Analyst

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2021, **146**, 2339

Solubility of folic acid and protonation of folate in NaCl at different concentrations, even in physiological solution

Emilio Bottari,[a] Antonietta D'Ambrosio,[a] Gaetano De Tommaso,[b] Maria Rosa Festa, *[a] Mauro Iuliano [b] and Martina Meschino[a]

Received 5th January 2021,
Accepted 8th February 2021

DOI: 10.1039/d1an00013f

rsc.li/analyst

The solubility of folic acid was determined at 25 °C in 1.00 mol dm^{-3} and in 0.15 mol dm^{-3} NaCl (physiological solution) spectrophotometrically by measuring the absorbance of saturated solution at different hydrogen ion concentrations. Five protonation constants of folate were determined both from the dependence of the solubility on the hydrogen ion concentration as well as from potentiometric titrations carried out in the presence of solid folic acid and in alkaline solution, in which folate is relatively soluble. Corresponding to the protonation constants, nuclear magnetic resonance and florescence spectra were also obtained at different hydrogen ion concentrations to determine the protonation positions in acid, neutral and alkaline solutions. An approach through circular dichroism was also applied to study the eventual polymerization of folate in alkaline solution.

Introduction

Folic acid or pteroil (mono)-glutamic acid or vitamin B9 (IUPAC name: (2*S*)-2-[4-{[(2-amino-4-hydroxypteridin-6-yl) methyl]amino}benzamido]pentanedioic acid belongs to the pteridines which are a family of heterocyclic compounds that are widespread in living organisms. Folic acid is necessary for the synthesis and methylation of DNA,[1] for homocysteine metabolism[2–5] and for several biochemical reactions, for example in the production of red globules in children and to prevent anemia and cancer.[6] These few examples show the interest focused on an investigation of the chemical behavior of folic acid.

From this point of view, it is necessary to start from the assumption that to transfer folic acid in the human body it is fundamental to know its solubility and its protolytic behavior.

The main literature investigating the protolytic properties and complex formation of a lot of potential ligands is collected by Powell,[7] but, in the case of folic acid, the information is incomplete and the data collected show high discrepancies; moreover, solubility is not considered.

From Fig. 1, where the structure of folic acid is reported, it can be deduced that folate (*i.e.* folic acid completely deprotonated) can be protonated in different positions.

Furthermore, from its structure, it is evident that folic acid solubility in water should be very limited and dependent on the hydrogen ion concentration.

Concerning solubility, we were able to find in the literature only the paper of Zhen *et al.*,[8] reporting a study carried out at different temperatures, but performed without evaluating the influence of the presence of an ionic medium, like NaCl. This means that the solubility of folic acid in physiological solution (*i.e.* 0.15 mol dm^{-3}) was not determined.

Powell,[7] in his collection on the protolytic behavior of folic acid, reported data obtained in different conditions, but only for one or three constants, not in agreement each other.

Only one dissociation constant was proposed by Albert[9] of pK_1 = 8.26, by Roos and Williams[10] of pK_1 = 7.98 and by Poe[11] of pK_1 = 8.38.

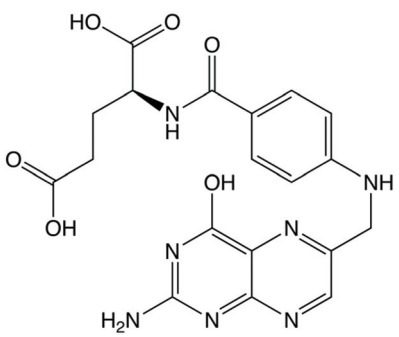


Fig. 1 Structure of folic acid.

[a]*Chemistry Department, Roma University "La Sapienza", P. le A. Moro 5, 00185 Rome, Italy. E-mail: mariarosa.festa@uniroma1.it*
[b]*Chemistry Sciences Department, Naples University "Federico II", Via Cintia – Monte Sant'angelo, 21 Naples, Italy*

Nayan and Dey[12] instead report three dissociable protons for folic acid but claim that only two can be obtained because it is insoluble at high acidity. However, from potentiometric data at 30 °C and in 0.01, 0.05, and 0.1 mol dm^{-3} KNO_3, they proposed three constants[12] of pK_1 = 8.30, pK_2 = 5.35 and pK_3 = 2.35 obtained in 0.1 mol dm^{-3} KNO_3. Zhen *et al.*,[8] by HPLC measurements at 25 °C without ionic strength, listed six protonation constants with pK_6 = 8.38.

More recently, Szakács and Noszál[13] explained their data, obtained from pressure-assisted capillary electrophoresis performed at 25 °C and 0.05 ionic strength (obtained with variable reagent compositions), by assuming the following constants: pK_1 = 2.38, pK_2 = 3.46, pK_3 = 4.98 and pK_4 = 8.08.

Fazary[14] studying the folic acid protonation constants in different aqueous solutions of dioxane explained the data assuming four constants.

Yoursef *et al.*[15] explained the potentiometric and conductometric data obtained at 25 °C in 0.2 mol dm^{-3} $NaNO_3$ by assuming three constants: pK_1 = 8.6, pK_2 = 5.1 and pK_3 = 3.63. On the other hand, Gottarelli *et al.*[16] explained their CD data determined for folate at unspecified hydrogen ion concentration by assuming the formation of polymeric species.

From an inspection of the above data, no agreement can be observed among the literature data either for solubility or for protolytic behavior of folic acid. However, data on folic acid solubility and protonation constants in 1.00 mol dm^{-3} and 0.15 mol dm^{-3} NaCl (physiological solution) are absent in the literature.

