The pK_a's of Saxitoxin

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Abstract: The pK_a's of saxitoxin and a number of its derivatives have been determined. In most cases, the determinations were made by both potentiometric titration and observation of the change in ^{13}C NMR chemical shift with pH. From these measurements it is clear that both dissociations of saxitoxin result from guanidinium groups. Thus the previous hypothesis that the first dissociation involved the ketone hydrate must be abandoned, and the more acidic pK_a (8.3) has been assigned to one of the guanidinium groups. This very unusual guanidinium moiety is involved in saxitoxin's blocking of the sodium ion channels of nerve cells.

The ionization constants of saxitoxin hydrate (**1**) have been investigated in view of their biological and chemical importance. Saxitoxin hydrate, one of the paralytic shellfish poisons, acts by blocking sodium ion conduction in nerves.[1] A model for this action has been proposed in which electrostatic forces and hydrogen bonding orient and hold a saxitoxin hydrate molecule in the sodium ion channel of the nerve,[2] and it was demonstrated that the action of saxitoxin is reduced at high pH.[3] Assignment of the dissociations of saxitoxin hydrate to specific structural moieties is then of considerable interest, so that knowledge of the dissociation behavior might be used to infer the bonding orientation.

Early chemical investigations suggested that the first dissociation of saxitoxin hydrate might result from a cyclol form, **1c**, of the carbamate.[4] Later data, primarily ^{13}C NMR, showed no evidence for a cyclol form in solution.[5] However, the possibility of a facile carbamate–cyclol equilibrium still existed. Also, the recent structural elucidation of several natural analogues of saxitoxin with distinct ion-exchange behavior reflecting on their pK_a's makes it highly desirable that the dissocition behavior of saxitoxin hydrate be fully explained.[6-8] In this report we provide an assignment for saxitoxins pK_a's based on a combination of aqueous titrations of derivatives of saxitoxin and ^{13}C NMR studies as a function of pH.

Results and Discussion

Potentiometric Titrations. Certain sites for dissociation can be eliminated by correlating the pK_a's of saxitoxin hydrate with those of its derivatives. Aqueous titration of saxitoxin hydrate showed pK_a's of 8.22 and 11.28, obtained at a concentration of 0.004 M. These values, given in Table I, were calculated by using an interative least-squares program modified to handle protonated nitrogen acids[9] and are in agreement with reported values of 8.24 and 11.60.[4] A titration in 0.1 M KCl gave values of 8.39 and 11.30 as a check on the activity corrections used for the calculations. The activity effects were thus demonstrated to be of much smaller magnitude than the effects of structural modification on the pK_a's.

Table I. Acid Dissociation Constants of Saxitoxin and Some of Its Derivatives by Potentiometric Titration in Water at 20 °C and ~0.004 M

compd	pK_{a_1}	pK_{a_2}
saxitoxin hydrate (**1**)	8.22	11.28
decarbamoylsaxitoxin hydrate (**2**)	8.10	10.84
β-dihydrosaxitoxin (**3**)	8.51	11.65
α-dihydrosaxitoxin (**4**)	8.64	11.82
decarbamoyl-α-dihydrosaxitoxin (**5**)	8.78	11.86
saxitoxin 1, in water–methanol		
(a) 16.3% methanol	8.25	11.57
(b) 33.3% methanol	8.28	11.70
(c) 52.1% methanol	8.30	12.28

Table II. ^{13}C NMR Chemical Shifts[a] for Saxitoxin Hydrate and Some of Its Derivatives

	compd						
carbon	**1**	**2**	**3**	**4**	**5**	T_1	1[b]
C-11	33.37	33.21	29.10	27.30	27.44	0.31	33.35
C-10	43.31	43.31	44.10	43.60	43.71	0.33	41.63
C-6	53.46	55.93	53.42	53.26	55.32	0.68	53.86
C-5	57.45	56.94	58.09	59.81	59.81	0.82	60.37
C-13	63.57	61.62	63.47	64.30	62.25	0.48	64.89
C-4	82.84	82.73	83.80	80.41	80.37	4.73	84.90
C-12	99.00	99.09	74.83	74.11	74.05	0.59	
C-2	156.43	156.37	156.16	156.10	156.19	4.02	156.56
C-8	158.28	158.27	158.09	158.65	158.57	3.54	164.73
C-14	159.30		159.31	158.95			159.14

[a] Relative to internal dioxane, 67.4 ppm from Me_4Si, at the pH attained upon solution. [b] Keto form at pH 10.18.

