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Phosphorous metabolites in boar spermatozoa
Identification of AMP by multinuclear magnetic resonance

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Abstract

Boar spermatozoa revealed three prominent resonances in the ^{31}P-NMR spectrum of intact cells. Two of these are known to be GPC and P_i, the third is a phosphomonoester (PME), the identification of which was carried out by proton-detected 2D ^{1}H,^{31}P and ^{1}H,^{13}C chemical shift correlation experiments with gradient selection. The PME was unambiguously assigned to adenosine 5′-monophosphate (AMP). The identification was confirmed by an AMP consuming enzymatic assay. Other physiologically relevant PME's, in particular inosine 5́-monophosphate (IMP) and sugar phosphates, were excluded. The intensity of the ^{31}P signal of AMP in boar sperm extract was much higher than those of ADP and ATP, and in intact cells only AMP but no ATP was visible.

Keywords: NMR, ^{1}H-; NMR, ^{13}C-; NMR, ^{31}P-; Heteronuclear single quantum correlation; Heteronuclear multiple quantum correlation; Gradient selection; Spermatozoon; Phosphomonoester; Adenosine 5′-monophosphate; Energy metabolism

1. Introduction

^{31}P-NMR spectroscopy has become a powerful tool for studying noninvasively the energy metabolism in vivo [1,2]. In addition, NMR detects resonances of metabolites which are not even expected or difficult to find by biochemical analyses. Many investigations performed on muscles have elucidated the changes of intracellular pH and the levels of phosphagen and inorganic phosphate (P_i) during contraction. Concerning adenine nucleotides, the three resonances of ATP are generally visible while those of ADP and AMP are not detectable in vivo because the cytoplasmic concentrations are low and the resonances are overlapped by either those of α- and γ-ATP or other phosphomonoesters [3].

Similar observations as in muscles have been reported in sperm from different species of invertebrates (sea urchin [4]; horseshoe crab [5]; lugworm [6]) and of vertebrates (trout [7]; carp and turkey [8]) but with the exception of mammals [9]. In mammalian semen or washed spermatozoa ATP and ADP resonances are difficult to detect in vivo and no phosphagen signal has been demonstrated so far. The spectra obtained from different mammalian spermatozoa only revealed the pronounced signal of a phosphodiester, a P_i signal, and a broad band of phosphomonoesters. In the case of boar spermatozoa the phosphodiester has been identified as glycerophosphorylcholine (GPC) [9,10] which was recently con-

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firmed by multinuclear magnetic resonance analysis [11]. The present work is focused on the identification of the phosphomonoesters in boar spermatozoa. From the ^{31}P chemical shifts, glycolytic phosphometabolites, nucleotide monophosphates, or analogues of phosphorylcholine are physiologically relevant candidates. The problems of bad signal resolution and of the pH-dependence of the ^{31}P chemical shifts [12] may explain why only few investigators have assigned the resonances to specific phosphomonoesters. This, however, is requested for the in vivo analysis of the energy metabolism in sperm since the concentration of phosphomonoesters appeared to be much larger than that of ADP or ATP.

Robitaille et al. [9] attributed the ^{31}P resonances of a phosphomonoester in boar sperm extract to fructose-1,6-biphosphate. However, the two signals expected at 4.52 and 4.65 p.p.m. at pH 8.7 [12] were not resolved at the magnetic field of 7 T used in their analysis. Similarly, Phadke et al. [13] investigated the cells of goat sperm originating from the cauda region of the epididymis and attributed three phosphomonoesters to phosphoglycerate, glucose 6-phosphate and AMP. Here we describe the identification of the phosphomonoesters in cell extracts of boar semen by the application of several homo- and heteronuclear NMR correlation methods. For optimum sensitivity and resolution the NMR spectra were recorded as lyophilizates of perchloric acid extracts of washed spermatozoa. The candidates for the PME compounds were proved by spiking and by the metabolization of the compound in a specific enzymatic assay.

2. Materials and methods

2.1. Samples and chemicals

Boar semen of proven fertility was provided by a local insemination centre (GFS-Ascheberg/Westphalia) and collected by using the gloved-hand technique. The gel fraction was removed by gauze filtration. Ejaculates of at least four boars were transported to our laboratory within 2 h after ejaculation and mixed. Sperm motility and concentration were assessed by microscopic observation.

