Acid–Base Properties of Adenosine 5′-*O*-Thiomonophosphate in Aqueous Solution**

Bin Song, Roland K. O. Sigel, and Helmut Sigel*

Abstract: The acidity constants of H_2-(AMPS)$^{\pm}$ were determined by potentiometric pH titrations in aqueous solution at 25 °C and $I = 0.1$ M ($NaNO_3$). Titrations with a combined single-junction glass electrode were hampered in the presence of AMPS by a "poisoning" effect; the problem could be avoided by use of two separated electrodes. The values of the acidity constants $pK^{H}_{H_2(AMPS)} = 3.72 \pm 0.03$ and $pK^{H}_{H(AMPS)} = 4.83 \pm 0.02$ are relatively close to each other; the buffer regions of the two equilibria overlap, and therefore a micro acidity constant scheme was developed and the constants for the various sites calculated. It is concluded that the thiophosphate-protonated species $(AMPS \cdot H)^-$ dominates at about 75% occurrence, while the form $(H \cdot AMPS)^-$, with the proton at the N1 site of the adenine residue, occurs at about 25%. Semiempirical AM1 and PM3 calculations including water as a solvent locate the proton in $(AMPS \cdot H)^-$ mainly on the terminal oxygen atoms rather than the sulfur. The acid–base properties of $H_2(AMPS)^{\pm}$ are considerably more complicated than those of the parent nucleotide, $H_2(AMP)^{\pm}$; for the latter the two (intrinsic) acidity constants are well separated and consequently practically all protons have left the N1 site before deprotonation at the monoprotonated phosphate group occurs. Finally, an estimate for the acidity constants of $H_2(ATP\gamma S)^{2-}$ is given.

Keywords
acidity · adenosinephosphates · adenosinethiophosphates · protonated isomers · semiempirical calculations

1. Introduction

Nucleotides participate in cellular metabolism as substrates or products in diverse biosynthetic pathways. The central role of nucleotides in living organisms[1] has led to their being widely studied,[2] and so-called nucleoside phosphorothioates[3] or thionucleotides,[4] in which an oxygen atom in the phosphate residue is replaced by sulfur, are nowadays widely employed as probes in all kinds of biological studies.[5, 6] Considering that most reactions in which nucleotides participate are also dependent on metal ions,[7] it is rather surprising to find that the acid–base[4, 8, 9] and metal-ion-binding properties[4, 10, 11] of thionucleotides have hardly been investigated. This contrasts with the situation for nucleotides themselves, for which much information concerning their metal-ion-binding properties has already been accumulated (see for example refs. [2,12,13]).

The first nucleoside phosphorothioate was synthesized in 1966,[3] and adenosine 5′-*O*-thiomonophosphate (AMPS, Figure 1),[14] the thionucleotide in the focus of the present study, shortly thereafter;[15] an improved synthesis was recently published.[16]

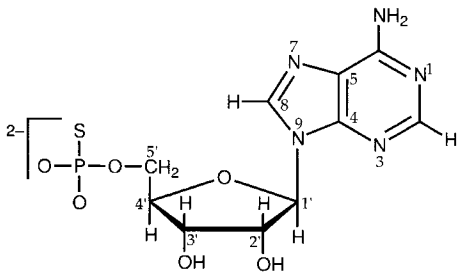

Figure 1. Structure of adenosine 5′-*O*-thiomonophosphate ($AMPS^{2-}$) in its *anti* conformation [14].

AMPS attracted our attention because no values quantifying the acid–base properties of the adenine residue exist, and the available literature values for the release of a proton from the monoprotonated thiophosphate group are relatively far apart, at $pK_a = 4.9$[9] and 5.3;[8] in fact, these values are actually micro acidity constants (see Section 2.1). Furthermore, and more important, the values given are already in the vicinity of the acidity constant for the deprotonation of the H^+(N1) site of twofold protonated adenosine 5′-monophosphate, $H_2(AMP)^{\pm}$, $pK_a = 3.84$.[17] Hence, considering that the latter nucleotide is the par-

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[**] Abbreviations: Ado, adenosine; AMP^{2-}, adenosine 5′-monophosphate; $AMPS^{2-}$, adenosine 5′-*O*-thiomonophosphate, also known as adenosine 5′-*O*-monophosphorothioate or adenosine 5′-[α-thio]monophosphate; ATP^{4-}, adenosine 5′-triphosphate; $ATP\gamma S^{4-}$, adenosine 5′-*O*-[γ-thio]triphosphate; En, ethylenediamine (=1,2-diaminoethane); $RibMP^{2-}$, D-ribose 5-monophosphate. Species which are named in the text without a charge either do not carry one or represent the species in general (i.e., independent of their degree of protonation); which of the two applies is always clear from the context.

