JOURNAL OF MATERIALS SCIENCE 14 (1979) 541–548

ESR study of degraded bovine bone

M. SCANDOLA*, P.E. REED
Department of Materials, Queen Mary College, London, UK

A comparison is made between the electron spin resonance (ESR) spectra obtained from bovine bone samples after (i) mechanical degradation, (ii) γ-irradiation, and (iii) mechanical degradation followed by γ-irradiation. Mechanical degradation is achieved by filing in either liquid nitrogen or air at room temperature. γ-irradiation is performed at 77 K. The stability of the radical species produced at 77 K is studied as a function of temperature. It is concluded that the spectra obtained in all instances arise from radicals formed both in the collagen and mineral phase of the bone, those from the collagen predominating. The relative radical concentrations from the collagen and mineral phase vary with the degradation method employed. It is observed that additional hydrogen atom radicals are produced only in the case of the γ-irradiated samples.

1. Introduction

Electron spin resonance (ESR) spectroscopy has been widely applied to a range of polymers to study degradation produced either by irradiation [1] or mechanically [2, 3]. ESR can identify free radicals resulting from the degradation in some instances and the quantity of free radicals formed. It has also been used to monitor the stability of free radicals formed as functions of both time and temperature. ESR is hence extensively used for mechano-chemistry investigations. Recently ESR techniques have been applied to studies of degradation in mineralized tissues, including bone, and a review in this area has been made by Ostrowsky *et al.* [4].

Molecular damage in polymers is induced by many kinds of radiation, ultra-violet, X-ray and γ-irradiation being commonly employed. Analyses of the spectra indicate that main chain scission is sometimes the origin of the molecular damage in polymers. However the reactivity of free radicals causes them to react readily with their environment to produce secondary radicals, so that primary radicals resulting from main chain fracture are not always observed. Such is frequently the case when damage is induced at room temperature. Consequently the irradiation is often performed at liquid nitrogen temperatures and ESR spectra are obtained from specimens maintained at 77 K.

Molecular degradation in polymers can also be induced mechanically, the primary radicals produced being often attributed to main chain fracture. While it is possible that all forms of mechanical deformation produce molecular scission, the ESR instrument sensitivity limitation permits the observation of only large quantities of such scissions. A commonly used technique, to produce sufficient free radicals, is to grind the specimen. The radical concentration is known to be a function of the surface area of powder produced [5], hence the finer the powder, the greater is the observed radical concentration.

Samples of bone have been subjected to ESR examination after either irradiation or grinding, or a combination of the two. Marino and Becker [6] ground human bone at room temperature and subsequently determined ESR spectra at both 114 K and room temperature. Wide magnetic field scans from 100 to 11 000 G produced only an apparent singlet resonance at $g = 2.008$. No hyperfine detail could be resolved in the spectrum, although they noted that the resonance saturated inhomogeneously. They showed that the radical concentration increased with the time of grinding, which reflects the increased surface area of the powder as the particle size is reduced. The nature of the magnetic species responsible for the absorption was unidentified.

*On leave of absence from C.N.R. Centre for Macromolecular Physics, University of Bologna, Italy.

Becker and Marino [7] had previously separated the mineral component from the bone powder and observed that the mineral component alone gave an ESR spectrum which contained at least 3 lines. Heat treatments applied to the mineral samples showed that the spectrum was a composite, which was considered to arise from surface absorbed water and H_3O^{2+} ions. Although doubts were later expressed on this interpretation, it is interesting to note that Becker and Marino observed completely reversible changes in the spectra between 119 K and room temperature. Similar changes have been observed in the present work.

Spectra obtained after irradiation [8, 9] have shown more detail than those previously obtained after mechanical degradation. Bone is a complex composite material, the major inorganic constituents of which are hydroxyapatite and amorphous calcium phosphate (mineral constituents), and the major organic constituent is collagen. Further inorganic and organic components exist in bone which include fluoride, sodium, chondroitin sulphate, keratan sulphate and protein other than collagen. ESR spectra obtained after irradiation of bone have been attributed to a superimposition of spectra from different radicals formed in the various bone constituents. Stackowicz *et al.* [9] obtained spectra from deproteinized human compact bone and demineralized human bone, which provided a basis for analysis of the spectrum from whole bone. They were able to establish that the whole bone spectrum from irradiated samples was a composite from radicals in the separate bone constitutents, with radicals formed in the organic components (probably collagen) contributing the major part.

