UDC 549.08:543.4:543.544.6

Using Physicochemical Methods for *in vitro* Studying Urinary Calculi

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(Received January 11, 2011; revised March 2, 2011)

Abstract

Results of the application of X-ray diffraction studies, IR spectroscopy, thermogravimetry, spectrophotometry to the *in vitro* investigation of the phase composition of urinary calculi and the use of X-ray spectral microanalysis for the determination of their elemental composition are presented. The methods of X-ray diffraction and thermogravimetry were optimized for the quantitative determination of crystallization water in oxalates, and Lawry method was optimized for the quantitative determination of protein in all the kinds of urinary calculi. An interconnection between the hardness of oxalates, urates and phosphates, protein content and the kinds of microstructure was revealed.

Key words: urinary calculi, physicochemical methods of analysis

INTRODUCTION

Among urologic diseases, urolithiasis occupies one of the first places: its fraction among the other urologic diseases in Russia on average is equal to 34.2 %. The treatment methods available do not save a patient from potential urolithiasis recurrence. In order to improve the methods for early diagnostics and treating the patients with recurrent urolithiasis, as well as to prevent urinary calculi formation process it is necessary, first of all, to have information concerning the phase and elemental composition of urinary calculi.

All the 45 species of urinary calculi known to date [1–4] were systematized and classified [1]. They are divided into four groups: phosphates, oxalates, urates, and others (organic and inorganic) compounds.

The methods of studying the urinary calculi could be divided into three groups. The first group includes the methods for determining the elemental composition: quantitative X-ray spectral microprobe analysis [2, 5], atomic emission spectral analysis [2, 6], scanning electron microscopy [3, 5, 7, 8], various electrochemical methods [2, 3], capillary electrophoresis [2, 3], chromatographic methods [2]. The second group includes methods for the determination of phase composition: XRD analysis [1–13], infrared spectroscopy (IR) [2, 5, 6, 14, 15], polarization microscopy [2, 3], thermogravimetry (TGA) [5, 6]. The third group includes methods for studying the morphology [2, 3] and microstructure [1, 5, 7, 9] of urinary calculi. Some of these methods, according to data available from the literature, are of limited use.

So, the method of thermogravimetry allowed the authors of [5] to compare to each other a series of oxalates only qualitatively concerning the content of crystallization water molecules therein. Very seldom one could find any application of the of XRD analysis method for quantitative determination of the phase composition for all the types of multiphase urinary calculi. The amount of crystallization wa-

ter in oxalates was determined only for separate single-crystal samples by means of single-crystal X-ray diffraction [13]. However, this method exhibits a significant limitation with respect to the application: it requires for high-quality single crystals only, those could be isolated from urinary calculi in very rare cases.

The purpose of this work consisted in the improvement and optimization of the methods of determining the composition of urinary calculi.

EXPERIMENTAL

The X-ray investigation of urinary calculi was carried out on a DRON-3 (graphite planar monochromator) and HZG-4 (Ni filter) diffractometers with CuK_α radiation at angles $2\theta = 5–50°$ (continuous mode, 1 °/min) and 5–100° (stepwise mode: pulse unset time 10 s, step value 0.02°). Sample preparation for diffractometry, depending on the size and hardness was carried out in several ways: by means grinding with the use of an agate mortar (without acetone and with acetone) and without grinding. In order to prevent the manifestation of possible texture the samples were rotated in the course of XRD registration, whereas some samples were measured again under different conditions. The unit cell parameters for the phases were refined by means of least-squares method from full-profile experimental data using Dicvol04 software [16]. As the external standard we used α-Al_2O_3 powder (American National Standards Institute): a = 4.759(1), c = 12.993(2) Å.

The qualitative X-ray diffraction phase analysis was performed using a JCPDS PDF-2 automatic database, ICSD structural database and original papers. The sensitivity of XRD phase analysis for the mentioned compounds amounted to 3–5 %.

