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PHYSICAL CHEMISTRY OF SOLUTIONS

d-Metal Folates and the Folic Acid–Imidazole Conjugate

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Abstract—Metal folates $MFol \cdot nH_2O$ ($M^{2+} = Mn^{2+}$, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}; $n = 4–6$), silver folate $Ag_2Fol \cdot 3H_2O$, and the folic acid–imidazole conjugate $H_2Fol \cdot 3Im \cdot 2H_2O$ were synthesized in aqueous solutions at a 1 : 1 M^{2+} : H_2Fol molar ratio and pH 5.5–6.6. The compositions of the resulting compounds were determined by chemical, thermal, and gravimetric analysis. The composition of copper(II) folate was confirmed by elemental analysis; the solubility product of nickel folate (9.65×10^{-9}) was estimated using solubility data. IR and electron absorption spectroscopy was used to show that oxygen atoms of carboxyl groups in folic acid and the pyridine nitrogen atom in imidazole are involved in bond formation in folates and the conjugate.

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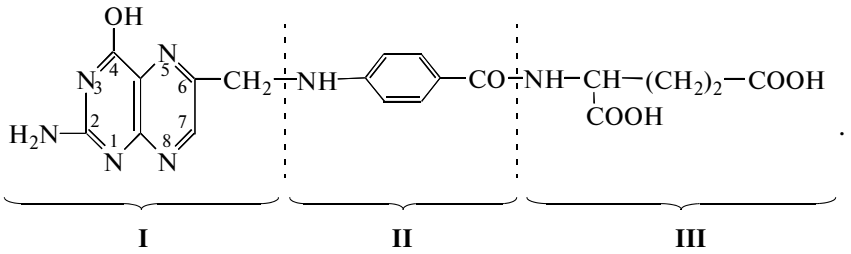
Coordination chemistry of *d* elements and ligands whose molecules simultaneously contain several donor atoms is both of fundamental and practical interest. Natural amino acids with oxygen- and nitrogen-containing donor groups are a classic example of these ligands. Folic acid, an essential group B vitamin (vitamin B_9), is one such compound. Along with vitamins B_6 and B_{12}, folic acid is involved in syntheses of protein, amino acids (methionine, serine, etc.), nucleic acids, purines, and pyrimidines; facilitates transport of iron through cell membranes inside the cells; and stimulates blood-producing functions of the organism.

Folic acid $C_{19}H_{19}N_7O_6$ (H_2Fol) consists of three structural units: 2-amino-4-oxy-6-methylpyridine residue (**I**), para-aminobenzoic (**II**), and *L*-glutamic (**III**) acids. Folic acid is an amphoteric substance with acidic properties being predominant: its basic properties are caused by the pteridine core, while the acidic properties are caused by enolic hydroxyl and carboxyl groups.



Due to its acidic properties, H_2Fol forms salts: highly soluble salts of alkali metals and poorly soluble ones of alkaline-earth metals and *d*-metals [1]. Poor solubilities of zinc, magnesium, calcium, and barium folates are used to remove impurities from synthetic folic acid [2, 3]. Thus, zinc folate residue at pH 6.5–7.0 is isolated from its solution and dissolved in lime-water; the solution with pH 10.6–10.8 is then treated with sulfuric acid solution until pH ~ 3 is reached to obtain folic acid residue with 85–90% purity. This process is repeated five times. According to a different procedure, pure folic acid is dissolved in an alkaline solution; the acid is converted to magnesium salt, which is subsequently precipitated in acidic solution to give rise to high-purity folic acid. The presence of incombustible (750–900°C) residue (3–3.5%) in some commercial forms of folic acid probably arises from the purification methods used.

Poorly soluble salts (metal folates) can be formed during storage of modern medical nutrition products [4] containing milk, molasses, sugar, vanillin, iron(II) and copper(II) sulfates, ascorbic and folic acids, and pyroxidine hydrochloride, which reduces product efficiency. It would probably be better to use synthesized folates instead of sulfates in these mixtures.

Folic acid containing various functional groups forms conjugates with some molecular substances. Thus, most folic acid in plants is conjugated to glutamic acid. Synthesized conjugates are used in tumor therapy.

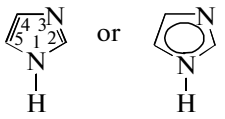
Tumor cell membranes often have an abnormally large number of receptors of folic acid or folates, which are involved in cell division and tumor development. The activity of folate receptors in most tumor cells is higher than that in normal cells [5]. This fact has rapidly increased the number of studies devoted to the synthesis of folic acid conjugates as objects that can be targeted against tumor cells.

