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Medical Applications of Zeolites

Krešimir Pavelić and Mirko Hadžija
Ruđer Bošković Institute, Zagreb, Croatia

I. INTRODUCTION

A particular structural feature of zeolites relative to other aluminosilicate materials, and other crystalline materials in general, is the existence of channels and/or cavities linked by channels. One of the characteristics that distinguishes zeolites from other porous materials is their variety of pore sizes and shapes. The size and shape of channels/cavities in zeolites therefore define the structural parameters of a given type of zeolite (1). Properties of zeolites, such as ion exchange, intercrystalline pores that discriminate between molecules of different dimension, strong acidic sites, and active reservoirs for metal-catalyzed reactions, have earned them extensive industrial uses. Consequently, fundamental zeolite research has become an area of great interest (2). The remarkable applicability of zeolites ranges from uses in biochemistry, the agroindustry, detergents, soil improvements, the nuclear industry, energy storage, and the textile industry (3).

Zeolites are among the most important inorganic cation exchangers. The aluminosilicate structure is negatively charged and attracts cations that come to reside inside the pores and channels. Zeolites have large empty spaces, or cages, within their structures that can accommodate large cations, such as Na^+, K^+, Br^+, and Ca^{2+}, and even relatively large molecules and cationic groups, such as water, ammonia, carbonate ions, and nitrate ions. The basic structure of zeolites is biologically neutral.

The ion-exchange process is reversible, allowing for adsorption of ions and molecules, making zeolites useful as filters for dust, toxin removal, and as chemical sieves. Zeolites can have water as part of their structure; after the water has been driven off by heating, the basic framework structure is left intact. Subsequently, other solutions can be put through the structure, and thus the zeolite acts as a delivery system for the new fluid. This process has been exploited and applied in medicine, farm animal feed, and other types of research. Nowadays zeolites compete with cation-exchange resins in water processing and in purification of wastewater and sewage. Zeolites have high cation exchange selectivities, good resistance to temperature and ionizing radiations, and excellent compatibility with the environment. Therefore, zeolites are widely used in modern technology as selective adsorbents, molecular sieves, and particularly as catalysts. It is obvious that the ion sieving and other remarkable properties of zeolites will be utilized in the near future for the environmental and health care industries. The reasons for this are as follows: (a) zeolites have known biological properties alone with long-term chemical and biological

stability (4); (b) they reversibly bind small molecules such as oxygen and nitric oxide (5,6); (c) they possess size and shape selectivities; (d) they offer the possibility of metalloenzyme mimicry; and (e) they have immunomodulatory activity (Fig. 1).

Researchers are even exploring the possibility that zeolites and feldspars played a major role in the beginning of "life," i.e., life may have begun by catalytic assembly on a mineral surface. Catalysis and mineral surfaces might have generated replicating biopolymers from simple chemicals supplied by meteorites, volcanic gases, and photochemical reactions (7). How could the first replicating and energy-supplying molecules have been assembled from simpler materials that were available on the early protocontinents? Concepts of catalysis that use organic compounds dispersed in aqueous "soups" require a mechanism for catalytic substrate. After catalysis, biochemically significant polymers such as polypeptides and RNA might have been protected from photochemical destruction by solar radiation. Assuming temperatures were not too high, energy-consuming replication/mutation of polymers could have led to the first primitive organisms.

A new concept is that certain materials have internal surfaces that are both organophilic and catalytic, allowing efficient capture of organic species for catalytic assembly into polymers in a protective environment. Indeed, caution is warranted in proposing that life began merely as a trivial scientific event on a mineral catalyst (7). Nucleic acids can be adsorbed onto and preserved by clays, which are layered aluminosilicates. It is known that environmental DNA can be stabilized by adsorption onto sand and clay particles, thereby becoming 100- to 1000-fold more resistant to deoxyribonuclease (DNase). Such adsorbed DNA may retain its transforming ability for weeks and even months (8).

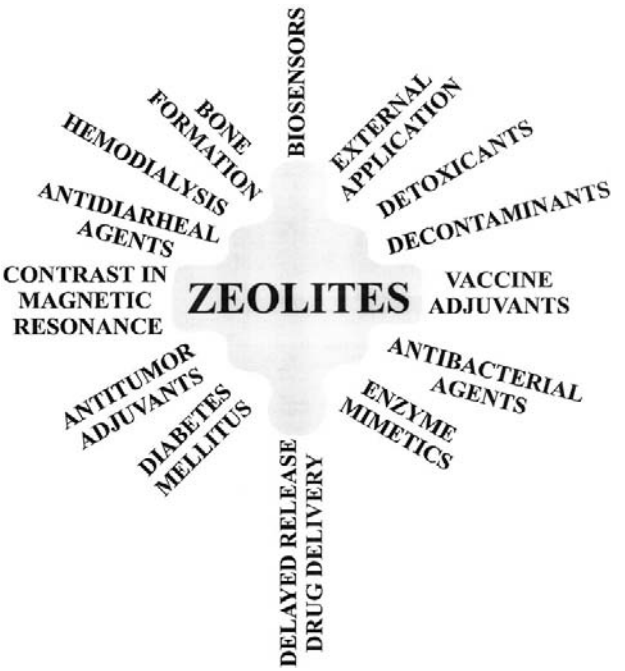

Fig. 1 Biomedical effects of zeolites. (From Ref. 86.)

Since many biochemical processes are closely related to some zeolite properties (ion-exchange, adsorption, and catalysis), we believe that natural and synthetic zeolites may lead to significant advances in biology, medicine, and in the pharmaceutical industry in the near future.

II. ANIMAL PRODUCTION, FOOD SUPPLEMENTS, AND ADDITIVES

Zeolites show remarkable selectivities for removing ammonia from water. Data support a favorable situation for potential applications in industrial and agricultural wastewater purification, aquaculture, animal feeding, agriculture, and horticulture (with use of natural zeolites as nitrogen fertilizers) (9).

Zeolites are already in use in the food industry. Zeolites saturated with CO_2 provide instantaneous carbonation to aqueous preparations. Beer is stabilized with NaA and LiX zeolites, which adsorb the proteins responsible for further degradation. The dealcoholization of beer is done with the help of dealuminated zeolite Y. The fatty acids of comestible oil are eliminated on zeolite X. Zeolites are also included in the formulation of toothpaste.

Ammonium adsorption from animal manures is one of the major applications of the zeolite clinoptilolite. The amount of NH_4^+ adsorbed increases as the pH and the initial NH_4^+ concentration increase. This behavior is an important characteristic of the zeolite that can be beneficial to minimizing N-losses via NH_3 volatilization during composting of N-rich animal manures (10).

Zeolites could be successfully used in agriculture. As soil additives, natural zeolites reduce the uptake of mercury by plants and the restriction of the entry of mercury into the food chain (11). An Italian chabazite-rich tuff can selectively remove considerable amounts of NH_4^+ from wastewater and, when exhausted, re-utilized for the correction of a soil to grow a common vegetable (tomato) and flower (Geranium) (11). In the case of urban wastewater, treatment with 1.25 g/l of zeolite lowers the NH_4^+ outflow remarkably. In the case of landfill wastewater, treatment with 200 g/l of zeolite strongly reduced the NH_4^+ outflow. When exhausted, the NH_4^+-enriched zeolite showed high efficiency in agriculture. Furthermore, clinoptilolite significantly inhibits the number of viable *Salmonella typhymurium* in soil and liquid microcosms (12). One of the secrets for the success of the famous Hungarian wine "Bull Blood" is linked to the nature of the cellars in the Eger's mountains: they are composed of zeolites that maintain a constant humidity level during the entire period of wine maturation.

A. Effect of Dietary Inclusions of Clinoptilolite

Dietary inclusions of clinoptilolite could be beneficial for animal production. Pigs fed clinoptilolite experience beneficial weight gains and are less subject to disease than pigs fed normal diets. They also show regular digestion, as well as an increase in appetite, and the meat content increases at the expense of the fat. Clinoptilolite, chabazite, mordenite, erionite, and phillipsite actively adsorb ammonia, carbon dioxide, hydrogen sulfide, and mercaptans and have a strong deodorizing effect. It is also possible that zeolites remove toxins and create changes in enzymology and immunological responses. All of these events have resulted from application of zeolites in the animal production industry.

Piglets aged 27 days were fed the natural clinoptilolite mannelite as 2% of their feed, corrected for nutrient dilution, for 4 weeks. Mannelite gave a tendency for higher growth

and lower feed-to-gain ratio. When the diet was not corrected for nutrient dilution, the piglets showed significantly higher growth and a better feed-to-gain ratio (corrected for differences in nutrient concentration of the diets) over the total experimental period. They were able to compensate for the energy-diluting effect of mannelite addition by increasing their feed intake. Authors concluded that dietary dilution of piglet feed with 2% mannelite significantly increased daily gain and decreased feed-to-gain ratio, corrected for nutrient dilution (13).

Klinofeed, containing 70% clinoptilolite, elevated nitrogen excretion in feces and lowered nitrogen excretion in urine. Protein retention was not significantly influenced by Klinofeed. Therefore, dietary inclusion of clinoptilolite for growing pigs changed the excretion in urine without altering protein deposition (14,15) (Fig 2).

Single-combed, 16-week-old pullets of three strains were fed a diet containing 135 g protein/kg with or without 50 g clinoptilolite/kg. Sterile river sand replaced clinoptilolite in the control diet in order to keep the diets isoenergetic. Significant dietary effects of feeding clinoptilolite were observed with improvement in number of eggs laid per hen, shell thickness, efficiency of food utilization, and droppings moisture content. No significant dietary effects between treatments were observed with body weight, age at first egg, egg weight, food intake of hen, and rate of amino acid absorption of radioactive lysine and methionine into the bloodstream (16).

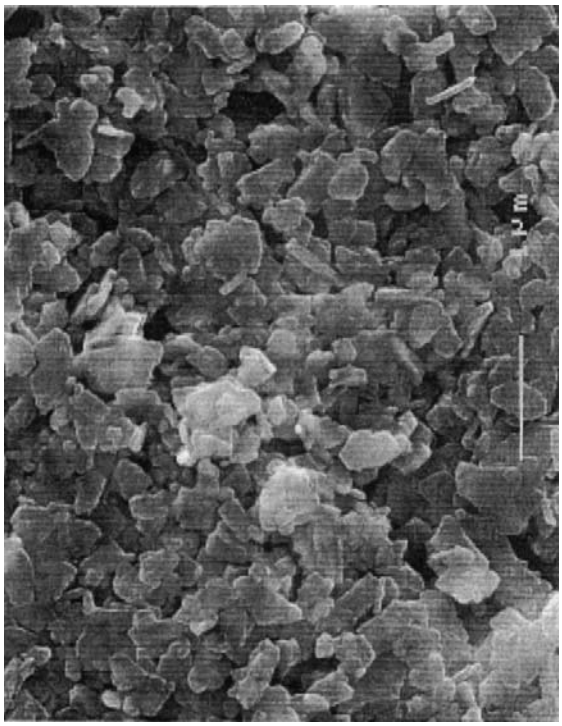

Fig. 2 Scanning electron microscopy photography of tribomechanically micronized clinoptilolite used for biomedical application. (From unpublished data.)

