Monatshefte für Chemie 130, 1431–1442 (1999)

Monatshefte für Chemie
Chemical Monthly

Investigation of a Microbially Produced Structural Modification of Magnesium-Ammonium Orthophosphate

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Summary. Infrared spectroscopic, X-ray diffraction, thermogravimetric and microscopic studies, atomic spectrometry, and ion chromatography trace elemental analyses of insoluble mineral crystals produced in a synthetic phosphate-containing medium during cultivation of the soil bacterium *Azospirillum brasilense* (strain Sp245) were carried out. The structure and chemical composition of the mineral, including the entrapped metal cations, the distribution of trace impurities of biologically important cations between the cultivation medium and the solid phase formed therein, and some aspects related to biomineralization, are considered and discussed.

Keywords. Magnesium-ammonium orthophosphate hydrate; Struvite; Trace impurities; Biomineralization; *Azospirillum brasilense*.

Untersuchungen einer microbiell erzeugten strukturellen Modifikation eines Magnesium-Ammonium-Orthophosphats

Zusammenfassung. IR-Spektroskopische, röntgendiffraktometrische, thermogravimetrische, mikroskopische, atomabsorptionsspektrometrische und ionenchromatographische Untersuchungen von unlöslichen mineralischen Kristallen, die sich während der Kultivierung des Bodenbakteriums *Azospirillum brasilense* (Stamm Sp245) in einem synthetischen phosphathältigen Medium abschieden, wurden durchgefürt. Die Struktur und chemische Zusammensetzung des Minerals sowie seiner eingeschlossenen Metallionen, die Verteilung von biologisch bedeutsamen Spurenelementen zwischen der Festphase und dem Kulturmedium sowie wichtige Aspekte der Biomineralisierung werden diskutiert.

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Introduction

The significant role of biological factors in the formation and transformation of minerals and their structural modifications has been emphasized in the literature [1–3]. Various microorganisms have been shown to be responsible for the process of biomineralization leading to the precipitation of minerals both in the cultivation medium [4, 5] and at the bacterial membranes [5, 6]. In particular, the formation of phosphate-containing insoluble biominerals [4, 7, 8] as well as of metal polyphosphates which play an important role in the microbial metabolism of phosphorus [9] and, on the other hand, solubilization of mineral phosphates [3, 10] are among the most common microbial transformations of inorganic phosphates.

In the present work, we performed infrared (IR) spectroscopic, X-ray diffraction (XRD) and thermogravimetric structural as well as microscopic investigations of mineral crystals that had been shown to form during cultivation of the nitrogen-fixing soil rhizobacterium *Azospirillum brasilense* (strain Sp245) in a synthetic phosphate-containing medium [11]. *A. brasilense* is known to colonize roots of higher plants, positively influencing their growth and development [12], in particular owing to the production of indole-3-acetic acid (a phytohormone of the auxin series) [13] and spirilobactin [14] (a low-molecular-weight siderophore involved in the transport of iron [15, 16] to both partners of the plant-microbe association). Since both of the latter substances are relatively strong complexing agents, we also studied the distribution of several biologically important microelements present in the cultivation medium between the solution and the solid phase crystallizing therein, as analyzed by atomic absorption/emission spectrometry (AAS/AES) and ion chromatography techniques [17]. The role of isomorphic admixtures in the formation of the crystal structure of the mineral formed as well as some aspects related to biomineralization and to the involvement of metal cations in the processes accompanying microbial metabolism [18–20] are also considered.

Results and Discussion

Considering the composition of the cultivation medium (see Experimental) which contains essential quantities of orthophosphates as well as their significant role in the formation of biominerals [8], it seemed reasonable to propose that the mineral crystals obtained are based on a water-insoluble orthophosphate phase. This assumption is corroborated by IR spectroscopy (Fig. 1, spectrum 1) showing features typical for orthophosphates [21] (see also Fig. 1, spectra 2–4). Comparison of the spectra of the initial air-dry sample (spectrum 1) and after its thermal treatment for 2.5 h at 250°C (spectrum 2) shows a noticeable decrease in absorption in the regions characteristic for adsorbed and bound water (the stretching ν(O–H) (for hydrogen-bonded hydroxyls) and bending δ(H–O–H) modes observed near 3200–3400 and 1630 cm^{-1}, respectively), in particular a disappearance of the broad band present as a shoulder near 3200 cm^{-1} in spectrum 1. This indicates the presence of crystallization water in the mineral (see below). Considering the absorption in the region 900–1300 cm^{-1} featuring ν(P–O) vibration modes [21] (see Fig. 1, spectra 1–4) it may be noted that a marked splitting of the band both for the initial crystals (spectrum 1) and for the calcined (*i.e.* anhydrous) sample

