Acid–Base Properties of Adenosine 5′-Monophosphate, Guanosine 5′-Monophosphate, and Inosine 5′-Monophosphate in Aqueous Solutions of Methanol

by **Farrokh Gharib***, **Mehran Abbaszadeh**, and **Mohammad Pousti**

Chemistry Department, Shahid Beheshti University, G. C., Tehran, Evin, Iran (fax: +98 21 22431661;
e-mail: f-gharib@sbu.ac.ir)

The protonation constants of adenosine 5′-monophosphate, guanosine 5′-monophosphate, and inosine 5′-monophosphate were determined in binary mixtures of H_2O containing 0, 10, 15, 20, 25, 30, 35, 40, 45, and 50% MeOH, using a combination of potentiometric and spectrophotometric methods at a constant temperature (25°) and constant ionic strength ($0.1\ mol \cdot dm^{-3}$ $NaClO_4$). The protonation constants were analyzed using the normalized polarity parameter (E_T^N), and *Kamlet*, *Abboud*, and *Taft* (*KAT*) parameters. A linear correlation of log *K vs.* the normalized polarity parameter was obtained. Dual-parameter correlation of log *K vs.* π^* (dipolarity/polarizability) and α (H-bond-donor acidity), as well as π^* and β (H-bond-acceptor basicity) also gives good results in various aqueous organic solvent mixtures. Finally, the results are discussed in terms of the effect of solvent on the protonation equilibria.

Introduction. – Adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and inosine 5′-monophosphate (IMP) are very important compounds due to their vital roles within living cells and in regulation of various functions in biological systems [1][2]. These compounds are the building blocks of both DNA and RNA, and are involved in a wide variety of processes such as cellular metabolism, cell bioenergetics, human cancer and immunodeficiency virus (HIV) markers, *etc.* [3][4]. The protonation constants of these nucleosides are among the most useful physicochemical features describing the extent of ionization of functional groups with respect to pH. This parameter is important in research areas such as pharmaceutical drug discovery and development, where it often has a vital role in understanding the pharmacodynamic properties of new drugs [5–9].

Many chemical reactions occur in solution. In a variety of chemical fields such as chemical synthesis, solvent extraction, liquid chromatography, *etc.*, binary solutions of H_2O and organic solvents are used. Aqueous organic solvents, mainly MeOH and EtOH, mixtures, have been widely used due to sparing solubility or insolubility of many compounds in pure H_2O as solvent. Further, any physicochemical property of solutions can be easily varied by changing the composition of H_2O or the organic solvent in the mixtures. However, chemists have usually attempted to understand solvent effects in terms of polarity, defined as the overall solution capability that depends on all possible (specific and non-specific) intermolecular interactions between solute and solvent molecules. Furthermore, the interaction of solvents, with different dielectric constants, with biological molecules is of importance in understanding the chemistry of *in vivo* processes such as enzyme interactions, the assembly of lipids in biomembranes, surfactant aggregation, and so forth [10].

In continuation of our previous work [11–16], in the present study the protonation constants of AMP, GMP, and IMP have been determined in different aqueous solutions of MeOH to examine the dependence of acid–base equilibria on solvent composition.

Results and Discussion. – The protonation constants of AMP, GMP, and IMP were determined spectrophotometrically based on the relation $A = f(\mathrm{pH})$ [17]. The measured absorbance, A (250–310 nm in 0.5-nm intervals), and $\mathrm{pC_H}$ from the spectrophotometric titration were conducted using the computer program Squad [18][19]. The data in the computer program were fitted to *Eqns. 3*, *5*, and *7* (see below) by minimizing the error square sum of the difference in the experimental absorbances and the calculated ones. The program allows calculation of the protonation constants with different stoichiometries. The number of experimental points (absorbances *vs.* $\mathrm{pC_H}$) was more than 35 (maximum 50) for each titration run. During the experiments, the solutions were stable, and the absorbance values did not change with time.

The results obtained using potentiometric/spectrophotometric titrations for the various acidity constants of the H-atom donors of the nucleosides (*Eqns. 1–8*), are listed in *Table 1*, together with some values reported in the literature [20][21]. In *Fig. 1*, the species mole fractions of the studied systems in different $\mathrm{pC_H}$ are shown in pure H_2O. The nucleoside 5′-monophosphates (NMPs) shown in *Fig. 2* can bind with two H-atoms at the phosphate group and one at the purine moiety. It was proposed [20] that $H_3(\mathrm{AMP})^+$, $H_3(\mathrm{GMP})^+$, and $H_3(\mathrm{IMP})^+$ release their first H-atoms from $P(O)(OH)_2$, the second one from $H^+(N1)$ in the case of AMP and $H^+(N7)$ from GMP and IMP, and the third one again from the phosphate group. A forth H-atom is released in the

Table 1. *Average Values of the Protonation Constants of AMP, GMP, and IMP at 25°, Constant Ionic Strength* ($0.1\ \mathrm{mol \cdot dm^{-3}\ NaClO_4}$), *and Different Aq. Solns. of MeOH*

