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Role of Water in Some Biophysical Properties of Skeletal Tissues

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In the course of our previous studies on the control system that governs bone growth in response to mechanical stress, we reached the conclusion that bone is a self-organizing system, which is based on certain solid-state properties of the matrix (Becker *et al.*, 1964; Becker and Brown, 1965). Specifically, the collagen fiber-apatite crystal structural relationship appears to form a stress-sensitive PN junction diode, which is capable of transducing mechanical stress into an electric control signal, which in turn stimulates and directs subsequent bone growth (Fig. 1). The system is a single closed-loop, negative feedback type with mechanical stress as the environmental input signal. The apatite-collagen junction transduces this to an electric current of amplitude that is proportional to the amount of deformation, with the polarity dependent on the direction of deformation. This current has been demonstrated to produce migration and parallel alignment of tropocollagen as well as osteoblastic response in the negative polarity area. This orientation of growth and fiber deposition is such that new bone is produced in the area of maximum stress.

Most of this work was done on human bone specimens, and we quickly noted that the reproducibility of the results depended on all the bone samples being in the same state of hydration. For example, phenomena such as photoconductivity were markedly altered if the samples were immersed for even a short time in Ringer's solution, normal saline, or distilled water. Certainly, according to the concept of the internal milieu (*milieu intérieur*), brief immersion in mammalian Ringer's solution should have little effect on

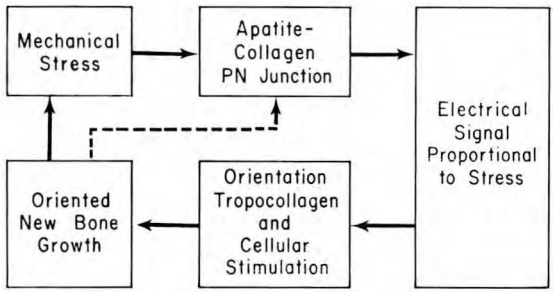

Fig. 1. Present concept of the control system that governs growth response of bone to mechanical stress.

these properties. Nevertheless, under these circumstances, we noted that these specimens demonstrated a surface type of photoconductivity, in contrast to the bulk photoconductivity that was demonstrated by untreated samples.

A review of the literature on the relationship between collagenous structures and water indicated that, except for the work of Berendsen, who utilized nuclear magnetic resonance (Berendsen and Migchelsen, 1965), this problem had not been studied by the use of modern physical techniques. Although the concept of free- and bound-, or structured, water compartments is well accepted, only indirect proof for their existence is available, and their quantitative relationship has not been determined (Szent-Györgyi, 1957).

Since we are concerned with electronic properties that are associated with each of the major components of the matrix (collagen and apatite), as well as with the additional structure that results from their precise molecular association with each other, we reasoned that the effect of water and various ions in solution is probably by way of the bound-water compartment in close association with the matrix components. We have applied two new techniques to the study of water that is associated with organized tissue: dielectric-constant measurements for quantitative determination of the various water compartments, and electron paramagnetic resonance to investigate possible structural relationships between the water molecules and the substrate.

Dielectric-Constant Measurements of Bone

Theoretically, if a sharp distinction exists physically between the free- and the bound-water compartments, one should be able to detect this by measurements of the dielectric constant of bone sam-

ples over a range of hydrations. The high dielectric constant of ordinary bulk water results in large part from the ability of the permanent dipole moment of the individual water molecules to follow the impressed electric field at radio frequencies. When water molecules are bound, their ability to make this orientational contribution is reduced, because of the retarding influence of the substrate. Thus, on an equal weight basis, it is expected that bound water will make a smaller contribution to the dielectric constant than will free water.

