Acid–base properties of the 5′-triphosphates of guanosine and inosine (GTP^{4-} and ITP^{4-}) and of several related nucleobase derivatives

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Received (in Cambridge, UK) 26th October 2000, Accepted 1st February 2001
First published as an Advance Article on the web 2nd March 2001

The acidity constants of $H_2(NTP)^{2-}$ [NTP^{4-} = guanosine 5′-triphosphate (GTP^{4-}) or inosine 5′-triphosphate (ITP^{4-})], which may lose three protons giving $(NTP - H)^{5-}$, were determined by potentiometric pH titrations (25 °C; $I = 0.1$ M, $NaNO_3$) and values for the deprotonation of $H_3(NTP)^-$ were estimated *via* micro-acidity-constant evaluations. Complete micro-acidity-constant schemes for both $H_3(GTP)^-$ and $H_3(ITP)^-$ are given. From the evaluations it follows that the ratios, *R*, of the twofold protonated and isocharged species $(H{\cdot}NTP{\cdot}H)^{2-}$ and $(NTP{\cdot}H_2)^{2-}$ for ITP and GTP, which carry one proton at N7 and one at the terminal γ-phosphate group or both protons at the triphosphate chain, respectively, are about 1 : 1 for the ITP system and about 10 : 1 for the GTP system. This confirms the higher basicity of N7 in the guanosine residue compared with that in the inosine moiety. For the evaluation of the indicated analysis it was also necessary to consider the acid–base properties of guanosine, inosine, adenosine, and of the 5′-triphosphates of adenosine, cytidine, uridine and thymidine; the corresponding acidity constants were in part taken from our earlier work and in part are also measured now.

1. Introduction

At present much activity in nucleotide chemistry centers on guanosine 5′-triphosphate (GTP^{4-}), which is utilized by so-called G-proteins in such diverse processes[1] as cellular signaling,[2] protein synthesis,[3] vesicular trafficking,[4] ion channel regulation[5] or nerve growth.[6] Therefore, it is surprising to note that apparently no detailed analysis of the acid–base properties of GTP^{4-} has been carried out,[7–10] though some of the macro acidity constants concerning the nucleobase residue and the triphosphate chain are available.[7–10]

In this paper we report a comprehensive set of the acidity constants of three-fold protonated GTP^{4-}, *i.e.* of $H_3(GTP)^-$, and also of its analogue inosine 5′-triphosphate (ITP^{4-}), where inosine = 2-deaminoguanosine (Fig. 1).[11–13] There are several instances where the buffer regions of the deprotonation reactions are overlapping, *i.e.* the acidity constants (pK_a values) are relatively similar. In these cases micro-acidity-constant analyses are presented, which allow a quantification of the intrinsic acid–base properties of the various sites. For comparison the acidity constants of several pyrimidine-nucleoside 5′-triphosphates ($PyNTP^{4-}$) are also considered.

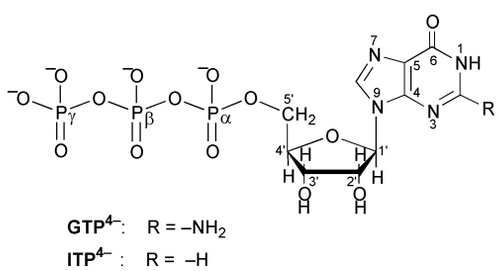

Fig. 1 Chemical structure of guanosine 5′-triphosphate (GTP^{4-}) and inosine 5′-triphosphate (ITP^{4-}) in their dominating *anti* conformation.[11–13]

2. Experimental

2.1 Materials

The sodium salts of GTP, ITP, CTP and UTP were purchased from Sigma Chemical Co. (St. Louis, MO) and also from Serva Feinbiochemica, GmbH (Heidelberg, Germany). The content of free inorganic phosphate initially present (determined with molybdate reagent as described previously; ref. 14) was below 3%.[15] The aqueous stock solutions of the NTPs were freshly prepared daily and the pH adjusted to about 8.2 with UTP, 8.5 with CTP, and 8.0 with GTP or ITP. The exact concentrations of the NTP solutions used in the titration experiments were newly measured (in the presence of an excess of HNO_3; see Section 2.3) each time by titrations with NaOH.

Sodium nitrate, HNO_3, NaOH (Titrisol), and potassium hydrogen phthalate (all *pro analysi*) were obtained from Merck AG, Darmstadt, Germany. The titers of the NaOH solutions used for the titrations were established with potassium hydrogen phthalate.

2.2 Potentiometric pH titrations

The pH titrations were carried out with a Metrohm E536 potentiograph equipped with an E655 dosimat and an EA121 or 6.0202.100 (JC) combined macro glass electrode. The buffer solutions [pH 4.00 or 4.64, 7.00, and 9.00; based on the NBS scale, now U.S. National Institute of Standards and Technology (NIST)] used for calibration were also from Metrohm

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DOI: 10.1039/b008673h

AG (Herisau, Switzerland). The direct pH-meter readings were used to calculate the acidity constants;[16,17] *i.e.* these constants are so-called practical, mixed or Brønsted constants.[16] Their negative logarithms given for aqueous solutions at $I = 0.1$ M and 25 °C may be converted into the corresponding concentration constants by subtracting 0.02 log unit[16] from the listed pK_a values. This conversion term contains both the junction potential of the glass electrode and the hydrogen ion activity.[16,18]

It should be emphasized that the ionic product of water (K_w) and the mentioned conversion term do *not* enter into the calculations because we evaluate the *differences* in NaOH consumption between solutions with and without ligand[16,17] (see also below).