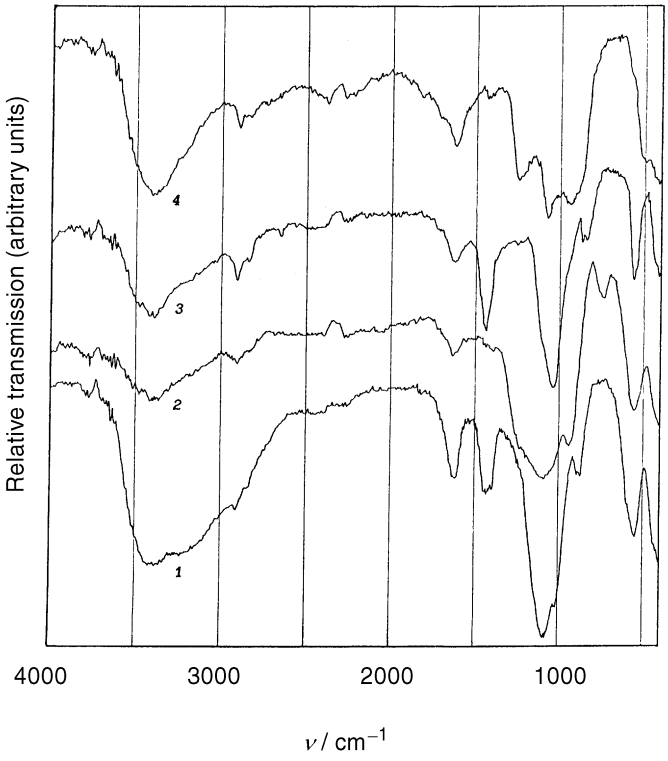

Fig. 1. Infrared spectra of mineral crystals produced during cultivation of *Azospirillum brasilense* Sp245 in a phosphate-containing synthetic medium in the air-dry state (1) and after heating at $T = 523$ K for 2.5 h (2) as well as of anhydrous Na_3PO_4 (3) and KH_2PO_4 (4) after heating for 2 h at 423 K and 523 K (KBr)

(spectrum 2) resembles rather the corresponding absorption for $H_2PO_4^-$ (spectrum 4 for KH_2PO_4) than the narrower band for PO_4^{3-} at *ca.* 1030 cm^{-1} (spectrum 3 for Na_3PO_4). This may be indicative of the presence of HPO_4^{2-} (and, probably, $H_2PO_4^-$) in the crystal lattice of the mineral (see below). It should be noted that a slow gradual increase in *pH* (from *ca.* 6.9 up to 7.6–7.9) detected during cultivation of azospirillum, resulting in the formation of relatively large crystals (see below), may well facilitate the formation of insoluble magnesium-ammonium hydrophosphates. A similar process occurs in an infected human urinary tract, where ammonia production by urea-cleaving bacteria results in a *pH* rise to above 7.5, facilitating supersaturation and precipitation of the mineral struvite ($MgNH_4PO_4 \times 6H_2O$) [22, 23].

An X-ray powder diffraction pattern of the mineral (Fig. 2; the corresponding interplanar spacing values *d* (in Å) and *hkl* indices (in parentheses) are also shown) displays a large number of peaks, the positions of which in general most closely correspond to those of stoichiometric struvite $MgNH_4PO_4 \times 6H_2O$ [4, 24]. Nevertheless, many of the lines are noticeably shifted, and there is a redistribution of their intensities as compared to the ASTM reference [24] which allowed us to refer the mineral studied to one of possible structural modifications of struvite (as noted by *Gibson* [4], struvite comprises a variety of forms). The differences between the

