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THE INTERACTIONS BETWEEN NUCLEIC ACIDS AND POLYAMINES

II. PROTONATION CONSTANTS AND ^{13}C-NMR CHEMICAL SHIFT ASSIGNMENTS OF SPERMIDINE, SPERMINE, AND HOMOLOGS *

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Macroscopic protonation constants have been determined by potentiometric titration for spermidine, spermine and for the four polyamines, 3,5-Spd, 4,4-Spd, 4,3,4-Spm and 4,4,4-Spm, which are homologs of spermidine and spermine. A method for calculation of microscopic protonation constants of polyamines based on data for mono- and diamines gives results for spermidine that agree well with the experimental macroscopic protonation constants and the protonation sequence of Kimberly and Goldstein. ^{13}C-NMR spectra of spermidine, spermine and six homologs have been obtained and used to assign specific resonances, correcting some ambiguity in the assignments for spermidine and some errors in the assignments for spermine.

1. Introduction

The need to understand the mechanisms by which spermidine and spermine [1] and their homologs [2] mediate critical physiological processes has led to substantial interest in establishing the physicochemical properties of these polyamines and their homologs [1,2]. Chief among the properties of these molecules needed for interpretation of their physiological behavior are NMR chemical shift data, and both microscopic and macroscopic protonation constants.

Precise values of the macroscopic protonation constants of diamines and symmetrical triamines of low molecular weight have been available for some time, and the careful work of Powell and co-workers [3–6] has made such data available for spermidine and some of its unsymmetrical homologs. In addition to confirming the protonation constants of spermidine and spermine, we have synthesized and determined protonation constants of several triamines and tetramines for which these data were unavailable.

We have also reexamined the ^{13}C-NMR assignments for spermine and spermidine. The ^{13}C-NMR spectra of both spermine [7] and spermidine [8–10] have been reported but there is disagreement about some of the assignments for spermidine and uncertainty regarding the assignments for spermine. Our evaluation of these assignments, which is based on chemical shifts we determined for other polyamines, has confirmed the rationale for the model for binding of 5'-AMP and spermidine we had proposed earlier [8].

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2. Nomenclature

We have used both common names (spermidine, I, and spermine, II) and Chemical Abstracts systematic names, e.g., *N,N'*-bis(3-aminopropyl)-1,4-butanediamine, in first use. We have systematically abbreviated the names of

$$\overset{a}{H_2}\overset{3'}{N}\overset{2'}{C}\overset{1'}{H_2}\overset{b}{C}\overset{1}{H_2}\overset{2}{C}\overset{3}{H_2}\overset{4}{C}\overset{c}{H_2}NH_2 \qquad (I)$$

$$\overset{a}{H_2}\overset{3'}{N}\overset{2'}{C}\overset{1'}{H_2}\overset{b}{C}\overset{1}{H_2}\overset{2}{C}\overset{3}{H_2}\overset{4}{C}\overset{c}{H_2}NHCH_2CH_2CH_2\overset{d}{N}H_2 \qquad (II)$$

homologs for tabulation and discussion. Thus $H_2N(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH_2$ is denoted 4,4,4-Spm and $H_2N(CH_2)_3NH(CH_2)_5NH_2$ is denoted 3,5-Spd. Nitrogens are denoted using the scheme of Kimberly and Goldstein [9].

3. Materials and methods

3.1. Source and purification

Spermidine (*N*-(3-aminopropyl)-1,4-butanediamine), spermine (*N,N'*-bis(3-aminopropyl)-1,4-butanediamine) and *N*-(3-aminopropyl)-1,3-propanediamine were obtained commercially and samples of *N*-(3-aminopropyl)-1,5-pentanediamine were provided by Dr. Keijiro Samejima of Tokyo Electrochemical Research Institute, and by Dr. E.F. Elsager of Park, Davis and Co., Ann Arbor, MI. The polyamines *N*-(4-aminobutyl)-1,4-butanediamine, *N,N'*-bis(4-aminobutyl)-1,4-butanediamine, and *N,N'*-bis(4-aminobutyl)-1,3-propanediamine were synthesized by methods to be reported elsewhere. All polyamines were purified by recrystallizing the hydrochloride salts thrice from 80–95% aqueous ethanol, and all were free of detectable impurities as judged by TLC [11].

