Spectrophotometric Determination of Acidity Constants of Group B Vitamins in Different Ionic Strengths at 25±0.1 °C

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ABSTRACT. The apparent acid dissociation constants of four water-soluble vitamins, folic acid (vitamin B₉ or B₀), thiamine (vitamin B₁), riboflavin (vitamin B₂) and pyridoxal (vitamin B₆) were determined spectrophotometrically in different ionic strengths at 25 °C±0.1. An accurate and sophisticated method based on chemometrical concepts was applied in order to determine acidity constants at different ionic strengths. For this purpose, spectrophotometric data were used. The spectra were recorded in the range 225-500 nm at different ionic strengths. The acidity constant was calculated by means of computer fitting of the pH-absorbance data with appropriate mass balance equations according to monoprotic, diprotic or triprotic acids. The computer program DATAN was used to extract the desired information from the spectral data. The outputs of the fitting processes were acidity constants, spectral profiles of pure forms, distribution diagrams, and other factor analysis data. The effect of ionic strength on the acidity constants is discussed.

Keywords: Vitamin B Group, Acidity Constants, DATAN, Distribution Diagrams, pKₐ Values, Ionic Strength
INTRODUCTION

Acid dissociation constants are useful physico-chemical parameters describing the extent of ionization of functional groups with respect to pH. These parameters are important in research areas such as pharmaceutical drug development, where knowledge of the ionization state of a particular functional group often is crucial in order to understand its pharmacokinetics and pharmacodynamics. 1

B vitamins form a wide organic-compound group that cannot be synthesized by humans. Since these compounds are necessary for the tropism of human beings, they need to be part of our daily intake. Vitamin B1 (Thiamine), vitamin B2 (Riboflavin), vitamin B6 (Pyridoxal) and vitamin B12 (folic acid) occur in living cells as essential substances for growth. Any deficiency of these in human nutrition will have adverse effects. Therefore, B vitamins are often supplemented to the diet as composite vitamin B tablets. 2

Folic acid, also known as vitamin B12, is also referred to as folacin or folate, while its chemical name is pteroylglutamic acid. 3 Folic acid is required for DNA synthesis and cell growth to take place, and it is important for the formation of red blood cells, for energy production, for the formation of amino acids. It is also required in protein metabolism and in treating folic acid anemia. 4 Deficiency of folic acid is a common nutritional problem of worldwide importance. Long-term deficiency may result in anemia and later in osteoporosis, as well as cancer of the bowel and cervix. 5

Thiamine, also called vitamin B1, assists in a great many bodily functions. It plays a key metabolic role in the cellular production of energy, primarily in glucose metabolism. 6-7 Thiamine is unstable at high pHs, 8-9 and in food it degrades by cooking of under mildly basic conditions. 9 Deficiency of vitamin B1 leads to beriberi, 10 a nutritional disease characterized by degenerative changes in the nervous system, including multiple peripheral neuritis; accompanied by generalized edema and serous effusions, with a tendency to cardiac hypertrophy and dilation. 11

Riboflavin (vitamin B2) is the prosthetic group of flavin enzymes, which are of great importance in general metabolism and particularly in metabolism of proteins. Vitamin B2 is required for the health of the mucous membranes in the digestive tract and aidin the absorption of iron and vitamin B6. 2 It is needed especially during periods of rapid growth, but also when protein intake is high. It is highly beneficial to the skin, hair and nails. 3 A shortage of this vitamin may manifest itself as cracks and sores at the corners of the mouth, eye disorders, inflammation of the mouth and tongue, and skin lesions. 6

Vitamin B6 (pyridoxal) is a common cofactor in enzymes that support amino acids metabolism. It controls the absorption, metabolism and conversion of amino acids into neurotransmitters, antibodies, digestive enzymes, muscles and tissues in the body. 12 Deficiency of vitamin B6 may make patients prone to nerve or blood disorders, and may cause convulsions in children. 13

In the present work, the protonation constants of vitamin B group in different concentrations of KNO3 were determined spectrophotometrically at 25 °C. The investigation of ionic strength influence on the acid-base behavior of simple organic compounds may contribute to a better understanding of the properties of complex substances such as natural organic matters.

