## Quantitative Evaluation of the Rate of Mineralization of Induced Skeletal Tissues by the Electron Spin Resonance Technique

In previous papers a new method for quantitative evaluation of the resorption rate of irradiated bone grafts by electron spin resonance (ESR) technique was described[1,2]. This method is based on the unusual stability of the radiation-induced paramagnetic centres derived from structural defects of bone hydroxyapatite, which exhibit a single asymetric ESR line ($H_{\parallel}$-$H_{\perp} \simeq 10$ gauss; $9_{\parallel} = 1.9952$, $9_{\perp} = 2.0024$). This signal persisted without any noticeable change of shape and intensity in bone samples prepared and irradiated in our laboratory more than three years ago, and kept at room temperature in air, notwithstanding whether the samples were wet or dry. The simple shape and stability of the signal makes it quite suitable for quantitative evaluation of the spin concentration in irradiated bone with acceptable accuracy. According to our experimental data, irradiated amorphous calcium phosphates do not exhibit an ESR signal of such shape and intensity. It is known, however, that ionizing radiation evokes in bone the formation of paramagnetic species connected not only with mineral (hydroxypatite), but also with organic constituents of bone, with much higher yields in the latter case[3,4]. Nevertheless, these entities identified as collagen radicals, due to their affinity to react with oxygen, disappear completely in the presence of air within 6–10 days after irradiation. Therefore, after this period, the only ESR signal observed is that connected with hydroxyapatite defects.

The present paper demonstrates that the ESR technique, as described in[1,2], may also be useful for quantitative evaluation of the rate of mineralization of skaletal tissues.

*Material and method.* Two sets of experiments with mice and guinea-pigs were performed. In the first experiment WISH cells were grafted intramuscularly into $B_{10}$LP mice treated with cortisone, according to the method previously described[5,6]. These xenogenic grafts evoke cartilage formation, which, beginning with 9–10th day, is gradually substituted by bone tissue[5,6]. The induced tissues were taken at various time intervals after WISH cells grafting, the adjacent soft tissues were removed, while the hard tissues were weighed when wet, lyophilized, weighed after lyophilization again, and pulverized mechanically (grain size ca 100–200 μm).

In the second experiment, the allogenic bladder mucosa of a guinea-pig was grafted intramuscularly. Induced bone tissue as well as soft tissues closely connected with it were isolated separately at various time intervals after grafting, and treated in the same way as described above.

In both experiments the histological sections of induced skeletal tissues were prepared at various time intervals after grafting.

Pulverized samples placed in special ampoules for ESR measurements were irradiated at room temperature in air in a $^{60}$Co source 'Gammacell 220' with a dose of 14 Mrads ± 6%, a dose rate equalled 0.206 Mrads per 1 h. In this range only a negligible dose-dependent effect may be expected[2]. After measurements carried out at an X-band (9500 MHz) with an EPR-2 spectrometer, the recorded spectra were doubly integrated, and the spin concentration corresponding to the area of the asymmetric ESR singlet line was calculated par 1 mg of fresh tissue. The ESR measurements were performed 10 days after irradiation, i.e. when symmetric ESR doublet arizing from bone collagen disappears completely. As a reference standard, a polycrystalline 1,1-diphenyl-2-picrylhydrazyl (DPPH) in NaCl previously calibrated was used.

*Results and discussion.* In both experimental systems skeletal tissue induction was found in 100% of cases.

Two weeks after grafting of WISH cells in mice, cartilage beginning to ossify was found on the periphery (Figure 1). In the next weeks the amount of cartilage decreased in favour of bone tissue, and in 4 weeks the hyaline cartilage disappeared almost completely.

In the guinea-pig induction system, bone induction does not include prior chondrogenesis[7,8]. Bone trabeculae were found on the 14th day after grafting of allogeneic transitional epithelium (Figure 2), and their amount increased gradually. Bone marrow-like tissue was associated with induced bone 3 weeks after grafting of WISH cells was ell as transitional epithelium.

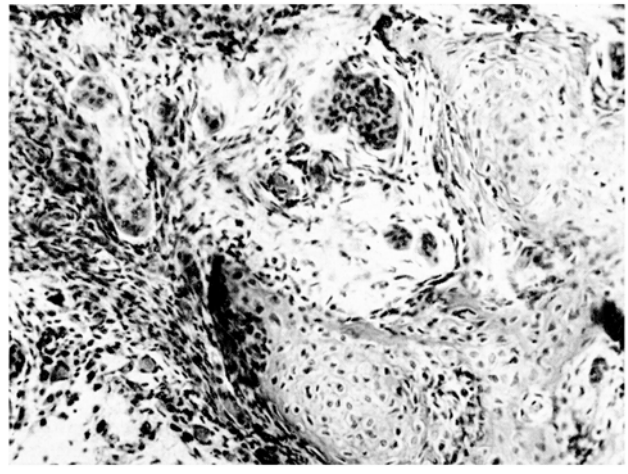

Fig. 1. The ossifying hyaline cartilage is seen 2 weeks after grafting of WISH cells in mice.

