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Comparison of the Acid–Base Properties of Ribose and 2′-Deoxyribose Nucleotides

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Dedicated to Professor Jan Reedijk on the occasion of his 65th birthday

Abstract: The extent to which the replacement of a ribose unit by a 2′-deoxyribose unit influences the acid–base properties of nucleotides has not hitherto been determined in detail. In this study, by potentiometric pH titrations in aqueous solution, we have measured the acidity constants of the 5′-di- and 5′-triphosphates of 2′-deoxyguanosine [i.e., of $H_2(dGDP)^-$ and $H_2(dGTP)^{2-}$] as well as of the 5′-mono-, 5′-di-, and 5′-triphosphates of 2′-deoxyadenosine [i.e., of $H_2(dAMP)^{\pm}$, $H_2(dADP)^-$, and $H_2(dATP)^{2-}$]. These 12 acidity constants (of the 56 that are listed) are compared with those of the corresponding ribose derivatives (published data) measured under the same experimental conditions. The results show that all protonation sites in the 2′-deoxynucleotides are more basic than those in their ribose counterparts. The influence of the 2′-OH group is dependent on the number of 5′-phosphate groups as well as on the nature of the purine nucleobase. The basicity of N7 in guanine nucleotides is most significantly enhanced (by about 0.2 pK units), while the effect on the phosphate groups and the N1H or $N1H^+$ sites is less pronounced but clearly present. In addition, ^{1}H NMR chemical shift change studies in dependence on pD in D_2O have been carried out for the dAMP, dADP, and dATP systems, which confirmed the results from the potentiometric pH titrations and showed the nucleotides to be in their *anti* conformations. Overall, our results are not only of relevance for metal ion binding to nucleotides or nucleic acids, but also constitute an exact basis for the calculation, determination, and understanding of perturbed pK_a values in DNAzymes and ribozymes, as needed for the delineation of acid–base mechanisms in catalysis.

Keywords: acidity · nucleic acids · nucleotides · purines · ribozymes

Introduction

Ribose and 2′-deoxyribose residues are the most prevalent sugar moieties in Nature.[1] They are usually part of nucleotides, which themselves are the building blocks of ribonucleic acids (RNA) and 2′-deoxyribonucleic acids (DNA).[1] It is well known that DNA is less sensitive to hydrolysis of the phosphate diester backbone than RNA due to the absence of the 2′-hydroxy group.[2,3] In a recent study on model mononucleotides,[4] it was shown that the presence or absence of the 2′-OH group affects the electronic properties of the nucleotide. Hence, the acid–base properties of the nucleobase residues in DNA and RNA are also expected to be affected. On this basis, it was concluded that RNA–RNA duplexes are more stable than DNA–DNA duplexes.[4] Surprisingly, however, crucial information on the effects, if any, exerted by the ribose and 2′-deoxyribose residues on the acid–base properties of nucleotides is still lacking.[5–7] Detailed knowledge on thus fundamental property of nucleobases is of relevance for several reasons: i) the potential to form hydrogen bonds,[8,9] as well as metal ion complexes,[10–12] is dependent on the acid–base properties; ii) only with this information can one compare certain properties of RNA and DNA;[13,14] iii) catalysis by several ribozymes,[15,16] as well as by DNAzymes,[17,18] is considered to be based on an acid–base mechanism with nucleobase functionalities acting as general acids and/or bases at physiological pH. The latter can only take place if the pK_a values of the nucleic acid building blocks are strongly perturbed, although the reasons for this remain largely unknown. Knowledge of the exact pK_a values of both deoxyribose and ribose mononucleotides, and of the ef-

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fects of any modification[8,9,19] or functionalisation, for example, of the 2′-OH group, would hence provide a basis for calculating[20] and rationalising the modulation of pK_a values in complex nucleic acid structures.

In the present study, we compare the acid–base properties of adenine and guanine nucleotides containing either a ribose or a 2′-deoxyribose moiety (Figure 1). To this end, the pK_a values were determined by potentiometric pH titrations and verified by NMR chemical shift measurements in D_2O. The results obtained by the two methods were found to be in good agreement. The results show that the basicity of N7 in the guanine nucleotides is affected most: replacing the 2′-OH group by a hydrogen atom increases the basicity of this site by about 0.2 p*K* units. The N1 site of adenine nucleotides also becomes more basic when ribose is replaced by 2′-deoxyribose, although the effect is small. The other acid–base sites are only slightly affected, although the terminal γ-phosphate groups of $dATP^{4-}$/$dGTP^{4-}$ are found to be more basic than those of ATP^{4-}/GTP^{4-} by about 0.1 p*K* units. The listed acidity constants also allow additional comparisons to be made, for example concerning the effects of differently sized phosphate chains on the acid–base properties of the nucleobase residues.

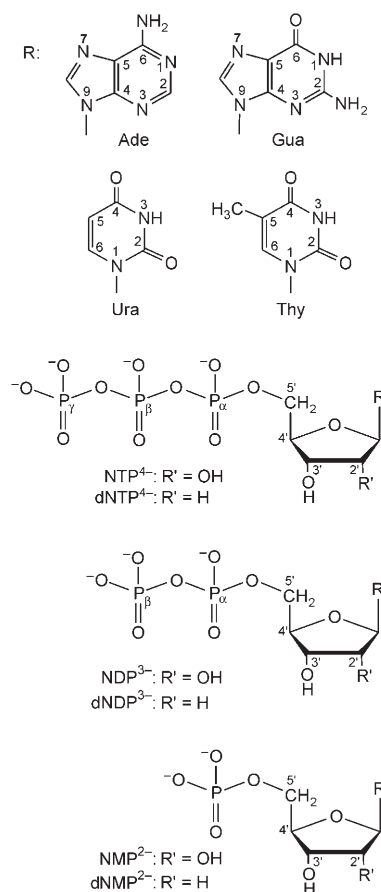
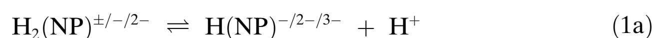


Figure 1. Chemical structures of the nucleobase residues adenine (Ade), guanine (Gua), uracil (Ura), and thymine (Thy), all of which occur in this study, are given at the top. Below, from top to bottom, the structures of the nucleoside and 2′-deoxynucleoside 5′-triphosphates (NTP^{4-}, $dNTP^{4-}$), 5′-diphosphates (NDP^{3-}, $dNDP^{3-}$), and 5′-monophosphates (NMP^{2-}, $dNMP^{2-}$) are shown. Attachment of the nucleobase residues (R) in the depicted orientation to the 1′-position of the (2′-deoxy)nucleoside 5′-phosphates results in the *anti* conformation. Besides the (2′-deoxy)nucleosides, adenosine (Ado), 2′-deoxyadenosine (dAdo), guanosine (Guo), and 2′-deoxyguanosine (dGuo), the following nucleotides (given with their abbreviations) have also been studied: adenosine 5′-mono-, di-, and triphosphate (AMP^{2-}, ADP^{3-}, ATP^{4-}); 2′-deoxyadenosine 5′-mono-, di-, and triphosphate ($dAMP^{2-}$, $dADP^{3-}$, $dATP^{4-}$); guanosine 5′-mono-, di-, and triphosphate (GMP^{2-}, GDP^{2-}, GTP^{4-}); 2′-deoxyguanosine 5′-mono-, di-, and triphosphate ($dGMP^{2-}$, $dGDP^{2-}$, $dGTP^{4-}$); uridine 5′-mono-, di-, and triphosphate (UMP^{2-}, UDP^{3-}, UTP^{4-}); and thymidine 5′-mono-, di-, and triphosphate ($dTMP^{2-}$, $dTDP^{3-}$, $dTTP^{4-}$).