Stackowicz *et al.* found that the admission of air to the whole bone sample, after irradiation under vacuum at room temperature, caused the radicals attributed to the collagen to decay preferentially to those formed in the mineral phase. The same effect could also be obtained by warming the irradiated specimens while they were held under vacuum. It was found that the radicals formed in the collagen were less stable than those in the mineral phase. Irradiation of human compact bone in air at room temperature was found to produce an ESR spectrum which was attributed to the mineral content alone.

The age of the bone is a further factor affecting the spectrum obtained. Termine *et al.* [8] showed that more lines became resolved in the spectrum of developing rat femora bones with increasing age (8 to 29 days). This was attributed to an increasing mineral content. Termine also obtained ESR spectra from irradiated samples of synthetic amorphous and crystalline calcium phosphate. These were found to be complex, containing many more lines than spectra obtained from irradiated deproteinized bone samples [9]. Attempts by Termine to reconstruct the spectrum from whole bone using spectra from the synthetic calcium phosphate and collagen were unsuccessful. A prominent feature of the spectra from synthetic calcium phosphate samples and irradiated whole bone are two lines of 508 G splitting, which are attributable to hydrogen atom radicals. In certain instances, superhyperfine detail has been observed in the hydrogen atom radical spectrum [10]. Fisher *et al.* have suggested that this splitting arises from interaction of the trapped hydrogen atom with the protons of trapped water molecules; Termine *et al.* have suggested that it arises from trapped hydrogen atoms in the immediate locality of apatite hydroxide anions. Termine *et al.* found that the hydrogen atom radical decayed at about 220 K and 273 K for amorphous and crystalline apatite respectively. They hence suggested that observations of the decay of the hydrogen atom radical line could be used to assess the crystalline apatite content in any given sample of whole bone.

2. Experimental

Bovine femoral cortical bone samples were used in the present investigation, taken from slaughtered animals probably between 2 and 4 years old. The bones from which the samples were cut had been stored in air in a refrigerator. Slices of approximately 40 mm × 5 mm × 5 mm were initially sawn from the larger bones. The slices taken were anterior sections with the major dimension in the longitudinal direction. While the sawing action during slice preparation produces some mechanical degradation at the sawn surfaces, the majority of mechanical degradation was achieved by filing. Clean, previously unused metal files of different coarseness were used to obtain powders of different particle size.

Filing was conducted either in air or in a liquid nitrogen bath. The powder produced was subsequently transferred to quartz tubes at essentially liquid nitrogen temperature. For low temperature measurements, the tubes were rapidly placed in the pre-cooled cavity of the ESR spectro-

meter. A Varian E-9 spectrometer, fitted with a variable temperature attachment, was used for all measurements. Incident microwave power of 0.2 mW was used for all measurements to avoid power saturation. A modulation frequency of 100 kHz was adopted for all tests.

Further samples of the same bovine bone were prepared for comparative γ-irradiation tests. Both bone slices and powdered samples were used. The bone slices were taken as previously described, with the same orientation but were of smaller cross-section to fit into the 4 mm diameter quartz spectrometer tubes. The powder samples were prepared by filing at 77 K. All samples (sliced and powdered) were then placed in quartz tubes in a dewar containing liquid nitrogen, transported and irradiated while immersed in liquid nitrogen, and finally transferred to the pre-cooled cavity of the ESR spectrometer for examination. A dose of 2 Mrad was applied to all samples.