All experiments with the NTPs were done in such a way that dephosphorylation was kept to a minimum.[19] There was no difference between the results of the reevaluated data obtained from earlier experiments carried out in the presence of $NaClO_4$ (*cf.* ref. 20) and those carried out now using $NaNO_3$ as background electrolyte. All measurements of the acidity constants were performed independently by two (or even three) persons with intervals of years.

All calculations were carried out with IBM-compatible desk computers with 80-486 or Pentium processors (connected with Epson Stylus 1000ESC/P 2 printers and a Hewlett-Packard Deskjet 1600 C Color Smart printer or a Hewlett-Packard 7475A plotter) by a curve-fitting procedure using a Newton–Gauss nonlinear least-squares program.

2.3 Determination of acidity constants

The acidity constants $K^{H}_{H_2(NTP)}$, $K^{H}_{H(NTP)}$, and K^{H}_{NTP} [eqns. (2)–(4)] of $H_2(NTP)^{2-}$ for the nucleoside 5′-triphosphates (NTP) GTP and ITP were determined by titrating an aqueous solution of HNO_3 or $HClO_4$ ($I = 0.1$ M; 25 °C) in the presence and absence of NTP under N_2 with 1 or 1.5 mL of NaOH. The differences in NaOH consumption between such a pair of titrations were used for the evaluations of the constants. Three sets of experiments were carried out: (*i*) $[HNO_3] = 10^{-3}$ M; $[NTP] = 5 \times 10^{-4}$ M; volume 50 mL; [NaOH] = 0.05 M (1.5 mL); $I = 0.1$ M ($NaNO_3$). (*ii*) $[HClO_4] = 1.6 \times 10^{-3}$ M; $[NTP] = 1.2 \times 10^{-3}$ M; volume 25 mL; [NaOH] = 0.05 M (1 mL); $I = 0.1$ M ($NaClO_4$). (*iii*) $[HNO_3] = 0.02$ M; $[NTP] = 2.8 \times 10^{-3}$ M; volume 20 mL; [NaOH] = 0.2 M (2 mL); $I = 0.1$ M ($NaNO_3$).

In the case of GTP the first set of experiments (*i*) was evaluated in the pH range 3.5–10.0 giving values for all three constants; for ITP the pH range 4.8–10.4 was used giving results for the second and third constants [eqns. (3) and (4)]. From the second set (*ii*) values for all three constants were also obtained for both NTPs in the pH range 3.5–8.5, and from the third set (*iii*) for both NTPs in the pH range 2.1–7.0 the first two constants [eqns. (2) and (3)] were calculated. The final results (see Section 3.1) are the averages of 14 titrations for $K^{H}_{H_2(ITP)}$ and of more than 40 (on average 50) titrations for all the other constants.

The acidity constants $K^{H}_{H_2(CTP)}$ and $K^{H}_{H(CTP)}$ of $H_2(CTP)^{2-}$ [eqns. (2) and (3)] were determined by titrating 50 mL of aqueous 1.3×10^{-3} M HNO_3 ($I = 0.1$ M, $NaNO_3$; 25 °C) in the presence and absence of 5×10^{-4} M CTP under N_2 with 1.5 mL of 0.05 M NaOH and by using again the differences in NaOH consumption between two such titrations for the calculations in the pH range 3.6–8.3. The average results from 15 titrations were identical to the values published previously for these acidity constants.[15]

The acidity constants $K^{H}_{H(UTP)}$ and K^{H}_{UTP} [eqns. (3) and (4)] were determined under exactly the same conditions as given above for $H_2(CTP)^{2-}$ but the calculations were in this case carried out in the pH range 4.8–10. In a further set of experiments 25 mL of aqueous 1.6×10^{-3} M $HClO_4$ ($I = 0.1$ M, $NaClO_4$; 25 °C) were titrated in the presence and absence of 1.2×10^{-3} M UTP under N_2 with 1 mL of 0.055 M NaOH and evaluated for the same two acidity constants in the pH range 4.8–8.5. The final results (Section 3.1) are the averages of at least 14 pairs of independent titrations.

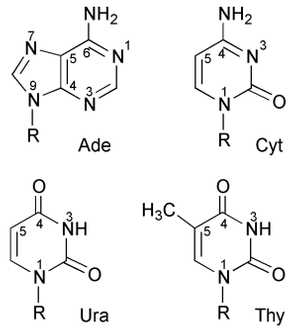

Fig. 2 Structure of the nucleobase residues adenine (Ade), cytosine (Cyt), uracil (Ura) and thymine (Thy) which occur in the nucleotides adenosine 5′-triphosphate (ATP^{4-}), cytidine 5′-triphosphate (CTP^{4-}), uridine 5′-triphosphate (UTP^{4-}) and thymine [= 1-(2′-deoxy-β-D-ribofuranosyl)thymine] 5′-triphosphate ($dTTP^{4-}$), respectively.[26]

3. Results and discussion

In the present study great care was taken to measure under conditions where no self-association of the nucleotides occurs.[21–24] Most measurements were made with solutions being 0.5 mM in nucleotide concentration; this guarantees[22,23,25] that indeed the properties of the monomeric species are studied.

