

# Nuclear Magnetic Resonance as a Tool for Determining Protonation Constants of Natural Polyprotic Bases in Solution<sup>1</sup>

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The acid-base properties of the tetramine 1,5,10,14-tetraazatetradecane  $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$  (spermine) in deuterated water have been studied at 40°C at various *pD* values by means of NMR spectroscopy. Both one-dimensional  $^{13}C\{^1H\}$  spectra and two-dimensional  $^1H/^{13}C$  heterocorrelation spectra with inverse detection have been recorded. A calculation procedure of general validity has been developed to unravel the effect of rapid exchange between the various species in equilibrium as a function of *pD* of the solution. The method of calculation used in this part of the new computer program, HYPNMR, is independent of the equilibrium model. HYPNMR has been used to obtain the basicity constants of spermine with respect to the  $D^+$  cation at 40°C. Calculations have been performed using either  $^{13}C\{^1H\}$  or  $^1H/^{13}C$  data individually, or using both sets of data simultaneously. The results of the latter calculations were practically the same as the results obtained with the single data sets; the calculated errors on the refined parameters were a little smaller. After appropriate empirical corrections for temperature effects and for the presence of  $D^+$  in contrast to  $H^+$ , the calculated constants are compared with spermine protonation constants which have been determined previously both from potentiometric and NMR data. © 1995 Academic Press, Inc.

Nuclear magnetic resonance spectroscopy (NMR) is being used more and more frequently for the determination of acid-base equilibrium constants ( $pK_A$ ) of substances of biological interest such as the amino acids

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(1,2), peptides (3,4), polyamines (5,6), antibiotics (7,8), or other substances containing acidic or basic groups. NMR measurements offer a particular advantage when compared to electrode potential measurements (the potentiometric titration method is widely used for the determination of  $pK_A$ 's): by concentrating on particular resonances, NMR spectroscopy can be used to follow the chemical shift, as a function of pH, of a particular substance present in a solution of physiological interest such as intracellular fluid, plasma, blood, and urine, which is a complex mixture often containing other acids and bases. The profiles of chemical shift as a function of pH obtained in this manner are independent of the presence of protolytic "impurities" so long as there is no direct interaction between the latter and the substance whose nucleus is under observation. That this is a significant advantage can be seen by recalling the importance in potentiometric work of avoiding contamination by carbon dioxide.

On the other hand, NMR measurements require an amount of experimental time that is strongly dependent on the concentrations of the species, the particular nucleus observed, and the pulse sequence employed. These factors need to be given careful consideration for the following reasons: species of biological interest may have concentrations in the micromolar range in the samples of interest, making measurements difficult if the nucleus being observed is less NMR sensitive than the proton; the determination of the  $pK_A$ 's of polyprotic molecules necessitates the collection of a large number of high-precision (large S/N ratio and digital resolution) experimental spectra.

Previous determinations of  $pK_A$  from NMR measurements have used as probes  $^1H$  or  $^{13}C$  nuclei, or  $^{15}N$  nuclei in labeled compounds (mainly peptides). They

have used one-dimensional spectra or 2D heterocorrelation spectra. Two-dimensional spectra can be acquired in direct detection mode or, more efficiently, in indirect detection mode (HMQC spectra). However, in many of these determinations the number of chemical shifts taken into consideration has been rather small; furthermore, the pK<sub>A</sub> value of a single amino acid residue of interest (considered to be the only protonation center even when there are others) has often been simplistically calculated from the Henderson–Hasselbach equation. Also, more often than not, measurements of spectra and of pH are made at different temperatures and no correction for isotope effects are made when dealing with D<sub>2</sub>O solutions.

Considering the enormous variety of systems that might be investigated it is clear that there is a need for a generally applicable computer program which can be used to determine equilibrium constants from data consisting of the variation of chemical shifts with pH. Some calculation procedures have been developed that are of limited application (9–11). A recent publication (12) describes the program EQNMR, which can be used for reactions of different types, such as protonation equilibria, metal ion hydrolysis, and ligand–metal interactions. Unfortunately, as far as we know, this method has only been tested on computer-generated data and not on experimental data.

In this work we have addressed the problem of determining the protonation constants of a biologically important substance, the tetramine 1,5,10,14-tetraazatetradecane H<sub>2</sub>N(CH<sub>2</sub>)<sub>3</sub>NH(CH<sub>2</sub>)<sub>4</sub>NH(CH<sub>2</sub>)<sub>3</sub>NH<sub>2</sub> (commonly called spermine). This molecule is present in a wide range of prokaryotic and eukaryotic cells, and its physiological role is well understood (13). In particular, we have recently demonstrated (14) that spermine is able to interfere with the processes by which protein kinase C (PKC)<sup>2</sup> associates with the acidic phospholipids in the cell membrane; this step is essential for the activation of the enzyme. In order to explain the mechanism by which the spermine acts we intend to study the complex(es) formed between spermine and phospholipids by NMR spectroscopy and, by determining geometry and conformation, to ascertain the nature of the interactions at the molecular level. An essential prerequisite for such a study is the determination of the protonation constants of spermine under the same conditions that will subsequently be used in the examination of its interactions with liposomes.