3. ESR spectra

Fig. 1 shows the first derivative spectra obtained from a sample filed at low temperature, successive spectra being recorded as the sample is warmed to room temperature. At 103 K (Fig. 1a, solid line) a six line spectrum is observed. The complexity of this spectrum is in marked contrast to the broad singlet observed by Becker and Marino from samples ground at room temperature. Heating to 123 K (Fig. 1b) produces little change in the spectrum, except for a decrease in its intensity. When the temperature of the sample is further raised to 223 K, a marked change in the spectrum begins to occur (Fig. 1c). The outer lines of the spectrum diminish in intensity and it changes to a predominantly two-line asymmetric spectrum. Further heating to 273 K (Fig. 1d) almost completes the conversion to the two-line spectrum. The spectrum obtained at 103 K is a composite, arising from at least two radical species.

Spectral subtraction of spectrum (d) from the initial spectrum at 103 K results in the modification to spectrum (a) shown by the dotted line. No lines are eliminated by the spectral subtraction, although the shoulder at $g = 2.003$ is smoothed out. The spectrum at 103 K could arise from only two different radical species, giving respectively the modified spectrum shown dotted in Fig. 1a and that shown in Fig. 1d. The radical species giving rise to the spectrum in Fig. 1a (dotted) is less stable than that giving rise to that shown in Fig. 1d. Although the spectrum at 103 K is a composite, it is dominated by the contribution from the radical species leading to Fig. 1a (dotted).

The first derivative spectrum obtained from samples filed in air at room temperature is shown in Fig. 2a. The spectrum is very similar to that

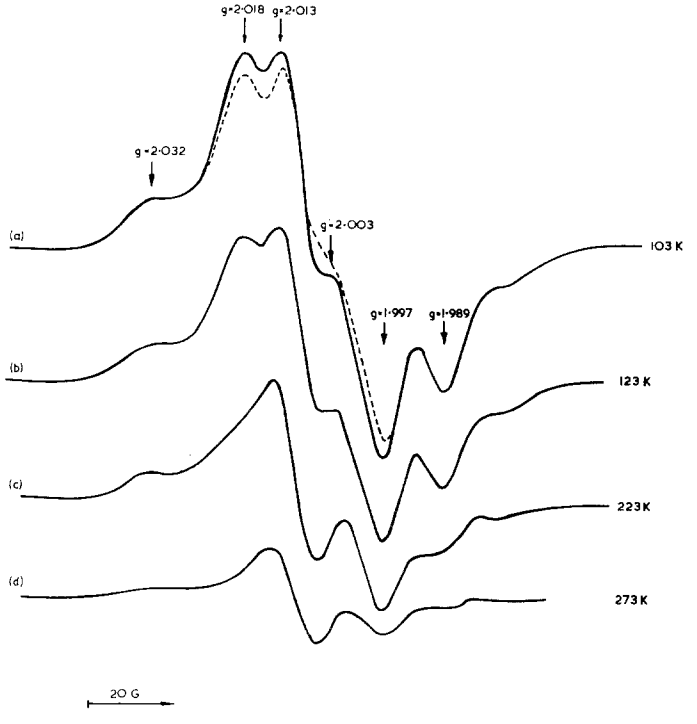

Figure 1 First derivative ESR spectra of filed bovine bone recorded at sample temperatures indicated after initial filing in liquid nitrogen. The dotted detail in (a) indicates the spectrum resulting after spectral subraction of (d).

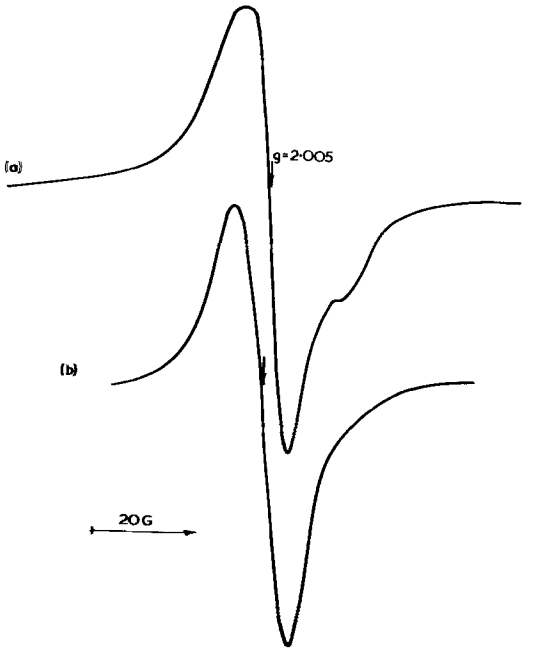

Figure 2 (a) First derivative ESR spectra from filed bovine bone, filed at room temperature in air, spectrum recorded at 293 K. (b) Sample prepared as in (a), but spectrum recorded after cooling bone powder to 103 K for 1 h.