Results

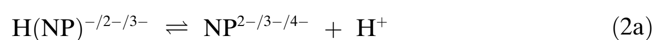
Potentiometric pH titrations: To cover the acid–base reactions of the nucleotides under focus in this study, we considered the pH range between about 2.5 and 10. The lower limit is determined by the deprotonation of the $N7H^+$ site of $H_2(GMP)^{\pm}$ (Figure 1), which occurs with $pK_a \approx 2.5$, while the upper limit is determined by the deprotonation of the N3H site of $dTTP^{4-}$, which has a pK_a of about 10.1 (see below). Consequently, the release of the primary protons from phosphate residues is beyond the scope of this study because these protons are released from the twofold protonated triphosphate chain, $P_3O_{10}H_2^{2-}$, of $H_3(ATP)^-$ and $H_3(GTP)^-$ with pK_a values of 1.7 ± 0.1 and 1.3 ± 0.2, respectively.[21] The release of the protons from the $P_2O_7H_2^-$ and $PO_2(OH)_2$ residues of related nucleoside 5′-di- and monophosphates occurs at even lower pK_a values.[22–24]

By using $H_2(GMP)^{\pm}$ as an example, three deprotonation reactions can occur in the aforementioned pH range, namely from the $N7H^+$ site, from the $PO_3(OH)^-$ group, and from the N1H unit. Hence, the following three general deprotonation reactions can be expressed for nucleoside 5′-mono-, di-, and triphosphates ($NP^{2-/3-/4-}$):



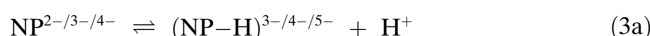
$$H_2(NP)^{\pm/-/2-} \rightleftharpoons H(NP)^{-/2-/3-} + H^+ \quad (1a)$$

$$K^H_{H_2(NP)} = [H(NP)^{-/2-/3-}][H^+]/[H_2(NP)^{\pm/-/2-}] \quad (1b)$$



$$H(NP)^{-/2-/3-} \rightleftharpoons NP^{2-/3-/4-} + H^+ \quad (2a)$$

$$K^H_{H(NP)} = [NP^{2-/3-/4-}][H^+]/[H(NP)^{-/2-/3-}] \quad (2b)$$



$$NP^{2-/3-/4-} \rightleftharpoons (NP-H)^{3-/4-/5-} + H^+ \quad (3a)$$

$$K^H_{NP} = [(NP-H)^{3-/4-/5-}][H^+]/[NP^{2-/3-/4-}] \quad (3b)$$

It should be noted that $(NP-H)^{3-/4-/5-}$ in Equilibrium (3a) is to be read as "NP *minus* H", meaning that the N1H site of a guanine residue or the N3H site of a uracil/thymine residue loses its proton. Depending on which nucleobase is considered, only some of the above equilibria hold. For guanine nucleotides, Equilibria (1a), (2a), and (3a) hold, whereas for adenine nucleotides only Equilibria (1a) and (2a) are of relevance, covering the deprotonation of the $N1H^+$ site [Eq. (1)] and of the monoprotonated phosphate group

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[Eq. (2); cf. also Figure 1]. In the case of uracil/thymine nucleotides, only Equilibria (2a) and (3a) need to be considered, quantifying the release of the proton from the monoprotonated phosphate residue [Eq. (2)] and the deprotonation of the N3H site [Eq. (3)].

In this study, we have determined a total of 12 new acidity constants, that is, those of $H_2(dGDP)^-$, $H_2(dGTP)^{2-}$, $H_2(dAMP)^{\pm}$, $H_2(dADP)^-$, and $H_2(dATP)^{2-}$ (Figure 1), by potentiometric pH titrations[25] (Table 1). The acidity constants of the corresponding ribose derivatives, which are needed for comparison and discussion, have been taken from the literature,[21–23,26–32] as have those of several related nucleotides.[21,24,27,29,33] All of these constants are summarised in Table 1.

In the 2′-deoxy series, only a few values have been published previously,[5–7] namely those for $H_2(dAMP)^{\pm}$ and $H_2(dGTP)^{2-}$ (as well as for $H_2(dGMP)^{\pm}$). Those for the guanine derivatives[34,35] are roughly about 0.1 to 0.2 pK units higher than the values listed in Table 1, the differences most likely originating from different calibration procedures of the electrodes used. The values measured by Stuehr et al.[36] for $H_2(dAMP)^{\pm}$: $pK^H_{H_2(dAMP)} = 3.99$ and $pK^H_{H(dAMP)} = 6.27$ (15 °C; $I = 0.1$ M, KNO_3) are in excellent agreement with ours listed in Table 1 (entry 4), despite the different temperatures used in the experiments. The internal consistency of the values listed in Table 1 is evident from the interrelations between the various acidity constants shown in Figure 2, where the $pK_{a/PO_2(OH)}$ and $pK_{a/N1H}$ values of the guanine derivatives are plotted in dependence on the $pK_{a/N7H}$ constants. The various acid–base sites are expected to affect each other in a systematic way, and this is nicely observed. A plot of $pK_{a/PO_2(OH)}$ versus $pK_{a/N1H}$ for the adenine nucleotides leads to the corresponding observation.

For completeness of Table 1 and for some comparisons, it is helpful to also include the acidity constants of the nucleosides. However, because we are aware[5–7] of only an estimated value ($pK_a = 3.8$)[37] for 2′-deoxyadenosine under the present conditions, we preferred to estimate the corresponding pK_a value for $N1H^+$ deprotonation ourselves, as described in the following. The influence of the 2′-OH group on the acid–base properties of guanine compounds is manifested in the acidity differences, $\Delta pK_a = pK^H_{H(dGuo)} - pK^H_{H(Guo)} = (2.30\pm0.04)-(2.11\pm0.04) = 0.19\pm0.06$ and $\Delta pK_a = pK^H_{H_2(dGMP)} - pK^H_{H_2(GMP)} = (2.69\pm0.03)-(2.48\pm0.04) = 0.21\pm0.05$ (Table 1; entries 1 and 3). These values are identical within their error limits, meaning that the difference in N7 basicity is the same for the guanosine/2′-deoxyguanosine and $H(GMP)^-$/$H(dGMP)^-$ pairs, even though the absolute pK_a values differ. Hence, the same equality can be expected for the adenosine/2′-deoxyadenosine and $H(AMP)^-$/$H(dAMP)^-$ pairs, from which it follows that: $pK^H_{H(dAdo)} = pK^H_{H(Ado)} + (pK^H_{H_2(dAMP)} - pK^H_{H_2(AMP)}) = (3.61\pm0.03) + [(3.97\pm0.02)-(3.84\pm0.02)] = 3.74\pm0.04$ (entry 2 in Table 1).