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ent compound of AMPS, it occurred to us that the release of the two protons from $H_2(AMPS)^{\pm}$ might occur in overlapping pH ranges and that therefore a detailed study was warranted. Such knowledge is relevant for both the general use of AMPS in biological studies[18] and for an understanding of its metal-ion-binding properties.[10, 11] By potentiometric pH titrations we have now determined the macro acidity constants of $H_2(AMPS)^{\pm}$ and evaluated these for the micro acidity constants of the various individual acidic sites.

2. Results and Discussion

The experimental conditions for the aqueous AMPS solutions ([AMPS] = 0.23 mM) used throughout this study were such that the well-known self-stacking of purine derivatives[19] was certainly negligible.[20]

2.1. Acidity constants of $H_2(AMPS)^{\pm}$: The AMP^{2-} analogue $AMPS^{2-}$ shown in Figure 1 is a tribasic species; it may bind two protons at the thiophosphate group and one at the N1 site of the adenine residue. The first proton from monoesterified derivatives of phosphoric acid is released in water with $pK_a \approx 1$;[21] for $H_3(AMP)^+$, $pK_a \approx 0.4$ was estimated.[14b] Hence, one may assume (also in accord with results obtained for H_3PO_4 and H_3PO_3S[22]) that the corresponding value for $H_3(AMPS)^+$ is even lower; in any case, to be on the safe side, we conclude only that $pK_a < 1.5$ for $H_3(AMPS)^+$ and indeed, during our experiments (pH 3.5–7.0) we never observed any indication of the formation of an $H_3(AMPS)^+$ species. The deprotonation steps that could be observed were for $H_2(AMPS)^{\pm}$, and these are expressed in the equilibria (1a) and (2b). The corresponding

$$H_2(AMPS)^{\pm} \rightleftharpoons H(AMPS)^- + H^+ \quad (1a)$$

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

$$H(AMPS)^- \rightleftharpoons AMPS^{2-} + H^+ \quad (2a)$$

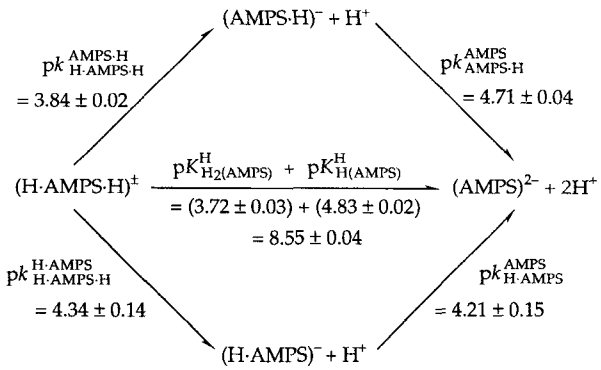
$$K^H_{H(AMPS)} = [(AMPS)^{2-}][H^+]/[H(AMPS)^-] \quad (2b)$$

acidity constants [Eqs. (1b, 2b)], measured in aqueous solution by potentiometric pH titrations (I = 0.1 M, $NaNO_3$; 25 °C), are listed in Table 1[23] together with some pertinent literature values for $H_2(AMP)^{\pm}$ (cf. ref. [17]) and monoprotonated D-ribose 5-monophosphate ($RibMP^{2-}$)[24] as well as adenosine.[14b]

Comparison of the various acidity constants listed in Table 1 shows that the pK_a of 3.72 is evidently largely caused by the deprotonation of the H^+(N1) site of the adenine residue of $H_2(AMPS)^{\pm}$, whereas the second deprotonation step, with a pK_a of 4.83, has largely to be attributed, in agreement with earlier work,[8, 9] to the deprotonation of the monoprotonated thiophosphate group. The two values given in the literature for this deprotonation step were determined by NMR shift measurements in aqueous solution containing 20% D_2O: pk_a = 5.3 (18 °C; I undefined)[8] and pk_a = 4.9 (30 °C; I undefined).[9] Considering that this NMR method actually measures micro acidity constants, k, and that the experimental conditions differ, these two values are in fair agreement with the present macroconstant, $pK^H_{H(AMPS)} = 4.83$.