3.1 Acidity constants of $H_3(ITP)^-$ and $H_3(GTP)^-$ in comparison with the constants of related species

In the pH range 1 to 11 $H_3(GTP)^-$ and $H_3(ITP)^-$ undergo four deprotonations. The corresponding pK_a values are listed in Table 1 together with the acidity constants of several related nucleosides and nucleotides.[26–28] To facilitate the comparisons the nucleobase residues of these compounds are shown in Fig. 2.[26] It may be added that the (macro) acidity constants determined now *via* potentiometric pH titrations for eqns. (2)–(4) (Table 1) agree well with a previous tabulation.[27]

The acidity constants for the $H_3(NTP)^-$ species are defined in the following equilibria.

$$H_3(NTP)^- \rightleftharpoons H_2(NTP)^{2-} + H^+ \quad (1a)$$

$$K^{H}_{H_3(NTP)} = [H_2(NTP)^{2-}][H^+]/[H_3(NTP)^-] \quad (1b)$$

$$H_2(NTP)^{2-} \rightleftharpoons H(NTP)^{3-} + H^+ \quad (2a)$$

$$K^{H}_{H_2(NTP)} = [H(NTP)^{3-}][H^+]/[H_2(NTP)^{2-}] \quad (2b)$$

$$H(NTP)^{3-} \rightleftharpoons NTP^{4-} + H^+ \quad (3a)$$

$$K^{H}_{H(NTP)} = [NTP^{4-}][H^+]/[H(NTP)^{3-}] \quad (3b)$$

$$NTP^{4-} \rightleftharpoons (NTP - H)^{5-} + H^+ \quad (4a)$$

$$K^{H}_{NTP} = [(NTP - H)^{5-}][H^+]/[NTP^{4-}] \quad (4b)$$

The first proton of $H_3(PuNTP)^-$ [eqn. (1)] is mainly released from one of the three primary sites of the twofold protonated triphosphate chain (Fig. 1); of course, the $-P_3O_{10}H_2^{2-}$ residue contains two more such primary sites, but these are protonated only at pH < 1 (*cf.* ref. 29)[30] and are therefore not considered here. The second constant [eqn. (2)] is primarily due to proton loss from the $(N7)H^+$ site (see Fig. 1); indeed, it should be noted that N7 is 1.05 log units more basic in guanosine ($pK^{H}_{H(Guo)} = 2.11$) than in inosine ($pK^{H}_{H(Ino)} = 1.06$; see Table 1).

Table 1 Negative logarithms of the acidity constants of several $H_3(PuNTP)^-$ species as determined by potentiometric pH titrations in aqueous solution at 25 °C and $I = 0.1$ M ($NaNO_3$ or $NaClO_4$) together with some related data that refer to the same conditions[a]

Acid	$pK^H_{H_3(PuNTP)}$ (or $pK^H_{H_2(PyNTP)}$) [eqn. (1)]	$pK^H_{H_2(NTP)}$ (or $pK^H_{H(Ns)}$) [eqn. (2)]	$pK^H_{H(NTP)}$ [eqn. (3)]	pK^H_{NTP} (or pK^H_{Ns}) [eqn. (4)]
$H(Guo)^+$		2.11 ± 0.04[b,c]		9.22 ± 0.01[b]
$H(Ino)^+$		1.06 ± 0.06[b,c]		8.76 ± 0.03[b]
$H(Ado)^+$		3.61 ± 0.03[d,e]		
$H_3(GTP)^-$	1.3 ± 0.2[f]	2.94 ± 0.02	6.50 ± 0.02	9.57 ± 0.02
$H_3(ITP)^-$	1.0 ± 0.2[g]	2.19 ± 0.05	6.47 ± 0.02	9.11 ± 0.03
$H_3(ATP)^-$	1.7 ± 0.1[h]	4.00 ± 0.01[d,i]	6.47 ± 0.01[i]	
$H_2(CTP)^{2-}$	1.7[j]	4.55 ± 0.03[d,i]	6.55 ± 0.02[i]	
$H_2(UTP)^{2-}$	2.0 ± 0.1[k]		6.48 ± 0.02[l]	9.57 ± 0.02[m]
$H_2(dTTP)^{2-}$	2.0[i]		6.52 ± 0.02[i]	10.08 ± 0.05[m,n]

[a] So called practical (or mixed) constants (see ref. 16) are listed; see Section 2.2. The errors given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. Those values for which no source is given have been determined in this study. For the sites at which the protons are located see also the text in Sections 3.1 to 3.3. [b] From ref. 25. [c] From ref. 23. [d] This value refers to the deprotonation of the $(N1)H^+$ site of the adenine residue; all the other values refer (largely; see text) to the deprotonation of the $(N7)H^+$ unit of the purines, except in the case of $H_2(CTP)^{2-}$ where the proton is at N3 (see ref. 27). [e] From ref. 12; see also ref. 25. [f] Rounded value from the scheme of Fig. 4; see also Section 3.2. [g] Rounded value from the scheme of Fig. 3; see also Section 3.3. [h] From ref. 28. [i] From ref. 15; the values for CTP have now been confirmed (see Section 2.3). [j] It is assumed that $pK^H_{H3(ATP)} \simeq pK^H_{H3(CTP)}$ because the effect of the protonated nucleobase residue on the release of the first proton from the twofold protonated triphosphate chain in $H_3(ATP)^-$ and $H_3(CTP)^-$ is expected to be very similar. [k] From ref. 19. [l] This result agrees well with our previous one (ref. 15). [m] This value refers to the deprotonation of the (N3)H site of a pyrimidine residue; all the other values in this column refer to the deprotonation of a purine-(N1)H site. [n] H. Sigel, unpublished result. The experiments were carried out as those described for UTP in Section 2.3.

