in the analysis by ^{1}H NMR. Deprotonation of site B modifies the electronic environment of the atoms around this site. However, if this deprotonation is followed by a change in the tautomeric form of site A, the chemical shifts of the protons of this site will also be changed. Eventually, unsuspected tautomerism can lead to wrong interpretations of the experimental data.

Asleeson and Frank,[11] from their ^{13}C NMR experiments, also concluded that the pK_2 and pK_3 have contributions from the B and C or D sites. They assumed that the 1st deprotonation is due exclusively to site A. Our results show that the site A proton is bonded to the oxygen of the amide group. After the 1st deprotonation, which our results indicate should occur on site B, the charge distribution on site A could, in turn, be completely modified because of changes in tautomeric forms that would change the ^{1}H and ^{13}C chemical shifts on site A.

Metal ions complexation by TC is also a field where there are disagreements among researchers. TC has several ways to complex metal ions. Depending on the solvents and conditions, different tautomeric species predominate in the medium and specific sites become more suitable for complexation of metal ions. This differentiation could explain the ability of TC to complex metal ions in different positions (see, for example, the Ca^{2+} and Mg^{2+} in the works of Caswell and Hutchison[7] and Lambs et al.[6]).

4. Conclusions—TC and its derivatives are a group of broad spectrum antibiotics that have been extensively studied in the last years. Their acid/base properties and chemical behavior in different mediums are extremely important to understand their mechanism of action and side-effects. However, tautomerism in these molecules has not received attention commensurate with its importance to the complete understanding of their chemical behavior in different mediums. We have explored all possibilities of tautomerism of TC by semiempirical calculations. We have shown that in solution, TC is in reality an equilibrium of different tautomers. This equilibrium can be displaced by varying the pH and the dielectric constant (solvent) of the system. We have also presented theoretical and experimental evidence that the discussion concerning the 4th deprotonation of TC is coherent. A theoretical estimation of the pK_4 of about 13 is in a good agreement with the potentiometric and spectrophotometric determinations of the pK_4 of about 12.

We conclude that TCycline seems to be a sort of a chameleon and highly adaptive molecule capable of modifying itself (its chemical bonds and of folding or extending itself) in a great diversity of manners in response to the environment in which it is immersed. And we extend this conclusion to the various TC derivatives with direct consequences to the understanding of their mechanisms of action.

6. Appendix

Potentiometric Measurements—Potentiometric titrations of 3 mmol L^{-1} aqueous solutions of TC HCl (Merck) were carried out with an automatic Methohm 670 apparatus coupled to a Metrohm Dosimat 665 autoburet. Experiments were carried out under nitrogen atmosphere and the temperature was kept constant at 25 °C. The ionic strength was maintained at 0.1 mol/L with sodium perchlorate. The electrode system was calibrated for [H^+] before the measurements, by titration of $HClO_4$ with standardized NaOH, and vice versa, at an ionic strength of 0.10 mol L^{-1} with $NaClO_4$ solution. The symbol pH therefore represents −log[H^+] in this paper. TC stock solutions were prepared just before use to avoid ligand degradation caused by oxygen and light. The acidity constants were calculated from the potentiometric titration data with the SUPERQUAD[36] computer program, based on pH values ranging from 2.0 to 11.5.

Spectrophotometric Measurements—A typical spectrophotometric titration involved 25.0 mL of a 0.5 mmol L^{-1} TC HCl solution being titrated with 0.08 mol L^{-1} NaOH. This solution, while being potentiometrically titrated, was continuously circulated with a peristaltic pump through a 0.1-cm continuos flow quartz cuvette installed in a model 8451A Diode Array Hewlett-Packard spectro-

photometer. This setup allowed simultaneous measurements of the volume of titrant (NaOH) added, of the hydrogen ion concentration, and of the absorbance of the solution. This titration was performed with increments of volume and time intervals, both pre-fixed (known as monotonic titration), to obtain an absorbance spectrum and the hydrogen ion concentration after each addition of the titrating solution. Results are shown in Figure 9. The data were treated with the program SQUAD[37] to calculate both pK_as and molar absorptivities of each one of the species.

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Acknowledgments

We thank Prof. Nivan Bezerra da Costa, Jr. for the suggestions and discussions during the development of this work. Financial support through grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), of Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), and Financiadora de Estudos e Projetos (FINEP) of Brazil are gratefully acknowledged.

JS980181R