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Comparative Analysis of Biogenous Carbonate Hydroxylapatite and of Its Synthetic Analog as a Material for Osteoplastics

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Abstract

A comparative analysis of biogenous and synthetic carbonate hydroxylapatite of various degree of crystallinity has been carried out. The analysis of chemical composition of enamel and dentine was carried out on an X-ray microanalyzer, that of the synthetic product on an X-ray fluorescence spectrometer. Crystallochemical characteristics were studied by X-ray diffractometry and molecular spectroscopy. Identity of structural characteristics and of the chemical composition of the specimens studied was demonstrated. Toxicological, mutagenic properties and histocompatibility of the synthetic apatite were studied. The material obtained was used for treatment of patients with defects of bone tissue.

INTRODUCTION

The mineral world is one of the most important components of the animal habitation milieu, and all living things have acquired in the process of evolution genetic relations with the environment [1]. “An organism represents an inseparable part of the Earth crust, its product, a part of its chemical mechanism”, – wrote V. I. Vernadskiy. Minerals of biogenous nature are greatly different from respective abiogenous objects, since so are also the mechanisms that bring about their advent. It is known that the mineral component of human bone tissue is represented by carbonate hydroxylapatite whose ideal formula is $Ca_5(PO_4)_3OH$ [2, 3]. However, this mineral turned out not to be universal because of inability to support various isomorphic substitutions in practically all structural positions due to the peculiarities of its crystalline structure [4]. For example, in deficit of Ca, its positions can be occupied about 20 elements, including K, Na, U, Sr, Th, Ba. Depending on the ecological situation in the regions, PO_4^{3-} may be partially substituted by CO_3^{2-}, HPO_4^{2-} and sometimes even SiO_4^{4-} and SO_4^{2-} ions, and groups OH^- by anions F^-, Cl^-, O^{2-} [5]. In samples of bone tissue there may be Sr, Fe, Cu (mass fraction of up to 0.014 %), and Sr (0.016 %) [6]. In normal situations, the mass fraction of Ca and P in bone tissues must be within the normal ratio of 2 : 1 [6, 7]. When such an equilibrium is disturbed, diseases may happen: *e. g.*, in an excess of P Boeck`s disease (disturbances of skeleton development), and in an excess of Ca hypercalciemia (salt deposits, diseases of joints *etc.*) [4].

Bone and tooth diseases are widespread and are often of endemic character; therefore, creation of biocompatible materials for osteoplastics remains an important problem in medicine. It is known that in order to fill in cavities and cysts of bone tissue, β-tricalcium phosphate and hydroxylapatite are used [7–9]. The processes of compatibility and bioadaptation of biological apatite compounds and of proposed synthetic samples require a thorough study. One of the most important approaches to this problem is modeling of the processes under study in controllable conditions with a subsequent comprehensive analysis of the experimental results.

The goal of the present work was investigation and comparative analysis of crystallochemical properties of carbonate hydroxylapatite of biological origin and of its synthetic analog for optimization of conditions for obtaining a bone-substituting material maximally close in its structure and composition to the biological apatite and meeting the requirements of artificial implants of bone tissue.

OBJECTS AND METHODS

The objects of study were well and poorly crystallized synthetic apatite and bioappatite – human tooth enamel and dentine. The synthetic material was produced by sedimentation from solution by means of neutralization of saturated $Ca(OH)_2$ solution with diluted phosphoric acid to $pH > 7.0$ according to equation [10, 11]

$$5Ca(OH)_2 + 3H_3PO_4 \rightarrow Ca_5(PO_4)_3OH + 9H_2O$$

The loose sediment of white color was filtered off, washed with distilled water and dried at various temperatures in order to obtain products with various degree of crystallinity. Chemical analysis of the end product was carried out on a fluorescence spectrometer CPM-25, calcination loss were estimated by the gravimetrical technique. The results of the analysis are presented in Table 1.

Taken as bioapatite were specimens of practically healthy teeth removed from patients due to trauma. Enamel and dentine were studied separately. Dentine was chosen as the most dynamic part of the mineralized tooth structure which was characterized by the most intense metabolism. In order to remove the protein component, dentin was treated in a solution of immobilized proteases which represent-

TABLE 1

Chemical composition of human teeth and of synthesized hydroxylapatite

Sample	SiO_2	Al_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	Ba	SO_3	Cl	F	Calcination loss	Σ, %
Tooth enamel	n/d*	n/d	n/d	0.38	48.30	0.63	0.02	37.84	n/d	0.05	0.36	0.85	n/d	88.43
Tooth dentine	»	»	»	1.89	46.40	0.94	0.03	37.00	»	0.08	0.20	0.76	»	87.30
Synthetic enamel	0.09	0.06	0.03	0.35	53.63	0.3	0.00	38.660	0.000	n/d	n/d	n/d	6.66	99.78
Synthetic dentine	0.13	0.08	0.03	0.35	52.18	0.3	0.00	38.490	0.000	»	»	»	8.26	99.82

