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Dielectric Determination of Bound Water of Bone

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ABSTRACT. The dielectric constant ϵ' and dielectric loss ϵ'' of human cortical bone have been measured for frequencies of 1, 10, 50 and 100 kc and for hydrations (h) from 0 to 65 mg-H_2O/g-bone. The absorption isotherm has also been determined. Curves of ϵ' and ϵ'' vs h are found to alter sharply at a particular value of the hydration, h_c, defining the 'critical hydration' of bone. The quantity h_c, ranges from 37 to 48 mg-H_2O/g-bone, depending on the density of the sample. It is interpreted as the amount of water necessary to occupy the primary absorption sites in bone, i.e., as the bound water of the system. The relationship of h_c with previously reported values of the bound water in hide collagen and synthetic hydroxyapatite is discussed.

1. Introduction

Water in biological systems is considered to be present in both structured or 'bound' forms and as free water (Szent-Gyorgyi 1957). The former is presumably associated with the biological substrate (protein [Klotz and Curmae 1948], DNA [Jacobson 1953]) in an orderly fashion, resulting in an ice-like structure with specific properties. Crystalline haemoglobin, for instance, demonstrates a marked increase in electrical conductivity and a decrease in activation energy when hydrated (Rosenberg 1962). Determinations of the amount of bound water present in purified proteins have been made by analysis of weight-hydration curves (Bull 1944). More recently, NMR techniques have substantiated the thesis that this water is structurally organized (Berendsen 1962). However, direct measurements of the absolute amount of bound water in organized tissues have not been reported. A determination of this value for bone appeared to be desirable for several reasons. First, the mineral phase of bone is important in the regulation of the internal ionic environment (Neuman and Neuman 1958) and second, the structured water may be an important factor in the reported electrical properties associated with the bone matrix (Bassett and Becker 1962, Becker, Bassett and Bachman 1964, Becker and Brown 1965, Becker and Marino 1966). The only qualitative study of the water of bone utilized a gravimetric technique, and made no attempt to measure the size of the bound water compartment (Robinson and Elliott 1957). In view of the highly polar nature of the water molecule and the fact that the degree of freedom of such a molecule should differ sharply depending on whether it is in the free or bound compartment, the property chosen for study was the complex dielectric constant ϵ.

In many cases the dielectric behaviour of water absorbed on organic and inorganic absorbents alters sharply at a particular amount of absorbate. Such is the case for water absorbed on protein powders (Rosen 1963), silica gel (Kurosaki 1954), magnesium hydroxide (Nelson, Newman, Tomlinson and Sutton 1959) and gamma alumina (Baldwin and Morrow 1962). For some absorbents alteration of the dielectric behaviour can be interpreted in terms of Brunauer, Emmett and Teller (BET) (1938) theory of multilayer absorption. In particular, the point of alteration is found to correspond to the amount of water needed to complete the BET monolayer as determined from the absorption isotherm (Kurosaki 1954, Nelson et al. 1959, Baldwin and Morrow 1962). However, for protein or heterogeneous systems such as calcified tissue, a variation in the binding energy of different sites may cause absorption on these materials to depart from BET-type behaviour (Rosen 1963).

The measured dielectric constant of a hydrated system receives a contribution from water which depends on the degree to which the water is structured or bound. Thus, a measurement of the dielectric constant simplifies the study of absorbed water by grouping together those water molecules which are in the same physical state. In the present work both the dielectric constant and the amount of water absorbed have been measured explicitly as a function of humidity.

2. Experimental

2.1. *Specimen preparation*

The origin and initial preparation of the samples has been described previously (Becker and Brown 1965). Clinically normal samples of dense human cortical bone were cut into regular geometrical shapes to facilitate measurement of the dielectric constant. In the preliminary preparation the samples were cut slowly, by hand, to avoid denaturation of the protein component. The final polishing was done with a graded series of abrasive papers. The dimensions in millimeters of the resulting (five) samples were: (1) $2{\cdot}27 \times 9{\cdot}77 \times 36{\cdot}90$; (2) $0{\cdot}92 \times 8{\cdot}03 \times 20{\cdot}84$; (3) $1{\cdot}96 \times 12{\cdot}09 \times 14{\cdot}16$; (4) $1{\cdot}96 \times 13{\cdot}34 \times 14{\cdot}11$; (5) $1{\cdot}84 \times 9{\cdot}67 \times 26{\cdot}20$. Care was taken to ensure that the samples were rectangular and as free as possible from surface defects. At no time were they immersed in solution or treated with any chemical agent. In the present study the largest dimension of all specimens was along the original long axis of the bone and all dielectric measurements were made at right angles to this axis.