Chemicals and enzymes were purchased from Boehringer, Mannheim and Merck, Darmstadt.

2.2. Extraction procedure

Freshly collected boar semen was centrifuged at 500 *g* for 20 min at room temperature and the supernatant removed. The spermatozoan pellet was gently resuspended in 160 mM NaCl-solution and centrifuged for 15 min. The procedure was repeated and the pellet was rapidly frozen in liquid nitrogen. 3.4 g of the frozen pellet was powdered under liquid nitrogen. The powder was mixed with 7.0 ml of 3 M PCA and homogenized with an Ultra Turrax (Janke und Kunkel, Staufen). After centrifugation (30,000 *g* for 20 min at 4°C) the supernatant was neutralized on ice with 3 M KOH containing 0.4 M KCl. $KClO_4$ was removed by centrifugation (10,000 *g* for 10 min at 4°C) and the extract was lyophilized.

2.3. Enzymatic AMP assay

For this objective the lyophilizate was dissolved in an aqueous test buffer (100 mM HEPES, 20 mM magnesium $(acetate)_2$, 50 mM KCl, pH 7.4) to a final volume of 3 ml. Then, the reagents ATP and phosphocreatine (PCr) were added from stock solutions to final concentrations of 0.8 mM and 1.6 mM, respectively. After addition of adenylate kinase (AK, EC 2.7.4.3, 20 U/ml) and creatine kinase (CK, EC 2.7.3.2, 16 U/ml) the following reactions took place:

$$\mathrm{AMP + ATP} \overset{AK}{\rightleftarrows} \mathrm{2ADP}$$

$$\mathrm{ADP + PCr} \overset{CK}{\rightleftarrows} \mathrm{ATP + Cr}$$

This two-step assay was pursued by ^{31}P-NMR spectroscopy at 30°C.

2.4. NMR measurements

A Bruker AM 360 instrument working at 145.8 MHz was used for in vivo ^{31}P-NMR measurements. Samples were measured in 10 mm o.d. sample tubes without spinning and field/frequency lock. The same instrument was used for recording the ^{31}P-NMR spectra of the lyophilizate employed for the enzyme test.

NMR spectra of the lyophilizates dissolved in D_2O were recorded on a Varian UNITY plus 600 spectrometer equipped with a 5 mm ^{1}H{X} pulsed-field gradient inverse detection probe operating at 600,

242.8 and 150.8 MHz for ^{1}H, ^{31}P and ^{13}C, respectively. The *z*-field gradients were of rectangular shape with a maximum gradient strength of 30 Gauss/cm. The ring down delay of the gradients was set to 50 μs. The samples were not spun. ^{1}H and ^{13}C chemical shifts were calibrated internally with 3-trimethylsilyl [2,2,3,3-D]-1-propionate (TSP). ^{31}P chemical shifts were expressed relative to 85% phosphoric acid, referenced to GPC at 0.51 p.p.m. [11].

2.4.1. 1D NMR spectra

The in vivo ^{31}P-NMR spectra, as well as those for the enzymatic test, were obtained with 50° pulses, 7 kHz spectral width, acquisition time 2.3 s, relaxation delay 0.5 s, 700 transients and processing with 15 Hz line broadening. No ^{1}H decoupling.

The ^{31}P-NMR spectrum of the lyophilizate at 242.8 MHz was obtained with 60° pulses, 12 kHz spectral width, acquisition time 2 s, relaxation delay 2 s, 4000 transients and processing with 10 Hz line broadening. ^{1}H WALTZ decoupling.

^{1}H-NMR spectra were obtained routinely using single pulses and presaturation of the HDO signal. 1D TOCSY [14] was performed with a 200 ms 90° EBURP-1 selective pulse [15] and with several spin-lock mixing times (for further details, see [16]).

2.4.2. 2D NMR spectra

2.4.2.1. HSQC with gradient selection and editing. The sequence is similar to the phase-cycled version of Davis [17] except that the editing period $\delta - 180(^{1}H,^{13}C) - \delta$ was applied after the variable evolution period [15]; $\tau = 1/4J$, $\delta = 1/2J$; ^{13}C decoupling with GARP. The gradient equation [18] was fulfilled by a 4:1 ratio of durations, 2 and 0.5 ms, respectively. The spectrum was recorded with a spectral width of 5 kHz and a 2K data size in f_2. 256 t_1 increments with 24 scans each, spectral width of 23 kHz in f_1. Homospoil pulse (8 ms) during the recycling time of 1.2 s. Processing with Gaussian functions in both dimensions. 2K × 2K Fourier transformation.