shown in Fig. 1d. It therefore appears inconsequential whether the powder is prepared at low temperature and then heated, or prepared at room temperature: the same radical species is obtained. This species is persistent, since annealing the filed sample at 373 K does not change the form of the room temperature spectrum and does not greatly diminish its intensity.

When the samples were prepared at room temperature and subsequently cooled to 103 K, the first derivative spectrum changed to give only a singlet, with the absorption line centred on $g = 2.003$ (± 0.003) as shown in Fig. 2b. Further investigation showed that the spectrum obtained depended on the lapse of time after cooling to 103 K. When the spectra were taken immediately after cooling, the first derivative spectrum was similar to that shown in Fig. 2a and contained two lines. After the sample had remained at 103 K for about one hour, the high field line had essentially disappeared and the singlet shown in Fig. 2b then appeared. Reheating to room temperature reversed the process. The extra line at high field did not immediately reappear on rewarming, but again only built up gradually over a period of several minutes.

Identical slow reversible behaviour was observed in the spectrum from samples originally prepared in liquid nitrogen, once the samples had been heated to about room temperature (Fig. 1d). On recooling the sample to 103 K, the spectrum shown in Fig. 1d slowly converted to a singlet. On reheating to 293 K, the spectrum shown in Fig. 1d reappeared. This similarity in behaviour strongly suggests that the radicals resulting from filing at room temperature (Fig. 2a) and the residual radicals remaining at room temperature in samples originally filed in liquid nitrogen (Fig. 1d) are the same.

It would be convenient if the radical species giving rise to the spectra shown could be unequivocally identified. However the complexity of bone renders this difficult, as reference to the literature readily indicates. Previous authors have sought to identify the different radical species in the whole bone by reference to similar degradation studies on bone constituents. Hence a number of ESR spectra from bone constituents already exist in the literature for reference purposes, although these mostly arise from irradiation studies. In the next section it will be shown that spectra arising following irradiation are similar in many respects to those arising from mechanical degradation.

Fig. 3 compares the reduced spectra shown in Fig. 1a with that from irradiated demineralized bovine cortical bone (shown dotted) at 105 K due to Termine *et al.* [8]. The accuracy of the reproduced spectrum due to Termine *et al.* is in some doubt, since it has been produced by considerable enlargement of a small published diagram. However the general width of the two spectra and position of the lines show considerable similarities. Hence it is thought that the less stable radical species present after low temperature mechanical degradation also arise from the organic constitutents as in the case of irradiation, probably from the collagen.

Further comparison of the persistent spectrum obtained from mechanically degraded bovine bone at room temperature with that obtained by Stachowicz from irradiated synthetic hydroxyapatite or deproteinized human compact bone shown in Fig. 4 shows that the three spectra are very similar in form. Hence the persistent radical species arising from mechanical degradation are attributed to the inorganic constituent of the bone, which, following Stachowicz, could be the hydroxyapatite.

