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Nuclear Magnetic Resonance Studies of Boar Seminal Plasma. Problems Encountered in the Identification of Small Molecules: Hypotaurine and Carnitine

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Two major components in boar seminal plasma were assigned by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The first, previously called substance X (see Ref. 1, *Biochim. Biophys. Acta* 1243, 101–109 (1995)), was identified with difficulty as hypotaurine. This pointed to general difficulties in the NMR assignments of small molecules in mixtures of substances, even at the highest magnetic fields. In contrast, the identification of the second component as carnitine was obtained in a straightforward manner by total correlation spectroscopy and proton-detected ^{13}C chemical shift correlation methods (gradient-selected heteronuclear single quantum coherence and heteronuclear multiple bond correlation). Carnitine is known as a transporter of fatty acids through membranes. Both compounds were ultimately confirmed by addition of the authentic compounds.

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INTRODUCTION

The analysis of low-molecular-weight compounds in animal semen with the aid of nuclear magnetic resonance (NMR) spectroscopic methods is a highly demanding task in reproductive physiology and pathophysiology.[2] Many of the constituents appear to be good candidates for proving the sperm energy charge[3] or the secretory functions of the epididymis and the accessory glands in mammals.[4] In the assignment of NMR signals for compound identification, however, one is confronted with many problems, e.g. very low concentrations of the substances, regions of overlapping resonances of the substance mixtures, and the intense solvent signal. ^{31}P NMR spectroscopy can be considered as relatively favourable because of its good sensitivity, its wide chemical shift range and the limited number of phosphorous-containing substances, whereas ^{1}H NMR spectroscopy requires the highest magnetic fields and multidimensional methods to obtain the resolution necessary for analysis. On the other hand, ^{13}C NMR spectroscopy demands high sensitivity which, apart from a high magnetic field, can be best obtained by proton-detected chemical shift correlation techniques.[5] The different requirements and methods for the answer to the questions raised by these nuclei were recently discussed by Lynch *et al*.[6] in relation to NMR studies on human seminal fluid.

Although instruments with the highest magnetic fields and numerous modern NMR techniques are available,[5–7] it was clear from our previous study[1] on boar seminal plasma that it is by no means simple to identify all the substances in a logical way, especially those with low molecular weight which give relatively little information from the ^{1}H and ^{13}C NMR spectra. Although knowledge of the chemical shifts, chemical shift correlations and coupling constants was available for a particular compound (designated as X in Ref. 1), we were only able to make a structural proposal of RCH_2CH_2R', where R,R′ remained definitively unknown. In the present work, we wanted to determine the molecular structure of X by a still more-detailed analysis of the chemical shift and coupling data, by an interpretation of the 'crossover' effect observed for the ^{1}H and ^{13}C chemical shift correlations, by pH titration and by search in a ^{13}C NMR data bank. Although we steadily approached the solution of the structure, this approach was strongly accelerated by the contact with people who had a large survey of the metabolites detected in various body fluids. The solution for the X compound was 'hypotaurine' (2-aminoethanesulfinic acid) (Scheme 1) which we were finally able to confirm from the ^{1}H and ^{13}C NMR spectra of an aqueous solution of the commercial substance, or better by its addition to the original seminal plasma sample, the so-called 'spiking' experiment.

We have recently been shown a preliminary note of van der Horst and Grooten[8] describing the occurrence of hypotaurine in seminal plasma of boar, bull and dog which was forwarded to us. Obviously, this has also happened to other authors since in none of the recent reviews on

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Abbreviations used: COSY, chemical shift correlation spectroscopy; E-BURP-1, pulse shape; GARP, pulse sequence for broad band decoupling; GPC, glycerophosphorylcholine; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; MLEV-17, pulse sequence for spin locking; TOCSY, total correlation spectroscopy; TSP, 3-trimethyl-silyl-2,2,3,3,-tetradeutero sodium propionate.

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endogenous metabolites in body fluids, was hypotaurine mentioned as constituent.[2,9] It is only very recently that the story of hypotaurine in spermatozoa has been taken up again and its importance for fertility emphasized.[10]

In the present work, not only was hypotaurine identified as a constituent of boar seminal plasma but also 'carnitine' (3-carboxy-2-hydroxy-N,N,N-trimethyl-1-propanaminium hydroxide). We shall demonstrate that the structure of the latter compound could be derived by straightforward NMR techniques including gradient-selected spectroscopy. Not only were the 'central' proton and carbon coupling patterns obtained, but also the 'closing' functional groups were established by correlations via homo- and heteronuclear long-range couplings. Obviously, the assignment of small organic molecules in seminal plasma requires different strategies from compound to compound.