The aim of this work is the determination of the solubility of folic acid as well as the study of the protonation constants of folate at 25 °C and in NaCl as ionic medium at two different concentrations: 1.00 and 0.15 mol dm^{-3} (physiologic solution). To attribute the protonation constants to the relative groups of the folate ion, ^{1}H-NMR and fluorescence spectra, at different hydrogen ion concentration, were obtained. The study has been completed to verify the eventual presence of associated species of folate in alkaline solution.

To obtain the desired results, a wide range of reagent concentrations is investigated. In order to minimize the activity coefficients of the reagents, the constant ionic medium method, proposed by Biedermann and Sillèn,[17] is adopted. This method permits one to substitute up to 15% of the ionic medium with the reagent ions without appreciable variation of the activity coefficients, so that concentrations can substitute for activities in all calculations.

For simplicity, solubility, as total concentration of $\sum$HnFol, is indicated with S, whereas s indicates the solubility of folic acid in neutral form. The total concentration of the reagents is indicated by C_x (where x is the considered reagent), whereas c_x is the free concentration. In this way, c_H represents the hydrogen ion concentration at equilibrium, while C_H is the analytical excess of H^+. L indicates completely deprotonated folate.

The protonation constants of folate are defined as follows: $c_{HnL} = K_n c_H{}^n c_{Hn-1L}$, where n can assume different values, as explained below.

Experimental

Details of experimental apparatus

All measurements were carried out in a thermostat room at 25.0 °C and in a thermostat at 25.00 ± 0.05 °C. Solutions containing folic acid or folate were protected from light or manipulated in dark vessels.

Solutions containing an excess of solid folic acid prepared for the determination of solubility were transferred in suitable glass bottles with an emeried plug and sealed with parafilm. The bottles were shaken mechanically in a thermostat at 25.0 ± 0.1 °C for 12 h, the time necessary to reach equilibrium. The equilibrated solutions were filtered through Millipore filter type 0.22 mm MCE Membrane (REF GSWP02500).

EMF (electromotive force) measurements of galvanic cells were performed with a Metrohm model 654 pH meter equipped with a glass electrode (no. 6.0102.000) from the same firm. The reference electrode (RE) was prepared according to Brown[18] (RE = Ag, AgCl/1.00 or 0.15 mol dm^{-3} NaCl, saturated with AgCl/1.00 or 0.15 mol dm^{-3} NaCl). In the absence of solid, constant values of EMF were obtained within a few minutes after each addition. In the presence of solid, equilibrium was reached in 15–30 minutes after each addition. The measured values were reproducible within ±0.2 mV.

The glass electrode (GE) response agreed with that of a hydrogen electrode to $-\log c_H \leq 9$. At higher $-\log c_H$ value, the EMF values obtained from GE were corrected by comparing with the response provided by the hydrogen electrode assumed as correct.

The EMF measurements were carried out in solutions at real equilibrium because data obtained from solutions prepared with different procedures agreed well. The real equilibrium of EMF measurements in alkaline solutions was further verified by the coincidence of data obtained from direct and back titrations.

The absorption spectra of solutions at different concentrations of the reagents and at different wavelengths were recorded with both a UV-visible Prixma spectrophotometer and a UV–visible Varian Cary 5000 spectrophotometer (Palo Alto, CA, USA), from 200 to 600 nm (optical path of 0.1, 0.5, 1, 2, 5 or 10 cm) at 25.0 °C, under a constant flow of nitrogen. The cuvettes, before each use, were treated with a mixture of H_2SO_4/H_2O_2 and rinsed with plenty of double-distilled water. Most of the measurements were carried out at 270 nm. Solutions of folic acid showed a maximum absorbance at λ = 270 nm. It was checked that the absorbance was dependent on the concentration of folic acid, following the Lambert and Beer law in the range 0.006 ≤ concentration × 10^3 ≤ 0.100 mol dm^{-3}. The molar absorption coefficient, ε, was determined and a value of ε = 3000 (mol dm^{-3})$^{-1}$ cm^{-1} was obtained.

All the ^{1}H-NMR spectra were recorded at 25 °C with a Bruker Unity spectrometer operating at a proton frequency of 400 MHz and located at the Department of Chemistry Science in Naples (Italy).

Fluorescence spectra were obtained using a JASCO spectrofluorimeter (model FP-750), from 200 to 600 nm (optical path of 0.2 cm).

The far UV-CD spectra were recorded with a JASCO spectropolarimeter (model J-715, Tokyo, Japan), from 200 to 600 nm (optical path of 0.2 cm) at 25.0 °C, under a constant flow of nitrogen.

Nitrogen (99.995%) from a cylinder, further purified by passing through 10% NaOH, 10% H_2SO_4, H_2O and the selected ionic medium and bubbling through the solutions, was used to eliminate oxygen and CO_2.

Reagents

Folic acid, a Fluka product ≥97% (HPLC), showed by thermogravimetric analysis the presence of 8% of water. It was purified by dissolving the commercial product in a moderate excess of NaOH and by adding, after filtration, a HCl (freshly distilled) solution to precipitate at $C_H \approx 10^{-3}$ M. The precipitate was washed until the absence of chloride and dried at 85 °C. The obtained purified product, analyzed by polarography, was free of cations and electroactive species. It showed, by thermogravimetric analysis, 93% purity, corresponding to the presence of two water molecules.

Sodium chloride, hydrochloric acid, and NaOH stock solutions were prepared and analyzed as described previously.[19]

Procedure

To achieve the set goal described above, different strategies were followed.

(1) In the first strategy, the dependence of solubility on the hydrogen ion concentration was studied in the range $1 \leq -\log c_H \leq 5$.