Catalytic hydrogenation of **1** in ethanol leads to a 60:40 mixture of β-dihydrosaxitoxin (**3**) and α-dihydrosaxitoxin (**4**) by ^{13}C NMR analysis. The stereochemistry may be assigned by examining the differences in chemical shift for the two isomers given in Table II. The resonances assigned to C-5 and C-13 in α-isomer **4** show a larger downfield shift when being compared with those of **1** and **3**, consistent with the release of steric compression resulting from replacement of the β-OH by H at C-12.

Pure α-isomer **4** was obtained by catalytic hydrogenation of **1** in 0.01 HCl and was found to have pK_a's of 8.64 and 11.82. Mixtures highly enriched in β-isomer **3** were obtained by reduction in less polar solvents. The preferential formation of the β-isomer under these conditions may be due to increased intramolecular hydrogen bonding between the carbamate and carbonyl at C-12, hindering attack from the β face. Titration of a mixture of **3** and **4**, containing less than 15% **4**, gave pK_a's of 8.51 and 11.65. For comparison with the reported[10] values of 8.3 and 11.5 for a mixture of unknown composition, a titration of a 50:50 mixture of **3** and

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(7) Boyer, G. L.; Schantz, E. J.; Schoes, H. K. *J. Chem. Soc., Chem. Commun.* **1978**, 889.
(8) Shimizu, Y.; Hsu, C.-p.; Fallon, W. E.; Oshima, Y.; Miura, I.; Nakanishi, K. *J. Am. Chem. Soc.* **1978**, *100*, 6791. Assignment of C-5 and C-6 in neosaxitoxin was based on selective irradiation and agrees with our assignment by double off-resonance decoupling. The pH-dependent ^1H NMR spectra suggested that the C-8 guanidine was responsible for the pK_a of 8.65 in neosaxitoxin, based on the change in chemical shift for the C-5–H between pH 8 and 9.
(9) Briggs, T. N.; Stuehr, J. E. *Anal. Chem.* **1975**, *47*, 1916.
(10) Schantz, E. J.; Mold, J. D.; Howard, W. H.; Bowden, J. P.; Stanger, D. W.; Lynch, J. M.; Wintersteiner, O. P.; Dutcher, J. D.; Walters, D. R.; Riegle, B. *Can. J. Chem.* **1961**, *39*, 2117.

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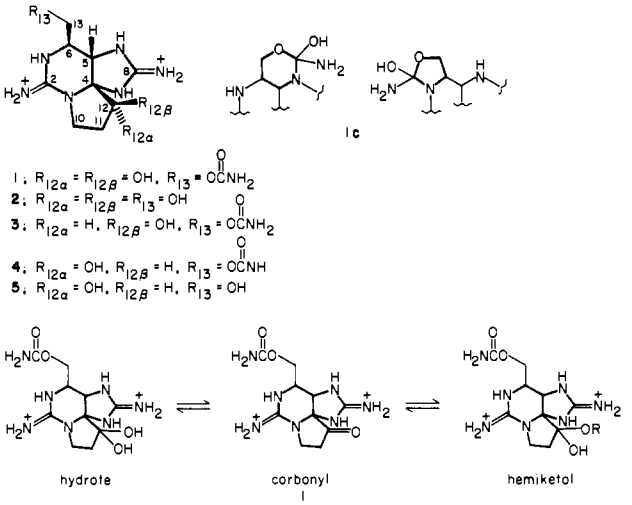
4 was done, yielding pK_a's of 8.50 and 11.78. The similarity of the pK_a's of both isomers of dihydrosaxitoxin to those of saxitoxin hydrate confirms that the first pK_a of **1** cannot be due to dissociation from one of the hydroxyls of the hydrate.

To reflect on the possibility of a cyclol form, originally postulated to explain the behavior of the pK_a of **1** when it was titrated in mixed water–ethanol solutions,[4] we prepared decarbamoylsaxitoxin hydrate (**2**) and decarbamoyl-α-dihydrosaxitoxin (**5**), which cannot exist in cyclol forms, by strong acid hydrolysis.[11] Titration of **2** and **5** showed pK_a's of 8.10 and 10.84 and 8.78 and 11.86, respectively. These results further establish that a cyclol form is not responsible for the first pK of saxitoxin hydrate.