3.2. Calibration of the pH meter

The pH meter was calibrated in terms of H^+ concentration by the method of Hedwig and Powell [3,12], with HCl, NaOH and ethanediammonium chloride, using the acid dissociation constants of ethanediammonium chloride reported by Everett and Pinsent [13]. The relationship of the concentration scale pH, (pH_c) to the standard pH_m (defined by the standard NBS * buffer) as calculated using Powell's computer program [12] combined with a linear regression analysis is given by eq. 1.

$$pH_m = 1.0027pH_c + 0.0294 \qquad (1)$$

The fit is quite good, the regression coefficient (multi *R*) being 0.9998 and the standard error of the regression line being 0.024. Treatment of this data using ZXSSQ, a nonlinear fitting program [14], confirmed that eq. 1 gives a better fit than do higher order equations.

3.3. Determination of the protonation constants

The hydrochloride salts of the polyamines at a concentration of approx. 6×10^{-3} M were titrated using the microtitration technique described by Margerum [15]. For all but two titrations, the background electrolyte was aqueous NaCl at a concentration such that the ionic strength was 0.10 at half titration. Titrations were performed in a water-jacketed vessel thermostatically maintained at 25.00 ± 0.02°C under a blanket of high-purity, water-saturated nitrogen, and were monitored with a Corning Model 135 digital pH meter fitted with a Corning ceramic junction calomel electrode and a Fisher 13-639-3 glass electrode. The standard 0.2 M NaOH titrant was added using a Gilmont digital micrometer buret.

3.4. ^{13}C-NMR

Spectra were acquired using polyamine concentrations from 0.1 to 0.3 M in water containing 10% ^2H_2O in 10-mm sample tubes with a JEOL FX-6 spectrometer and a Varian XL-200 spectrometer. The pH of each sample was adjusted by dropwise addition of concentrated NaOH and measured using a calibrated combination electrode. Alkaline solutions were prepared in a

* National Bureau of Standards.

nitrogen-filled glovebag to prevent absorption of carbon dioxide.

4. Results

4.1. Macroscopic protonation constants

Protonation constants for spermidine, spermine and several analogs calculated using Hedwig and Powell's [3,12] nonlinear regression analysis of the titration curves are given in table 1. These constants are concentration constants. Each log K value is the mean of at least four values, each of which was determined from a separate titration, and the uncertainty is the standard deviation of the mean.

Although the slope and the intercept of eq. 1 differ slightly from the values reported by Hedwig Powell [3], the values we obtained for the protonation constants of spermidine and spermine generally agree closely with theirs. The deviation is largest for the fourth protonation constant of spermine, for which our value is 0.26 log K units higher. For the remaining protonation constants, the difference between our value and Powell's is between 0.01 log K unit and 0.10 log K units.

4.2. Estimation of microscopic constants

Microscopic constants for protonation of each base site in spermidine and spermine were estimated by correcting the protonation constants of isolated primary and secondary amines for the cumulative base-weakening effects of the remaining nitrogens. This approach is essentially the same as that used by Clark and Perrin [16] to estimate macroscopic protonation constants of organic bases, but there is one significant difference. Instead of associating a given macroscopic constant with the single most favorable corresponding microscopic protonation step as did the earlier workers, we estimated all of the microscopic constants and combined them in the usual manner to

Table 2

Base values for estimating microscopic protonation constants of polyamines

log K values – Monoamines		
Type of amine	Log K	
Primary	10.65	
Secondary	10.95	
Base-weakening effect of nitrogens		
Intervening carbons [a]	Δlog K protonated N	Δlog K unprotonated N
3	−1.61	−0.43
4	−0.91	−0.23
8	−0.05 [b]	0.00 [a]
12	0.00 [b]	0.00 [b]

[a] Intervening nitrogens counted as carbons.
[b] Estimated by extrapolation of data for shorter chain diamines.

Table 1

Stepwise protonation constants of polyamines at 25°C
Uncertainties represent the S.D. of the mean.