B. Effect on Chicken Aflatoxicosis

Clinoptilolite incorporated into the diet can reduce the deleterious effects of aflatoxin because it strongly adsorbs aflatoxins and zearalenone (17). Mineral adsorbents based on natural zeolite and bentonite may be used in animal diets to prevent poisoning caused by mycotoxins. Clinoptilolite incorporated into the diet at 1.5% and 2.5% was evaluated for ability to reduce the deleterious effects of 2.5 mg total aflatoxin on broiler chickens. When compared with the controls, aflatoxin treatment significantly reduced serum total protein, albumin, inorganic phosphorus, uric acid, total cholesterol, and the hematocrit, red blood cell count, mean corpuscular volume, hemoglobin, thrombocyte count, and monocyte count, while increasing the white blood cell and neutrophil counts. The addition of clinoptilolite to the aflatoxin diet reduced the adverse effects of aflatoxin and should be helpful in solving the aflatoxicosis problem in poultry (18).

Clinoptilolite incorporated into the diet at 50 g/kg reduces the deleterious effects of aflatoxin in growing Japanese quail chicks from 10 to 45 days of age. While aflatoxin decreased food consumption and body weight gain from the third week onward, addition of clinoptilolite significantly reduced the negative aflatoxin effects on food consumption ratio (19). Similar effects were observed with some synthetic zeolites. Zeolites NaX, NaY, NaA, and CaA were evaluated in vitro for their ability to sorb aflatoxin B1 from an aqueous solution. Zeolite NaA was selected for testing in vivo because of its high affinity and its stable association with aflatoxin. This artificial zeolite almost totally protected growing broiler chicks against the effects caused by aflatoxin (20).

An important question is whether zeolites influence vitamin and microelement adsorption. Neither amino acids (tryptophan and phenylalanine) nor vitamins (A, D, E) are adsorbed by clinoptilolite. Natural zeolites have a low efficiency for binding vitamin B_6 in vitro. This process is dependent on crystallinity and the mineralogical composition of the zeolitic samples. On the other hand, vitamin B_6 is tightly bound to the clay mineral bentonite. Cu, Zn, Co, and Mn are bound less tightly to zeolite than to bentonite. These data suggest that the bentonite material would reduce micronutrient availability more than zeolite (21).

Zeolites could have some effects on egg characteristics. Clinoptilolite from Greece improves both the albumen weight and yolk weight. The beneficial effect of clinoptilolite on egg and albumen weight was independent of hen age and the type of diet (isonitrogenous, or isonitrogenous + isoenergetic) (22). Finally, the mycotoxin cyclopiazonic acid strongly adsorbs onto the surface of a naturally acidic phyllosilicate clay, which was not confirmed by in vivo experiments (23).

III. RADIOPROTECTION

Many researchers have demonstrated the ability of several natural zeolites to take up certain radionuclides (e.g., ^{90}Sr, ^{137}Cs, ^{60}Co, ^{45}Ca, and ^{51}Cr). Zeolite mordenite has effectively decontaminated soils contaminated with ^{137}Cs and ^{90}Sr (25). Clinoptilolite shows a significant protective effect reducing radiocesium-137 accumulation in male broiler chickens exposed to alimentary contamination. The reduction of radiocesium in meat ranged between 60% and 70% and in edible organs it was greater than 50%.

Clinoptilolite supplementation in food eliminated ^{137}Cs deposition in some organs and tissues. After dietary administration with 2.5%, 5.0% and 10% zeolite, ^{137}Cs elimination increased and the radionuclide deposition in liver, kidneys, and femoral musculature decreased. The clinoptilolite decontamination effects were observed with

preventive administration, as well as with sorbent administration from 24 h after a single contamination of brown rats (26,27).

Akyuz showed that clinoptilolite from the deposits of Cankiri-Corum Basin is an excellent sorber for both cesium and strontium ions and can be used for the treatment of radioactive wastewater and other decontamination purposes (28). Similar properties of clinoptilolite from other deposits are known as well.

IV. REMOVAL OF HEAVY METALS AND ORGANOPOISONING

Heavy metals released in wastewater are among the most worrisome pollution problems due to their cumulative effects along the food chain. The natural zeolites clinoptilolite, phillipsite, and chabazite are particularly useful in selectively eliminating ammonia and heavy metals such as Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, and, partially, Cr^{3+}. Generally, clinoptilolite is stable in an acidic environment and shows high selectivity for many heavy metals. Malion et al. showed that the particle size of zeolite does not affect the actual metal uptake at the equilibrium point. However, metal removal is greatly affected when the contact of the solid/liquid phases is short, which is an essential parameter for treatment of wastewater. Kinetic curves showed very clearly the selectivity of zeolite for lead ions, and significant amounts of cadmium could be removed as well (29).

Although mercury is a well-known poison to human and animal health, its use in many industrial processes (e.g., catalysis, pigments, batteries, etc.) and even in agriculture (e.g., antifungals) is still rather extensive. This creates serious environmental problems, including especially the pollution of aquatic systems, which leads to mercury involvement in hydrological-hydrochemical, and biological cycles. Remarkable removal rates of mercury from aqueous solutions by NaCl-pretreated pure heulandite crystals and NaCl-pretreated clinoptilolite-containing rock samples have been observed (108). Therefore, chemical pretreatment plays a critical role, and it could be proposed that natural zeolite materials be used to remove heavy metals from aqueous solutions. The metal binding is attributed to ion-exchange adsorption and surface precipitation processes.

The preventive effect of zeolites on the intoxication of organophosphate poisoning has been described (105). Zeolite tuff containing 61% clinoptilolite has been shown to prevent and eliminate organophosphate poisoning. The organophosphate poison substance XX can strongly inhibit enzyme cholinesterase in erythrocytes, and in the stomach, brain, and liver. This effect can be strongly diminished after pretreatment with zeolite (1 g/kg 5 min before intoxication). The duodenum and colon are exceptions whereby the cholinesterase activity was not significantly restored. The low resorption rate from the gastrointestinal tract, weaker clinical signs of intoxication, and longer life span for the onset of specific therapy are facts that create conditions for inclusion of natural zeolites in the arsenal of rational prevention and therapy of organophosphate poisoning.

V. ANTIMICROBIAL EFFECTS

The antimicrobial effects of zeolites are well known. These properties have been used in different situations, including when a balloon catheter is employed for controlling urinary tract infection. Uchida et al. showed a bactericidal effect against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* in vitro (31). The antibacterial effects correlated with the concentration of zeolite and the size of the catheter. It was efficient for various urological patients who needed long-term use of a balloon catheter for lower urinary tract obstruction and for neurogenic bladder. All patients had been catheterized with indwelling silicon balloon catheters for 3–6 months and had suffered with compli-

cated urinary tract infection. No patient was taking antibiotics during the trial. Therefore, the antibacterial effect of zeolites for balloon catheters might be useful for patients who need long-term indwelling balloon catheters.

Metal-exchanged zeolites have been proposed in the last decade for controlled release of agents against microbial pollution (32). Zeolites containing copper ions exhibit good antibacterial activity for both gram-negative and gram-positive bacteria, and the effect developed in a short period of time. A new antifungal material, Ag-zeolite (Zeonic), was combined with a commercial tissue conditioner as a successful agent against *Candida albicans* growth and/or acid production. The inhibitory effects of these materials on fungal growth were decreased by the presence of a saliva coat, particularly with zeolite specimens and special tissue conditioner. Test specimens containing 2–5% Zeonic showed a significantly greater effect on the delay in rapid decline of pH as compared with the other specimens examined. These results suggest that zeolite-combined tissue conditioner would be a potential aid in denture plaque control (33). A silver zeolite mouth rinse was prepared by suspending zeolite powder into phosphate-buffered saline at a concentration of 3% (w/w). A double-blind crossover clinical study confirmed that silver zeolite significantly reduced plaque formation compared to the placebo (34).

Tissue conditioners containing silver-exchanged zeolite showed a strong in vitro antimicrobial effect on *Candida albicans*, and also on nasocomial respiratory infections of *S. aureus* and *P. aeruginosa*. All microbes were killed whether they had been immersed in saliva or not. Based on these findings and a double-blind clinical study the drug Zeomin was registered for dental purposes (35).

A new type of antibacterial temporary filling material in dentistry was incorporated into urethane acrylate monomer paste. These materials exhibited prominent in vitro antibacterial activity against *Streptococcus mutans* and *Streptococcus mitis*. The Ag-Zn-zeolite in these materials was able to release very small but detectable amounts of Ag and Zn even 4 weeks after the immersion started. The larger the amount of Ag-Zn-zeolite that was incorporated, the greater the release of silver and zinc. However, it appears that increasing antibacterial activity is not promoted by the higher ratio of Ag-Zn-zeolite (36).

An important question is whether antimicrobial silver-zeolite alters the dynamic viscoelastic properties of various tissue conditioners. Results suggest that incorporating silver-zeolite does not affect some tissue conditioner's inherent dynamic viscoelastic properties, although some reports claim that other tissue conditioners investigated may be found to have changed their properties as a consequence of the inclusion of silver-zeolite (37).

VI. HEMODIALYSIS, ANESTHESIOLOGY, AND HEMOPERFUSION

Zeolites, because of their impurity removal properties, have been used in hemodialysis (38). Ammonia removal from a recirculating dialyzate stream is a major challenge in developing a truly portable, regenerable hemodialysis system. Three zeolites—type F, type W, and clinoptilolite—were found to have good ammonia ion–exchange capacity. Zeolite ion–exchange capability was regulated by flushing the column with 2 mol/L sodium chloride after an ion–exchange run. Atomic absorption spectroscopy of the column eluent showed that no detectible (< 1 ppm) Si or Al had leached from the zeolite (39).

There is a clinical study that shows complete selective adsorption of the inhalation anesthetic desflurane by high silica zeolites in a special adsorber inserted at the outlet of a

scavenging system of an anesthesia machine. In comparison with charcoal filters, zeolites allow almost complete desorption at moderate temperatures followed by condensation of the desflurane to the liquid phase. Results show that about 85% of the adsorbed desflurane could be recovered as liquid with high purity via desorption (40).

Although zeolites effectively absorb nitrogen dioxide during the delivery of inhaled nitric oxide, there was re-formation of nitrogen dioxide from nitric oxide and oxygen. This initial production of nitrogen dioxide was very rapid and could not be prevented by the use of zeolite scavenger. The authors concluded that zeolite had no practical effect as scavengers in this delivery system (41).

Zeolites also can be used as a cartridge in hemoperfusion. Clinoptilolite as a cartridge for hemoperfusion columns was evaluated based on selected indices of blood biochemistry in sheep. Commercial hemoperfusion columns Hemasorb 400C were filled with a sodium form of natural zeolite-clinoptilolite partially saturated with potassium chloride. During 2 h of hemoperfusion, a significant decrease in the numbers of leukocytes and thrombocytes was found. Synthetic zeolites 4A and BX were incorporated in the drainage tube. Pentosan polysulfhate sodium bound to a carrier synthetic zeolite was incorporated in the drainage tube, which was then tested for its anticoagulant properties during perfusion with Tris buffer solution, citrated plasma, and blood. The amount of pentosan polysulfhate sodium released from the tube walls during perfusion with human citrated plasma was enough to exert an anticoagulant effect on the streaming plasma (42).