MeOH [%]	AMP		GMP			IMP			Ref.
	$\log K_2$	$\log K_1$	$\log K_2$	$\log K_1$	$\log K_{-H}$	$\log K_2$	$\log K_1$	$\log K_{-H}$	
0	3.63 ± 0.03	6.53 ± 0.02	2.55 ± 0.04	6.09 ± 0.03	9.41 ± 0.02	1.47 ± 0.04	6.02 ± 0.03	9.32 ± 0.04	a)
10	3.61 ± 0.03	6.57 ± 0.02	2.53 ± 0.05	6.12 ± 0.03	9.51 ± 0.05	1.43 ± 0.02	6.17 ± 0.05	9.38 ± 0.05	a)
15	3.60 ± 0.04	6.61 ± 0.04	2.52 ± 0.05	6.18 ± 0.04	9.57 ± 0.06	1.36 ± 0.02	6.31 ± 0.05	9.45 ± 0.05	a)
20	3.57 ± 0.03	6.69 ± 0.01	2.51 ± 0.04	6.25 ± 0.06	9.63 ± 0.06	1.30 ± 0.01	6.45 ± 0.06	9.56 ± 0.07	a)
25	3.57 ± 0.02	6.74 ± 0.05	2.49 ± 0.04	6.29 ± 0.05	9.64 ± 0.05	1.23 ± 0.03	6.65 ± 0.04	9.64 ± 0.06	a)
30	3.56 ± 0.04	6.77 ± 0.04	2.48 ± 0.02	6.42 ± 0.05	9.71 ± 0.06	1.20 ± 0.02	6.89 ± 0.05	9.76 ± 0.06	a)
35	3.56 ± 0.02	6.81 ± 0.06	2.47 ± 0.03	6.50 ± 0.06	9.77 ± 0.04	1.13 ± 0.03	7.04 ± 0.06	9.84 ± 0.05	a)
40	3.53 ± 0.03	6.91 ± 0.05	2.46 ± 0.02	6.57 ± 0.04	9.87 ± 0.05	1.05 ± 0.04	7.12 ± 0.06	9.95 ± 0.04	a)
45	3.52 ± 0.03	7.03 ± 0.04	2.44 ± 0.03	6.75 ± 0.04	9.95 ± 0.06	1.01 ± 0.02	7.44 ± 0.04	10.02 ± 0.04	a)
50	3.51 ± 0.04	7.11 ± 0.06	2.42 ± 0.04	6.86 ± 0.02	10.01 ± 0.07	0.96 ± 0.03	7.51 ± 0.04	10.11 ± 0.05	a)
0	3.84	6.21							[20]
0			2.48	6.25	9.49				[20]
0						1.30	6.22	9.02	[20]
0							6.24	8.83	[21]

a) This work.