Saturated solutions of folic acid in the selected ionic media and at different hydrogen ion concentrations are filtered through a Millipore filter. The obtained clear solution is analyzed. Its UV spectrum is used to determine the solubility, S, after suitable calibration. The free hydrogen ion concentration ($-\log c_H$) of the same solution is obtained from EMF measurements, using a GE, after suitable calibration.

(2) A second strategy is performed by measuring the EMF of the following cell:

$$\text{RE/solution } T_1\text{/G.E.} \qquad \text{(I)}$$

where RE and GE are reference and glass electrodes, respectively. Solution T_1 of cell (I) has the following general composition:

C_H mol dm^{-3} in H^+; (1.00 or 0.15 − C_H) mol dm^{-3} in Na^+; 1.00 or 0.15 mol dm^{-3} in Cl^-.

At 25 °C, in millivolt units and in constant ionic medium, the EMF of cell (I) is:

$$E_I = E_I^\circ + 59.16 \log c_H + Ej \qquad (1)$$

where E_I°, a constant, and Ej, the liquid junction potential, are determined in the absence of folic acid, so that $C_H = c_H$.

A first approach of this strategy was carried out in the presence of an excess of solid folic acid, so that in the second part of each titration, an excess of solid folic acid in the selected ionic medium is added to solution T_1. After reaching equilibrium, the solution is titrated by a stock NaOH solution in the selected ionic medium, so that $-\log c_H$ increased gradually, by keeping the presence of solid folic acid. The knowledge of solubility s, concentration of added NaOH and EMF measurement constitute the basis to obtain the folate protonation constant K_3, as explained in the section describing the results.

A second approach of the same strategy is carried out by measuring the EMF of cell (I) in the range $7.2 \leq -\log c_H \leq 10$, where folate is relatively soluble. In this case, in the second part of the titration, a solution of folate, at known concentration and with known C_H, in the selected ionic medium, is added. To the resulting solution, a stock NaOH solution in the selected ionic medium is added stepwise in order to increase gradually $-\log c_H$. Successively, to verify the real equilibrium of the investigated solutions, back titrations are carried out with addition of acid solution to decrease again $-\log c_H$ in a stepwise manner. The knowledge of C_H, $-\log c_H$ and the total concentration of folate, C_L, allows calculation of the protonation function as explained in the section describing the results.

The data obtained by means of the above strategies are used to obtain the solubility of folic acid and protonation constants of folate in both adopted ionic media, as explained in the next sections.

To obtain information regarding the sites of protonation of folate ion or of folic acid, ^{1}H-NMR and fluorescence measurements are performed in the range $0 \leq -\log c_H \leq 10$.

An inspection of Fig. 1 relative to completely deprotonated folate shows different possibilities for the proton allocation: both to the carboxylic groups, to the nitrogen amino acidic group and to the nitrogen of the pteridine rings.

Finally, an approach with CD is also performed to verify the eventual dimerization of folate in alkaline solution.

Results

The solubility and protonation constants of folate at 25 °C and in two different ionic media are obtained from UV spectra and EMF measurements, carried out at different $-\log c_H$ ranges. To obtain information on the position of the protonated groups, ^{1}H-NMR, UV and florescence measurements are performed.

At first, as the investigation of solubility and protonation constants is carried out with different methodologies, the obtained results are presented in different sections:

1. UV and EMF results to obtain the solubility s and K_3, K_4 and K_5.

2. Results of EMF titrations in the presence of solid folic acid.

3. EMF titration of folate clear solutions in the range $7.2 \leq -\log c_H \leq 10$.

Solubility s and K_3, K_4 and K_5

In the range $0.1 \leq C_H \leq 1 \times 10^{-5}$ mol dm^{-3}, solutions are prepared with the following composition:

(a) 50 solutions (each repeated three times) in ionic medium 1.00 mol dm^{-3} NaCl: C_H mol dm^{-3} in H^+; (1.00 − C_H) mol dm^{-3} in Na^+; 1.00 mol dm^{-3} in Cl^-, in the range $0.1 \leq C_H \leq 1 \times 10^{-5}$ mol dm^{-3} are prepared.

(b) 35 solutions (each repeated three times) in ionic medium 0.15 mol dm^{-3} NaCl: C_H mol dm^{-3} in H^+; (0.15 − C_H) mol dm^{-3} in Na^+; 0.15 mol dm^{-3} in Cl^-, in the range 0.025 ≤ C_H ≤ 1 × 10^{-5} mol dm^{-3} are prepared.

Both (a) and (b) solutions are mixed with an excess of solid folic acid and debated up to equilibrium.

Each equilibrated solution is filtered through 0.25 μm Millipore filter to obtain a clear solution, without Tyndall effect. At least three samples of the clear solution are analyzed spectrophotometrically and, by comparing the absorbance with the corresponding standard, the value of S is obtained. Spectra recorded after 24 h remained identical. Next, −log c_H is measured potentiometrically by means of GE.

The obtained experimental data are plotted in Fig. 2.

Fig. 2 shows a minimum of −log S and −log S', corresponding to the solubility s of folic acid and two branches, where −log S and −log S' increase by decreasing or increasing −log c_H, respectively.

On these bases, the material balance of S, in both constant ionic media, can be written as follows:

$$S = K_4 c_H s + s + K_3^{-1} c_H^{-1} s \quad (2)$$

In eqn (2), s = c_{H3L}, *i.e.* the concentration of H_3Fol, representing the minimum of solubility, so that, in the range 1 ≤ −log c_H ≤ 5, eqn (2) can be rewritten:

$$\log S = \log s + \log(K_4 c_H + 1 + K_3^{-1} c_H^{-1}) \quad (3)$$

Following the graphic method proposed by Sillèn,[20] eqn (3) can be normalized in the following way:

$$\Psi = \log(u + 1 + Ru^{-1}), \quad (4)$$

where log S − Ψ = log s; log u = log K_4 + log c_H; and log K_3 = log K_4 − log R.