VII. EXTERNAL APPLICATION

The fact that zeolites can protect polymers from ultraviolet degradation opens a wide spectrum of external application of zeolites in cosmetics and dermatology. Zeolite powder has been found to be effective in the treatment of athlete's foot and to reduce the healing time of wounds and surgical incisions (3). Anecdotal information indicates that the cuts of mine and mill workers exposed to on-the-job zeolite dust heal remarkably quickly. There are some reports that tribomechanically micronized clinoptilolite helps healing of ulcer cruris and decubitus, and has some benefits in the treatment of psoriasis (J. Lipozenčić, V. Vučevac, M. Stipošek, S. Ivković, personal communication 1999). In Cuba, it is a common practice to dust the cuts of horses and cows with clinoptilolite to hasten the healing process (3).

VIII. BONE FORMATION

Silicon in trace amounts enhances bone formation, and the silicon-containing zeolite A increases eggshell thickness in hens. Zeolites have interesting effects on bone structure and formation. Zeolite A is a synthetic zeolite that may have therapeutic utility in osteoporotic individuals because of its ability to stimulate bone formation. There was no significant absorption of aluminium from the aluminum-zeolite treatments of beagle dogs (30 mg/kg). The concentrations of silicon and aluminum were determined by graphite furnace atomic absorption (43).

Zeolite A increases proliferation, differentiation, and transforming growth factor β (TGFβ) production in normal, adult human osteoblast-like cells in vitro. In concentrations from 0.1 to 100 μg/mL, zeolite A induces a dose-dependent increase in DNA synthesis of normal human osteoblast-like cells. Zeolite A also increases alkaline phosphatase activity and osteocalcin release. TGFβ is a potent mitogen for osteoblasts. Zeolite A treatment increases the steady-state mRNA levels for TGFβ1 and induces the

release of the latent form of TGFβ protein into the conditioned medium within 6 h. In conclusion, zeolite A induces the proliferation and differentiation of cells of the osteoblast lineage (44).

IX. EFFECTS ON GASTROINTESTINAL DISORDERS

As discussed earlier, the low resorption rate from the gastrointestinal tract, weaker clinical signs of intoxication, and longer time span for the onset of specific therapy are factors that create conditions for inclusion of natural zeolite in the arsenal of rational prevention and therapy of organophosphate poisoning (45). Observations were made first in animal nutrition. Ten percent of clinoptilolite or mordenite as dietary supplements for swine and poultry showed that animals generally grew faster, and the number and severity of intestinal diseases were reduced. In 1997, a new antidiarrheic drug for humans was introduced based on the physical and chemical properties of a purified natural clinoptilolite. A series of physical, chemical, technological, pharmacological, microbiological, and clinical studies was successfully conducted to meet the requirements of the Cuban Drug Quality Agency (46).

Zeolites can adsorb cholera toxin and *Escherichia coli* enterotoxins. A variety of common inorganic adsorbents, including aluminas, zeolites, phyllosilicate clays, silica, and carbon, can adsorb cholera toxin and heat-labile *E. coli* enterotoxin. These inorganic adsorbents have a beneficial role for children suffering spontaneous diarrhea by reducing enterotoxin activity (47).

A. Antacid Activity

It has been established through pharmacological and clinical studies that natural clinoptilolite from the Tasajeras deposit in Cuba does not cause any biological damage in humans. The structural stability of natural clinoptilolite during its transit through the gastrointestinal tract as compared to synthetic zeolites (48), the use of purified natural clinoptilolite as a gastric alcalinizant (49), and the use of antacids containing sodium carbonate (48) all suggested that a study of a Na_2CO_3-clinoptilolite (combination product formed via hydrothermal transformation) as an improved antacid was warranted. Rivera and coworkers showed that Na_2CO_3-clinoptilolite has more than twice the neutralization capacity of clinoptilolite. Rivera's experiments suggest that a 400-mg dose of such hydrothermally transformed zeolite will be able to increase the stomach pH to the value expected of an antacid treatment. In addition, UVspectroscopy demonstrated that the zeolite did not affect the concentration and stability of the enzyme pepsin in synthetic gastric juice (48).

Lam et al. performed a theoretical study of the physical adsorption of aspirin on natural clinoptilolite (50). Because the aspirin molecule is larger than the dimensions of the zeolite channels, only interaction with the external surface of zeolite is possible. The aspirin molecule was oriented to the cavities in three principal directions: 8-ring window model, 10-ring window model, and surface model. Their results support the possibility of the physical adsorption of aspirin by clinoptilolite (50). The best results were obtained for a pure silicon structure, followed by a structure with one hydrogen cation and then a structure with one sodium cation. At the same time, the presence of more aluminum atoms and compensating cations in the structures did not significantly alter the results. The aspirin molecule might therefore be adsorbed in gastrointestinal juices in a stronger fashion to an acid zeolite than to a sodium zeolite (50).

Based on these observations the gastric antacid Neutacid has been registered (49). As a nutriceutical, it consists mostly of the naturally occurring zeolite clinoptilolite, along with vitamins and minerals having antioxidative properties (51). It also possibly reduces the gastrointestinal toxic burden by affecting the anaerobic fermenting processes after digestion of food and by removing harmful metabolites after medical treatment of cancer and/or liver and kidney failure (52).

X. IMMUNOLOGICAL CONSEQUENCES

Many authors have noticed the appearance of autoimmune diseases after exposure to silicate materials (53). Although controversial, silicon breast implants have been implicated in occurrence of autoimmune and other diseases (54). Asbestos and fibrous zeolites have been implicated in pathogenesis of mesothelioma, lung fibrosis, and some autoimmune disorders (55).

Čolić and Pavelić (24) proposed that silicate materials act as superantigens. Superantigens are a group of bacterial and viral toxins, such as staphylococcal enterotoxin, that can induce cell death in certain populations of T cells that express Vβ T-cell receptors. Superantigens induce contacts of Vβ T cells and antigen-presenting major histocompatibility complex (MHC) II cells such as macrophages. This results in transient overactivation with subsequent activation-induced Vβ T-cell death. Such antigens can indeed enhance or reduce the immune response to a large number of antigens involved in a significant number of diseases. T cells expressing the Vβ repertoire represent between 5% and 20% of the total immune system and are involved in numerous autoimmune disorders (56) (Fig. 3).

Superantigens are in fact implicated in the pathology of numerous autoimmune and other relevant diseases. On the other hand, superantigens are also currently being tested in clinical trials as immunomodulators for treatment of cancer and autoimmune disorders (57). Preliminary in vitro experiments with asbestos and peripheral blood mononuclear cells indeed showed activation-induced Vβ T-cell death. Removal of MHC class II DP/DR-positive cells prevented the effect of asbestos on proliferation/death of Vβ T cells. We propose that such experiments should be performed with other silicate materials.

There are alternative mechanisms for the bioactivity of silicate materials. It was shown that intraperitoneal application of silica results in prevention of diabetes development in the NOD mice model and in other models of type I diabetes (109). It was also shown that silica killed macrophages needed for the activation of proinflammatory $CD4^+$ Th1 cells. This resulted in the activation of anti-inflammatory $CD4^+$ Th2 cells. Numerous authors have shown that activation and subsequent death of macrophages upon ingestion of silicate materials results an overproduction of free-radical species (58). Such macrophage death might be phenotype nonspecific, which can also be tested in vitro and in vivo.

Finally, it was also shown that asbestos and silica induced changes in human alveolar macrophages (107). Silicate materials in vitro induced stimulation of the proinflammatory macrophage phenotypes, which activated $CD4^+$ Th1 cells. These authors did not study long-term exposure effects and consequently did not show activation-induced cell death of such phenotypes.

At least three different mechanisms for the action of silicate materials can be proposed: (a) they act as superantigens, with specific activation-induced cell death of Vβ T cells; (b) they nonspecifically kill all macrophages, which also results in lack of activation of T cells; and (c) they activate and then cause activation-induced cell death of the

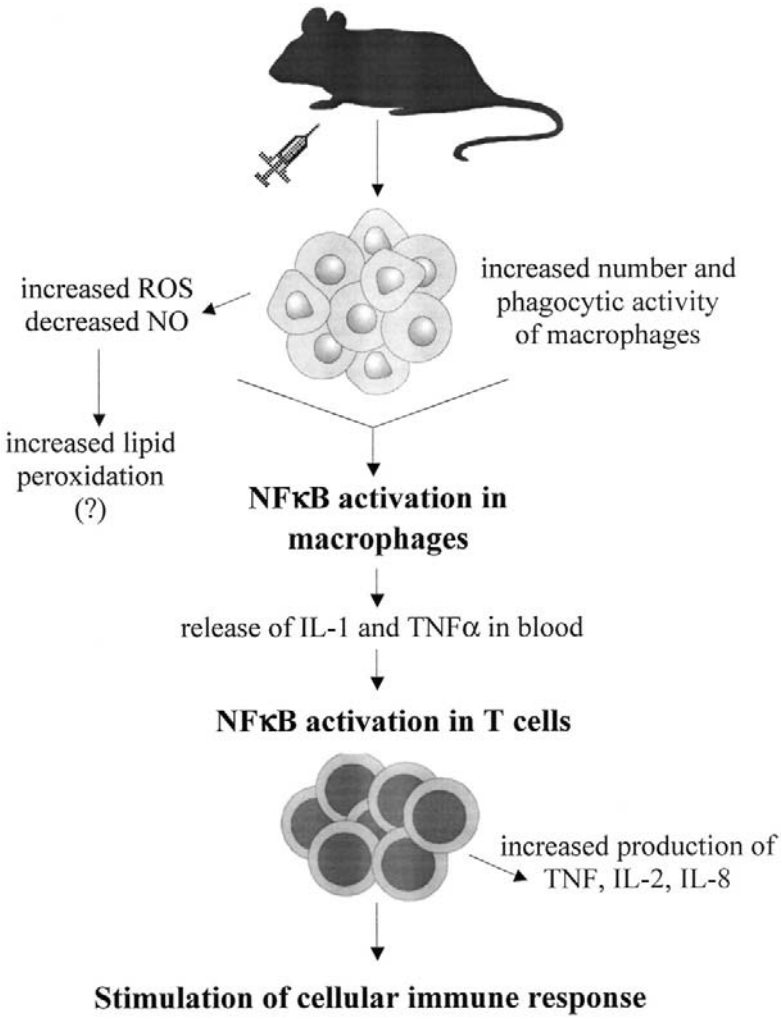

Fig. 3 Clinoptilolite-induced stimulation of cellular immune response. (From Ref. 106.)

macrophage phenotypes that activate proinflammatory $CD4^+$ Th1 T cells. These hypotheses can easily be tested with current immunological techniques and model systems.

A. Immunomodulatory Effect

Clinoptilolite has provoked the accumulation of macrophages in the peritoneum (106). The number of peritoneal macrophages after treatment was 7 times higher than in control mice. The concentration of O_2^- was 10 times higher in macrophages of treated mice than in controls. Since $O2^-$ release was calculated for 10^6 cells, the increased release was not the result of increased number of macrophages, but represents truly increased activity. Production of NO by peritoneal macrophages isolated from treated mice, and cultivated for another 24 hours ex vivo, was greatly decreased.