EXPERIMENTAL

Seminal plasma of boar was deproteinized with cold PCA solution according to Ref. 1. The pH was adjusted with KOH solutions. After centrifugation and lyophilization, the resulting powder was dissolved in D_2O. The test compounds were commercial and used without further purification.

NMR spectra were recorded on a Varian UNITY plus 600 spectrometer equipped with a 5 mm $^1H\{^{15}N–^{31}P\}$ pulsed field gradient indirect probe operating at 600 and 150.8 MHz for ^1H 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 measuring temperature was 30°C. The samples were not spun. Calibration was made internally with TSP.

Spectra measurement

One-dimensional ^{1}H NMR. Spectra were obtained with 50° pulses, a 5 kHz window, 3.5 s acquisition time and 32 K data points.

Selective one-dimensional TOCSY. Data were acquired using the pulse sequence of Kessler *et al*.,[11] except that a 200 ms E-BURP-1 selective pulse[12] was used. This pulse was shaped in 1 dB steps using a scaling attenuator. With digitization as a regular sequence of 256 rectangular elements, a good phase purity and uniform excitation intensity was obtained within the specified bandwidth. The pulse length of the trimpulses was 2 ms. The MLEV-17[13] spin-lock field (10 kHz) was applied in an array between 80 and 150 ms. The recycling time was 1 s.

Two-dimensional J-resolved ^{1}H NMR. Spectra were obtained by the standard spin–echo sequence with a spectral width of ± 25 Hz in f_1 and 5 kHz in f_2. One hundred and twenty-eight experiments were performed with size 2 K in t_2. The recycling time was 1.5 s. Prior to Fourier transformation, the data were zero-filled to 512 W in t_1 and apodized by means of a sine-bell function in both directions. The spectra were tilted by 45° and symmetrized.

HSQC with gradient selection and editing. The pulse sequence for inverse single quantum editing was similar to the phase-cycled version of Davis[14] except that the editing period δ-180(^{1}H,^{13}C)-δ was applied after the variable evolution period. The delays $\tau = 1/(4J)$, $\delta = 1/(2J)$ corresponded to an editing of CH_2 negative, CH/CH_3 positive. J was set at a value of 140 Hz for the one-bond C,H couplings. The ^{13}C nuclei were decoupled with the GARP technique.[15] The gradient equation[16] 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 2 K data size in f_2. Five hundred and twelve t_1 increments with 16 scans each were made. A homospoil pulse was used during the recycling time of 1 s, a gaussian function in both dimensions and a 2×2 K Fourier transformation.

Gradient-selected HMBC. The pulse sequence of Bax and Summers[17] was applied. The low pass J-filter (Δ_1) was optimized for an average C,H coupling of 140 Hz (3.6 ms); the Δ_2 delay was optimized for 8 Hz (62 ms). The coherence pathways were selected with three pulsed-field gradients according to Hurd and John[18] with the area ratios $g_1:g_2:g_3 = 2:2:-1$ (p-type selection yielding a magnitude mode spectrum). The pulsed field gradients were of 2 ms duration. The data were accumulated as 512×2 K points with 28 acquisitions taken per t_1 increment and processed by sine-bell functions in both directions followed by a 2×2 K Fourier transformation.

RESULTS AND DISCUSSION

In a previous publication[1] inositol, citrate, lactate and GPC have been identified as prominent constituents in boar seminal plasma. However, resonances were left in the ^{1}H and ^{13}C NMR spectra corresponding to further endogenous metabolites of this biofluid. In the present work we have used higher magnetic fields for NMR measurements and, at the same time, made the following changes in the sample preparation. (a) The seminal plasma, after treatment with PCA, was neutralized with KOH instead of K_2CO_3. This preventive measure was according to Sherry *et al*.[19] who had observed that taurine, a possible candidate for the X compound, was derivatized to its carbamate in extract solutions during neutralization with bicarbonate. (b) The lyophilizates were titrated to pH 7. (c) TSP was added as internal standard to make chemical shifts independent of the salt concentrations (and thus magnetic susceptibility differences) which varied in the series of extract preparations.