The family of theoretical curves of eqn (4) is superimposed on the plot of experimental data and the two plots are moved parallel along both axes to obtain the best fit. In this position, applying the mathematical positions, relative to curve A, the values of log s, log K_4 and log K_3 are obtained for 1.00 mol dm^{-3} NaCl ionic medium. Similarly, from curve B, the values of log s', log K'_4 and log K'_3 are obtained for 0.15 mol dm^{-3} NaCl ionic medium.

The values of s, s', and first approximation of log K_3, log K'_3 and log K_4, log K'_4 obtained at 25 °C and in 1.00 and 0.15 mol dm^{-3} NaCl as ionic media, respectively, are collected in Table 1.

However, due to the uncertainty of the solubility determination, the values obtained for the protonation constants are considered as a first approximation and they will be refined in the section "Refinement".

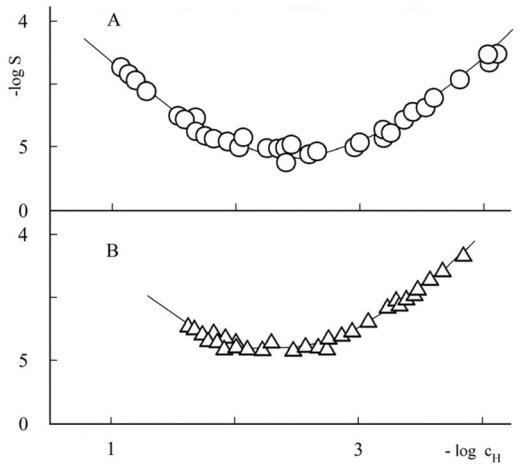


Fig. 2 The dependence of log S (A) and log S' (B) on −log c_H. Points in (A) and (B) are obtained in 1.00 and in 0.15 mol dm^{-3} NaCl respectively. The curves are the theoretical ones in the position of best fit.

Refinement of protonation constants of Table 1

To finish K_3, a second procedure is applied, where EMF measurements are carried out in the presence of solid folic acid. The experimental data C_{OH} and −log c_H are elaborated as follows.

The constant K_3 is defined according to the equation:

$$K_3 = c_{H3L}(c_{H2L} \cdot c_H)^{-1}$$

which, assuming c_{H3L} = s, becomes:

$$\log K_3 = \log s - \log(c_{H2L} \cdot c_H) \quad (5)$$

From the reaction occurring in the presence of the solid:

$$H_3L + OH^- = H_2L^- + H_2O$$

c_{H2L} can be calculated from the concentration of added NaOH, increased by the H_3L dissociated protons c_{H2L} = C_{OH} + c_H, and, remembering that s is a constant, it can be written as a constant K''_3 including s solubility constant:

$$\log K''_3 = \log K_3 - \log s = -\log(c_{H2L} \cdot c_H) \quad (6)$$

In Table 2, it can be observed that the values of −log (c_{H2L}·c_H) obtained for two different titrations are constants.

The log K_3 value can be calculated from the average value of log K''_3 and log s from Table 1:

$$\log K_3 = \log K''_3 + \log s = 8.35\text{–}5.2 = 3.15 \pm 0.05$$

As can be observed, log K_3 obtained by applying this procedure agrees well with that of Table 1 within ±0.05. The

Table 1 The values of −log s, −log s', log K_3 and log K_4 obtained as described in the text. M stands for mol dm^{-3}

	1.00 M NaCl	0.15 M NaCl
−log s	5.20 ± 0.05	—
−log s'	—	5.05 ± 0.05
log K_3	3.10 ± 0.08	—
log K'_3	—	3.00 ± 0.08
log K_4	1.8 ± 0.1	—
log K'_4	—	1.7 ± 0.1

The limits of error were estimated from the maximum shift possible for the calculated curve and experimental points for which agreement was still acceptable.

Table 2 Two examples of titrations with the presence of solid folic acid in 1.00 mol dm^{-3} NaCl. The constant values $-\log(c_{H_2L} \cdot c_H)$

	Titration A			Titration B	
$-\log(C_{OH} + c_H)$	$-\log c_H$	$\log K''_3$	$-\log(C_{OH} + c_H)$	$-\log c_H$	$\log K''_3$
3.14	5.24	8.38	3.19	5.15	8.34
3.11	5.26	8.37	3.14	5.22	8.36
3.07	5.30	8.37	3.11	5.26	8.37
3.03	5.33	8.36	3.07	5.29	8.36
3.01	5.35	8.36	3.04	5.31	8.35
2.99	5.37	8.36	3.01	5.34	8.35
2.95	5.39	8.34	2.98	5.37	8.34
2.93	5.41	8.34	2.96	5.39	8.34
2.91	5.43	8.34	2.94	5.41	8.34

Average value of $\log K''_3 = 8.35 \pm 0.02$.

obtained average value provides the refined value of $\log K_3 = 3.12 \pm 0.05$.

A similar procedure is carried out for data obtained using 0.15 mol dm^{-3} NaCl.

To refine $\log K_4$, the points obtained in the increasing curve of Fig. 2 (acid branches) were elaborated, to test the eventual presence of a further protonation.

In this way, the data obtained using 1.00 mol dm^{-3} NaCl, in the range $-\log c_H \leq 2$, can be expressed using the following equation:

$$S = s + sK_4c_H + sK_4K_5c_H^2 \quad (7)$$

elaborated as follows:

$$\rho = \log\{[(S/s) - 1]c_H^{-1}\} = \log K_4 + \log(1 + \log K_5c_H) \quad (8)$$

Eqn (8) can be normalized in the form: $P = \log(1 + u)$, where $\rho - P = \log K_4$ and $u = K_5c_H$.

