ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF BONE AND ITS MAJOR COMPONENTS

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A PRELIMINARY paper reported the detection of electron paramagnetic resonance (EPR) signals in non-irradiated human and amphibian bone[1]. Whole cortical bone was used in that investigation and the signals were described as multiple and anisotropic. The study was undertaken to obtain evidence to confirm the theory that some properties of bone result from a semiconducting *P-n* diode structure formed by the association between the mineral crystals and the collagen fibres of the matrix[2,3]. While the preliminary results neither confirmed nor refuted this thesis we wish now to report on a much more extensive study from which several interesting conclusions may be drawn.

While the literature is replete with reports of EPR studies on irradiated biological materials, we have found only scanty references to work on non-irradiated bone, collagen or mineral. Slager and Zucker[4] found no resonances in bone prepared for use in bone banks, and Cole and Silver[5] reported no resonances in a single deciduous tooth. Both these investigations were concerned primarily with the effects of irradiation and non-irradiated samples were employed only as controls. In discussing our preliminary communication, Commoner[6] stated that the resonance in whole bone was most likely due to the bone mineral, presumably apatite. Vinokurov and Zaripov[7] reported the presence of EPR signals in geological apatite. However, we have been unable to find any reports of similar studies on bone mineral. Likewise we have found no mention in the literature of EPR signals from non-irradiated collagen, derived either from bone or from soft tissue, except for the report by Abagyan[8] of complex signals from gelatine. Commoner and Ternberg[9,10], Swartz and Molenda[11] and Mallard and Kent[12] have all reported signals arising from whole, non-irradiated animal tissues of various types. However, none of these reports dealt with bone or with any type of collagen *per se*.

Bone samples. In the present investigation, powdered samples of human bone, collagen and mineral were used exclusively to avoid the complication which accompanied the previously reported anisotropic signals from whole bone. General sample procurement and treatment was as reported in detail in a previous publication, including the use of similar techniques for preparation of bone mineral and bone collagen. It is important to note that the extractions of collagen and mineral involve the use of aqueous solutions and subsequent washing in water. Once the basic samples were prepared they were stored in Petri dishes under normal laboratory conditions relative to temperature, humidity and illumination. In the present experiments these basic samples were reduced to powder by scraping with a sharp glass fragment (microscope slides were routinely used and discarded as soon as they showed signs of wear). The bone samples were held by hand in a paper tissue and no metallic objects were used in any part of the preparation procedure. This method resulted in a fine granular powder, uncontaminated by metallic or glass fragments, which was used for all samples except for the rat tail tendon. In this case the

fibres were obtained by stripping away from the bony structures; the portion of the tendon which had been grasped by the metallic clamp was then cut off and discarded. The fibres were permitted to equilibrate to room conditions, then sectioned into pieces of less than 1 mm length using sharp scissors. Dehydrated samples of all materials were prepared by vacuum drying at a pressure of 10μ of mercury for a minimum of 2 weeks. During the first few days of this period the vacuum chamber was repeatedly flushed with dry nitrogen. This procedure was primarily intended to produce the maximum water loss without producing heat denaturation of the protein components.

EPR techniques. Samples were examined in 4 mm optically dense quartz tubes; each tube was previously checked for resonances utilizing the same instrument settings to be subsequently applied to the sample. Tubes with any major resonances in the $g2$ vicinity were discarded. In order properly to compare signals of the two components with the whole bone signal, tubes were filled according to a 2 : 1 ratio of mineral to collagen. This resulted in the whole bone sample tubes containing 150 mg of material, the mineral tubes 100 mg and the collagen tubes 50 mg of material. A Varian '*X*-band' spectrometer with 100 k.c. field modulation and phase detection with a time constant of 0·3 sec was used. Variable temperature studies were done using a Varian variable temperature attachment utilizing a constant flow of dry nitrogen at the desired temperature; g values were calculated from a determination of the klystron frequency using a Hewlett–Packard wavemeter, type '*X*532*B*'. The first derivative of the absorption signal was recorded.

