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High resolution proton magnetic resonance spectroscopy of human cervical mucus

Ulrike Sahrbacher [a], Anja Pehlke-Rimpf [a], Gerhard Rohr [b], Waltraud Eggert-Kruse [c], Hans Robert Kalbitzer [a,d,*]

[a] *Department of Biophysics, Max-Planck-Institute for Medical Research, Heidelberg, Germany*
[b] *Department of Internal Medicine IV, Mannheim, University of Heidelberg, Heidelberg, Germany*
[c] *Department of Gynaecological Endocrinology and Reproductive Medicine, Women's Hospital, University of Heidelberg, Heidelberg, Germany*
[d] *Institute of Biophysics and Physical Biochemistry, University of Regensburg, D-93040 Regensburg, Germany*

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Abstract

High resolution ^{1}H NMR spectroscopy is a powerful method for qualitative and quantitative analysis of the highly viscous human cervical mucus (CM). Up to 23 compounds could be identified in this study and can be observed in the ^{1}H NMR spectra of native mucus without the need of any complicated preparative chemistry. Storage conditions could be excluded as a possible reason for variations observed between different samples. pH values decreased after freezing and storing at 253 K. For NMR studies, lyophilization proved to be most useful, allowing the determination of the water content, replacement of H_2O by D_2O, and most importantly, absolute quantification of low molecular mass compounds. In a small collective of women the concentrations of some of the small constituents of the CM are strongly correlated; an example is the mutual positive correlation of taurine, citrate and creatinine. In conclusion, high resolution ^{1}H NMR spectroscopy is a valid method to investigate mucus composition and to determine absolute concentrations of low molecular mass compounds in CM. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cervical mucus; NMR; Fertility; Sterility

Abbreviations: NMR, nuclear magnetic resonance; CM, human cervical mucus; ITP, isotachophoresis; HPLC, phase high-performance liquid chromatography; HMG, human menopausal gonadotropin; TSP, trimethylsilylperdeuteropropionic acid; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; DMA, dimethylamine; GABA, γ-aminobutyrate; Crea, creatinine; Cit, citrate; Tau, taurine.

* Corresponding author. Tel.: +49-941-943-2595; fax: +49-941-943-2479.
E-mail address: hans-robert.kalbitzer@biologie.uni-regensburg.de (H.R. Kalbitzer).

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1. Introduction

Human cervical mucus (CM) is produced by cervical epithelial cells and is an important barrier for the upper genital tract against ascending genital infections. Depending on the cyclical changes, it acts as a barrier or transport medium for spermatozoa. It consists of a viscous and an aqueous phase. The extremely large mucins with a high carbohydrate content [1] are responsible for the high viscosity of this material. Depending on the viscosity and the water content, which varies between 85 and 98% [1,2], sperm migration changes [3]. Abnormality in the physical properties of the mucus may cause subfertility (cervical factor).

There have been many investigations about the glycoproteins, their polypeptide backbone and their side chains [1,2], the electrolyte composition [4] and the free sugars [5,6] contained in CM. The lipid composition of CM was investigated [7] and different proteins and enzymes could be identified [8]. Gladdines et al. [9] investigated the aqueous phase of the mucus with capillary isotachophoresis (ITP) and reversed-phase high-performance liquid chromatography (HPLC) and were able to identify eight low-molecular mass substances. No standard clinical chemical method could be established. Composition of the aqueous phase is not yet understood and except for a possible role of the lactate concentration [9], only the water content is known to be of importance for CM function [1,2].

The high viscosity of CM makes the application of standard biochemical methods difficult. Already in 1966, Odeblad [10,11] used NMR-spectroscopical methods to develop a possible model of mucus function. With the instrumentation then available he was able to measure relaxation times, but no high resolution spectra were obtained. His results are not easily reproducible and have been controversially discussed [12].

NMR-spectroscopy has been developed into a method that can provide valuable information on the content of low molecular substances in biological fluids [13–15]. A preliminary study has been published including the analysis of CM [16]. We have now analyzed the molecular composition of the aqueous phase of human CM in detail. A method has been established to determine the absolute concentrations in CM which allows a direct comparison of different samples.

2. Materials and methods

2.1. Sample preparation

CM samples were obtained from women presenting with subfertility (at least 1 year, median 5 years) at the outpatient Infertility Unit of the University of Heidelberg. The hormonal influence on mucus properties was standardized by pretreatment with oral estrogens [17,18]. The patients were seen between the 9th and 14th day of the menstrual cycle after at least 5 days of sexual abstinence and after oral administration of ethinyl estradiol (80 μg/day) for at least 7 days before evaluation of CM. Any medication with a potentially negative effect on the rheological properties of the mucus, e.g. clomiphene citrate, was stopped one cycle before the collection of the sample. Samples for intraindividual comparison were obtained from patients who were treated with human menopausal gonadotropin (HMG) (for IVF treatment according to standardized protocols).

The cervix was exposed with a sterile speculum, cleaned of excess debris with a large cotton swab, and CM was carefully obtained from the endocervix by means of a special device (Aspiglaire®, IMV, l'Aigle, France). Samples contaminated with blood or with vaginal secretions were discharged. The patients were free of signs or symptoms of genital tract infection.

For biochemical analysis of the CM by NMR-spectroscopy, CM was used either fresh, directly after it had been obtained from the endocervix, or after freezing and storage in sterile plastic tubes at 253 K (0 °C = 273.15 K). For the general assignments of the NMR signals, several specimens from different donors were mixed to obtain a sample volume of more than 500 μl and to obtain a representative sample composition. This mixed preparation (500 μl) was used to fill a 5 mm NMR tube, again with an Aspiglaire® to avoid bubbles which are fatal for shimming of the sample, and 200 μl of D_2O were added to all samples for

providing the lock signal. Under these conditions insoluble high molecular components floated at the surface of the sample outside the receiver coils. Alternatively, for improving the signal-to-noise ratio, 1500 μl of mixed samples were freeze-dried and rediluted in 500 μl D_2O.