In this communication we report the proton and carbon NMR spectra obtained on a variety of solutions of spermine at different pD values in D<sub>2</sub>O solution. The observed chemical shifts were processed by means of a sophisticated computer program of general validity,

HYPMNR; the actual spectra, which are rather complicated on account of there being five nonequivalent carbon atoms and also five nonequivalent protons, require a quite sophisticated calculation procedure. Moreover, the four pK<sub>A</sub> values of spermine lie in the interval 8–11 so that the four buffer regions are superimposed on one another. For this reason simple computational procedures based on the approximation that each species is formed successively as the pD rises cannot be used.

Both 1D <sup>13</sup>C{<sup>1</sup>H} spectra and 2D <sup>1</sup>H/<sup>13</sup>C spectra were obtained, the latter with the aid of inverse detection (HMQC spectra). Two-dimensional spectra had to be taken because of overlap of some proton signals in the 1D spectra, which rendered difficult the identification and chemical shift determination of the CH<sub>2</sub> protons in the α position with respect to the amino groups. Use of the inverse detection technique made it possible to collect the spectra in the relatively short time of 1–2 h despite the low concentration (~50 mM) of the substance.

## MATERIALS AND METHODS

### Materials

The tetrahydrochloride of spermine was obtained from Sigma Chemical Co. (U.S.A.; purity, 95–98%) and was kept in a desiccator at –20°C over silica gel. Elemental analysis gave the following results: C, 34.29, H, 8.49, N, 15.96; C<sub>10</sub>H<sub>30</sub>N<sub>4</sub>Cl<sub>4</sub> requires C, 34.50, H, 8.68, N, 16.09%. As, in addition, its <sup>1</sup>H NMR spectrum did not reveal the presence of appreciable quantities of impurities, the commercial product was used without further purification. D<sub>2</sub>O, KOD, and DCl were obtained from Merck (Germany; Uvasol grade, 99.9%), sodium 3-(trimethylsilyl)propionate-*d*<sub>4</sub> (TSP) was supplied by Wilmad (U.S.A.).

### Methods

*Calibration of the pH meter.* A Radiometer Model PHM 26C pH meter with a combined glass electrode (Radiometer Model GK2322C) was calibrated at 40°C in terms of deuterium ion activity,  $a_D = 10^{-pD}$ , by means of two buffer solutions in D<sub>2</sub>O: KD<sub>2</sub>PO<sub>4</sub> (0.025 *m*) + K<sub>2</sub>DPO<sub>4</sub> (0.025 *m*), and KDCO<sub>3</sub> (0.025 *m*) + K<sub>2</sub>CO<sub>3</sub> (0.025 *m*), for which the standard reference values are pD = 7.39 and pD = 10.60, respectively (15). Potassium salts were used in preference to sodium salts in order to reduce the errors in potentiometric measurements of pD that may be caused by interference from Na<sup>+</sup> above pD = 9.

The pD of each sample was varied by means of the addition of small measured quantities, of the order of a few microliters, of a concentrated solution of KOD or DCl to 0.5 cm<sup>3</sup> of a stock solution containing 50 mM of

<sup>2</sup> Abbreviations used: PKC, protein kinase C; TSP, sodium 3-(trimethylsilyl)propionate-*d*<sub>4</sub>.

the tetrahydrochloride of spermine. The concentrations of the resulting solutions were calculated by assuming additivity of volume. The *pD* values of each solution were measured to a precision of  $\pm 0.01$  units both before and after the spectrophotometric measurement. The largest discrepancy between the measurements was 0.02 *pD* units.

The ionic strength, *I*, was not maintained at a constant value by means of the addition of an inert salt, despite the fact that *I* varied significantly from sample to sample (from  $\sim 0.2$  M, when the spermine was deprotonated, up to  $\sim 0.5$  M when it was completely protonated). This is justified by noting that Davies's equation (16)

$$\log \gamma_i = -Az_i^2[I^{1/2}/(1 + I^{1/2}) - 0.3I]$$

gives approximately constant activity coefficients in the range of ionic strengths used in our experimental measurements. In particular, the values of  $\log \gamma_i$  for a monovalent cation such as  $D^+$  come out between  $-0.13$  and  $-0.14$  so that we can convert from activity,  $a_D$ , to concentration,  $[D^+]$ , by using Eq. [1]

$$-\log[D^+] = pD - 0.135. \quad [1]$$

**NMR determinations.** Natural abundance spectra were recorded on an AMX-400 WB Bruker spectrometer, operating at 9.395 T with a reverse  $^1H/BB$  5-mm probe. All spectra were acquired at a temperature of 40°C (controlled by a Eurotherm 3000 VT control unit) and using TSP as an internal standard for Chemical Shift reference. Experimental parameters for  $^1H$  nucleus were as follows: spectral frequency = 400.13 MHz, spectral width = 5600 Hz (14 ppm), 32K data points in quadrature detection, pulse width = 4  $\mu s$  (45° flip angle), acquisition time = 2.916 s. HDO signal was suppressed by 1 s of presaturation pulse.

Experimental parameters for  $^{13}C$  nucleus were as follows: spectral frequency = 100.61 MHz, spectral width = 6942 Hz (69.0) ppm, 32K data points in the time domain and 64K data points in the frequency domain, pulse width = 5  $\mu s$  (11.3  $\mu s$  at 90° flip angle), acquisition time = 2.36 s, number of scans = 256. Spectra were acquired using WALTZ-16 sequence for proton decoupling ( $PW_{90^\circ} = 90 \mu s$ ). FIDs were processed with enhancement multiplication (line broadening = 1 Hz).