(*A*) *Whole bone powder*

Hydrated. At room temperature all samples showed a clearly defined single resonance at $g2{\cdot}001$ ($\pm 0{\cdot}005$) with a line-width of 10 $\pm$ 1 gauss. This resonance saturated at about 100 dB (Fig. 1). The total signal amplitude decreased very slowly with storage under dark conditions. In general a decline to 30 per cent of the original value was apparent after 90 days. The other signal parameters, however, remained unchanged.

Dehydrated. Vacuum drying resulted in approximately a four-fold increase in signal amplitude, but again the other parameters of the signal remained unchanged.

Resonance signal variations with temperature. The amplitude of both hydrated and dehydrated samples had the same temperature dependence in the interval $-150°$ C–$+20°$ C (Fig. 2). In this region the curve was found to be reproducible. On heating, however, hydrated bone showed a discrete shift to a smaller amplitude in the vicinity of $+65°$ C (Fig. 3), while dehydrated bone showed a more gradual transition to smaller amplitudes over the range $+20°$ C to $+95°$ C. In both cases, the decrease is much greater than one would expect on the basis of a simple Curie dependence. The decrease in signal amplitude at $+65°$ C appeared to be irreversible. Each sample demonstrated this phenomenon one time only and, provided a sample was not carried beyond $+100°$ C (with resultant loss of volatile components), when returned to $+20°$ C did not regain the original signal amplitude. Further heating to $+200°$ C caused the hydrated sample signal to increase in magnitude while the dehydrated sample signal remained essentially constant. Heating of a hydrated sample in a $+800°$ C flame for 3 min caused the signal to increase by a factor of 100. In all heating experiments including the burning in a $+800°$ C flame, the g-value and line-width of the observed resonance remained constant.

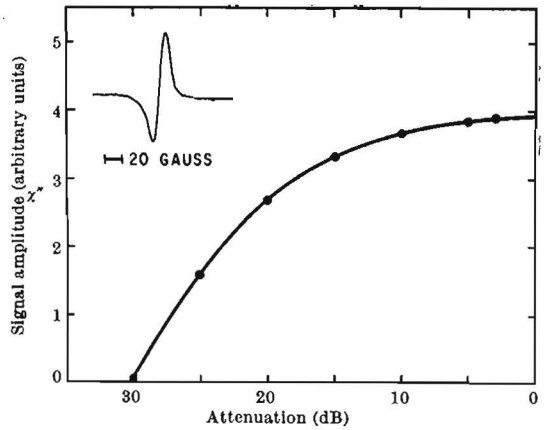

Fig. 1. Saturation of resonance from hydrated whole bone powder. The insert shows the standard resonance observed from this material

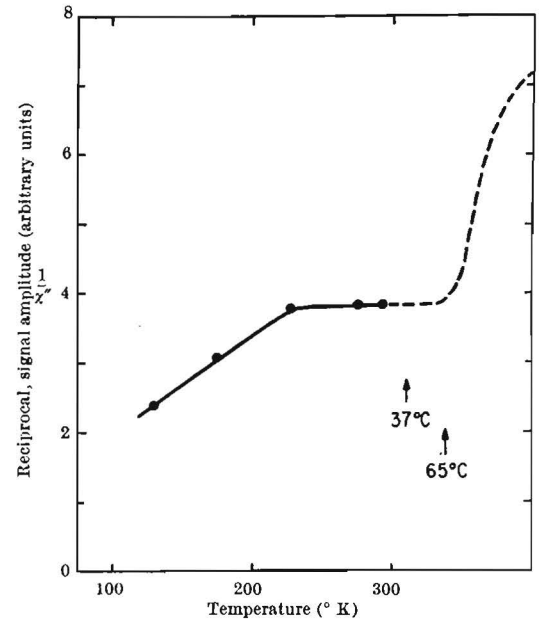

Fig. 2. Temperature dependence of reciprocal of signal amplitude from hydrated and dehydrated whole bone powder. The solid portion of the graph is stable and reproducible. The large sample temperature gradient (10° C) and temperature controller 'dead band' at present preclude assigning definite points to the curve above $+200°$ K, that is, slope of dotted line depends on details of heating experiment. However, the abrupt signal change in vicinity of $+65°$ C was routinely observed and is approximately as depicted