2.4.2.2. Gradient-selected HMBC. The pulse sequence of Bax and Summers [19] was used.

$^{1}H,^{13}C$ correlation: low-pass J-filter with $\Delta_1 = 3.6$ ms, $\Delta_2 = 62$ ms (optimized for $^{2,3}J \sim 8$ Hz). Coherence pathway selection with three pulsed-field gradients [20] with the area ratios $g_1:g_2:g_3 = 2:2:-1$ (p-type selection). The pulsed field gradients were of 2 ms duration with maximum gradient strength. Data were accumulated as 512 × 2K points with 44 acquisitions taken per t_1 increment. Spectral width of 30 kHz in f_1 and 5 kHz in f_2. Processing with sine-bell functions in both directions. 2K × 2K Fourier transformation.

$^{1}H,^{31}P$ correlation: $\Delta_2 = 71$ ms (optimized for $^{3,4}J \sim 7$ Hz). Area ratios of the pulsed-field gradients $g_1:g_2:g_3 = 1.25:1.25:-1$. 256 increments with 48 scans each. Homospoil pulse (8 ms) during the recycling time of 1 s. Spectral width of 12 kHz in f_1 and 5 kHz in f_2. Processing with sine-bell functions in both dimensions. 2K × 2K Fourier transformation.

3. Results and discussion

3.1. NMR identification of the phosphomonoester

Fig. 1a shows the in vivo ^{31}P-NMR spectrum of motile boar spermatozoa. Several rather broad resonances could be detected of which those at 0.51 and 2.3 p.p.m. were assigned earlier to GPC and P_i, respectively [9–11]. A third signal was observed at approx. 3.6 p.p.m. corresponding to the ^{31}P spectral range where phosphomonoesters (PME's) are expected [12]. For the following assignment a PCA extract of the sperm cells was prepared which gave a ^{31}P-NMR spectrum of much narrower line widths and therefore better resolution than was observed with the intact spermatozoa.

Fig. 1b shows the ^{31}P-NMR spectrum of the lyophilizate of the PCA extract of washed spermatozoa recorded in D_2O at pH 8.5. In contrast to the in vivo spectrum where no ADP and AMP were visible, low-intensity signals were observed in the −5 to −20 p.p.m. range. The chemical shift change of P_i from 2.3 to 3.31 p.p.m. was indicative that in the intact cells (Fig. 1a) pH < 7 [12]. Obviously, when compared with Fig. 1a, the PME signals occurring at 5.05, 4.47 and 4.10 p.p.m. were also displaced toward high frequency, corresponding to the effect of ionization of the secondary phosphate. However, the signal at 4.47 p.p.m. was largely dominant in the extract spectrum (Fig. 1b) (> 90%). The signal with

line width of 10 Hz was unlikely to be composed of two resonances since at 14 T a chemical shift difference as small as 0.04 p.p.m. should be resolved. In the following the identification of the prominent PME compound is reported. The use of D_2O simplified the recording of the ^{1}H-NMR spectrum as well as that of the ^{1}H,^{31}P and ^{1}H,^{13}C chemical shift correlated 2D NMR spectra needed for identification. Also, the choice of pH 8.5 where the ionization of the secondary phosphate is complete in most cases facilitated the comparison of the ^{31}P, ^{1}H and ^{13}C chemical shift data with literature values.

As already noted in the introduction, the ^{31}P chemical shift value of the observed PME (4.47 p.p.m. in the extract at pH 8.5) cannot be assigned with certainty to a unique compound, and many candidates with similar chemical shifts needed to be considered ($\delta \pm 0.05$ p.p.m. of the deprotonated forms from [12]): phosphorylethanolamine (4.42); phosphorylserine (4.50); ribose-5-phosphate (4.50); D-myo-inositol-