As all sources of oxygen dissociation thus have been eliminated as candidates for the first pK, the mixed solvent titration behavior of saxitoxin hydrate was reexamined. Earlier work showed that the first pK of **1** increased from 8.24 in water to 8.50 in 20% ethanol and to 9.05 in 50% ethanol. Different results have been found in methanol–water solutions for which standard buffers have been established, allowing more careful standarization of the pH electrode.[12] The first pK of **1** showed little change, rising from 8.22 in water to 8.30 in 52% methanol. The second pK, in contrast, showed a large change from 11.28 to 12.28 in water vs. that in 52% methanol. Extensive work[13] has shown that acids in which the proton dissociates from oxygen such as carboxylic acids and phenols exhibit upward trends in pK with increasing alcohol content (decreasing polarity) while nitrogen acid (proton dissociating from nitrogen) has pK values which decrease to a minimum at about 80% alcohol and only then show an increase. This is true even for complex amines such as EDTA and *p*-(dimethylamino)azobenzene which have strong charge interactions and are highly charge delocalized.[14,15]

It is not known whether guanidines as a group are exceptions to this generalization about nitrogen acids, as no reports have appeared on mixed-solvent titrations of simple guanidines. Saxitoxin hydrate may be a special case, however, in that a hydrate–carbonyl–hemiketal equilibrium may exist in mixed alcohol–water systems as shown. Our ^{13}C NMR evidence suggests that the hydrate–carbonyl equilibrium is strongly affected by pH in water. The carbonyl–hemiketal equilibrium should be affected by solvent composition. The behavior of the pK values of **1** in



alcohol–water mixtures therefore may not reflect dissociation affects but rather changes in the species dissociating; this in turn could affect the pK in unpredictable ways. Thus we conclude that the pK behavior of saxitoxin in mixed solvents cannot be used for unambiguous structural assignments.

Table III. pH-Dependent ^{13}C Chemical Shifts, $\Delta\delta$/pH, of Some Model Guanidines

	carbon atom				
	C-1	C-2	C-4	C-5	CH_3
ethylguanidine (**8**)	3.23[a]	0.03			0.74
butylguanidine (**9**)	3.58[a]	0.09	0.26	0.27	0.74
2-amino-4-methyl-2-imidazoline (**10**)		3.98[a]	2.49	3.23	0.54
2-amino-3,4,5,6-tetrahydropyrimidine (**11**)		1.05[a]	1.00	0.74	

[a] Guanidine carbon.

^{13}C NMR Studies. Characteristic changes in chemical shift occur in both ^{13}C and ^1H NMR spectra with deprotonation of charged nitrogen acids. Our interest in the behavior of the guanidine moieties of saxitoxin favored the choice of ^{13}C, though recently an investigation of the pK behavior of neosaxitoxin was done with ^1H NMR.[8] Investigations of the dissociation of several biologically interesting compounds have been made by ^{13}C NMR,[16] but very few data are available for guanidines. The only report[1m] involved work on *sym*-tetramethylphenylguanidine (**6**) and *sym*-tetramethylbenzylguanidine (**7**). It was found that the guanidino carbon of **6** shifts downfield by 1.5 ppm while the methyl carbons in **6** and the benzylic carbon in **7** shift upfield by 2.0 and 5.8 ppm, respectively, when the guanidine is deprotonated.

In view of the lack of data, the pH-dependent chemical shifts of two acyclic models, ethylguanidine (**8**) and butylguanidine (**9**), and two cyclic guanidines, 2-amino-4-methyl-2-imidazoline (**10**) and 2-amino-3,4,5,6-tetrahydropyrimidine, were examined (Table III). All resonances were seen to shift downfield on deprotonation. The discontinuous nature of the $\Delta\delta$/pH (change in chemical shift with change in pH) seen in amines and amino acids[16] is reflected in the results for the acyclic models **8** and **9**. In each case the largest change is seen for the guanidine carbon. The cyclic compounds, **10** and **11**, do not show the strong β effect seen in the acyclic models. Instead, large changes in shift were seen in the α-carbons.

To allow optimization of the pulse parameters used in obtaining pH dependent spectra, we measured spin–latice relaxation times for decarbamoyl-α-dihydrosaxitoxin (**5**, Table II). Peak assignments previously in doubt for C-5 and C-6 were made by using double off-resonance decoupling.[8,18] The 57.45-ppm resonance was found to be coupled to a single proton of shift δ 4.73, and the 53.46-ppm resonance was coupled to a proton having a shift of δ 3.80. Comparing the calculated shifts with the reported values[5] of δ 4.77 for the hydrogen on C-5 and δ 3.87 for the hydrogen on C-6 allows definitive assignement as given in Table II for C-5 and C-6.