The low $pK^H_{H_2(ITP)}$ value of 2.19 for $H_2(ITP)^{2-}$ in Table 1 suggests thus some contribution from the mentioned second triphosphate proton. This problem will be further addressed in Sections 3.2 and 3.3.

The third constant [eqn. (3)] given in column 4 of Table 1 refers to the loss of the last triphosphate-bound proton in $H(GTP)^{3-}$ or $H(ITP)^{3-}$ and is identical to the values found for $H(ATP)^{2-}$ ($pK^H_{H(ATP)} = 6.47$) and also closely similar to those for monoprotonated pyrimidine-nucleoside 5′-triphosphates ($pK^H_{H(PyNTP)} = 6.50 \pm 0.05$).[15,31] The highest values in Table 1 [eqn. (4)] refer to ionization from the (N1)H site of the nucleobase in GTP^{4-} and ITP^{4-} (in the case of UTP^{4-} and $dTTP^{4-}$ the (N3)H is deprotonated) to yield species at pH > 10 of an overall −5 charge, *i.e.* $(NTP - H)^{5-}$. All these values are consistent with the patterns found for nucleotides and their nucleobases,[7–10,20,32] and the site attributions agree with previous conclusions.[11,27,33]

Comparison of the acidity constants of the nucleosides with those of their corresponding nucleotides (Table 1) reveals the effect of the 4-fold negatively charged triphosphate chain on the deprotonation of the (N1)H site.

$$pK^H_{GTP} - pK^H_{Guo} = (9.57 \pm 0.02) - (9.22 \pm 0.01) = 0.35 \pm 0.02$$

$$pK^H_{ITP} - pK^H_{Ino} = (9.11 \pm 0.03) - (8.76 \pm 0.03) = 0.35 \pm 0.04$$

As one might expect, the release of the proton from (N1)H in $PuNTP^{4-}$ is inhibited by the triphosphate chain and the effect is very similar to that observed with the pyrimidines and their (N3)H site.

$$pK^H_{UTP} - pK^H_{Urd} = (9.57 \pm 0.02) - (9.19 \pm 0.02; \text{ from ref. } 34) = 0.38 \pm 0.03$$

$$pK^H_{dTTP} - pK^H_{Thy} = (10.08 \pm 0.05) - (9.69 \pm 0.03; \text{ from ref. } 34) = 0.39 \pm 0.06$$

It may be emphasized that the consistency of the above differences provides the necessary confidence in the evaluation of further differences between acidity constants carried out in the next two sections.

It is also interesting to observe that the effect described above also operates when a positively charged $(N1)H^+$ site is involved as in $H_2(ATP)^{2-}$ and H(adenosine)$^+$.

$$pK^H_{H_2(ATP)} - pK^H_{H(Ado)} = (4.00 \pm 0.01) - (3.61 \pm 0.03) = 0.39 \pm 0.03$$

That the release of this H^+ is inhibited to the same extent by the monoprotonated 3-fold negatively charged triphosphate group as above by the 4-fold negatively charged triphosphate chain is likely to be due to the formation of Na^+ complexes[7] for the latter species,[35] which neutralizes in part the extra charge.

3.2 Estimations of some acidity constants in the low pH range

As indicated in Section 3.1, it is expected that the macro acidity constant $pK^H_{H_2(ITP)}$ for the $H_2(ITP)^{2-}$ species also contains a contribution from the release of one of the primary phosphate protons and thus does not refer solely to the $(N7)H^+$ site. With this in mind, we estimate a micro acidity constant[36] for reaction (5) (loss of a proton from N7 of phosphate protonated ITP),

$$(H{\cdot}ITP{\cdot}H)^{2-} \rightleftharpoons (ITP{\cdot}H)^{3-} + H^+ \qquad (5)$$

where $(H{\cdot}ITP{\cdot}H)^{2-}$ represents a species that carries a proton each at N7 and the terminal γ-phosphate group. The *difference* between the pK_a values for the deprotonation of the $(N7)H^+$ site in the guanine and hypoxanthine moieties is expected to be independent of the presence of the triphosphate chain because the effect of this chain on the acid–base properties of N7 should be identical in both moieties, *i.e.* this difference should be the same if calculated for the pK_a values of the two nucleosides (Table 1), Ino and Guo, or for those of the two NTPs. Hence, $\Delta pK_a = pK^H_{H(Guo)} - pK^H_{H(Ino)} = (2.11 \pm 0.04) - (1.06 \pm 0.06) = 1.05 \pm 0.07$, and therefore $pk^{ITP{\cdot}H}_{H{\cdot}ITP{\cdot}H} = pK^H_{H_2(GTP)} - \Delta pK_a = (2.94 \pm 0.02) - (1.05 \pm 0.07) = 1.89 \pm 0.07$. This micro acidity constant for equilibrium (5) is in excellent agreement with the result from ^{1}H-NMR shift measurements in D_2O ($pK^D_{D_2(ITP)} = 2.40 \pm 0.15$)[23] if the corresponding value is transformed[37] into H_2O as solvent: $pk^{ITP{\cdot}H}_{H{\cdot}ITP{\cdot}H} = 1.92 \pm 0.15$. This agreement proves that the above given reasoning is correct and it proves further that any contribution from a primary phosphate proton towards $pK^H_{H_2(GTP)}$ is insignificant (this is also confirmed in Section 3.3).