Compound abbreviation	log K_1	log K_2	log K_3	log K_4	Electrolyte	Ionic strength
3,4-Spd	10.83 ± 0.03	9.83 ± 0.01	8.35 ± 0.02		NaCl	0.10
3,4-Spd	10.93 ± 0.03	9.87 ± 0.01	8.40 ± 0.01		KNO_3	0.12
3,5-Spd	11.02 ± 0.02	10.09 ± 0.02	8.65 ± 0.02		KNO_3	0.12
4,4-Spd [a]	11.04 ± 0.01	10.09 ± 0.01	8.99 ± 0.01		NaCl	0.10
3,4,3-Spm	10.86 ± 0.06	10.05 ± 0.01	8.82 ± 0.01	7.95 ± 0.01	NaCl	0.10
4,3,4-Spm	10.62 ± 0.04	9.70 ± 0.01	9.18 ± 0.01	8.07 ± 0.01	NaCl	0.07
4,4,4-Spm	11.25 ± 0.03	10.40 ± 0.02	9.57 ± 0.01	8.72 ± 0.01	NaCl	0.10

[a] One determination only.

Table 3
Logarithmic microscopic protonation constants for spermidine and spermine

Spermidine					
k_a	10.22	k_b	10.29	k_c	10.42
k_{ab}	9.11	k_{ac}	10.37	k_{ba}	9.04
k_{bc}	9.74	k_{ca}	10.17	k_{cb}	10.04
k_{abc}	9.69	k_{acb}	8.43	k_{bca}	8.99
Spermine					
$k_a = k_d$	10.22	$k_b = k_c$	10.29		
$k_{ab} = k_{dc}$	9.11	$k_{ac} = k_{db}$	10.24	$k_{ad} = k_{da}$	10.22
$k_{ba} = k_{cd}$	9.04	$k_{bc} = k_{cb}$	9.61	$k_{bd} = k_{ca}$	10.17
$k_{abc} = k_{dcb}$	9.56	$k_{abd} = k_{cda}$	10.17	$k_{acb} = k_{bdc}$	8.43
$k_{abcd} = k_{bcda}$	8.99	$k_{abdc} = k_{acdb}$	8.38		

calculate the macroscopic constants. For molecules such as spermidine and spermine in which a number of microscopic constants contribute significantly to some of the macroscopic constants, it is necessary to proceed in this manner to obtain accurate estimates of the macroscopic constants. As the basis for calculating microscopic constants, we used the critical values of protonation constants of aliphatic monoamines and diamines compiled by Smith and Martell [17], which refer to an ionic strength of 0.1 and a temperature of 25°C. The data set for estimation of the microscopic protonation constants is listed in table 2 and the microscopic constants for spermidine and spermine are given in table 3.

4.3. ^{13}C-NMR spectra

The measured chemical shifts of the protonated polyamines, together with new or modified assignments, are presented in table 4. The chemical shifts of unprotonated spermidine, 3,3-Spd and 3,5-Spd were also measured to aid in assignments. The measured chemical shifts are in substantial agreement with those reported for spermidine [9], but the chemical shifts found for spermine are approx. 0.8 ppm farther downfield than the values reported by Weser et al. [7]. We attribute this difference primarily to a difference in the assigned chemical shift of the dioxane reference peak, 66.4 ppm assigned by Weser et al. [7] vs. 67.4 ppm (measured) in this work.

5. Discussion

5.1. Protonation constants

Consistent with the decrease in the mutual base weakening of amino groups associated with increased separation, the homologs of both spermidine and spermine exhibit a trend toward greater basicity as the number of carbon atoms increases. As the similarity of the protonation constants of 3,4-Spd in 0.1 M NaCl and in 0.12 M KNO_3 shows, small changes in the concentration and the nature of the background electrolyte have relatively little effect on the measured values of the protonation constants. Although the protonation constants are slightly greater in the latter medium, the increase is not large enough to preclude comparison of protonation constants of the polyamines in table 1.

Whereas a macroscopic protonation constant describes the average proton affinity of all of the molecules in which a given number of base sites have already been protonated, each of the corresponding microscopic constants describes the proton affinity of a particular base site in a molecule in which the previously accepted protons are located on specific base sites. Because both the macroscopic constants and the microscopic constants refer to the proton affinities of the same group of base sites, albeit in different ways, the macroscopic constants of a polybasic molecule are related mathematically to its microscopic constants. Hence, a simple way to test a set of micro-

Table 4

^{13}C-NMR chemical shifts of protonated polyamines in 0.1–0.3 M solution in 10% $^2H_2O/H_2O$, dioxane reference