The quantitative XRD phase analysis was performed according to the method of coefficients based on a comparative assessment of the intensities of the strongest diffraction reflexes from separate phases of two-phase sample [1, 10]:

$I = kx/[\rho S(x_i m_i^*)]$

Here I is the intensity of a diffraction reflex; x is the mass fraction of a compound in the mixture, %; x_i is the mass fraction of the i-th component of the compound, %; m_i^* are the molecular absorption coefficients of the i-th component; ρ is the density, determined as

$\rho = 1.6606\ Mz/V$

where M is the molecular mass of the compound; z is the number of formula units per unit cell; V is the unit cell volume equal to $a[bc]$ (a, b, c being unit cell parameters), Å^3. The ratio between the two strongest diffraction reflexes corresponding to the two different compounds **1** and **2** in the two-phase mixture could be expressed as it follows:

$I_1 : I_2 = [x_1/(\rho_1 S(x_i m_i^*)] : [x_2/(\rho_2 S(x_j m_j^*)] = K(x_1/x_2)$

Further, in order to assess the correctness of the calculation from the experimental reflex intensities those we determined on the X-

TABLE 1

Comparison of the results of X-ray diffraction (XRD), infrared spectral (IR) and thermogravimetric analysis (TGA) technique for oxalates containing whewellite (WW) and weddellite (WD)

Methods of analysis	Sample number								
	1	2	3	4	5	6	7	8	9
XRD, % (WW/WD)	0/100	40/60	59/41	60/40	62/38	72/28	76/24	82/18	100/0
IR, % (WW/WD)	0/100	41.7/58.3	58.7/41.3	60.4/39.6	61.5/38.5	73.3/26.7	77.1/22.9	82.2/17.8	100/0
TGA, % ($\Sigma\Delta m$)	40	37	ND	32	ND	ND	ND	ND	28
n^*	2.20(4)	1.75(4)	ND	1.50(4)	ND	ND	ND	ND	1.00(4)
n^{**}	2.18(2)	ND	2.25(2)	ND	ND	2.27(2)	2.29(2)	2.22(2)	ND

Notes. 1. $\Sigma\Delta m$ is oxalate mass change; n^* is the total number of water molecules in the weddellite and whewellite, calculated according to the TGA; n^{**} is the number of water molecules in the weddellite obtained *via* calculating the theoretical values for the intensity of diffraction reflexes. 2. In brackets the standard deviation value is presented. 3. ND – no data.

ray diffraction profiles inherent in the most common two-phase mixtures (those we prepared) with the content of one component x_i = 0, 20, 50, 80, 100 % we found the experimental values of K which were compared with the calculated values and then averaged. The repeatability limit (r) for the results amounted to 0.2 %. This allowed us to calculate the value of the proportionality factor (K) for different two-phase urinary calculi (for example, for the system of whewellite–weddellite K = 1.25) (Table 1). In a similar manner, we performed the calculation for the three-phase mixture:

$I_1 : I_2 : I_3 = [x_1/(\rho_1 S(x_i m_i^*)] : [x_2/(\rho_2 S \times (x_j m_j^*)] : [x_3/(\rho_3 S(x_q m_q^*)] = K_1 x_1 : K_2 x_2 : K_3 x_3$

The IR spectral phase analysis was performed using EQ 5.5 FT-IR spectrometer.

The qualitative analysis was based on the determining the characteristic peaks of stretching vibrations for the functional groups, particularly oxalate groups: C=O (1700 cm^{-1}), C–O (~1100 cm^{-1}), H_2O (3200–3400 cm^{-1}) [14].

The quantitative determination of urinary calculi composition was performed by means of formula

$x = 100(H_2a + zH_2b)/(H_2a - zH_2b) + (zH_1b - H_1a)$

where H_1a, H_2a, H_1b, H_2b are the peak height values for the first and second reference standard at the wavelength equal to a and b [14], respectively, mm; x is the content of compound **1** in a concrement with mixed composition, %; z is the ratio between peaks of compounds **1** and **2**, respectively, on the spectrum of urinary calculus under investigation for the quantitative determination of the first component (compound **1**). The repeatability limit (r) for oxalate amounted to 0.4 % (see Table 1), which corresponds to data presented in [14].