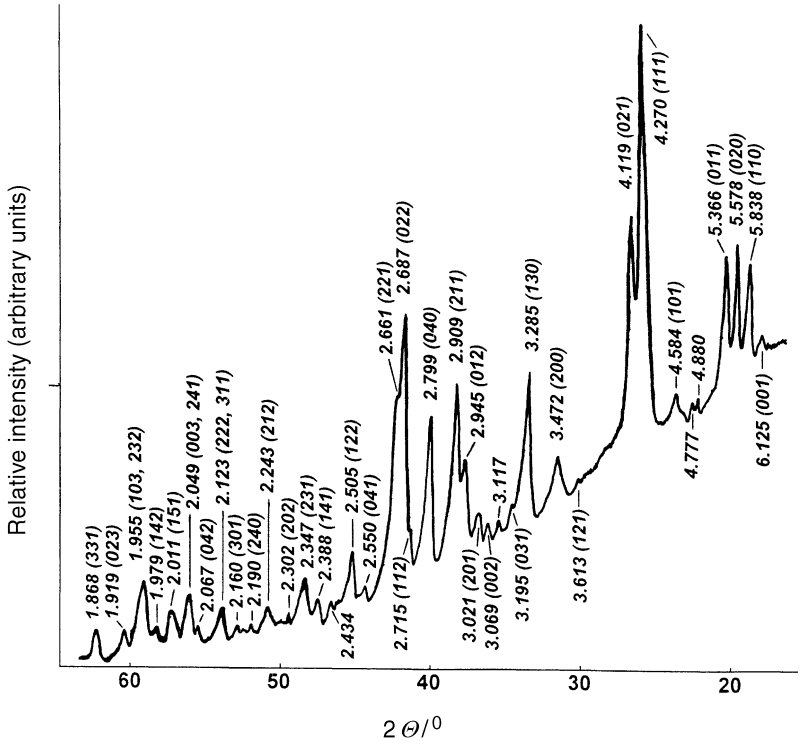

Fig. 2. X-Ray diffraction pattern for the mineral produced during cultivation of *Azospirillum brasilense* Sp245 in a phosphate-containing synthetic medium (the interplanar spacing values d(Å) and *hkl* indices (in parentheses) are given at the corresponding peak positions)

diffractogram in Fig. 2 and the ASTM reference [24] may be caused by the presence of isomorphic admixtures of other cations in the mineral (see below) as well as of hydrophosphate anions HPO_4^{2-} (as a substitute for phosphate in struvite; see above) decreasing the molar fraction of cations, resulting in distortions in the crystal lattice relative to stoichiometric struvite [24].

The noticeable right-hand asymmetric rise of the diffractogram (“halo”) in Fig. 2 indicates the presence of a mixture of an amorphous phase with a crystalline phase; note that in diluted aqueous solutions both crystalline and amorphous ammonium-magnesium phosphates have been shown to form [25].

Thermal analysis of the mineral shows that decomposition of the main phase, beginning at a temperature above 100°C (Fig. 3a), is almost complete at 200°–250°C, gradually continuing up to *ca.* 400°C. There is a single strong endothermal peak centred at *ca.* 140°C on the differential thermal analysis (DTA) curve (Fig. 3b), which may obviously be attributed to practically simultaneous removal of crystallization water and ammonia [25, 26]. The total weight loss according to TGA is 55 wt.% corresponding to the formation of pyrophosphate. Thus, the appearance of a relatively small peak at *ca.* 740 cm^{-1} in the IR spectrum of the mineral after heating at 250°C for 2.5 h (see Fig. 1, curve 2), which is absent in

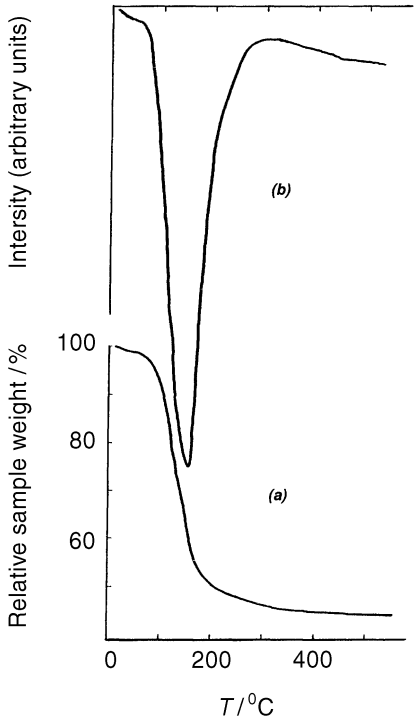

Fig. 3. (a) Thermogravimetric (TGA) and (b) differential thermal analysis (DTA) curves for the crystalline phase produced during cultivation of *Azospirillum brasilense* Sp245 in a phosphate-containing synthetic medium (air-dry sample, initial weight 200 mg, heating rate 10 K/min)