Saturated salt solutions (Lange 1949) giving the desired relative humidities were used to control the bone water content. Samples were supported above the salt solutions by thin gauze sheets and were permitted to reach equilibrium with the controlled atmosphere.

2.2. *Apparatus*

Measurements of ϵ' and ϵ'', the dielectric constant, and dielectric loss (Debye 1929) were made at frequencies 100, 50, 10, and 1 kc, employing a capacitance measuring assembly, and a micrometer-driven dielectric sample cell. The cell was fitted with side plates especially fabricated to allow a determination of ϵ' and ϵ'' for completely dry bone in a controlled atmosphere (dry nitrogen).

The increase in capacitance and conductance across the terminals of the sample cell due to the insertion of the sample were measured. ϵ' and ϵ'' were then calculated using cell constants, due allowance being made for the fraction of the volume of the cell not occupied by the sample. Individual capacitance measurements could be made to an accuracy of 0·25%. The resulting relative error of the computed dielectric constants varied with the thickness-to-area ratio of the samples but was generally less than 3%. ϵ'' was measured to $\pm 2\%$ or $\pm 0{\cdot}07$ whichever is greater.

The humidities maintained by the saturated salt solutions (range 12%–82%) were measured to an accuracy of $\pm 1{\cdot}5\%$ by means of a wide-range humidity sensing element, and matching electric hygrometer indicator. An airtight humidity chamber was constructed for this purpose from Plexiglas and fitted with a threaded aluminium cap in which the sensing element was mounted. The ratio of the volume of the chamber to the surface area of the solution was about 50 cm. In general, the measured humidities maintained by the solutions in the room temperature interval 21°C ± 1°C agreed with the published values which are given for 20°C. During the experiment the room temperature was maintained at 21°C ± 1°C and the room humidity below 30%.

2.3. *Procedure*

The general procedure was as follows: the prepared sample was first vacuum dried at a pressure of 10 microns and its dry weight, volume and dielectric constant determined. It was then equilibrated with the atmospheres maintained by the saturated salt solutions. When equilibrium was attained at a particular humidity the sample was removed and the dielectric and weight measurements were made in an average time of eight minutes. The sample was then transferred to the next highest humidity. In order to avoid hysteresis effects, all measurements on a given sample began at the lowest attainable humidity (12%) and proceeded stepwise through to the highest humidity (82%). Each sample was treated as a specific entity and all measurements were made directly on the sample for which data are reported. Thus, for each sample, the results were a series of curves, ϵ' vs frequency, ϵ'' vs frequency, with hydration, or water content as a parameter.

In view of the method used, two restrictions were imposed: (*a*) humidities above 82% were not employed, (*b*) dielectric readings were taken at four frequencies only. Both restrictions are necessary in order that the assumption of equilibrium during the measurement be a reasonable one.

There is an inherent ambiguity in any definition of ' dry ' bone, and as a result there are a number of competing criteria. One may, for instance, heat to constant weight at 105°C and define bone so treated as ' dry '. However, there are no assurances that the resulting material will have the same physical, chemical or electrical properties as bone dried in another manner. Indeed there is some evidence to the contrary (Bull 1944). Also, the protein fraction of bone is known to undergo denaturation at about 64°C, and this may have an important effect on the absorption properties of bone. In view of these considerations, dry bone is defined for the purpose of this study as bone in

equilibrium with a water-free atmosphere at 10 microns pressure and room temperature. This procedure is believed to result in a minimum amount of irreversible change of the sample. In practice, reproducible measurements of the dielectric constant are obtained over several dehydration-hydration cycles. For the sample sizes of interest it is found by weight monitoring that two months are required for the drying process. During this time at least two processes are taking place: the sample is ' degassed ' and diffusible oils from the sample are settled out on to an absorbent paper layer. After this time no weight change is seen to $\pm 0{\cdot}3$ mg. All samples for which data are reported were dried for a minimum of two months.