Hypotaurine

Figure 1 shows the 600 MHz ^{1}H NMR spectrum of the lyophilizate of boar seminal plasma (pH 7) between 1 and 6 ppm. Furthermore, the regions where the multiplets of the X compound are contained were expanded (Fig. 1(a)). From a two-dimensional *J*-resolved spectrum (Fig. 2) the cross-sections at the chemical shift positions of 2.66 and 3.37 ppm clearly revealed two (pseudo)triplets corresponding to a $[AX]_2$ spin system with the partial structure RCH_2CH_2R' (two vicinal H,H couplings ~6.8 Hz). A two-dimensional C,H chemical shift correlation experiment (not shown) assigned the carbons which were part of the ethylene fragment. As already mentioned, a 'crossover' effect of the ^{1}H and ^{13}C chemical shifts was observed: the ^{13}C resonance at high frequency (58.0 ppm) correlated with the methylene protons at low frequency (2.66 ppm), that at low frequency

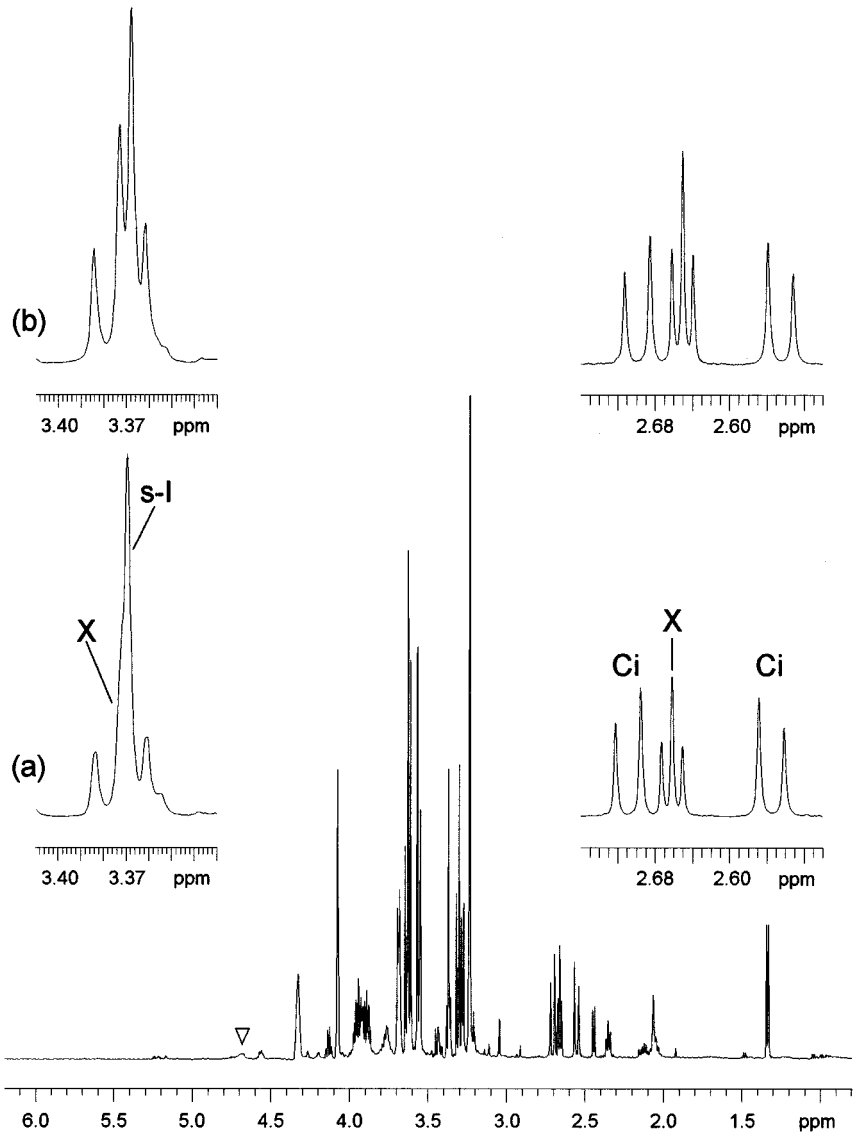

Figure 1. ^{1}H NMR spectrum of the aliphatic region of boar seminal plasma in D_2O. The HDO signal (▽) was presaturated. Expansions (a) contain the (pseudo)triplets of hypotaurine X. The same expansions are shown in (b) after addition of the authentic material to the seminal plasma ('spiking'). Key: Ci, citrate; s-I, scylloinositol (cf. Ref. 1).