Figures 1, 2, and 3 show plots of the change in ^{13}C chemical shift vs. pH for α-dihydrosaxitoxin (**4**), decarbamoyl-α-dihydrosaxitoxin (**5**), and saxitoxin hydrate (**1**). The data allow the ^{13}C NMR resonances for C-2 and C-8 to be clearly differentiated. For **5** the change in chemical shift for the 158.57-ppm resonance is 5.40 ppm between pH 6.83 and 11.47. This is larger than that seen for the deprotonation of the model guanidines. The difference is probably due to the much higher pK_a's of the models which preclude total deprotonation even in 2.0 M potassium hydroxide. On this basis the first dissociation can be assigned to one of the guanidines.

The second dissociation for decarbamoyl-α-dihydrosaxitoxin (**5**) can be assigned to the 156.14-ppm guanidine by noting that the small upfield change in chemical shift between pH 8 and 10.4

(11) Ghazarossian, V. E.; Schantz, E. J.; Schnoes, H. K.; Strong, F. M. *Biochem. Biophys. Res. Commun.*, **1976**, *68*, 776.
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(17) Kalinowski, H. O.; Dessler, H. *Org. Magn. Reson.* **1975**, *7*, 128.
(18) Calculations were done by using $\delta^H = (\delta^1 J^2 + \delta^2 J^1)/(J^1 + J^2)$ where δ is proton shifts in H, δ^H, the proton observed, δ^1, the upfield decoupling frequency, δ^2 the downfield decoupling frequency, and J represents the respective residual couplings.

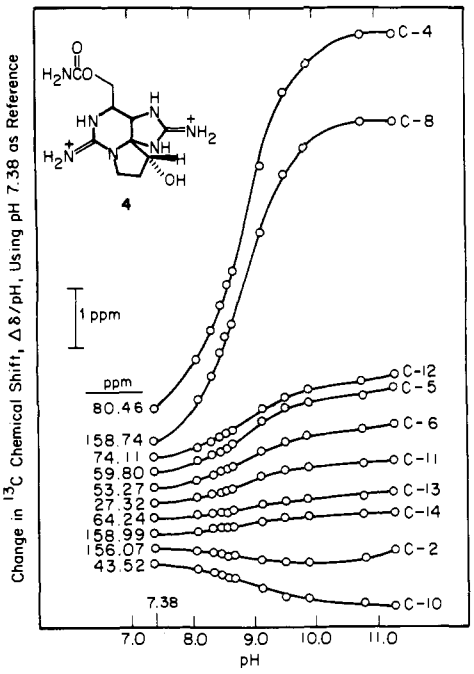

Figure 1. Change in chemical shift of the carbons of α-dihydrosaxitoxin (**4**) as a function of pH, Δδ/pH.

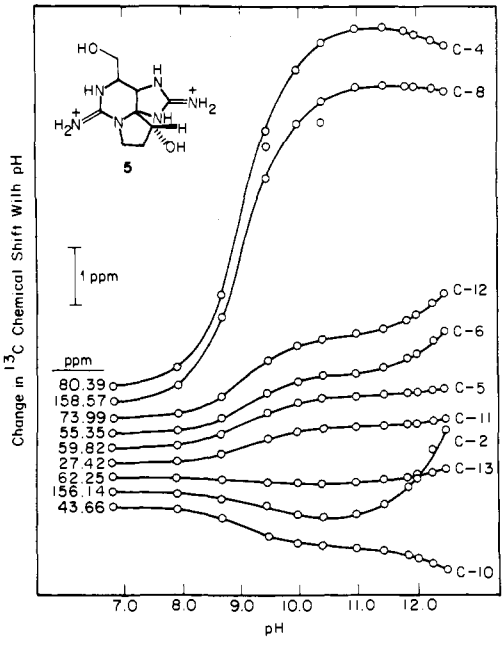

Figure 2. Change in chemical shift of the carbons of decarbamoyl-α-dihydrosaxitoxin (**5**) as a function of pH, Δδ/pH.

reverses above pH 10.5. In the pH region corresponding to K_2, this resonance shifts strongly downfield as expected for deportonation of the second guanidine. The initial upfield shift is the result of an interaction between the two charged guanidines.