$^1H/^{13}C$  heterocorrelated experiments were run in the reverse detection mode. Spectra were acquired with HDO signal suppression by 0.7 s presaturation and with  $BB$   $^{13}C$  decoupling using GARP (17) sequence (pulse width = 100  $\mu s$ ). Experimental parameters were as follows: spectral width  $^1H$  dimension ( $F_2$ ) = 1500 Hz, spectral width  $^{13}C$  dimension ( $F_1$ ) = 6942 Hz; 4K data points in  $F_2$  and 128 in  $F_1$ ; proton pulse width

= 8  $\mu s$  (90° flip angle), carbon pulse width = 9.8  $\mu s$ ; acquisition time = 1.365 s; number of scans = 16; number of dummy scans = 128. Owing to the very narrow spectral width in  $F_2$ , which allowed a large digital resolution, the spectra were acquired by changing the carrier wave from the resonance frequency of the HDO (during the presaturation period) to the center of the  $^1H$  spectral window (during all the remaining steps of the pulse sequence). FIDs were processed doubling the number of points by zero filling in  $F_1$  dimension and with SIN function (shifted by  $\pi/2$  rad) filtering in both dimensions. The FT was performed in the *magnitude* mode.

Spectra were recorded for 20 solutions, prepared as described above, with *pD* values between 6.80 and 12.38. The precision of the chemical shift measurements was estimated to be 0.001 and 0.002 ppm for  $^1H$  and  $^{13}C$ , respectively.

**Data analysis.** The determination of the equilibrium constants was done using HYPNMR, a new computer program derived from an early version of HYP-ERQUAD (18), modified to process NMR data in place of spectrophotometric data. In the original program all the free reagent concentrations, including that of the hydrogen ion, are calculated from experimental values of the analytical (total) concentrations. As in the present work the concentration of the deuterium ion is derived from experimental emf measurements (see Eq. [1]), a different approach is needed for the calculation of the free concentrations of the reagents other than the hydrogen ion.

**Concentrations of species in a solution at known *pH* (or *pD*).** Let there be in solution  $n_r$  reagents A, B, . . . , with total concentration of  $T_A, T_B, \dots$  able to form  $n_s$  species.<sup>3</sup> At equilibrium the concentration,  $C_j$ , of the *j*th species in a solution is defined in terms of the stoichiometric coefficients  $a_j, b_j, \dots, h_j$  and the cumulative formation constant  $\beta_j$ .

$$C_j = [H_{h_j}A_{a_j}B_{b_j}\dots] = \beta_j[A]^{a_j}[B]^{b_j}\dots[H]^{h_j}, \quad [2]$$

where  $a_j, b_j, \dots$  are the number of units of each reagent present in the *j*th species, and  $h_j$  is the number of hydrogen ions (protons or deuterons) present in the species. A positive, negative, or zero value of the stoichiometric coefficient  $h_j$  implies a protonated, deprotonated, or unprotonated species, respectively. The total concentration of each reagent in the solution is constrained by a set of conditions of mass balance

$$T_A = [A] + \sum_j a_j C_j = [A] + \sum_j a_j \beta_j [A]^{a_j} [B]^{b_j} \dots [H]^{h_j}$$

<sup>3</sup> The electrical charges carried by the species are omitted for convenience.

$$T_B = [B] + \sum_j b_j C_j \quad [3]$$

$$= [B] + \sum_j b_j \beta_j [A]^{a_j} [B]^{b_j} \dots [H]^{h_j} \dots$$

If the concentration [H] can be derived from a pH (or pD) measurement and the formation constants are known, the set of  $n_r$  mass balance equations (Eqs. [3]) can be solved for the free concentrations [A], [B], . . . and hence all the species concentrations in the solution can be obtained using Eq. [2].

*Least-squares refinement of equilibrium constants and chemical shifts.* In the computer analysis of the NMR spectra the exchange between the various species present in an equilibrium has been assumed to be rapid on the NMR time scale. As a consequence, the observed chemical shift,  $\delta$ , for a particular nucleus is the average of the chemical shifts ( $d_j$ ) of that nucleus in the various species present, weighted according to their fractional populations  $f_j$ :

$$\delta = \sum_j f_j d_j, \quad [4]$$

where

$$f_j = x_j C_j / T_x \quad [5]$$

In Eq. [5]  $T_x$  is the total concentration of the reagent containing the nucleus under consideration and  $x_j$  is the stoichiometric coefficient of reagent X in the  $j$ th species.