The thermogravimetric analysis of oxalates was performed using Q-derivatograph (thermocouple Pt/Pt–Rh, T_{ini} ~ 28 °C, T_{fin} = 600 °C, scan rate (heating) equal to 10 °C/min; quartz crucible, sensitivity 50, the mass of weighed sample portions amounting to about 100 mg. For the analysis we used the samples of oxalates containing 100, 60, 40 and 0 % of weddellite. In the course of the first stage of the analysis, removing water from the system took place (at 135–137 °C). Further, an organic component was removed, whose content in the samples being observed almost the same and approximately equal to 18 % (at 140–600 °C).

The content of water was calculated from changing the mass of the samples. The repeatability limit r = 0.04 (see Table 1).

The protein content in urinary calculi with different compositions was determined by means of Lowry method employing an SF-26 spectrophotometer (at the wavelength λ = 750 nm).

For plotting the calibration curve we used six standard solutions of tryptophan $C_{11}H_{12}N_2O_2$ with the concentration equal to 8–48 μg/mL. In order to find out the leaching time (by the example of the two samples) we investigated protein segregated from the grinded concrement sample portion (the sample No. 1 – 0.1 g, the sample No. 2 – 0.15 g) in 0.1 M NaOH solution within a discrete time interval ranging within 24–144 h (Table 2). It was established experimentally that during 24, 48 and 72 h, protein liberated not completely, however during 120 and 144 h the liberated protein decomposed. Thus, the maximum amount of protein is liberated *via* leaching for 96 h (see Table 2). Repeatability limit r = 0.1 %. It should be noted that the authors of [5], who used the method of Lowry for the same purpose, performed leaching for 8 h, but this is not enough for the complete extraction of protein from urinary calculi. The authors of [2], determined the protein content by means of Benedict method, and to extract the protein components from the concrements they used a mixture of chloroform and ethanol at a ratio of 1 : 1. This method allows researchers to determine the protein concentration at a range of 0.12 mg in a sample, whereas the Lowry method is more sensitive, and the range of protein concentrations in the sample amounts to 10–100 μg.

TABLE 2

Effect of leaching time on the protein concentration in the solution, μg/mL

Leaching time, h	Sample No. 1	Sample No. 2
24	18.91	44.50
48	22.12	50.34
72	24.03	59.78
96	25.57	62.29
120	25.14	61.48
144	24.55	60.24

The X-ray spectral analysis (with studying the microstructure) was performed on a Quanta 400 X-ray spectrometer. We used Si–Li detector with an ultrathin window, which allows performing the quantitative analysis of elements with atomic number $N \geq 4$. The repeatability limit $r = 0.1$ % [5].