*Not determined.

ed a complex of proteolytic enzymes obtained from bacterial raw material and linked to a polymeric template by azide bonds. Hydrolysis of dentine proteins was carried out until a constant concentration of degradation products was obtained. Chemical analysis of enamel and dentin was performed on an X-ray microanalyzer KAMEBAKS-micro (see Table 1). The phase composition of, and the structural information about the synthesized and the biological apatite were obtained by means of powder diffraction (device DRON-UM 1, CuK_α) and infrared spectroscopy (Specord-75 IR). For subsequent studies, synthesized specimens were selected in which no admixtures of other phases, *e. g.* $CaCO_3$, were found.

Tests for histocompatibility were carried out. Toxicological and mutagenic properties of the synthetic material were studied. Pure 200 mesh granules and granules incorporated into the structure of low-molecular gel that served as the shape-forming component were used. All the studies were carried out in accordance with the requirements of the Pharmaceutical Committee of the Russian Ministry of Health. Mutagenic characteristics were detected with the help of the method of recessive lethals on *Drosophila melanogaster* whose food contained an addition of gel with apatite. The method envisages the possibility of activating in both parents a character whose transmission to offspring in a double dose leads to their death. The cytotoxicity of the synthesized materials with various degrees of crystallinity was studied on a fibroblast L 929 strain culture. Cells were cultivated in the medium RDM-1640 with addition of gentamycin, glutathione and embryonic calf serum. When the cultivation was over, the cells“ viability was estimated by the method of reduction of tetrazolium nitroblue with subsequent analysis on a spectrophotometer. The results of these studies demonstrated that the specimens synthesized by us had no cytotoxicity.

Reaction of tissues to the presence of carbonate hydroxylapatite was studied by means of its implantation. Under ether anesthesia subfascially through a cut of tissues in the region of left scapula a sample was introduced to the rats of Wistar strain weighing 140–160 g. In 1–2 months after the operation, the animals were decapitated under general anesthesia and tissues surrounding the carbonate hydroxylapatite were excised. Thereupon, the tissues were subjected to the standard histological treatment. Sections were stained with hematoxylin-eosin, toiluoidin blue (pH 5.6) and by the combined histochemical method (colloid iron – SCHIK – hematoxylin). The study was carried out in the usual and in polarized light. The results were treated by the conventional methods of biological statistics. In the course of morphological study of the tissue surrounding the synthesized carbonate apatite, it was established that all the layers of epidermis and subcutaneous tissue reacted in the normal physiological way to the surgical trauma and to the presence of the implant. The skin and its accessories had a typical structure, although the reticular layer in the region of the surgical scar was loose and consisted of thinner collagen and reticular fibers than those of intact skin. In all the cases, the wounds of experimental animals healed by primary adhesion. When examined in polarized light at the level of the aponeurotic plate, carbonate apatite granules surrounded by macrophages and histiocytes were observed. There were no symptoms of any inflammatory reaction (polymorphonuclear leucocytes, edema, tissue debris). Neither were there any symptoms of allergic reaction – aggregation of mast cells.

RESULTS AND DISCUSSION

Among the minerals known to be found in nature, it is only apatite that is a through mineral in living and inorganic nature, a mineral of fertility, mineral of life. The structures of enamel and dentin correspond to the structure of carbonate hydroxylapatite which is well crystallized in the case of enamel (large size of coherent scattering blocks and presence of ordering in the structure) and a low degree of crystallinity in the case of dentine (small blocks and a high structure defectiveness) (Fig. 1). In crystallograms of dentine and of weakly crystallized synthetic specimen, only the most intense reflexes corresponding to crystallographic directions 002, 211, 210 (see Fig. 1, curves *3*, *4*) appear. The difference in crystallinity of