Folic acid is known to form a complex with polysaccharides [6] through binding between the carboxylic group of folic acid and the hydroxyl group of polysaccharide.

Pavich et al. [7] synthesized and studied the absorption and luminescence spectra of folic acid–*L*-alanine–NH_2Phen (NH_2Phen is the amino substituted phenanthrolene) and folic acid–*L*-alanine–europium chelate (NH_2Phen)Eu(BTFA)$_3$ conjugates (BTFA is benzoyl trifluoroacetone), as well as the spectra of individual components. In a molecule of primary conjugate H_2Fol–NH_2Phen, the absorption bands of folic acid at 361 and 286 nm shift hypsochromically to 335 and 278 nm, respectively. The absorption band of NH_2Phen (263 nm) in the conjugate does not shift. It was shown that the synthesized europium conjugate binds to folate receptor of some tumor cells.

Eremin et al. [8] described a way to obtain associates of folic acid with composite nanoparticles (Zn)ZnS and (Cd)CdS in aqueous solutions. The folic acid–(Cd)CdS associate is characterized by high fluorescence intensity and storage stability. The folic acid–silver nanoparticles associate [9] was synthesized using folic acid, which is characterized by its fluorescence ability, to simultaneously reduce Ag^+ ions and modify the resulting silver nanoparticles. There is a currently rising interest in using these associates in biology and medicine as biomarkers for diagnosing and targeted drug delivery into cells. Hence, researchers from the Purdue's Weldon School of Biomedical Engineering coated the surface of gold nanorods with folate and achieved fixation of gold on the tumor cell membrane. Gold hydrosols stabilized in solution by sodium folate [10] can also be used to treat cancer diseases. After analyzing the IR spectroscopy data and comparing them to the literature findings, the authors put forward a hypothesis that gold nanoparticles are stabilized by sodium folate due to the binding between gold and the NH_2 group of folate anion.

We found no data on the composition of *d*-metal folates in the available literature, except for that of silver folate $C_{19}H_{17}O_6N_7Ag_2$ [11]. There are reports about mixed-ligand copper(II) and potassium salts, such as copper(II) potassium folate–glycinate [12] and copper(II) potassium folate–*L*-glutaminate [13], etc., which exhibit anti-inflammatory and analgesic activity. Since folic acid acts as a weak acid, it is expected to interact with molecular compounds having basic properties. One such compound an be imidazole $C_3H_4N_2$, a five-membered heterocycle containing two nitrogen heteroatoms



Im,

which exhibits amphoteric properties (is an acid with respect to $N_{(1)}$ and a base with respect to N_3), but basic properties predominate.

This study was aimed at finding the conditions for synthesizing folates of some *d*-metals, determining their compositions, and investigating their properties, as well as synthesizing a product of reaction between folic acid and imidazole.

EXPERIMENTAL

Folates of *d*-metal ions (Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) and silver ions, as well as the folic acid–imidazole conjugate were used for the study. The inventivetive tools used to to analyze and study properties of the synthesized salts and the conjugate were thermal analysis, thermogravimetry, elemental analysis, pH potentiometry, spectrophotometry, and IR spectroscopy.

Precipitates of *d*-metal folates were isolated from aqueous solutions with pH 5.5–6.6 at a M^{2+} : H_2Fol molar ratio of 1 : 1. Preliminary experiments demonstrated that folic acid acts as a dibasic acid when reacted with cations of alkaline and alkali metal elements, or mono- and bivalent *d* cations in this pH range. When synthesizing folates, we added a solution of metal chloride or nitrate (Mohr's salt being used to synthesize iron(II) folate) to a folic acid solution with pH 6.7–7.0. A weighed sample of the acid was dissolved in 0.1 mol/L NaOH solution at ≈ 1 : 2 H_2Fol : NaOH ratio; pH in the resulting mixture was 5.5–6.5. The isolated metal folates are brightly colored: copper folate forms a green precipitate, cobalt folate forms a dark yellow precipitate, etc.