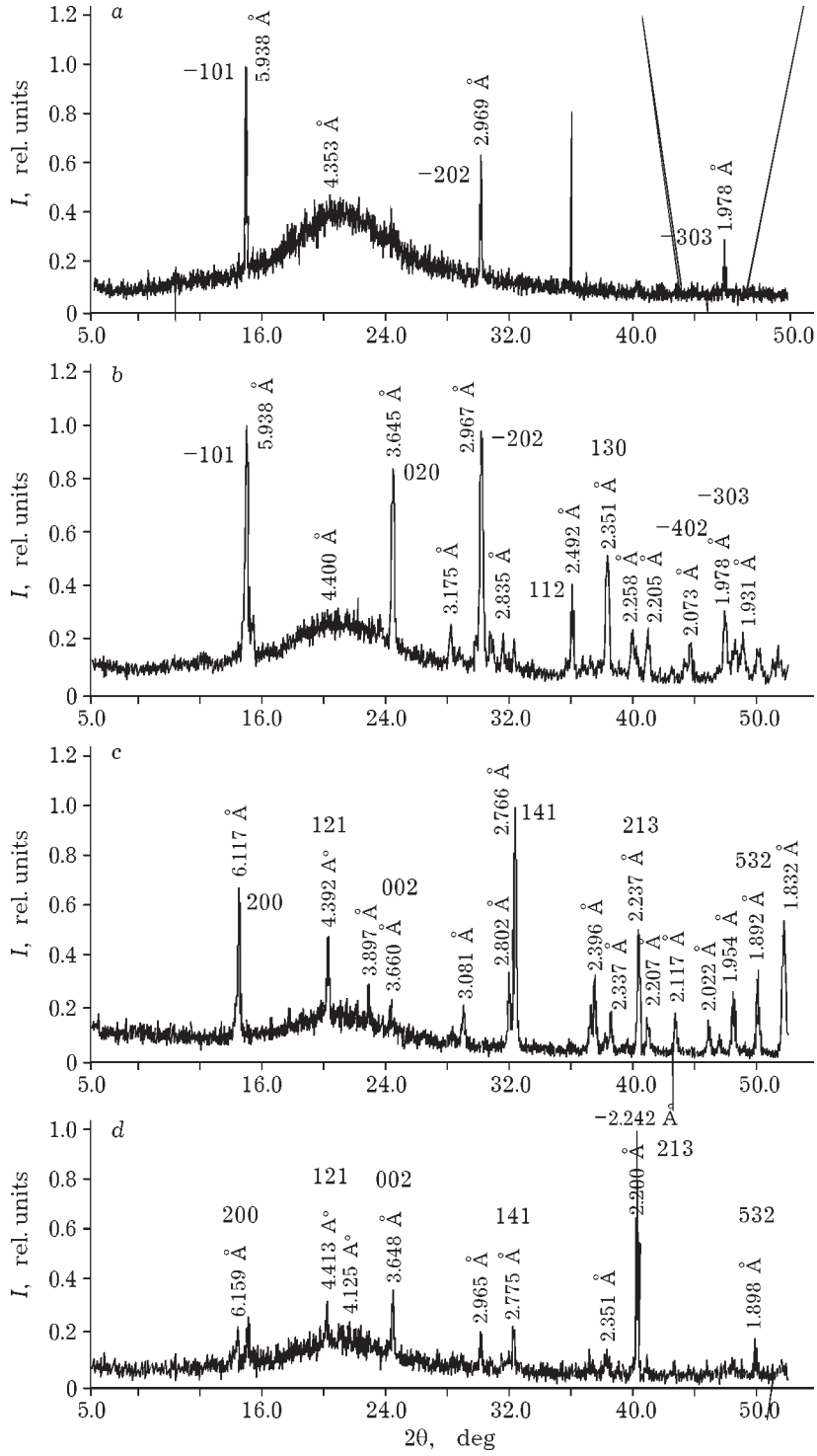

Fig. 1. XRD profiles for single-phase urinary calculi consisting of whewellite (*a*, *b*) and weddellite (*c*, *d*). Interplanar distances and symbols for the individual reflexes are presented.

RESULTS AND DISCUSSION

We analyzed urinary calculi taken from patients with urolithiasis (more than 300 people). The analysis of data published and the results of our study demonstrated that among urinary concrements oxalates are predominant, so as the objects of inquiry we chose whewellite ($CaC_2O_4 \cdot H_2O$), weddellite ($CaC_2O_4 \cdot nH_2O$), and the mixture of whewellite and weddellite, as well as the other compositions of urinary calculi.