According to Sillèn,[20] the normalized curve is superimposed on the experimental points and the two plots are moved parallel to both axes to reach the best fit. In this position, by taking into account the mathematical positions, the refined values of $\log K_4$ and $\log K_5$ are obtained. A similar elaboration is carried out for the data of 0.15 mol dm^{-3} NaCl, where eqn (8) is similarly written: $\rho' = \log\{[(S'/s') - 1]c_H^{-1}\} = \log K'_4 + \log(1 + \log K'_5c_H)$.

From Fig. 3, where ρ (Fig. 3A) and ρ' (Fig. 3B) are plotted *versus* $-\log c_H$, the agreement between the experimental data and normalized curve can be observed.

In Table 3 the refined values of the constants are reported for both ionic media.

EMF titrations of folate in alkaline solution

The first and second protonation constants of folate, respectively $\log K_1$, $\log K_2$ and $\log K'_1$, $\log K'_2$, are obtained from EMF measurements carried out in the range $7.2 \leq -\log c_H \leq 10$, where it is possible to obtain a suitable concentration of folate, in both adopted ionic media.

After the first part of the procedure carried out with the galvanic cell (I), a solution of the following composition is added:

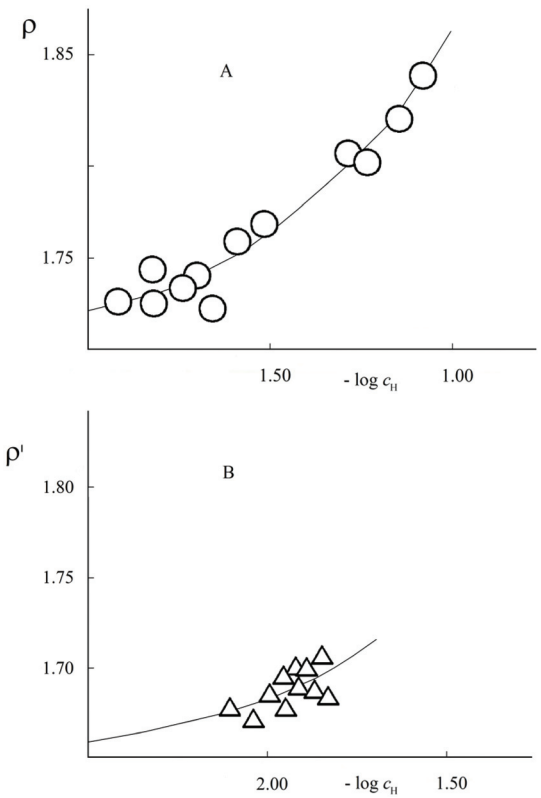


Fig. 3 The dependence of ρ and ρ' on $-\log c_H$. Points obtained for (A) 1.00 mol dm^{-3} NaCl and (B) 0.15 mol dm^{-3} NaCl. Curves are the normalized ones in the position of best fit.

Table 3 The refined values of $\log K_3$ and $\log K_4$ obtained as described in the text. M stands for mol dm^{-3}

	1.00 M NaCl	0.15 M NaCl
$\log K_3$	3.12 ± 0.08	—
$\log K'_3$	—	3.10 ± 0.08
$\log K_4$	1.70 ± 0.05	—
$\log K'_4$	—	1.65 ± 0.05
$\log K_5$	0.7 ± 0.1	—
$\log K'_5$	—	0.9 ± 0.15

The limits of error were estimated from the maximum shift possible for the calculated curves and experimental points for which agreement was still acceptable.

C_L mol dm^{-3} in folate; C_H mol dm^{-3} in H^+, ([1.00 or 0.15] – C_H) mol dm^{-3} in Na^+; [(1.00 or 0.15)–(3 – C_H) C_L] mol dm^{-3} in Cl^-.

When equilibrium is reached, an alkaline solution, C_{OH}, in the selected ionic medium is added to increase gradually $-\log c_H$ until $-\log c_H \leq 10$. To verify the real equilibrium of the investigated solutions, back titrations are performed adding an acid solution, C_H, in the selected ionic media, until about $-\log c_H \sim 8$.

It seems reasonable to assume that in this range only the species HL and H_2L can exist.

The constants $K_1 = c_{HL}(c_L \cdot c_H)^{-1}$ and $K_1K_2 = c_{H2L}\ (c_L \cdot c_H^{\ 2})^{-1}$ are obtained from the elaboration of the material balances of the analytical excess of hydrogen ions, C_H and of the total concentration of folate C_L.

By taking into account the mass action law and in the constant ionic medium, it is written:

$$C_H = c_H + c_H c_L K_1 + 2c_H^{\ 2} c_L K_1 K_2 \quad (9)$$

and

$$C_L = c_L + c_H c_L K_1 + c_H^{\ 2} c_L K_1 K_2 \quad (10)$$

Rearranging eqn. (7) and (8), the protonation function of folate, $\dot{p}$, as number of protons per folate can be calculated:

$$\dot{p} = (C_H - c_H) C_L^{\ -1} = (c_H K_1 + 2c_H^{\ 2} K_1 K_2)\ (1 + c_H K_1 + c_H^{\ 2} K_1 K_2)^{-1} \quad (11)$$

The protonation functions of folate $\dot{p}$ and $\dot{p}'$ obtained in the investigated ionic media are plotted in Fig. 4A and B, respectively.