Since the quantities  $f_j$  depend on the values of the equilibrium constant  $\beta_j$ ,  $\delta$  is a function of both  $d_j$  and  $\beta_j$ . These parameters may be refined by minimizing the error square sum  $U$ , over the whole set of experimental determinations.

$$U = \sum_i w_i r_i^2 = \sum_i w_i (\delta_i^{\text{obs}} - \delta_i^{\text{calcd}})^2, \quad [6]$$

where  $\delta_i^{\text{obs}}$  are the observed chemical shifts,  $\delta_i^{\text{calcd}}$  are the corresponding quantities calculated using an estimated set of  $d_j$  and  $\beta_j$  values, and  $w_i$  is the weight assigned to the  $i$ th experimental observation. The minimization of the function  $U$  is carried out by the Gauss–Newton–Marquardt nonlinear least-squares method. Shifts of the parameters are calculated by solving the so-called normal equations (formalism as outlined previously) (18)

$$\mathbf{J}^T \mathbf{W} \mathbf{J} \mathbf{s} = \mathbf{J}^T \mathbf{W} \mathbf{r}, \quad [7]$$

where  $\mathbf{J}$  is the Jacobian matrix whose elements are the partial derivatives of the chemical shifts with respect to the parameters. All the required derivatives are calculated using analytical expressions.  $\mathbf{s}$  is a shift vector and  $\mathbf{r}$  is a vector containing the residuals  $r_i = \delta_i^{\text{obs}} -$

$\delta_i^{\text{calcd}}$ .  $\mathbf{W}$  is a weight matrix, which may be a unit matrix; an alternative weighting scheme is possible, where the weight matrix is still diagonal, but each term,  $w_{ii}$ , is set equal to the inverse of the estimated variance of the  $i$ th chemical shift measurement.

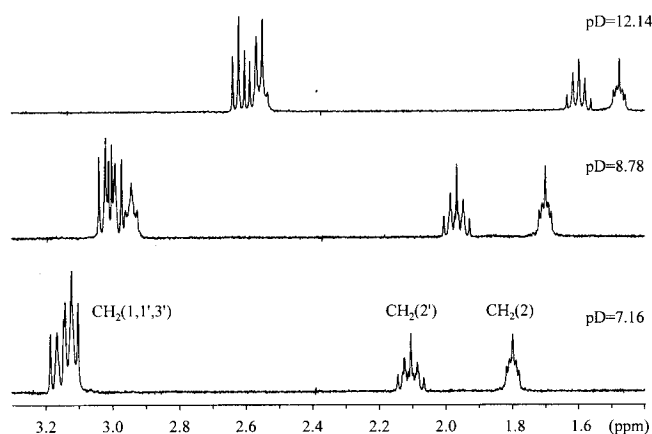
The computer program HYPNMR was written in FORTRAN in such a way as to be applicable to the widest possible range of NMR data, of chemical and of spectroscopic models. There are no limits, other than that imposed by the quantity of conventional memory available, on the number of reagents, the number of formation constants, the number and type of resonating nuclei, and the number of experimental spectra. This flexibility has been achieved by run-time allocation of memory for all the arrays used in the program. The program can also be used for "incomplete" NMR data in which the chemical shift of one or more nuclei may be assigned in some spectra but not in others.

The program has four main parts. In the first part data are read in. The data consist essentially of (i) specification of the chemical model by defining the stoichiometric coefficients,  $a_j, b_j, \dots, h_j$ , of the species assumed to be present together with estimated values of the corresponding formation constants  $\beta_{ij}$ ; (ii) a list of refinement keys used to define which of the parameters (equilibrium constants  $\beta_j$  and/or chemical shifts  $d_j$ ) should be refined and which should be kept constant; (iii) specification of the contribution from each species (including the free reagents) to the overall observed chemical shifts,  $\delta$ ; (iv) for every solution: the molar concentrations of the reagents A, B, . . . , the value of pH (or pD)<sup>4</sup> and the measured chemical shifts.

In the second part of the program the chemical shifts,  $d_i$ , of the individual nuclei which contribute to the overall shifts are calculated by solving the Eqs. [4] by the method of linear least squares. The fractional populations,  $f_j$ , are derived from the concentrations of the species, which in turn are derived from the current values of the formation constants and from the free reagent concentrations obtained by solving the system of mass balance equations (Eqs. [3]).

The sum of squares residuals,  $U$ , is minimized in the third section of the program. The process involves iterative refinement of the equilibrium constants using the Gauss–Newton–Marquardt method (19,20). With each iteration new values for the parameters and their standard deviations are obtained. The iterations are terminated when two convergence criteria are satisfied: (i)  $U$  decreases by less than 0.01% of its value and

<sup>4</sup> If the emf cell is calibrated in terms of H<sup>+</sup> (or D<sup>+</sup>) activity, the equilibrium constants will follow the "mixed activity–concentration" convention; but if the calibration is performed in terms of concentration, or if the observed pH (or pD) values are corrected for the appropriate activity coefficients, all the equilibrium constants will be strictly molar concentration ratios.



**FIG. 1.**  $^1\text{H}$  NMR spectra of 50 mM spermine solutions at 40°C in  $\text{D}_2\text{O}$  at the three pD values indicated. Chemical shifts are relative to TSP, used as an internal standard. Assignments are reported according to Ref. (22).

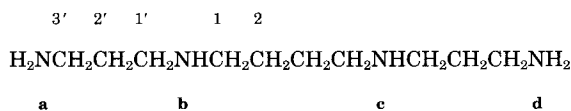
(ii) the elements of the shift vector  $\mathbf{s}$  (Eq. [7]) are less than 1% of the standard deviation of the relevant parameter.