From Fig. 1, *a* and *b* it is seen that whewellite can be either textured (see Fig. 1, *a*), *i. e.*, exhibit a preferred orientation of crystallites along one direction (in this case, the texture haze zonal axis <010>), or non-textured (see Fig. 1, *b*). Among a great number of the samples of urinary calculi those we studied, no textured weddellite was found. Different crystalline state of urinary calculi (single crystals, polycrystals, and texture) should affect their hardness. So, among all the studied oxalates just whewellite exhibits the highest hardness. Its diffraction pattern indicates the formation of texture, whereas the data concerning the microstructure of urinary calculi indicate tight interlacing the crystal formations both at the periphery, and at the centre [5]. According to the results of X-ray microanalysis, the outer part of the sample contains calcium in a higher amount than its content in the inner part of the sample, whereas the content of carbon, phosphorus, and oxygen, on the contrary, is less than that for the inner part [5].

The statistical analysis we carried out for the oxalate samples studied does not exclude any connection between the hardness of oxalate and the content of water molecules therein. Whewellites, as a rule, are harder comparing to weddellites. Comparing the typical diffraction patterns of whewellite and weddellite (see Fig. 1, *b*–*d*), one could observe significant differences in the intensity of diffraction reflexes for weddellite (some reflexes three-fold differ from each other), which could, to all appearance, indicate a different content of crystallization water molecules in the weddellite. (It should be noted that the sample preparation and the analysis of diffraction reflexes exclude manifesting any texture and its impact on the redistribution of the reflection intensity.) On the other hand, the unit cell parameters for whewellite and weddellite vary to a significant extent, with exceeding the limits of the standard deviation (a similar phenomenon was observed by the authors of [13]). In this case, no significant change in the integrated intensity of the reflexes is observed (within about 10 %). So, for example, for weddellite (space group $14/m$) $a = 12.30-12.37(2)$ Å, $c = 7.19-7.40(1)$ Å; for whewellite (space group $P2_1/c$) $a = 6.25-6.29(1)$ Å, $b = 14.47-14.64(3)$ Å, $c = 9.75-10.01(1)$ Å. The data obtained indicate that there are several different compositions, which could be caused due by both/either by an isomorphic substitution of components in the whewellites and weddellites (the chemical analysis confirms the presence of Mg, Na and Fe ions in oxalates [12]) and/or by a different content of water molecules in the weddellite. The latter should play a significant role in changing the intensities of the diffraction reflexes.

In order to estimate the number of water molecules in the weddellite we first developed and used an XRD technique. On the basis of the structural data for weddellite $CaC_2O_4 \cdot nH_2O$

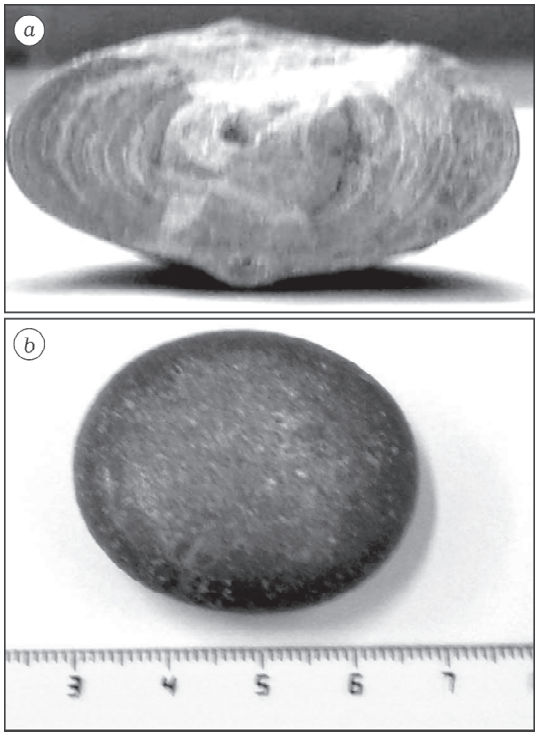

Fig. 2. Photographic image of the urate-based urinary concrement (uric acid): *a* – appearance, *b* – cross section.