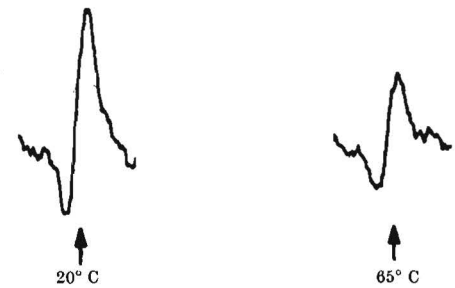

Fig. 3. Comparison of resonance signal amplitude from whole bone powder at room temperature and at $+65°$ C. The shift to a lower amplitude appears to occur rather abruptly in the vicinity of $+65°$ C

(*B*) *Bone mineral powder*

Hydrated. At room temperature a complex resonance (Fig. 4*A*) was observed, composed of several unresolved resonances with the largest slightly to the low field side of $g2$. In addition three equally spaced, low amplitude resonances have been found farther to the low field side. The low amplitude resonances are relatively independent of temperature, and disappear when the apatite is heated above $+150°$ C. Storage in the dark for 90 days did not result in any appreciable decline in signal amplitude.

Dehydrated. At room temperature there was a slight increase in total resonance amplitude compared with hydrated apatite, but there was considerable improvement in resolution indicating the presence of three separate resonances in the $g2$ area (Fig. 4*B*). These are subsequently

identified as apatite resonances I, II and III with resonance III having a g-value very close to $g2$ and I and II being displaced towards the low field side of III. A careful inspection of the resonance observed in the hydrated sample reveals that the same three resonances are probably present in the one complex signal but with such low resolution as to be practically indistinguishable. The three low amplitude, low field resonances are still present but reduced in amplitude.

Resonance signal variations with temperature. (Fig. 5.) Both hydrated and dehydrated samples displayed the same series of changes with temperature. On cooling from room temperature to $-50°$ C there was a slight increase in total signal amplitude showing roughly an inverse dependence on temperature. In the vicinity of $-100°$ C a decrease in signal amplitude occurs and continues until at $-154°$ C the total signal amplitude is approximately 50 per cent of that at room temperature. These changes between room temperature and $-154°$ C are completely reversible. Heating the samples from room temperature to $+150°$ C results in a monotonic decrease in amplitude of the total resonance. At $+150°$ C, resonance I is no longer observable or combined with resonance II. The amplitude of resonance II is reduced sufficiently to render resonance III quite prominent. Between $+150°$ C and $+250°$ C there is a further reduction in the resonance representing the combination of I and II while resonance III demonstrates a real increase in amplitude. This same process (decrease in amplitude resonances I and II, increase in resonance III) continues up to $+300°$ C. If the heating process is halted at this point, and the sample cooled to room temperature and examined, a signal much different than the previous room temperature signal is seen.

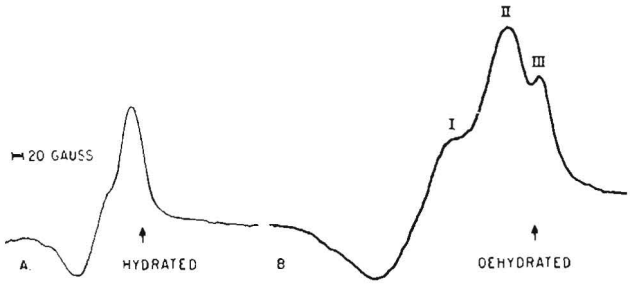

Fig. 4. Resonance signals obtained at room temperature from extracted bone mineral, hydrated (A) and dehydrated (B). The three low amplitude resonances towards the low field side of $g2$ are not pictured. There appear to be three resonances in the vicinity of $g2$; the numbers assigned to them on the dehydrated resonance (B) correspond to the numbers in the text. The scale of the horizontal axis of B is twice that of A