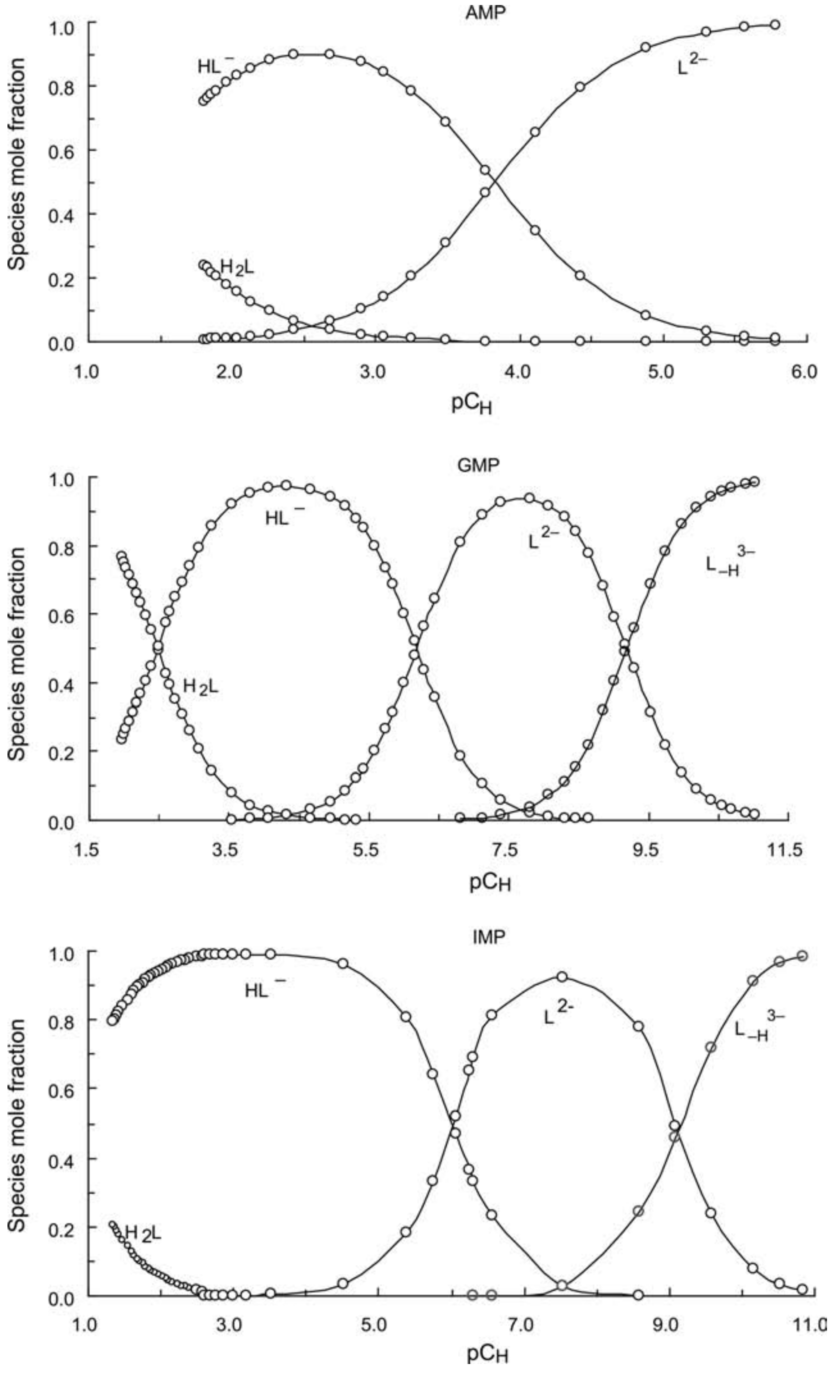

Fig. 1. *Distribution diagrams of the different species of AMP, GMP, and IMP* (from top to bottom) *in H_2O at 25° and ionic strength of 0.1 mol · dm^{-3} $NaClO_4$*

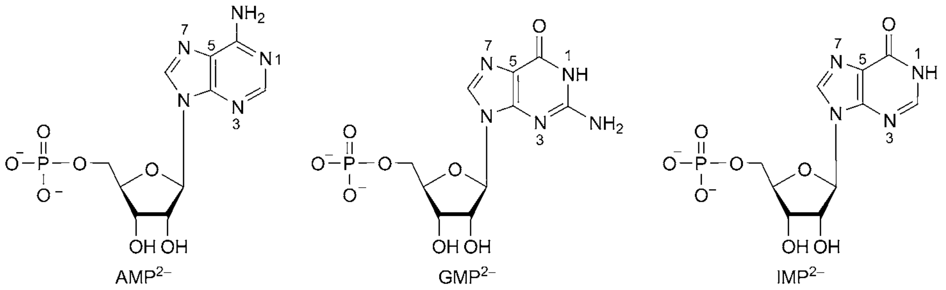

Fig. 2. *Chemical Structures of AMP^{2-}, GMP^{2-}, and IMP^{2-}*

alkaline pH range from H(N1) site in GMP and IMP. The first deprotonation of the nucleosides from the $P(O)(OH)_2$ (p$K < 1$) and the last from the ribose groups (p$K >$ 12) [20] are not considered further in this work. The given acidity constants agree well, as far as available, with those reported in recent compilations (*Table 1*) [20][21]. The differences are possibly due to the different experimental methods and the different background electrolytes used. These steps are expressed by the following equilibria:

$$H_2(NMP) + H^+ \rightleftharpoons H_3(NMP)^+ \tag{1}$$

$$K_3 = [H_3(NMP)^+]/[H^+][H_2(NMP)] \tag{2}$$

$$H(NMP)^- + H^+ \rightleftharpoons H_2(NMP) \tag{3}$$

$$K_2 = [H_2(NMP)]/[H^+][H(NMP)^-] \tag{4}$$

$$(NMP)^{2-} + H^+ \rightleftharpoons H(NMP)^- \tag{5}$$

$$K_1 = [H(NMP)^-]/[H^+][H(NMP)^{2-}] \tag{6}$$

$$(NMP - H)^{3-} + H^+ \rightleftharpoons (NMP)^{2-} \tag{7}$$

$$K_{-H} = [(NMP)^{2-}]/[H^+][(NMP - H)^{3-}] \tag{8}$$

Solvent Effect. – The protonation constants of AMP, GMP, and IMP in H_2O/MeOH mixtures have different behaviors. log K_2 values of the nucleosides decrease, but those of log K_1 and log K_{-H} of the bases increase with increasing proportion of organic solvent in the mixtures (*Table 1*). In general, the standard *Gibbs* energy of protonation equilibria consists of two terms: an electrostatic term, which can be estimated by the *Born* equation, and a non-electrostatic term, which includes specific solute–solvent interaction [22]. When the electrostatic effects predominate, then, in accordance with the *Born* equation, *Eqn. 9*, the plot of log K *vs.* reciprocal of the dielectric constant of the media, $1/\varepsilon$, should be linear.