As a control of the obtained values, we can compare the difference for the $H(AMP)^-$/$H(dAMP)^-$ pair, $pK^H_{H_2(dAMP)} - pK^H_{H_2(AMP)} = (3.97\pm0.02)-(3.84\pm0.02) = 0.13\pm0.03$ (Table 1, entry 4) with that observed for the cytosine nucleotides,[33] that is, $pK^H_{H_2(dCMP)} - pK^H_{H_2(CMP)} = (4.46\pm0.01)-(4.33\pm0.04) = 0.13\pm0.04$. These two values are equal, which is to be expected because both the purine and

Table 1. Comparison of the negative logarithms of the acidity constants [Eqs. (1–3)] as determined by potentiometric titrations in aqueous solution for guanosine/2′-deoxyguanosine and adenosine/2′-deoxyadenosine nucleotides, together with corresponding data for some related nucleosides and nucleotides (25 °C; $I = 0.1$ M, $NaNO_3$).[a,b]

No.	Acid	pK_a values for the sites		
		$N1H^+$ or $N7H^+$	$PO_2(OH)^-$	N1H or N3H
	N/dN	N/dN	N/dN	N/dN
1[c,d]	$H(Guo)^+$/$H(dGuo)^+$	2.11±0.04/2.30±0.04		9.22±0.01/9.24±0.03
2[c,e]	$H(Ado)^+$/$H(dAdo)^+$	3.61±0.03/3.74±0.04		
3[c,f]	$H_2(GMP)^{\pm}$/$H_2(dGMP)^{\pm}$	2.48±0.04/2.69±0.03	6.25±0.02/6.29±0.01	9.49±0.02/9.56±0.02
4[c,g]	$H_2(AMP)^{\pm}$/$H_2(dAMP)^{\pm}$	3.84±0.02/3.97±0.02	6.21±0.01/6.27±0.04	
5[h,i]	$H_2(CMP)^{\pm}$/$H_2(dCMP)^{\pm}$	4.33±0.04/4.46±0.01	6.19±0.02/6.24±0.01	
6[h]	$H(UMP)^-$/–		6.15±0.01/–	9.45±0.02/–
7[h]	–/$H(dTMP)^-$		–/6.36±0.01	–/9.90±0.03
8[j]	$H(NMP)^-$/$H(dNMP)$		6.20±0.05/6.27 ±0.05	
9[k,g]	$H_2(GDP)^-$/$H_2(dGDP)^-$	2.67±0.02/2.91±0.07	6.38±0.01/6.46±0.03	9.56±0.03/9.64±0.04
10[l,g]	$H_2(ADP)^-$/$H_2(dADP)^-$	3.92±0.02/4.00±0.03	6.40±0.01/6.45±0.01	
11[m]	$H(UDP)^{2-}$/–		6.38±0.02/–	9.47±0.02/–
12[m]	–/$H(dTDP)^{2-}$		–/6.44±0.01	–/9.93±0.02
13[n]	$H(NDP)^{2-}$/–		6.39±0.02/–	
14[o,g]	$H_2(GTP)^{2-}$/$H_2(dGTP)^{2-}$	2.94±0.02/3.16±0.05	6.50±0.02/6.64±0.02	9.57±0.02/9.66±0.04
15[o,g]	$H_2(ATP)^{2-}$/$H_2(dATP)^{2-}$	4.00±0.01/4.14±0.02	6.47±0.01/6.62±0.03	
16[o]	$H(UTP)^{3-}$/–		6.48±0.02/–	9.57±0.02/–
17[o]	–/$H(dTTP)^{3-}$		–/6.52±0.02	–/10.08±0.05
18[p]	$H(NTP)^{3-}$/–		6.49±0.05/–	

[a] So-called practical (or mixed) constants[25] are listed; see the Experimental Section. [b] The error limits are *three times* the standard error of the mean value (3σ) or the sum of the probable systematic errors, whichever is the larger. [c] From ref. [26]. [d] From ref. [27]. [e] See the Results Section. [f] From ref. [28]. [g] This study. [h] From ref. [29]; the deprotonation site in $H_2(CMP)^{\pm}$ is $N3H^+$. [i] From ref. [33]; the deprotonation site in $H_2(dCMP)^{\pm}$ is $N3H^+$. [j] Average of the five values measured for $H(UMP)^-$ (6.15±0.01),[29] $H(CMP)^-$ (6.19±0.02),[29] $H(AMP)^-$ (6.21±0.01),[26] $H(IMP)^-$ (6.22±0.01),[26] and $H(GMP)^-$ (6.25±0.02),[26] and of the three values measured for $H(dGMP)^-$ (6.29±0.01),[28] $H(dAMP)^-$ (6.27±0.04),[g] and $H(dCMP)^-$ (6.24±0.01).[33] [k] From ref. [30]; see also refs. [22,31,32]. [l] From ref. [23]. [m] From ref. [24]. [n] Average of the five values measured for $H(UDP)^{2-}$ (6.38±0.02),[24] $H(CDP)^{2-}$ (6.39±0.02),[24] $H(ADP)^{2-}$ (6.40±0.01),[23] $H(IDP)^{2-}$ (6.38±0.02),[k] and $H(GDP)^{2-}$ (6.38±0.01).[k] [o] Taken from the list given in ref. [21]. [p] Average of the five values measured for $H(UTP)^{3-}$ (6.48±0.02), $H(CTP)^{3-}$ (6.55±0.02), $H(ATP)^{3-}$ (6.47±0.01), $H(ITP)^{3-}$ (6.47±0.02), and $H(GTP)^{3-}$ (6.50±0.02); see list in ref. [21].

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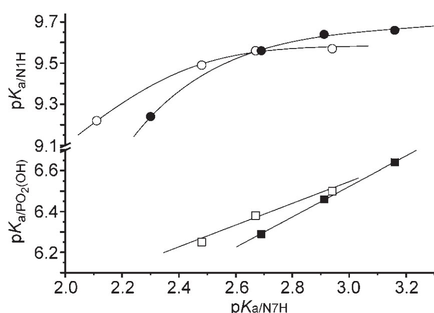

Figure 2. Interrelations between the various acidity constants for the guanine derivatives. Plots of the $pK_{a/N1H}$ (upper part) and $pK_{a/PO_2(OH)}$ (lower part) values in dependence on $pK_{a/N7H}$ (constants from Table 1). The lines represent the calculated best fits. Upper part: The open circles refer to guanosine, GMP, GDP, and GTP (from left to right) and the filled circles to the corresponding 2′-deoxy compounds. Lower part: The open squares refer to GMP, GDP, and GTP (from left to right) and the filled squares to the corresponding 2′-deoxy compounds.

pyrimidine moieties in $H_2(AMP)^{\pm}/H_2(dAMP)^{\pm}$ and $H_2(CMP)^{\pm}/H_2(dCMP)^{\pm}$ are in the *anti* conformation. Consequently, the $N1H^+$ and $N3H^+$ sites are directed away from the sugar ring and its 2′-OH/H position. In contrast, $N7H^+$ in $H_2(GMP)^{\pm}/H_2(dGMP)^{\pm}$ is spatially much closer to 2′-OH/H, and hence the effect is larger, that is, $pK^H_{H_2(dGMP)} - pK^H_{H_2(GMP)} = (2.69\pm0.03)-(2.48\pm0.04) = 0.21\pm0.05$ (Table 1, entry 3).