THEORY

The theory and application of the physical constraints method have been thoroughly discussed by Kubista et al. 14-22 In the following, the general principal will be outlined briefly.

Spectra of each vitamin at different pH values and at different ionic strengths are digitized and arranged in a data matrix A, which is decomposed into an orthonormal basis set by NIPALS or any equivalent method: 

\[
A = TP' + E = TP' + \sum p_i' \approx \sum i \sum p_i' \tag{1}
\]

The orthogonal target vectors \( t_i \), and orthonormal projection vectors \( p_i' \) are mathematical constructs that cannot be directly related to component spectra.
and concentrations. The symbol $r$ represents the number of independent spectroscopic components, which corresponds to the number of light-absorbing chemical species. It is determined by visual inspection of the $t$ and $p'$ vectors, or by applying statistical methods, such as the $\chi^2$-test. $E$ is an error matrix.

By assuming linear responses, the spectra in matrix $A$ are linear combinations of the concentrations, $C$, and spectral responses, $V$, of the chemical components.

$$A = CV + E$$

If the spectral profiles of the components are known, the concentration of each component can be calculated easily, for example, by least squares minimization. If standards are not available, it is generally assumed that the components' spectral responses cannot be separated, which precludes their identification. This is due to ambiguity in determining the rotation matrix, $R$, in the following equations; from Eqs. (1) and (2) follows that there is a square matrix $R(r \times r)$ that satisfies

$$T = CR$$

$$P' = R^{-1}V$$

Since $A = CV + E$, $C = (R^{-1})^T(CR^T) = TR^{-1}$. If $R$ can be determined, the spectral responses $V$ and concentrations $C$ of the components can be calculated from the target $T$ and projection $P'$ matrices:

$$C = TR^{-1}$$

$$V = RP'$$

The thermodynamic expressions that describes the concentration of the components is the main constraint used to determine $R$, from which thermodynamic parameters, acidity constants, and components spectral responses and concentrations of all species are calculated. So, according to these facts, the strategy for determining the rotation matrix $R$ is as follows. The concentrations of the chemical species are calculated, using equilibrium expressions and various trial values of the acidity constants, and fitted to the calculated target vectors according to Eq. (3). The accuracy of this fitting depends crucially on the trial values of the acidity constants, and the best fit determines their values and the elements of matrix $R$.

**EXPERIMENTAL**

**Reagents**

All the chemicals were of analytical reagent grade. Four given solutions (as working solutions, folic acid, thiamine, riboflavin and pyridoxal) were prepared in 100 mL volumetric flasks by dissolving 2.00, 2.00, 2.50 and 3.00 mg of each compound in water, respectively, and the solutions were used for pH titration. Titration of each vitamin was carried out at five fixed ionic strengths with NaOH solution. The starting points of pH titrations were pH 2.00, which were set using concentrated solutions of HCl and NaOH. The concentrated NaOH solution was also used for titrations, to avoid dilution of the working solutions.

To maintain the ionic strength at a desired value a high concentrated solution of KNO$_3$ was used for all titrations. All experiments were carried out at 25 °C. For all of the above-mentioned solutions, doubly distilled water used throughout and the solutions were kept in brown volumetric flasks to protect from light.

**Apparatus and software**

The pH values were measured by model 300 HANA pH-meter using a combined glass electrode. The glass electrode was calibrated on the basis of the proton concentration at each constant ionic strength according to the procedure described elsewhere. The calibration was repeated at each ionic strength. The calibration procedure was as recommended by the IUPAC for glass electrodes.

Absorption spectra were measured on an Agilent 8453 UV-Visible Diode-Array spectrophotometer using the Agilent UV-Visible ChemStation Software for data acquisition. A cell of 10 mm optical path was used for all measurements.