(36.1 ppm) with the methylene protons at high frequency (3.37 ppm). Table 1 displays the ^{1}H and the ^{13}C chemical shifts of the methylene groups of X measured under the altered experimental conditions. In addition, Table 1 gives the one-bond C,H coupling constants.

Additional information could be obtained by recording the NMR spectra of boar seminal plasma at varying pH (Fig. 3). Differential proton chemical shift changes towards low frequencies occurred for both resonances between pH 7 and 12, with an inflection point at about 9.5. This led to the conclusion that one or more titrating protons must be in the molecule, and that at least one amino group could be assumed for R,R′. We were therefore directed to aliphatic (poly)amines with two chemically non-equivalent carbon atoms of identical intensity. This immediately excluded spermine and spermidine, detected earlier in seminal fluid,[20] and also putrescine, since a more complex ^{1}H NMR spectrum was expected as a consequence of its $[(AX)_2]_2$ spin system and lack of a C,H chemical shift 'crossover' effect (Table 1). Although we do not understand this 'crossover' effect in detail we believe that the main contribution arises from the electric fields[21] of the charged groups on the ^{13}C chemical shifts. In the following this effect was very useful for the elimination of a large number of compounds, including β-alanine (Table 1).

In the series of polyamines[22] further compounds with two chemically non-equivalent carbon atoms needed discussion: 2,2′-imino-bis-(ethylamine) and 2,2′,2″-nitrilo-tris-(ethylamine). In aqueous solution at neutral pH the primary amino groups were fully protonated and caused a 'crossover' effect (Table 1). However, neither the ^{1}H nor ^{13}C chemical shifts corresponded to those of the unknown compound (Table 1), a fact which was also demonstrated by 'spiking'. The ammonium salt $^{+}N(CH_2CH_2NH_2)_4Cl^{-}$ was also considered, but this compound was not available to us and therefore no NMR spectrum or 'spiking' could be performed. It was nevertheless excluded by the argument that the ^{13}C signal should show fine structure since the one-bond ^{13}C,^{14}N coupling must be observable due to the highly symmetrical nitrogen environment. For comparison, in $^{+}N(CH_2CH_3)_4Cl^{-}$ a triplet with a coupling of 3 Hz was well resolved (own measurement). On the other hand, there appears to be no straightforward way to exclude a compound such as $^{-}O–N^{+}(CH_2CH_2NH_2)_3$ since it is not

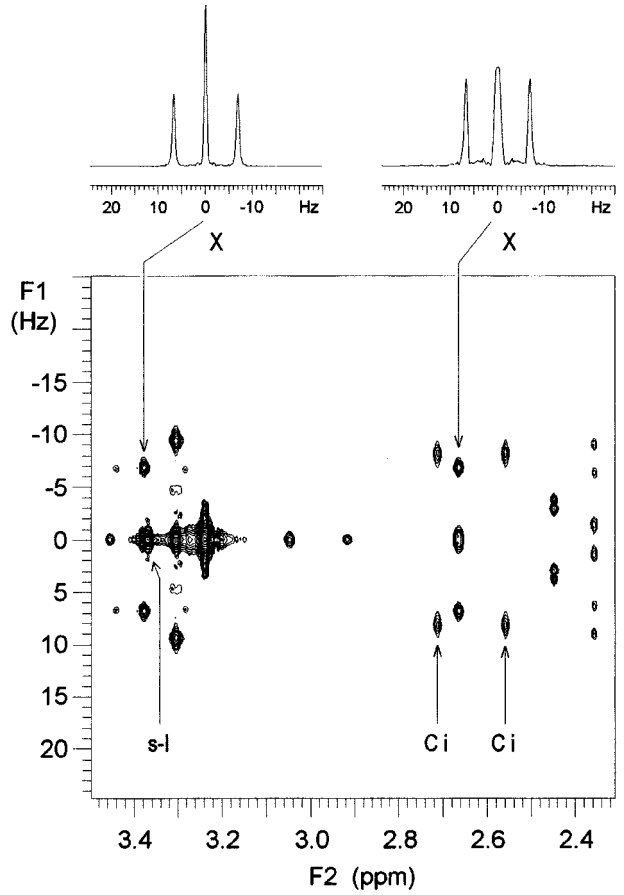

Figure 2. Two-dimensional J-resolved ^{1}H NMR spectrum of part of the aliphatic region of boar seminal plasma, showing the contour plot and the cross-sections at 2.66 and 3.37 ppm of hypotaurine X.

commercially available and since the parent $^-O–N^+(CH_3)_3$ which is widespread in the animal kingdom[23] did not show a resolved triplet for the carbon resonance.