It is interesting to note that replacement of one of the oxygens of the phosphate group of AMP by a sulfur atom reduces its basicity by approximately 1.4 log units. This observation is in agreement with the conclusion of Jaffe and Cohn[8] that the pK_a values of thiophosphates "... are at least 1 pH unit below the pK_a of the parent compounds".

2.2. Micro acidity constants scheme for $H_2(AMPS)^{\pm}$: The macro acidity constants for $H_2(AMPS)^{\pm}$ listed in Table 1 are separated only by about 1.1 log units; this means that equilibria (1a) and (2a) actually overlap somewhat. To be able to quantify correctly the intrinsic basicities (or acidities) of the proton-binding sites, we calculated the necessary microconstants. This evaluation (Figure 2) was carried out in analogy to a similar problem discussed previously in ref. [17a].



$$K^H_{H_2(AMPS)} = k^{AMPS\cdot H}_{H\cdot AMPS\cdot H} + k^{H\cdot AMPS}_{H\cdot AMPS\cdot H} \quad (3a)$$

$$\frac{1}{K^H_{H(AMPS)}} = \frac{1}{k^{AMPS}_{AMPS\cdot H}} + \frac{1}{k^{AMPS}_{H\cdot AMPS}} \quad (3b)$$

$$K^H_{H_2(AMPS)} \cdot K^H_{H(AMPS)} = k^{AMPS\cdot H}_{H\cdot AMPS\cdot H} \cdot k^{AMPS}_{AMPS\cdot H} = k^{H\cdot AMPS}_{H\cdot AMPS\cdot H} \cdot k^{AMPS}_{H\cdot AMPS} \quad (3c)$$

Figure 2. Equilibrium scheme defining the micro acidity constants (k) and showing their interrelation with the macro acidity constants (K) and the connection between $(AMPS\cdot H)^-$ and $(H\cdot AMPS)$ and the other species present. In $(AMPS\cdot H)^-$ the proton is bound to the thiophosphate group, and in $(H\cdot AMPS)^-$ it is at N1 of the adenine residue (Figure 1); $(H\cdot AMPS\cdot H)^{\pm}$, also often written as $H_2(AMPS)^{\pm}$, carries a proton at N1 and another at the thiophosphate group. The arrows indicate the direction for which the acidity constants are defined. Use of the value measured for $H_2(AMP)^{\pm}$, $pK^H_{H_2(AMP)} = 3.84 \pm 0.02$ (see Table 1), for the microconstant $pk^{AMPS\cdot H}_{H\cdot AMPS\cdot H}$ permits calculation of the other microconstants from Equations (3a), (3b), and (3c). The error limits of the various constants were calculated according to the error propagation after Gauss; the limits correspond to three times the standard error (see Table 1). For further details see Section 2.2.

Table 1. Negative logarithms of the acidity constants [Eqs. (1,2)] of the twofold protonated $H_2(AMP)^{\pm}$ and $H_2(AMPS)^{\pm}$ species (see Figure 1) as well as of the related monoprotonated adenosine and D-ribose 5-monophosphate species determined by potentiometric pH titrations in water at 25 °C and I = 0.1 M ($NaNO_3$) [a].

Acid	pK_a for the sites (N1)H^+	-$P(O)_2(X)H^-$ [b]	Ref.
H(adenosine)$^+$	3.61 ± 0.03	–	[14b]
H(RibMP)$^-$	–	6.24 ± 0.01	[24]
$H_2(AMP)^{\pm}$	3.84 ± 0.02	6.21 ± 0.01	[17a]
$H_2(AMPS)^{\pm}$	3.72 ± 0.03	4.83 ± 0.02	this work

[a] So-called practical (or mixed) constants [23] are listed; see Section 4.2. The error limits given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] X = O for $RibMP^{2-}$ and AMP^{2-}; X = S for $AMPS^{2-}$.