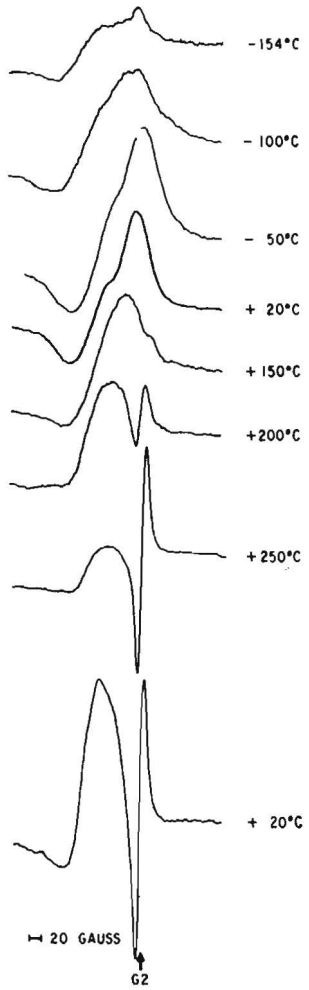

Fig. 5. Variations in the resonance signals from bone mineral (dehydrated in vacuum) with temperature changes. Instrument gain was reduced 50 per cent for the $+250°$ C temperature recording

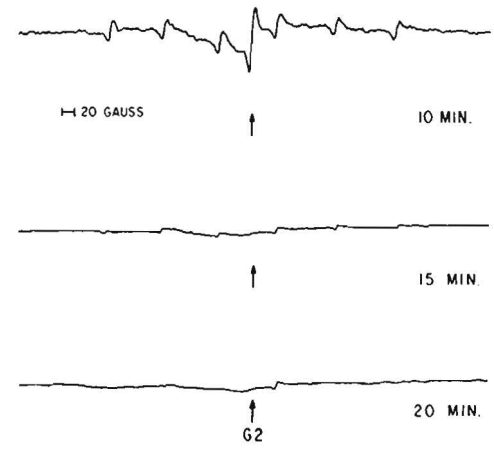

Fig. 6. Alterations in the resonance from bone mineral with exposure to $+800°$ C temperature for varying periods of time

Detailed experiments on bone mineral in the temperature range $+300°$ C to $+800°$ C using a muffle furnace are in process. However, the following preliminary observations have been made (Fig. 6).

(1) By heating to constant weight at $+400°$ C the broad low field resonance may be eliminated leaving essentially resonance III with a line-width of 10 gauss; g-value determinations were within the same range as the $g2$ signal from whole bone.

(2) By equilibrating mineral showing only a $g2$ resonance in distilled water for 24 h, the low field resonance may be partially restored.

(3) Transient heating in a $+800°$ C flame causes a decreaso in the $g2$ resonance and the appearance of six satellite lines.

(4) Further flame heating causes the complete disappearance of the $g2$ line and a diminution of the satellite lines.

(5) Heating to constant weight at $+800°$ C causes the satellite lines to disappear completely with the exception of the first on the high field side.

The 'resolving' of the $g2$ resonance in apatite by heating to $+400°$ C enabled studies of its temperature dependence in the range $-154°$ C to $+200°$ C to be made (Fig. 7).

(C) Collagen

All samples of collagon, whether from rat tail tendon or human bone, that were immersed in water as a routine part of the preparation procedure displayed approximately the same complex signal: a broad peak towards the low field side of $g2$, a slightly narrower asymmetric peak closer to $g2$ and a partially resolved peak at $g2$. These resonances are hereafter referred to respectively

as I, II and III (Fig. 8*A*). Samples dehydrated in vacuum were essentially the same except for a slight increase in amplitude and resolution of the *g*2 peak (Fig. 8*B*). The only samples of collagen not immersed in water during preparation were those from rat tail tendon. These were found to have only the *g*2 peak (Fig. 8*C*). If these samples were immersed in water and air dried they displayed a resonance identical to hydrated bone collagen (Fig. 8*D*). Subsequent vacuum drying produced a resonance similar to that of vacuum dried bone collagen. Bone collagen stored under dark conditions for 90 days demonstrated approximately a 30 per cent loss in signal amplitude.