	Polyamines		^{13}C-NMR chemical shifts (ppm vs. tetramethylsilane)							
3,4-Spd	$^{+}NH_3\,^{3'}CH_2\,^{2'}CH_2\,^{1'}CH_2\,^{+}NH_2\,^{1}CH_2\,^{2}CH_2\,^{3}CH_2\,^{4}CH_2\,^{+}NH_3$	pH 4.1:	3' 37.7	2' 24.7	1' 45.5	1 48.0	2 23.6	3 24.8	4 39.9	
		pH 11.7:	39.6	31.6	46.9	49.1	26.4	29.8	41.3	
3,3-Spd	$^{+}NH_2\,^{3}CH_2\,^{2}CH_2\,^{1}CH_2\,^{+}NH_2CH_2CH_2CH_2\overset{+}{N}H_3$	pH 5.0:	3 37.7	2 24.7	1 45.6					
		pH 11.0	39.3	31.3	46.9					
4,4-Spd	$^{+}NH_3CH_2CH_2CH_2CH_2\overset{+}{N}H_3\,^{1}CH_2\,^{2}CH_2\,^{3}CH_2\,^{4}CH_2\,^{+}NH_3$					1 49.7	2 23.6	3 24.9	4 40.0	
3,5-Spd	$^{+}NH_3\,^{3'}CH_2\,^{2'}CH_2\,^{1'}CH_2\,^{+}NH_2\,^{1}CH_2\,^{2}CH_2\,^{3}CH_2\,^{4}CH_2\,^{5}CH_2\,^{+}NH_3$	pH 4.7:	3' 37.7	2' 24.7	1' 45.4	1 48.4	2 25.9	3 23.6	5 40.3	4 27.1
		pH > 11:	39.8	32.1	47.1	49.4	29.2	24.8	41.6	32.8
3,4,3-Spm	$^{+}NH_3\,^{3'}CH_2\,^{2'}CH_2\,^{1'}CH_2\,^{+}NH_2\,^{1}CH_2\,^{2}CH_2\,^{3}CH_2\,^{+}CH_2\,^{+}NH_2(CH_2)_3NH_3$		3' 37.7	2' 24.7	1' 45.5	1 48.0	2 23.7			
4,2,4-Spm	$^{+}NH_3\,^{4'}CH_2\,^{3'}CH_2\,^{2'}CH_2\,^{1'}CH_2\,^{+}NH_2\,^{1}CH_2\,^{2}CH_2\,^{+}NH_2(CH_2)_4NH_3$					1' 48.4	2' 23.6	3' 24.9	4' 39.9	1 44.0
4,3,4-Spm	$^{+}NH_3\,^{4'}CH_2\,^{3'}CH_2\,^{2'}CH_2\,^{1'}CH_2\,^{+}NH_2\,^{1}CH_2\,^{2}CH_2\,^{3}CH_2\,^{+}NH_2(CH_2)_4NH_3$				1 45.5	1' 48.0	2' 23.6	3' 24.9	4' 40.0	2 23.6
4,4,4-Spm	$^{+}NH_3\,^{4'}CH_2\,^{3'}CH_2\,^{2'}CH_2\,^{1'}CH_2\,^{+}NH_2\,^{1}CH_2\,^{2}CH_2\,^{3}CH_2\,^{4}CH_2\,^{+}NH_2(CH_2)_4NH_3$					1 + 1' 47.9	2 2' 23.6	3' 24.9	4' 39.9	

scopic constants is to compare the values of the macroscopic constants derived from them to the directly measured values of the macroscopic constants. Table 5 shows that our estimated values of the microscopic constants of spermidine and spermine meet this test well. The largest discrepancy between the experimental and calculated macroscopic constants is 0.12 log K units and for most of the protonation constants the discrepancy is much smaller. Considering the simplicity of the method used to estimate the microscopic constants, this high level of agreement is surprisingly good.

Calculation of the microscopic constants for spermidine and spermine makes it possible to predict the protonation sequences of these polyamines. Fig. 1 shows that as the extent of protonation increases, the basicity of the secondary nitrogens in both polyamines varies in a systematic way. Above pH 10.5, where less than 0.75 equivalents of H^+ have been added, the basicity of N-b in spermidine and spermine is comparable to that of N-a, but as the extent of protonation increases further, the degree of protonation of N-b falls progressively behind that of N-a. The microscopic constants in table 3 and the substituent effects in table 2 suggest that the decrease in the basicity of N-b in both polyamines reflects the concerted base-weakening effects of increased protonation at N-a and N-c.