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Figure 2 summarizes the equilibrium scheme for $H_2(AMPS)^{\pm}$ defining the microconstants (k) and giving their interrelation with the macro acidity constants (K). There are three independent equations, (3a), (3b), and (3c) (Figure 2), but four unknown microconstants.[17a, 25] However, by assuming that the monoprotonated thiophosphate and phosphate groups of $H_2(AMPS)^{\pm}$ and $H_2(AMP)^{\pm}$, respectively, have the same effect on the deprotonation of the $H^+(N1)$ site of the adenine residue, one may conclude that $pk^{AMPS\cdot H}_{H\cdot AMPS\cdot H} = pK^{H}_{H_2(AMPS)} = 3.84 \pm 0.02$.[17a] Consequently, the other three microconstants can now be calculated. The corresponding results are given on the arrows in Figure 2.

These microconstants now permit the estimation of the ratio R of the monoprotonated species $(H\cdot AMPS)^-$ and $(AMPS\cdot H)^-$ (Figure 2), which carry the proton at N1 or at the thiophosphate group, respectively [Eq. (4)]. The values for the

$$R = \frac{[(AMPS\cdot H)^-]}{[(H\cdot AMPS)^-]} = \frac{k^{AMPS\cdot H}_{H\cdot AMPS\cdot H}}{k^{H\cdot AMPS}_{H\cdot AMPS\cdot H}} = \frac{10^{-3.84\pm0.02}}{10^{-4.34\pm0.14}}$$
$$= 10^{0.50\pm0.14} = 3.16 = \frac{76}{24} \approx \frac{3}{1}\left(\frac{4.4}{1}; \frac{2.3}{1}\right) \quad (4)$$

ratio given in parenthesis are the upper and lower limits, respectively, calculated from the error propagation. Overall it is evident that the species $(AMPS\cdot H)^-$ is dominant at about 75% occurrence, while $(H\cdot AMPS)^-$ forms only at about 25%. Certainly, this result is an estimate, but still it proves i) that both tautomeric forms of $H(AMPS)^-$ occur simultaneously in appreciable amounts and ii) that the $(AMPS\cdot H)^-$ species dominates and therefore largely determines $pK^{H}_{H(AMPS)}$, as already indicated in Section 2.1.

2.3. Some considerations on the acid–base properties of the thiophosphate group: That one proton in $H_2(AMPS)^{\pm}$ is bound at the N1 site of the adenine residue and the other at the thiophosphate group follows clearly from the acidity constants listed in Table 1. However, while protonation of the adenine residue at N1 is unequivocal, the site of protonation at the thiophosphate group is less clear (see also Section 2.4). Considering that the phosphorus in a phosphate monoester has a tetrahedral structure,[26] the three terminal oxygens in the phosphate residue (Figure 3) have identical properties; that is, each of them in the dianion carries two thirds of a charge unit. If one of these three oxygens is replaced by a sulfur atom, assuming that a proton does not bind at all to the sulfur atom one might expect a decrease of the pK_a value by 0.18 pK units (because in $ROPO_3^{2-}$ the proton finds three possibilities to bind, and in $ROPO_2S^{2-}$, under the assumption made, only two). On the other hand one might argue that owing to the lower electronegativity of sulfur compared with oxygen, the charge density at the remaining two terminal oxygens increases. If this were the case then the basicity, that is, the affinity for protons, of these two oxygens should increase. Consequently, considering that these two aspects operate in opposite directions, one might expect that the value for $pK^{H}_{H(AMPS)}$ would be about the same as for $pK^{H}_{H(AMP)}$ or, if the second of the two effects is somewhat larger, a higher basicity for $AMPS^{2-}$ might even be expected. However, exactly the opposite is observed; the acidity of the monoprotonated thiophosphate group in $(AMPS\cdot H)^-$ is *lower* than that of the monoprotonated phosphate residue in $H(AMP)^-$ by about 1.5 pK_a units [expression (5)]. Indeed, this

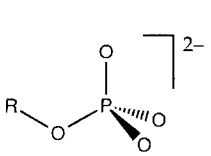

Figure 3. Tetrahedral structure of a phosphate residue.

$$\Delta pK_a = pK^{H}_{H(AMP)} - pk^{AMPS}_{AMPS\cdot H} = (6.21 \pm 0.01) - (4.71 \pm 0.04)$$
$$= 1.50 \pm 0.04 \quad (5)$$

ΔpK_a value agrees well with observations made by Jaffe and Cohn[8] in water containing 20% D_2O, who found $pK_a = 6.7$ for $H_2PO_4^-$ and $pK_a = 5.4$ for $H_2PO_3S^-$ ($\Delta pK_a = 1.3$), as well as with the results of Mäkitie and Konttinen[27] for the same two acids in aqueous solution at $I = 0.1$ M (KCl) and 25 °C, namely $pK^{H}_{H_2PO_4} = 6.76$ and $pK^{H}_{H_2PO_3S} = 5.38$, and hence $\Delta pK_a = 1.38$.