The question arises as to how long is required to establish equilibrium at a given humidity. In order to answer this the following experiment was performed. Two samples were vacuum dried, and then placed in separate humidity chambers that had been modified to allow conductivity measurements while the sample was being hydrated. The humidity in one chamber was maintained at 50% and in the other at 98%. The direct current conductivity was monitored periodically for two weeks and, as can be seen from fig. 1, equilibrium was attained for the worst case after approximately one week. The same results were obtained in another experiment in which the weight of the sample was monitored. In view of this, and since in the actual experiment no sample was subjected to a humidity change of more than about 12%, one week was allowed for equilibrium to be attained before a measurement was taken at a given humidity.

3. Results

3.1. *Dielectric measurements*

Typical results for the dielectric constant are given in fig. 2. As can be seen, the ϵ' vs log f plots are sensitive to water content. In all cases, for a given hydration, h (mg of water per gramme of dry bone), ϵ' decreased continuously

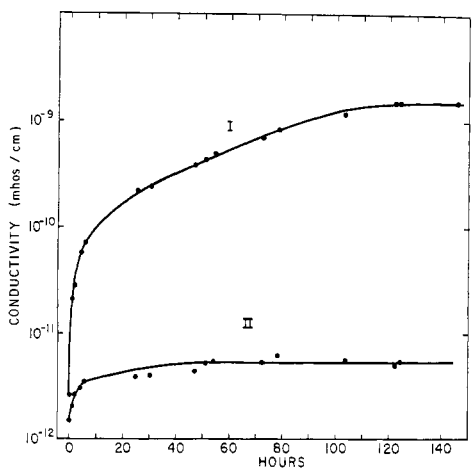

Fig. 1. Conductivity of bone as a function of time. Both samples were initially vacuum dried. I shows equilibrium being attained with humidity of 98%, II with 50%.

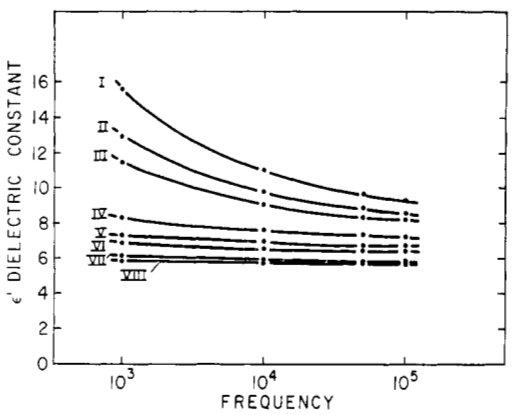

Fig. 2. Dielectric constant of bone as a function of frequency with hydration [given in (mg-H_2O/g-bone) for all eight values]: I—61·6; II—56·4; III—53·8; IV—39·8; V—29·9; VI—21·4; VII—7·0; VIII—0.

with increasing frequency. When the results are plotted as in fig. 3, with frequency as the parameter, the points are found to lie reasonably well along two straight line segments having different slopes. After excluding the point corresponding to $h = 39{\cdot}8$, a best fit straight line can be calculated for each line segment. The intercept of the lower line gives an extrapolated value of the dielectric constant of completely dry bone. The intersection of the two lines defines a quantity h_c, the ' critical hydration ' of bone.

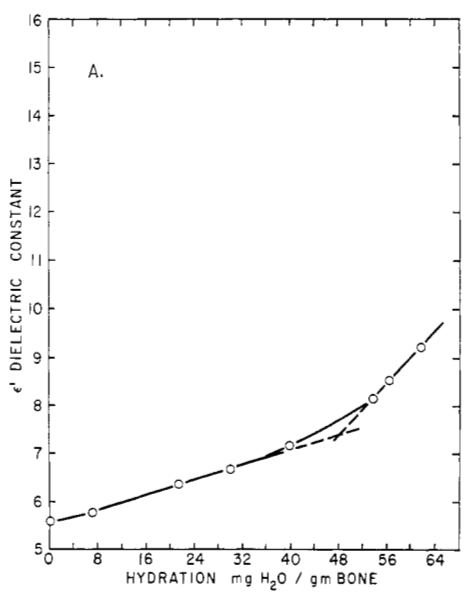

Fig. 3 (a)