Methanol extraction of the sample was performed by freeze-drying the sample and redissolving it in 500 μl of perdeutero methanol. Chloroform extraction of CM was obtained by diluting the native mucus sample twofold by addition of 99.8% $CDCl_3$. After short (10 min) centrifugation the chloroform phase was filled in a sample tube and the spectrum of the apolar phase was recorded using the ^{2}H-signal $CDCl_3$ for the field/frequency lock.

Fresh specimens from individual probants were first examined in the native state after addition of suitable quantities of D_2O (final volume 700 μl). Afterwards they were freeze-dried and rediluted in 500 μl D_2O. Five microliters of 0.1 M trimethylsilylperdeuteropropionic acid (TSP) were added as internal reference compound.

2.2. NMR spectroscopy

^{1}H NMR spectra were obtained with a Bruker AM-500 spectrometer operating at 500.13 MHz. Spectra were measured at 296 ± 2 K in 5-mm-diameter NMR tubes. A selective presaturation pulse of 1 s duration was used to suppress the water signal. For optimal sensitivity, 708 free induction decays (FIDs) were added. To estimate absolute concentrations of metabolites, fully relaxed spectra were recorded with a repetition time of 12 s and 908 scans. A spectral width of 6024 Hz allowed recognition of all signals in the aliphatic and aromatic region.

Assignment of unknown NMR peaks was based on standard approaches, including comparison of chemical shifts and ^{1}H-coupling constants. For the identification of most of the signals the accumulation of 250 FIDs was sufficient. Accumulation of 4600 FIDs was necessary only for the identification of the weak signals in the aromatic region. ^{1}H chemical shifts are given relative to TSP used as internal standard at 0 ppm. If necessary, DQF-COSY [19] and TOCSY [20] were used for the identification of spin systems. Phase-sensitive detection in t_1 direction was obtained using the TPPI method [21].

A hypothesis was verified by adding a solution of the expected substance to the sample. The test solutions were pH-controlled and adapted in NaCl concentration (0.9%) to avoid unexpected pH-dependent signal shifts.

2.3. pH titrations

The pH dependence of chemical shifts was recorded in mixed samples lyophilized and redissolved in D_2O. In some cases an appropriate quantity of the pure substance was added to the sample to improve the signal-to-noise ratio. The pH values were determined using a combination glass electrode (Spintec®). As usual, the pH values and calculated pK values were not corrected for the isotope effect. To vary the pH value, appropriate quantities of 1% NaOD or DCl solutions were added. Titration curves were fitted to the modified Henderson–Hasselbalch equation (see e.g. [22]) assuming a suitable number of pK values.

2.4. Quantification of signals

Several specimens from different patients and those from one patient taken at different days of the cycle were measured seperately to compare the concentration of the identified substances. Each sample was weighed before and after lyophilization for the calculation of the water content and for the correction of the concentrations after redilution. For the determination of concentrations, only fully relaxed spectra were used. After filtering and Fourier transformation the spectra were carefully phase and baseline corrected. Integrals are given relative to the integral of TSP with a known concentration.

3. Results

3.1. Assignment of resonance lines

The basic resonance assignment was performed in samples concentrated by freeze-drying and dissolved in D_2O with the methods described above. As usual the hypothesis to identify unknown signals was based on the chemical shifts, the multiplet structure, coupling constants and coupling patterns elucidated by time-shared homodecoupling or two-dimensional DQF-COSY and TOCSY experiments. The identity of components was proven at least once by adding the expected substance in low quantities to the mucus sample and by comparing these signals with the signals to be assigned (Fig. 1). (Fig. 1 shows an overview of the total aliphatic region with the assignment of the most prominent peaks. Smaller peaks are only visible in a detailed view.) Using this method the compounds listed in Table 1 could be unambiguously assigned. On the basis of these reference spectra the corresponding resonances could be identified in the spectra of samples with unknown composition.

From the 20 proteogenic amino acids, 13 amino acids (Ala, Arg, Gly, Glu, His, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val) can regularly be observed in CM. The remaining amino acids probably occur at concentrations below the limit of detection of about 10 μM. Other low-molecular mass compounds usually detected are acetate, citrate, cholin, creatinine, glucose, lactate, succinate, and taurine. Signals arising from fructose could not be identified by 1D- or 2D-NMR spectroscopy. NMR spectra were carefully screened for some