We then turned our attention to sulphur-containing amino acids. Since the 'crossover' effect was maintained at high pH after deprotonation of the amino group(s) and because of the hypothesis that a charged group was required for 'crossover', we searched for an acidic group stronger than a carboxylic function, e.g. a sulfonic or a sulfinic acid group, with very low pK_a at the edge of the measured pH titration range (Fig. 3). Taurine (pK_as = 1.5; 8.7)[24] appeared as a good candidate since it has been found in the NMR spectra of human seminal fluid[3] and was also found in a ^{13}C NMR data bank. However, the spiking experiment failed, and as a consequence several other sulphur-containing amino acids related to cysteine, e.g. hypotaurine, thiotaurine and S-sulfocysteamine (not part of the ^{13}C NMR data bank mentioned) were examined. Finally, we confirmed hypotaurine (pK_as = 2.2; 9.6)[24] by spiking. Figure 1(b) shows the effect of adding free hypotaurine to the lyophilizate solution. The increase in the intensity of the triplet at 2.66 ppm was clearly visible when the spectral sections of Fig. 1(a) and (b) (right) were compared and reference was taken to the adjacent citrate resonances. The increase of the triplet at 3.37 ppm (Fig. 1(b), left) was also evident though slightly hampered due to the overlap of the central line by the singlet resonance of scyllo-inositol.

The individual proton assignments in X (Table 1) were made by analogy to those in taurine.[19] The carbon assignments followed from the subsequent C,H chemical shift correlation. The large one-bond C,H coupling of 145 Hz (Table 1) confirmed the assignment of the N-CH_2 carbon (compare 145 Hz for $CH_3{}^+NH_3$ and 138 Hz for CH_3SR).[25]

The occurrence of hypotaurine and other sulphur-containing amino acids in the seminal plasma of boar was demonstrated earlier by electrophoresis experiments.[8] Indeed, hypotaurine was found[8] to be the main component of all amino acids, which was in accordance with the present NMR results (~10 mM, determined from the ^{1}H NMR signal intensities referenced to the citrate concentration;[1] experiments were carried out with four boars and the results were fairly similar). Recent results[10] point to a key role for hypotaurine in fertility since it is present all the time before and after fertilization and also is in contact with gametes and the embryo. Hypotaurine appears to be a most important candidate for testing pathological cases in boar

Table 1. NMR parameters of hypotaurine in boar seminal plasma and of selected compounds examined for its identification[a]

Compound[b]	Assignment	Chemical shift (ppm) ^{1}H	^{13}C	^1J(C,H) (Hz)
Hypotaurine (X)	1-CH_2	2.66	58.0	137
	2-CH_2	3.37	36.1	145
$H_3\overset{+}{N}$-$\overset{3}{C}H_2$-$\overset{2}{C}H_2$-$\overset{1}{C}O_2^-$	2-CH_2	2.47	36.2	128
β-Alanine	3-CH_2	3.10	39.2	145
$H_3\overset{+}{N}$-$\overset{1}{C}H_2$-$\overset{2}{C}H_2$-$\overset{3}{C}H_2$-$\overset{4}{C}H_2$-$\overset{+}{N}H_3$	1,4-CH_2	2.98	42.4	143
Putrescine	2,3-CH_2	1.69	27.5	129
$HN(\overset{2}{C}H_2$-$\overset{1}{C}H_2$-$\overset{+}{N}H_3)_2$	1-CH_2	3.04	41.5	143
2,2′-Imino-bis-(ethylamine)	2-CH_2	2.85	47.9	136
$N(\overset{2}{C}H_2$-$\overset{1}{C}H_2$-$\overset{+}{N}H_3)_3$	1-CH_2	3.09	39.4	144
2,2′,2″-Nitrilo-tris-(ethylamine)	2-CH_2	2.83	52.8	136
$H_3\overset{+}{C}$-$\overset{2}{C}H_2$-$\overset{1}{C}H_2$-SO^-_3	1-CH_2	3.19	50.1	137
Taurine	2-CH_2	3.36	38.0	147

[a] All samples at pH 7–7.4 in D_2O. Proton and carbon chemical shifts referenced to internal TSP.
[b] Structures are drawn with the charges as met at neutral pH.