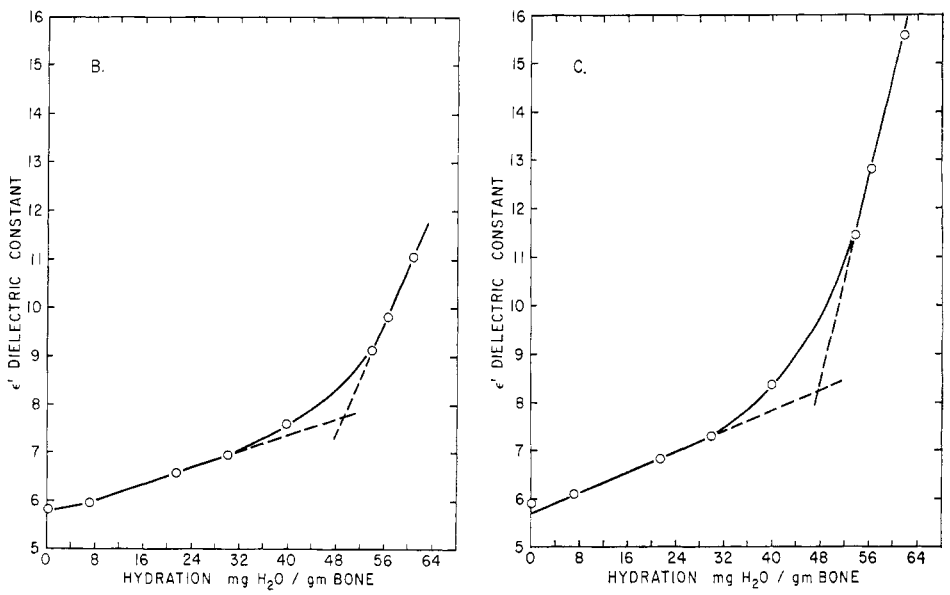

Fig. 3. Dielectric constant of bone as a function of hydration: (*a*) 100 kc, (*b*) 10 kc, (*c*) 1 kc.

Results for ϵ'', the dielectric loss, are given on fig. 4. Again the dependence on water content is evident. The large resistance of low water-content bone causes the corresponding values of ϵ'' to be of the same order as the experimental error. For higher water contents, no dielectric loss maxima were seen, even for samples on which more detailed measurements could be taken.

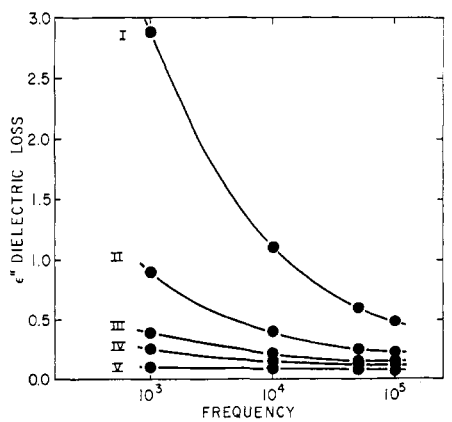

Fig. 4. Dielectric loss of bone as a function of frequency with hydration of: (given in mg-H_2O/g-bone for all five values): I—53·8; II—39·8; III—29·9; IV—21·4; V—0.

When ϵ'' is plotted against hydration, the points again fall along two straight line segments whose intersection similarly defines h_c (fig. 5). The qualitative behaviour of both ϵ'' and ϵ' vs h is the same, and since the values for the latter