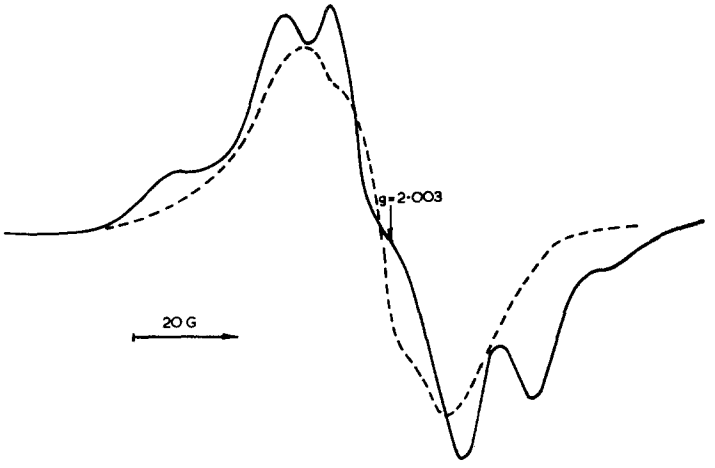

Figure 3 Comparison of first derivative ESR spectrum shown in Fig. 1a (solid line) for filed bovine bone at low temperature with that from irradiated demineralized cortical bone (shown dotted). Both spectra recorded at 103 K.

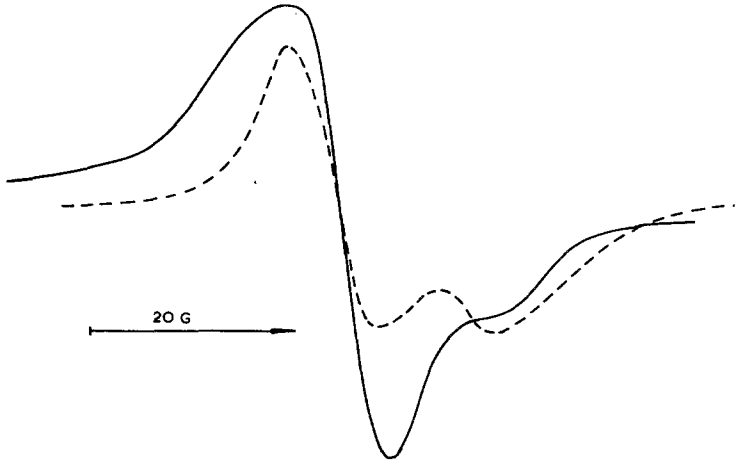

Figure 4 Comparison of first derivative ESR spectrum from mechanically degraded bovine bone at room temperature with that obtained by Stachowicz from irradiated synthetic hydroxyapatite (dotted).

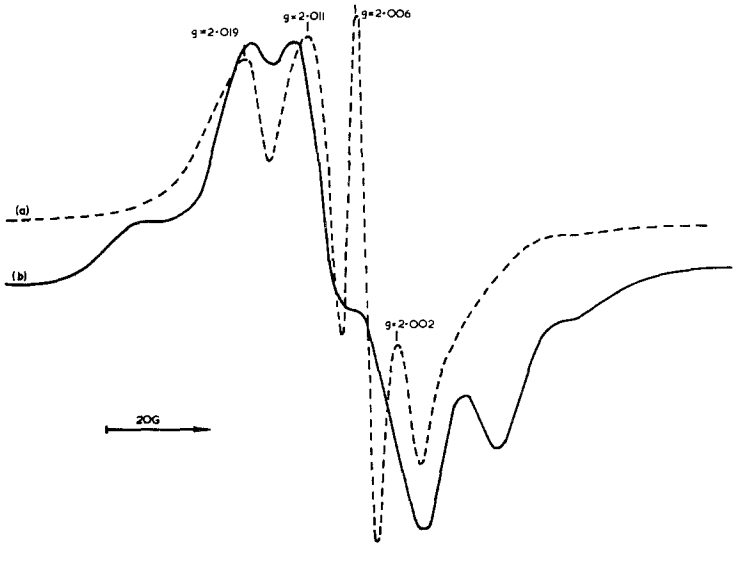

Figure 5 (a) First derivative ESR spectrum from γ-irradiated bovine bone slices after irradiation at 77 K, spectrum recorded at 103 K. (b) First derivative ESR spectrum from filed bovine bone powder after filing in liquid nitrogen, spectrum recorded at 103 K (Fig. 1a).