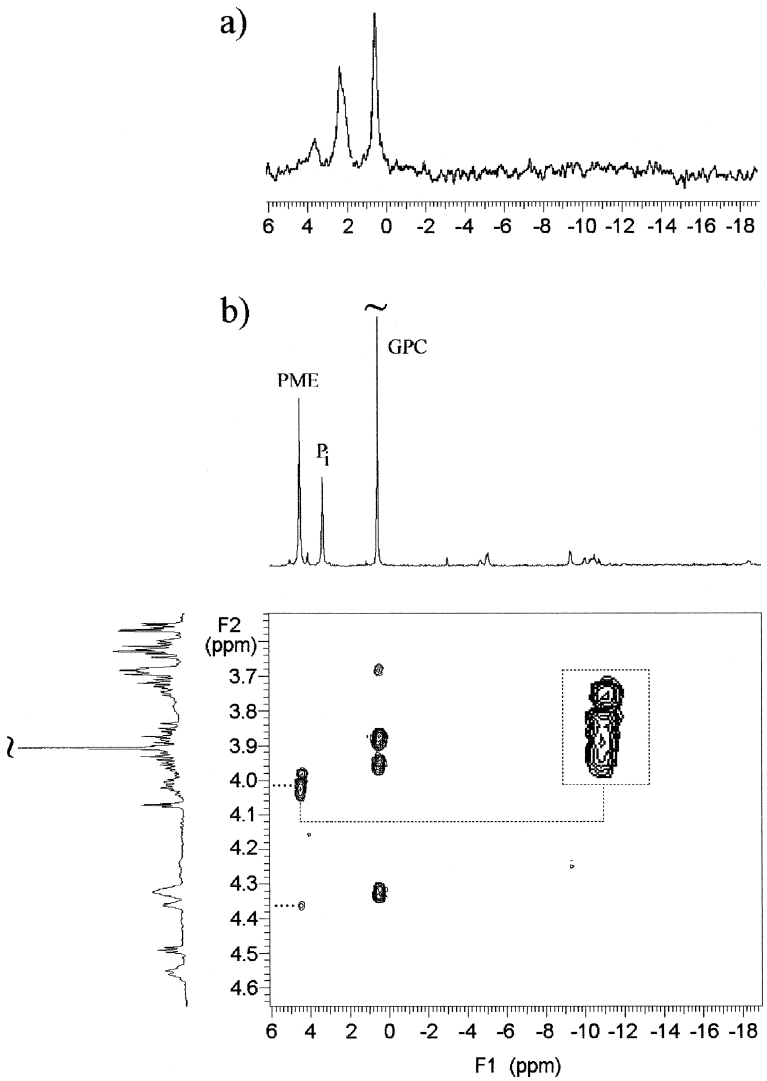


Fig. 1. a: in vivo ^{31}P-NMR spectrum (145.8 MHz) of motile boar spermatozoa washed with 160 mM NaCl and measured at 22°C. b: ^{31}P-NMR spectrum (242.8 MHz) of a PCA extract of the sperm cells at pH 8.5 and 30°C in ^2H_2O. Below: 2D ^{1}H,^{31}P chemical shift correlation (gs-HMBC). The inset shows an expansion for the PME cross-peak at δ (H5′,5″) ~ 4 p.p.m. The experiment was optimized for a P,H coupling constant of 7 Hz. Assignments: GPC, glycerol 3-phosphorylcholine; P_i, inorganic phosphate; PME, phosphomonoesters.

1-phosphate (4.60); fructose 6-phosphate (4.46); 3-phosphoglycerate (4.69). In addition, the nucleotides AMP (4.41), GMP (4.39), TMP (4.32), UMP (4.37) as well as IMP (4.36 [21]) should be examined. IMP is of particular physiological relevance in mammalian muscles where it accumulates during contraction when ATP is successively dephosphorylated to ADP and AMP followed by deamination of AMP [21]. It was thus evident that only the additional analysis of the ^{1}H- and ^{13}C-NMR spectra could help in the assignment of the searched compound. However, heteronuclear chemical shift correlated spectra provide the chemical shifts of both ^{1}H and ^{13}C, or ^{31}P, and therefore give more reliable assignments [22].