The cells of lymph nodes of mice fed 28 days with clinoptilolite (l g/day) provoked strong *graft-versus-host* reaction. Such treatment increased the concentration

of lipid-bound sialic acid in serum, but lipid peroxidation in the liver was decreased. Since it is a marker for inflammation, sialic acid may have a regulatory role in immunological processes. Results with clinoptilolite are probably associated with inflammatory processes, i.e., activation of macrophages. This is supported by elevated $O2^-$ in the peritoneal macrophages of clinoptilolite-fed mice. It is possible that factors activating and influencing the proliferation or increasing the synthetic capacity of the phagocyte system might cause a change in serum sialic acid. It is also possible that macrophages participate in this process indirectly by releasing tumor necrosis factor α (TNFα) and interleukin-l or is connected with an elevation of acute phase proteins.

Based on our recent results, we propose a mechanism of clinoptilolite in vivo action (59,106). Clinoptilolite caused local inflammation at the place of application that attracted peritoneal macrophages. Macrophages were activated, which has been shown by increased $O2^-$ production. We suggest that activated macrophages produced TNFα that, together with the other stimulants (e.g., other cytokines, reactive oxigene species (ROS), or changed intracellular calcium concentration), stimulated splenic T cells. Since products of the genes that are regulated by neurofibrillary κB (NFκB) also cause its activation, this type of positive regulatory loop may amplify and perpetuate a local inflammatory response. Our hypothesis is that clinoptilolite acts the same way after oral administration, affecting intestinal macrophages. Our results are in agreement with the accumulating evidence that zeolites could play an important role in regulation of the immune system as well as with the report that silica, silicates, and aluminosilicates act as nonspecific immunomodulators similarly to superantigens (59).

Surprising results were reported by Korkina et al. (60). They found that natural clinoptilolite exhibits a high hemolytic activity and cytotoxicity. The ability of macrophages to induce phagocytosis was decreased. Modification of the clinoptilolite surface by ammonia ions led to a decrease in its cytotoxic properties. Ethanol, mannitol, and sodium azide had no effect, whereas catalase reduced the ability of clinoptilolite to damage the membranes of macrophages and red cells.

B. Immunization

Silica and related substances, such as silicate, have been proven to possess "adjuvant" effects. Silicate as a superantigen in vitro induced polyclonal human T-cell activation. Aikoh et al. (61) observed activation-induced cell death in human lymphocytes after stimulation with chrysotile, a type of silicate. Activation-induced cell death occurred through Fas-Fas ligand interaction in lymphocytes after stimulation with silicate in a concentration with which no acute cytotoxicity has been detected.

Rabbits and mice injected with inactivated *Trypanosoma gambiense* vaccine adsorbed onto zeolite, which has strong adsorptive capacity due to its cationic exchange properties, were completely protected from a challenge inoculation of homologous viable parasites. The protective ability was remarkable at the first to second weeks after the last immunization and then slightly decreased although a high level of agglutination titer remained in immune serum. It is interesting that other inactive vaccines that were prepared with artificial zeolites showed little protective effect on mice (62,63).

XI. EFFECTS ON DIABETES MELLITUS

Zeolites are of potential use in the treatment of diabetes. Our unpublished data concerning alloxan-induced diabetic mice showed that natural clinoptilolite could prevent or diminish some late complications of diabetes, namely, development of polyneuropa-

thies. Although the natural, finely ground clinoptilolite did not significantly decrease the blood glucose levels in our animals, there were some indications that zeolite did in fact sorb a small amount of the glucose. The hydrothermal transformation of natural, purified clinoptilolite using $FeSO_4$ has been shown to cause selectivity for glucose adsorption (64).

The quantities of consumed water and excreted urine in diabetic mice are shown in Fig. 4 and 5. Alloxan-induced diabetic mice spent 24 h in metabolic cages during 6 days of clinoptilolite application. The measured volume of drinking water and excreted urine was decreased, and on day 6 these parameters were reduced by 50%.

Clinoptilolite showed positive effects on many diabetic symptoms. Some biochemical parameters in sera of the treated diabetic mice are given in Table 1. Significant differences between zeolite-treated and nontreated diabetic mice were noticed only in the amount of total Ca in sera. Nontreated diabetic animals had 1.92 mM/L Ca in sera, whereas clinoptilolite-treated diabetic mice had a higher concentration of Ca in sera, ranging from 2.15 to 2.3 mM/L. Iron (Fe_2^+)–containing, natural clinoptilolite interacts with glucose with formation of an iron–glucose complex in the clinoptilolite. The mechanism of action of the Fe_2^+–clinoptilolite–glucose interaction is a strong adsorption governed by the reactive characteristics of glucose (64,65).

It is well known that administration of silica prevents almost completely the onset of spontaneous diabetes in young BB rats (66). Administration of silica particles prevents β-cell destruction in nonobese mice given cyclophosphamide (67). Since silica is highly specific in its action against macrophages, this observation indicates an important role of these cells in the pathogenesis of the disease. An additional use of zeolites related to diabetes is the use of ultrastable zeolite Y for removal of toxic preservatives (i.e., phenol and *m*-cresol) in pharmaceutical preparations of insulin (68).

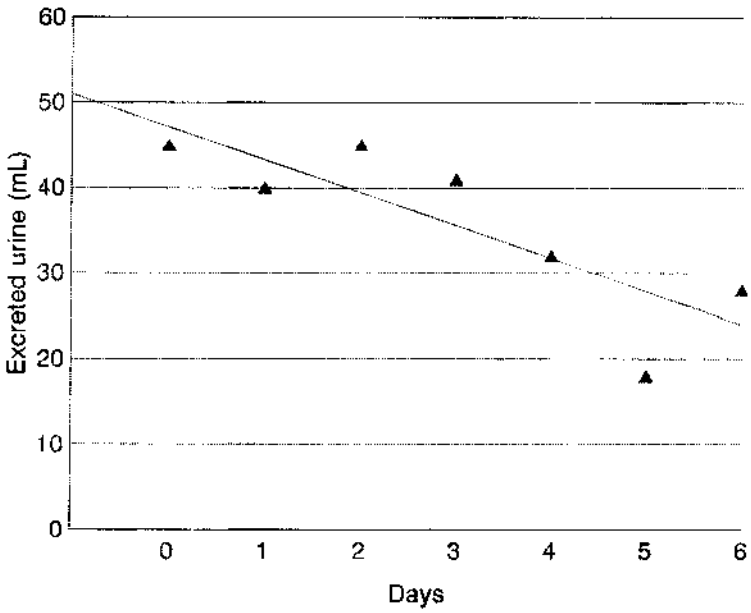

Fig. 4 Quantity of excreted urine of diabetic CBA mice through 24 h (over 6 days). (From unpublished data.)

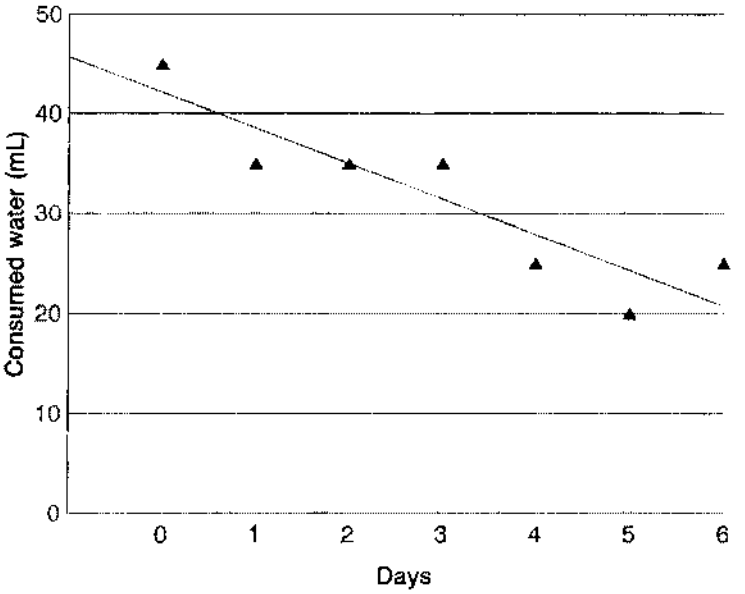

Fig. 5 Quantity of consumed water in diabetic CBA mice through 24 h (over 6 days). (From unpublished data.)

XII. CONTRAST MEDIUM FOR MAGNETIC RESONANCE IMAGING

Some zeolites show use as promising contrast media in diagnosis. Gadolite zeolite (Gd^{3+}-zeolite) is a promising contrast medium for enhancement of the gastrointestinal tract in MRI (69). The oral suspension gadolite shows excellent enhancement of the dog gastrointestinal system. No toxicity or absorption of gadolite was observed in dogs receiving doses up to four times the anticipated human dose daily for 14 consecutive days. Phase II and III multicenter clinical trials showed that gadolite oral suspension is a highly effective, safe, and well-tolerated contrast agent for clinical use as a gastrointestinal contrast medium for MRI. Certainty in MR diagnosis increased significantly in a double-blind study between pre- and postgadolite images (70). In clinical trials, gadolite

Table 1 Serum Clinical Chemistry Parameters in Diabetic Mice Treated with Clinoptilolite

Parameters	24 h	7 days	3 months	6 months
AF (U/L)	72.2 ± 1.8	125 ± 7.2	113 ± 11.6	46.6 ± 8.4
Glucose (mM/L)	18.8 ± 6.8	22.3 ± 4.2	19.6 ± 4.9	16.0 ± 2.2
AST (U/L)	70.6 ± 11.9	99.6 ± 24.7	90 ± 21.8	74 ± 23.9
ALT (U/L)	26.6 ± 2.7	29 ± 3.7	25 ± 3.2	35.9 ± 7.8
Ca (mM/L)	2.3 ± 0.3	2.15 ± 0.07	2.17 ± 0.06	2.24 ± 0.1

U, international units; AF, alkaline phosphatase; AST, aspartate aminotransferase; AL, alanin; aminotransferase; Ca, total calcium.
Source: Unpublished data.

significantly improved the efficacy scores for all groups and for all pulsing sequences. Efficacy scores and signal intensities generally increased with concentration and volume. No gadolinium was detected in blood or urine specimens, and no significant adverse events were reported.

XIII. EFFECTS ON CELL CULTURES

In vitro studies on different cell culture systems revealed interesting effects upon addition of clinoptilolite and other zeolites. Capiaumont et al (71) studied the effect of clinoptilolite on hybridoma cell cultures. One of the factors that limit the proliferation of eukaryotic cells in vitro is ammonia. It is believed that ammonia is toxic for mammalian cell proliferation and secretion. Passing culture media through a clinoptilolite removed ammonia and resulted in better cell growth, but did not display better specific antibody secretion. In vitro experiments involving contact of the silicate with cultured murine Ehrlich cells resulted in modifications in the surface chemistry of Al, Mg, and Fe from the silicates, and changes in cellular iron content (72).

Bauman et al. (73) reported that natural clinoptilolite increases the concentrations of sphingoid bases in the yeast *Yarrowia lipolytica*. High-performance liquid chromatography analysis of sphingoid bases obtained by acid hydrolysis of complex sphingolipids from *Y. lipolytica* showed that their concentrations markedly rose upon the addition of the zeolite. The largest increase among the identified molecular species of sphingoid bases was seen in C18 phytosphingosine, whose levels rose 6-fold and 22-fold after culturing cells for 24 and 36 h, respectively, in the presence of tribomechanically micronized clinoptilolite. Ion-exchange capacity probably was not responsible for the observed change in sphingolipid metabolism. It is interesting that zeolite affected cell size and shape. *Y. lipolytica* cells grown in the absence of zeolite were oval shaped with an average cell size of 0.7–2.7 μm, whereas when cultured with zeolite, they were round-shaped and larger, having an average cell size of 1.3–2.9 μm (71,73).