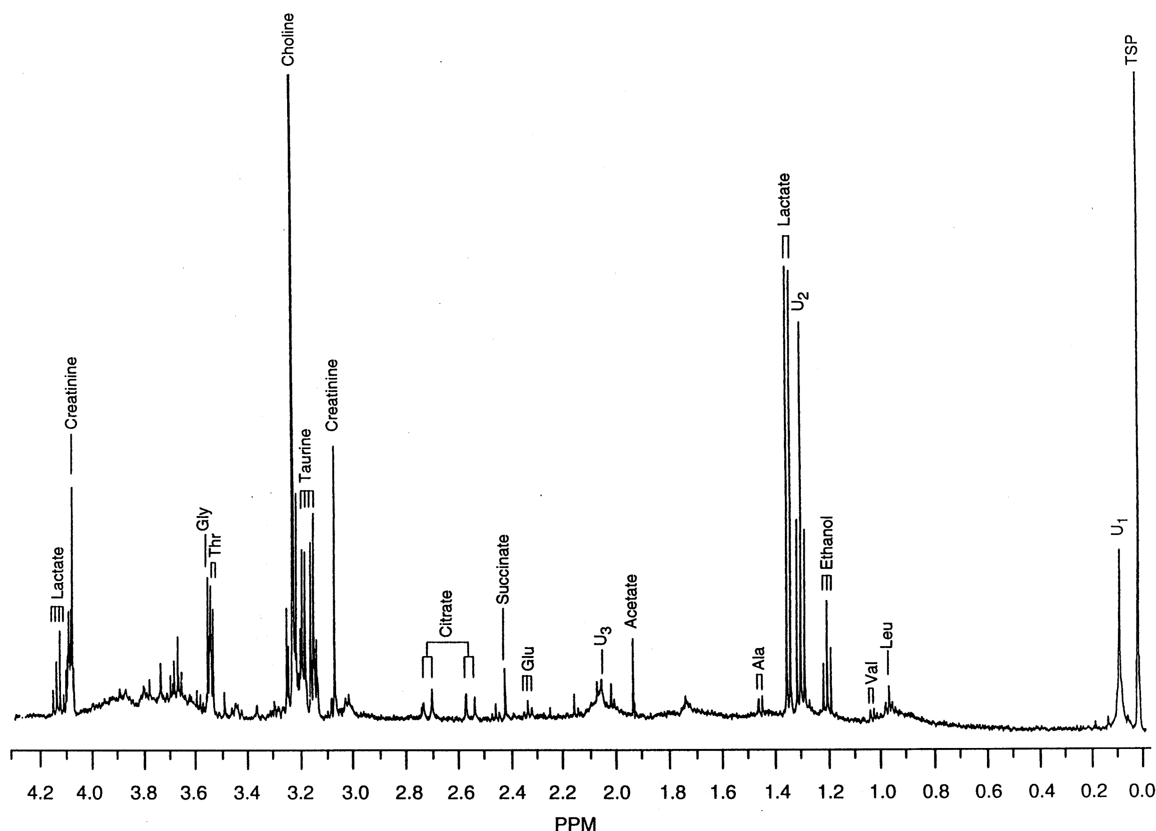

Fig. 1. ^1H NMR spectrum of human cervical mucus. 500 MHz NMR spectrum of human cervical mucus freeze-dried and redissolved in D_2O. Temperature 296 K, total acquisition time 13 hrs. The assignments indicated were obtained as described in Materials and Methods. U_1, U_2 and U_3 represent not yet identified broad signals always present in the spectra of cervical mucus.

Table 1
Low molecular mass compounds identified in CM by ^{1}H NMR spectroscopy[a]

Compound	^{1}H chemical shifts δ (ppm relative to TSP)						pK_a[b]
Acetate	1.92 ($\mathbf{CH_3}$)						
Alanine	3.78 ($\mathbf{H}^{\alpha}$)	1.22 ($\mathbf{H}_3^{\beta}$)					10.5
Arginine	3.61 ($\mathbf{H}^{\alpha}$)	1.83 ($\mathbf{H}_2^{\beta}$)	1.68 ($\mathbf{H}_2^{\gamma}$)	3.25 ($\mathbf{H}_2^{\delta}$)			
Choline	3.21 ($\mathbf{CH_3}$)$_3$	3.53 ($\mathbf{CH_2}$)	4.01 ($\mathbf{CH_2}$)				
Citrate	2.54 ($\mathbf{C}$H$\mathbf{H}$)$_2$	2.70 (CH$\mathbf{H}$)$_2$					
Creatinine	4.07 ($\mathbf{CH_2}$)	3.75 ($\mathbf{CH_3}$)					
Ethanol[c]	3.66 ($\mathbf{CH_2}$)	1.18 ($\mathbf{CH_3}$)					
α-D-Glucose	5.24 (C^1$\mathbf{H}$)	3.53 (C^2$\mathbf{H}$)	3.73 (C^3$\mathbf{H}$)	3.41 (C^4$\mathbf{H}$)	3.85 (C^5$\mathbf{H}$)	3.85 (C^6$\mathbf{H}$)	
						3.76 (C^6$\mathbf{H}$)	
β-D-Glucose	4.63 (C^1$\mathbf{H}$)	3.24 (C^2$\mathbf{H}$)	(n.d.) (C^3$\mathbf{H}$)	(n.d.) (C^4$\mathbf{H}$)	(n.d.) (C^5$\mathbf{H}$)	3.89 (C^6$\mathbf{H}$)	
						3.73 (C^6$\mathbf{H}$)	
Glutamate	3.95 ($\mathbf{H}^{\alpha}$)	2.14 ($\mathbf{H}^{\beta}$)	2.09 ($\mathbf{H}_2^{\gamma}$)				
		2.17 ($\mathbf{H}^{\beta}$)					
Glycine	3.59 ($\mathbf{H}_2^{\alpha}$)						
Histidine	3.89 ($\mathbf{H}^{\alpha}$)	3.11 ($\mathbf{H}^{\beta}$)	7.74 ($\mathbf{H}^{\delta 2}$)	7.06 ($\mathbf{H}^{\varepsilon 1}$)			
		3.22 ($\mathbf{H}^{\beta}$)					
Lactate	4.12 ($\mathbf{CH}$)	1.33 ($\mathbf{CH_3}$)					
Leucine	3.72 ($\mathbf{H}^{\alpha}$)	1.43 ($\mathbf{H}_2^{\beta}$)	1.68 ($\mathbf{H}_2^{\gamma}$)	0.96 ($\mathbf{H}_2^{\delta}$)			10.3
				0.99 ($\mathbf{H}_2^{\delta}$)			
Lysine	3.73 ($\mathbf{H}^{\alpha}$)	1.89 ($\mathbf{H}_2^{\beta}$)	1.47 ($\mathbf{H}_2^{\gamma}$)	1.72 ($\mathbf{H}_2^{\delta}$)	3.12 ($\mathbf{H}_2^{\varepsilon}$)		10.6
Phenylalanine	3.93 ($\mathbf{H}^{\alpha}$)	3.08 ($\mathbf{H}^{\beta}$)	7.34 ($\mathbf{H}_2^{\delta}$)	7.43 ($\mathbf{H}_2^{\varepsilon}$)	7.39 ($\mathbf{H}^{\zeta}$)		9.5
		3.14 ($\mathbf{H}^{\beta}$)					
Proline	4.23 ($\mathbf{H}^{\alpha}$)	2.36 ($\mathbf{H}^{\beta}$)	2.02 ($\mathbf{H}_2^{\gamma}$)	3.34 ($\mathbf{H}^{\delta}$)			10.0
		2.09 ($\mathbf{H}^{\beta}$)		3.42 ($\mathbf{H}^{\delta}$)			
Serine	3.80 ($\mathbf{H}^{\alpha}$)	3.92 ($\mathbf{H}^{\beta}$)					
		3.95 ($\mathbf{H}^{\beta}$)					
Succinate	2.41 ($\mathbf{CH_2}$)$_2$						
Taurine	3.26 ($\mathbf{H}_2^{\alpha}$)	3.43 ($\mathbf{H}_2^{\beta}$)					9.2
Threonine	3.53 ($\mathbf{H}^{\alpha}$)	4.22 ($\mathbf{H}^{\beta}$)	1.31 ($\mathbf{H}_3^{\gamma}$)				10.5
Tyrosine	3.95 ($\mathbf{H}^{\alpha}$)	3.21 ($\mathbf{H}^{\beta}$)	7.22 ($\mathbf{H}_2^{\delta}$)	6.92 ($\mathbf{H}_2^{\varepsilon}$)			10.2
Valine	3.60 ($\mathbf{H}^{\alpha}$)	2.27 ($\mathbf{H}^{\beta}$)	1.00 ($\mathbf{H}_3^{\gamma}$)				10.1
			1.04 ($\mathbf{H}_3^{\gamma}$)				