$$\Delta\log K = (121.6n/r)(1/\varepsilon - 0.0128) \tag{9}$$

where r is the common radius of the ions, and n is the square summation of the charges involved in the protonation equilibria. For example, $n = 2$ for the charge type $HL^- \rightleftharpoons H_2L$, $n = 4$ for $L^{2-} \rightleftharpoons HL^-$, and $n = 6$ for the charge type $H_{-1}L^{3-} \rightleftharpoons L^{2-}$. Therefore, a change in polarity of the medium should have a major effect on the protonation constants of the bases. However, the linearity of the correlation between log K of the bases with $1/\varepsilon$ of $MeOH/H_2O$ mixtures is not good in all cases (with correlation coefficients between 0.96 and 0.97; *Fig. 3*). This indicates that the protonation constants depend not only on electrostatic forces but also on the solute–solvent interactions of the different species in the mixtures [23]. Therefore, it is necessary to elucidate the nature of solute–solvent interactions for a better understanding of solvent effects.

When the N(1) (in the case of AMP) and N(7) sites of the purine moieties of GMP and IMP are protonated, there are changes from a negatively charged species to a neutral one ($HL^- + H^+ \rightleftharpoons H_2L$), and, so, the medium has a minor influence on this protonation process, which depends on the solvation of the species by the solvents of the mixtures. This behavior explains why the influences of the percentages of MeOH on the log K_2 values are small in all cases. However, in protonation of the phosphate groups of all bases, a negatively charged species are created ($L^{2-} + H^+ \rightleftharpoons HL^-$) and, therefore, any variation in the polarity of the medium exerts a strong influence in protonation of the bases, which leads to a considerable change in the log K_1 values. The same rule is valid for log K_{-H} of GMP and IMP.

It is very difficult to interpret the variation of the protonation-constant values of the bases with respect to the percentage of MeOH in the mixtures using the dielectric constant, ε, of the solutions as a single parameter. However, it is now understood that the anion and cation species are selectively solvated by acidic or basic solvents, respectively [23]. In fact, anion solvation is closely related to the electron-pair acceptability or *Lewis* acidity of solvents, and tends to become stronger with the increase in acceptor number of the medium [24]. However, the solvent molecules approach a cation with their negative charge. Therefore, cation solvation is closely related to the electron pair-donor capacity or *Lewis* basicity of the solvents and tends to become stronger with the increase in donor number of the medium. This behavior in the proposed aqueous MeOH solutions indicates again why the values of log K_2 decrease, and log K_1 and log K_{-H} increase when the organic-solvent percentage increases in the mixtures.

To obtain a quantitative method for evaluation of the solute-solvent interaction on protonation or other equilibrium constants, during the last two decades, several empirical solvent scales have been devised [25]. Among these scales (more than 40), the most comprehensive are the solvatochromic ones, but only a few of them have found wider application in correlation analysis of solvent effects. A quantitative determination of the solvent polarity, E_T, has been introduced by *Dimroth* and *Reichardts*, based on the solvatochromic behavior of pyridinium *N*-phenoxide betaine dye [25]. This dye is the most solvatochromic compound reported to date [25]. This scale now has been revised and normalized to E_T^N, known as the normalized polarity parameter, due to the introduction of SI units. E_T^N is related to the ability of a solvent to