The salts were subjected to thermal analysis to determine the content of the oxide: the salts were exposed to 900°C for 2–3 h (silver folate decomposed to metallic silver). The water, folate anion, and metal oxide were thermogravimetrically determined in the salts, since water removal, folate decomposition, and formation of proper metal oxide occur in different temperature ranges (Table 1). The folate anion was also determined permanganatometrically using the empirical titer of potassium permanganate found by titrating a solution with the known concentration of $H_2Fol \cdot 2H_2O$ in 3 N H_2SO_4. Under our conditions,

Table 1. Analysis of thermoanalytical curves of cobalt(II), copper(II), and zinc folates; folic acid; and its conjugate with imidazole ("f" stands for found; "c" stands for calculated)

No.	Type of effec	Temperature range, °C	Weight loss (percent of the initial value), %		Relevant process
			f	c	
		$CoFol \cdot 6H_2O$			
1	Endotherm	141.7	16.2	17.81	Loss of water
2	Endo- and exotherms	301–674	71.4	72.47	Loss of folate ion
3	Exotherms and endotherms	674–926	13.1	13.24	Formation of oxide Co_3O_4
		$CuFol \cdot 5H_2O$			
1	Endotherm	102–127	15.4	15.18	Loss of water
2	Set of exotherms	261–600; 600–900	70.5; 14.4	74.10; 13.41	Loss of folate ion; formation of CuO
		$ZnFol \cdot 5H_2O$			
1	Endotherm	98–166	12.6	15.13	Loss of water
2	Set of endo- and exotherms	302–700; 700–900	73.3; 14.8	73.88; 13.68	Loss of folate ion; formation of oxide
		$H_2Fol \cdot 2H_2O$			
1	Endotherm	164.7	7.8	7.54	Loss of water
2	Set of exo- and endotherms	234–700 414.2	90.9	92.46	Decomposition and combustion of H_2Fol
		$H_2Fol \cdot 3Im \cdot 2H_2O$			
1	Endotherm	92.2	5.6	5.28	Loss of water
2	Exotherm	238.4	30.0	29.96	Loss of 3Im
3	Endotherm and exotherm	405, 671–720	62.2	64.76	Loss of H_2Fol

1 mL of 7.14×10^{-3} N $KMnO_4$ solution corresponded to 1.44×10^{-3} g of folate anion (determination error was 2.5–4.0%). Table 2 shows the results of analyses of the synthesized folates. The values of oxide weight contents in salts are average results of thermal analysis and thermogravimetry; the two folate anion content values were obtained by redox method and thermogravimetry. Elemental analysis was carried out for copper(II) folate.

For $CuC_{19}H_{17}N_7O_6 \cdot 5H_2O$, anal. calcd. (%): N, 16.53; C, 38.45; H, 4.56; O, 29.68.

Found (%): N, 16.58; C, 37.87; H, 4.35; O, 28.42.

The solubility of nickel folate $NiC_{19}H_{17}N_7O_6 \cdot 6H_2O$ determined in 0.1 mol/L NaCl solution was 5.01×10^{-4} mol/L.

When synthesizing the conjugate, we added a weighed sample of folic acid $H_2Fol \cdot 2H_2O$ to a small volume (~10 mL) of aqueous Im solution (pH ~ 9) to achieve the H_2Fol : Im molar ratio of 1 : 3. As folic acid was dissolved incompletely, the mixture was kept on a water bath for several minutes. The transparent solution was cooled (pH in the mixture was 6.8–7.3); addition of 30 mL of acetone yielded a well-filtrable gelatinous precipitate. The precipitate was washed with acetone and air dried; the yield of the product was ~75%. The conjugate was analyzed thermogravimetrically and permanganatometrically to determine water, imidazole, and folic acid. Potassium permanganate did not oxidize the aromatic imidazole ring under the selected conditions.

RESULTS AND DISCUSSION

The low solubility of folic acid in water (1 mg/100 mL at 0°C), ethanol, acetone, benzene, diethyl ether, and chloroform is attributed to the presence of strong intermolecular hydrogen bonds and makes it difficult to study its interaction with metal ions. Folic acid is soluble in alkali solutions, carbonates, and concentrated hydrochloric acid. When synthesizing salts, pH in solution needs to be higher than 5.5 so that metal folate, rather than folic acid (which becomes gelatinous at pH 4.7), could be isolated. It follows from the folic acid species distribution diagram (for protonation constants of dibasic acid equal to $\log B_1 = 6.75$, $\log B_2 = 11.40$ [14]) that $HFol^-$ are dominant species (50–60%) in the folic acid solution (H_2Fol : NaOH ≈ 1 : 2) at pH 6.7–7.0 and are involved in folate