The last-mentioned conclusion also allows us now to estimate a value for $pK^H_{H_3(GTP)}$ [eqn. (1)], *i.e.* for the deprotonation of $H_3(GTP)^-$ or the release of the final primary proton from the

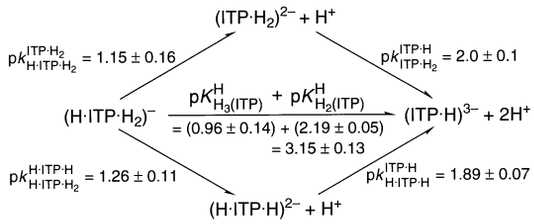

Fig. 3 Equilibrium scheme for $(H{\cdot}ITP{\cdot}H_2)^-$ defining the micro acidity constants (k) and showing their interrelation with the macro acidity constants (K) and also the interrelation between $(ITP{\cdot}H_2)^{2-}$ and $(H{\cdot}ITP{\cdot}H)^{2-}$ and the other species present. In $(ITP{\cdot}H_2)^{2-}$ one proton is at the γ-phosphate group and the other at one of the primary sites at the triphosphate chain, while in $(H{\cdot}ITP{\cdot}H)^{2-}$ one proton is at N7 and the other at the γ-phosphate group (Fig. 1). $(H{\cdot}ITP{\cdot}H_2)^-$ is also often written as $H_3(ITP)^-$; it carries one proton at N7 and the two others at the triphosphate residue. The arrows indicate the direction for which the acidity constants are defined. For the origin of the various constants see the text in Section 3.3.

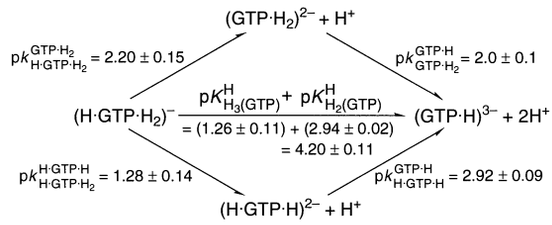

Fig. 4 Equilibrium scheme for $(H{\cdot}GTP{\cdot}H_2)^-$ defining the micro acidity constants (k) and showing their interrelation with the macro acidity constants (K) and also the interrelation between $(GTP{\cdot}H_2)^{2-}$ and $(H{\cdot}GTP{\cdot}H)^{2-}$ and the other species present. In $(GTP{\cdot}H)^{2-}$ one proton is at the γ-phosphate group and the other at one of the primary sites at the triphosphate chain, while in $(H{\cdot}GTP{\cdot}H)^{2-}$ one proton is at N7 and the other at the γ-phosphate group (Fig. 1). $(H{\cdot}GTP{\cdot}H_2)^-$ is also often written as $H_3(GTP)^-$; it carries one proton at N7 and the two others at the triphosphate residue. The arrows indicate the direction for which the acidity constants are defined. For the origin of the various constants see text in Section 3.3.

triphosphate chain. This estimate is based on the known value[28] for $pK^{H}_{H_3(ATP)}$ by taking into account the different distances of $(N1)H^+$ in $H_3(ATP)^-$ and $(N7)H^+$ in $H_3(GTP)^-$ (see Fig. 1) from the triphosphate chain. This distance effect is evidently represented by $\Delta\Delta pK_a = \Delta pK_{a/N7} - \Delta pK_{a/N1}$, where $\Delta pK_{a/N7} = pK^{H}_{H_2(GTP)} - pK^{H}_{H(Guo)} = (2.94 \pm 0.02) - (2.11 \pm 0.04) = 0.83 \pm 0.04$ and $\Delta pK_{a/N1} = pK^{H}_{H_2(ATP)} - pK^{H}_{H(Ado)} = (4.00 \pm 0.01) - (3.61 \pm 0.03) = 0.39 \pm 0.03$ (see Table 1), and hence, $\Delta\Delta pK_a = (0.83 \pm 0.04) - (0.39 \pm 0.03) = 0.44 \pm 0.05$. Based on $pK^{H}_{H_3(ATP)}$ one obtains for $pK^{H}_{H_3(GTP)} = pK^{H}_{H_3(ATP)} - \Delta\Delta pK_a = (1.7 \pm 0.1) - (0.44 \pm 0.05) = 1.26 \pm 0.11$; this result rounded to 1.3 ± 0.2 is listed in column two of Table 1.