Why is $ROPO_3^{2-}$ more basic than $ROPO_2S^{2-}$? Is it because the proton is partially sulfur-bound to the latter species, or is it because the proton in $ROPO_3H^-$ is chelated by hydrogen bonds in a six-membered ring including a water molecule to one of the other two (negatively charged) terminal oxygens and that this inhibits the release of the proton? Clearly, replacement of one of the two oxygens by a sulfur would destabilize such a hydrogen-bonded chelate and thus facilitate the release of the proton from a $-OP(O_2S)H^-$ group. Frey and Sammons[28] explained the observations described—the relative strengths of analogous sulfur and oxyacids—quite generally, with the assumption that "in aqueous solution a negative charge localized on sulfur is less unstable than one localized on oxygen ... because the larger size and polarizability of sulfur relative to oxygen ... (allow) the charge density in a thiolate anion to be less than that in an oxyanion".

2.4. Where is the proton in $(AMPS\cdot H)^-$ located? Regarding this question, the review by Frey and Sammons[28] summarizing the experimental evidence concerning the bond order and charge localization in nucleoside phosphorothioate anions, as well as of some related work,[29] is helpful. For $AMPS^{2-}$ it was concluded that the P–S bond is a single bond with a negative charge localized on sulfur and that the two terminal P–O bonds approach bond orders of 1.5, so each oxygen carries half a charge unit.[28, 29a]

That the negative charge density is high on the sulfur atom was also concluded for thiophosphate by Katchalski et al. from changes in the absorption spectrum; these authors considered tautomeric equilibria but made no final suggestion regarding the location of the proton.[30] It is thus interesting that Hidaka et al. concluded that two PO_3S^{3-} ions coordinate to [*trans*-Co$(En)_2]^{3+}$ through the S atoms.[31] On the other hand, Frey and Sammons[28] placed the proton in HPO_3S^{2-} at an oxygen in accord with the infrared and Raman spectroscopic studies of Steger and Martin,[32] whose P–S stretching frequencies[32a] of HPO_3S^{2-} and PO_3S^{3-} are consistent with single bonds,[29b] and who found no sign of an S–H group in HPO_3S^{2-}.[28, 32]

With the above-mentioned situation in mind, semiempirical calculations were carried out for thioacetic acid and monoprotonated methylthiophosphate, which is the most simple model for $(AMPS\cdot H)^-$ (Figure 1). We included thioacetic acid ($pK_a = 3.33$)[33] in the calculations because i) practically the same ΔpK_a value (1.42) is observed compared with acetic acid

($pK_a = 4.75$)[33] as given in Section 2.3 for the phosphoric/thiophosphoric acids and also because ii) it has been unequivocally shown by Raman and infrared spectroscopy[34] that the right-hand side of the tautomeric equilibrium $CH_3C(S)OH \rightleftharpoons CH_3C(O)SH$ is heavily favored at room temperature, that is, "the acid is substantially completely in the thiol rather than the thione form";[34b] this also applies to related acids[35] like monothioformic acid.[35a] Indeed, calculations based on the PM 3 method of Stewart,[36] including water as a solvent by applying the SM 3 procedure of Cramer and Truhlar,[37] yielded a reaction enthalpy at standard conditions of $-32\ kJ\,mol^{-1}$ for the tautomeric equilibrium $CH_3C(S)OH \rightleftharpoons CH_3C(O)SH$. A corresponding result of $-20\ kJ\,mol^{-1}$ was obtained with the AM 1 calculation technique of Dewar et al.[38] considering the presence of water with the SM 2 method.[39] It is satisfying to see that both semiempirical calculation procedures, despite the difference in the actual values, favor the $CH_3C(O)SH$ tautomer at 25 °C, in accord with the experimental observations described. This gives one the confidence to apply the same two calculation techniques to the tautomeric equilibrium $CH_3OP(S)(O)OH^- \rightleftharpoons CH_3OP(O)_2SH^-$; the results are 38[36, 37] and $53\ kJ\,mol^{-1}$,[38, 39] meaning that the proton is now preferably oxygen-bound, which is in agreement with the suggestion of Frey and Sammons for HPO_3S^{2-} and $H_2PO_3S^-$.[28]