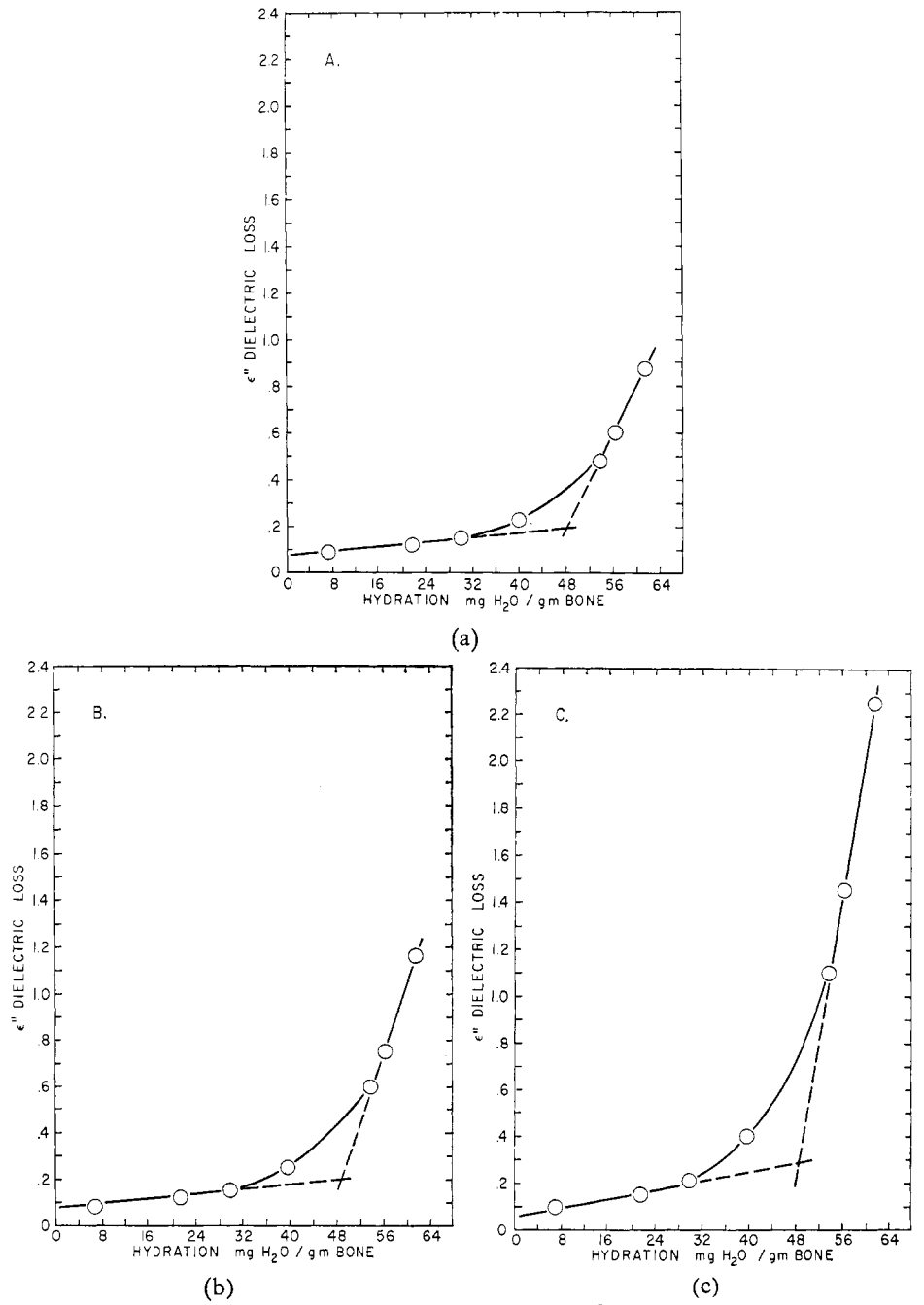

Fig. 5. Dielectric loss of bone as a function of hydration: (*a*) 100 kc, (*b*) 50 kc, (*c*) 10 kc.

curve are more accurately known, only the ϵ' curves are used to determine the magnitude of h_c.

The value of h_c for the sample discussed (Sample No. 1) together with relevant data for the other samples examined are given in table 1. The density given in table 1 was determined simply from measurements of the linear dimensions of the sample. Thus, it takes no account of the various kinds of spaces or cavities in bone, and is only a measure of the relative compactness of the various samples.

3.2 *Absorption*

The average absorption isotherm for bone is given in fig. 6. It is seen to be linear over a rather wide range of humidities. For values of the humidity above 80%, the curve is very steep and accurate measurements could not be taken.

An increase in the linear dimensions of the samples with increasing hydrations was observed. Relative to the dimensions at zero water content, the average

Table 1. Measured and extrapolated values of dielectric constant of dry bone and critical hydration. Values are averaged over frequencies 100, 10 and 1 kc.

Sample number	Density g/cc	Measured dielectric constant for dry bone	Extrapolated dielectric constant for dry bone	h_c (mg-H_2O/g-bone)
1	$1{\cdot}76 \pm 0{\cdot}01$	5·7	5·6	49 ± 1
2	$1{\cdot}86 \pm 0{\cdot}01$	5·8	5·7	42 ± 4
3	$1{\cdot}88 \pm 0{\cdot}02$	6·0	5·9	46 ± 2
4	$1{\cdot}89 \pm 0{\cdot}02$	6·3	5·6	45 ± 2
5	$1{\cdot}90 \pm 0{\cdot}02$	6·5	6·3	37 ± 5