Zeolite NaY improved ethanol production from glucose in *Saccharomyces bayanus* and *S. cerevisiae* by enhancing invertase activity of yeast cells. The authors postulated that the zeolite acted as a pH regulator, permitting the fermentation of high glucose concentrations (74). There are some data about the effect of zeolites on neuronal cells. To study their cytotoxicity, clays (layered aluminum silicates) were added to cultures of primary murine spinal cord neurons and differentiated N1E-115 neuroblastoma cells. Both bentonite and montmorillonite clays caused complete cell lyses in neuronal cultures within 60 min of addition. Erionite zeolite, on the other hand, had no effect. None of the clays appeared to be cytotoxic to the differentiated cells, even though bentonite and montmorillonite were closely associated with the cell membrane (75).

XIV. BIOSENSORS

Biosensors—which fuse the exquisite sensitivity and specificity of living systems with the processing power of microelectronics—are simple, inexpensive measurement systems (76). Recently, significant activity has been centered on the development of different types of zeolites biosensors. One of these contains chemically modified electrodes. The emphasis has been on improving the selectivity of electroanalytical measurements. Compared with other electrode concepts, the distinctive feature of a chemically modified electrode is that generally a thin film of a selected chemical is bound to or coated on the electrode surface to endow the electrode with the chemical, optical, electrical, transport, and other desirable

properties on the film in a rational manner. Applications of such electrodes in research and chemical analysis are quite numerous (77).

A new biosensor for the amperometric detection of hydrogen peroxide was developed based on the coimmobilizatioin of horseradish peroxidase and methylene green on a zeolite-modified glassy carbon electrode, avoiding the commonly used bovine serum albumen glutaraldehyde (78). The large specific surface area of the zeolite matrix resulted in high enzyme and mediator loading. The detection and quantitative determination of hydrogen peroxide is of importance in many areas ranging from industry to the clinical laboratory. A sensitive hydrogen peroxide sensor can form a suitable basis for amperometric oxidase-based sensors for biologically important substances. Horseradish peroxidase can catalyze four kinds of reactions, i.e., peroxidation, oxidation, dismutation, and hydroxylation. Liu et al. (78) reported that, due to the hydrophilic character of zeolites, the soluble enzyme and methylene green can be retained in the zeolite film, greatly improving the stability of the sensor. The sensor exhibited high sensitivity and could be used for more than 2 months. In the past, montmorillonite and α-zirconium phosphate were used by the same research group to prevent the soluble mediator from leaching out of the enzyme electrodes (79,80). However, the pore sizes of these materials are too small to allow direct adsorption of enzymes. Some types of zeolites, such as modified type Y zeolite, which has both micropores and mesopores, and exhibits ion exchange properties, were used successfully as a coimmobilization matrix to incorporate enzyme and electron transfer mediator into a glassy carbon electrode surface.

As an enzyme immobilization matrix, a zeolite's major attribute is that it entraps the enzymes on its internal surface only by physical adsorption, without covalent linkages, which can partially deactivate the enzyme (78). Studies by Wang and Walcarius (81) showed that the incorporation of zeolite particles within glucose oxidase–containing carbon paste leads to improved amperometric biosensing of glucose. Kotte et al. (30) reported the use of zeolites for immobilizing positively charged mediators. Marko-Varga (82) brought to our attention the implication of modified zeolites for improving tyrosinase activity.

Generally, from the work of Liu (78) as well as others we can conclude that modified zeolites could be an attractive matrix for coimmobilizing peroxidase and methylene green (or perhaps other enzymes and bioactive molecules), and a reliable, low-cost, highly sensitive H_2O_2 sensor may be developed. As such, the zeolites are not only an effective support for enzyme immobilization but are also helpful for the improvement of sensor stability.

XV. EFFECT ON TUMOR GROWTH

Finely ground clinoptilolite could serve as a new adjuvant in anticancer therapy. Such treatment of mice and dogs suffering from a variety of tumor types led to improvement in the overall health status, a prolonged life span, and a decrease in tumor size. Local application of clinoptilolite to skin cancers of dogs effectively reduced tumor formation and growth. In vitro tissue culture studies showed that finely ground clinoptilolite inhibits protein kinase B (c-Akt), induces expression of $p21^{WAF1/CIP1}$ and $p37^{KIP1}$ tumor suppressor proteins, and blocks cell growth in several cancer cell lines. Since previous studies have indicated that exposure of cells to silicate particles leads to activation of MAP Kinase (MAPK), protein kinase C and stress-activated protein kinase/JNK (83), it was interesting to further analyze whether clinoptilolite treatment also affects mitogenic and survival signaling pathways in tumor cell models.

A. Cell Signaling and Apoptosis

The most significant results were detected measuring the activity of Akt protein in tumor cells in vitro. Akt, or protein kinase B, has been recently shown to mediate survival signals downstream of phosphoinositide-3 kinase by phosphorylating Bad (this is a protein family) proteins (84). An increase in Akt phosphorylation was observed in response to serum, epidermal growth factor (EGF), or insulin treatment (Fig. 6). The addition of a clinoptilolite-pretreated medium containing 10% fetal bovine serum (FBS) to the cells decreased Akt phosphorylation in comparison to the cells treated with only serum-containing media. The addition of growth factors EGF and platelet-derived growth factor (PDGF) restored cell activity. Determination of the activity of Akt at various times after the addition of clinoptilolite-pretreated medium with 10% FBS showed a slight decrease in pAkt level after 5 min. This decrease was more pronounced after 30 and 60 min of treatment. However, the addition of clinoptilolite pretreated medium without serum to the cells increased activity of Akt compared only to the serum-starved cells. Combined overnight treatment of the cells with EGF and clinoptilolite-pretreated medium decreased Act activity, indicating that inhibition of Akt might be linked to clinoptilolite inhibition of the EGF-triggered pathways. MAPK activity was increased temporarily in serum-starved cells treated with clinoptilolite. In contrast, addition of clinoptilolite-pretreated medium plus 10% serum slightly decreased MAPK activity compared to serum-treated cells or cells incubated only with clinoptilolite-pretreated medium. Media pretreated with clinoptilolite added to the cells either alone or in combination with serum caused no change in JNK activity.

Inhibition of cell growth was due to programmed cell death, i.e., apoptosis. DNA fragments isolated from zeolite-treated cervical carcinoma cells (HeLa) exhibited significant degradation in comparison to DNA from untreated cells.

B. Proliferation of Tumor Cell Lines in vitro

Studies performed in tissue culture in vitro indicate that natural clinoptilolite treatment affects proliferation and survival of several cancer cell lines of human origin (84). Addition of clinoptilolite inhibited cell proliferation in a concentration dependent manner, in part due to induction of inhibitors of cycline-dependent kinases, inhibition of B/Akt expression, and induction of programmed cell death (84). The growth of HeLa (cervical carcinoma), CaCo-2, HT-29, MCF-7, and SKBR-3 (mammary carcinomas) and mouse fibrosarcoma cells after 3 days of treatment was significantly inhibited with a dose of 50 mg/ml. The growth of normal fibroblasts was slightly stimulated. Similar results were observed measuring ^{3}H-thymidine incorporation assay in the presence of 10% fetal bovine serum in mouse fibrosarcoma cells (Fig. 7).

C. Tumor Growth in vivo

The range of effects on tumor growth in vivo are diverse, ranging from negative antitumor response, to normalization of biochemical parameters, prolongation of life span, and decrease in tumor size. The best results in animal models were observed in the treatment of skin cancer in dogs, suggesting that adsorption of some active components is responsible for clinoptilolite activity (direct contact action) (84).

Clinoptilolite, administered by gastric intubation to mice injected with melanoma cells, significantly reduced the number of melanoma metastases. In mice fed clinoptilolite for 28 days, the concentration of lipid-bound sialic acid in serum was increased, but lipid

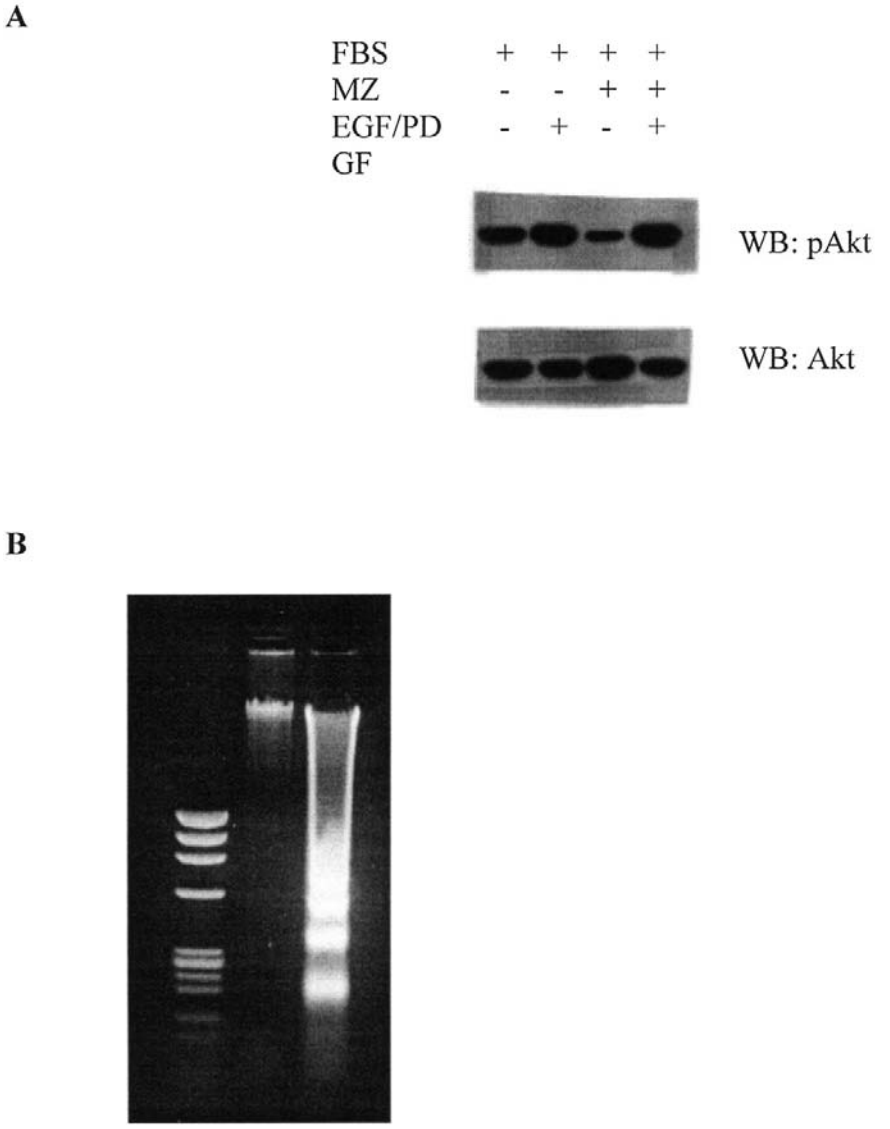

Fig. 6 (A) Activity of Akt protein 5 min after addition of the clinoptilolite-pretreated medium to murine fibrosarcoma cells. pAkt, phosphorylated Akt; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MZ, clinoptilolite. (B) Apoptotic DNA fragments in 1.5% agarose gel. Lane 1 DNA molecular weight marker IX; lane 2 DNA isolated from untreated HeLa cells; lane 3 DNA isolated from the clinoptilolite-treated HeLa cells; degraded, low molecular weight DNA fragments. (From Ref. 84.)