To conclude, the reliability of such semiempirical calculations should not be overestimated, but they appear to indicate the correct trend. In other words, they confirm the experimental observation that $CH_3C(O)SH$ is quite stable and consequently they suggest also that $CH_3OP(O)_2SH^-$ is the less stable tautomer, meaning that at 25 °C $CH_3OP(S)(O)OH^-$ either strongly dominates or at least exists to a large fraction in the $CH_3OP(S)(O)OH^- \rightleftharpoons CH_3OP(O)_2SH^-$ equilibrium. Hence, it appears that in $(AMPS{\cdot}H)^-$ the proton is mainly oxygen-bound, although it is still possible that a minor fraction of the sulfur-bound tautomer also occurs.

3. Conclusions

From an acid–base point of view AMPS is clearly a more complicated molecule than its parent nucleotide AMP. The two pK_a values for $H_2(AMP)^{\pm}$ are separated by about 2.4 pK_a units (see Table 1); this means that practically all protons have left the N1 site (compare with $pK^H_{H(Ado)} = 3.61$[14b]) before deprotonation at the still monoprotonated phosphate group (compare with $pK^H_{H(RibMP)} = 6.24$[24]) proceeds (see Figure 4, upper part). This is very different for $H_2(AMPS)^{\pm}$; here the two deprotonation reactions overlap, and consequently at about pH 4.28, where the $H(AMPS)^-$ species reaches its maximum concentration (see the lower part of Figure 4), approximately 25% of the protons are at the N1 site and 75% at the thiophosphate group;[40] in the latter the protons appear to be mainly on oxygen atoms (as discussed in Section 2.4).

As for $H_2(ATP\gamma S)^{2-}$ (a thio analogue of ATP, which is also widely employed as a probe in biological studies, e.g., refs. [5a,6a,18a,18e,41]), a similar rigorous evaluation of its acid–base properties still needs to be made. Based on the available acidity constants for $H_2(AMP)^{\pm}$ (Table 1) and $H_2(ATP)^{2-}$ (cf. ref. [42]) and the differences observed between $H_2(AMP)^{\pm}$ and $H_2(AMPS)^{\pm}$ (see Table 1) one may estimate values for $H_2(ATP\gamma S)^{2-}$: $pK^H_{H_2(ATP\gamma S)} = 3.88$ and $pK^H_{H(ATP\gamma S)} = 5.09$ (25 °C; $I = 0.1$ M); the estimated error limits are ± 0.1 pK unit. The estimated $pK^H_{H(ATP\gamma S)}$ value for $H(ATP\gamma S)^{3-}$ is lower by $\Delta pK_a = 1.4$ than that measured for $H(ATP)^{3-}$ ($pK^H_{H(ATP)} = 6.47$);[42] this difference is identical with the one obtained by Jaffe and Cohn[8] in their ^{31}P NMR shift experiments for the same species. It is evident that in this case also some overlap in the pH ranges of the two deprotonation reactions occurs, a situation which could be analyzed analogously to the treatment presented here for $H_2(AMPS)^{\pm}$.

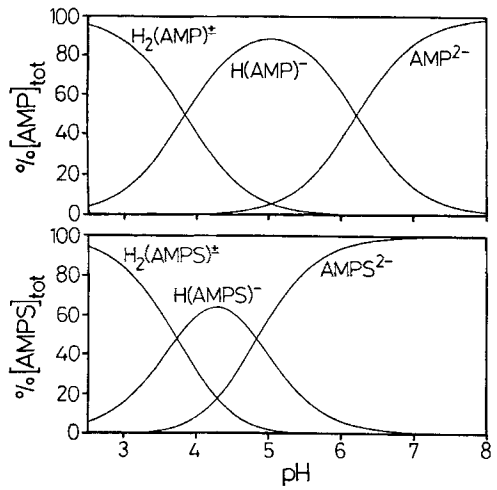

Figure 4. Effect of pH on the concentration of the species present in a diluted aqueous solution of AMP (top) or AMPS (bottom) at 25 °C; $I = 0.1$ M, $NaNO_3$. The results are plotted as the percentage of the total AMP or AMPS present. The calculations are based on the acidity constants listed in Table 1.