Uniform specimens of adult human cortical bone were vacuum dried and then were equilibrated to constant weight in a successive series of controlled humidities. The gain in weight or hydration was measured as milligrams of water per gram of dried whole bone. Dielectric constants were determined at three different frequencies at each humidity value. The results indicated that, at any one frequency, the curve of the dielectric constant versus hydration could be reconstructed as two straight lines with differing slopes (Fig. 2). The break in the curve was interpreted as indicating the transition

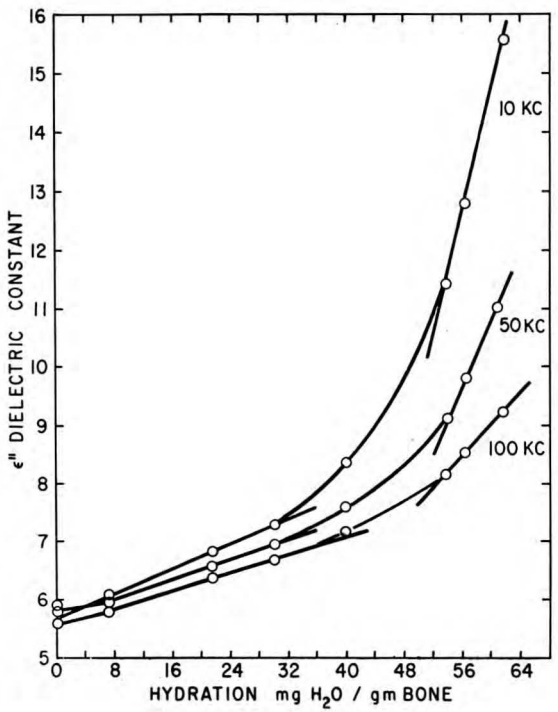

Fig. 2. Dielectric constant versus hydration of whole human cortical bone.

between the completely filled bound-water compartment and the beginning of entry of water molecules into the free compartment.

Measurements at the various frequencies produced a family of curves, with the break in the slope occurring over a rather narrow range of water contents, 37 to 48 milligrams of water per gram of bone. We interpret these results to substantiate the existence of two separate water compartments in bone, with the bound-water compartment averaging 57 percent of the maximum total water content of bone equilibrated at 82 percent relative humidity.

Electron Paramagnetic Resonances in Bone

We reported the presence of electron paramagnetic resonances in whole human cortical bone as well as in separated bone collagen and apatite (Becker and Marino, 1966). However, certain anoma lies became apparent at an early stage in that study, which we ascribed to the use of the aqueous solutions in the chemical separation of the two components of the matrix. The spectrum obtained for apatite, as well as for collagen, differed strongly from that of the original whole bone (Fig. 3). The apatite was prepared by refluxing it with 90 percent ethylenediamine for 72 hours and subsequently washing it in tap water and distilled water in order to remove the solvent. Collagen was prepared by demineralization of whole human cortical bone in 5 percent formic acid, after which a

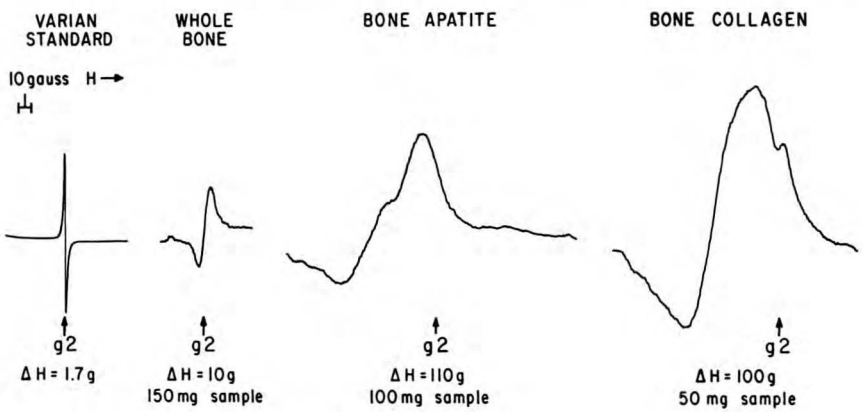

Fig. 3. Electron paramagnetic resonance spectra of whole bone, bone collagen, and bone apatite. The line width of the resonance in gauss measured between points of half-maximum absorption is indicated by ΔH.

similar washing procedure was used. The spectra shown in Fig. 3 were taken after the materials had evaporated to dryness at room temperature and humidity (21°C, 40 percent).

The signal from whole bone is a simple symmetric singlet at g2.001, but that of apatite is a complex asymmetric signal, which is composed of several separate resonances. Bone collagen also exhibits a complex signal. In both instances, vacuum drying at room temperature produced a slight enhancement of the resonance with no decrease in the complexity but with a better separation of the component resonances. It appeared to be desirable to determine, if possible, the resonance associated with the apatite and the collagen in their native unaltered state.