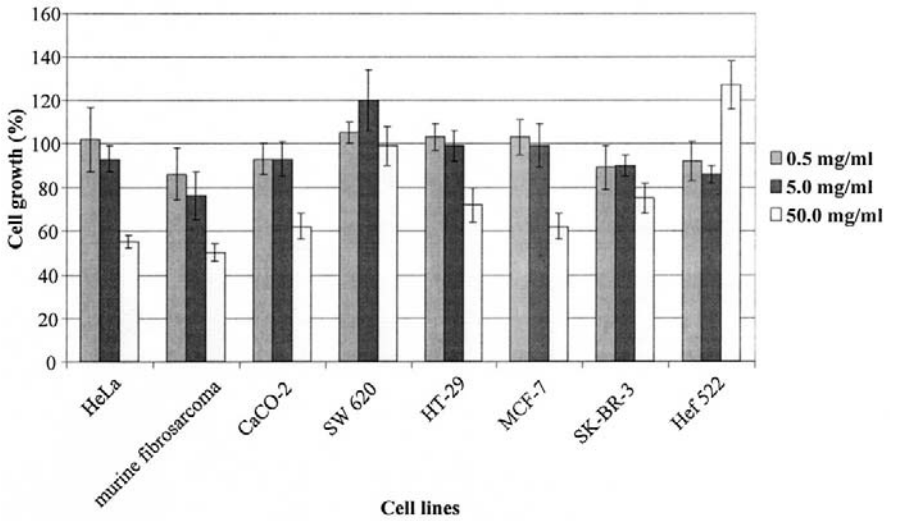

Fig. 7 Effect of the medium pretreated with 0.5, 5.0, and 50.0 mg/ml clinoptilolite on growth of various cell lines. (From Ref. 84.)

peroxidation in liver was decreased. The lymphocytes from lymph nodes of these mice provoked significantly higher "allogeneic" graft-versus-host reaction. After intraperitoneal application of clinoptilolite, the number of peritoneal macrophages, as well as their production of superoxide anion, was increased. However, nitric oxide generation was totally abolished. At the same time, translocation of p65 (NFκB subunit) to the nucleus of splenic cells was observed (59) (Fig. 8).

Subsequent studies were performed on murine transplantable tumors, melanoma B16, and three different types of mammary carcinomas (84). Tumor growth was significantly inhibited in animals suffering from anaplastic mammary carcinoma in groups

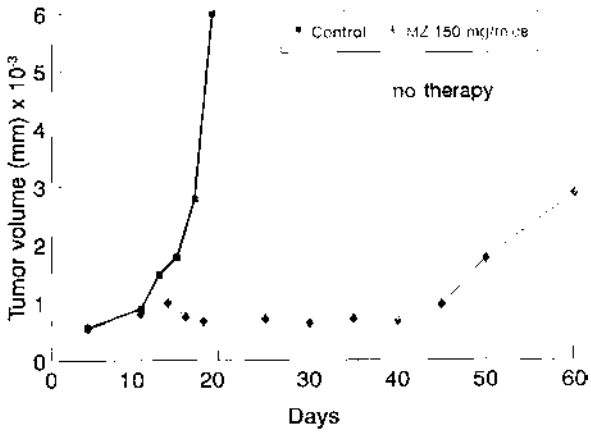

Fig. 8 Growth rate of melanoma B16 treated with 150 mg clinoptilolite/mouse per day. (From Ref. 84.)

of mice fed with food supplemented with clinoptilolite starting from 15 days prior to tumor transplantation until the animal's death, and animals fed with zeolite from the day of tumor transplantation until the animal's death. However there was no difference in mice survival among the control and zeolite-treated groups. There was also no effect of clinoptilolite on two mammary carcinomas that were histologically different from the previous.

Mice bearing melanoma B16 were fed clinoptilolite for 30 days, five times per day. Tumor volume was markedly lower in 5 of 80 mice. Despite the fact that the tumors started to grow rapidly after therapy with clinoptilolite was discontinued (between days 30 and 60 after tumor transplantation), the mice lived statistically much longer when treated with 200 and 150 mg clinoptilolite than did the control animals (84).

Interesting results have been obtained with dogs (84). Of 22 dogs suffering from various kinds of spontaneous tumors and treated with clinoptilolite, 14 responded to therapy, i.e., the tumor disappeared completely or the tumor size was significantly reduced. Three dogs had prostate tumors; one of these was studied via ultrasound and found to also have a prostate cyst. This dog was conspicuously quiet, without appetite, and lethargic prior to treatment. When conventional therapies did not work, clinoptilolite therapy was started. After just 2 days of treatment the dog became active; on the third day it began eating normally, and on the fourth day the dog urinated normally. On day 10 the cyst and the tumor were reduced in size, and after 1 month they had disappeared completely. Although the prostate became only insignificantly smaller, the dog showed no signs of illness. Furthermore, the very high pretherapy serum values for aspartate aminotransferase (497 μM/L) and alanine aminotransferase (433 μM/L) decreased after 1 month of clinoptilolite therapy to normal levels (16 and 43 μM/L), and remained in the normal range for the entire observation period (5 months).

In addition to the effect of clinoptilolite on the primary disease, all dogs, even those in which the primary disease was not cured, responded to zeolite therapy in a positive way. After about 7 days they displayed general constitutional and behavioral improvement that lasted even after therapy was discontinued. The same was observed for some of the hematological and serum clinical parameters measured before and after therapy. Hematocrit decreased to the normal range in one case, very high total serum bilirubin values fell to the normal range in two cases, and serum urea concentration changes were noted in another two cases. Elevated pretherapy values of aminotransferase, alanine aminotransferase, and alkaline leukocyte phosphatase all normalized after therapy was started in most cases (84) (Fig. 9).

Some other minerals could be of benefit for tumor protection. Long-term ingestion of hydroxyapatite (a diet containing hydroxyapatite at 2.5% or 5% for up to 7 months of treatment) could induce an apparent inhibition of the incidence of spontaneous mammary tumors in mice (85). This inhibition could be at least partially ascribed to its prevention of a decreased metabolic turnover as reflected by the higher excretion of urinary components in tumorous mice given hydroxyapatite. Hydroxyapatite contains abundant calcium and phosphorus, and has the characteristic of adsorbing various types of macromolecules. The removal of toxic substances from the body is one of the most efficient ways to protect against cancer.

There is some concern about the possibility of zeolite dissolution or degradation during use, which could lead to solubilization or particulate colloidal suspensions containing Si or Al, the major building blocks of zeolites. Patzer et al. (39) analyzed the final effluents from each ion-exchange run for the presence of Si and Al using atomic absorption spectroscopy. They detected neither within the limits of detection of the

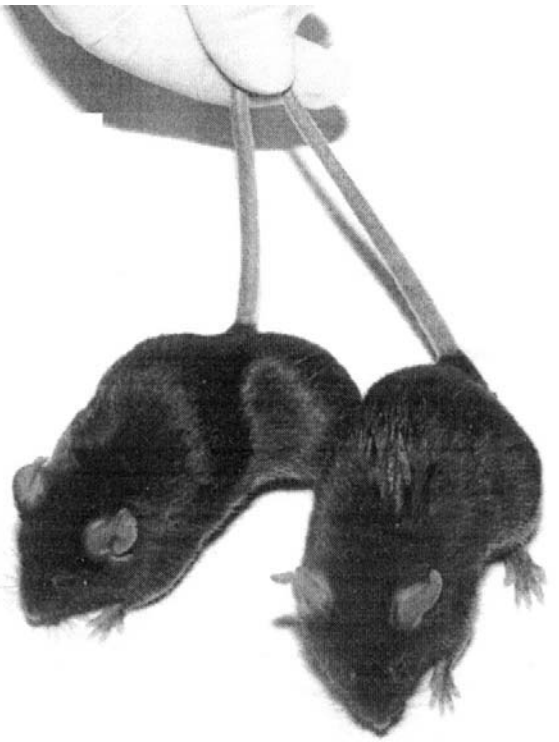

Fig. 9 Clinoptilolite-treated (right) and nontreated (left) C57BL/6 mice bearing melanoma B16BL6. (From unpublished data; for details, see Ref. 84.)

instrument (1 ppm). Although this is encouraging, additional monitoring of Si and Al in the effluent from a clinical scale column is warranted (28).

XVI. TOXICOLOGY OF CLINOPTILOLITE

In human medicine, zeolites have been used as antidiarrheal remedies (46), for the external treatment of skin wounds and athletes foot, and in kidney dialyses for the removal of ammonia ions from body fluids (3,86). There were not many data showing the systemic effects of zeolites on physiological systems of the body. The beneficial effects of zeolites on hematopoiesis (87), and various disease states, including tumors (84), have been observed. No toxic effects were observed in our toxicology study of clinoptilolite. The physical status of examined animals showed no evidence of any harmful reaction during the studies (Fig. 10).

Clinoptilolite is well suited for these applications because of its large pore space, high resistance to extreme temperatures, and chemically neutral framework. There are a few toxicology studies of clinoptilolite obtained from different locations. The conclusion from all of them is that natural clinoptilolite is not toxic and can be used in human as well as in veterinary medicine. Here we will describe the preclinical toxicology of clinoptilolite from Vranje, southern Serbia, by setting the "limit" test. This refers to administering high doses of clinoptilolite (2×200 and 2×500 mg/mouse per day orally by gavage) for 6, 14, and 30 days. Since the clinoptilolite did not cause death of mice in

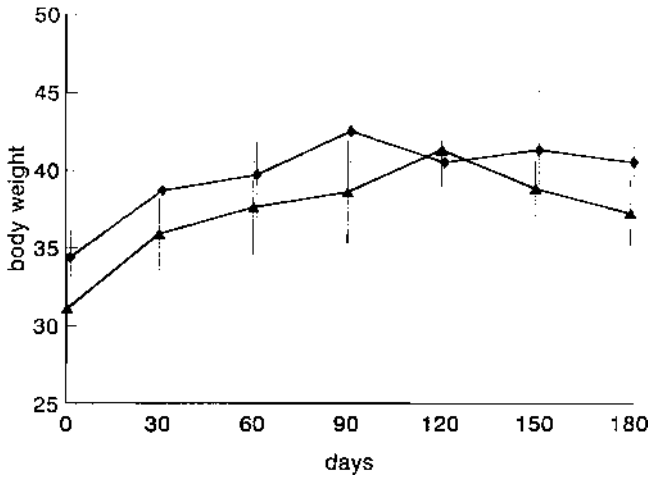

Fig. 10 The body weight gain of control (diamond) and clinoptilolite treated (triangle) male CBA mice fed with 25% of clinoptilolite during 6 months. (From unpublished data.)

this limit test, an "up-and-down" test on mice was performed, with daily doses ranging from 60 to 4000 mg/per mouse. Again, no toxicity was observed. Classical acute, subacute, and chronic toxicity studies on mice and rats of both sexes (separately) were performed in our lab. The duration of the study was as follows: acute, 1 month; subacute, up to 3 months; chronic toxicity, up to 6 months. Animals were monitored for phenotypic changes, changes in behavior and survival, changes in body weight, amount of food and water consumed, changes in hematological and serum clinical chemistry parameters (erythrocytes, leukocytes, platelets, hematocrit, hemoglobin, glucose, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, bilirubin, inorganic phosphorus, and calcium), and urine clinical chemistry parameters (glucose, proteins, urobilinogen, bilirubin, nitrites, erythrocytes, leukocytes, pH, and specific gravity). Pathohistological analysis of liver, spleen, kidney, brain, lung, testes, ovary, duodenum, eye, stomach, large and small intestine, muscles, myocardium, pancreas, thymus, and axillary lymph nodes was carried out on sacrificed experimental and control mice.