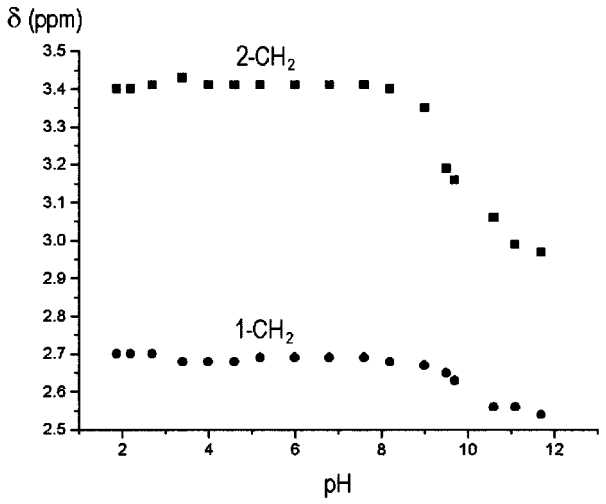

Figure 3. The pH dependence of the ^{1}H chemical shifts of hypotaurine in boar seminal plasma measured in D_2O at 30°C.

spermatozoa and its NMR characterization and quantification must be considered to be essential. Since in hypotaurine, as in taurine,[19] carbamate formation can be expected during neutralization of the PCA extracts with bicarbonate,[1] the decision to perform neutralization with KOH is retrospectively justified.

Carnitine

In contrast to hypotaurine, another component of boar seminal plasma, carnitine Car, could be identified by purely NMR spectroscopic methods, without trial and intuition. First the corresponding proton–proton coupling pattern was detected by selective one-dimensional TOCSY.[11, 26] Figure 4 shows, as an example, the spectral editing of the ABKMX spin system where the transitions of the AB part at ~2.46 ppm corresponding to the 2-CH_2 protons were selectively irradiated. Using a rather long mixing time, coherence transfer occurred not only to the X spins at 4.58 ppm corresponding to the 3-CH protons, but also to the KM spins at 3.45 ppm corresponding to the 4-CH_2 protons. The relative signal intensities of the three proton groups were in agreement with the structure. Furthermore, a small intensity singlet response was observed at 3.25 ppm which was indicative of coherence propagated via the long-range coupling to the trimethyl amino protons. In the original ^{1}H NMR spectrum (Fig. 4) the latter resonance signal was overlapped by those of other compounds containing the same trimethylammonium group (mostly GPC).[1] The gradient-selected HSQC spectrum with multiplicity editing[14] is shown in Figure 5. The proton and carbon nuclei are correlated via ^{1}J. Starting from the proton assignments, the resonances of C-2 to C-4 were located indicating the correct multiplicities and reasonable chemical shifts (C-2 (45.7 ppm, t), C-3 (66.8 ppm, d) and C-4 (73.0 ppm, t)). As expected[5] the gradient-selected HMBC spectrum (Fig. 6) showed much less T_1 noise than its phase-cycled analogue. The correlations via ^{1}J were perfectly suppressed by the low-pass filter. Apart from the correlation of the 2-CH_2 protons with C-3 (via ^{2}J) and C-4 (via ^{3}J), that with C-1 of the carboxyl group (via ^{2}J) was also clearly observable. The assignment of C-1 as part of a carboxyl group was also confirmed by the chemical shift of 181.5 ppm. The 4-CH_2 protons showed the expected correlations with C-2 (via ^{3}J) and C-3 (via ^{2}J). However, it was essential to find another cross-peak at ~57 ppm in the region of the ^{13}C resonances of trimethylammonium groups (Fig. 6). This gave further confidence for the attribution of such a group to the unknown compound. The 3-CH proton confirmed the