It can be observed that points obtained at folate concentration $C_L = 4$ and 2×10^{-3} mol dm^{-3} in 1.00 mol dm^{-3} NaCl (Fig. 4A) and 2 and 1×10^{-3} mol dm^{-3} in 0.15 mol dm^{-3} NaCl (Fig. 4B) fall, respectively, on the same curve. The presence of polymeric species is excluded in the investigated folate concentration range and in the investigated hydrogen ion concentration range.

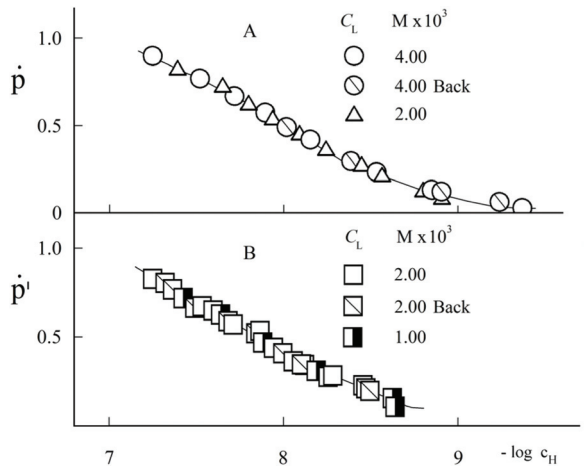


Fig. 4 Protonation function (A) $\dot{p}$ (1.00 mol dm^{-3} NaCl) and (B) $\dot{p}'$ (0.15 mol dm^{-3} NaCl) *versus* $-\log c_H$. Curves are the normalized ones in the position of best fit.

The values of the constants $\log K_1$ and $\log K_2$ (1.00 mol dm^{-3} NaCl) and $\log K'_1$ and $\log K'_2$ (0.15 mol dm^{-3} NaCl) are obtained whether applying the graphic method proposed by Sillèn[18] or by elaborating the primary data using the PC program HYPERQUAD.[21]

According to the graphic method, eqn (11) can be normalized with the following:

$$P = (Ru + 2u^2)\ (1 + Ru + u^2)^{-1} \quad (12)$$

where $\log u = \log c_H + \frac{1}{2}\log K_1K_2$ and $\log K_1 = \log R + \frac{1}{2}\log K_1K_2$.

By superimposing the family of normalized curves (eqn (12)) on the experimental points and shifting the two plots parallel to the abscissa, the best fit is obtained. At this point, and on the basis of the above positions, the values of the constants are found.

Next, the experimental data, elaborated using the PC program HYPERQUAD, give values of $\log K_1$, $\log K_1K_2$, $\log K'_1$ and $\log K'_1K'_2$ in good agreement with those obtained graphically.

In Table 4 the values of the constants obtained with both methods are presented.

Protonation positions of folate and absence of polymeric species

An inspection of Fig. 1 shows different possible positions of protonation of folate: one "phenol" group, many nitrogen atoms in different positions and two carboxylic groups.

To try to attribute the protonation sites of folate corresponding to the protonation constants obtained from solubility and EMF measurements, ^{1}H NMR and fluorescence spectra were obtained at different $-\log c_H$ values. In Table 5 the proton chemical shifts of aromatic and pteridinic rings as a function of pH are reported.

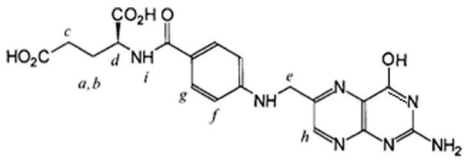
The chemical shift of H(h) undergoes an increase with pH up to 8.0. This is compatible with a dissociation involving a nitrogen of the pteridine ring (K'_5). Subsequently, for pH values greater than 8.0, a decrease in the chemical shift is observed. This behavior is in agreement with the ionization of phenol group of pteridinic group (K'_1). The H(f) signal

Table 4 Values of $\log K_1$, $\log K_2$, $\log K'_1$ and $\log K'_2$ at 25 °C and in the respective ionic media. M = mol dm^{-3}

	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl
	1.00 M Graphical	1.00 M PC	0.15 M Graphical	0.15 M PC	1.00 M Proposed values	0.15 M
$\log K_1$	8.02 ± 0.05	8.08 ± 0.02	—	—	8.05 ± 0.03	—
$\log K'_1$	—	—	7.85 ± 0.05	7.95 ± 0.03	—	7.90 ± 0.05
$\log K_2$	6.00 ± 0.05	6.02 ± 0.05	—	—	6.01± 0.03	—
$\log K'_2$	—	—	5.76 ± 0.07	5.80 ± 0.05	—	5.78 ± 0.05

The limits of error were estimated from the maximum shift possible for the calculated curve and experimental points for which agreement was still acceptable.

Table 5 Proton chemical shifts belonging to aromatic and pteridinic rings



pH	H(*a*), H(*b*), ppm	H(*c*), ppm	H(*h*), ppm	H(*g*), ppm	H(*f*), ppm
0.0	1.91, 2.06	2.32	8.62	7.60	7.20
1.0	2.12, 2.31	2.53	8.65	7.62	6.90
8.0	2.00, 2.09	2.31	8.75	7.63	6.83
10.0	1.98, 2.04	2.29	8.65	7.65	6.81
12.0	1.97, 2.03	2.29	8.61	7.63	6.82

decreases to pH 8. This can be explained by the protonation of nitrogen bound to the aromatic ring (K'_2). The lower value of the protonation constant with respect to that of the phenol group can be attributed to the influence of the pteridinic ring. Finally, H(*g*) does not undergo significant variations with pH; therefore, the dissociation on nitrogen of the glutamic residue is excluded. The chemical shifts relating to the aliphatic hydrogens of the glutamic acid residue show variations for values of pH < 8. This behavior is in agreement with the deprotonation of the carboxyl groups (K'_3 and K'_4 constants).