Local tolerance as well as repeated-dose dermal tolerance testing was performed on mice and rats to ascertain whether the zeolite is tolerated at the sites that may come into contact with the product as a result of its administration (58).

The results of all of these studies were that oral (in diet) administration of clinoptilolite to mice and rats for 6 and 12 months, respectively, caused no change that could be considered a toxic effect of treatment. The clinoptilolite equalized (regulated) and shortened the pregnancy period. The number of pups per litter was increased in clinoptilolite-treated mice. This is likely the reason for decreased body weight of pups. Higher mortality of pups between days 8 and 21 of the neonatal period was observed. However, there were no differences between control and treated animals that would suggest reproductive toxicity attributable to the clinoptilolite during the period of organogenesis.

The clinoptilolite was neither toxic nor allergenic to skin (84). Martin-Kleiner et al. (87) compared the effects of two preparations of clinoptilolite differing in particle size on serum chemistry and hematopoiesis in mice. One preparation was a powder obtained from tribomechanical treatment (MTCp) of the clinoptilolite and another was normally ground

clinoptilolite (NGCp). Young adult mice were supplied with food containing 12.5%, 25%, or 50% clinoptilolite powder. Control animals received the same food, ad libitum, without the clinoptilolite. Clinoptilolite ingestion was well tolerated, as judged by comparable body masses of treated and control animals. A 20% increase of the potassium level was detected in mice receiving the zeolite-rich diet, without other changes in serum chemistry. Erythrocyte, hemoglobin, and platelet levels in peripheral blood were not significantly affected. NGCp caused leukocytosis, with concomitant decline of the granulocyte-macrophage colony-forming unit (GM-CFU) content in the bone marrow, which was attributed to intestinal irritation by rough zeolite particles. The MTCp preparation caused similar, albeit less pronounced, changes. In a limited experiment, mice having transplanted mammary carcinoma in the terminal stage showed increased potassium and decreased sodium and chloride levels, severe anemia and leukocytosis, decreased bone marrow cellularity, and diminished content of hematopoietic progenitor cells in the bone marrow. The clinoptilolite preparation ameliorated the sodium and chloride decline, whereas the effects of hematopoiesis were erratic (87).

In experiments on healthy CBA/H Zgr mice of both sexes older than 6 months, various parameters were controlled that were anticipated to cause eventual clinoptilolite toxicity, which was added daily to the food or by gavage. The effect of the clinoptilolite was studied as a function of body weight gain (Fig. 10). Body weight gain was slightly elevated for both treated and control animal groups, and differences were not statistically significant.

According to research protocol, every 14 days the mice spent 24 h in metabolic cages. During this time, the quantities of food and water consumed and urine and feces excreted were measured. The results showed that, on average, nontreated (control) mice ate 3.3 g of food, drank 3.8 ml of water, excreted 1.7 g of feces, and urinated 1.3 ml of urine. Over the course of 6 months, there were no differences between clinoptilolite-treated (per os) and untreated mice.

A. Changes in Hematological Parameters

During the toxicity study, the animals were monitored for hematological parameters. The number of erythrocytes in mice treated and not treated with zeolite for 6 months was not different. Also, leukocyte counts in the mice were not significantly different. Lymphocyte and leukocyte mononuclear cells made up about 90% of the white cell population in mouse peripheral blood. The platelet count also was not different in control and in clinoptilolite-treated mice (Table 2) in spite of intensive megakariocytogenesis in the spleen. Hemoglobin concentrations in both groups of mice were slightly lower than published values, but the two groups were not statistically different (Table 2).

Table 2 Hematology Parameters in Blood Sample

Group of mice	ERC(10^{12}/L)	L (10^9/L)	Hgb (g/L)	TRC (10^9/L)
Control	5.2 ± 0.3	4.7 ± 0.3	102.3 ± 11.3	327 ± 29
30th day	5.6 ± 0.6	4.1 ± 0.9	88.7 ± 4.5	375 ± 56
90th day	5.2 ± 0.6	3.8 ± 0.8	87.7 ± 2.4	376 ± 46
180th day	5.3 ± 0.7	5.9 ± 0.1	87.8 ± 13.1	316 ± 50

ERC, erythrocyte; L, leukocytes; Hgb, hemoglobin; TRC, thrombocytes.
Source: M. Hadžija, S. Križanac, toxicology study, Zagreb, 1999, unpublished data.

In general, clinoptilolite particles have been found to cause less irritation and tissue damage than the rod- and fiber-shaped particles of other natural zeolites such as erionite or mordenite, which resemble the asbestos fibers in morphology.

B. Serum Clinical Chemistry Parameters

Mice were killed at various times during the 6-month toxicological study. The serum obtained was analyzed for a number of biochemical parameters that would indicate the degree of damage to vital organs and metabolic function. The parameters were alkaline phosphatase (AF), glucose, aspartate aminotransferase (AST), alanine aminotransferase (AL), and total calcium (Ca). The results presented in Table 3 indicate that there were no changes in these parameters during the 6 months. The value of AST was somewhat raised during days 1 and 7 of the experiment, which could be explained by adjustment of the mice to the new type of diet (maybe less food was consumed during the first month). But after 6 months there were no changes (Table 3). Urine analysis did not show any changes in glucose, bilirubin, ketonic bodies, erythrocytes, urobilinogen, nitrites, or leukocytes.

C. Interaction of Clinoptilolite with Small Intestine

Clinoptilolite is resistant to degradation by gastric and intestinal juices, and its major constitutive elements are not absorbed from the gut into circulation significantly (M. Čolić, personal communication). Overnight incubation of clinoptilolite in acidic or weakly alkaline media at 37°C resulted in minimal amounts of soluble silicon (50–60 mg/L) (B. Subotić, personal communication). No traces of silicon have been detected in the serum of Wistar rats or CBA mice (I. Hršak, personal communications) receiving clinoptilolite in food. However, zeolite particles were found in the first and second layers of duodenal cells (Fig. 11). Cefali et al. (88) found elevated levels of silicon and aluminum in the plasma of experimental dogs ingesting synthetic zeolite A as a single dose. Likewise, Roland et al. (89) showed increased excretion of Si and Al in hens receiving zeolite A by intubation. It should be noted that zeolite A is soluble, particularly in acidic media (90).

Results of clinoptilolite added to food have been contradictory. The ion-exchange behavior of zeolites in the gut is complicated and a matter of growing interest. Many people want to see ion exchange in the gastrointestinal tract, but this capacity has been limited. Martin-Kleiner et al. (87) found that the urea concentration in serum was surprisingly high in mice even when fed zeolites, whereas the concentration of creatinine in all experimental animals was normal. A provisional explanation was that the food was contaminated with urine because mice ate food crumbs from the bedding. In additional

Table 3 Serum Clinical Chemistry Parameters in Mice Treated with Clinoptilolite

Parameters	24 h	7 days	3 months	6 months
AF (U/L)	92.4 ± 8.5	108.3 ± 11.4	41.2 ± 3.3	42.8 ± 0.60
Glucose (mM/L)	5.8 ± 0.8	5.3 ± 0.6	6.6 ± 0.9	6.0 ± 1.8
AST (U/L)	70.8 ± 4.3	76.2 ± 12.6	132.0 ± 41.9	70.8 ± 13.6
ALT (U/L)	28.6 ± 2.3	25.4 ± 3.1	59.0 ± 38.7	40.4 ± 4.3
Ca (mM/L)	2.14 ± 0.09	2.51 ± 0.12	2.38 ± 0.04	2.2 ± 0.2

AF, alkaline phosphatase; AST, aspartate aminotransferase; AL, alanine aminotransferase; Ca, total calcium.
Source: M. Hadžija, S. Križanac, toxicology study, Zagreb 1999, unpublished data.

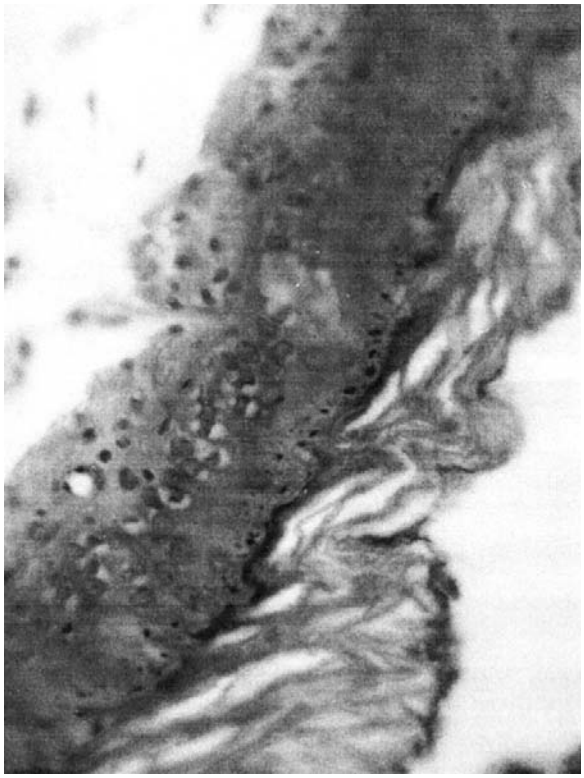

Fig. 11 Photomicrograph of duodenum. (From unpublished data.)

experiments, urea in serum was equally high in all four groups of mice, whether supplied with zeolite or not. For that reason, any possible effects of zeolite on ammonia (NH_3) uptake and turnover, attributable to ion-exchange phenomena in the gastrointestinal tract, were obscured. Cattle are fed urea, but in food containing 50% clinoptilolite the ammonium concentration in the rumen and in the portal vein was reduced (91).

Carbohydrates, starch, disaccharides, lactose, and glycogen are a major source of energy in the body. Digestion of carbohydrate begins in the mouth by α-amylase from salivary glands. Acidity of the stomach inactivates α-amylase. Digestion of carbohydrate is continued in the small intestine by pancreatic α-amylase. The products of luminal hydrolysis are substrate (disaccharide) for enzymes from the brush border of enterocytes. α-Glucosidase is one of four disaccharidases. This complex of enzymes is involved in degradation of oligosaccharides from the digestive tract and in one step of glycogen degradation in the liver. Our results showed (data not published) that clinoptilolite had no influence on the catabolic concentration of α-glucosidase on the brush-border enterocytes, but neutral α-glucosidase was significantly reduced in the liver during the first 24 h of treatment.