The reliability of the constants assembled in Table 1 is further confirmed by the following comparison. The acidity constants measured for the deprotonation of $N1H^+$ in the cyclic 3′,5′-phosphate diester, $H(cAMP)^{\pm}$ ($pK_a = 3.80\pm0.02$; estimated error),[38] and in the $H_2(AMP)^{\pm}$ species ($pK^H_{H_2(AMP)} = 3.84\pm0.02$; Table 1, entry 4) bearing a 5′-$OPO_2(OH)^-$ residue are virtually identical. They are even more so if one converts the given concentration constant[38] for $H(cAMP)^{\pm}$ into the so-called practical constant (Table 1; footnote [a]),[25] giving $pK^H_{H(cAMP)} = (3.80\pm0.02) + 0.02 = 3.82\pm0.02$. In fact, comparing $pK^H_{H(cAMP)} - pK^H_{H(Ado)} = (3.82\pm0.02)-(3.61\pm0.03) = 0.21\pm0.04$ with $pK^H_{H_2(AMP)} - pK^H_{H(Ado)} = (3.84\pm0.02)-(3.61\pm0.03) = 0.23\pm0.04$ shows that the basicity increasing effects of the two singly negatively charged phosphate residues (lying at about the same distance from N1) are identical within the error limits.

^{1}H NMR shift measurements: Potentiometric pH titrations allow the determination of acidity constants in an exact manner, but this method does not provide any information about the sites from which the protons are released. This, however, is not the case with ^{1}H NMR shift experiments.[39] Although the assignment of the sites for the "simple" systems considered here is not a problem because the binding sites of the protons are known (see the preceding section and Table 1), we measured the chemical shift data to check the compatibility of the two methods and to possibly gain additional structural information. For the NMR experiments, we selected the 2′-deoxyadenosine 5′-phosphates for two reasons: i) compared to the 2′-deoxyguanosine 5′-phosphates, one more proton is available at the nucleobase residue, that is, H2 (see Figure 1); ii) some information on other adenine nucleotides is available,[39–41] which could be helpful for making comparisons. Hence, we measured the chemical shifts of the various protons in dependence on pD for $H_2(dAMP)^{\pm}$, $H_2(dADP)^-$, and $H_2(dATP)^{2-}$ in D_2O, with the aim of fitting these shift data to the acidity constants determined in water.

Acidity constants valid for H_2O as solvent can be transformed into the corresponding acidity constants valid for D_2O as solvent[8,9] by means of Equation (4):[42]

$$pK_{a/D_2O} = (1.015\,pK_{a/H_2O}) + 0.45 \quad (4)$$

Transformation of the acidity constants of the 2′-deoxyadenine series obtained by potentiometric pH titrations in H_2O (Table 1) by applying Equation (4) gave the following results:

$$D_2(dAMP)^{\pm}: \quad pK^D_{D_2(dAMP)} = 4.48 \text{ and } pK^D_{D(dAMP)} = 6.81 \quad (5)$$

$$D_2(dADP)^{-}: \quad pK^D_{D_2(dADP)} = 4.51 \text{ and } pK^D_{D(dADP)} = 7.00 \quad (6)$$

$$D_2(dATP)^{2-}: \quad pK^D_{D_2(dATP)} = 4.65 \text{ and } pK^D_{D(dATP)} = 7.17 \quad (7)$$

The dependences on pD of the chemical shifts of H2, H8, H1′, H2′, H2″, H5′, and H5″ for all three $D_2(dNP)^{\pm/-/2-}$ systems are shown in Figure 3. The fits with the abovementioned pK_{a/D_2O} values are excellent for all three systems. The solid curves in Figure 3 are the calculated best fits of the experimental data by applying the above pK_{a/D_2O} values [Eqs. (5)–(7)]. The chemical shift data for the $H_2(dNP)^{\pm/-/2-}$, $H(dNP)^{-/2-/3-}$, and $dNP^{2-/3-/4-}$ species obtained by these calculations are assembled in Table 2, together with those for the $H_2(AMP)^{\pm}$ system,[39] which was studied earlier (note that for simplification we write here, as well as in Table 2, the proton (H^+) instead of the deuteron (D^+)).

The resonances were assigned by means of [^{1}H,^{1}H]-ROESY (rotating frame nuclear Overhauser effect spectroscopy) experiments. For example, of the two multiplets between $\delta = 2.5$ and 2.9 ppm, attributable to H2′ and H2″, only the downfield resonance shows a cross-peak with H8 of the adenine residue as well as with H5′. This pattern befits H2′, as this proton is located above the sugar plane. The upfield resonance of H2″, on the other hand, exhibits stronger couplings with H1′ and H4′, since all three are located below the sugar plane. The other resonances could be assigned analogously.

Comparison of the chemical shifts of the various protons in the $H_2(dNP)^{\pm/-/2-}$ species and $H_2(AMP)^{\pm}$ (Figure 3 and Table 2) reveals that the chemical shifts of H2 are practically identical (average $\delta_H = 8.443$ ppm). This means that the re-

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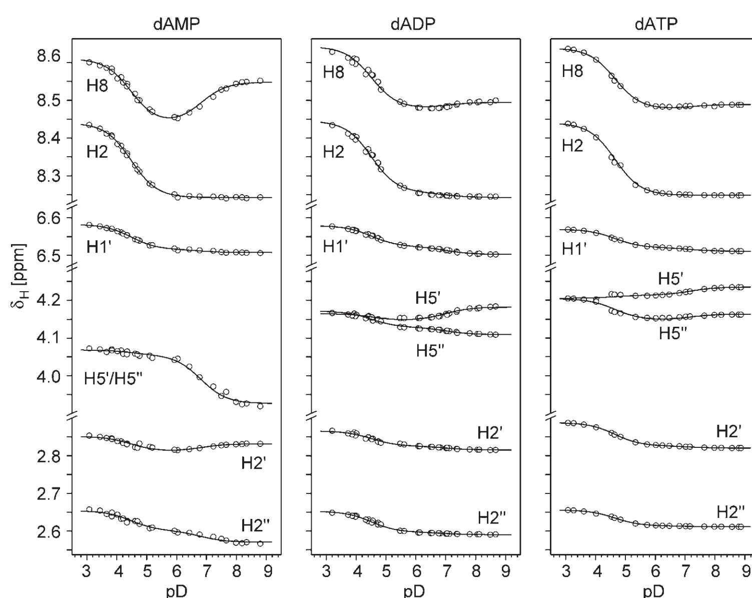

Figure 3. Variation of the chemical shift δ_H for protons of dAMP, dADP, and dATP (1 mM) in dependence on pD (25 °C; I = 0.1 M, $NaNO_3$; see also the Experimental Section). The solid curves are the calculated best fits of the experimental data using the pK_{a/D_2O} values given in the text [Eqs. (5)–(7)].