On the basis of the changes in chemical shift with pH for C-5 and C-6, we can assign the specific guanidine resonances in **5**. The resonance assigned to C-5 changes only between pH 8 and 10.5, the region where the guanidine resonating initially at 158.57 ppm is deprotonated. In contrast, the C-6 resonance, which also shifts through the first dissociation, shows a second shift above pH 11.0 corresponding to the second dissociation. Since deprotonation of the C-2 guanidine would be expected to influence C-6 more than C-5, the second dissociation can be assigned to the C-2 guanidine. Therefore the resonance at 156.14 ppm in **5** can be assigned to C-2.

The first dissociation in **5** thus occurs from the C-8 guanidine, and the resonance at 158.57 ppm can be assigned to C-8. That

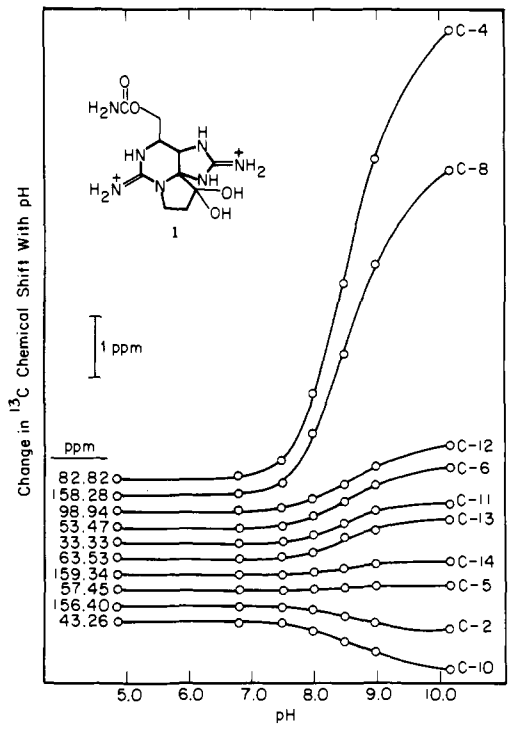

Figure 3. Change in chemical shift of the carbons of saxitoxin (**1**) as a function of pH, Δδ/pH.

C-6 does shift through the pH region of the first dissociation is more likely a result of interaction between the guanidines than an anomalously large β effect from the C-8 guanidine.

By analogy, we can assign the resonances for the guanidines of saxitoxin hydrate (**1**) and α-dihydrosaxitoxin (**4**). The upfield shift in C-10 for all three compounds is similar to the shifts reported in the literature and noted earlier. Also, the downfield shifts for the other carbons are all in reasonable agreement with those of the model compounds except for C-4 which will be discussed below.

Comparing Figures 1 and 2, we can see that the two reduced compounds are very similar, with only slight differences in the behavior of C-5 and C-13. Two important differences can be seen when the dihydro compounds **4** and **5** are compared with saxitoxin hydrate (**1**). The change in position of the C-4 resonance for **1** is much larger than for **4** and **5**, while the change for C-5 is much smaller for **1** in comparison with those for **4** and **5**. In addition, the resonance for C-8 of decarbamoly-α-dihydrosaxitoxin (**5**) becomes very broad between pH 8.6 and 10.0 and is split at pH 10.42 into two peaks at 163.33 and 163.67 ppm. The peaks coalesce at higher pH. Carbon-4 shows similar behavior, splitting into two peaks at 84.51 and 84.75 ppm at pH 9.48 and again coalescing at higher pH. Our model compounds showed some peak broadening but no splitting or coalescence.

The depressed pK_1 for these compounds is not a result of the interaction of the two charged guanidines since the ^{13}C chemical shift vs. pH profile shows discrete changes for K_1 and K_2. An investigation of the macroscopic and microscopic dissociations of diamines and diaminocarboxylic acids shows that in cases where the interaction of the charged amino group lowers the first dissociation from nitrogen, clean breaks in the ^{13}C chemical shift vs. pH profile do not occur.[19]

A possible explanation for (a) the differences between the dihydro compounds **4** and **5** and saxitoxin hydrate (**1**), (b) the splitting of C-4 and C-8, and (c) the abnormally low first pK of these compounds is that the C-8 guanidinium ion is not fully resonance stabilized. Were bond fixation to occur between C-8 and N-9, deprotonation and removal of charge at N-9 would be expected to effect C-4 greatly, resulting in a large pH dependent

(19) Sayer, T. L.; Rabenstein, D. L. *Can. J. Chem.* **1976**, *54*, 3392.