4. ESR spectra – irradiated samples

Fig. 5 shows (dotted) the first derivative spectrum obtained from bovine bone slices after irradiation at low temperature. Spectra shown have also been recorded at low temperature. The spectrum from the irradiated sample is compared with that from a filed sample (solid line). The base lines of the compared spectra are displaced for clarity of presentation. The spectrum obtained from the unirradiated sliced bone was very weak and the spectrum shown by the dotted line in Fig. 5 arises predominantly from irradiation damage. The spectra arising from irradiation and mechanical degradation are seen to be very similar and are thought to arise from the same radical species. The general width of the spectrum and number of lines present are thought to be the same. However, the spectra differ in detail. The outer lines at high and low field are less marked in the spectrum obtained after irradiation. Also the centre peak at $g = 2.006$ in the first derivative curve is much larger and the minimum negative slope occurs at a lower field value in the spectrum from the irradiated sample. Since the centre of the composite spectrum is affected by the persistent spectrum, all these differences would be consistent with a larger contribution to the composite spectrum from radicals giving rise to the persistent spectrum in the case of the irradiated sample. It is therefore suggested that, although radicals are formed in both the organic and inorganic constituents by both irradiation and filing, mechanical degradation at 77 K results in a relatively higher proportion of radicals in the organic components.

Filing the bone at 77 K to produce a powder prior to irradiation results in a first derivative spectrum which is shown in Fig. 6 (dotted) and compared again with that from simply filed bovine

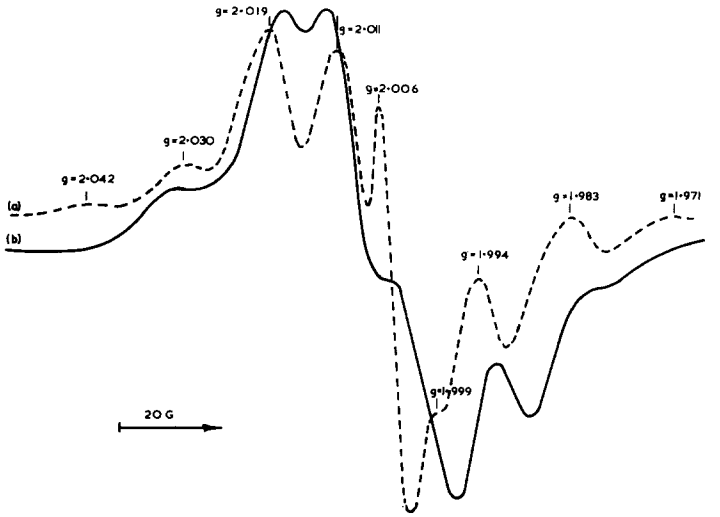

Figure 6 (a) First derivative ESR spectrum from bovine cortical bone after filing and γ-irradiation at 77 K; spectrum recorded at 103 K. (b) As (a), but sample only filed and not additionally irradiated.

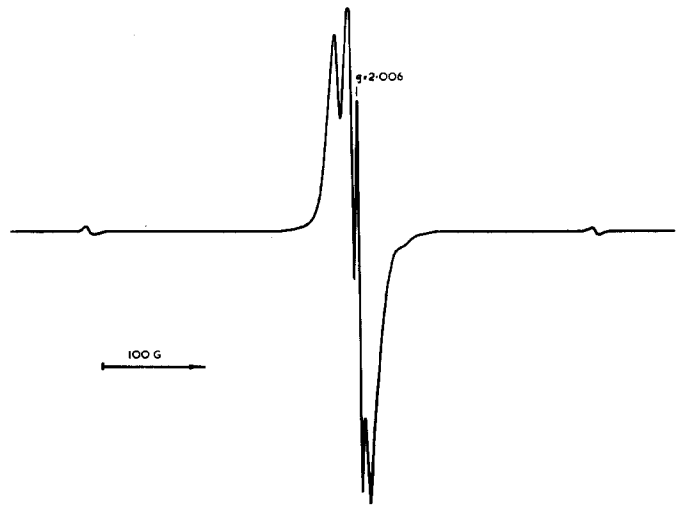

Figure 7 Full first derivative ESR spectrum from γ-irradiated bovine bone slices, showing hydrogen lines. Samples prepared at 77 K spectrum recorded at 103 K.

bone, spectra again being recorded at low temperature. Comparison of the spectra in Figs. 5 and 6 for the irradiated bone slices and filed and irradiated powder respectively reveals interesting differences. Filing the bone prior to irradiation enhances the outer lines of the spectrum and also diminishes the relative intensity of the lines in the centre of the spectrum. ($g \simeq 2.006$ and $g \simeq 2.000$). Hence Figs. 4a, 5a and 5b show respectively progressive stages of diminishing proportion of radicals giving rise to the persistent spectrum, due to the different treatments imposed on the bone.