Table 2. Chemical shifts (in ppm) of the protons for protonated, that is, deuterated, and free forms of dAMP, dADP, and dATP, as determined in D_2O from the experiments that yielded the data plotted in Figure 3 (25 °C; I=0.1 M, $NaNO_3$).[a] The shift differences, $\Delta\delta$, resulting from increasing deprotonation of the species are also listed, that is, $\Delta\delta_2=\delta_{H_2(NP)}-\delta_{H(NP)}$ and $\Delta\delta_1=\delta_{H(NP)}-\delta_{NP}$. The corresponding data for AMP are from the literature[39] and are given for comparison (25 °C; I=0.1 M, $NaNO_3$).[b]

NP	H	$\delta_{H_2(NP)}$	$\delta_{H(NP)}$	$\delta_{(NP)}$	$\Delta\delta_2$	$\Delta\delta_1$
AMP	H2	8.447 ± 0.002	8.243 ± 0.002	8.245 ± 0.001	0.204 ± 0.003	−0.002 ± 0.002
	H8	8.637 ± 0.002	8.463 ± 0.002	8.605 ± 0.002	0.174 ± 0.003	−0.142 ± 0.003
	H1′	6.206 ± 0.001	6.129 ± 0.001	6.127 ± 0.001	0.077 ± 0.001	0.002 ± 0.001
	H5′	4.161 ± 0.004	4.137 ± 0.003	4.009 ± 0.003	0.024 ± 0.005	0.128 ± 0.004
	H5″	4.129 ± 0.004	4.105 ± 0.003	3.974 ± 0.004	0.024 ± 0.005	0.131 ± 0.005
dAMP[c]	H2	8.440 ± 0.002	8.243 ± 0.003	8.243 ± 0.002	0.197 ± 0.004	0.000 ± 0.004
	H8	8.610 ± 0.004	8.437 ± 0.005	8.549 ± 0.005	0.173 ± 0.006	−0.112 ± 0.007
	H1′	6.582 ± 0.002	6.516 ± 0.002	6.507 ± 0.001	0.066 ± 0.003	0.009 ± 0.002
	H2′	2.851 ± 0.003	2.812 ± 0.003	2.832 ± 0.003	0.039 ± 0.004	−0.020 ± 0.004
	H2″	2.654 ± 0.004	2.602 ± 0.004	2.570 ± 0.004	0.052 ± 0.006	0.032 ± 0.006
	H5′/H5″	4.068 ± 0.005	4.055 ± 0.006	3.926 ± 0.005	0.013 ± 0.008	0.129 ± 0.008
dADP	H2	8.445 ± 0.004	8.256 ± 0.004	8.243 ± 0.004	0.189 ± 0.006	0.013 ± 0.006
	H8	8.642 ± 0.006	8.478 ± 0.005	8.495 ± 0.005	0.164 ± 0.008	−0.017 ± 0.007
	H1′	6.579 ± 0.001	6.524 ± 0.002	6.503 ± 0.001	0.055 ± 0.002	0.021 ± 0.002
	H2′	2.866 ± 0.002	2.825 ± 0.002	2.815 ± 0.002	0.041 ± 0.003	0.010 ± 0.003
	H2″	2.653 ± 0.002	2.597 ± 0.002	2.590 ± 0.001	0.056 ± 0.003	0.007 ± 0.002
	H5′	4.168 ± 0.004[d]	4.138 ± 0.010[d]	4.182 ± 0.002	0.030 ± 0.010	−0.042 ± 0.010
	H5″	4.168 ± 0.004[d]	4.138 ± 0.010[d]	4.109 ± 0.002	0.030 ± 0.010	0.029 ± 0.010
dATP	H2	8.440 ± 0.005	8.249 ± 0.004	8.249 ± 0.004	0.191 ± 0.006	0.000 ± 0.006
	H8	8.638 ± 0.003	8.476 ± 0.002	8.489 ± 0.002	0.162 ± 0.004	−0.013 ± 0.003
	H1′	6.569 ± 0.001	6.520 ± 0.001	6.511 ± 0.001	0.049 ± 0.001	0.009 ± 0.001
	H2′	2.888 ± 0.001	2.826 ± 0.001	2.820 ± 0.001	0.062 ± 0.001	0.006 ± 0.001
	H2″	2.656 ± 0.001	2.613 ± 0.001	2.611 ± 0.001	0.043 ± 0.001	0.002 ± 0.001
	H5′	4.204 ± 0.004[d]	4.213 ± 0.003	4.235 ± 0.003	−0.009 ± 0.005	−0.022 ± 0.004
	H5″	4.204 ± 0.004[d]	4.148 ± 0.003	4.163 ± 0.003	0.056 ± 0.005	−0.015 ± 0.004

[a] The chemical shifts were calculated by using the values of the acidity constants (pK_{a/D_2O}) given in the text. [b] The specified error ranges for the calculated chemical shifts (δ) and the shift differences ($\Delta\delta$) are twice the standard deviation (2σ) in each case. [c] The resonance signals for H5′ and H5″ are not separated in this case. [d] At low pH, the resonance signals of H5′ and H5″ are not separated. The values given above for the limiting shifts are the averages (with an estimated error limit) of the two fits shown in Figure 3.

placement of -OH by -H at the 2′-position of the sugar residue does not affect the electron density at the H2-position of the adenine moiety. Likewise, deprotonation of the $PO_2(OH)^-$ group does not affect H2 of any of the adenine species, as exemplified by $H(dAMP)^-$. In contrast, deprotonation of the neighbouring $N1H^+$ strongly affects H2, to about the same extent in all four systems ($\Delta\delta$ ≈0.2 ppm; Table 2, column 6).

The chemical shift of H8 is also quite constant for all of the $H_2(dNP)^{\pm/-/2-}$ species, including $H_2(AMP)^{\pm}$ (Table 2). For all adenine nucleotides, deprotonation of the $N1H^+$ site leads to a significant downfield shift (average $\Delta\delta_H$ = 0.168 ppm). Deprotonation of the $PO_2(OH)^-$ groups leads to a strong change in the electron density at H8, but in the opposite sense. In the case of $H(AMP)^-$ and $H(dAMP)^-$, a significant so-called[41] "wrong way" downfield shift occurs (Table 2, column 7), which is practically unobserved with $H(dADP)^{2-}$ and $H(dATP)^{3-}$. This seems to be a general phenomenon, as indicated by analogous previous observations[43] of a weak downfield shift of the signal of H8 for $H(ATP)^{3-}$ and $H(ITP)^{3-}$ (see also point (ii) in the Discussion).

The chemical shifts of H1′ in $H_2(AMP)^{\pm}$ and $H_2(dAMP)^{\pm}$ differ significantly due to the different neighbouring 2′-OH/H sites. Likewise, deprotonation of the $N1H^+$ site affects H1′ relatively strongly in all four adenine nucleotides (Figure 3; Table 2, column 6), whereas the deprotonation of $PO_2(OH)^-$ has little or no influence (Table 2, column 7). Similarly, in all of the species, the signals of H2′ and H2″ show a significant upfield shift upon deprotonation of the $N1H^+$ site (Figure 3). In contrast, deprotonation of $PO_2(OH)^-$ has different effects: H2′ and H2″ of $H(dADP)^{2-}$ and $H(dATP)^{3-}$ are hardly affected by the distant terminal phosphate, their respective signals displaying only minor upfield shifts, whereas in $H(dAMP)^-$ both resonances

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are much more strongly affected by the close proximity of the $PO_2(OH)^-$ group. The signal of H2″ thus displays an upfield shift, whereas that of H2′ displays a "wrong way" downfield shift (Figure 3) (see also point i) in the Discussion).