the spectra of the other samples (curves 1, 3, 4), may be attributed to the bending O–P–O (δ(O–P–O)) [21] and symmetric stretching ν_s(P–O–P) vibrations [25] of pyrophosphate $P_2O_7^{4-}$ (and, possibly, some other polyphosphates; see below). In addition, the removal of ammonia during thermal decomposition of the NH_4^+ cation leads to a decrease in the absorption intensity of stretching ν(N–H) and bending δ(H–N–H) vibrations (3145 and 1400 cm^{-1}, respectively [21]), contributing to the hypochromic effect in the corresponding spectral regions (cf. curves 1 and 2 in Fig. 1).

The data of elemental analyses of the crystalline mineral for the elements present in the cultivation medium in the form of the corresponding salts (except molybdenum) obtained by AES/AAS and IC are presented in Table 1. It can be seen that Mg, N, and P are, in fact, the main constituents showing the mineral to be based on magnesium-ammonium orthophosphate. Nevertheless, the contents of these three elements differ noticeably from the theoretical values corresponding to stoichiometric $MgNH_4PO_4 \times 6H_2O$ (99, 57, and 126 mg/g for Mg, N, and P, respectively). The data of Table 1 allow the general formula for the mineral to be derived. Considering also the content of Na and K (other elements are present in essentially lower quantities and may obviously be regarded as trace impurities), the general exact formula may be written as $Mg_{0.51}(NH_4)_{0.74}K_{0.03}Na_{0.01}H_{1.20}PO_4 \times 2.53H_2O$. Taking into account the magnesium-ammonium orthophosphate hydrate

Table 1. Elemental analyses data for the mineral crystals formed during cultivation of *Azospirillum brasilense* Sp245

Element	Method[a]	Content±standard deviation (mg per gram of solid)	Distribution coefficient[b]
Na	AES	0.96±0.01	1.2
K	AES	6.12±0.01	3.2
Mg	AAS	72.73±0.43	1800
Ca	AAS	0.367±0.001	51
Fe	AAS	0.587±0.005	80
Mn	AAS	1.153±0.007	32
N	Kjeldahl	61±1	47
P	IC (as PO_4^{3-})	183±3	185
Cl	IC (as Cl^-)	0.30±0.03	0.09
S	IC (as SO_4^{2-})	0.039±0.002	0.5

[a]AES (AAS): atomic emission (absorption) spectrometry, *Kjeldahl*: the conventional *Kjeldahl* method, IC: ion chromatography; [b]dimensionless coefficients (k_d) calculated for each element as $k_d = c_{cr}/c_{sol}$ where c_{cr} and c_{sol} are the w/w concentrations of an element in the crystalline phase and in the cultivation medium, respectively

entity only, the latter composition could be represented by a formula essentially close to $Mg_2(NH_4)_3H_5(PO_4)_4 \times 10H_2O$. Thus, the presence of HPO_4^{2-} (*ca.* 75%) and $H_2PO_4^-$ (*ca.* 25%) as substitutes for PO_4^{3-} in stoichiometric struvite is evident (see above), which can in part account for the differences in their XRD patterns (see Fig. 2 and Ref. [24]). The molar Mg:P ratio of the mineral being close to 1:2 accounts for the formation of higher polyphosphates [25, 26] along with $Mg_2P_2O_7$ upon heating. Also, the IR spectrum of the mineral studied (see Fig. 1, spectrum 1), although in general resembling those of *e.g.* urinary struvite [23] or of a corresponding mineral from Hamburg, Germany [27], shows some noticeable differences from each of the latter (see above), which also do not coincide in some details.

It is also interesting to note that according to *Mazaeva et al.* [28] the stoichiometric struvite hexahydrate is formed in aqueous solutions at 20°C; at 30°C the hydrate loses one water molecule, and at 50°C the trihydrate is formed, all products being poorly soluble. Thus, the somewhat lower water content in the mineral studied (see above) as compared to the stoichiometric struvite may in part be caused by the cultivation temperature of 32°C (see Experimental).