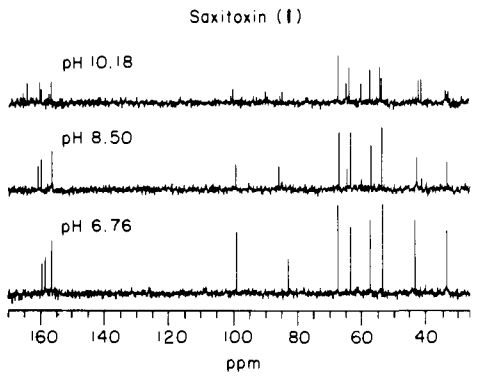

Figure 4. ^{13}C NMR spectra of saxitoxin (**1**) in strong alkali (pH 10.18) and upon reacidification (pH 6.76).

shift. Conversely with little positive charge delocalized to N-7, little change in C-5 would be expected. Reduction of the hydrate would lead to less bond fixation, a smaller effect on deprotonation at C-4, and consequently a larger effect at C-5 and a higher pK due to more charge delocalization. The splitting of the guanidine resonance would be due in turn to slow tautomerization between the two deprotonated forms. The nonresonance-stabilized guanidine argument has been used to explain another anomalously low guanidine pK, that of phakellin.[20]

The appearance of a new set of peaks was observed in the spectrum of saxitoxin hydrate at alkaline pH. Decomposition was feared, and the titration was stopped at pH 10.18 and the sample reacidified. The reacidified solution showed a spectrum identical with that of the original, indicating no decomposition. Further inspection suggests that the new resonances (Figure 4) arise from alkali-catalyzed conversion of the hydrate to the ketone. Noise limitations preclude the assignment of any downfield resonance to the carbonyl carbon; however, the downfield shifts for C-5 and C-13 are consistent with release of steric compression by removal of the α-OH at C-12. The large upfield change seen for C-4 between the hydrate and keto forms must be ascribed to the highly polarized nature of the aminal carbon. This represents the first time saxitoxin, and not its hydrate, has been observed. When precautions are taken to exclude oxygen, saxitoxin appears to be suffciently stable in alkali to allow its reactivity to be explored. The new resonances seen in Figure 4 should be contrasted with the constancy shown in Figure 5. Decarbamoyl-α-dihydrosaxitoxin (**5**) can exhibit no hydrate–ketone equilibrium, and all we see are pH-dependent shifts with no new resonances.

In summary, all the data are now consistent with, and clearly establish that, both pK_a's of saxitoxin result from guanidinium ion dissociations. The first pK_a, 8.3, can be assigned to the guanidinium ion involving C-8, and the second pK_a, 11.3, is assigned to the C-2 guanidinium ion.

Experimental Section

Methods. Measurements of pH were performed by using a Corning 130 pH meter and a Corning 476050 electrode which was carefully checked for stability. The pH of the ^{13}C NMR samples was adjusted in the sample tubes and measured with a Broadley-James 9008 electrode under an argon atmosphere. ^{13}C NMR spectra were recorded at 45.29 MHz on a Bruker 180 MHz magnet and Nicollet 1180 computer at 20 °C in 50% D_2O–50% H_2O. This mixture was found to prevent the disappearance of the C-11 resonance seen in D_2O due to rapid exchange of the C-11 protons. Adjustments in pH were made with carbonate free KOH (J.T. Baker, Dilute-It), and corrections for pD were not made. All chemical shifts are reported vs. an internal dioxane standard determined to be 67.40 ppm vs. external Me_2Si. T_1 measurements were performed by inversion-recovery and calculated by using the method of Levy and Peat.[21] Chromatographic purifications were done by using medium-presure liquid chromatography monitored with a Waters Associates R401 refractive index detector and common thin-layer chromatographic spot tests.[22] Field desorption mass spectroscopic characterization of **5** was provided by the Space Science Laboratory, University of California, Berkeley.