The H5′ and H5″ resonances of the dAMP species are overlapped over the whole pH range, whereas the corresponding resonances in AMP run in parallel with a separation of about 0.033 ppm (Table 2, column 7). All of these resonances show a significant upfield shift as a result of the two deprotonation steps, the effect of $N1H^+$ deprotonation being small. On the other hand, the H5′ and H5″ resonances of $H_2(dADP)^-$ and $H_2(dATP)^{2-}$ (Figure 3) have the same chemical shift at low pH but separate upon deprotonation; the former first undergoes an upfield and then a downfield shift, whereas the latter is only shifted upfield. The resonance of H5′ of $H_2(dATP)^{2-}$ is downfield shifted as a result of both deprotonation reactions, whereas the resonance of H5″ is upfield shifted upon deprotonation of $N1H^+$ but downfield shifted upon the release of H^+ from $PO_2(OH)^-$. The divergent chemical shift behaviour for H5′ and H5″ of the different (2′-deoxy)adenosine nucleotides, as well as the "wrong way" downfield shifts, are of the utmost interest (Figure 3) as they directly reflect the nucleotide conformation, as discussed in the following section.

Discussion

The various changes in chemical shifts illustrated in Figure 3 and listed in Table 2, as well as the acidity constants assembled in Table 1, allow many comparisons to be made, a few of which are indicated below:

i) The fact that the chemical shift of H2 in all of the adenine nucleotides is strongly affected by the deprotonation of the $N1H^+$ site but is essentially unaffected by the release of the proton from $PO_2(OH)^-$ confirms that the *anti* conformation prevails in each of the species (Figure 1).[39–41] The observed deshielding of H2′ in $H(dAMP)^-$ upon deprotonation of $PO_2(OH)^-$ is consistent with this interpretation, since it can be taken as an indication of an even higher preference for the *anti* conformation (see also point (ii) below).

ii) The very strong "wrong way" downfield shift[41] of the resonance of H8 upon deprotonation of $H(AMP)^-$ (Table 2, column 7),[39] as we have also observed for $H(dAMP)^-$ and to a much lesser extent for $H(dADP)^{2-}$ and $H(dATP)^{3-}$ (Figure 3), has received much attention and has been the subject of many interpretations. The once proposed hydrogen-bond formation between $PO_2(OH)^-$ and N7, that is, "macrochelate" formation (see also below in point (ix)), cannot be the reason (or at least the sole reason) for this, because a similar "wrong way" shift is observed in the case of $H(TuMP)^-$.[39] In $TuMP^{2-}$ (= tubercidin 5′-monophosphate), N7 is replaced by a CH unit, and hence no hydrogen-bond formation is possible. It thus appears that the anisotropy of the phosphate group,[44] together with an increasing preference for the *anti* conformation about the glycosyl bond upon deprotonation of phosphate,[40,41] is responsible for the observed "wrong way" downfield shift of the resonance of H8. The same explanation also holds for the "wrong way" downfield shifts observed for the signals of the other protons (Figure 3), meaning that the (terminal) phosphate group and the nucleobase moiety face each other in the *anti* conformation, thereby affecting the chemical shift of the H8 proton.

iii) A further interesting observation is the chemical shift separation between the signals of H5′ and H5″ upon deprotonation of $H(dADP)^{2-}$ and $H(dATP)^{3-}$ (Figure 3). This indicates that "free" rotation about the C4′-C5′ bond is hampered, possibly because of some degree of bridging between the phosphate group and N7 by a water molecule in $dADP^{3-}$ and $dATP^{4-}$ (see also point (ix) below).

iv) If one considers the acidity constants listed in Table 1, the most obvious feature is clearly the effect of the substitution of the 2′-OH group on the sugar ring by a hydrogen atom on the acid–base equilibria of the nucleobase and phosphate moieties. Looking at the effect of the 2′-OH on $N7H^+$ deprotonation within the guanine series, one obtains the following acidity differences: $H(dGuo)^+/H(Guo)^+$, 0.19 ± 0.06 (from entry 1); $H_2(dGMP)^{\pm}/H_2(GMP)^{\pm}$, 0.21 ± 0.05 (from entry 2); $H_2(dGDP)^-/H_2(GDP)^-$, 0.24 ± 0.07 (from entry 9); and $H_2(dGTP)^{2-}/H_2(GTP)^{2-}$, 0.22 ± 0.05 (from entry 14). These ΔpK_a values are identical within their error limits, giving an average of $\Delta pK_a = 0.22\pm0.03$. This means that the 2′-deoxy derivative is always more basic by about 0.2 pK units and that the length and charge of the phosphate residue has no significant influence on the deoxyribose/ribose pairs, as is also nicely seen in Figure 4.

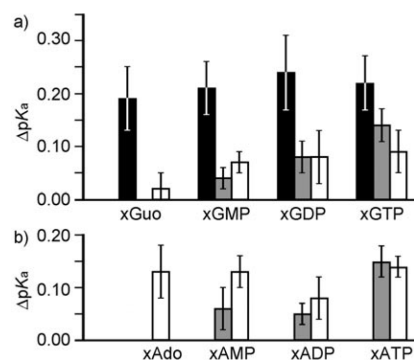

Figure 4. Variations in the acidities of the respective deprotonation sites within the different a) guanine and b) adenine derivatives upon replacement of the 2′-OH by a hydrogen atom. The plots show the resulting increases in basicity, that is, the ΔpK_a values for the $N7H^+$ sites (black bars), the phosphate groups (grey bars), and the $N1H/N1H^+$ sites (empty bars). The error bars represent 3σ; see footnote [a] of Table 1.

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v) The differences in acidity of the $N1H^+$ sites between the $dAP^{2-/3-/4-}$ and $AP^{2-/3-/4-}$ species amount, on average, to only $\Delta pK_a = 0.12 \pm 0.05$, but here again the deoxyribose derivatives are more basic than the ribose compounds. Obviously, the influence of the 2′-OH group is smaller (compared to the guanine series) because the N1 site is more distant from C2′ than N7 (see Figure 4b).

vi) An influence of the phosphate group(s) is observed for all of the acid–base sites in the systems discussed if one considers the absolute values of the acidity constants (Figure 5). For example, on addition of an increasing number of phosphate groups to the guanosine 5′-phosphate (GP) and the 2′-deoxyguanosine 5′-phosphate (dGP) series at C5′, the basicity of N7 increases concomitantly: $pK^H_{H(dGuo)} = 2.30 \pm 0.04 < pK^H_{H_2(dGMP)} = 2.69 \pm 0.03 < pK^H_{H_2(dGDP)} = 2.91 \pm 0.07 < pK^H_{H_2(dGTP)} = 3.16 \pm 0.05$ (Figure 5a). It is evident that the addition of the first 5′-phosphate group to the nucleoside has the largest effect ($\Delta pK_a = 0.39 \pm 0.05$), and that thereafter the effect decreases (0.22 ± 0.08) and levels off with the third phosphate group (0.25 ± 0.09) within the error limits. A similar situation is observed for the corresponding ribose and adenine series (Figure 5b).