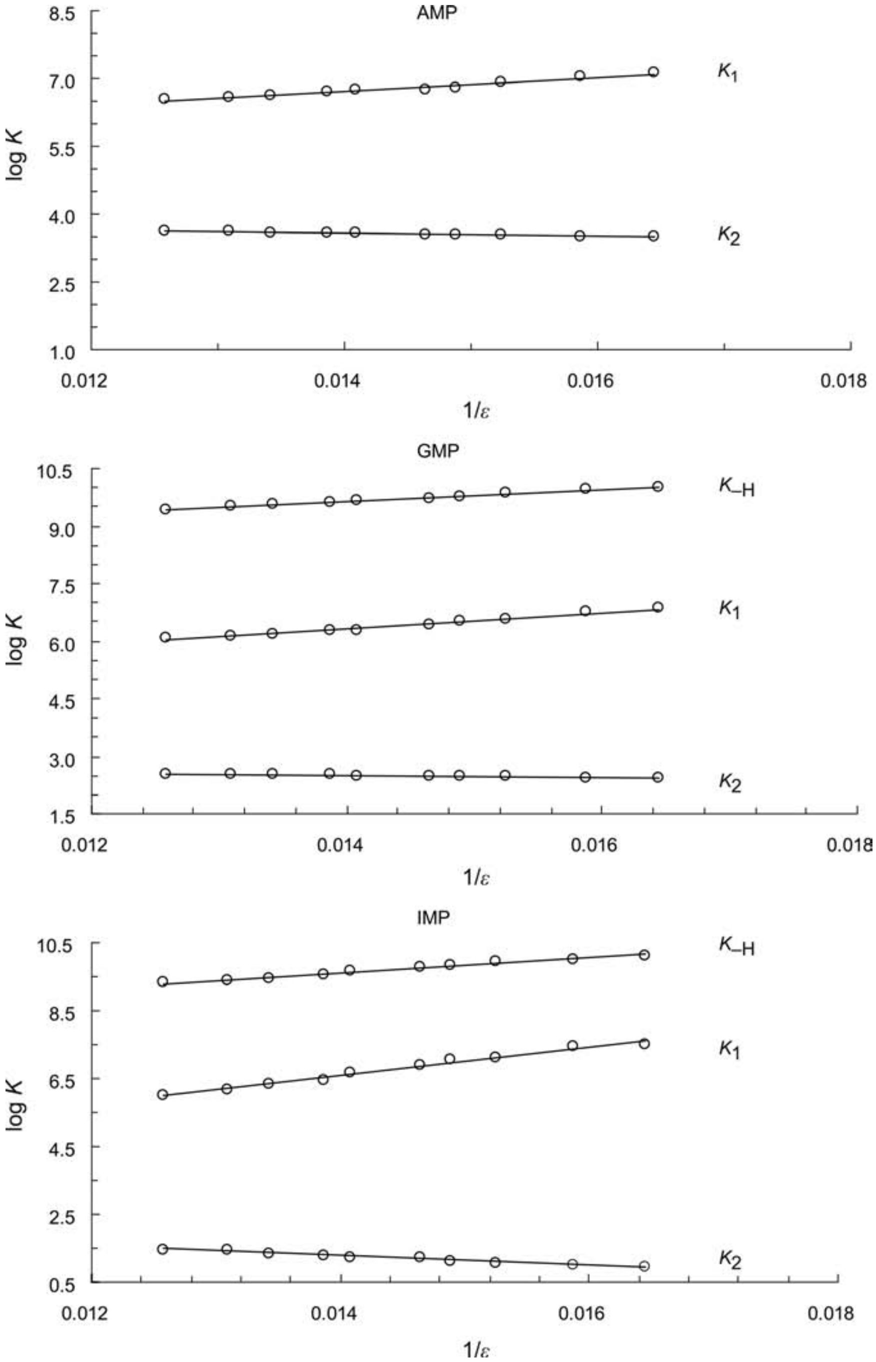

Fig. 3. *The plots of log* K *values of AMP, GMP, and IMP* vs. *the reciprocal of the dielectric constants of the different solvents mixtures at 25° and an ionic strength of 0.1 mol·dm^{-3} NaClO$_4$*

stabilize charge separation in the dye and has the value of zero for Me_4Si, the least polar solvent, and 1.0 for H_2O, the most polar solvent. According to this approach, the protonation-constant values (in logarithm scale) were correlated with E_T^N as a single linear regression analysis using the computer program *Microsoft* Excel Solver and Linest [26]. A linear correlation of log K *vs.* E_T^N was obtained in different aqueous solutions of MeOH in all cases:

$$\log K_2(\mathrm{AMP}) = 2.87\,(\pm 0.05) + 0.77\,(\pm 0.06)\; E_T^N \tag{10a}$$

$$N = 10,\ \mathrm{rss} = 6.47 \times 10^{-4},\ \mathrm{ose} = 9.00 \times 10^{-3}$$

$$\log K_1(\mathrm{AMP}) = 10.09\,(\pm 0.42) - 3.66\,(\pm 0.46)\; E_T^N \tag{10b}$$

$$N = 10,\ \mathrm{rss} = 3.82 \times 10^{-2},\ \mathrm{ose} = 6.91 \times 10^{-2}$$

$$\log K_2(\mathrm{GMP}) = 1.75\,(\pm 0.06) + 0.81\,(\pm 0.06)\; E_T^N \tag{10c}$$

$$N = 10,\ \mathrm{rss} = 7.23 \times 10^{-4},\ \mathrm{ose} = 9.51 \times 10^{-3}$$

$$\log K_1\mathrm{GMP}) = 10.91\,(\pm 0.62) - 4.97\,(\pm 0.68)\; E_T^N \tag{10d}$$

$$N = 10,\ \mathrm{rss} = 8.24 \times 10^{-2},\ \mathrm{ose} = 1.02 \times 10^{-1}$$

$$\log K_{-\mathrm{H}}(\mathrm{GMP}) = 13.14\,(\pm 0.31) - 3.78\,(\pm 0.34)\; E_T^N \tag{10e}$$

$$N = 10,\ \mathrm{rss} = 2.02 \times 10^{-2},\ \mathrm{ose} = 5.02 \times 10^{-2}$$

$$\log K_2(\mathrm{IMP}) = -1.94\,(\pm 0.24) + 3.48\,(\pm 0.26)\; E_T^N \tag{10f}$$

$$N = 10,\ \mathrm{rss} = 1.24 \times 10^{-2},\ \mathrm{ose} = 3.94 \times 10^{-2}$$

$$\log K_1(\mathrm{IMP}) = 16.03\,(\pm 0.80) - 10.23\,(\pm 0.88)\; E_T^N \tag{10g}$$

$$N = 10,\ \mathrm{rss} = 1.37 \times 10^{-1},\ \mathrm{ose} = 1.31 \times 10^{-1}$$

$$\log K_{-\mathrm{H}}(\mathrm{IMP}) = 14.31\,(\pm 0.0.42) - 0.042\,(\pm 0.46)\; E_T^N \tag{10h}$$

$$N = 10,\ \mathrm{rss} = 3.77 \times 10^{-2},\ \mathrm{ose} = 6.86 \times 10^{-2}$$

where N, rss, and ose represent the number of the mixed solvents, the residual sum of squares, and the overall error, respectively.