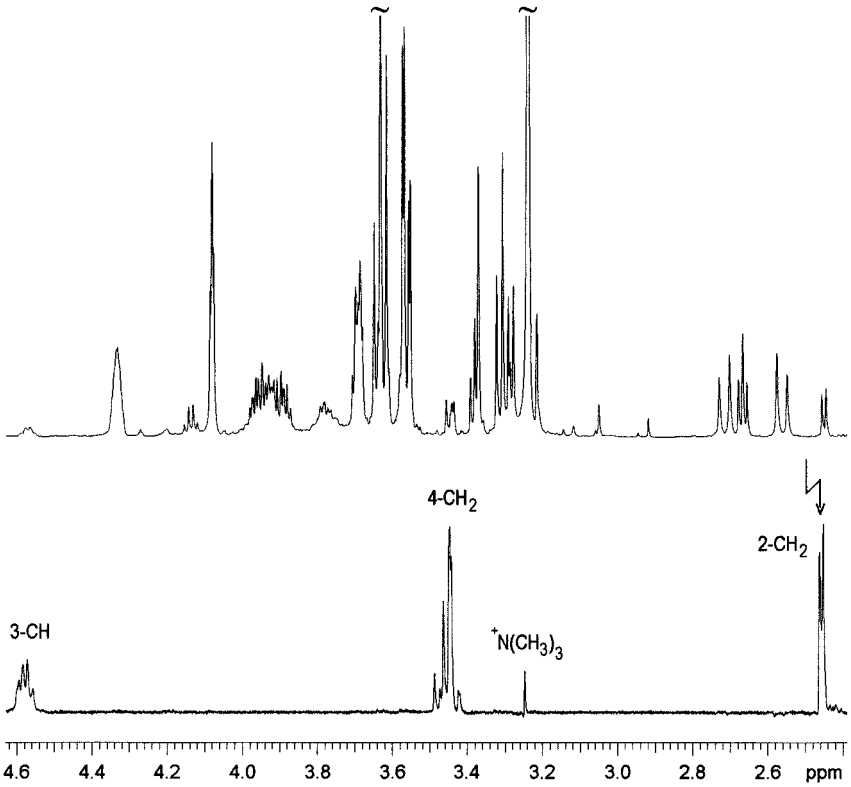

Figure 4. ^{1}H TOCSY spectrum of boar seminal plasma in D_2O. The selective irradiation (ν_1) was at the 2-CH_2 protons of carnitine. The spin–lock mixing time of 140 ms provided the full ^{1}H coupling pattern. Above: normal ^{1}H NMR spectrum.

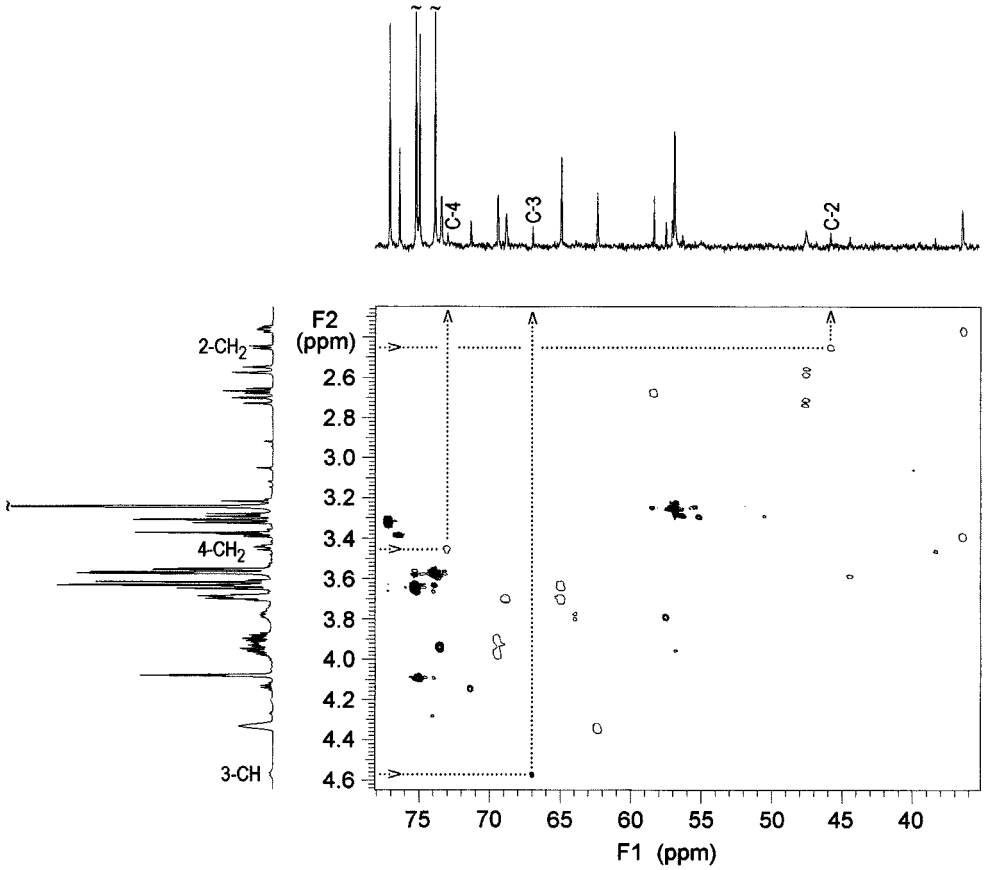

Figure 5. Gradient-selected HSQC-edited spectrum of boar seminal plasma in D_2O. Negative levels are shown with only one contour line to allow distinction from positive peaks. The one-bond C,H correlations for carnitine are drawn out.