vii) The basicity of the terminal phosphate group increases in the series $H(NMP)^- < H(NDP)^{2-} < H(NTP)^{3-}$ ($pK^H_{H(NMP)} = 6.20 \pm 0.05$; $pK^H_{H(NDP)} = 6.39 \pm 0.02$; $pK^H_{H(NTP)} = 6.49 \pm 0.05$; Table 1, entries 8, 13, and 18). This trend is to be expected due to the increasing negative charge (and in accordance with the values plotted in Figures 4 and 5). The good agreement of the pK_a values of the corresponding nucleotides, giving the above three averages, demonstrates that the nature of the nucleobase has no significant impact on the phosphate basicity. For the 2′-deoxynucleotides, only an average value for the deoxynucleoside 5′-monophosphates can be defined (entry 8) as insufficient data are available for the di- and triphosphates. The average $pK^H_{H(dNMP)} = 6.27 \pm 0.05$, encompassing the results for the 5′-monophosphates of 2′-deoxyguanosine, 2′-deoxyadenosine, and 2′-deoxycytidine, illustrates (if compared with $pK^H_{H(NMP)} = 6.20 \pm 0.05$) that the effect of the 2′-OH/H site is again small.

viii) The pK_a value for $H(dTMP)^-$ (entry 7) deserves special attention because, at 6.36 ± 0.01 (entry 7), it is slightly higher than $pK^H_{H(dNMP)}$. Because its sister nucleotide $H(UMP)^-$ behaves "normally", it is evident that besides the 2′-OH/H interchange, the methyl group at C5 also affects the deprotonation of the phosphate group to some extent. Indeed, once the thymine residue is far away, as in $H(dTTP)^{3-}$, the pK_a value (6.52 ± 0.02; entry 17) falls into the pK_a range defined by the NTPs ($pK^H_{H(NTP)} = 6.49 \pm 0.05$; entry 18).

ix) With the preceding observation in mind, the following numbers are clearly notable: $pK^H_{H(dGTP)} - pK^H_{H(GTP)} = (6.64 \pm 0.02) - (6.50 \pm 0.02) = 0.14 \pm 0.03$ and $pK^H_{H(dATP)} - pK^H_{H(ATP)} = (6.62 \pm 0.03) - (6.47 \pm 0.01) = 0.15 \pm 0.03$. The question then arises as to why the effect of the 2′-OH/H is significantly larger in these purine nucleoside 5′-triphosphates than it is in the case of the corresponding di- and monophosphates or the $H(UTP)^{3-}/H(dTTP)^{3-}$ pair. The only conceivable explanation is that the triphosphate chain can fold back and that the proton at the terminal γ-phosphate group forms a hydrogen bond to N7. The formation of such a "macrochelate" is corroborated by the fact that the N7 site of $dNTP^{4-}$ is more basic than that in NTP^{4-}. This "macrochelate" may thus be formed to a greater extent, thereby inhibiting the release of the proton from the $P_3O_{10}H^{3-}$ residue in the dNTPs. The formation of such a macrochelate is consistent with the finding of a recent study on dAMP,[45] in which it was concluded on the basis of density functional theory calculations that a water molecule bridges a phosphate oxygen and N7, forming a so-called semi-chelate. This is also consistent with our NMR results, as discussed in points (ii) and (iii) above. Analogously, the high degree

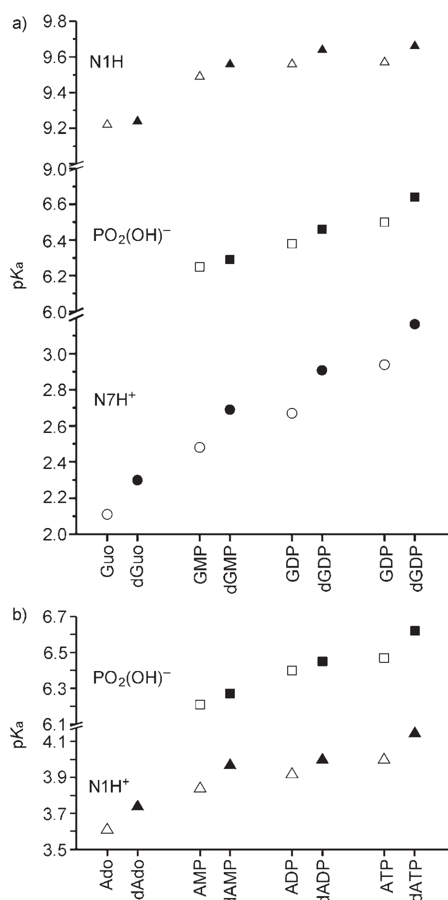

Figure 5. Trends in the acidity constants (pK_a values) of the different ribose (empty symbols) and deoxyribose (filled symbols) derivatives containing a a) guanine or an b) adenine residue. The pK_a values of the $N7H^+$ sites (circles), the terminal phosphate groups (squares), and the N1H sites (triangles) are plotted. It can clearly be seen that the basicity of each site increases with the addition of each phosphate group as well as with the replacement of the 2′-OH by a hydrogen atom.

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of macrochelate formation in $M(NTP)^{2-}$ complexes of divalent metal ions is well known.[10,31]

x) Of further interest is the effect of 2′-OH/H on the deprotonation reaction at the N1H site of the guanine nucleotide species. The difference between the pK_a values of $dGP^{2-/3-/4-}$ and $GP^{2-/3-/4-}$ (Table 1; column 5, entries 3, 9, 14) amounts, on average, to $\Delta pK_a = 0.08 \pm 0.02$ (see also Figure 4a). This effect is clearly small, but, within the error limits, corresponds to that observed for the $N1H^+$ deprotonation of the adenine nucleotides, $\Delta pK_a = 0.12 \pm 0.05$ (see also point v). This agreement is comforting because the distance between the N1 and the 2′-OH/H sites is the same in both series and thus the effect (despite the different charges) should also be the same.

Conclusions

Several conclusions regarding the fundamental properties of (2′-deoxy)nucleotides may be drawn from this study. The NMR measurements have confirmed that all of the purine nucleotides considered here predominantly exist in their *anti* conformation in aqueous solution over the measured pH range. Substitution of the 2′-OH group in ribose by a hydrogen atom enhances the basicity of all basic sites in the nucleosides and nucleotides studied, that is, the pK_a values of $N7H^+$, $PO_2(OH)^-$, and N1H or $N1H^+$ are somewhat higher in the 2′-deoxynucleotides compared to their ribose counterparts. This is because the substitution of the 2′-OH by a hydrogen atom makes the whole nucleotide slightly less hydrophilic, thereby affecting its solvation by water molecules and consequently also its deprotonation reactions. Along the same lines, it has recently been concluded, on the basis of density functional theory calculations, that water solvation affects the properties of 2′-deoxynucleoside 5′-monophosphates.[45]

Overall, the N7 site of guanine is clearly the most markedly affected, with the basicity enhancement amounting to 0.22 ± 0.03 pK units (Figure 4). In other words, the effects of the replacement of the ribose by a 2′-deoxyribose moiety on N1H/$N1H^+$ and on the phosphate groups are significantly smaller. Only when the phosphate chain is long enough to form a macrochelate with N7, that is, with the triphosphates, do these effects become more marked once more (about 0.15 pK units), as is reflected in the acidity constants of the monoprotonated γ-phosphate groups (Table 1, entries 14 and 15).