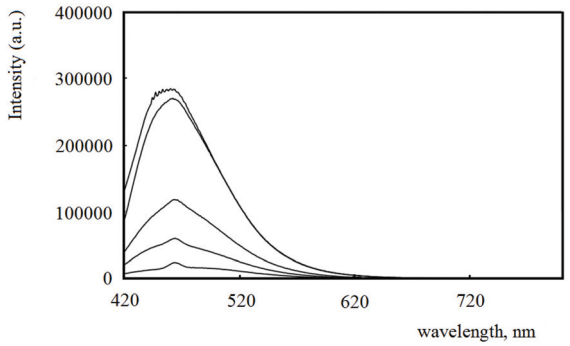


Fig. 5 Fluorescence measurements for 5.0×10^{-6} mol dm^{-3} folic acid (optical path = 1.0 cm) at different pH: 1.0, 3.0, 6.0, 8.0, 9.5 (curves from bottom to top).

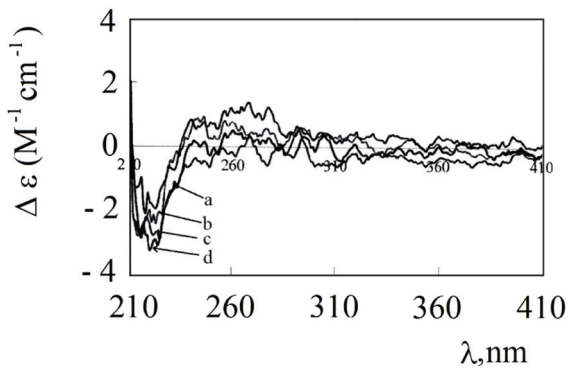


Fig. 6 Measurements on 3.04×10^{-5} mol dm^{-3} folic acid in 1.0 mol dm^{-3} NaCl. CD spectra at different pH values (a: 4.55, b: 8.13, c: 9.77, d: 10.53) with optical path of 1.0 cm.

Fluorescence spectra at different pH values are shown in Fig. 5. An increase in fluorescence up to pH 8 is observed, connected to an increase in the electron density on the pteridine ring, influenced by the acid dissociation of the groups.

The increase of fluorescence shown in Fig. 5 can be explained by comparison with aniline, which is not fluorescent, but its dissociation provokes an increase of fluorescence.

Furthermore, CD UV-visible spectra at different pH values confirm the absence of polymeric species (Fig. 6).

Conclusions

Proposed folic acid solubility and protonation constants of folate, determined in two different ionic media, even in physiological solution, are collected in Tables 2–4.

It is difficult to compare our results with the literature data, because of the different conditions and approaches used. In any case, most authors do not determine all five constants and solubility in physiological ionic medium. Only Zhen *et al.*[8] report the folic acid solubility at 25 °C ($-\log s$ = 5.8), but in unknown ionic medium.

Concerning the constants, the values of $\log K_1$ can be considered in agreement with literature data by taking in account the different experimental conditions.

Gottarelli *et al.*[16] claim that polymeric species are observed by CD measurements on folic acid solutions, but the number of protons bound to folate and pH of the studied solutions are not specified. However, the absence of polymeric species of folate is supported by ^{1}H-NMR and CD spectra of Fig. 6 and from the protonation functions of folate plotted in Fig. 4A and B.

Solubility and potentiometric data are not able to give structural information relative to the position of the protonated groups, but obtained ^{1}H-NMR, fluorescence and CD spectra and literature data can suggest some hypotheses.

To propose a correlation among protonation sites in folate and the attribution of K_n values, the structure of folic acid can be considered as formed of a pteridinic ring (formed by pyrimidine and pyrazine) + aniline + glutamic acid.

From literature data, 4-methylaniline has pK = 5.28,[22] the pK values of glutamic acid attributed to carboxylic groups are pK_2 = 4.17 and pK_3 = 2.31,[23] whereas, according to Malin *et al.*, pyrazine has pK = 0.65.[23]

These values could agree with $\log K_2$ = 6 which can be attributed to nitrogen of the benzenic ring and, taking into account the influence of the pteridinic ring, the above values could agree respectively with $\log K_3$ = 3.1 and $\log K_4$ = 1.8 attributed to the carboxylic groups. It seems reasonable to assume that the last protonation involves the pyrazine nitrogen, comparing the values of $\log K_5$ = 0.8 and the constant of pyrazine pK = 0.65.[24]

This hypothesis can also explain the low solubility of H_3Fol, which should have a positive (benzenic nitrogen) and a negative (one of the carboxylic groups) charge. Solubility increases at lower pH for the protonation of pyrazine ring nitrogen and of the second carboxylic group.

The folic acid solubility dependence on pH seems similar to that of ethylenediaminetetraacetic acid (EDTA). It is known that EDTA in the form H_4Y is slightly soluble, even if it has two positive charges on both nitrogen atoms and two negative charges on the carboxylic groups. Anderegg[25] reports the values of $\log K_5$ and $\log K_6$ relative to the protonation of two carboxylic groups, increasing its solubility at high acidity.

The above formulated hypothesis is supported by the results of Tyagi and Penzkofer,[26] who, from fluorescence spectroscopic measurements, did not perform a protolytic investigation, but explained their fluorescence data assuming the presence of a form with two protons in acid solution, a neutral form and an anionic form in alkaline solution.

In this way, the neutral form, corresponding to the minimum solubility of folic acid, can be H_3Fol, while the alkaline form corresponds to an absence of protonation on folate and the deprotonation of "phenol" OH.