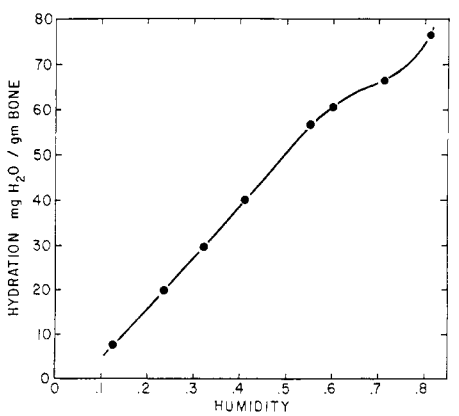

Fig. 6. Average absorption isotherm of bone. Temperature, 21°C ± 1°C.

increase for bone in equilibrium with humidity at 82% was 2·5% for dimensions at right angles to the collagen fibres, and 0·5% along the fibres.

4. Discussion

The literature contains many references to what are called the ' bound water ' and ' free water ' compartments of bone. The bound water compartment is generally thought to be of a relatively fixed size and to contain water which is ordered or structured by individual (primary) binding sites in the tissue. The free water compartment contains the less strongly held water found in bone. It is thought to be present by virtue of a dipolar interaction with bound water in amounts determined by atmospheric humidity in the case of excised bone, and metabolic processes in the case of bone *in vivo*. Our experiment clearly indicates that the equilibrium water content of bone is determined solely by the humidity of the atmosphere with which it is in equilibrium (cf. fig. 6), as indeed is the case for all other hydroscopic materials which have been reported.

The experimentally determined quantity, h_c, may be interpreted as the bound water of bone (Rosen 1963, Kurosaki 1954, Nelson et al. 1959, Balwin and Morrow 1962): for low equilibrium water contents the absorbed water is structured or bound, so that it is essentially able to make only an electronic or molecular contribution to the measured dielectric constant of the system. That is, there is no contribution from re-orientation of the water dipole moments in the electric field. For progressively higher water contents, the absorbed water is less strongly held, i.e. is relatively ' free ' and, therefore able to make a significant contribution to the dielectric constant, resulting in the steep rise seen in fig. 3: h_c is then the dividing point between the two regions.

In addition, there is the possibility that the observed dielectric dispersion contains a contribution from a form of interfacial polarization. For instance, at high water contents surface ions from bone mineral may acquire a certain local mobility in the electric field and thus produce a dispersion which could account in part for the high dielectric constants seen at high water contents. Whatever the mechanism (or combination of mechanisms) that explains the steep rise seen in fig. 3, it appears permissible to identify h_c with the amount of water required to fill the primary absorption sites in bone (PASB).

The results give no information concerning the nature of the PASB. It is thought that absorption on proteins involves an interaction between water molecules and local dipoles along the protein molecule, and that absorption on the mineral hydroxy-apatite results from the electric field asymmetry at the surface of the individual crystals (producing the ' hydration shell '). Thus, absorption in bone may correspond to a superposition of the two effects. Indeed, on the basis of this assumption reasonably good agreement with the work of previous investigators is obtained as follows: absorption isotherms for synthetic hydroxy-apatite (Neuman, Toribara and Mulryan 1953) and hide collagen (Bull 1944) have previously been reported. Both isotherms were

fitted to a BET-type equation resulting in values, for the amount of water necessary to occupy the primary absorption sites, of 17·6 mg/g for hydroxyapatite and 95·2 mg/g for collagen. If, for simplicity, a gramme of bone is considered to consist of 65% apatite and 35% collagen by weight, then one arrives at the value (0·65) (17·6) + (0·35) (95·2) = 44·8 for the water necessary to occupy the PASB, as compared with the experimentally determined range of 37 to 48. Hence, it is suggested that absorption of water by bone may be considered a superposition of absorption by collagen and apatite separately, with collagen playing the dominant role in terms of amount absorbed.

It should be pointed out that even though, (*a*) absorption on collagen and apatite separately has been found to obey a BET-type equation, and (*b*) the results reported here are interpretable in terms of the BET constants for collagen and apatite, this does not necessarily imply that absorption on bone follows a BET-type equation. It may or may not, and the absorption isotherm given in fig. 6 is not sufficiently detailed to answer the question. A system of any two BET absorbers will, in general, not be a BET absorber. The exception is when, (*c*), the BET constant related to the heat of absorption, is the same for each absorber. If the absorption isotherm of bone is found to fit a BET-type equation, the value of the bound water that is thus determined would be expected to agree with the value of h_c reported here. If absorption in bone is found to be non-BET, then one may conclude only that the heats of absorption on collagen and apatite are different and no definitive statement concerning the amount of bound water can be made.