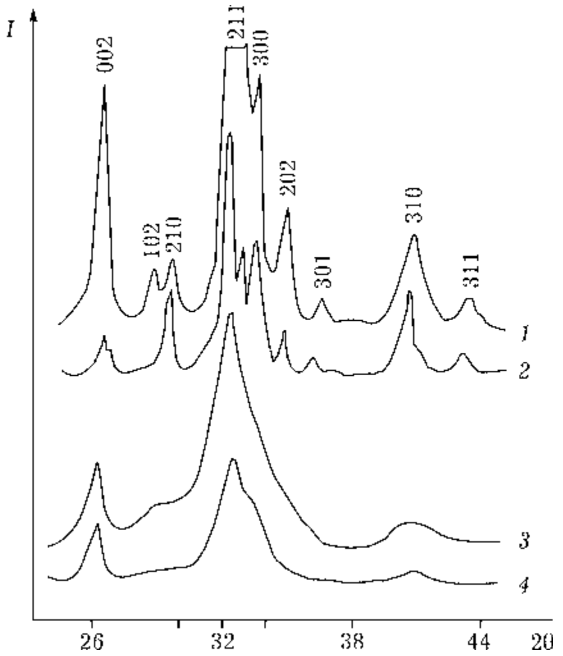

Fig. 1. Fragments of apatite roentgenograms: *1* – synthetic well-crystallized apatite; *2* – human tooth enamel; *3* – synthetic poorly crystallized apatite; *4* – human dentine.

the specimens studied is reflected also in IR spectra with the help of which we obtain additional information about the degree of substitution of positions PO_4^{3-} by CO_3^{2-} ions (Fig. 2). In the structure of physiogenous apatites, as a rule, the carbonate ion is present in a definite amount [2], whereas in pathogenic, *e.g.* urine concrements in association with oxalates, biogenous apatite may be carbonate-free [12].

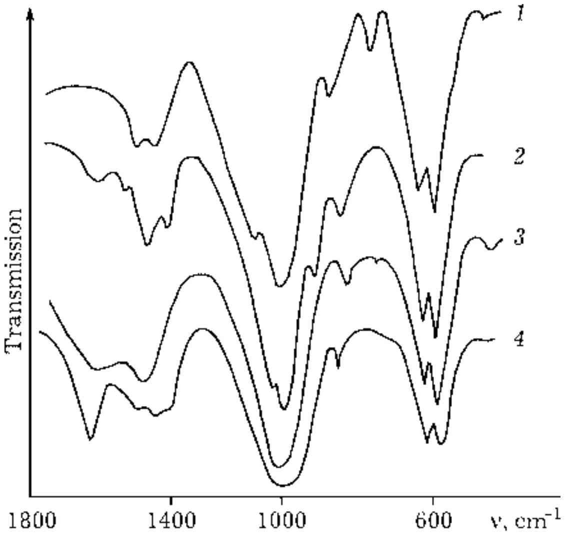

Fig. 2. Fragments of IR spectra of apatite: *1* – synthetic well-crystallized apatite; *2* – human tooth enamel; *3* – synthetic poorly crystallized apatite; *4* – human tooth dentine.

We tried to obtain synthetic materials maximally similar in their structure to physiogenous carbonate hydroxylapatite. According to IR spectroscopy data, the fraction of CO_3^{2-} ions in the structure of synthetic apatite depends on the pH of its sedimentation solution. At pH 7, carbonate-free apatite is crystallized on whose IR spectrum bands of carbonate ions do not appear (Fig. 3, curve *1*). The degree of resolution of such a spectrum by components is higher, the band of OH vibrations at 630 cm^{-1} which is visible only as an arm in the spectrum of poorly crystallized carbonate-containing specimen can be seen clearly. (see Fig. 3, curve *2*). Increasing the pH value, one can control the degree of isomorphic substitution of PO_4^{3-} by CO_3^{2-} in the structure up to the formation of high-carbonate hydroxylapatite in a maximally alkaline medium. The degree of crystallinity of the end product depends not only on the pH of its sedimentation medium. The rate of crystal sedimentation and the temperature of the end product formation are also important. Varying these parameters, one can obtain a material with preset characteristics.

Chemical analysis of enamel and dentine of a healthy tooth of a 29-year-old patient and of synthetic specimens of apatite demonstrated a similarity of their composition (see Table 1), and the data of roentgenography

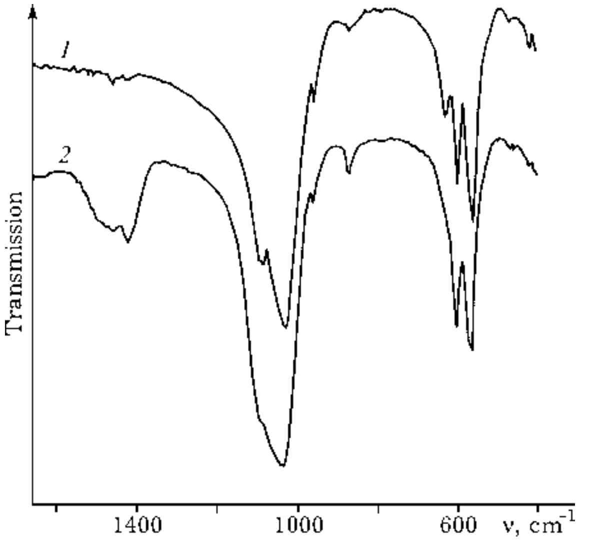

Fig. 3. Fragments of IR spectra of synthetic apatite obtained at pH 7 (*1*) and 12 (*2*).

and IR spectroscopy showed a similarity of their structure. The lowered calcium and phosphorus content in biological specimens as compared to artificial ones is accounted for by the presence of a certain amount of organic component and of water molecules.