Table 2. Data of chemical, thermal, and thermogravimetric analysis of metal folates, folic acid, and its conjugate ("f" stands for found; "c" stands for calculated)

Formula of the compound	H_2O, %		M_xO_y, %		Fol^{2-}, %	
	f	c	f	c	f	c
$MnC_{19}H_{17}N_7O_6 \cdot 6H_2O$	15.8	17.93	12.6	12.66	69.2, 72.2	72.95
$FeC_{19}H_{17}N_7O_6 \cdot 5H_2O$	13.1	15.38	13.60	13.19	73.7	75.11
$CoC_{19}H_{17}N_7O_6 \cdot 6H_2O$	16.2	17.81	13.1	13.24	71.8, 71.4	72.47
$NiC_{19}H_{17}N_7O_6 \cdot 6H_2O$	17.6	17.82	12.6	12.32	70.6, 69.7	72.50
$CuC_{19}H_{17}N_7O_6 \cdot 5H_2O$	15.4	15.18	13.9	13.41	72.0, 70.5	74.10
$ZnC_{19}H_{17}N_7O_6 \cdot 5H_2O$	12.6	15.13	14.0	13.68	72.0, 73.3	73.88
$Ag_2C_{19}H_{17}N_7O_6 \cdot 3H_2O$	5.8	7.61	30.4 (Ag)	30.42	59.2, 60.9	61.96
$H_2C_{19}H_{17}N_7O_6 \cdot 2H_2O$	7.8	7.54	–	–	90.9	92.46
$H_2C_{19}H_{17}N_7O_6 \cdot 3C_3H_4N_2 \cdot 2H_2O$	5.6	5.28	–	–	61.2, 62.2	64.76

formation with replacement of the proton in hydrofolate ion with a metal ion:

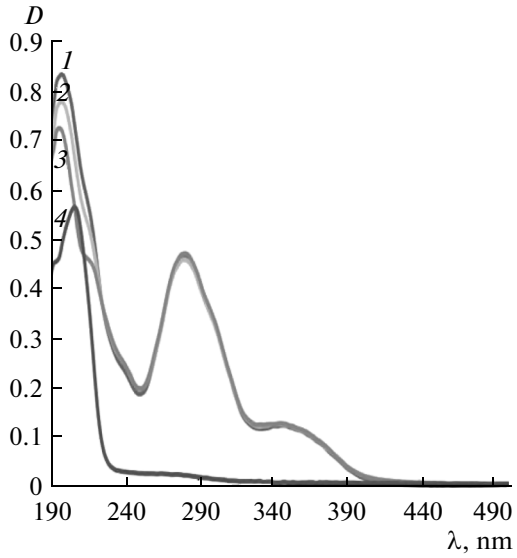
$$M^{2+} + HFol^- = MFol\downarrow + H^+,$$

pH in the mixture decreases to 6.5–5.5.

d-Metal folates are poorly soluble. The solubility product of nickel folate $NiC_{19}H_{17}N_7O_6 \cdot 6H_2O$ was estimated from the solubility value in 0.1 mol/L NaCl solution and pH of a saturated solution (5.42) using the formula $K_{sp} = (c_{salt})^2/f = 9.65 \times 10^{-9}$ ($f = 1 + B_1[H^+] + B_2[H^+]^2$).

The IR spectra of the resulting folates do not feature the absorption band of carbonyl group C=O of folic acid (1694.4 cm^{-1}). Instead, there are two bands at 1605 and 1404 cm^{-1} caused by asymmetric and symmetric valence vibrations of ionized carboxylic groups. These findings also support the fact that salt precipitates contain no folic acid impurity when metal folates are synthesized from a solution at pH 5.5–6.5. The data of elemental analysis of silver folate [11] are indicative of an increased content of folate anion in the salt.

During synthesis of the conjugate, poorly soluble folic acid is dissolved in imidazole solution at H_2Fol : Im molar ratio of 1 : 3 (0.16 mol/L imidazole solution has pH ~ 9.5; according to its distribution diagram [15], imidazole exists as a neutral molecule at this pH) and pH in the mixture decreases to 7. The formation of the conjugate $H_2Fol \cdot 3Im \cdot 2H_2O$ is probably related to the donor–acceptor interaction of pyridine nitrogen atom $N_{(3)}$ in the ring of three imidazole molecules with carboxyl residues of glutamic acid and the hydroxyl group of the pteridine ring in folic acid molecule. The electronic absorption spectra of the conjugate and the mixture of initial components at the H_2Fol : Im molar ratio of 1 : 3 (figure) are almost identical to the absorption spectrum of folic acid (λ_{max} = 346, 280, and 198 nm); the absorption band of imidazole at 206 nm corresponding to $\pi \rightarrow \pi^*$ transitions is not seen in the mixture and in the conjugate; a partial protonation of the pyridine nitrogen atom of imidazole in the presence of folic acid can probably cause hypsochromic shift. A slight hyperchromic effect in