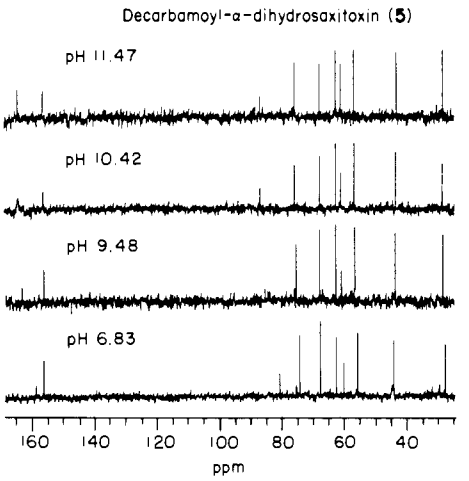

Figure 5. ^{13}C NMR spectra of decarbamoyl-α-dihydrosaxitoxin (**5**) from pH 6.83 to pH 11.47.

Reductions of Saxitoxin Hydrate. Saxitoxin hydrate (**1**), purified by elution from Bio Rex 70 (H^+ form) with 0.25 M HOAc,[23] acidic alumina (Woelm) with 85% ethanol, and Bio Gel P-2 with 0.05 M HOAc, was reduced with hydrogen over PtO_2 under the following conditions: (a) as the dihydrochloride in ethanol following literature procedures[5] to provide a 60:40 ratio of β-dihydrosaxitoxin (**3**) to α-dihydrosaxitoxin (**4**) analyzed by intensity of the ^{13}C NMR spectra, (b) as the dihydrochloride (46.7 mg) in 0.01 M HCl (10 mL) with 20 mg PtO_2 for 21 h (pure **4** was obtained, as analyzed by ^{13}C NMR), (c) as the $CH_3(CH_2)_5PO(OH_2)$[24] salt (33.6 mg, from the diacetate by lyophilization) in DMF (15 mL) with PtO_2 (50 mg) for 48 h. The solution was centrifuged to remove the catalyst, evaporated in vacuo to dryness, and partitioned between 0.5 M HCl and chloroform–2-propanol (5:1). Lyophilization of the aqueous layer yielded a residue which was further purified on Bio Rex 70 (H^+ form). ^{13}C NMR showed the presence of predominately **3** contaminated by approximately 15% of **4**. Separation of the two isomers by thin-layer chromatography or medium-pressure liquid chromatography has not yet been successful.

Decarbamoylsaxitoxin hydrate (**2**) was prepared as reported[11] with purification on Bio Rex 70 (H^+ form), acidic alumina, and Bio Gel P-2.

Decarbamoyl-α-dihydrosaxitoxin (**5**). α-Dihydrosaxitoxin (**4**, 56.3 mg) was refluxed in 6 M HCl under a flow of argon for 4 h with only slight discoloration. After the solution was cooled, it was evaported in vacuo and the residue purified by one pass through a 0.6 × 25.0 cm column of Woelm acidic alumina, eluting with 85% ethanol. ^{13}C NMR showed complete decarbamoylation; field desorption MS, m/e 241, (MH^+, $C_9H_{16}N_6O_2$).

^{13}C NMR Model Compounds 8, 9, 10, and 11. The preparation of 2-amino-4-methyl-2-imidazoline (**10**) and 2-amino-3,4,5,6-tetrahydropyrimidine (**11**) has been reported previously.[25]

n-Butylguanidine (**9**) was prepared by treating *n*-butylamine with *S*-methylisothiourea hydroiodide in 85% ethanol; picrate, mp 151.5–153 °C (lit.[26] mp 154.5 °C). Ethylguanidine (**8**) was a commercial sample. Conversion to the hydrochlorides was effected by partioning between 0.5 M HCl and chloroform–2-propanol (5:1).

Titrations. Samples for all titrations were weighed by the Analytical Laboratory, Department of Chemistry, University of California. Weighing boats were sealed in a dry inert atmosphere due to the extremely hygroscopic nature of the compounds. Generally 3 mg of sample, dissolved in 2 mL of degassed H_2O and continually flushed with argon, was titrated by adding small portions of KOH from a 0.25 mL Gilmont micrometer buret. Temperature was controlled to ±0.2 °C. The pK_a values were determined by using the program of Briggs and Stuehr.[9] The program was modified by changing the the charge balance equations and the Debye–Hückel activity expression to allow its use on charged acids and in mixed methanol–water solutions. Experimental difficulties limit the accuracy of the pK_a values reported to ±0.07 pH unit. The values reported are mixed, not thermodynamic, dissociation constants.

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Standard KH_2PO_4–Na_2HPO_4 and borax buffers for the mixed methanol–water titrations were made according to Bates, Paabo, and Robinson,[11] and the electrode was checked for stability and slope in the 16.3, 33.3 and 52.1 wt % methanol buffers. The titration samples were made up by weight with appropriate amounts of degassed, distilled methanol and water and titrated with KOH (J.T. Baker Dilute-It) diluted to the appropriate solvent composition.