As a result of disturbance of metabolic processes, in the organism a decrease in tooth Ca^{2+} and ${PO_4}^{3-}$ content and an increase H_2O, CO_3^{2-} and some other elements and groups that occupy the positions of Ca^{2+} and PO_4^{3-} can take place. The decrease Ca^{2+} and PO_4^{3-} fractions is not only their loss, but also a result of increase in the number of isomorphic substitutions depending on many factors, including the human habitation milieu. Due to isomorphism, in the enamel structure there appear defects, stresses, and, as a result, microcrevices (because of difference in the radii of the substituting ions and in the forces of their interaction with neighboring atoms), which results in caries and increases the susceptibility of the teeth. Synthetic apatite specimens were used for tests in several patients suffering from a heightened susceptibility of enamel to external influences. Daily massage of teeth using a fine-dispersed powder of apatite with a structure identical to that of enamel decreased considerably the enamel sensitivity. At present, apatite-based preparations find a wide field of application in dentistry for the treatment of parodontosis, jaw cysts, implantation of constructions intended for fixation of tooth prostheses *etc*. [13].

A preliminary clinical study of bone-substituting material was carried out on 14 patients at the age of 17 to 44 years, including 4 who had been treated for various extremity injuries and had defects (cavities, cysts) of metaphyses, diaphyses, bones, and 10 patients having osteomyelitis foci of various localization as a result of chronic osteomyelitis. The patients“ bone tissue defects were filled-in with modified carbonate hydroxylapatite incorporated into the structure of a biocompatible gel. Subsequent observation of the operation results for 6 months to 6 years has demonstrated that in all the cases the osteogenesis process went closely to physiological time course [14]. Carbonate hydroxylapatite became replaced by a normal bone tissue. A complete concrescence of the newly formed tissue with the bone took place. No sequestration reaction was observed.

The organism consists of a large number of components and elements whose functions are manifold. For example, calcium and phosphorus in the form of apatite participate in construction of mineral skeleton structure. The mineral component of bone tissue is in the state of continuous renewal in which two cell types take part which fulfill opposite functions of building and destroying the bone tissue [6, 15]. Calcium salts are continuously being eliminated from the human body and therefore must be continuously re-supplied there. Calcium metabolism in the body is very complicated, since its salts have different absorption capacity in the intestine. Assimilation of phosphoric calcium salts is a easier process than assimilation of its other compounds. As it was told above, a necessary condition for replenishment of calcium reserves in the organism is a definite proportion of calcium and phosphorus, *i. e.* the amount of Ca must be twice as large as that of P. It is only at this proportion that the two elements are optimally assimilated and fulfill their function [6]. In the new generation of food supplements intended for normalization of calcium metabolism in the organism an important component is carbonate hydroxylapatite, and these preparations are recommended for pathological states such as systemic osteoporosis, extremity fractures, diseases of joints, for strengthening of teeth *etc.* [14, 15].

Studies of resistance of the mineral component of bones and teeth depending on the geochemistry of environment have been extremely scarce. Meanwhile, in the environmental deficit of Ca and excess of some competitor elements (Sr, Se, U, Th, Ba *etc.*), an isomorphic substitution Ca → Sr or Ba in the apatite structure is probable, which can provoke some pathological conditions in human organism [16]. In this regard, a detailed study of peculiarities of composition, structure and properties of this unique mineral of various genetic belonging is very important for understanding the processes of apatite formation and of calcium metabolism in the human organism, since, being a mineral of life, it can become a cause of diseases or even of death. In the case of disturbance of metabolic processes or in the presence of infectious bacteria capable of synthesizing calcium phosphates in blood [17], apatite is sedimented on the walls of blood vessels, on heart valves, in kidneys and other tissues and organs [4, 18, 19], which results in formation of urine, salivary, tooth concrements, in calcification of cardiac valves, lungs *etc.* [20].

CONCLUSION

As a result of studies, it has been demonstrated that by means of directed synthesis it is possible to obtain carbonate hydroxylapatite with crystallochemical characteristics analogous to those of biogenous apatite. The positive results of tests of the synthesized specimens as fillers for elimination of defects of bone tissues, additions to tooth-pastes permit hoping that improvement of the methods of obtaining materials similar to biogenous apatite is a promising trend in obtaining bone-replacing materials fit for application in dentistry, traumatology and reconstructive surgery.

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