In conclusion, the following scheme could represent a hypothesis for the protonation of folate:

Basic form, soluble	Neutral form, low solubility	Acid form, increased solubility
OH "phenol" Nitrogen and carboxyl	Benzenic nitrogen and one carboxyl	The second carboxyl and pyrazine nitrogen
Deprotonated $Fol^{3-} = L$	Protonated $H_3Fol = H_3L$	Protonated $H_5Fol^{+2} = H_5L$

The basic and acid forms are more soluble then H_3L, because they have three or two charges, while the neutral form is slightly soluble even if it has one negative (carboxylic groups) and one positive (nitrogen pyrazine) charge.

To better clarify the proposed folate protonation sequence, Fig. 7 shows the relative positions correlated to the protonation constants.

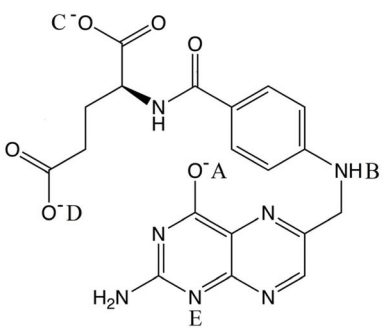


Fig. 7 Protonation sites to folate: A (first protonation, "phenol" group, K1), B (second protonation, benzenic ring, K2), C (third protonation, one carboxylic group, K3), D (fourth protonation, the second carboxylic group, K4), E (fifth protonation pyrazine nitrogen, K5).

Finally, the increased solubility of folate ($S \sim 0.01$ mol dm^{-3}) in alkaline solution ($\approx$0.03 mol dm^{-3} excess NaOH) could allow the investigation of the complex formation of folate with several cations, even under physiological conditions, which to date has been seldom studied.[7]

Author contributions

Author E. Bottari: planning of the work and program of all measurements, control of references. Author A. D'Ambrosio: equilibria solid – solution in different ionic media, UV–visible spectra. Author G. De Tommaso: NMR measurements. Author M.R. Festa: general conducting of the work, collection of EMF and NMR data, final results. Author M. Iuliano: CD, florescence spectra and related interpretations. Author M. Meschino: performances of EMF measurements.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1 V. Papper, O. Pokholenko, Y. Wu, Y. Zhou, P. Jianfeng, T. W. J. Steele and R. S. Marks, Novel photochrome aptamer switch assay (PHASA) for adaptive binding to aptamers, *J. Fluoresc.*, 2014, **24**, 1581–1591.
2 G. Litwack, Vitamins and Hormones: Folic acid and Folate, in *Vitamins and Hormones Series*, Academic Press, New York, 2008, Vol. 79.
3 S. Brustolin, R. Giugliani and T. M. Félix, *Braz. J. Med. Biol. Res.*, 2010, **43**, 1.
4 M. Lucock, Folic acid: Nutricional Biochemistry, Molecular Biology, and Role in Desease Processes, *Mol. Genet. Metab*, 2000, **71**, 121.
5 W. Herrmann, Significance of hyperhomocysteinemia, *Clin Lab*, 2006, **52**, 367.
6 R. B. Rucker, J. W. Suttie, D. B. Mc Cotmick and L. J. Machlin, *Handbook of Vitamins*, Marcel Dekker Inc., U. S.A, 2001.
7 K. J. Powell, *The IUPAC Stability Constants database*, Academic Software, in cooperation with Royal Society of Chemistry, 2000.
8 Z. Wu, X. Li, C. Hou and Yu Qian, *J. Chem. Eng. Data*, 2010, **55**, 3958.
9 A. Albert, *Biochem. J.*, 1953, **54**, 646.
10 J. Roos and D. Williams, *J. Inorg. Nucl. Chem.*, 1977, **39**, 367.

11 M. Poe, *J. Biol. Chem.*, 1973, **248**, 7025.
12 R. Nayan and A. K. Dey, *Z. Naturforsch.*, 1970, **25**, 1453.
13 Z. Szakács and B. Noszál, *Electrophoresis*, 2006, **27**, 3399.
14 A. E. Fazary, *J. Chem. Eng. Data*, 2013, **58**, 2219.
15 W. M. Yoursef, K. Alenezi, A. H. Naggar, T. M. Hassan, S. Z. Bortata and O. A. Farghaly, *Int. J. Electrochem. Sci.*, 2017, **12**, 1146.
16 G. Gottarelli, E. Mezzina, G. P. Spada, F. Carsughi, G. Di Nicola, P. Mariani, A. Sabatucci and S. Bonazzi, *Helv. Chim. Acta*, 1996, **79**, 220.
17 G. Biedermann and L. G. Sillèn, *Ark. Kemi*, 1953, **5**, 425.
18 A. S. Brown, *J. Am. Chem. Soc.*, 1934, **56**, 646.
19 E. Bottari, M. R. Festa and L. Gentile, *J. Chem. Eng. Data*, 2011, **56**, 4751.
20 L. G. Sillèn, *Acta Chem. Scand.*, 1956, **10**, 186.
21 P. Gans, A. Sabadini and A. Vacca, *Talanta*, 1996, **43**, 1739.
22 L. Fossey and F. F. Cantwell, *Anal. Chem.*, 1985, **57**, 922.
23 E. Bottari, M. R. Festa and R. Jasionowska, *Chem. Scr.*, 1989, **29**, 85.
24 J. M. Malin and R. E. Shepherd, *J. Inorg. Nucl. Chem.*, 1972, **34**, 3203.
25 G. Anderegg, IUPAC, Critical survey of stability constants of EDTA complexes. Chemical Data Series No. 14 (1978).
26 A. Tyagi and A. Penzkofer, *Chem. Phys.*, 2010, **367**, 83.