In order to compare the shape of the crystals of the biomineral under study with that of synthetic crystals which formed in the corresponding cell-free cultivation solution during artificial slow gradual alkalization of the medium from the initial *pH* of *ca*. 6.9 up to 7.8 over 18 h using dialysis of a diluted NaOH solution, their microphotographs were obtained (Fig. 4a, b). It is evident that, besides an approximately 5-fold smaller size of the synthetic crystals (Fig. 4b) as compared to those of the biomineral, the former belong to a different group of crystallographic symmetry, whereas the biomineral crystals (Fig. 4a) have almost the same shape as and crystallographic faces topologically equivalent to those of the natural struvite described by *Strübel* and *Zimmer* [29] (Fig. 4c). This is indicative of an essential

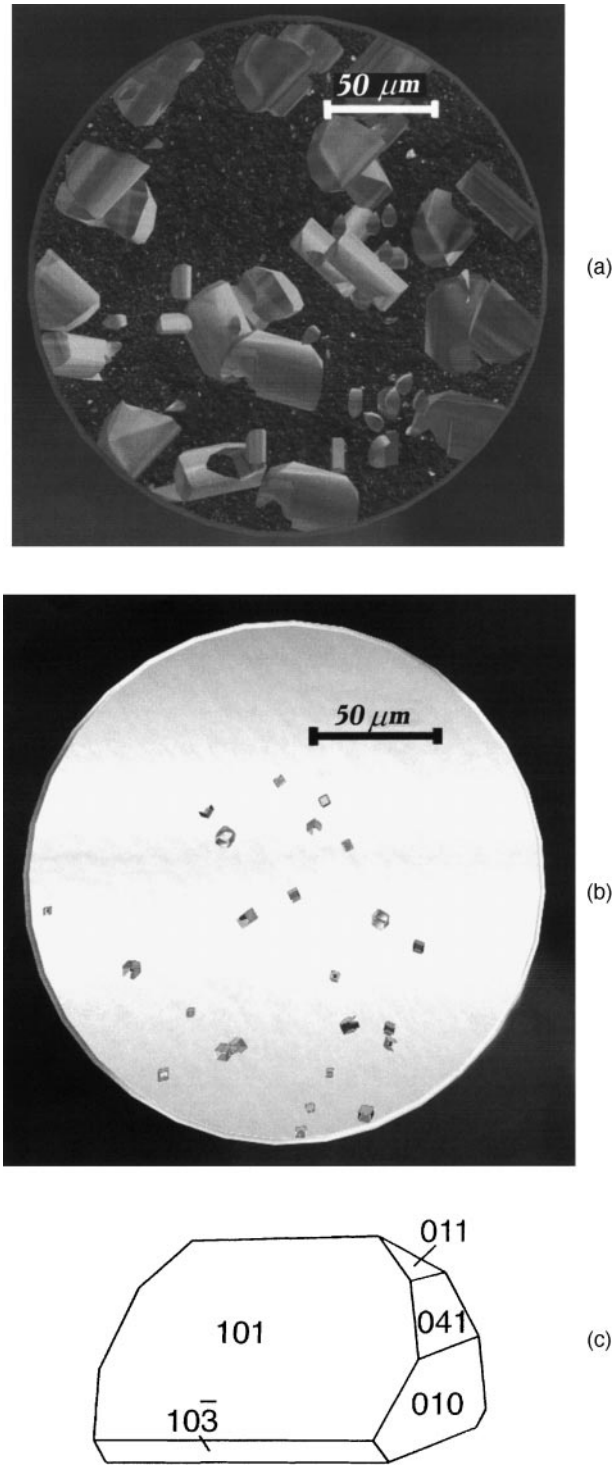

Fig. 4. Microphotographs of (a) mineral crystals produced during cultivation of *Azospirillum brasilense* Sp245 in a phosphate-containing synthetic medium and (b) crystals formed in the corresponding cell-free cultivation solution during slow gradual alkalization of the medium for 18 h using dialysis of a diluted NaOH solution (initial *pH*: *ca*. 6.9, final *pH*: 7.8) as well as (c) the shape of a struvite crystal described by *Strübel* and *Zimmer* [29]

role of a set of specific microconditions created in the medium in the process of cultivation of azospirillum for the formation of a specific phase structure (*i.e.* struvite). Along with the medium composition and the aforementioned very slow gradual rise in *pH*, the excessive formation of slime intimately associated with the crystals which had to be removed from the latter using ultrasonic treatment (see Experimental) may have also been essential, *e.g.* controlling the diffusion of the solution components to the crystallites surface.