If one convergence criterion is not satisfied we return to the second section of the program. Otherwise control is passed to the fourth part of the program in which the results of the calculation are presented in the form of tables. These include the final values of the formation constants and chemical shifts, their standard deviations, the correlation coefficients between formation constants, the sample standard deviation, the concentrations of each species in every solution, and a comparison between the observed and calculated chemical shifts. The last two items can also be obtained in the form of graphs.<sup>5</sup>

## RESULTS

### NMR Investigations

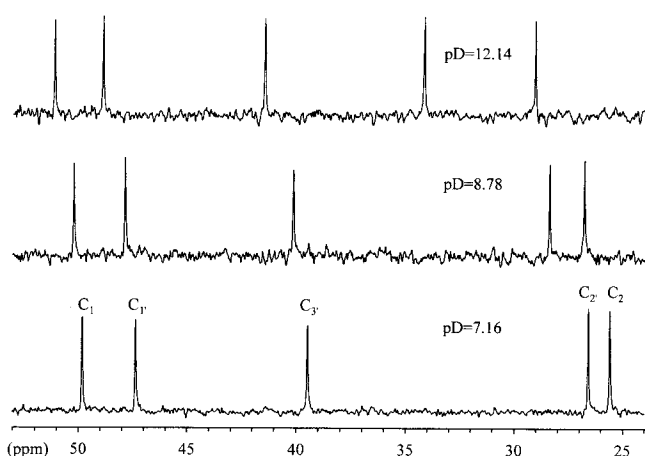
The molecule of spermine has five sets of chemically equivalent protons, so that only five distinct  $^1\text{H}$  NMR signals can be observed. Likewise, only five distinct  $^{13}\text{C}$  signals are to be expected. Each signal derives from two methylene groups. The designation of the atoms is given in Scheme 1 and is in accord with Onasch *et al.* (21).



**SCHEME 1**

Some  $^1\text{H}$  spectra of spermine solutions are illustrated in Fig. 1, together with assignments according to

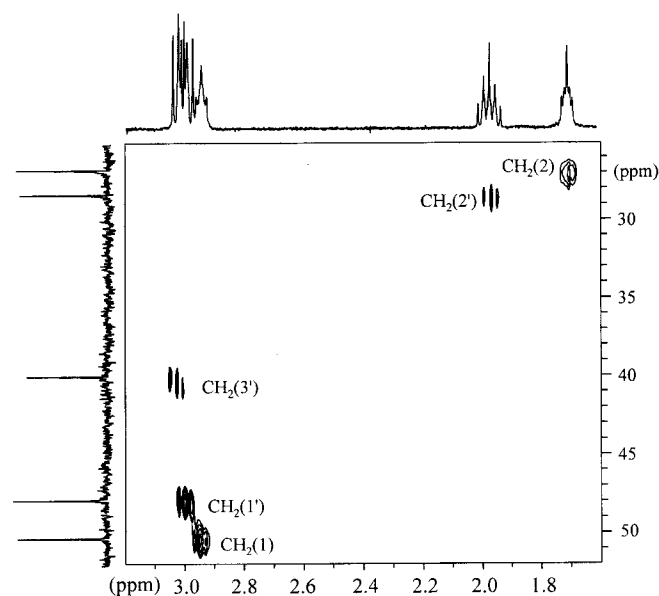
<sup>5</sup> To obtain a copy (gratis) of the program HYPNMR please contact A. Vacca (e-mail: vacsab@chimati1.chim1.unifi.it).



**FIG. 2.**  $^{13}\text{C}$  NMR spectra of 50 mM spermine solutions at 40°C in  $\text{D}_2\text{O}$  at the three pD values indicated. Chemical shifts values are relative to TSP, used as an internal standard. Assignments are reported according to Ref. (22).

Scheme 1 and Ref. (22). The assignments are based on heteronuclear  $^1\text{H}/^{13}\text{C}$  correlation experiments conducted in inverse detection mode (23). The complexity of the multiplets at low field is the result of second-order effects due to closeness of the chemical shifts of the  $\text{CH}_2$  groups labeled 1, 1', and 3' and to the strong coupling with their respective vicinal  $\text{CH}_2$  groups.

By contrast, the  $^{13}\text{C}\{^1\text{H}\}$  spectra of spermine show five completely resolved signals, as illustrated in Fig. 2. These spectra are from the same solutions as were



**FIG. 3.**  $^1\text{H}/^{13}\text{C}$  heteronuclear correlated spectrum (HMQC) of a 50 mM spermine solution at 40°C in  $\text{D}_2\text{O}$  at pD = 8.78. Chemical shifts are given relative to TSP, used as an internal standard. Assignments are reported according to Ref. (22).