Absorption spectra of aqueous solutions of (*1*) H_2Fol : Im = 1 : 3 ($c_{Fol} = 1.67 \times 10^{-5}$, $c_{Im} = 5 \times 10^{-5}$ mol/L; pH 6.5; λ_{max} = 198, 280, and 346 nm); (*2*) $H_2Fol \cdot 3Im \cdot 2H_2O$ ($c_{Fol} = 1.67 \times 10^{-5}$, $c_{Im} = 5 \times 10^{-5}$ mol/L; pH 5.9; λ_{max} = 198, 280, and 346 nm); (*3*) H_2Fol ($c_{Fol} = 1.67 \times 10^{-5}$ mol/L; pH 6.8; λ_{max} = 196, 280, and 346 nm); and (*4*) Im ($c_{Im} = 1.25 \times 10^{-4}$ mol/L; pH 7.2; λ_{max} = 206 nm).

the mixture and in the adduct is observed at 198 nm as compared to absorption of the initial components (H_2Fol and Im).

The shift of intense bands of bending vibrations of the ring (1099.9 and 1053.9 cm^{-1}) towards higher frequencies (1101.7 and 1056.2 cm^{-1}) and the intact band of stretching vibrations of the N–H bond of the pyrrole group (3050–3300 cm^{-1} [16]) when proceeding from unbound imidazole Im (3143.5 cm^{-1}) to its conjugate $H_2Fol \cdot 3Im \cdot 2H_2O$ (3143.5 cm^{-1}), indicate that the pyridine nitrogen atom $N_{(3)}$ is involved in binding to protonated carboxylic groups of folic acid and probably the hydroxyl group. The IR spectrum of the conjugate still contains the band of stretching vibrations of the carboxyl group C=O (1694.4 cm^{-1}) of H_2Fol, but new bands emerge at 1605 and 1404 cm^{-1}, which are responsible for the asymmetric and symmetric stretching vibrations of COO^- groups; these bands are also present in metal folates (1602.5–1604.6 cm^{-1}).

An analysis of thermogravimetry data (Table 1) shows the conjugate and salts lose water of crystallization at the temperature range of 92–142°C, while folic acid loses water at 165°C. Thermal decomposition in folate anion with complex structure is accompanied by a set of endotherms (301–419°C) and exotherms (360–620°C). The decomposition of folic acid takes place in a temperature range of 234–700°C and is accompanied by several exo- and one endotherm at 414°C. The conjugate loses imidazole (235°C) with an exotherm effect and folic acid is decomposed at 405–700°C (with endoterms and exotherms).

Thus, the combination of analytic, spectral, and thermogravimetry data confirms the formation of the folic acid–imidazole conjugate. The synthesized conjugate $H_2Fol \cdot 3Im \cdot 2H_2O$ can be regarded as a molecular charge-transfer complex (CTC) (CTCs of azoles have been poorly studied). The charge transfer band for this complex shifts from the UV region to the visible spectral region (figure); the solution is yellow. It is almost only aromatic compounds that act as donors in organic CTCs [17]. According to the modern views, molecular complexes can contain ions, free radicals, ion radicals, and excited molecules. Molecular complexes also include hydrogen-bonded complexes.

Isaev [18] performed quantum-chemical simulation of proton transfer in some molecular systems similar to the system discussed in this study. In the hydrogen-bonded molecular complex MeIm–$H^+\cdots H_2O\cdots CH_3COO^-$ (MeIm–H^+ is protonated methylimidazole) imitating the proton donor–chain–acceptor system, a water molecule acts as a bridge to transfer a proton from protonated methylimidazole to organic acid anion. In this ionic molecular complex, N and O atoms are involved in hydrogen bonds. The pK_a values of the molecular complex components need to be similar to ensure mutual proton exchange. The presence of proton exchange in conjugate $H_2Fol \cdot 3Im \cdot 2H_2O$ can be confirmed by the fact that its IR spectrum contains absorption bands of both non-dissociated and dissociated carboxylic groups of folic acid.

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