In order to study the distribution of elements between the cultivation medium and the solid phase gradually formed therein in the course of *A. brasilense* cultivation, we also compared the trace elemental composition of the mineral with the concentrations of the elements in solution. The resulting distribution coefficients (k_d) for each element calculated as $k_d = c_{cr}/c_{sol}$, where c_{cr} and c_{sol} are the concentrations of an element in the crystalline phase and in solution, respectively, are also presented in Table 1. These data clearly show that, along with the three main constituents of the precipitate (Mg, N, and P), also calcium, iron, and manganese most essentially accumulate in the crystals from the solution (other elements feature by much lower k_d values being close to or under unity; see Table 1).

Note that the ASTM reference sample of struvite [24] was shown to contain 0.1 to 1 mg/g Na and 0.01 to 0.1 mg/g each of Ca and Fe (as well as Si). This corresponds to the amount of sodium found in the biomineral under study (see Table 1), whereas the content of both Ca and Fe in the latter is significantly higher. Manganese(II), together with iron(II), was also reported to be a usual isomorphic admixture in struvite [30].

In general, the ion exchange capability of hydroxo groups in acidic phosphates has long been known [31, 32]. In particular, the k_d values for the sodium and potassium cations have been shown to be $k_d(Na^+) = 0$ and $k_d(K^+) = 3$ for the ammonium salt of a phosphate- and molybdate-containing heteropolyacid in which the ammonium ion can be readily exchanged for other cations (cf. the data in Table 1, particularly, $k_d = 3.2$ found for potassium) [32].

As for metals other than alkaline, it has recently been shown [33] that inorganic calcium phosphate-based granules, which formed as a separate compartment (different from magnetosomes) in cells of magnetotactic bacteria, could incorporate Zn, Fe, and Al. It has also been noted that amorphous minerals, being more likely to incorporate extraneous ions than crystalline materials, may form precursor phases for biomineralization in biological systems [33]; this may well have been the case with the biomineral studied here (see above).

Since metal cations are known to play a significant and specific role in regulating the microbial and plant enzymatic activity [34–37] and may be involved in redox or other chemical transformations of microbial and plant metabolites (see *e.g.* Refs. [18–20, 38–43] and references reported therein), the above results may be essential for the environmental processes accompanying the *A. brasilense* metabolism under natural conditions. 'Immobilization' of metal cations entrapped within such an insoluble solid phase gradually forming during cultivation may obviously lead to their exclusion from the processes of microbial and plant metabolism which would involve only soluble or solubilized metal species (*e.g.* hydrated ions and/or complex forms). On the other hand, taking into account the

ability of azospirilla to form plant-microbe associations, the above considered accumulation of cations in an insoluble phase might in principle present an alternative to the known schemes of resistance of plants to heavy metals [44]. In addition, the structural modification of struvite studied could also contribute to the formation of the soil texture under appropriate environmental conditions in the process of biomineralization. Besides its aforementioned formation in an infected human urinary tract [4, 22, 23], struvite occurs as one of the main components of urolites in combination with hannayite ($Mg_3(NH_4)_2(HPO_4)_4\times 8H_2O$) and newberyite ($MgHPO_4\times 3H_2O$). It is noteworthy that all the three minerals are found in the form of large well-shaped crystals in guano [29], which is indicative of their predominantly biological origin; however, isomorphic admixtures of Fe and Mn are characteristic neither for newberyite nor for hannayite.

Conclusions

Physicochemical and microscopic investigation of insoluble mineral crystals formed in a synthetic phosphate-containing cultivation medium of the soil bacterium *Azospirillum brasilense* (strain Sp245) using IR spectroscopy, X-ray diffraction and thermal analysis as well as elemental analyses by atomic spectrometry and ion chromatography have shown the mineral to have the exact formula $Mg_{0.51}(NH_4)_{0.74}K_{0.03}Na_{0.01}H_{1.20}PO_4\times 2.53H_2O$ (excluding trace impurities), which may be represented, in terms of the magnesium-ammonium orthophosphate hydrate entity only, by an approximate composition of $Mg_2(NH_4)_3H_5(PO_4)_4\times 10H_2O$. Its structure has been found to be close to that of the mineral struvite ($MgNH_4PO_4\times 6H_2O$; ASTM file No. 15–762) which is known to have a variety of forms. It has been shown that a gradual increase in *pH* of the medium from 6.9 up to *ca*. 7.8, occurring during cultivation of azospirillum facilitates the slow process of biomineralization in the presence of orthophosphates, leading to the formation of relatively large struvite-like crystals based on magnesium-ammonium orthophosphate hydrate containing entrapped cations available from the medium. The data of trace elemental analyses of the mineral and their comparison with the composition of the cultivation medium show that Ca, Fe, and Mn accumulate in the solid phase precipitating from the solution, thus being obviously excluded from metabolic processes.