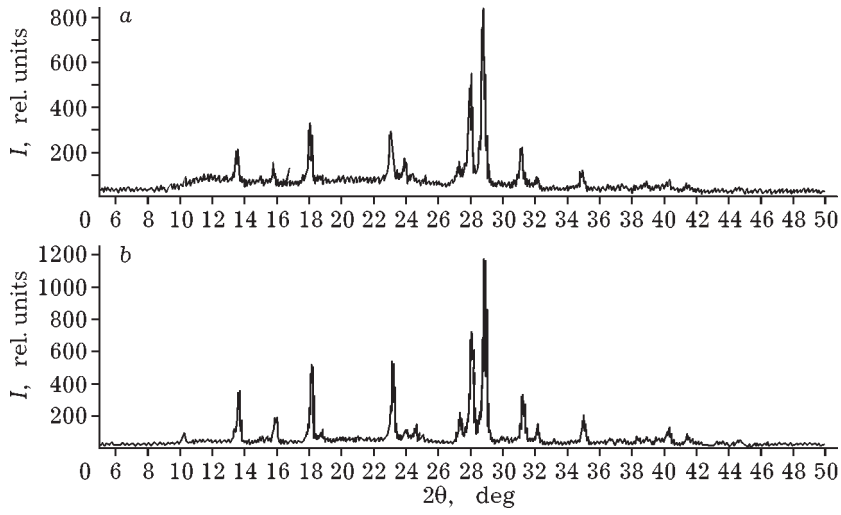

Fig. 3. XRD profiles for peripheral (*a*) and central (*b*) parts of urate-based urinary concrements.

(n = 2.375) [16] we performed the calculation of the theoretical values for the intensity of diffraction reflexes according to diffraction crystallography formulas, assuming a different content of water molecules therein (n = 2.0, 2.25, 2.375, 2.5). The parameters of the cell and the coordinates of the atoms were conserved constant, whereas hydrogen atoms were not taken into account. The analysis of the calculation results allowed us to determine reflexes (121, 141, 213, 532) those are the most sensitive with respect to the variations in the value of n. For example, it is seen that the reflex 213 varies to a most significant extent. The same reflexes exhibit a significant change also on the experimental X-ray diffraction patterns (see Fig. 1, *c*, *d*) for the samples with a similar crystallite size (coherent scattering regions), according to the half-width observed for the diffraction reflexes.

The comparative analysis of the experimental data concerning the integrated intensity of the reflexes for weddellites with a known content of crystallization water molecules [16, 17], and theoretical calculations allowed us to pro-

TABLE 3

Elemental composition of urate concrements (see Figs. 2, 3), mass %

Components	Periphery	Central part		
		White band	Yellow band	Dark ring
C	35.58	35.92	39.66	42.36
N	36.22	17.85	31.83	32.04
O	27.96	41.26	26.98	25.42
Na	0	0.18	0.17	0.13
Si	0	0.03	0.04	0
P	0	0.10	0	0
Cl	0	0.02	0	0.01
Ca	0	4.59	0.25	0.04
Fe	0	0.07	0.08	0
Ni	0	0	0.06	0
F	0	0	0.94	0
Protein concentration	5.05	–	–	55.23