Figs. 7 and 8 show the full first derivative spectra obtained from irradiated bone slices and irradiated powder from bovine bone, all preparations and measurements being made at 103 K. The two outer lines of 508 G splitting due to hydrogen atom radicals are clearly visible in both cases. Such lines were not observed in spectra obtained from purely mechanically degraded samples. Thus the hydrogen atom radicals are apparently a product of irradiation alone. Fisher *et al.* [10] suggest that the most likely mechanism for hydrogen atom formation is the protonation of the radiation-produced electron by water molecules and hydroxide groups in the hydroxyapatite lattice.

Further comparison of the hydrogen atom radical peaks in Figs. 7 and 8 shows that the hydrogen line in the spectrum from filed and irradiated samples is larger in comparison with the central portion of the spectrum than that found in the spectrum from irradiated sliced whole bone. Although double integration techniques have not been applied to estimate the proportion of hydrogen atom radical species present, it is clear that any such calculation must show a higher proportion present in the case of the irradiated powder sample. Earlier comparison of the spectra obtained from the two types of irradiated sample (sliced or powdered) led to the suggestion that the contribution from radicals in the mineral phase was greatest in the case of the sliced whole bone. By comparison with the results of Fisher *et al.* [10] on irradiated basic calcium phosphate, it is possible that the spectrum detail in the region of $g = 2$ arising from the mineral phase is due to the radicals PO_4^{2-}, and O^-. The hydrogen line in Figs. 7 and 8 is largest in the case of the filed and irradiated sample, when the contribution from the possible PO_4^{2-} radical is least. It seems unlikely therefore, that the hydrogen atom radical and other species in the mineral phase giving rise to the detail around $g = 2$, originate from the same mechanism. If they did, then it would be expected that their relative contributions would increase in the same sense.

Further examination of the hydrogen atom radical spectrum from the sliced and powdered samples shows that its form differs in the two cases. Fig. 9 shows the first derivative spectra for the high field hydrogen line in each case. The line from the powdered sample is 10 G wide and

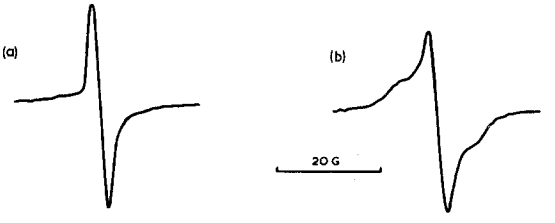

Figure 9 Detail of the hydrogen line spectra shown in Figs. 6 and 7 for (a) filed sample (b) whole bone, after γ-irradiation.

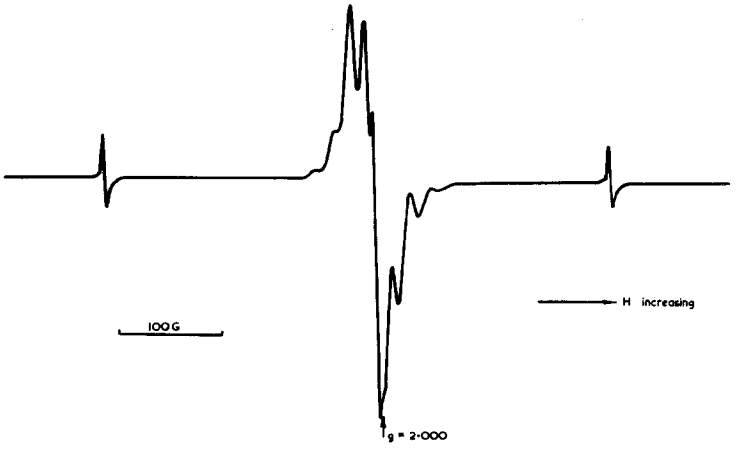

Figure 8 Full first derivative ESR spectrum from filed and γ-irradiated bovine bone, showing hydrogen lines. Sample prepared at 77 K spectrum recorded at 103 K.