^{13}C NMR/pH Studies. For the model compounds the hydrochlorides were dissolved in 50% D_2O–50% H_2O and 2.0 M KOH to provide spectra of the protonated and deprotonated forms. Spectra at intermediate pH values were run for *n*-butylguanidine (**9**) and 2-amino-4-methyl-2-imidazoline (**10**) which showed chemical shift values between the fully protonated and deprotonated values as expected. The 2 M KOH spectra may not reflect the maximum shift possible for these model compounds due to the high p*K* values of normal guanidines. The pH-dependent spectra of **1**, **4** and **5** were run on samples of 126.8, 56.3, and 44.0 mg, respectively, dissolved in 3 mL of 50% D_2O–50% H_2O, and the pH was adjusted with 2.0 M KOH. Reacidification with 2.0 M HCl in each case gave spectra indentical with the original.

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Chemical and Spectroscopic Studies of the Binuclear Copper Active Site of *Neurospora* Tyrosinase: Comparision to Hemocyanins

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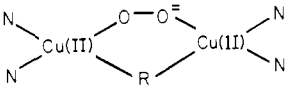
Abstract: This paper reports the results of a series of chemical and spectroscopic studies on derivatives of the binuclear copper active site of *Neurospora crassa* tyrosinase. Parallel results are also reported for *Limulus polyphemus*, the hemocyanin which exhibits spectral properties most similar to those found for tyrosinase. The chemistry and spectroscopy of half met [Cu(II)Cu(I)], 2-mercaptoethanol, dimer (EPR-detectable met) [Cu(II)Cu(II)], met (EPR-nondetectable met) [Cu(II)···Cu(II)], and oxy I derivatives of tyrosinase are presented and shown to be quite similar to the results for hemocyanin in terms of geometric and electronic structure, but with higher accessibility to exogenous ligands. The involvement of the effective structure of the oxy "type 3" copper active site (*Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2094–2098) in substrate reactions is then discussed.

Introduction

Tyrosinase is one of a series of metalloproteins and enzymes (hemocyanin, tyrosinase, laccase, ceruloplasmin, and ascorbic acid oxidase) which contain a strongly coupled binuclear copper active site[1a] but perform different biological functions. This "type 3" copper unit[1b] is characterized by its lack of any EPR signal for formally cupric ions and as the site of interaction with dioxygen. Particularly strong parallels exist between hemocyanin and tyrosinase in that both contain only one binuclear copper unit[2] (laccase, ceruloplasmin, and ascorbate oxidase contain additional copper sites) and both reversibly bind oxygen to produce oxyhemocyanin and oxytyrosinase.[3] These exhibit very similar absorption features which are unique when compared to simple copper complexes: an intense band at $\sim$350 nm ($\epsilon \approx 20000$ M^{-1} cm^{-1}) and a reasonably intense absorption at $\sim$570 nm ($\epsilon \approx 1000$ M^{-1} cm^{-1}). However, while hemocyanin functions only as an oxygen carrier, tyrosinase is an enzyme which functions both as a monooxygenase (monophenol + $O_2 \rightarrow$ *o*-diphenol + H_2O) and as a 2-electron oxidase (2 *o*-diphenol + $O_2 \rightarrow$ 2 *o*-quinone + $2H_2O$). Resonance Raman studies using UV laser excitation into the 350-nm absorption of oxytyrosinase produced a 755-cm^{-1} vibration which shifted upon oxygen isotopic substitution.[4] This peroxide type stretching frequency, as well as the M–L vibrational spectrum was extremely similar to resonance Raman vibrational features observed earlier for oxyhemocyanins.[5] Thus the enzymatic activity of tyrosinase, in contrast to hemocyanin, cannot simply be related to differences in oxygen activation but must be associated with detailed differences in the binuclear copper active site.

Recently, a series of hemocyanin derivatives has been prepared which allows the active site to be systematically varied and subjected to detailed spectroscopic study.[6] These derivatives (met apo [Cu(II)–], half met [Cu(II)Cu(I)], met (EPR-nondetectable met) [Cu(II)···Cu(II)], dimer (EPR-detectable met) [Cu(II)-Cu(II)] and oxy I) allowed an effective structural model of the



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binuclear copper active site to be developed[1a,6a] which also explains

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