[a] Data were obtained at 296 K from CM freeze-dried and redissolved in D_2O.
[b] Only pK_a values which may influence the chemical shift values observed in the CM (average uncorrected pH value 9.2) are given.
[c] Only found in a few samples.

compounds possibly occurring in CM or already described [9] by comparison with the test spectra of pure compounds dissolved in CM. From the compounds tested, creatine, dimethylamine (DMA), γ-amino-butyrate (GABA), hippuric acid, hydroxybutyrate, and hypoxanthine are definitely not present in CM in concentrations above the detection limit.

Interestingly, in some spectra ethanol could be identified. To exclude contamination by still biologically active microbiological contamination, these specimens were incubated at 310 K for several days, which did not increase the ethanol concentration. Incubation of ethanol-negative samples at the same temperature did not lead to ethanol in measurable quantities; even clearly microbially contaminated samples with positive nitrite assay did not develop an ethanol signal after several days.

CM is expected to contain different lipids, which do not show well-resolved NMR spectra in aqueous solution. Various methods were applied for characterizing these substances by NMR spectroscopy including chloroform extraction and

methanol extraction of the freeze-dried mucus. The latter method proved to be especially well-suited to observe the cholesterol contained in the sample (Fig. 2) and the cholesterol signals could easily be assigned after this preparation.

For systematic studies the reproducibility of the measurements and the dependence of the results on the sample preparation are important. Samples were measured directly after they were collected from the patient and at different timepoints after storage at 277 K. Storage of mucus samples at 277 K for several days has only minor effects on the spectrum; the intensities of the signals from low molecular mass compounds remain unchanged. However, since the pH values measured with a glass electrode increase with time, pH-sensitive resonances shift also with time. In addition, a slight increase of the intensity of the unresolved signals in the region between 3.5 and 4.0 ppm and of the broad signals at 0.07 and 2.04 ppm could be observed. Spectra of the same samples before and after storing at 253 K showed comparable differences: the pH increased and the intensities of unresolved resonances changed slightly. In addition, after freezing a clear separation of the viscous and aqueous phase could be observed. This turned out to be advantageous for NMR-spectroscopy since the low-viscosity phase could be placed in the receiving area of the receiver coils, thus giving spectra with a better resolution. For many studies, lyophilization of the samples showed to be most useful because: (1) Weighing the sample before and after drying allows calculation of the water content of the sample, an important parameter determining the rheological properties at different states of the menstrual cycle. (2) H_2O could be replaced by D_2O giving spectra of higher quality. (3) The absolute concentrations in the sample could be increased by using a smaller volume for rehydration than the original

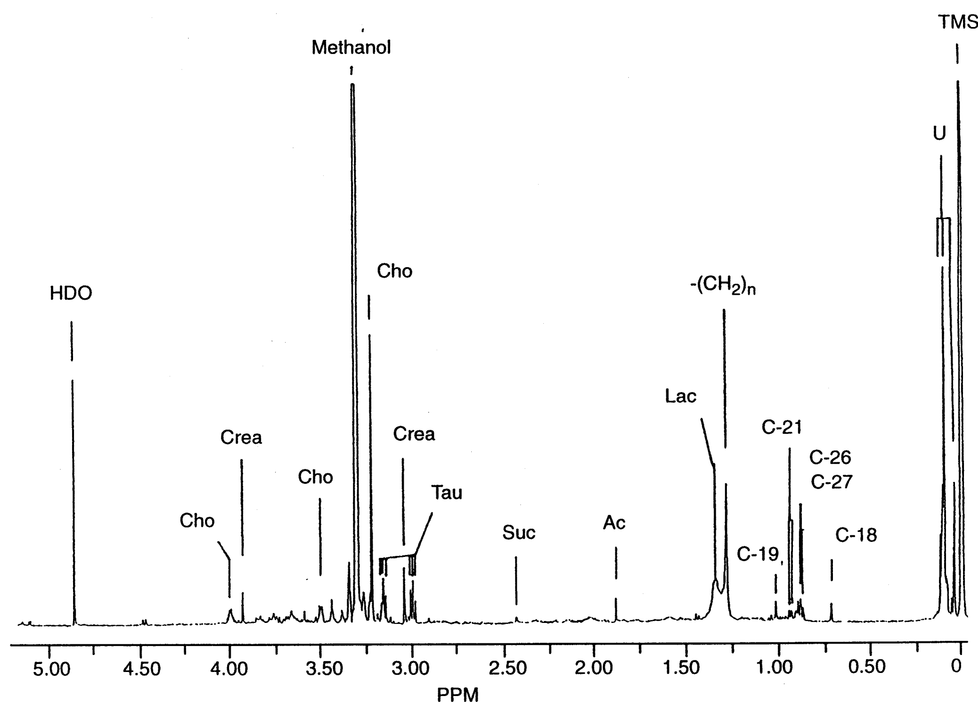

Fig. 2. Methol extraction of cervical mucus. 500 MHz NMR spectrum of human cervical mucus freeze-dried and redissolved in perdeutero methanol. TMS was added as internal reference. Temperature 296 K, total acquisition time 6 min. The assignments indicated were obtained as described in Materials and Methods. Cho, choline, Crea, creatinine, Tau, taurine, Suc, succinate, Ac, acetate, Lac, lactate, -(CH_2)n, methylene resonances of fatty acids, C-18, C-19, C-21, C-26, C-27, cholesterol resonances, U, not yet identified broad signal always present in the spectra of cervical mucus (see Fig. 1).