The normalized polarity parameters (E_T^N) for all of the H_2O/MeOH mixtures used in this work were obtained from the plot of the property *vs.* the mole fraction of the organic solvent of the values that have been reported in the literature for some other percentages of aqueous solutions of MeOH used in this study [27], and the dielectric constants, ε, values were obtained from the literature [28] for the differently mixed solvents (*Table 2*). The normalized polarity parameter is a blend of pure polarity (dipolarity/polarizability) and H-bonding interactions. To obtain the magnitude of the effect of these interactions on the protonation constant, a dual-parameter correlation of log *K vs.* the *KAT* parameters [29][30] was obtained:

$$\log K_2(\mathrm{AMP}) = 2.83\,(\pm 0.11) - 0.24\,(\pm 0.17)\alpha + 0.43\,(\pm 0.27)\pi^* \tag{11a}$$

$$N = 10,\ \mathrm{rss} = 4.37 \times 10^{-4},\ \mathrm{ose} = 7.90 \times 10^{-3},\ r^2 = 0.97,\ f = 107.40$$

$$\log K_1(\mathrm{AMP}) = 11.62\,(\pm 0.28) + 0.85\,(\pm 0.41)\alpha - 5.38\,(\pm 0.66)\pi^* \tag{11b}$$

$$N = 10,\ \mathrm{rss} = 2.61 \times 10^{-3},\ \mathrm{ose} = 1.93 \times 10^{-2},\ r^2 = 0.99,\ f = 444.81$$

$$\log K_2(\mathrm{GMP}) = 1.66\,(\pm 0.07) + 0.17\,(\pm 0.10)\alpha + 0.60\,(\pm 0.0.16)\pi^* \tag{11c}$$

$$N = 10,\ \mathrm{rss} = 1.49 \times 10^{-4},\ \mathrm{ose} = 4.62 \times 10^{-3},\ r^2 = 0.99,\ f = 352.85$$

$$\log K_1(\mathrm{GMP}) = 13.18\,(\pm 0.33) + 1.38\,(\pm 0.49)\alpha - 7.70\,(\pm 0.78)\pi^* \tag{11d}$$

$$N = 10,\ \mathrm{rss} = 3.64 \times 10^{-3},\ \mathrm{ose} = 2.28 \times 10^{-2},\ r^2 = 0.99,\ f = 602.92$$

$$\log K_{-\mathrm{H}}(\mathrm{GMP}) = 11.77\,(\pm 1.78) + 1.43\,(\pm 1.10)\beta - 2.67\,(\pm 1.08)\pi^* \tag{11e}$$

$$N = 10,\ \mathrm{rss} = 4.01 \times 10^{-3},\ \mathrm{ose} = 2.39 \times 10^{-2},\ r^2 = 0.99,\ f = 290.96$$

$$\log K_2(\mathrm{IMP}) = -2.14\,(\pm 0.23) + 1.09\,(\pm 0.34)\alpha + 2.00\,(\pm 0.55)\pi^* \tag{11f}$$

$$N = 10,\ \mathrm{rss} = 1.83 \times 10^{-3},\ \mathrm{ose} = 1.62 \times 10^{-2},\ r^2 = 0.99,\ f = 534.85$$

$$\log K_1(\mathrm{IMP}) = 16.95\,(\pm 0.69) - 2.79\,(\pm 1.02)\alpha - 6.60\,(\pm 1.62)\pi^* \tag{11g}$$

$$N = 10,\ \mathrm{rss} = 1.59 \times 10^{-2},\ \mathrm{ose} = 4.76 \times 10^{-2},\ r^2 = 0.99,\ f = 538.05$$

$$\log K_{-\mathrm{H}}(\mathrm{IMP}) = 12.10\,(\pm 1.55) + 2.21\,(\pm 0.95)\beta - 3.66\,(\pm 0.94)\pi^* \tag{11h}$$

$$N = 10,\ \mathrm{rss} = 3.03 \times 10^{-3},\ \mathrm{ose} = 2.08 \times 10^{-2},\ r^2 = 1.00,\ f = 791.61$$