Signal variation with temperature

Bone collagen. Over the temperature range −154°–+150° C, resonance I appeared to increase slightly. Resonance II displayed a slight increase below −50° C, remained constant in amplitude between −50° C and +65° C, and decreased between +65° C and +150° C. Resonance III exhibited a constant decline from −154° C to +150° C. Since this resonance is poorly resolved, we cannot be certain whether it follows a strictly Curie dependence.

The signal complex composed of peaks II and III displayed a rather abrupt decrease in signal amplitude in the vicinity of +65° C. This phenomenon is somewhat easier to observe in the dehydrated bone collagen samples than in the hydrated. When samples that had been carried to +150° C were returned to room temperature, signals I and III were found to be relatively unchanged while signal II remained diminished in amplitude.

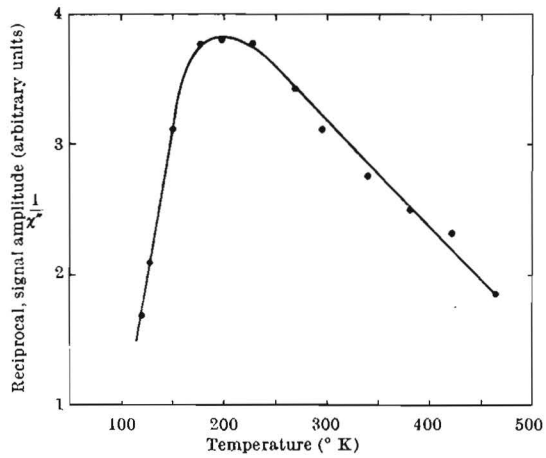

Fig. 7. Temperature dependence of reciprocal of signal amplitude from bone mineral treated to show only the *g*2 signal (heated to +400° C)

Rat tail tendon. The *g*2 signal was relatively independent of temperature between −154° C and +65° C. At the latter temperature an abrupt shift to a smaller amplitude is observed. Heating over +100° C produces an increase in signal amplitude that may well be ascribed to the loss of volatile materials.

In general, on an equal weight basis the signal amplitude from human bone collagen is approximately three times as great as that from rat tail tendon.

Bone mineral. The resonance signals obtained from bone mineral appear to have considerable significance both in lending support to the thesis that the material is hydroxyapatite and in substantiating some concepts of its structure.

In order to explain the low calcium–phosphorus ratio in many hydroxyapatites it has been postulated that $2H_3O^{++}$ may be substituted for Ca^{++} in the crystal lattice, up to a maximum of four hydronium ions per unit cell. One of the chief successes of this theory is in explaining the observed water loss of apatite as a function of temperature. Experimentally, it is found, when heating apatite to constant weight, that weight is lost in a series of three

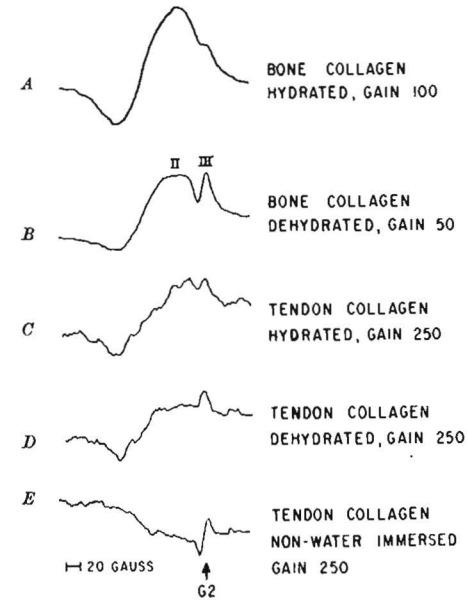

Fig. 8. Resonance signals from various types of collagen at room temperature. The numbers on resonance *B* (dehydrated bone collagen) correspond to those in the text. Resonance I, the broad low field signal, is not pictured in the figure

discrete steps terminating at temperatures of +110° C, +400° C and +600° C (ref. 13). These changes have been attributed to an initial loss of surface absorbed water, a loss of substituted hydronium ions and, thirdly, to liberation of hydroxyl radicals from the unit cell.