Of course, the reasoning given in the preceding paragraph can also be applied to $H_3(ITP)^-$ if the equilibrium (6) is

$$(H{\cdot}ITP{\cdot}H_2)^- \rightleftharpoons (H{\cdot}ITP{\cdot}H)^{2-} + H^+ \qquad (6)$$

considered, where $(H{\cdot}ITP{\cdot}H_2)^-$ carries one proton at N7 and two protons at the triphosphate chain. The corresponding micro acidity constant (see also below) is then given by $pk^{H{\cdot}ITP{\cdot}H}_{H{\cdot}ITP{\cdot}H_2} = pK^{H}_{H_3(GTP)} = 1.26 \pm 0.11$.

3.3 Micro acidity constant schemes for $H_3(ITP)^-$ and $H_3(GTP)^-$

In Fig. 3 we have summarized the equilibrium scheme for $H_3(ITP)^-$ defining the microconstants (k) and giving their interrelation with the macro acidity constants (K). By use of the values deduced in Section 3.2 for $pk^{H{\cdot}ITP{\cdot}H}_{H{\cdot}ITP{\cdot}H_2}$ and $pk^{ITP{\cdot}H}_{H{\cdot}ITP{\cdot}H}$ it is possible to calculate a value for the global acidity constant $\mathbf{p}K^{H}_{H_3(ITP)} = 0.96 \pm 0.14$ [eqn. (1)] (lower part of the scheme in Fig. 3). This result rounded to 1.0 ± 0.2 is also listed in column two of Table 1.

In principle it should now also be possible to calculate values for the other microconstants depicted in the upper part of Fig. 3 by following the routes described previously.[36,38,39] However, as the error limits of some of the acidity constants are rather large, we prefer in the present case to make a further sophisticated estimate: The release of the third primary proton from the triphosphate chain in $H_2(UTP)^{2-}$ is evidently unaffected by the uridine moiety as this residue is uncharged. Consequently, the corresponding acidity constant is a good estimate for the microconstant of equilibrium (7); this means

$$(ITP{\cdot}H_2)^{2-} \rightleftharpoons (ITP{\cdot}H)^{3-} + H^+ \qquad (7)$$

$pk^{ITP{\cdot}H}_{ITP{\cdot}H_2} = pK^{H}_{H_2(UTP)} = 2.0 \pm 0.1$ (Table 1). By employing this value, the final micro acidity constant of the upper cycle in the scheme of Fig. 3 is now easily calculated.

In Fig. 4 the micro acidity constant scheme for $H_3(GTP)^-$ is depicted. It may be emphasized that the value for $pk^{GTP{\cdot}H}_{H{\cdot}GTP{\cdot}H}$ is calculated[37] from the result of ^{1}H-NMR shift measurements in D_2O ($pK^{D}_{D_2(GTP)} = 3.41 \pm 0.09$)[23] to give 2.92 ± 0.09. This result proves the conclusion presented before (Sections 3.1 and 3.2) that the global constant $pK^{H}_{H_3(GTP)}$ (Table 1, column 3) and the microconstant $pk^{GTP{\cdot}H}_{H{\cdot}GTP{\cdot}H}$ are identical within experimental error; the same applies to $pK^{H}_{H_3(GTP)}$ and the derived microconstant $pk^{H{\cdot}GTP{\cdot}H}_{H{\cdot}GTP{\cdot}H_2}$ (lower cycle in Fig. 4).

Of course, for the present case $pk^{GTP{\cdot}H}_{GTP{\cdot}H_2} = pK^{H}_{H_2(UTP)} = 2.0 \pm 0.1$ has also to hold for the reasons outlined above, and thus the remaining microconstant in the upper pathway of Fig. 4 can be calculated. Moreover, the basicity difference of N7 in GTP and ITP can now be established: $\Delta pK_{a/N7/NTP} = pk^{GTP{\cdot}H_2}_{H{\cdot}GTP{\cdot}H_2} - pk^{ITP{\cdot}H_2}_{H{\cdot}ITP{\cdot}H_2} = (2.20 \pm 0.15) - (1.15 \pm 0.16) = 1.05 \pm 0.22$ (Fig. 3 and 4) and be compared with $\Delta pK_{a/N7/Ns} = pK^{H}_{H(Guo)} - pK^{H}_{H(Ino)} = (2.11 \pm 0.04) - (1.06 \pm 0.06) = 1.05 \pm 0.07$. The agreement between $\Delta pK_{a/N7/NTP}$ and $\Delta pK_{a/N7/Ns}$ is excellent, as it should be because the relative basicity of N7 in a guanine residue *versus* that in a hypoxanthine residue has always to be the same. The result of this comparison is quite satisfying as it proves that the micro acidity constants given in the schemes of Fig. 3 and 4 are self-consistent with each other.

Finally, the results of Fig. 3 and 4 allow us now to estimate the ratios R of the twofold protonated and isocharged species $(H{\cdot}NTP{\cdot}H)^{2-}$ and $(NTP{\cdot}H_2)^{2-}$ for ITP and GTP, which carry one proton at N7 and one at the terminal γ-phosphate group or both protons at the triphosphate chain, respectively [eqns. (8) and (9)].