contains no further hyperfine details, while that from the whole bone is 30 G wide and contains extra detail. Such similar superhyperfine detail has been previously observed by Fisher *et al.* [10] in γ-irradiated basic calcium phosphate and by Termine *et al.* [8] in *X*-irradiated human enamel and synthetic hydroxyapatite. Both Fisher and Termine used powdered or crushed samples. It does not appear, therefore, that the appearance of the superhyperfine detail only from the whole bone samples in the present experiments can be attributed merely to the structural anisotropy and orientation of the sample.

5. Conclusions

Free radicals are formed in bovine bone by both mechanical degradation and irradiation. Preliminary analysis of the complex ESR spectra obtained indicates that at least two radical species exist as a consequence of either method of degradation. The radicals giving rise to the ESR spectra have not been positively identified. At least one of the radical species is less stable, decaying at 223 K, while the remaining radical is very persistent. Identification of the radicals obtained has been attempted by comparing the spectra obtained with previously published data. On this basis, it is tentatively suggested that the less stable radical species could arise from the collagen, while the more persistent radical species is possibly associated with the hydroxyapatite. This tentative assignment of the source of radical species giving rise to the less stable and persistent spectra has been adopted in the following conclusions. There is evidence to suggest that a higher proportion of radicals are generated in the collagen during mechanical degradation at low temperature than when γ-irradiation is applied. γ-irradiation at low temperature leads to the production of free radicals similar to those produced by mechanical degradation but also produces hydrogen atom radicals. The latter are completely absent from purely mechanically degraded samples. The origin and location of the hydrogen atom radical have not been identified.

In bovine bone samples that have been mechanically degraded at room temperature, only radicals originating from the hydroxyapatite have been observed. If degradation of the collagen occurs at room temperature, the stability of radicals originating from this source must be short lived when exposed to air at room temperature. This observation is consistent with data reported by Stachowicz for irradiated human bone. The radicals formed in the mineral phase are very persistent. Heat treatment to 378 K does not eliminate them.

The structure of bone tissue is complex and it is to be expected that ESR studies of molecular damage due to degradation will also be complex. It has now been established that, at least at low temperature, and except for the hydrogen atom radical, mechanical degradation produces similar molecular damage to that from γ-irradiation, although there are significant differences in the contributions from the different radical species in the two instances.

References

1. D. CAMPBELL, *Macromol. Rev.* **4** (1970) 91.
2. H. H. KAUSCH, *J. Macromol. Sci. Rev. Macromol. Chem. C* **4** (1970) 243.
3. E. H. ANDREWS and P. E. REED, "Molecular Fracture in Polymers". Advances in Polymer Science, 27 (Springer Verlag, Heidelberg, 1978).
4. K. OSTROWSKI, A. DZIEDZIC-GOCLAWSKA, W. STACHOWICZ and J. MICHALIK, *Clin. Orth. Rel. Res.* 97 (1973) 213.
5. D. N. BACKMAN and K. L. DeVRIES, *J. Polymer Sci. A1* **7** (1969) 2125.
6. A. A. MARINO and R. O. BECKER, *Nature* **218** (1968) 466.
7. R. O. BECKER and A. A. MARINO, *ibid.* **210** (1966) 583.
8. J. D. TERMINE, I. PULLMAN and A. S. POSNER, *Arch. Biochem. Biophys.* **122** (1967) 318.
9. W. STACHOWICZ, K. OSTROWSKI, A. DZIEDZIC-GOCLAWSKA and A. KOMENDER, *Nukleonika* **15** (1970) 131.
10. M. V. FISHER, R. E. MORGAN, G. O. PHILLIPS and H. W. WARDALE, *Rad. Res.* **46** (1971) 229.

Received 28 February and accepted 19 July 1978.