The data were preprocessed using MATLAB software, version 6.5 (Mathworks, Natick, USA) and the deconvolution of the obtained data matrix.
was performed using DATAN version 3.1.

RESULTS AND DISCUSSION

The electronic absorption spectra of group B vitamins were recorded in different ionic strengths and at various pH values. Sample spectra of each vitamin at different pH values and at five ionic strengths are shown in Figs. 1-4. The principal component analysis of all absorption data matrices obtained at various pH values shows the different number of factors for each vitamin. The number of factors could be attributed to the number of dissociation equilibria of each vitamin. The pKₐ values of group B vitamins were investigated spectrophotometrically at 25 °C in five different ionic strengths. The acidity constants of these vitamins in several ionic strengths were evaluated by the DATAN program using the corresponding spectral absorption-pH data. From inspection of the experimental spectra, it is hard to guess even the number of protolytic species involved. The number of calculated projection vectors with clear spectral features, as compared to noise, shows the presence of four, three, two and three spectroscopically distinguishable components for folic acid, thiamine, riboflavin and pyridoxal, respectively.

The output of DATAN comprises pKₐ values, the number of principal components, projection vectors(loadings), diagrams of the concentration distribution, and the spectrum of each assumed species. The obtained pKₐ values are listed in Table 1. The pKₐ values correspond to the pH dependent variation of absorption spectra in all ionic strengths. One of the most important outputs of the program is the calculated spectrum of different forms of each vitamin at each ionic strength. The most important features of the distribution diagrams are the pH limits of the evolving and disappearing of components. Some typical distribution diagrams are shown in Fig. 5.

Consider the cationic form of pyridoxal (Scheme 1), which has two dissociable protons bound to distinctly different sites, the phenolic oxygen and the ring nitrogen.

Fig. 1. Absorption spectra of Folic Acid, in different concentrations of KNO₃: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.
Fig. 2. Absorption spectra of Thiamine in different concentrations of KNO₃: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.

Fig. 3. Absorption spectra of Riboflavin in different concentrations of KNO₃: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.
Either of two protons could dissociate first as the pH is raised. However, the two microscopic dissociation constants are distinctly different. At 25 °C in the neutral (monoprotonated) form 80% of the molecules carry a proton on the N, while the remaining 20% are protonated on the less basic O.[28] The obtained pK₁ and pK₂ by computer fitting of spectral data are listed in Table 1. The previously reported values of pK₁ and pK₂ at pure water are 4.64 and 8.89, respectively.[23]

As it is clear from Table 1, the pKₐ's are dependent on the ionic strength and this in turn is due to the dependence of the activity factors on the ionic strength. The ionic strength due to vitamin and also buffer solution constituents is negligible, so essentially all ionic strength is due to KCl salt. This clearly shows that the reported values and the obtained values are more or less the same, within the experimental and instrumental errors.

Riboflavin consists of a heterocyclic isoalloxazine ring attached to the sugar alcohol, ribitol. It is stable to heat but extremely sensitive to light. One of the products of photolysis is lumichrome.[28] Freshly prepared solution of this vitamin was used as a titration solution to determine the corresponding acidity constant, avoiding thereby the photolysis of riboflavin. As is clear from the structural scheme of riboflavin (Scheme 2), which has a similar group to phthalimide, it has a dissociable proton bound to the ring nitrogen. The pKₐ value obtained in this work (Table 1) for the similar functional group is between 9.5 and 11,[30] and the previously reported pKₐ value for riboflavin was 10.2. Since the change of ionic strength is associated with change of pKₐ values in the case of riboflavin these variations are small and probably a more sensitive probe such as fluorescence spectroscopy is needed to determine the dependence of the pKₐ's on the ionic strength.