A strikingly similar series of changes takes place in the EPR signal with temperature. Heating to +150° C eliminates the three low amplitude resonances and diminishes resonance II. Heating to +400° C almost completely eliminates I and II, leaving only the *g*2 resonance (III), and heating from +400° C to +800° C reveals the complex series of changes previously described. Prolonged heating at +800° C eliminates all resonances except the first high field satellite line.

Substituted hydronium ions (or similar type units) can produce a resonance signal. If it is postulated that the 'surface absorbed' water is somewhat similarly structured and capable of producing a resonance, then a high degree of correlation may be noted between the weight changes with temperature and the EPR signal changes with temperature, resonance II arising from surface-absorbed water and resonance I from H_3O^{++}. Further evidence for attributing the resonances in apatite, with the exception of the *g*2 resonance, to some form of water is provided by the work of Siegel *et al.*[14] on γ-irradiated ice. Among the resonances they found is one that is strikingly similar to the one we have found, showing resonance I (including the low field resonances) and resonance II, and differing only in that the unresolved resonance at *g*2 appears to be missing. While Siegel attributes the resonance to water the possibility remains that it is due to H_3O^{++} (or, alternatively, that water and not H_3O^{++} is substituted in the crystal lattice). In addition it should be noted that the broad low field resonance I may be enhanced by immersing the apatite in water for 24 h.

Vinokurov[7], investigating the EPR signal of blue geological apatites, finds in addition to Mn^{++} lines a relatively intense line at *g*2 of width 10 gauss, with six satellite lines symmetrically displaced of lesser intensity. He finds that the lines disappear at about +500° C, and attributes them to *F*-centre electrons localized at halogen ion vacancies (the position normally occupied by hydroxide in hydroxyapatite). As previously described, the same spectra with the notable absence of Mn^{++} lines are found in bone apatite in transient heating, and could presumably be produced by heating to constant weight in

the temperature range +400° C to +800° C. Significantly, the temperature at which the seven line spectra in bone apatite disappear, +800° C, is also the temperature at which the hydroxyl ion is driven off, collapsing the unit cell, and converting bone apatite into tricalcium phosphate. Commercially purchased tricalcium phosphate is found to have no resonance. Thus, the $g2$ resonance in apatite is tentatively identified as being directly associated with either the hydroxyl radical itself, or electrons localized in hydroxyl vacancies (F-centres).

It is difficult to explain the observed anomalous temperature dependence of the $g2$ signal (Fig. 7) on the basis of strictly localized electrons as postulated by Vinokurov[7]. If one assumes that the trapped electrons do not participate in the resonance an alternative model that does fit the observations would be an excess of conduction electrons in equilibrium with the traps at +200° K (the observed signal minima), all traps would be considered filled, the resonance arising from the excess free electron population. Heating above +200° K would release increased numbers of electrons from the traps, thereby augmenting the resonance. Cooling below +200° K also leads to augmentation of the signal, derived in this case from the population of excess free electrons by virtue of the classical Curie dependence. On the basis of this picture, one may expect that there exists a temperature range in which all the traps are emptied, yet which lies below the temperature at which the onset of conversion to tricalcium phosphate begins (+600° C). In such a range, the resonance amplitude would be independent of temperature, except for the Curie dependence.

From a consideration of the data we conclude that resonance II (and the three low field resonances) results from surface-absorbed water possessing a structure capable of producing a resonant signal. Resonance I is tentatively identified with either H_3O^{++} ions or water in substitution positions within the lattice. The $g2$ resonance would appear to be derived from hydroxyl radicals, F-centre electrons, or conduction electrons. The similarity of the high temperature spectra sequence to that observed in geological apatite is striking but obviously not conclusive.

Collagen. All samples of tendon collagen initially demonstrated only the $g2$ resonance and within the confines of the experimental procedures did not indicate the presence of paramagnetic ions either as functional constituents or as impurities. Following exposure to aqueous solutions and subsequent drying (in air or vacuum) the resonance became practically identical with that of bone collagen, except in magnitude.