The *KAT* equation contains non-specific as well as specific solute–solvent interactions separately, and the latter should be subdivided into solvent–*Lewis* acid interactions (H-bond acceptor (HBA) solute, and H-bond donor (HBD) solvent) and solvent–*Lewis* base interactions (HBD solute, HBA solvent). In general, these parameters constitute more comprehensive measures of solvent polarity than the dielectric constant, ε, alone, because they reflect more reliably the complete picture of all intermolecular forces acting between solute and solvent molecules. This approach has been widely and successfully applied in the correlation analysis of all kind of solvent-dependent processes [10]. Using the solvatochromic solvent parameters, α, β,

Table 2. *Solvatochromic Parameters and Dielectric Constants of Different Aqueous MeOH Mixtures at 25°*

MeOH [%]	α	β	π^*	E_T^N	ε
0	1.23	0.49	1.14	1.00	79.5
10	1.19	0.51	1.13	0.96	76.4
15	1.17	0.53	1.12	0.94	74.5
20	1.14	0.54	1.10	0.92	72.1
25	1.11	0.56	1.09	0.91	71.0
30	1.08	0.57	1.07	0.89	68.3
35	1.06	0.59	1.06	0.88	67.2
40	1.04	0.60	1.04	0.87	65.6
45	1.02	0.62	1.02	0.85	63.0
50	1.01	0.63	1.00	0.84	60.8

and π^*, which have been introduced in previous report [16], the multiparameter equation, *Eqn. 12*, has been proposed for use in the so-called linear solvation energy relationship.

$$\log K = A_0 + a\alpha + b\beta + p\pi^* \tag{12}$$

where A_0 represents the regression value, and π^* is the index of the solvent dipolarity/polarizability, which is a measure of the ability of a solvent to stabilize a charge or a dipole by its own dielectric effects. The α coefficient represents the solvent H-bond donor (HBD) acidity, in other words, it describes the ability of a solvent to donate a H-atom to a solute H-bond. The β coefficient is a measure of a solvent H-bond acceptor (HBA) basicity, and describes the ability of a solvent to accept a H-atom from a solute to a solvent H-bond. The regression coefficients a, b, and p are the relative susceptibilities of the solvent-dependence of log K on the indicated solvent parameters.

The procedure used in the regression analysis involves a rigorous statistical treatment to find out which parameter in *Eqn. 12* is best-suited to the H_2O/organic solvent mixtures. So, a stepwise procedure and least-squares analysis were applied to select the significant solvent properties to be influenced in the model and to obtain the final expression for the protonation constants. Therefore, the *KAT* equation was reduced to single, dual, and multi parameters for correlation analysis of log K in various solvent mixtures. The computer program used can give the values of A_0, a, b, p, and some statistical parameters including r^2 coefficient, standard deviation of any parameter, given in brackets, and the overall standard error, ose of log K. The *KAT* parameters have been taken from our previous report [16].

Although the solvent polarity is identified as the main reason of the variation of log K values in H_2O/organic solvent mixtures, the results, showing single-parameter correlations of log K values individually with π^*, did not give good results in all cases. However, the correlation analysis of log K values with dual-parameter equations (*Eqn. 12*), indicates significant improvement with regard to the single- or multi-parameter models which is also consistent with the results obtained with normalized polarity parameter, E_T^N, regression analysis.

In correlation analysis of the dual parameter of the *KAT* equation of log K_2, the coefficient of π^* has a major role in all cases (*ca.* 64, 78, and 72% for AMP, GMP, and IMP, resp.). The positive sign of π^* shows that log K_2 values should decrease with decreasing the polarity of the medium, which is consistent with obtained results. This is supported by the positive sign of α, indicating that a decrease in the HBD acidity of the solvent causes a decrease in the solvation tendency of the neutral species formed. The coefficient of π^* in correlation analysis of log K_1 has a major role for all cases (more than 70%); its negative sign indicates that a decrease in the polarity of the solutions increases the log K_1 values. The coefficient of π^* in the correlation analysis of the dual parameter of the *KAT* equation, in the case of log $K_{-\mathrm{H}}$ of GMP and IMP, is negative and again has a major role (more than 60%). This indicates that log $K_{-\mathrm{H}}$ values should increase with decreasing polarity of the medium. This is also supported by the positive sign of β, which indicates that an increase in the HBA basicity of the solvent increases the solvation tendency of the produced anion and makes it more likely.

Experimental Part

Generals. AMP, GMP, and IMP were obtained from *Fluka* as anal. reagent-grade materials and used without further purification. MeOH was from *Merck* (reagent grade) and was used as received. $NaClO_4$ was from *Merck* and was dried under vacuum at r.t. for at least 72 h before use. NaOH soln. was prepared from a titrisol soln. (*Merck*). $HClO_4$ was from *Merck* and was used as supplied. All dil. solns. were prepared from double-distilled H_2O with a conductance equal to 1.3 ± 0.1 µS.

The electromotive force (emf) was measured with a *Metrohm model 781* pH ion-meter. A combined glass-pH electrode (model 6.0258.000) was modified by replacing its aq. KCl soln. with 0.01 $\mathrm{mol \cdot dm^{-3}}$ NaCl + 0.09 $\mathrm{mol \cdot dm^{-3}}$ $NaClO_4$ saturated with AgCl. The electrode was soaked for 15–20 min in a H_2O/org. solvent mixture before the potentiometric measurements. All titrations were carried out in a 80-ml thermostated double-walled glass vessel.