Experimental

Materials

The bacterium *Azospirillum brasilense*, strain Sp245 (Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov), was cultivated with extensive aeration using a laboratory shaker at $T = 305$ K for 18 h in a synthetic medium containing (g/l, with respect to anhydrous salts): KH_2PO_4 2.0, K_2HPO_4 3.0, NaCl 0.1, $MgSO_4$ 0.2, $CaCl_2$ 0.02, sodium malate 5.0 (prepared by titrating a malic acid solution with alkali up to *pH* 6.86), Na_2MoO_4 0.002, NH_4Cl 0.5, $FeSO_4$ 0.02, $MnSO_4$ 0.1, and yeast extract (Serva) 0.1. The initial *pH* value of the cultivation medium was 6.86, and its change with time in the course of cultivation was controlled with an OP 264/1 digital *pH*-meter (Hungary). All chemicals used were of analytical or reagent grade. After centrifugation at $T = 277$ K, mineral crystals were completely separated from

organic matter (slime and microbial cells) using a UD-10 ultrasonic disintegrator (Poland), washed thoroughly with doubly distilled water, and dried in air at ambient temperature. The yield of the dry crystals slightly varied reaching up to 4 wt.% of the raw centrifugate. Synthetic crystals (for comparative microscopic observation) were obtained by artificial slow gradual alkalization of the initial cell-free cultivation medium for 18 h from $pH \cong 6.9$ up to 7.8 using dialysis of a 1 m*M* NaOH solution; they were washed and dried as described above.

Methods

Infrared (IR) spectra were recorded using a Specord IR 75 spectrophotometer (C. Zeiss, Germany); samples were pressed in disks with KBr. The X-ray diffraction (XRD) pattern was obtained with a DRON-3.0 diffractometer (Russia); FeK_α radiation was used (scanning rate: $2^\circ \cdot \mathrm{min}^{-1}$). Thermogravimetric (TGA) and differential thermal (DTA) analyses were performed on an OD-103 derivatograph (MOM, Hungary) for the air-dry sample (initial weight 200 mg; temperature range 293–873 K; heating rate 10 K/min). Microphotographs of the biomineral and of the synthetic crystals formed in the same initial cell-free cultivation solution during its gradual alkalization using dialysis of a diluted NaOH solution for 18 h were obtained using a phase-contrast microscope Jenaval (Germany) with a 300-fold magnification.

Metal cations added to the cultural medium were determined in the mineral after its digestion in a Parr Acid Digestion Bomb No. 4745 (total volume 23 ml, Parr Instruments Company, USA) by flame atomic emission (FAES; for Na and K) and absorption spectrometry (FAAS; for Mg, Ca, Fe, Mn) using an acetylene-air flame (spectrometer Perkin-Elmer, Model 3110; autosampler Perkin-Elmer, Model AS-60, USA). Total phosphorus (in the form of PO_4^{3-}) was determined by ion chromatography (IC) using a modified procedure similar to that proposed recently for the determination of total nitrogen [45]. Chlorine (as Cl^-) and sulfur (as SO_4^{2-}) admixtures were also analysed using the IC method. Total nitrogen was determined by the conventional *Kjeldahl* method [46]. Unless otherwise indicated, all experimental procedures and measurements were carried out at ambient temperature (295±3K).

Acknowledgements

The authors would like to thank Dr. *I. Ya. Evtushenko*, *N. N. Bylinkina*, and *O. E. Koblova* (Saratov) for their skilful assistance in experimental work and *S. S. Khmelev* (Saratov) for many helpful discussions. This work was supported in parts by the EC (INTAS Project No. 96-1015) and the *Russian Foundation for Basic Research* (RFBR grant No. 95-03-08295a).

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Received May 10, 1999. Accepted May 17, 1999