The ability to calculate the protonation sequence is of particular interest for spermidine, because the protonation sequence of this polyamine has been the subject of three experimental studies, the conclusions of which are not consistent. On the basis of calorimetric measurements, Anichini and co-workers [18] concluded that the nitrogen atom which is protonated in the first step is a primary amine. This conclusion was supported

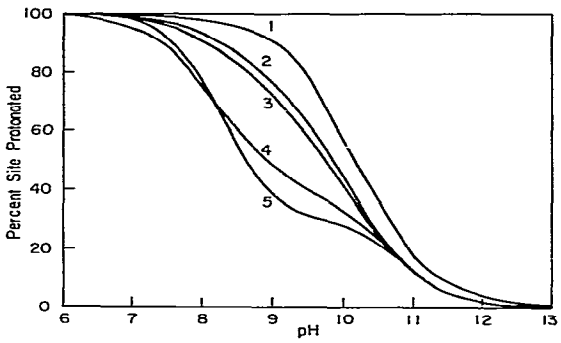

Fig. 1. Calculated protonation sequences of spermidine and spermine. (1) N-c, spermidine; (2) N-a, spermidine; (3) N-a and N-d, spermine; (4) N-b and N-c, spermine; (5) N-b, spermidine.

by Delfini and co-workers [10], who studied the protonation sequence using ^{13}C-NMR and ^{1}H-NMR. On the other hand, Kimberly and Goldstein [9] concluded from their ^{13}C-NMR study that in monoprotonated spermidine, N-a and N-b are each 32% protonated and N-c is 43% protonated. Our microscopic constants predict that the extent of protonation of these three sites corresponds to 28, 31 and 40%, in excellent agreement with the results of Kimberly and Goldstein. The level of agreement between our microscopic constants and the results of Kimberly and Goldstein for diprotonated spermidine is nearly as good. Here, the experimental results are that the extent of protonation at N-a, N-b and N-c is 67, 42 and 86%, respectively, while the microscopic constants predict that the extent of protonation at these three sites is 75, 36 and 89%, respectively. Such agreement between the predicted and experimental proton distributions in spermidine not only provides independent support for the protonation se-

Table 5

Comparison of macroscopic constants determined (Meas.) in 0.1 M NaCl with values calculated (Calc.) from microscopic constants

Compound	log K_1		log K_2		log K_3		log K_4	
	Meas.	Calc.	Meas.	Calc.	Meas.	Calc.	Meas.	Calc.
3,4-Spd	10.83	10.79	9.83	9.92	8.35	8.31	–	–
3,4,3-Spm	10.86	10.86	10.05	10.10	8.82	8.94	7.95	7.98

quence proposed by Kimberly and Goldstein but also gives additional evidence of the validity of this method of estimating microscoping constants.

5.2. ^{13}C chemical shifts

Determination of the ^{13}C chemical shifts of protonated symmetrical and umsymmetrical triamines which are homologs of spermidine, together with the determination of the change in the ^{13}C chemic·l shift on deprotonation of 3,3-Spd made it possible to assign ^{13}C chemical shifts unambiguously in these compounds and in spermidine, spermine and homologs of the tetramine. In this analysis the following principles were used for preliminary assignments.:

(1) α-carbon atoms (those with amine substituents) have larger downfield ^{13}C shifts than carbon atoms farther removed [19,20];

(2) carbons α to secondary amines have larger downfield shifts than those α to primary amines [19];

(3) the chemical shift of carbons β to a protonated amine changes most on deprotonation [19,20]. The assignments are developed in detail below and in table 4.

5.2.1. Spermidine and analogs

The assignment of the absorption at 45.6 ppm to carbon 1 in 3,3-Spd, that at 37.7 ppm to carbon 3 and that at 24.7 ppm to carbon 2 follows directly from these general principles; so also do the assignments for 4,4-Spd of the absorption at 47.9 ppm to carbon 1 and that at 40.0 ppm to carbon 4. The assignments of the 23.6 ppm resonance to carbon 2 which is β to a secondary amine and that at 24.9 ppm to carbon 3 which is β to a primary amine, follow directly from the data of Sarneski et al. [19], which indicate that (in monoamines) the resonance of carbon β to a protonated secondary amine is about 1.3 ppm upfield from that of carbon β to a protonated primary amine.