$$R_{ITP} = \frac{[(H{\cdot}ITP{\cdot}H)^{2-}]}{[(ITP{\cdot}H_2)^{2-}]} = \frac{k^{H{\cdot}ITP{\cdot}H}_{H{\cdot}ITP{\cdot}H_2}}{k^{ITP{\cdot}H_2}_{H{\cdot}ITP{\cdot}H_2}} \qquad (8a)$$

$$= \frac{10^{-1.26 \pm 0.11}}{10^{-1.15 \pm 0.16}} = 10^{-0.11 \pm 0.19} = \frac{0.78 \pm 0.35}{1} \qquad (8b)$$

$$= \frac{44}{56}\left(\frac{30}{70}; \frac{53}{47}\right) \qquad (8c)$$

$$R_{GTP} = \frac{[(H{\cdot}GTP{\cdot}H)^{2-}]}{[(GTP{\cdot}H_2)^{2-}]} = \frac{k^{H{\cdot}GTP{\cdot}H}_{H{\cdot}GTP{\cdot}H_2}}{k^{GTP{\cdot}H_2}_{H{\cdot}GTP{\cdot}H_2}} \qquad (9a)$$

$$= \frac{10^{-1.28 \pm 0.14}}{10^{-2.20 \pm 0.15}} = 10^{0.92 \pm 0.21} = \frac{8.32 \pm 4.02}{1} \qquad (9b)$$

$$= \frac{89}{11}\left(\frac{81}{19}; \frac{93}{7}\right) \qquad (9c)$$

The ratio in eqn. (8c) corresponds to the approximate percentages of the $(H{\cdot}ITP{\cdot}H)^{2-}$ and $(ITP{\cdot}H_2)^{2-}$ species. The first ratio given in parentheses represents the lower limit following from $0.78 - 0.35 = 0.43$ [eqn. (8b)] and the second ratio the

upper limit which follows from 0.78 + 0.35 = 1.13. For GTP and eqn. (9c) the analogous procedure holds.

The results of eqns. (8c) and (9c) confirm the assumptions expressed at the beginning: in the case of $H_2(ITP)^{2-}$ the two isomers occur within the error limits in a ratio of about 1 : 1, while for $H_2(GTP)^{2-}$ the isomeric ratio is close to 10 : 1, confirming that the $(H{\cdot}GTP{\cdot}H)^{2-}$ species strongly dominates.

4. Conclusion

For the twofold negatively charged $H_2(ITP)^{2-}$ species which predominate in the pH range 1.3 < pH < 3.3, the N7 and the phosphate-protonated isomers occur in about equal amounts [eqn. (8)], while for $H_2(GTP)^{2-}$ the N7-protonated species dominate by about 10 : 1 [eqn. (9)]. Of course, both nucleoside 5′-triphosphates bear a γ-phosphate-bound proton that is not lost until neutral solutions are reached (Table 1); at the physiological pH of about 7.4 approximately 90% of both NTPs occur in the form of NTP^{4-}. The (N1)H site begins to lose its proton only at pH > 8.5.

The presented micro-acidity-constant evaluations, especially regarding the basicity of N7 in GTP and ITP, should prove helpful if hydrogen-bond formation or metal-ion binding is considered in a quantitative way. For example, for $Zn(GTP)^{2-}$ and $Zn(ITP)^{2-}$ it is expected that macrochelate formation, *i.e.* the interaction of a phosphate-coordinated Zn^{2+} with N7 (*cf.* Fig. 1), is more pronounced for the former species.

Acknowledgements

The competent technical assistance of Mrs Rita Baumbusch and Mrs Astrid Sigel in the preparation of this manuscript and research grants over several years from the Swiss National Science Foundation (H. S.), which made this study possible, are gratefully acknowledged.