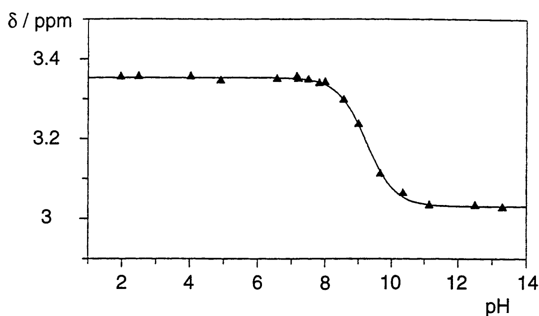

Fig. 3. pH-dependence of chemical shifts of the taurine resonances. The pH-dependence of the chemical shifts of the α-methylene protons was determined in a mucus sample prepared as described in Fig. 1. Only the mean position of the two methylene signals is shown. The pH was varied by addition of appropriate amounts of NaOD or DCI to the sample. The data were fitted to a modified Henderson-Hasselbalch-equation (see e.g. Hausser and Kalbitzer), the pK_a value of the amino group obtained from this analysis was 9.2.

one. Comparison of spectra from samples before and after lyophilization showed only the differences originating from freezing the samples as discussed above.

3.2. pH-dependent shifts

The mean pH value measured with a combination glass electrode in the untreated, native mucus samples studied was 8.26 ($s = 0.34$, $n = 29$). In the freeze-dried D_2O samples of this study an uncorrected pH value of 9.22 ($s = 0.32$, $n = 29$) was determined. Chemical shifts of compounds with functional groups which can be protonated or deprotonated in dependence on the pH of the solution are usually pH-dependent. Since the relevant pK_a values are dependent on the composition of the solution, they have to be measured under the conditions used. The pH dependence of chemical shifts was followed for taurine, lactate, choline and the amino acid signals of alanine (Hβ), valine (Hγ), threonine (Hγ), leucine (Hδ), lysine (Hδ and Hε), proline (Hγ), phenylalanine (Hδ, Hε, Hζ) and tyrosine (Hδ, Hε). From these measurements the corresponding pK_a values could be calculated (Table 1). As an example, the titration curve of taurine is shown (Fig. 3). Taurine has a pK value in the physiologically relevant range between pH 7 and 10. In addition, the taurine NMR signal has a rather high intensity in CM, its characteristic signals are easy to identify and show large pH-dependent chemical shift changes. Therefore, the taurine signal represents a convenient internal pH probe, which allows the exact determination of the actual pH of the sample by measuring its chemical shift.

Choline and lactate can be used as internal frequency standards in the physiologically interesting pH range in CM. No pH-dependent changes of the chemical shifts could be seen for choline singlet resonance at 3.210 ppm (Fig. 4) in the pH range between pH 2.4 and 12.5 (the barely visible downfield shift at low pH represents the known small pH-dependence [23] of chemical shift of the used reference compound TSP). The same is true for lactate, whose methyl doublet resonance at 1.329 ppm is easy to detect and does not shift in the pH range from pH 7 to 13.3 (Fig. 5). With a pK value of 3.4 it could be used at acidic pH values to directly determine the pH value from the pH dependence of its chemical shift.

The resonance positions of some of the amino acids are also dependent on the pH (Table 1) at the pH values around 9.2 found in the freeze-dried samples redissolved in D_2O (note that this value is not corrected for the isotope effect, see below). For an automated recognition of reso-

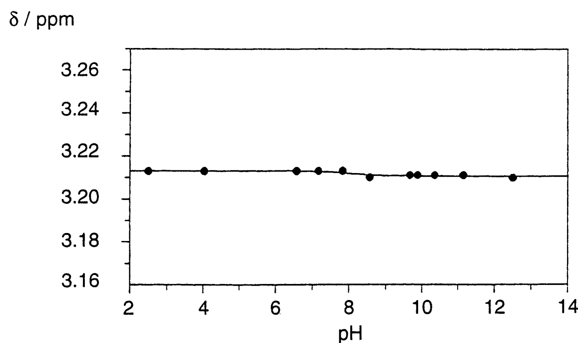

Fig. 4. pH-dependence of chemical shifts of cholin resonances. The pH dependence of chemical shifts of the cholin methyl resonance was determined in a mucus sample prepared as described in Fig. 1. The pH was varied by addition of appropriate amounts of NaOD or DCI to the sample. The data were fitted to a modified Henderson-Hasselbalch-equation (see e.g. Hausser and Kalbitzer).

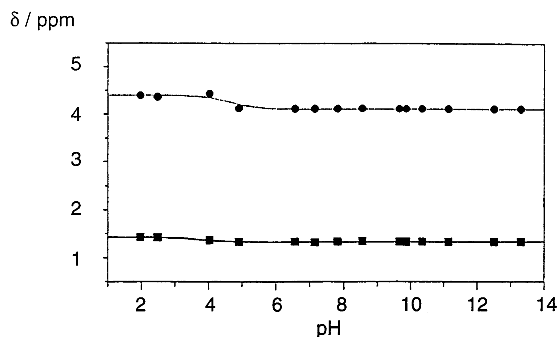

Fig. 5. pH-dependence of chemical shifts of the lactate resonsnces. The pH-dependence of chemical shifts of the methine (o) and methyl (U) resonances of lactate were determined in a mucus sample prepared as described in Fig. 1. The pH was varied by addition of appropiate amount of NaOD or DCI to the sample. The data were fitted to a modified Henderson-Hasselbalch-equation (see e.g. Hausser and Kalbitzer), pK_a value of the carboxy group of lactate obtained from this analysis was 3.4.

nances (which represents an ultimate goal if clinical applications are envisaged), this effect has to be taken into account; the method of choice would be to measure the actual pH from the taurine chemical shifts and to predict the resonance positions of the amino acid resonances from the known pK_a values.