The weakly basic portion of thiamine (Scheme 3) or of its coenzyme form is protonated at low pH, largely on N-1 of the pyrimidine ring.[31,32] The reported pKₐ value is ~4.9.[28]

The hydrogen atom in the 2-position of the thiazolium ring, between the sulfur and the nitrogen atoms, dissociates as H⁺ during catalysis and the
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Table 1. The acidity constants of vitamins at different ionic strengths at 25 °C

<table>
<thead>
<tr>
<th>KNO_3 (M)</th>
<th>Pyridoxal</th>
<th>Riboflavin</th>
<th>Thiamine</th>
<th>Folic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK_a1</td>
<td>pK_a2</td>
<td>pK_a1</td>
<td>pK_a1</td>
</tr>
<tr>
<td>0.00</td>
<td>4.92</td>
<td>9.19</td>
<td>10.64</td>
<td>4.76</td>
</tr>
<tr>
<td>0.01</td>
<td>4.96</td>
<td>9.42</td>
<td>10.64</td>
<td>4.98</td>
</tr>
<tr>
<td>0.05</td>
<td>5.05</td>
<td>9.46</td>
<td>10.62</td>
<td>5.05</td>
</tr>
<tr>
<td>0.10</td>
<td>5.06</td>
<td>9.45</td>
<td>10.58</td>
<td>5.07</td>
</tr>
<tr>
<td>0.30</td>
<td>5.12</td>
<td>9.44</td>
<td>10.57</td>
<td>5.23</td>
</tr>
</tbody>
</table>

*Fig. 5. Concentration distribution diagrams of (I) Folic Acid, (II) Thiamine, (III) Riboflavin and (IV) Pyridoxal, in aqueous solution at 25 °C and zero ionic strength.*

*pK_a value of this proton has been estimated as ~18, which means that it cannot be as a acidic proton.* The portion that can be protonated next to the NH_2 group is the pyrimidine ring. The pK_a value that we obtained in this report (Table 1) is comparable with

Scheme 1.

Scheme 2.
previously reported values.\textsuperscript{28}

The \( pK_{a1} \) and \( pK_{a2} \) values have shown a fair dependence to ionic strength. As it can be seen from Table 1 the higher ionic strength the higher \( pK_a \) values.

The folic acid, as shown in Scheme 4, has a complicated structure and allocates the obtained acidity constants to specific groups. The previously reported \( pK_a \) values are 4.82 (related to N-5 site) and 10.5 (related to N-3 site, transferring from O-position to N-position during tautomerism).\textsuperscript{28}

Three \( pK_a \) values were obtained, which are listed in Table 1. As the structural scheme shows, folic acid possesses two carboxylic groups apart from the two acidic positions, as discussed above. It can be assumed that the obtained \( pK_a \) value relates to one of the two carboxylic groups, and the other two \( pK_a \) values can be compared with reported values, 4.82 and 10.5 for \( pK_{a1} \) and \( pK_{a2} \), respectively.\textsuperscript{23} It is surprising, to note that, the dependence of the \( pK_a \) 's values of the folic acid on the ionic strength did not show a regular pattern. This, unlikely, is related to the no systematic changing of the absorption spectra in the course of titration, Fig. 1. And in turn these irregularities return to this fact that folic acid solution has not enough stability and as it cleared by the manufacturer companies, the solution and the pure compound must be kept in cool and dark place.

As shown by Table 1 and Figs. 1-4, changes in ionic strengths have more or less observable effects on the spectral data of the four vitamins, which means that the acidity constants for \( pK_{a1} \) and \( pK_{a2} \) change mildly by changing ionic strength. Of course, they show some irregular variations, which may be due to experimental and instrumental errors in some cases. So, variation in ionic strength which have some effects on the acidity constants of these vitamins can influence on the ionic state of these compounds as well as on the functionality of the enzymes which benefits the catalytic properties of these molecules as coenzymes.

CONCLUSION

The dissociation constants of the group B vitamins were calculated with spectrophotometric titrations using a chemometric method. The striking advantage of the proposed method is using of the whole spectral information in the computation process which enable us to have more precise and accurate thermodynamics constants in comparison to the classical methods such as single wavelength approach. The effect of the ionic strength on the acidity constants is investigated and it reveals the complex relations of the dissociation constants to the ionic strength. The results show good consistency with the previous reported results.

REFERENCES

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