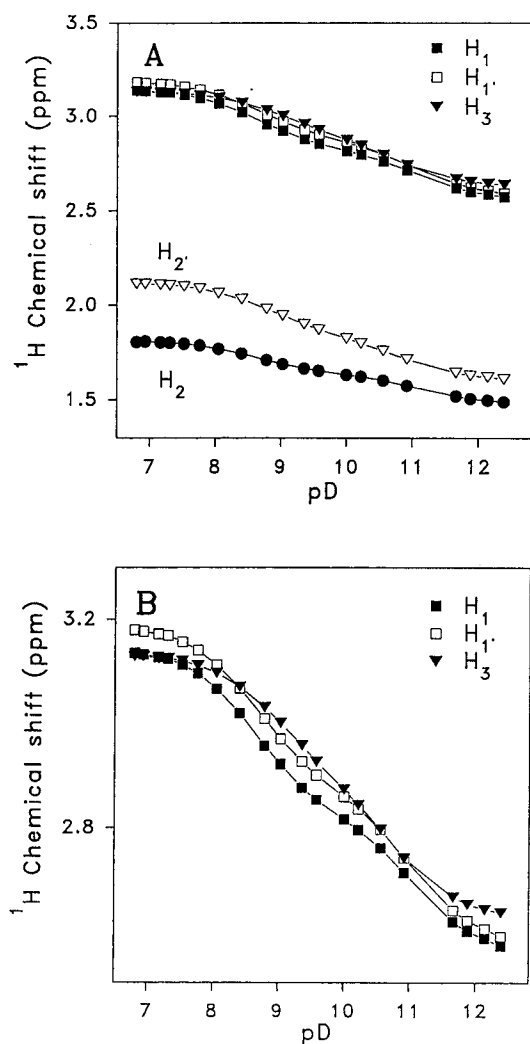


FIG. 4. (A) pD dependence of <sup>1</sup>H NMR chemical shifts of the five nonequivalent sets of protons of spermine in D<sub>2</sub>O solution at 40°C. (B) Expanded region for the curves corresponding to methylene groups 1, 1', and 3'.

used to obtain the spectra in Fig. 1. The assignment of the resonances (22) was unambiguous and the experimental chemical shifts are in good agreement with those calculated using the semiempirical formula of Sarneski *et al.* (24).

The two-dimensional heteronuclear correlation contour plot obtained from a solution at pD = 8.78 is shown in Fig. 3. The resonances due to protons with similar chemical shifts which are not resolved in the 1D spectra are completely resolved in the 2D spectra.

The spermine molecule is effectively completely protonated at pD = 6.80. As pD increases there is a gradual deprotonation and the proton resonances shift to higher field, as shown in Fig. 4 (where the assignments follow the numbering in Scheme 1). The peaks due to the 2 and 2' protons are well separated, but the peaks

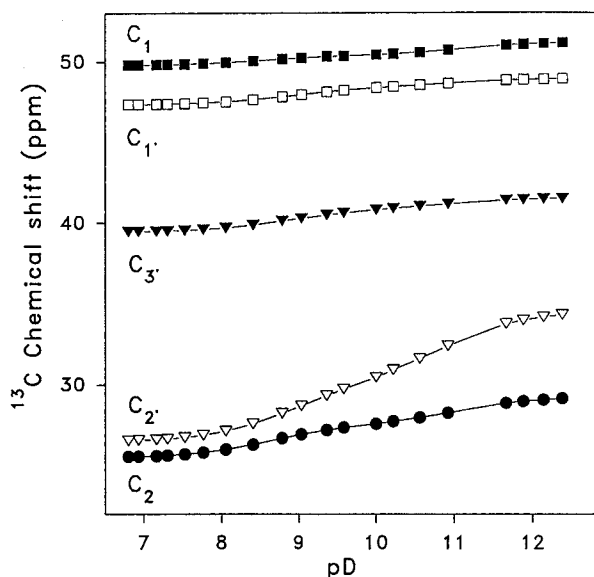


FIG. 5. pD dependence of <sup>13</sup>C NMR chemical shifts of the five non-equivalent methylene groups of spermine in D<sub>2</sub>O solution at 40°C.

due to H<sub>1</sub>, H<sub>1'</sub>, and H<sub>3</sub> exhibit a rather tangled pattern. This notwithstanding, the 2D heterocorrelation experiments have ensured that there can be no doubt as to the actual assignments.

As pD rises the carbon resonances move to lower field as illustrated in Fig. 5. Since there is no overlap between the <sup>13</sup>C peaks, the curves for each peak are well defined.

#### Basicity Constants and Chemical Shifts

The stepwise (deuteron) basicity constants of spermine, obtained by refining <sup>1</sup>H and <sup>13</sup>C chemical shifts simultaneously (200 data points), are given in Table 1. Both the values for the constants and their error limits were derived from the results of the refinement procedure by methods which have been described previously (25). It may be noted that the precision of the values obtained here from NMR data is comparable to the

TABLE 1  
Logarithms of the Stepwise Basicity Constants (pK<sub>A</sub>) of Spermine (L) Determined at 40°C in D<sub>2</sub>O

Equilibrium	log K
L + D <sup>+</sup> = DL <sup>+</sup>	11.20 (2) <sup>a</sup>
DL <sup>+</sup> + D <sup>+</sup> = D <sub>2</sub> L <sup>2+</sup>	10.30 (4)
D <sub>2</sub> L <sup>2+</sup> + D <sup>+</sup> = D <sub>3</sub> L <sup>3+</sup>	9.05 (4)
D <sub>3</sub> L <sup>3+</sup> + D <sup>+</sup> = D <sub>4</sub> L <sup>4+</sup>	8.18 (2)

<sup>a</sup> Values in parentheses are estimated standard deviations on the last significant digit.