3.3. Quantification of the low-molecular mass compounds

Integration and calculation of relative concentrations was possible for signals in nonoverlapping regions. Absolute quantification requires knowledge of the number of protons contributing to a peak and therefore was only possible for identified signals. A sufficiently accurate integration of the following signals was possible: lactate (methyl and methine resonance), acetate (methyl resonance), succinate (methylene resonances), citrate (methyl resonances), taurine (methyl resonances), choline (methyl- and methylene-group), creatinine (methyl-group and ring proton), glucose (C1-proton), alanine (H_3^β), threonine (H_3^γ) and glutamate (H_2^γ) and the unidentified signals U1–U4.

A measure for the integration error can be obtained for a compound with more than one resonance line by determining its concentration separately for its different resonance lines, which, in the ideal case, should be equal. A typical example is the citrate, where the integration error determined from these data was 0.002 mM. However, the absolute error of the concentrations is probably larger and mainly due to the error introduced by the addition of the internal reference TSP.

3.4. Inter- and intraindividual variation of the concentration of small metabolites

For the understanding of the role of variations of the constituents of CM and their possible importance for clinical diagnosis, a quantification is mandatory. In this study, a small collective of women ($n = 7$) was studied whose hormonal influence on mucus properties was standardized by treatment with estrogen (see Section 2). In addition, for an assessment of the intraindividual variations, a number of samples were obtained from two patients who were treated with HMG according to a standard stimulation protocol.

As Table 2 shows, the metabolite concentrations vary by almost two orders of magnitude in the mucus samples taken from different patients or from the same patients at different times. These variations cannot be due to changes of the water content of the mucus, which varies only in the range from 96.91 to 99.25%. As is obvious from Table 3, the concentrations of the various constituents are clearly correlated. As an example, Fig. 6 shows the correlation between the creatinine (Crea), citrate (Cit) and taurine (Tau) concentrations [Crea], [Cit], and [Tau], which can be described by the linear relation $[Crea] = 0.45 \times [Tau]$ and $[Cit] = 0.2 \times [Tau]$. The correspondent correlation coefficients are 0.980 ([Crea], [Tau]), and 0.999 ([Cit], [Tau]).

Concentrations of all constituents correlate well with the concentration of the non-aqueous phase if only samples from one patient taken at different time points during one cycle (I1–I5) are compared. The correlation coefficients are in a range from 0.829 ([Glu], dry mass/total mass) and 0.999

Table 2
Concentration of low molecular mass compounds in CM

Compound	All samples ($n = 15$)			Ethinylestradiol (EE_2)-treated probands A–F ($n = 7$)			HMG treated proband H ($n = 3$, from the same proband)			HMG treated proband I ($n = 5$, from the same proband)			Plasma reference
	Mean (mM)	s (mM)	Range (mM)	Mean (mM)	s (mM)	Range (mM)	Mean (mM)	s (mM)	Range (mM)	Mean (mM)	s (mM)	Range (mM)	Range (mM)
Acetate	0.36	0.69	0.03–2.59	0.16	0.16	0.03–0.44	0.10	0.06	0.05–0.17	0.81	1.12	0.03–2.59	0.060
Alanine	2.13	4.85	0.09–18.43	0.42	0.31	0.09–0.92	0.52	0.46	0.20–0.85	5.15	7.65	0.09–18.43	0.326
Choline	5.25	11.52	0.16–43.08	2.04	2.87	0.23–7.10	0.97	0.70	0.16–1.44	11.04	17.93	0.16–43.08	2.01–4.47
Citrate	1.43	4.00	0.04–15.84	0.23	0.18	0.04–0.61	0.12	0.07	0.05–0.19	3.89	6.68	0.04–15.84	0.099
Creatinine	2.22	5.25	0.10–21.02	0.71	0.69	0.17–2.14	0.30	0.19	0.10–0.48	5.49	8.69	0.10–21.02	0.050–0.093
Glucose	0.83	1.4	0.03–4.50	0.23	0.28	0.03–0.84	0.08	0.03	0.06–0.10	2.71	1.86	0.03–4.50	3.5–5.2
Glutamate	1.44	3.38	0.06–11.99	0.19	0.13	0.09–0.41	0.23	0.24	0.06–0.40	3.92	5.44	0.06–11.99	0.06
Lactate	25.98	66.97	0.64–262.21	4.52	3.41	1.33–10.33	2.48	2.72	0.64–5.60	70.12	109.64	0.64–262.21	0.76
Succinate	0.17	0.30	0.01–1.18	0.08	0.09	0.02–0.23	0.14	0.20	0.01–0.37	0.31	0.49	0.01–1.18	0.0088
Taurine	7.75	20.46	0.30–72.45	0.79	0.39	0.35–1.32	0.82	0.74	0.30–1.34	17.50	30.77	0.30–72.45	0.162
Threonine	0.13	0.12	0.02–0.33	0.10	0.12	0.02–0.33	n.d.			0.20	0.13	0.02–0.33	0.143
Water content (%)	97.98	0.65	96.91–99.25	98.17	0.48	97.37–98.86	98.11	1.15	96.96–99.25	97.64	0.50	96.91–98.26	

Table 3
Correlations between different low molecular mass compounds in CM ($n = 5$) from one patient (I1–5) taken at different time points during one cycle[a]

	Lactate	Taurine	Choline	Creatinine	Alanine	Glutamate	Citrate	Glucose	Succinate	Acetate	H_2O[b]	100-H_2O (%)[c]
Lactate	1											
Taurine	0.9774	1										
Choline	0.9837	0.9984	1									
Creatinine	0.9855	0.9986	0.9999	1								
Alanine	0.9977	0.9714	0.9760	0.9786	1							
Glutamate	0.9973	0.9874	0.9900	0.9919	0.9968	1						
Citrate	0.9854	0.9983	0.9998	0.9999	0.9779	0.9920	1					
Glucose	0.9230	0.8104	0.8399	0.8420	0.9236	0.8906	0.8445	1				
Succinate	0.9947	0.9916	0.9952	0.9961	0.9885	0.9984	0.9965	0.8857	1			
Acetate	0.9642	0.8897	0.9012	0.9060	0.9717	0.9447	0.9056	0.9939	0.9346	1		
Dry compartment (% total mass)	0.9792	0.9988	0.9991	0.9990	0.9705	0.9889	0.9993	0.8291	0.9941	0.8917	−1	1