Our method of preparing apatite involved refluxing whole-bone chips with ethylenediamine until all of the organic matrix had been broken down and extracted. Usually, the remaining material was subsequently washed, first in tap water and then in distilled water, to remove the residual ethylenediamine. It appeared to be quite possible that the apatite, released from its bonding to the collagen fibers, structured some inorganic ions that were available in the washing water. We, therefore, determined the spectrum of apatite that had been removed from the refluxing apparatus and dried without washing. A simple singlet at g2.001 with a line width of 10 gauss was detected (Fig. 4).

Dried ethylenediamine was found to have no detectable electron paramagnetic resonance signal; however, the possibility that the line is caused by a residual organic content must be considered. A distorted and broadened signal, although still centered at g2, was produced when the extracted apatite was washed in mammalian Ringer's solution. Washing with distilled water produced a complex, multiresonance signal, which was very similar to the signal from gamma-irradiated ice described by Siegel *et al.* (1960). The signal that came from the irradiated ice was attributed to the formation of some form of electronically unbalanced water (the authors suggested HO_2^- but H_3O^+ cannot, of course, be excluded).

Ability of Bone Apatite to Structure Ions

The known presence of the aforementioned ions in distilled water renders it possible that the apatite is capable of structuring such

units, with the subsequent formation of the observed signal. Interestingly, if the distilled water in which apatite has been washed is returned to the refluxing apparatus and reprocessed for 24 hours, it acquires a blue color, not unlike that displayed by the blue geologic apatites. The electron paramagnetic resonance signal from this material is basically unchanged from the signal that is received before the second refluxing. Regardless of the aqueous solution utilized for washing the extracted apatite, the observed signals did not diminish in intensity or alter in line width or complexity after the apatite was vacuum dried at 10 microns for several months (or after dehydration in two changes of dioxane for 24 hours).

It appears, therefore, that apatite is capable of structuring a variety of inorganic ions from solution in such a fashion that an electron paramagnetic resonance signal is produced, and extraction under moderately severe physical conditions is resisted. The marked difference between the resonances obtained from whole bone and unwashed, extracted apatite, on the one hand, and those observed from extracted apatite that was washed in mammalian Ringer's solution, on the other, is interesting. If the concept that apatite is deposited from the extracellular fluid is correct, one would expect

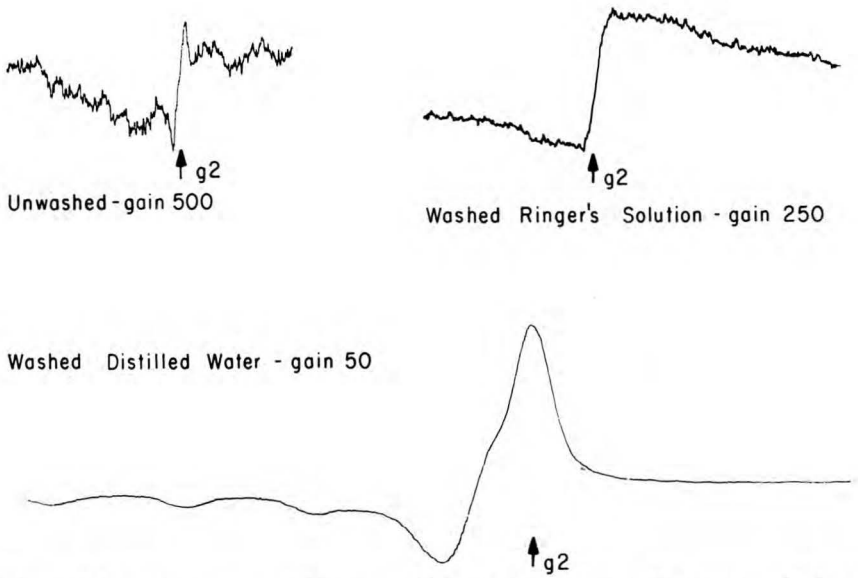

Fig. 4. Electron paramagnetic spectra of bone apatite with different methods of washing.

the apatite that was washed in Ringer's solution to demonstrate a signal similar to that of whole bone or unwashed apatite.