TABLE 2  
Refined Values (ppm) of  $^1\text{H}$  and  $^{13}\text{C}$  NMR Chemical Shifts for the Various Species  
of Spermine ( $\text{D}_n\text{L}^{n+}$ ,  $n = 0-4$ ) at  $40^\circ\text{C}$  in  $\text{D}_2\text{O}^a$

Nucleus	L	$\text{DL}^+$	$\text{D}_2\text{L}^{2+}$	$\text{D}_3\text{L}^{3+}$	$\text{D}_4\text{L}^{4+}$
$\text{H}_2$	1.481 (4)	1.592 (9)	1.639 (6)	1.706 (8)	1.811 (2)
$\text{H}_{2'}$	1.603 (4)	1.717 (9)	1.841 (7)	1.989 (8)	2.118 (2)
$\text{H}_3$	2.628 (4)	2.73 (1)	2.904 (8)	3.050 (8)	3.133 (2)
$\text{H}_{3'}$	2.576 (4)	2.76 (1)	2.877 (7)	3.002 (9)	3.184 (2)
$\text{H}_1$	2.558 (4)	2.74 (1)	2.828 (7)	2.950 (9)	3.141 (2)
$\text{C}_2$	29.230 (5)	28.11 (4)	27.49 (1)	26.74 (4)	25.514 (3)
$\text{C}_{2'}$	34.491 (6)	32.39 (9)	30.09 (5)	28.02 (7)	26.497 (3)
$\text{C}_3$	41.534 (4)	41.15 (2)	40.77 (1)	40.07 (3)	39.452 (3)
$\text{C}_{3'}$	48.953 (4)	48.62 (1)	48.37 (1)	47.76 (2)	47.356 (3)
$\text{C}_1$	51.210 (4)	50.62 (2)	50.380 (7)	50.20 (1)	49.802 (3)

<sup>a</sup> Values in parentheses are estimated standard deviations on the last significant digit.

precision usually achieved when using potentiometric data. The calculated chemical shifts of the nonequivalent nuclei in each of the five forms of spermine ( $\text{D}_n\text{L}^{n+}$ ,  $n = 0-4$ ) are given in Table 2.

By using the  $^1\text{H}$  data only, the refinement converged to the following set of stepwise basicity constants:  $\log K_1 = 11.25 \pm 0.05$ ,  $\log K_2 = 10.42 \pm 0.08$ ,  $\log K_3 = 9.14 \pm 0.07$ , and  $\log K_4 = 8.23 \pm 0.04$ . On the other hand, the refinement of the  $^{13}\text{C}$  data gave the following results:  $\log K_1 = 11.20 \pm 0.03$ ,  $\log K_2 = 10.30 \pm 0.06$ ,  $\log K_3 = 9.05 \pm 0.05$ , and  $\log K_4 = 8.18 \pm 0.04$ . Comparison of these two sets of values with one another and with those reported in Table 1 (derived from the refinement of the whole set of experimental data) shows that (i) corresponding values of  $\log K$  in the various sets are the same within the estimated uncertainty intervals; (ii) the results achieved using the combined  $^{13}\text{C}/^1\text{H}$  experiments are very slightly more precise than those obtained from  $^1\text{H}$  or  $^{13}\text{C}$  data alone in the sense that all the errors on the calculated equilibrium constants are slightly smaller.

The chemical shifts of the individual species calculated in both cases do not show appreciable discrepancies with respect to those reported in Table 2, obtained from the simultaneous refinement of both  $^1\text{H}$  and  $^{13}\text{C}$  data points.

## DISCUSSION

Two-dimensional  $^1\text{H}/^n\text{X}$  heterocorrelation spectra (23) offer the advantage of resolving peaks that are overlapped in the one-dimensional proton NMR spectrum. Also, many chemical shifts may be determined in one spectrum, both for the proton and for the X nucleus. Nevertheless for stability constant work the chemical shifts must be determined with high precision, which implies that the spectra must have a high S/N ratio and a large dynamic range (high-resolution A/D conversion). In the case of the  $^{13}\text{C}$  or  $^{15}\text{N}$  dimension

this can only be achieved by long data-collection times, 10 h or more for a single spectrum, even when using labeled compounds and HMQC detection. To achieve adequate precision in a reasonable time the solutions used with NMR spectroscopy have to be at least an order of magnitude more concentrated than the solutions typically used in potentiometry.

In this work we have chosen to use HMQC  $^1\text{H}/^{13}\text{C}$  spectra obtained from spermine samples containing  $^{13}\text{C}$  at natural abundance to derive the proton chemical shifts. The  $^{13}\text{C}$  chemical shifts were obtained from 1D  $^{13}\text{C}\{^1\text{H}\}$  spectra. It was thus possible to sacrifice some of the digital resolution in the  $t_2$  dimension of the 2D spectra and so reduce their data collection time considerably. We believe that these are the best conditions that could have been used in order to achieve the required precision in the experimental data.