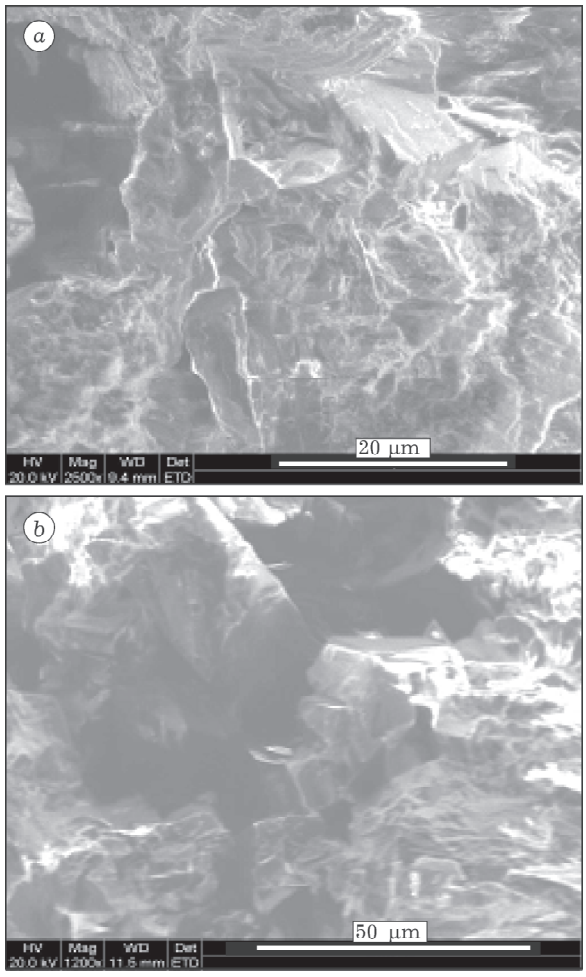

Fig. 4. Photographic images for the microstructure of the urate-based urinary concrement (uric acid): *a* – periphery, *b* – central part.

pose the relationships those connect the n and the value of corresponding diffraction reflex intensity I (in %), as it follows:

$I_{121} = 66.54n - 74.71$

$I_{141} = 7.12n + 16.65$

$I_{213} = 6.40n + 11.92$

$I_{532} = 4.69n + 4.18$

It should be noted that the strongest reflex taken for 100 %, was not included in group of the selected reflexes chosen. Further, the n values obtained were averaged ($r = 0.02$). The results of the calculation, on the one hand, are in a good agreement with the data presented in [13], whereas on the other hand, these data do not contradict the TGA results (see Table 1).

The appearance of urate concrements disintegrated from a urinary bladder, and the sectional view of the concrement is demonstrated in Fig. 2.

Figure 3 demonstrates the XRD patterns of its peripheral and central parts. It can be seen that the outer part of the sample is presented by anhydrous uric acid whereas the interior part is presented by uric acid dihydrate. The section of the concrement (see Fig. 2, *b*) exhibits interchanging the growth bands, and one could observe the white bands of the concrement to contain much more calcium comparing to the yellow bands (Table 3). The structure of the peripheral part consists of elements those form the basis of uric acid (C, N, O) (see Table 3), whereas the central part is more diverse in composition. The comparative analysis of the microstructure of different parts of the urate sample leads to the conclusion that the central part is much more friable than the peripheral one; therefore it is less hard (Fig. 4). A similar situation was observed for another urate concrement (anhydrous uric acid) disintegrated from a kidney [10]. No texture formation was revealed among the urate renal concrements.

Among phosphate concrements, there were no textured samples revealed, too. It was found that in the samples of the concrements the fraction of amorphous structures is much higher (at least in the peripheral part) than that of crystalline formations. In addition, judging by a high half-width value of the diffraction re-

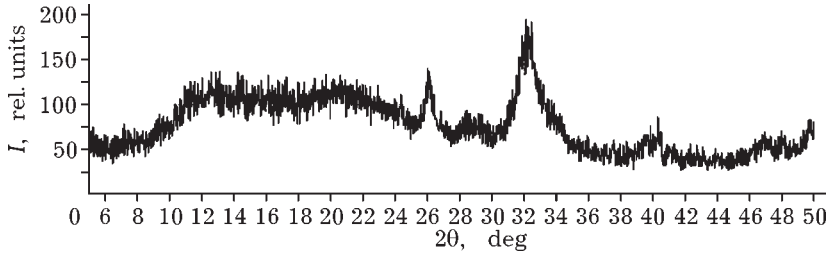

Fig. 5. XRD profile of phosphate urinary concrement (carbonateapatite).