[a] Pearson–Bravais correlation coefficient was calculated using the program Excel version 5 (Microsoft).
[b] Water Content (% total mass)
[c] Dry Compartment (% total mass)

Table 4
Correlations between different low molecular mass compounds in CM from seven women (A–G) after EE_2 treatment[a]

	Lactate	Taurine	Choline	Creatinine	Alanine	Glutamate	Citrate	Glucose	Succinate	Acetate	H_2O[c]	100-H_2O (%)[d]
Lactate	1											
Taurine	0.3890	1										
Choline	0.8035	0.9993	1									
Creatinine	0.8289	0.9160	0.9986	1								
Alanine	0.9755	0.5118	0.7875	0.8024	1							
Glutamate	0.8982	0.0817	–[b]	0.1446	0.8287	1						
Citrate	0.8095	0.8641	0.9795	0.9679	0.7507	0.2542	1					
Glucose	0.8733	–[b]	0.8977	0.8451	0.8263	0.9299	0.8757	1				
Succinate	0.7486	0.6678	0.9487	0.7772	0.8104	0.2477	0.6115	0.5332	1			
Acetate	0.4212	0.4285	0.7954	0.7201	0.4596	–[b]	0.7624	0.6378	0.3358	1		
Dry compartment (% total mass)	0.7540	−0.1298	0.2786	0.2929	0.6909	0.9425	0.3452	0.5679	0.2911	−0.1096	−1	1

[a] Pearson–Bravais correlation coefficient was calculated using the program Excel version 5 (Microsoft).
[b] Not enough values to calculate correlations.
[c] Water Content (% of total mass)
[d] Dry Compartment (% of total mass)

for [Cit] and the relative content of dry mass to total mass (Table 3). The different compounds are also well correlated between each other with correlation coefficients of at least 0.810 for [Gluc], [Tau] and maximal 0.999 for [Choline] and [Crea]. These correlations cannot be found if samples from different patients (A–G) are compared. The only compound with a good correlation to the nonaqueous phase is [Glu] with a correlation coefficient of 0.9423 (Table 4). Correlations between the different compounds vary between 0.082 for [Tau] and [Glu] and 0.999 for [Tau] and [Choline]. For a more detailed analysis, the study of a much larger collective would be necessary.

4. Discussion

CM is a complex material which is difficult to analyze due to its extremely high viscosity, which varies considerably during the menstrual cycle. Oral application of ethinylestradiol allows standardization (to a certain degree) of the mucus independent of the cyclical changes and achievement of a good functional quality with a cervical index of 10 according to Insler et al. [17,18]. This mucus is equivalent to normal periovulatory mucus. Most analytical methods require elaborate material preparations before investigation [24–26], which might be the origin of preparation

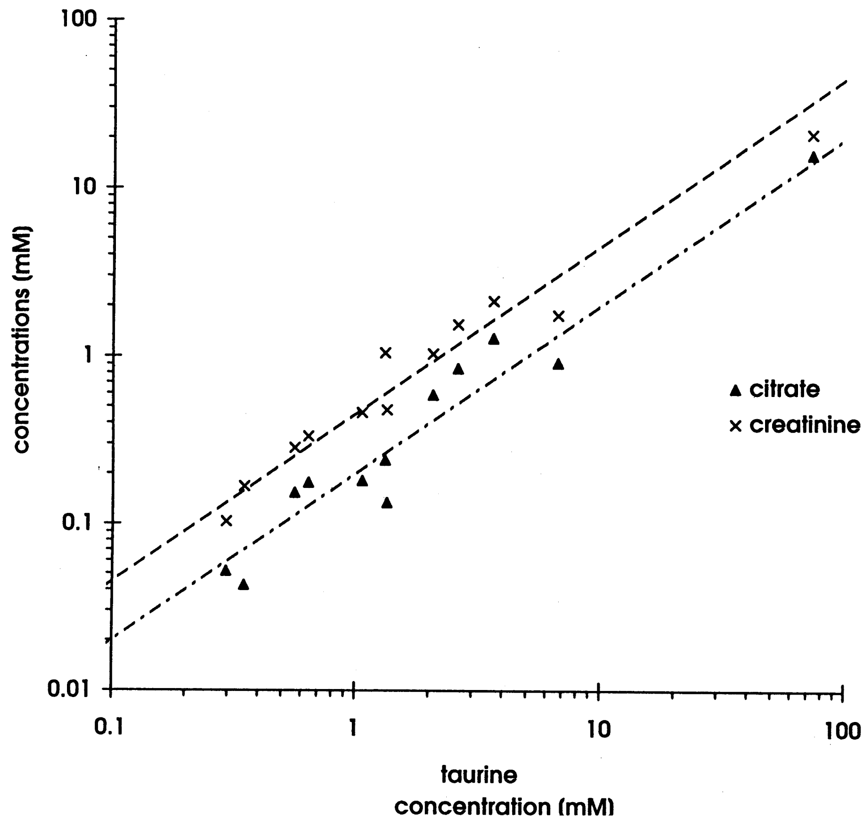

Fig. 6. Correlation of creatinine, citrate and taurine concentrations. The creatine and the citrate concentrations [Crea] and [Cit] are plotted as function of the taurine concentration [Tau] for mucus samples of seven treated probands A–G pretreated with ethinylestradiol and mucus samples of two HMG treated probands H and I taken at different times. The straight line represents the best linear fit of the data with [Crea] = 0.45 × [Tau] and with [Cit] = 0.20 × [Tau].

artefacts. Examplarily, the influence of such artefacts has been described for electron microscopy in detail [27]. In contrast to these methods, NMR-spectroscopy allows the investigation of the mucus as it is. Spectra of fresh CM samples can easily be obtained. Because of a macroscopically observable change of the sample for some applications, an immediate investigation must be recommended.

The high viscosity of the material gives rise to many problems in conventional biochemical methods. Most conventional protein fractionation techniques are not applicable because of the high content of carbohydrates. The only technical problems observed in NMR-spectroscopy were shimming problems for samples with an extremely high viscosity.