The 2D ^{1}H,^{31}P chemical shift correlation spectrum of the extract from a gradient selected HMBC experiment [19] is shown in Fig. 1b. The measurement was optimized for a P,H coupling of 7 Hz. On the f_2-axis, a portion of the 1D ^{1}H-NMR spectrum of the extract is presented. As expected, the latter was extremely complex with severe overlap of the resonances. If the

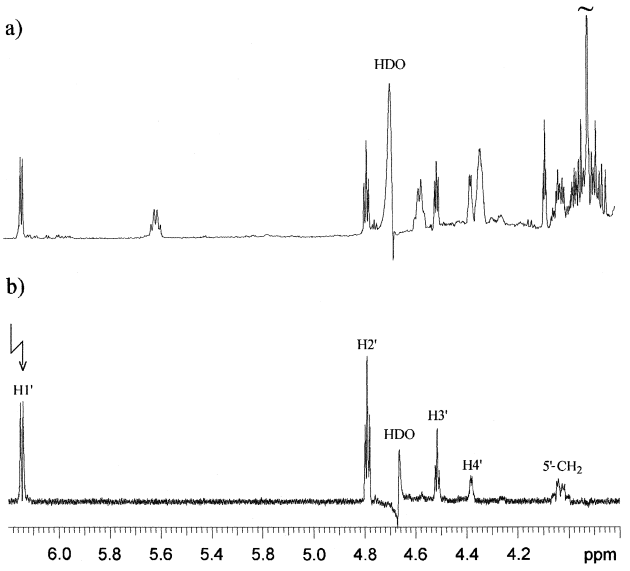
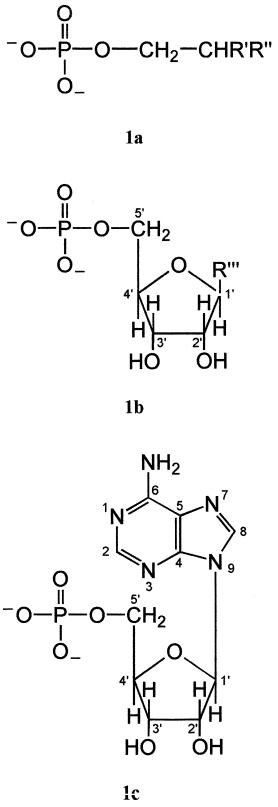


Fig. 2. a: section of the normal ^{1}H-NMR spectrum of the extract. The HDO solvent signal was suppressed by presaturation. b: 1D TOCSY spectrum obtained by selective irradiation (↓) of the doublet at 6.13 p.p.m. The spin-lock mixing time was 140 ms. The full proton coupling pattern of the ribose moiety is visible.



Scheme 1.

Table 1
^{1}H- and ^{13}C-NMR data of the prominent PME compound in boar cell extracts [a], identified as AMP (lc)

Assignments	Chemical shifts(p.p.m.) [b,c]		Coupling constants(Hz) [d]
	^{1}H	^{13}C	
2CH	8.24 (s)	155.9	$^{3}J(H1',H2') = 5.5$
4C	–	152.0	$^{3}J(H2',H3') = 5.5$
5C	–	121.6	$^{3}J(H3',H4') = 4.3$
6C	–	158.6	
8CH	8.57 (s)	143.1	
1′CH	6.13 (d)	90.0	
2′CH	4.78 (dd)	77.4	
3′CH	4.50 (dd)	73.6	
4′CH	4.37 (m) [e]	87.5 (d)	$^{3}J(P,C4') = 8.8$
5′CH_2	~ 4.0 (m) [e]	66.5 (d)	$^{2}J(P,C5') = 4.1$

[a] Measured as lyophilizates in $^{2}H_2O$ at pH 8.5 (meter reading) and $T = 30$°C.
[b] Measured relative to internal TSP. For comparison with literature values, take notice of $\delta_H(TSP) = \delta_H(TMS)$, but $\delta_c(TSP) = \delta_c(TMS) + 1.7$ p.p.m.
[c] ^{1}H-NMR: s, singlet; d, doublet; m, second order multiplet. ^{13}C-NMR: d, due to P,C coupling.
[d] Only those values are given which could be read directly from the extract spectra.
[e] Multiplets are complex because of additional P,H couplings.

correlation peaks obtained at the ^{31}P chemical shift of 0.51 p.p.m. and arising from several three- and four-bond P,H couplings [23] confirmed the presence of GPC, those at $\delta(^{31}P) = 4.47$ p.p.m. needed discussion for the assignment of the PME.