Reference to table 1 shows that the measured values of the dielectric constant of dry bone are slightly but consistently larger than the extrapolated values. A possible explanation is that bone, vacuum dried in the manner previously described, contains a residual water content which augments the measured value of ϵ'. Such water would thus represent the most strongly bound water to be associated with the system. The amount of water, having the dielectric properties of bulk water, that is necessary to produce the observed increment is not large (~ 1 mg). However, for strongly bound water unable to make a significant dipolar contribution, the amount required to account for the difference would be greater. Thus, on the basis of this experiment it is not possible to relate the observed difference to a specific amount of residual water.

Alternatively, extrapolation to zero water content may not be a valid procedure. That is, the dielectric constant of bone corresponding to hydrations in the range 0 to 8 mg-H_2O/g-bone may define a third linear line segment (and a second critical hydration) as, for instance, has been found for water absorbed on silica gel (Kurosaki 1954).

The results obtained indicate that water absorbed on bone exists in at least two separate states, one more strongly bound to the underlying matrix than the other. The bound or structured water compartment by direct measurement ranges from 37 to 48 mg-H_2O/g-bone, depending on the density of the samples.

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Résumé

Détermination de l'eau liée dans les os par la méthode diélectrique

On a mesuré la constante diélectrique ϵ' et la perte diélectrique ϵ'' de l'os cortical humain pour les fréquences de 1, 10, 50 et 100 kHz et pour les hydratations (h) de O à 65 mg H_2O/g os. On a aussi déterminé l'isotherme d'absorption. On a trouvé que les courbes de ϵ' et ϵ'' en fonction de h changent brusquement pour une valeur particulière de l'hydratation, h_c, qui définit " l'hydratation critique " de l'os. La quantité h_c varie entre 37 et 48 mg H_2O/g os, dépendant de la densité de l'os. On interprète cette valeur comme la quantité d'eau, indispensable pour occuper les emplacements d'absorption primaire dans les os, c'est-à-dire comme l'eau liée du système. On discute re rapport de h_c aux valeurs publiées antérieurement pour l'eau liée dans le collagène de la peau et dans l'hydroxyapatite synthétique.

Zusammenfassung

Dielektrische Bestimmung des gebundenen Wassers im Knochen

Es wurden die Dielektrizitätskonstante ϵ' sowie der dielektrische Verlust ϵ'' des menschlichen Kortikalknochens für Frequenzen von 1, 10, 50 und 100 kHz und für Hydratationswerte (h) von 0 bis 65 mg H_2O/g Knochen gemessen. Die Absorptionsisotherme wurde ebenfalls bestimmt. Es wurde gefunden, dass die ϵ'- und ϵ''-Kurven als Funktionen von h sich bei einem besonderen Hydratationswert, h_c, scharf verändern, wobei h_c die kritische Hydratation des Knochens definiert. Die h_c-Menge variiert zwischen 37 und 48 mg H_2O/g Knochen, in Anhängigkeit von der Dichte der Probe. Diese Menge wird als diejenige Wassermenge gedeutet, die zur Besetzung der primären Absorptionsstellen im Knochen unentbehrlich ist, d.h. als das gebundene Wasser des Systems. Es wird die Beziehung von h_c zu den früher veröffentlichten Werten des gebundenen Wassers im Hautkollagen und im synthetischen Hydroxyapatit erörtert.

Резюме

Определение связанной воды в кости диэлектрическим методом

Диэлектрическая константа ϵ' и диэлектрическая потеря ϵ'' человеческой корковой кости измерялись для частот 1, 10, 50 и 100 кгц и для гидратации (h) от 0 до 65 мг H_2O/г кости. Определялась также изотерма абсорбции. Оказалось, что/кривые ϵ' и ϵ'' в зависимости от h изменяются круто при определенном значении/гидратации, h_c, определяющем " критическую гидратацию " кости. Количество h_c колеблется от 37 до 48 мг H_2O/г кости, в зависимости от плотности пробы. Это количество интерпретируется как количество воды, необходимое для занятия первичных мест абсорбции в кости, т.е. как связанная вода системы. Обсуждается отношение h_c к определенным раньше значениям связанной воды в коллагене кожи и в синтетическом гидроксиапатите.

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