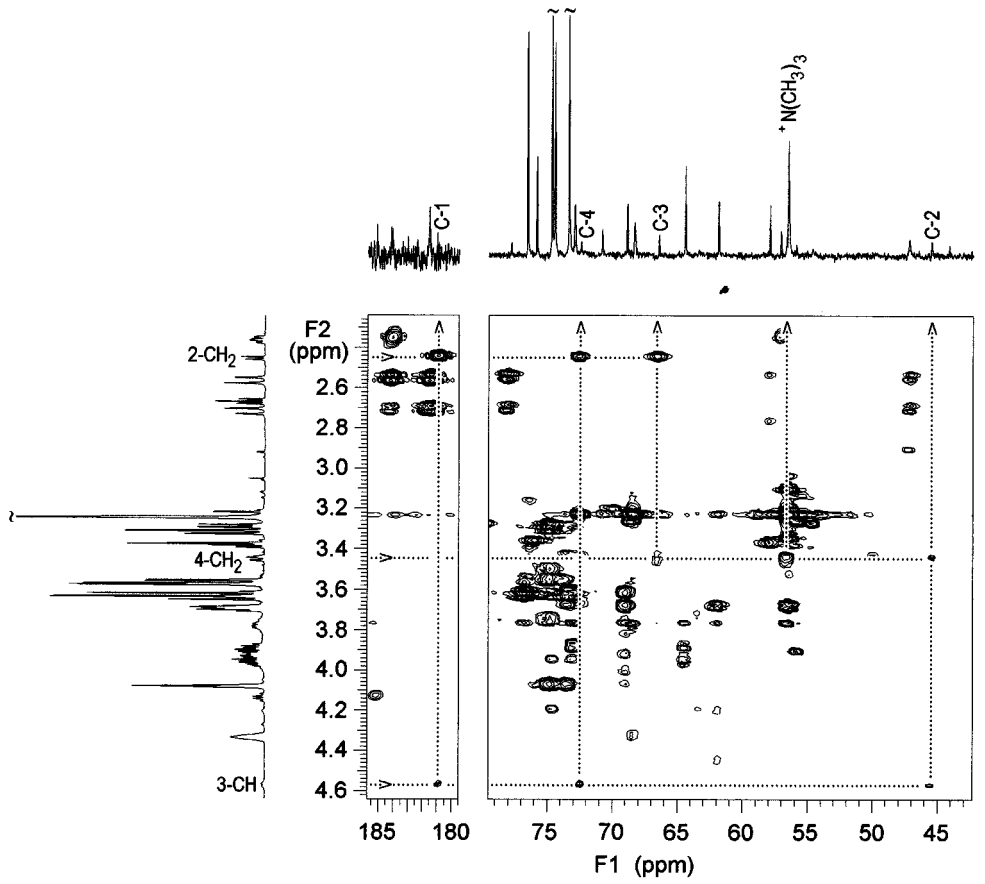

Figure 6. Gradient-selected HMBC spectrum of boar seminal plasma in D_2O. The correlations indicated are from the C,H long-range couplings in carnitine.

expected correlation network. Finally, the third functional group attached to C-3 was designated to be a hydroxyl group because of the characteristic chemical shift of C-3 at 66.8 ppm, and also from the absence of additional homo- (Fig. 4) or heteronuclear (Fig. 6) long-range couplings with 3-CH (in the case of acetylcarnitine an additional correlation of 3-CH within the carbonyl chemical shift range would have been expected).

The presence of carnitine has been found in different reproductive organs of mammals[4, 27] and recently also in the ^{1}H NMR spectra of PCA extracts of organs of rats.[28] Carnitine and acetylcarnitine play a central role in the transport of fatty acid through the membranes. The fact that spermatozoa contain high activities of acetyltransferase[4] supports the assumption that carnitine is involved in sperm anabolic or catabolic fat metabolism.

Acknowledgements

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