Notes and references

1 D. G. Lambright, J. P. Noel, H. E. Hamm and P. B. Sigler, *Nature*, 1994, **369**, 621.
2 (*a*) C. A. Parent and P. N. Devreotes, *Science*, 1999, **284**, 765; (*b*) H. R. Mott, D. Owen, D. Nietlispach, P. N. Lowe, E. Manser, L. Lim and D. E. Laue, *Nature*, 1999, **399**, 384; (*c*) S. W. Jeong and S. R. Ikeda, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 907.
3 (*a*) N. H. Keep, M. Barnes, I. Barsukov, R. Badii, L.-Y. Lian, A. W. Segal, P. C. E. Moody and G. C. K. Roberts, *Structure*, 1997, **5**, 623; (*b*) E. Cabib, J. Drgonova and T. Drgon, *Annu. Rev. Biochem.*, 1998, **67**, 307.
4 F. Schimmoller, I. Simon and S. R. Pfeffer, *J. Biol. Chem.*, 1998, **273**, 22161.
5 (*a*) D. E. Clapham, *Nature*, 1996, **379**, 297; (*b*) T. Kobayashi, K. Ikeda, H. Kojima, H. Niki, R. Yano, T. Yoshioka and T. Kumanishi, *Nature Neuroscience*, 1999, **2**, 1091; (*c*) H. A. Lester and A. Karschin, *Annu. Rev. Neurosci.*, 2000, **23**, 89.
6 J. W. Gysbers, S. Guarnieri, M. A. Mariggio, T. Pietrangelo, G. Fano and M. P. Rathbone, *Neuroscience*, 2000, **96**, 817.
7 R. M. Smith, A. E. Martell and Y. Chen, *Pure Appl. Chem.*, 1991, **63**, 1015.
8 *IUPAC Stability Constants Database*, Release 3, Version 4.02 (compiled by L. D. Pettit and H. K. J. Powell), Academic Software, Timble, Otley W. Yorks, UK, 1999.
9 *NIST Critically Selected Constants of Metal Complexes*, Reference Database 46, Version 5.0 (data collected and selected by R. M. Smith and A. E. Martell), US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, USA, 1998.
10 *Joint Expert Speciation System* (*JESS*), Version 6.0 (joint venture by K. Murray and P. M. May) Division of Water Technology, CSIR, Pretoria, South Africa, and School of Mathematical and Physical Sciences, Murdoch University, Murdoch, Western Australia, 1999.
11 R. B. Martin and Y. H. Mariam, *Met. Ions Biol. Syst.*, 1979, **8**, 57.
12 R. Tribolet and H. Sigel, *Eur. J. Biochem.*, 1987, **163**, 353.
13 K. Aoki, *Met. Ions Biol. Syst.*, 1996, **32**, 91.
14 H. Sigel, *Coord. Chem. Rev.*, 1990, **100**, 453.
15 H. Sigel, R. Tribolet, R. Malini-Balakrishnan and R. B. Martin, *Inorg. Chem.*, 1987, **26**, 2149.
16 H. Sigel, A. D. Zuberbühler and O. Yamauchi, *Anal. Chim. Acta*, 1991, **255**, 63.
17 M. Bastian and H. Sigel, *J. Coord. Chem.*, 1991, **23**, 137.
18 H. M. Irving, M. G. Miles and L. D. Pettit, *Anal. Chim. Acta*, 1967, **38**, 475.
19 R. Tribolet, R. Malini-Balakrishnan and H. Sigel, *J. Chem. Soc., Dalton Trans.*, 1985, 2291.
20 H. Sigel, *J. Inorg. Nucl. Chem.*, 1977, **39**, 1903.
21 K. H. Scheller, F. Hofstetter, P. R. Mitchell, B. Prijs and H. Sigel, *J. Am. Chem. Soc.*, 1981, **103**, 247.
22 N. A. Corfù, R. Tribolet and H. Sigel, *Eur. J. Biochem.*, 1990, **191**, 721.
23 N. A. Corfù and H. Sigel, *Eur. J. Biochem.*, 1991, **199**, 659.
24 (*a*) H. Sigel, *Biol. Trace Elem. Res.*, 1989, **21**, 49; (*b*) O. Yamauchi, A. Odani, H. Masuda and H. Sigel, *Met. Ions Biol. Syst.*, 1996, **32**, 207.
25 H. Sigel, S. S. Massoud and N. A. Corfù, *J. Am. Chem. Soc.*, 1994, **116**, 2958.
26 Abbreviations and definitions (see also Fig. 1 and 2): Ado, adenosine; Guo, guanosine; *I*, ionic strength; Ino, inosine; Ns, nucleoside; NTP^{4-}, nucleoside 5′-triphosphate; pK_a, negative logarithm of a general acidity constant; $PuNTP^{4-}$, purine-nucleoside 5′-triphosphate; $PyNTP^{4-}$, pyrimidine-nucleoside 5′-triphosphate; Thy, thymidine [= 1-(2′-deoxy-β-D-ribofuranosyl)thymine]; Urd, uridine. Species written in the text without a charge either do not carry one or represent the species in general (*i.e.* independent from their deprotonation degree); which of the two possibilities applies is always clear from the context.
27 R. B. Martin, *Met. Ions Biol. Syst.*, 1996, **32**, 61.
28 R. Tribolet and H. Sigel, *Eur. J. Biochem.*, 1988, **170**, 617.
29 This conclusion is based, *e.g.* on $pK^{H}_{H_2(UMP)} = 0.7 \pm 0.3$ (*cf.* ref. 30) and $pK^{H}_{H_3(AMP)} = 0.4 \pm 0.2$ (*cf.* refs. 12, 23 and 25).
30 S. S. Massoud and H. Sigel, *Inorg. Chem.*, 1988, **27**, 1447.
31 H. Sigel, *Eur. J. Biochem.*, 1987, **165**, 65.
32 H. Sigel, *J. Am. Chem. Soc.*, 1975, **97**, 3209.
33 R. B. Martin, *Acc. Chem. Res.*, 1985, **18**, 32.
34 C. F. Moreno-Luque, E. Freisinger, R. Griesser, J. Ochocki, B. Lippert and H. Sigel, *J. Chem. Soc., Perkin Trans. 2*, submitted for publication.
35 If one assumes for the stability of the $Na(GTP)^{3-}$ and $Na(ITP)^{3-}$ complexes that $\log K^{Na}_{Na(ATP)} = \log K^{Na}_{Na(GTP)} = \log K^{Na}_{Na(ITP)} = 1.31$ (ref. 7) one calculates with $pK^{H}_{H(NTP)} = 6.50$ for $[PuNTP^{4-}] = 5 \times 10^{-4}$ M and $[Na^+] = 0.1$ M that at pH 8.0 two thirds of $PuNTP^{4-}$ are actually present as $Na(PuNTP)^{3-}$. See also footnote 51 in ref. 21.
36 R. B. Martin, *Met. Ions Biol. Syst.*, 1979, **9**, 1.
37 R. B. Martin, *Science*, 1963, **139**, 1198.
38 H. Sigel, S. S. Massoud and R. Tribolet, *J. Am. Chem. Soc.*, 1988, **110**, 6857.
39 B. Song, R. K. O. Sigel and H. Sigel, *Chem. Eur. J.*, 1997, **3**, 29.