The resonance produced in rat tail collagen by water immersion (II) could be removed by heating to +150° C, while with bone collagen it was only partially removed. The line shape intensity and position of the remaining resonance (II), in +150° C heated bone collagen, together with spectroscopic data[15], strongly indicate that it is a result of residual apatite. Since the preparation of bone collagen involves prolonged exposure to aqueous solutions it must be considered possible that its unaltered, *in vivo*, resonance is also a similar single $g2$ peak. The data indicate that the broad resonance, II, observed after such exposure results from water structured on or within the collagen fibres which is structured in such a fashion as to be capable of producing an electron resonance. Since the water is apparently quite tightly bound to the collagen structure one may well question the relationship between structural or electronic data determined on collagen prepared in such a fashion with native unaltered collagen *in vivo*. In addition the data lead to the conclusion that both bone collagen and rat tail tendon collagen are not in free equilibrium with the water of the extracellular fluid. This is scarcely surprising for the type of bone collagen utilized (which is probably representative of 'unexchangeable bone'), but it is quite unexpected in the case of the tendon collagen.

The abrupt decline in signal magnitude in the vicinity of +65° C is indicative of a corresponding alteration in electronic structure. This phenomenon may, however, not be as abrupt as it would appear to be at first glance. For example, the curve of signal variation against temperature for bone (Fig. 2) shows a similar abrupt change at +65° C, but this transition terminates a temperature sequence from −50° C to +65° C within which the signal amplitude does not change with temperature. Below −50° C the signal follows a Curie dependence quite exactly. One has, therefore, rather than an abrupt anomaly occurring at a single temperature, an anomaly spread over a rather wide temperature range which is terminated abruptly at +65° C. It is interesting to reflect on the fact that the collagen *in vivo* exists in this anomalous state and that the temperature at which the state is terminated coincides quite well with the temperature at which the well-known 'shrinkage' phenomenon occurs.

Bone. The previously reported resonance signal at $g2$ is confirmed by the present investigation. The signal described in the present article is much greater in amplitude and much better defined than that previously reported because the total mass of specimen present in the powdered form is many times greater than the mass of the intact specimens used in the previous experiments.

The temperature dependence of the signal amplitude parallels that of collagen so well that the signal must be considered to derive, at least in part, from collagen. It is certainly not due solely to the presence of the apatite. While we cannot state with certainty the state of hydration of the apatite present in whole bone, all available evidence[13] indicates that at least that portion of the hydration represented by the substituted hydronium ions is present. One might therefore expect to see at least some of apatite resonance I in addition to the $g2$ resonance. However, none is found. In consideration of these facts it is surprising that the whole bone signal observed was consistently a relatively narrow, symmetrical intense line at $g2$. In general, the results are in accord with the thesis that the apatite–collagen relationship in bone produces a new unit with properties that are more than the simple additive sum of the properties of both components. If one may somewhat arbitrarily compare the single $g2$ signal of whole bone, with the corresponding signal of rat tail tendon collagen and that of apatite which has been heated to +400° C, few differences can be seen on superficial examination. However, the saturation curve for whole bone (Fig. 1) shows the onset of saturation at 10 dB, while a similar curve for both collagen and apatite reveals saturation in the vicinity of 15 dB, indicating some basic dissimilarity. In addition, the temperature dependences of bone powder and heated apatite are quite different.

To sum up, EPR observations have been made on powdered samples of whole human bone, bone collagen, bone mineral and rat tail tendon collagen.

(1) All substances examined revealed resonance signals.

(2) The resonance signal from bone mineral is quite similar to that derived from geological apatite within a certain temperature range and is attributed to certain types of structured water as well as F-centre electrons localized at the hydroxyl ion position and possibly conduction electrons.

(3) There is a distinct difference between the signals exhibited by water immersed and non-water immersed collagen. This is at present attributed to the ability of the collagen fibre to 'structure' absorbed water to a high degree.

(4) Collagen and bone exhibited an anomaly in the curve of signal against temperature at +65° C. The possible relationship between this and the shrinkage phenomenon is indicated.

(5) No paramagnetic species were found in any of the materials, either as functional components or as contaminants.

(6) The resonance signal in bone is not attributable to either apatite or collagen alone or to a simply additive sum of the resonances of both of these substances in combination.

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