D. Zeolite and Reproduction

Clinoptilolite was given to the mice as a powder mixed with standard food at the ratio of 25% clinoptilolite. The treatment was continued during the prepregnancy and

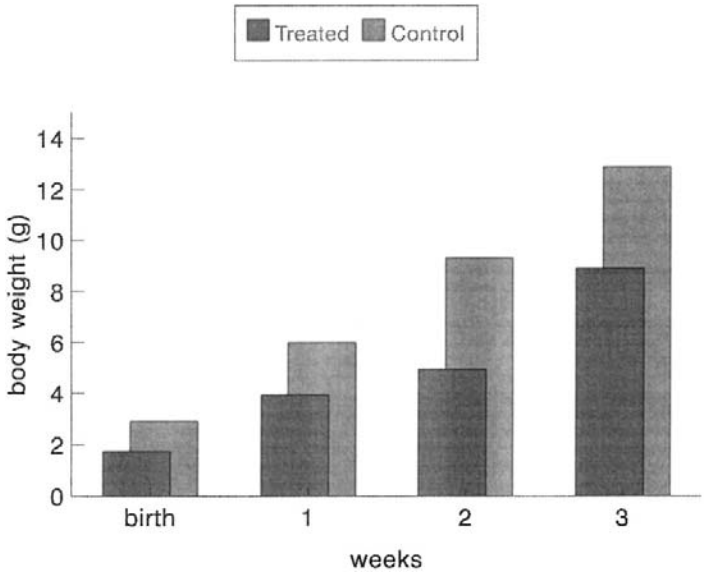

Fig. 12 The average of born body weight and a gain in body weight of control and clinoptilolite-treated mice. (From M. Slijepčević, A reproductive/development toxicity study, Zagreb 1998, unpublished data).

pregnancy periods, and during the lactation period. Special consideration was given to the teratological influence of zeolite on organogenesis (from 6 to 16 days of pregnancy). The prepregnancy period in mice treated with clinoptilolite was shorter than in control (nontreated) mice. The number of pups per litter (both before and after birth) was increased in clinoptilolite-treated mice. In addition, the average body weight of pups was lower (on average 100 mg per pup). In conclusion, the clinoptilolite equalized (regulated) and shortened the prepregnancy period. The number of pups per litter was increased in clinoptilolite-treated mice. Decreased birth weight may have been a consequence of increased number of pups per litter (Fig. 12). Regarding other parameters, including teratogenesis, clinoptilolite-treated mice were not different from control mice and did not show negative results, which suggests no attributable toxicity of clinoptilolite on reproduction (Table 4).

Table 4 Duration of Prepregnancy and Pregnancy Period (days)

	Prepregnancy and pregnancy period (cycles)			
Groups	1st	2nd	3rd	4th
Treated[a]	24.0 ± 3.8	24.3 ± 1.0	25.5 ± 1.9	26.4 ± 1.8
Control	56.8 ± 8.1	32.5 ± 11.2	43.2 ± 4.8	21.2 ± 0.4

[a] Mice fed with the food supplemented with 25% of the clinoptilolite.
Source: M. Slijepčević, reproductive development toxicity study, Zagreb 1998, unpublished data.

XVII. DRUG CARRIERS AND DELIVERY SYSTEMS

Techniques developed to introduce active sites such as metals into zeolite channels and cavities have resulted in "ship-in-a-bottle" complexes that allow for unique chemistries (90,92,93). One of potentially many exciting pharmacological applications of such zeolites and mesoporous silicates could be the encapsulation of different ions and molecules with delayed-release properties. Many proteins (enzymes) can indeed be encapsulated, and the enzymes when released often show almost full activity. The following subsections shall describe the potential applications in more detail. M. Čolić (personal communication) recently used clinoptilolite with zinc and silver inside the pores and salicylic acid adsorbed on the zeolite surface for acne and scar treatment. Zeolites are also used as support matrices for enzymes and antibodies (90,94). Glucose oxidase has been fixed onto zeolite 4A and zeolite X. Coenzymes were stabilized on 4A. Thermal stability of trypsin adsorbed onto zeolite 4A is highly enhanced.

A. Surfactant-Modified Zeolites as a Drug Carrier

Adsolubilization of drugs by zeolite–surfactant complexes may lead to potential new uses, such as new drug delivery systems and controlled-release agricultural chemicals. The enhanced coadsorption of organic molecules on solid–surfactant complexes is ascribed to the partition of organic solutes between the aqueous bulk phase and admicelles on the solid surface. This has been termed surface solubilization or adsolubilization (95). Zeolite–surfactant complexes are capable of incorporating hydrophobic compounds, such as surfactant micelles, to solubilize water-insoluble compounds in the hydrophobic core. Hayakawa et al. (96) described the efficient adsolubilization of the drug chloroquin by zeolite–surfactant complexes. The zeolites used were P-type zeolite with a sodium counterion and X-type zeolites with sodium or calcium counterions. The cationic surfactants used were hexadecyl-, tetradecyl-, and dodecyltrimethylammonium bromide. Zeolite–surfactant complexes incorporated more chloroquin than zeolite alone, and the elution rate depended on the length of the surfactant chain and the ionic strength of the eluate.

B. Microcapsulation and Slow Release

The use of microcapsules containing a urease-zeolite preparation may be a potential route to urea removal. The use of zeolite ion exchangers, and zeolite W in particular, can alleviate the problems encountered with zirconium phosphate, including a negative calcium balance. Unlike zirconium phosphate, zeolite W is nonselective toward calcium ions and is stable at the high pH found in the intestinal tract. The application of enzyme envelopes to zeolite particles is an immobilization procedure that does not involve the use of colloidal silica and can reduce the amount of ingested material by as much as 25%. Cattaneo and Chang (97) showed that cellulose acetate butyrate microcapsules, containing a urease-zeolite preparation, remove up to 80% of urea in less than 1 h (97).

Commercial zeolite Y acts as a slow-release carrier for a number of antihelmintic drugs. Administration to rats, dosed with *Nipostrongylus brasiliensis*, of pyrantel and/or fenbendasole and to pigs, dosed with *Ascaris* and *Oesophagostomum*, of dichlorvos loaded onto zeolite Y was more successful in killing adult worms than administration of the pure drug alone. The results indicate that zeolite Y is a suitable vehicle for the slow release of some antihelmintics. Slow release of drug from the zeolite matrix also improved the drug's efficiency (98).

C. Zeolites as Models for Biomimetic Oxidation Catalysts

Zeolite-encapsulated metal complexes (including so-called ship-in-a-bottle complexes) may be a method of rationally designing inorganic enzymatic catalysts. Zeolites resemble enzyme structures in that the channels and cages created by the aluminosilicate framework have internal voids on the order of molecular dimensions, whereas those created by the tertiary protein structure of an enzyme have a molecular-sized substrate binding site. This has led to the development of remarkable zeolite mimics of enzyme functions. Within a cage, a molecule may be activated by oxide ions of the framework, associated protons, or other exchangeable cations. By taking advantage of the structural similarities, one can develop some exciting new catalysts, which combine the attractive features of the robust, chemically inert zeolite with the tremendous selectivity and activity of enzymes (99). Zeolites can therefore be viewed as sterically demanding supports for active sites, not only as acid catalysts or ion exchangers.

Some researchers have shown that the structure of the encapsulated complex is almost identical with the free complex. However, encapsulation does modify properties such as vibrational and electronic spectral behavior (100).

Since several papers have been published on biomimetic oxidation using clay-intercalated and zeolite-encapsulated catalysts, the idea of building into a mineral host the ability to mimic certain biological processes is realistic. Zeolite-encapsulated complexes have been used as an oxygen carrier, mimicking hemoglobin, cytochrome P450, and iron-sulfur proteins (110). The use of Y-zeolite-encapsulated Co^{II}salen has shown that the entrapped complex binds oxygen reversibly and can separate oxygen from nitrogen in dry air. The zeolite framework prevents complexes from dimerizing and degrading. The zeolite complexes are more efficient reversible oxygen carriers than the homogeneous dissolved complexes that degrade in solution.

One of the important properties of zeolite-encapsulated molecules is their size and shape selectivities. They have shown a clear preference for oxidation of the smaller of two substrates (101). Such selectivity presumably arises from the molecular sieving action of the zeolite support. From a review published by Bedioui (101) about zeolite encapsulation, a number of generalizations can be made: (a) turnover numbers increase when the catalyst is encapsulated; (b) turnover numbers decrease with increased loading of the zeolite with complex; (c) the dimensions and shape of the environment of the active sites control shape and regioselectivity, as well as reactant diffusion; and (d) high yields of oxygenated products in biomimetic oxidations are most often obtained with pure and highly diluted entrapped complexes (101).

Corma et al. (102) have reported that the bulky 2,4,6-triphenylpyrylium ion (TPP) can be prepared inside zeolite Y supercages through a ship-in-a-bottle synthesis that relies on the diffusion of much smaller synthetic precursors. After encapsulation, TPP remains mechanically immobilized inside the zeolite Y supercages, but it still can interact with smaller molecules through the cavity windows.

Encapsulation of pyrilium ions inside zeolite Y and the resulting stability in aqueous solutions has enabled Domenech et al. (103) to test this material as an electrocatalyst for the oxidation of dopamine and norepinephrine. These compounds play an essential role in neurochemistry as neurotransmitter catecholamines. Since the loss of neurotransmitter-containing neurons may result in serious diseases such as parkinsonism, the determination of such compounds in real biological systems is an obvious target in neurochemical studies.

2,4,6-Triphenylpyrylium ions entrapped in supercages of Y zeolite exert a remarkable catalytic effect toward the electrochemical oxidation of dopamine and norepinephrine

(neurotransmitter catecholamines) in neutral aqueous media. This novel observation is an example of the new opportunities that stabilization of organic species by encapsulation inside the rigid framework of microporous materials can offer development of new electrochemical sensors with high selectivity and increased sensitivity.

D. Inorganic Layered Double Hydroxides as Nonviral Vectors

Other inorganic supramolecules, such as layered double hydroxides (LDHs), can act as biomolecular reservoirs and gene and drug carriers. LDHs are so-called anionic clays, consisting of cationic brucite-like layers and exchangeable interlayer anions, that could be used as inorganic matrices for encapsulating functional biomolecules. The biomolecules can be incorporated between hydroxide layers by a simple ion-exchange reaction to form bio-LDH nanohybrids. The hydroxide layers can serve as a reservoir to protect intercalated DNA from DNase degradation, and the charge neutralization enhances transfer of the DNA-LDH hybrid into mammalian cells through endocytotic means (104). Once within the cells, slightly acidic lyzosomes dissolve the LDH and release the intercalated molecule. This inorganic-biomolecular hybrid system can deliver drugs or genes from the noncytotoxic carrier. Choy et al. (104) demonstrated that antisense oligonucleotides enter into cells through this mechanism and participate in the process of cell division. Antisense Myc were intercalated into LDH and successfully transferred into the promyelocytic leukemia cell line HL-60. Myc-LDH hybrid strongly inhibited cell proliferation in comparison with antisense Myc only. LDH itself is noncytotoxic and can act as a new inorganic carrier, completely different from existing nonviral vectors in terms of their chemical nature (104).

To conclude, more studies on additional other biomedical aspects of zeolites are certain to be performed in the future. The most urgent needs, as far as biomedical applications of microporous and mesoporous materials are concerned, are (a) cost-effective processing of clinoptilolite with small particle size (2 μm or less) and (b) synthesis of crystalline mesoporous silicate and aluminosilicate materials of small particle size (submicrometer). These challenges will hopefully be met by the members of the materials science community.

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