The strong cross-peak observed at δ (^{1}H) ~ 4.0 p.p.m., after expansion in the f_1 and f_2 axes (inset in Fig. 1b), was indicative of diastereotopic methylene protons undergoing vicinal couplings with the phosphorous atom. The contribution of two coupling protons was confirmed by integration within the ^{1}H NMR spectrum. However, the recording of a ^{1}H-coupled ^{31}P-NMR spectrum did not resolve a fine structure, presumably due to an unfavourable coupling to line width ratio. A further cross-peak was observed at $\delta(^{1}H) = 4.37$ p.p.m. (Fig. 1b), indicating a small P,H long-range coupling. In conclusion, the partial structure **1a** (Scheme 1) could be anticipated. A convenient way of identification of further protons being part of the PME was achieved by 1D TOCSY. Fig. 2b shows the result of a selective irradiation of the proton doublet at 6.13 p.p.m. From its high-frequency chemical shift position relative to the other proton signals edited, this resonance was indicative of the anomeric proton of a sugar moiety. The TOCSY spectrum obtained with a long spin-lock mixing time gave the resonances of all protons within the same coupling network, namely four additional proton multiplets, including those two which were already detected due to their ^{1}H,^{31}P correlations. The individual

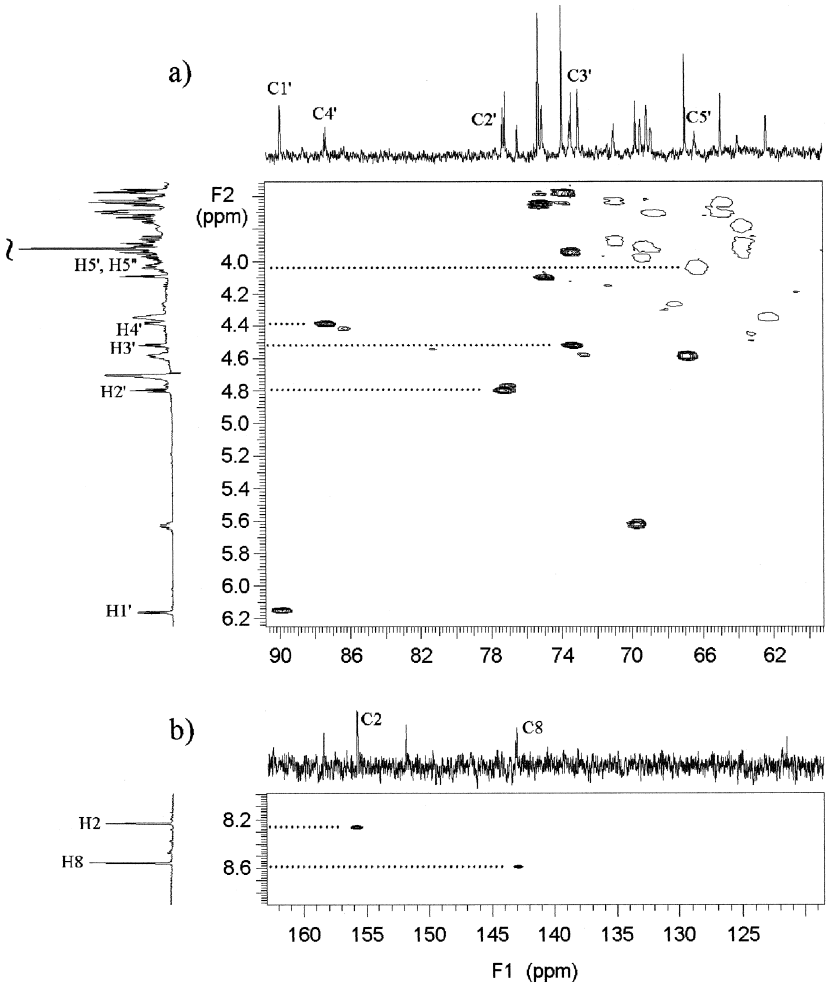


Fig. 3. 2D ^{1}H,^{13}C chemical shift correlation (gs-HSQC) of the extract. The sequence gives correlations over one bond with editing: CH_2 peaks negative (one contour line) and CH/CH_3 peaks positive. The experiment was optimized for a C,H coupling constant of 140 Hz. On the left: 1D ^{1}H-NMR spectrum. On the top: 1D ^{13}C-NMR spectrum. The lower part shows the aromatic region.