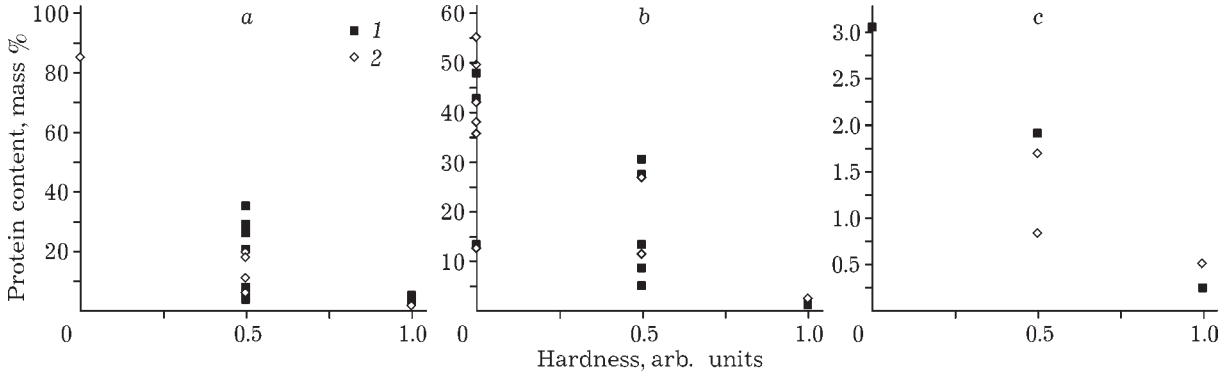

Fig. 6. Relationship between urinary calculi hardness and protein concentration at the periphery (*1*) and in the central part (*2*): *a* – oxalates, *b* – urates, *c* – phosphates.

flexes (as the result, a small size of the crystallites), the phosphate concrements, in particular apatite (hydroxyapatite or carbonate apatite) ones exhibit a low level of crystallinity (Fig. 5). The central part of the phosphate concrements is characterized by a higher hardness compared with the periphery.

It should not be excluded that the hardness of urinary calculi could depend not only on the presence of texture and the content of crystallization water molecules in oxalates, but also on the amount of protein in the composition. In order to determine protein in all the species of urinary calculi we used Lowry method.

The experimental conditions were the same and included the preparation of samples for analysis, leaching within 96 h; the sample portion mass was equal to 0.1–0.15 g, the optical density was measured using a spectrophotometer (at λ = 750 nm). Indeed, when comparing the hardness of a concrement with the content of proteins demonstrates that the greater the hardness of the concrement, the lower the content of protein (see Fig. 4). Conventionally, all urinary calculi could be divided into three groups of hardness, (arbitrary units): 0 soft concrements, 0.5 hard concrements, 1 very hard concrements. Maximum protein content is inherent in urinary uric acid concrements (Fig. 6, *b*), whereas the minimum one is inherent in phosphate urinary calculi (see Fig. 6, *c*), although the total amount of organic component therein is greater (especially as apatites are concerned). This is indicated by a great background in the diffraction profile and an amorphous halo at nearer angles (see Fig. 5). In the central part of oxalate and urate concrements, the protein content is greater compared to its content at the periphery, whereas an opposite picture is observed for phosphates.

CONCLUSION

We first developed and applied XRD technique for quantitative determining the phase composition of all the types of two-phase urinary calculi, as well as thermogravimetry and XRD analysis for assessing the amount of crystallization water in oxalates. Optimal modes are established for determining the content of protein in all the species of urinary calculi by means of the Lowry method, which allows researchers to increase the level of extraction for the protein components from the concrements as well as the accuracy in determining their number.

It is for the first time established that there is an influence of the texture and the features of the microstructure upon the hardness of oxalates, as well as an effect of the protein on the hardness urate calculi of all the compositions. It is established that the volume distribution of protein in urinary calculi: for oxalate and urate calculi in the central part of the concrement the content of protein is greater than that at the periphery, whereas an opposite picture is observed for phosphates.

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