It has recently been shown that fundamental properties of mononucleotides, such as metal ion binding, can be directly extrapolated to large RNA and DNA molecules.[13] Therefore, a possible consequence of a greater degree of "macrochelate" formation in 2′-deoxypurine nucleoside 5′-triphosphates, involving the protonated γ-phosphate group and the N7 site, is that a greater degree of formation of the analogous macrochelates might be expected in the corresponding metal ion complexes of the 2′-deoxynucleotides as compared to those of the ribose species. Similarly, one may predict that within nucleic acids, N7 of guanine in DNA will have a somewhat higher metal ion affinity than N7 of guanine in RNA.

Experimental Section

Materials: Nitric acid and potassium hydrogen phthalate (both pro analysi) were purchased from Merck KGaA (Darmstadt, Germany). The nucleotides dAMP, dADP, dATP, dGDP, and dGTP were obtained from Sigma-Aldrich Co. (St. Louis MO, USA). Sodium hydroxide solution (Fixanal) and sodium nitrate (*puriss p. a.*) were purchased from Riedel-de-Haën GmbH (Seelze, Germany); tetramethylammonium bromide (97%) was purchased from Fluka AG (Buchs, Switzerland). D_2O (99.998% D), NaOD (40% in D_2O; 99.9% D), and DNO_3 (65% in D_2O; 99.5% D) were obtained from Armar Chemicals (Doettlingen, Switzerland). The buffer solutions used (pH 4.00, 7.00, 9.00) were based on standard reference materials (SRM) of the US National Institute of Science and Technology (NIST) and were purchased from Metrohm AG (Herisau, Switzerland). All solutions were prepared using deionised, ultra-pure (TKA GenePure, Niederelbert, Germany), CO_2-free water.

The concentration of the NaOH solution used was determined using potassium hydrogen phthalate. Stock solutions of the nucleotides were freshly prepared daily. The exact concentrations of these ligand solutions were determined in each experiment by evaluation of the appropriate titration pair, that is, the difference in NaOH consumption between HNO_3 solutions with and without the ligand (see below).

Potentiometric pH titrations: The pH titrations were performed with an E536 potentiograph connected to an E665 dosimat and a 6.0253.100 Aquatrode-plus combined macro glass electrode (all from Metrohm AG, Herisau, Switzerland). The instruments were calibrated using the buffer solutions mentioned above. The acidity constants determined at $I = 0.1$ M ($NaNO_3$) and 25 °C are so-called practical, mixed, or Brønsted constants,[25] which may be converted into the corresponding concentration constants by subtracting 0.02 from the measured pK_a values.[25] The ionic product of water (K_w) does not enter into our calculations because the differences in NaOH consumption between solutions with and without nucleotides are evaluated.

Determination of the equilibrium constants: The acidity constants $K^H_{H_2(AP)}$ [Eq. (1)] and $K^H_{H(AP)}$ [Eq. (2)] of $H_2(dAMP)^{\pm}$, $H_2(dADP)^-$, and $H_2(dATP)^{2-}$ as well as $K^H_{H_2(GP)}$ [Eq. (1)], $K^H_{H(GP)}$ [Eq. (2)], and K^H_{GP} [Eq. (3)] of $H_2(dGDP)^-$ and $H_2(dGTP)^{2-}$ were determined by titrating 50 mL of aqueous 1.667 mM HNO_3 (25 °C; $I = 0.1$ M, $NaNO_3$) under N_2 with up to 2 mL of 0.06 M NaOH in the presence and absence of 0.6 mM dAMP; 0.21, 0.30, and 0.41 mM dADP; 0.34 mM dATP; 0.13, 0.19, and 0.27 mM dGDP; and 0.13, 0.16, 0.21, and 0.27 mM dGTP. It should be emphasised that in cases in which more than one ligand concentration was applied, the calculated acidity constants showed no dependence on the nucleotide concentration. Clearly, self-association is of no relevance under the given experimental conditions.[26]

The experimental data were evaluated by means of a curve-fitting procedure using a Newton–Gauss nonlinear least-squares program that utilised the difference in NaOH consumption between the aforementioned pairs of titrations, that is, with and without nucleotide, at increments of 0.1 pH units. The acidity constants of $H_2(dAP)^{\pm/-/2-}$ were calculated within the pH range 3.5 to 8.0 and those of $H_2(dGP)^{\pm/-/2-}$ between pH 3.0 and 10.0. This corresponds to an initial degree of neutralisation of about 25%, 24%, 19%, 55%, and 41% for the equilibria $H_2(dAMP)^{\pm}/H(dAMP)^-$, $H_2(dADP)^-/H(dADP)^{2-}$, $H_2(dATP)^{2-}/H(dATP)^{3-}$, $H_2(dGDP)^-/H(dGDP)^{2-}$, and $H_2(dGTP)^{2-}/H(dGTP)^{3-}$, respectively. The final degrees of neutralisation of the equilibria $H(dAMP)^-/dAMP^{2-}$, $H(dADP)^{2-}/dADP^{3-}$, $H(dATP)^{3-}/dATP^{4-}$, $dGDP^{3-}/(dGDP{-}H)^{4-}$, and $dGTP^{4-}/(dGTP{-}H)^{5-}$ amounted to about 98, 97, 96, 70, and 68%, respectively.

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The final acidity constants quoted for each of the nucleotides are the averages of at least six independent pairs of titrations. All error limits are three times the standard error of the mean value (3σ) or the sum of the probable systematic errors, whichever is the larger.

^{1}H NMR shift experiments: ^{1}H NMR spectra of the dNPs (1 mM) in D_2O, in dependence on pD, were recorded on a Bruker AV2-500 spectrometer (500 MHz) with a TXI probehead at 25 °C and $I = 0.1$ M ($NaNO_3$). The centre peak of the tetramethylammonium ion triplet (ca. 0.5 mM) at $\delta = 3.174$ ppm (D_2O) relative to 3-(trimethylsilyl)propane-1-sulfonate was used as an internal reference.[39,46] $(CH_3)_4N^+$ has been repeatedly proven to be a reliable internal reference in the presence of nucleotides and their derivatives.[8,9,39,46,47] [^{1}H,^{1}H]-ROESY spectra were recorded under the same conditions using a mixing time of 100 ms.

The pD of the solutions was adjusted by dotting with a glass stick using relatively concentrated DNO_3 or NaOD solutions and by measuring the actual pD by means of a Hamilton Minitrode glass electrode (Hamilton AG, Bonaduz, Switzerland) connected to a Metrohm 605 digital pH meter (Metrohm AG, Herisau, Switzerland). The final pD of the D_2O solutions was obtained by adding 0.40 to the pH meter reading.[48] The ^{1}H NMR signals of the adenine nucleotides were assigned as previously.[39,46]

The experimental data were analysed by means of the Newton–Gauss nonlinear least-squares method. Results were obtained with the aid of a computer-based curve-fitting program, which was based on the general equation published previously.[8,39] This equation relates the observed chemical shift, δ_{obsd}, with the $pK^H_{H_2(dNP)}$ and $pK^H_{H(dNP)}$ values and the chemical shifts for the species $H_2(dNP)^{\pm/-/2-}$, $H(dNP)^{-/2-/3-}$, and $dNP^{2-/3-/4-}$.

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