As could be shown, storage at 277 or 253 K and lyophilization have no or minimal effect on the obtained spectra. Especially, the concentrations of NMR-visible low molecular mass compounds described here are not changed in the limits of error (approximately 10 μM). The only effect is an increase in pH (probably by removal of CO_2 during the freezing/thawing process) and a change of viscosity since the high molecular mass compounds separate partly from the solution. These effects are most pronounced after freeze-drying of the samples. This means that investigation of a larger series of samples is more practicable if they can be stored and investigated together. Lyophilization and resolubilization with D_2O proved to be a well-suited method to investigate the mucus samples and was used for most experiments described here. However, D_2O spectra also grant the optimal spectral quality, although it is feasible to measure the spectra of fresh, native CM only if relative concentrations of the constituents are of interest. Modern superconducting spectrometers are stable enough for short time measurements without locking the field to D_2O. As internal ppm-reference, the signals from lactate at 1.329 ppm and cholin at 3.210 ppm could be used. Since the water content of the mucus samples only varies by a few percent, absolute concentrations could be obtained by integration of the water peak. Somewhat more precise absolute concentrations can be obtained by adding a well-defined quantity of a reference compound such as TSP dissolved in D_2O (for providing a lock substance). Simultaneously, precise pH values can be obtained from the chemical shift of the taurine resonances. Compounds with a concentration of > 10 μM can be detected with a minimal sample volume of 200–300 μl. However, one should have in mind that NMR-spectroscopy in native material determines the physiologically more important concentration of free compounds in solution. If compounds are strongly bound to large molecules they are not directly visible by NMR-spectroscopy; for detecting them a pretreatment of the sample such as denaturing and removal of proteins would be necessary.

An advantage of NMR-spectroscopy is the possibility to discover substances which were not expected in the investigated material; an example is the identification of ethanol in some of the specimens. The reason for the occurrence of ethanol in some of the mucus samples is completely unclear. We do not think (but cannot exclude completely) that it is a simple impurity, because care has been taken to always use the same standard procedure, and ethanol was only found in a few samples. Production of ethanol by a microbial contamination is also not very likely as the concentration of ethanol did increase after incubation of the samples for longer times. A remote possibility for explaining the ethanol content of the CM may be that it represents the consequence of ethanol consumption of the probant in the time before taking the mucus sample. This hypothesis will be tested experimentally in future.

Gladdines et al. [9] described the quantitation of 34 substances including electrolytes by HPLC and ITP in combination. Compared to these results NMR-spectroscopy provides broad new information about the composition of the mucus. In comparison to Gladdines et al. [9], the amino acid composition could be studied in detail, and 13 different amino acids could be identified in the NMR-spectra. In the study of Gladdines et al. [9], hippurate and hypoxanthine have been described to occur in most mucus samples in detectable concentrations. They could not be detected in our samples with NMR-spectroscopy. Unfortunately, Gladdines et al. did not quantify exactly the con-

centrations of the compounds identified by HPLC. The concentrations of substances were only given by the relative height of the UV-ab- sorption peak after separation by HPLC. It is therefore not clear if the concentrations of the two compounds are high enough to be detected by NMR spectroscopy (>10 μM). An alternative explanation for this discrepancy would be that there was an error in the identification of these compounds by Gladdines et al. [9] or that the two compounds are strongly bound to the macromolecular matrix of the mucus. However, the titration of mucus with hippurate and hypoxanthine gave no indication for a strong interaction with the mucus since the two compounds were easily visible in the spectra.

Altogether, the signals of 23 compounds could be assigned here in the NMR spectra. A number of resonances could not yet be assigned and can provide further biochemical information if identified. The NMR spectroscopy is in principle suited for routine analysis of body fluids since there are automated sample changers available. Especially, in the environment of specialized laboratories a large number of mucus samples could be analyzed. As we have shown, sensitivity is not an issue, especially because the newly developed cryoprobes promise a substantial increase in sensitivity.

As could be shown here, two-dimensional NMR-techniques are helpful for the identification of more complex molecules. These methods however are time-consuming and can be applied for questions like the existence of sugars other than glucose. Neither with one- nor with two-dimensional methods is the existence of fructose could be shown as it was described by Linden et al. [5]. However, since the fructose signals are to be expected in a spectral range containing many other resonances, this finding only excludes its presence in high concentration (mM). The fructose concentration in CM is probably rather low. It has been supposed that fructose is important as an energy supply for sperm migration [5]. Whether the low doses observed here are sufficient is questionable.

The pH value of CM is of importance for sperm–mucus interaction [28]. We observed that pH values increased while storing samples and after lyophilization and resuspension in D_2O. This underlines the importance of investigating samples immediately after they have been obtained from the endocervix. The evaluation of the taurine chemical shifts allows the direct determination of the pH value by NMR-spectroscopy as additional information without further methods, saves much time, and excludes possible contaminations of the samples.

As shown in Table 2, the concentrations of constituents vary by almost two orders of magnitude in different samples. This is in agreement with the range of the relative concentrations found by Gladdines et al. [9]. A similar variance can be observed in the intraindividual comparison. The concentrations of the constituents determined by NMR-spectroscopy do not seem to be related to the values described for blood plasma, which excludes that they simply represent a transsudate. In the CM the mean concentrations of lactate, succinate, citrate, and creatinine are higher than standard plasma reference values by more than a factor of ten. Comparing samples of one patient at different time points we found a strong correlation of all constituents to the water content. This means that all observed constituents are regulated in a constant manner. However, if samples from different patients are compared, even after hormonal standardization, only glutamate and to a lower extent lactate are correlated to the water content. All other constituents seem to vary independently from the water content and thus from viscosity. The other correlated substances have no obvious biochemical relations. This needs to be confirmed in future studies with higher numbers of samples including those from women with a normal menstrual cycle.

The data clearly show that CM samples are still variable between individuals in spite of 7 days of pretreatment with ethinylestradiol. The strong correlations between different substances implicate that the strict regulation of these constituents is of biological relevance and might be important in the control of CM function in fertility. Identification of factors important for CM function will be helpful to understand the mechanisms of sperm–mucus interaction and might be a basis for the development of contraceptives on cervical level in the future.

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