Structuring of Ions by Tendon Collagen

As we have mentioned, the complex signal obtained from bone collagen prepared by demineralization in 5 percent formic acid is not diminished by vacuum drying. It, therefore, seemed to be possible that collagen also was structuring ions from the aqueous washing solutions. Since all other methods of demineralization are productive of structural alterations in the collagen fibers (Morris and Benton, 1956), we elected to study another well-organized collagenous structure, tendon, which could be obtained without chemical separation. The human peroneus longus is a true tendon, which runs for most of its length in a typical tendon sheath.

Segments of human peroneus longus approximately 9–10 centimeters long were resected, the tendon sheath and associated paratenon were removed, and the tendons were placed in the extended position in a sterile petri dish. Short segments were cut and minced with a scalpel or razor blade as necessary for study. Specimens prepared immediately after resection had water contents incompatible with spectrometer sensitivity considerations; however, after they were dried at room temperature and humidity for 24 hours, they revealed a symmetric singlet of 10-gauss line width at g2.006 (Fig. 5).

Subsequent drying did not materially alter this signal. When this material was washed in either distilled water or mammalian Ringer's solution and was dried at room temperature, a complex signal was produced, which was remarkably similar to that of apatite-distilled water, although it was considerably lower in magnitude. Dehydration by vacuum or dioxane for 24 hours resulted only in a sharpening of the trace; the original g2 signal was still present, but the complex signal was not materially diminished.

One might conclude, if tendon collagen may be considered to be analogous to bone collagen before mineralization, that collagen, like apatite, is capable of structuring ions from aqueous solutions in an intimate fashion. The exact similarity between the signals from collagen washed in mammalian Ringer's solution and that washed in distilled water is of interest for two reasons. If the distilled-water tracing is the result of the uptake of electronically unbalanced water

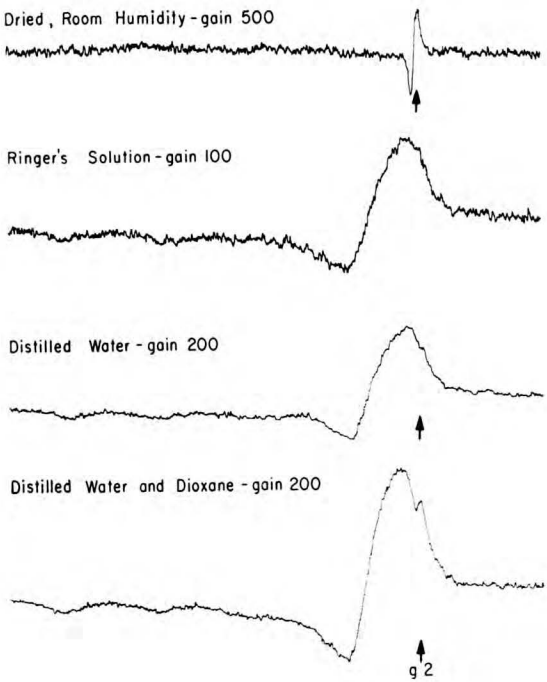

Fig. 5. Electron paramagnetic resonance of human tendon (peroneus longus).

molecules, then perhaps similar units are preferentially absorbed from the mammalian Ringer's solution. This is certainly surprising in view of the variety of other ions available. Furthermore, if tendon is in free communication with the extracellular fluid, one would expect no alteration beyond the original g2 signal on immersion in mammalian Ringer's solution.

Source of Functional Types of Collagen

The electron paramagnetic resonance signals obtainable from bone, bone apatite, bone collagen, and tendon collagen all seem to challenge the applicability of the *milieu intérieur* concept of these structures. All of these materials are capable of structuring a variety of inorganic ions (including electronically active water molecules) on their surfaces in rather tightly held configurations, which are capable of producing electron paramagnetic resonance signals. It is logical to conclude that this phenomenon occurs in conjunction with the structuring of the bound-water shell.

Might not the functional characteristics of the collagenous matrix

be determined by specific variations in the aforedescribed structuring? If this is a tenable thesis, one might postulate that specific collagenous structures are formed and sequestered within active membranes of some type. The preferential passage of certain ions through the membranes might produce the varying functional types of collagen that are found in living organisms, all of which would, nevertheless, show similar molecular and chemical structures on isolation and analysis.

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