The values of the stepwise basicity constants of

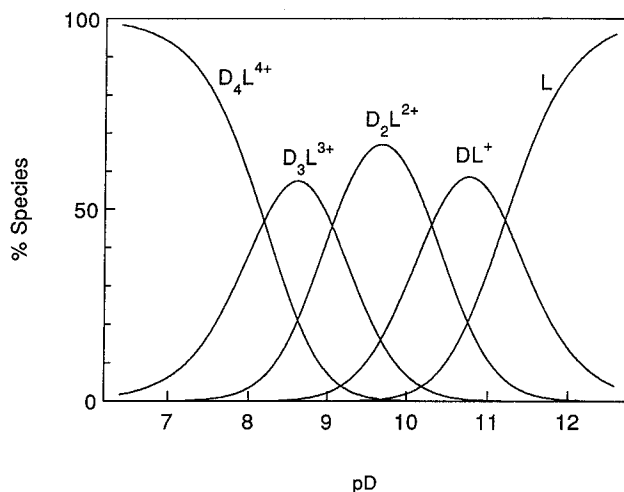


FIG. 6. Distribution diagram of the species formed in the system spermine (L)-deuterium ion in  $\text{D}_2\text{O}$  as a function of pD at  $40^\circ\text{C}$ .

TABLE 3  
Comparison between the Logarithms of the Stepwise Basicity Constants (pK<sub>A</sub>) of Spermine Previously Published and Those Obtained in the Present Work

Method	log K <sub>1</sub>	log K <sub>2</sub>	log K <sub>3</sub>	log K <sub>4</sub>	T, °C	Ionic strength	Ref.
Potentiometry	10.97	10.27	9.04	8.03	27	0.1 M NaCl	26
Potentiometry	10.86 (6) <sup>a</sup>	10.05 (1)	8.82 (1)	7.95 (1)	25	0.1 M NaCl	27
Potentiometry	10.80 (1)	10.02 (1)	8.85 (1)	7.86 (1)	25	0.1 M NaCl	28
Potentiometry	10.83	9.95	8.77	7.90	25	0.1 M KNO <sub>3</sub>	29
NMR <sup>15</sup> N	11.5 (2)	11.0 (2)	9.8 (1)	8.9 (5)	32		26
NMR <sup>13</sup> C	11.5 (5)	11.3 (1)	10.0 (1)	9.3 (1)	21		26
NMR <sup>1</sup> H/ <sup>13</sup> C	11.03 (7)	10.09 (8)	8.84 (8)	7.93 (8)	25		<sup>b</sup>

<sup>a</sup> Values in parentheses are estimated standard deviations on the last significant digit.

<sup>b</sup> This work: values are corrected for the different temperature and the deuterium isotope effect (see text).

spermine are as to be expected for an aliphatic linear tetramine in which the chains of methylene groups are relatively long, that is, the constants are relatively high and close to each other. The consequence of this is that the pH (or pD) zones in which the variously protonated species exist are extensively overlapped, as the species distribution diagram, Fig. 6, shows.

The results of previous determinations (26–29) of the basicity constants of spermine are given in Table 3. In order to compare our results with those reported previously we must take into account (i) the different solvent and therefore the different Lewis acid (D<sup>+</sup> in contrast to H<sup>+</sup>) and (ii) the different temperature.

The first correction is made by using the semiempirical formula for the deuterium isotope effect, due to Dagnall *et al.* (30),

$$\log K_{(\text{H}_2\text{O})} = \log K_{(\text{D}_2\text{O})} - 0.63 \pm 0.07$$

while the second correction can be done by applying Van't Hoff's equation in the form

$$\log K(T_1) = \log K(T_2) - \frac{\Delta H^0}{R \ln 10} \frac{T_2 - T_1}{T_1 T_2},$$

where  $T_1$  and  $T_2$  are 298 and 313 K, respectively. The values of the four protonation enthalpies for the successive stages are taken from the calorimetric work of Palmer and Powell (31) to be  $-55.0$ ,  $-52.0$ ,  $-52.0$ , and  $-47.9$  kJ mol<sup>-1</sup>. Our corrected values of log  $K$ ,<sup>6</sup> also shown in Table 3, are in satisfactory agreement with values previously determined by potentiometry, bearing in mind that the background medium was different. The discrepancies between our values and those obtained by <sup>13</sup>C and <sup>15</sup>N NMR spectroscopy measure-

ments in a previous study (26) are significantly larger. This is probably to be attributed to the lack of both activity coefficient and deuterium isotope effect corrections in that investigation. It may also be noted that the reagent concentration was much higher than in the present work (0.79 M compared to 0.050 M), which, in turn, is larger by an order of magnitude with respect to what was used in potentiometric studies. This is because the NMR signal is "weak" so that NMR studies are practicable only with relatively strong solutions.

We conclude that NMR spectroscopy offers a valid alternative to potentiometry for the study of biologically significant basicity constants. <sup>13</sup>C NMR is likely to be easier to use than <sup>1</sup>H NMR in the sense that signals are likely to be better separated. <sup>13</sup>C NMR may also give more precise pK<sub>A</sub> values, as was the case in the present study. On the other hand <sup>1</sup>H NMR may be difficult to use because of overlap of the signals due to chemically nonequivalent nuclei. Although the problem of overlap can be alleviated by working at higher detection frequencies, we have shown that the problem of overlap is not insuperable: it can be overcome by using a two-dimensional NMR technique. Combining both <sup>1</sup>H and <sup>13</sup>C NMR data in a simultaneous analysis of the experimental data appears to yield the most precise results. The computer program HYPNMR, which is based on well-tested mathematical and statistical algorithms, provides an effective tool with which to obtain a sound quantitative definition of the equilibrium model.

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<sup>6</sup> The errors associated with the corrected values were calculated from the errors of the constants and the estimated errors of the correction terms by means of the rules of propagation of variance.

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