In the unsymmetrical 3,5-Spd the matching of resonances at 45.4, 37.7 and 24.7 ppm with carbons 1′,3′ and 2′, respectively, is very like that in 3,3-Spd. So also is the assignment of the 45.5, 37.7 and 24.7 ppm resonances of spermidine (3,4-Spd) to carbons 1′,3′ and 2′. There is little complication here; the assignments correct those of Bunce and Kong [8] and agree with those of Kimberly and Goldstein [9]. The assignment of the 48.0 ppm resonance of spermidine to carbon 1 follows from assignment of the 47.9 ppm resonance to the carbon (C-1) next to the secondary amine nitrogen in the four carbon chain of 4,4-Spd; in 3,5-Spd the corresponding carbon (C-1) in the five-carbon chain is shifted slightly further downfield. Similarly, the assignment of the 39.9 ppm resonance to carbon 4 can be made with confidence: it is very similar to carbon 4 in 4,4-Spd. The assignments of the 24.8 ppm resonance to carbon 3 and of the 23.6 resonance to carbon 2 follow also from the assignments in 4,4-Spd. The change in chemical shift on protonation for C-3, a carbon β to a primary amine, is found to be greater than that for C-2, a carbon β to a secondary amine, consistent with the observations of Sarneski et al. on primary and secondary monoamines [19]. This fixes the assignment left uncertain by Kimberly and Goldstein [9].

The changes in ^{13}C assignments for spermidine from those used in the earlier study of spermidine–5′-AMP interactions by Bunce and Kong [8] do not affect the conclusions and the model for interaction presented there.

The remaining assignments for 3,5-Spd (homospermidine) are made with reference to the chemical shifts of 1,5-diaminopentane found by Rabenstein and Sayer [20] (similar values were reported by Sarneski et al. [19]) where chemical shifts of the fully protonated diamine are 40.7 ppm for C-1 (corresponding in 3,5-Spd, to C-5, 40.3 ppm), 27.5 ppm for C-2 (corresponding in 3,5-Spd to C-4, 27.1 ppm), and 23.8 ppm for C-3 (corresponding in 3,5-Spd to C-3, 23.6 ppm). Carbon 1, α to a secondary amine, is 48.4 ppm and C-2 is 25.9 ppm. These assignments are confirmed by the NMR spectrum at high pH: the chemical shift for carbon 4, β to the primary amine is greater than for carbon 2, β to a secondary amine.

5.2.2. Spermine and homologs

There can be no ambiguity concerning the assignment of the resonances at 45.5, 37.7 and 24.7 ppm to carbons 1′,3′ and 2′ in the three-carbon chain of spermine, since their chemical shifts are almost identical to those of the corresponding

carbons of spermidine. The remaining chemical shifts, 48.0 and 23.7 ppm, correspond closely to those of carbons 1 and 1′ and 2 and 2′ of 4,4,4-Spm. The assignments of the remaining resonances in 4,4,4-Spm and in the four-carbon chains in 4,2,4-Spm and 4,3,4-Spm follow directly from the assignments for 4,4-Spd. Carbon 4′ at 39.9 ppm is like C-4 in 4,4-Spd at 40.0 ppm and C-3′, like C-3 in 4,4-Spd is at 24.9 ppm.

In 4,2,4-Spm, carbon 1 in the center group has no close counterpart in other polyamines examined here, but its situation and absorption are roughly equivalent to the central carbons in *N*,*N*′-diethylethanediamine, with absorption at 44.0 [19].

5.3. Applications to polyamine studies

The microscopic protonation constants and the ^{13}C-NMR chemical shift assignments of spermidine and spermine will have value as points of reference in studies of the interactions of these polyamines with other substances. While full experimental verification of the microscopic constants of spermidine and spermine remains to be accomplished, the fact that the microscopic constants generate the correct values of the macroscopic constants of both polyamines, and the excellent agreement of the predicted proton distribution of monoprotonated and diprotonated spermidine with the experimental values of Kimberly and Goldstein provide strong support for the validity of this method for estimating the microscopic constants. The ability to calculate protonation sequences of polyamines should provide guidance in the assignment of NMR chemical shifts for other polyamines and insight concerning the nature of their protonated species in solution.

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