Spectrophotometric measurements were performed with a UV/Vis *Shimadzu 2100* spectrophotometer with a *Pentium 4* computer and using thermostated 10-mm quartz cells. The measurement cell was of the flow type. A peristaltic pump allowed circulation of the soln. under study from the potentiometric cell to the spectrophotometric cell, so the absorbance and the emf of the soln. could be measured simultaneously. To exclude CO_2 from the system, a stream of purified N_2 was passed through a NaOH soln. and then bubbled slowly through the reaction soln.

Procedure. All measurements were performed at 25° and a constant ionic strength (0.1 $\mathrm{mol \cdot dm^{-3}}$ $NaClO_4$. The protonation constants were determined from the measurements of absorbance *vs.* emf by titration of 25 ml of the nucleosides ($(1.0-3.0) \cdot 10^{-3}$ $\mathrm{mol \cdot dm^{-3}}$) with 0.1 $\mathrm{mol \cdot dm^{-3}}$ NaOH soln. both with the same ionic strength and mole fraction of org. solvent (0–50% MeOH).

In the first step, the electrode system calibration was performed by *Gran*'s method [31]. For this purpose, a measured amount of an acidic soln., to be used at the same temp., ionic strength and solvent composition in later experiments, was placed in the double-walled thermostated vessel. The electrode was immersed in the soln. in the vessel, and the acidic soln. was titrated with a strong base (0.1 $\mathrm{mol \cdot dm^{-3}}$ NaOH). The potential was allowed to stabilize after each addition of the titrant, and the recorded emf values were then used to obtain E°. The procedure was continued to pH of *ca.* 2. In the second step, 25 ml of an acidic soln. (0.01 $\mathrm{mol \cdot dm^{-3}}$ $HClO_4$) of the nucleosides ($(1.0$ to $3.0) \cdot 10^{-3}$ $\mathrm{mol \cdot dm^{-3}}$) at the same temp., ionic strength, and solvent composition was titrated with a NaOH soln. (0.1 $\mathrm{mol \cdot dm^{-3}}$). The emf and the absorbance (in the interval of (250 to 310) nm) values were then determined. The procedures were repeated with different proportions of the org. solvent.

The recorded emf values were then converted to $\mathrm{p}C_\mathrm{H}$ ($-\log [\mathrm{H}^+]$) by a method described in [32]. The measured potential of the cell, E_cell, glass elec./$HClO_4$-$NaClO_4$ in H_2O/org. solvent // NaCl-$NaClO_4$/Ag-AgCl, can be formulated as

$$E_{\mathrm{cell}}\ [\mathrm{mV}] = E^{0}_{\mathrm{cell}} + k \cdot \log\ [\mathrm{H}^{+}] + k \cdot \log\ \gamma_{\mathrm{H}^{+}} + E_{\mathrm{LJ}} \quad (13)$$

where E^{0}_{cell} is the standard potential of the cell, E_{LJ} is the liquid–junction potential, $k = 2.303$ RT/F in which R, T and F have the usual meanings, and $\gamma_{\mathrm{H}^{+}}$ is the activity coefficient of H^{+}. Difficulties in computing the activity coefficients of H^{+} in various aqueous mixtures of organic solvents lead to measurement of emf *vs.* H^{+} concentration in solution. Because the ionic strength of the solution is kept constant, the activity coefficient of H^{+} is constant too. The non-ideality of solutions is then included in E'_{a} (the specific constant of the potentiometric cell in the acidic region):

$$E_{\mathrm{cell}} = E'_{\mathrm{a}} + k \cdot \log\ [\mathrm{H}^{+}] \quad (14)$$

where E'_{a} is $E^{0}_{\mathrm{cell}} + k \cdot \log\ \gamma_{\mathrm{H}^{+}} + E_{\mathrm{LJ}}$. The use of a glass electrode (with an aqueous inner solution) in nonaqueous media introduces a deviation from ideality. But it has been shown that the deviation is negligible, and the glass electrode is always usable in such media to measure H^{+} concentrations with a linear relation of E_{cell} *vs.* log $[\mathrm{H}^{+}]$ [33][34]. The H^{+} concentration can be expressed as

$$[\mathrm{H}^{+}] = (M_{\mathrm{HClO_4}} V_0 - M_{\mathrm{NaOH}} V_1)/(V_0 + V_1) \quad (15)$$

where $M_{\mathrm{HClO_4}}$ and M_{NaOH} are the molarities of $\mathrm{HClO_4}$ and NaOH, resp., V_0 and V_1 are the initial volume of $\mathrm{HClO_4}$ and the added volume of NaOH soln., resp. Finally

$$\mathrm{pC_H} = (E'_{\mathrm{a}} - E_{\mathrm{cell}})/k \quad (16)$$

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