4. Experimental Section

4.1. Materials: Two different lots of the dilithium salt of $AMPS^{2-}$ were obtained from Sigma, St. Louis, MO, and a further sample of the dilithium salt came from Serva Feinbiochemica, Heidelberg (Germany). Throughout the studies no differences between the three samples could be detected. The curve fits carried out in connection with the determination of the pK_a values of $H_2(AMPS)^+$ proved the absence of any impurity with acid–base properties. In fact, the very high purity of the commercial AMPS was confirmed by a referee, who ran a ^{31}P NMR spectrum of a sample from Sigma; we are most grateful to the referee for this action. The aqueous stock solution of $AMPS^{2-}$ was freshly prepared daily, and the pH was adjusted to about 7.5.

Potassium hydrogenphthalate, HNO_3, NaOH (Titrisol), and sodium nitrate (all pro analysi) were from Merck, Darmstadt (Germany). All solutions were prepared with bidistilled CO_2-free water. The titer of the NaOH used for the titrations was established with potassium hydrogenphthalate.

4.2. Potentiometric pH titrations: The pH titrations were carried out with a Metrohm E 536 potentiograph, E 655 dosimat, and 6.0202.100 (JC) combined single-junction macro glass electrodes or separated electrodes (a 6.0133.100 Metrohm pH measuring electrode with a 6.0726.100 Ag/AgCl reference electrode). The buffer solutions (pH 4.64, 7.00, 9.00; based on the NBS scale, now U. S. National Institute of Standards and Technology (NIST)) used for calibration were also from Metrohm AG, Herisau (Switzerland). Direct pH-meter readings were used in the calculations of the acidity constants; these constants are so-called practical, mixed, or Brønsted constants [23a]. Their negative logarithms given for aqueous solutions at $I = 0.1$ M ($NaNO_3$) and 25 °C may be converted into the corresponding concentration constants by subtracting 0.02 from the listed pK_a values [23a]; this conversion term contains both the junction potential of the glass electrode and the hydrogen-ion

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activity [23,43]. It should be emphasized that the ionic product of water (K_w) and the conversion term mentioned do not enter into the calculations because the differences in NaOH consumption between pairs of solutions, i.e. with and without ligand [23], were used in the evaluations (see also below).

At the beginning our potentiometric pH titrations were seriously hampered by the use of the above-mentioned combined glass electrodes: as soon as the ligand AMPS was added to the titration vessel, "poisoning" of the electrode could be observed; this means that during the titration the plotted pH vs. volume of NaOH curve became unsteady (in this connection see also ref. [44]). The problem increased every time a solution containing the ligand was titrated. An improvement in the titration curves of the solutions with the ligand could be achieved firstly by rigorous exclusion of dioxygen from the titration vessel right from the beginning and addition of the necessary HNO_3 only shortly before starting the titration, and secondly by washing the electrode after each ligand titration for 45 s in 50 mM HNO_3, flushing it with water, and then leaving it for 10 min in 3 M KCl solution to recover. Finally we found that the problem could be avoided and that excellent titration curves could be obtained with the two separated electrodes mentioned, a pH-measuring electrode in combination with a reference electrode, but still carrying out the treatments described above. The results listed in Section 2 are the averages obtained from all experiments.

4.3. Determination of the acidity constants of $H_2(AMPS)^{\pm}$: The acidity constants $K^{H}_{H_2(AMPS)}$ and $K^{H}_{H(AMPS)}$ of $H_2(AMPS)^{\pm}$ were determined by titrating 50 mL of aqueous 0.56 mM HNO_3 and $NaNO_3$ ($I = 0.1$ M; 25 °C) in the presence and absence of 0.23 mM $AMPS^{2-}$ under N_2 with 1 mL of 0.03 M NaOH, and by means of the difference in NaOH consumption between two such titrations for the calculations.

The acidity constants were calculated with an IBM-compatible PC (with an 80486 processor) connected to a Hewlett-Packard 7475 A plotter and a Brother M1509 printer, with a curve-fit procedure that used a Newton–Gauss nonlinear least-squares program within the pH range of about 3.7 to 7, which corresponds to a neutralization degree of about 50% for the equilibrium $H_2(AMPS)^{\pm}/H(AMPS)^{-}$ and to one of more than 99% for the equilibrium $H(AMPS)^{-}/AMPS^{2-}$. The results given in Table 1 are the averages of 27 and 34 independent pairs of titrations for $pK^{H}_{H_2(AMPS)}$ and $pK^{H}_{H(AMPS)}$, respectively.

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