coupling relations could be obtained from a COSY spectrum or from TOCSY spectra with gradually increasing mixing time (not shown). The TOCSY spectrum edited in Fig. 2b and assigned as indicated was in favour of a five-membered sugar ring, and the measured ^{1}H chemical shifts and H,H coupling constants (Table 1) proposed the partial structure **1b**, i.e., a 1′-substituted ribose-5′-phosphate. A 2′-deoxy-ribose sugar could be excluded since the appearance of a further proton would have changed the H,H coupling network. This argument was also valid for the elimination of fructose 6-phosphate existing dominantly (81%) in the β-furanose form [24]. If the TOCSY spectrum was able to eliminate most of the compounds mentioned above as candidates for the PME in boar cell extract, there was no intention to further push the structural identification at that point since, in particular for nucleotides, it is well known [25] that the ^{1}H chemical shifts vary strongly with concentration, temperature and pH.

Fig. 3 shows the gs-HSQC spectrum with gradient selection and multiplicity editing. The ^{1}H and ^{13}C chemical shifts were correlated via the one-bond C,H couplings. The proton resonances of the sugar moiety which were identified before, led to the assignment of five ^{13}C resonances C1′ to C5′. The editing clearly confirmed that C5′ was bound to two protons with almost degenerate chemical shift (Fig. 3a). It once more excluded a 2′-deoxy-ribose derivative since C2′ was bound to only one proton. The resonances of C4′ and C5′ appeared as doublets in the ^{1}H decoupled ^{13}C-NMR spectrum (Fig. 3a, on the top). This gave

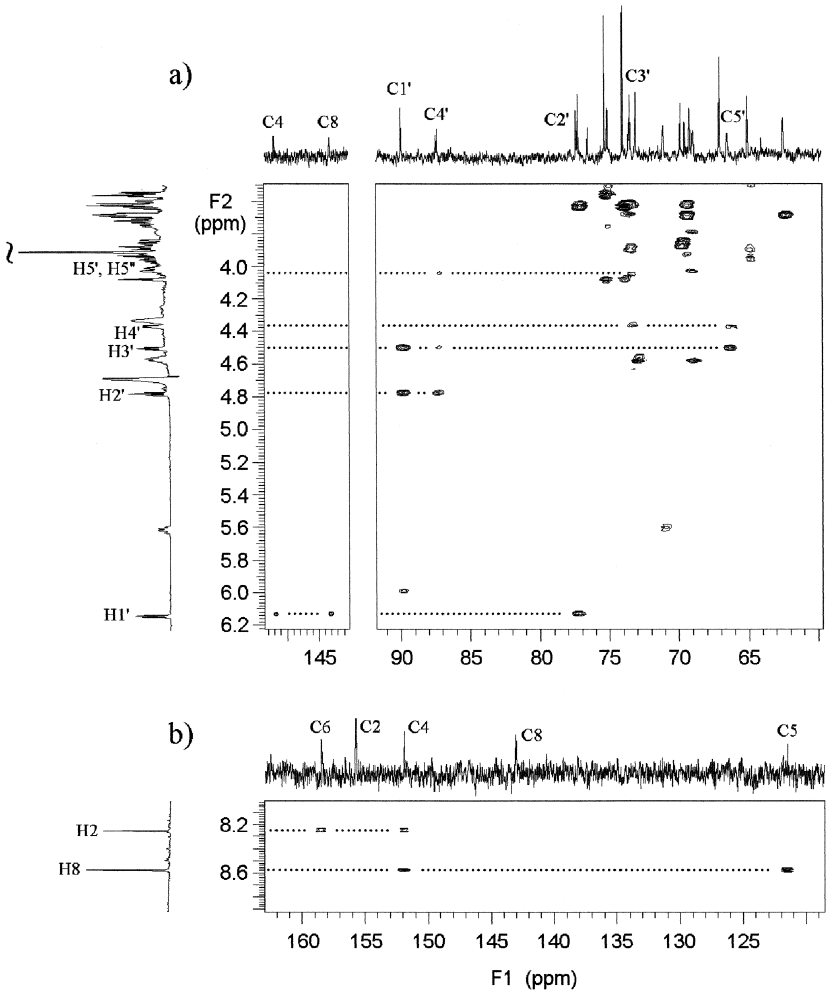


Fig. 4. 2D ^{1}H,^{13}C chemical shift correlation (gs-HMBC) of the extract. The correlation peaks are due to two- and three-bond C,H couplings. The measurement was optimized for a coupling constant of 8 Hz. The lower part shows the aromatic region.

the indication for a C,P coupling over three and two bonds, respectively, and confirmation of structure **1b** (a cyclic 3′,5′-phosphate would reveal four couplings). The heteronuclear coupling constants are given in Table 1, together with the ^{13}C chemical shift data.

Fig. 3b shows the aromatic part of the gs-HSQC spectrum. Two one-proton singlets were observed at 8.24 and 8.57 p.p.m. correlating via $^{1}J_{C,H}$ with the carbons at 155.9 and 143.1 p.p.m. Obviously, the three other carbon resonances observed in the aromatic region should correspond to quaternary carbons. It was however not clear if the latter were part of the same molecule in this mixture of compounds. The gs-HMBC spectrum of Fig. 4b showed that both aromatic protons gave responses to the quaternary carbons (via $^{2,3}J_{C,H}$) proving that all five carbon resonances arise from the same heteroaromatic moiety. The number of C-atoms immediately excluded any pyrimidine bases such as cytosine or uracil. Comparing the NMR data of Table 1 with ^{1}H [25] and ^{13}C [26] literature values clearly indicated the presence of adenine. Indeed, the ^{1}H chemical shifts were most similar in solutions of high (0.01 M) dilution [25]. From the other purine bases, guanine could be excluded since only one singlet from H8 was expected in the aromatic chemical shift range. Also, inosine could be excluded, most definitively due to the chemical shift reversal of the C2 and C4 resonances [27].

The question whether adenine is linked to the structure **1b** forming AMP could not be answered by ^{1}H NMR spectroscopy since there are no readily observable proton-proton couplings between the ribose and the base [28]. However, affirmation was obtained by looking at the gs-HMBC spectrum (Fig. 4a). Indeed, the anomeric proton H1′ showed two responses from the vicinal couplings with C4 and C8 of the adenine moiety. This information, linking the different parts of the molecule must be considered as very important for the structural identification of AMP (**1c**) in the extracts of spermatozoa.

3.2. Identification of the phosphomonoester by a specific enzyme assay

Fig. 5a shows the ^{31}P-NMR spectrum of the extract after the addition of ATP and PCr (see Section 2), the signals for GPC, P_i and PME derived from the extracted intracellular compounds (cf. Fig. 1b). After further addition of the enzymes AK and CK (Fig. 5b) the ^{31}P resonance assigned to a PME dissappeared within 20 min, metabolizing to ATP with a corresponding two-fold decrease of the reagent PCr. Due to the specificity of this AK reaction [29,30] PME was assigned to AMP.

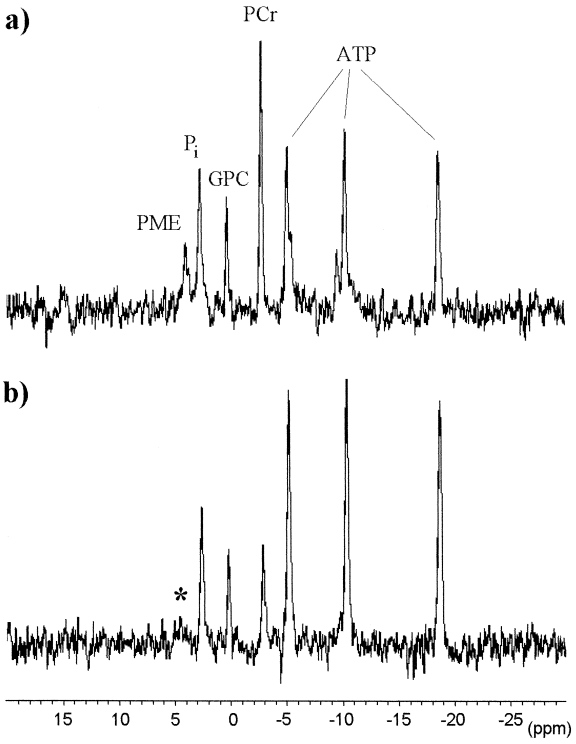


Fig. 5. a: ^{31}P-NMR spectrum (145.8 MHz) of the extract recorded at 30°C after addition of ATP and phosphocreatine (PCr); b: after further addition of the enzymes AK and CK and a 20 min incubation. The asterisk indicates the disappearence of the PME signal.

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