Spin concentration in induced skeletal tissues after irradiation with a dose of 14 Mrads

| Animals | Inductor | No. of grafts | Days after grafting | Average spin concentration ($\times 10^{12}$ spins/mg) in induced skeletal tissues |
|---|---|---|---|---|
| Mice | WISH cells | 44 | 14 | 7.9 |
| | | 30 | 21 | 29.1 |
| | | 18 | 28 | 38.4 |
| Guinea-pigs | Urinary bladder mucosa | 10 | 14 | 22.7 |
| | | 16 | 21 | 30.5 |
| | | 10 | 28 | 43.2 |


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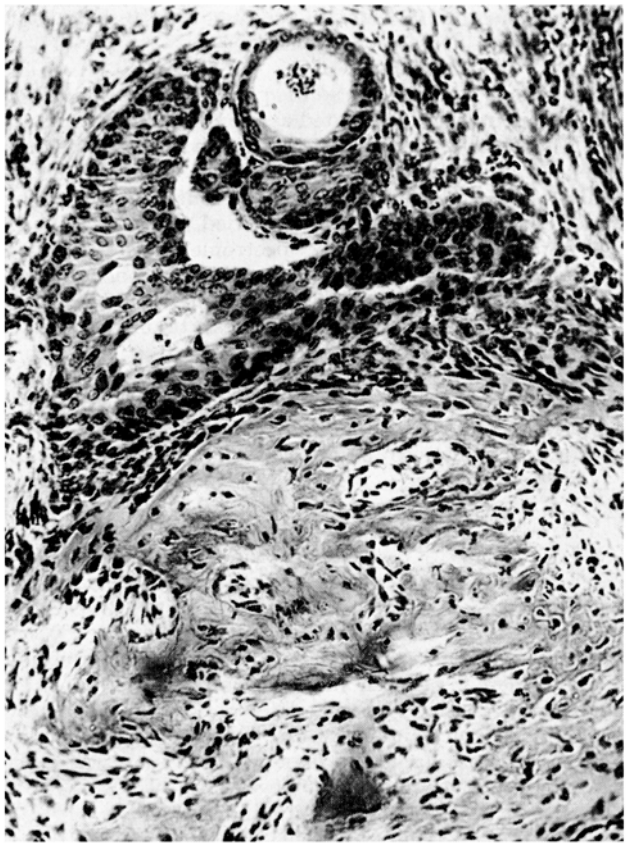

Fig. 2. Bone induction in the vicinity of transitional epithelium in guinea-pig 2 weeks after grafting.

The results obtained in the ESR study are presented in the Table. In both experiments spin concentration increases with the age of the induced skeletal tissues. There is a distinct correlation between the degree of cartilage ossification and spin concentration in the induction system of mice. The spin concentration in bone, induced in the guinea-pig, is higher than in the mouse at the beginning of the induction process (2 weeks after grafting); but 28 days after grafting, the values of spin concentration in both experimental systems are similar. The relatively small amount of bone tissue at an early stage of induction in mice after WISH cells grafting causes lower spin concentration. When ossification of the cartilage proceeds, the amount of mineralized tissue and spin concentration increase gradually, but when cartilage disappears almost completely (28 days after grafting), the spin concentration in the induced skeletal tissues in mice reaches its highest level. It should be repeated at this point that, in the guinea-pig induction system, bone trabeculae are formed without previous cartilage formation[7,8]; hence the spin concentration is higher from the beginning than in mice. In the experiment with guinea-pigs no ESR signal arizing from hydroxyapatite was ever observed in irradiated soft tissues adjacent to the induced bone.

The conclusion from this work is that, using ESR technique, one can detect and describe the kinetics of mineralization connected with induced bone formation.

*Zusammenfassung.* Es wird gezeigt, dass die entwickelte ERS-Technik auf Probleme der Mineralisation an Knochengewebe angewandt werden kann. Die Kinetik des Mineralisationsvorganges in Verbindung mit induzierter Knochenbildung wird beschrieben.

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## Protection of Chlorophyll a from Photo-Oxidation by β-Carotene in Binary Mixture of Organic Solvents

This article deals with the optical and photochemical behaviours of chlorophyll a (CHL), in the absence and presence of β-carotene (CAR), in binary mixture of organic solvents, to find some information about the interaction between both pigments, which is controlled by solvent.

When CHL soution is irradiated with the red band of CHL under aerobic conditions, CHL is bleached by photo-oxidation. Figure 1 shows the changes in the amount of bleached CHL after 30 min irradiation with the volume ratio of methanol to pyridine, + methanol $V_{me}/$, ($V_{py}$, + $V_{me}$) in the absence and presence of CAR. The bleached CHL, in free CHL solution, gradually increases with increasing $V_{me}/V_{py}$ (curve 1). However, in the presence of CAR, the photobleaching of CHL is considerably protected by CAR[1,2] (curve 2). The protection first increases with increasing $V_{me}/$, ($V_{py}$, + $V_{me}$) and reaches a maximum at the volume ratio of 0.4, then decreases to zero. This is shown in curve 3 as the difference between curves 1 and 2. Since the fluorescence of CHL is not quenched by oxygen, the singlet state is not responsible for the photo-oxidation. CHL in the triplet state may react with oxygen to be oxidized. CAR is an efficient quencher for the triplet state of CHL but not for the fluorescent state[3]. The direct transfer[4,5] of triplet energy of CHL to CAR is thought to be an important mechanism of the protective action of CAR. The triplet CAR produced reacts with oxygen or dissipates its energy as heat. On the other hand, the triplet CHL may form a triplet-triplet complex with oxygen in the triplet ground state. According to the spin conservation law, the singlet, triplet and quintet states are allowed for this complex. The singlet complex dissociates into singlet CHL and singlet oxygen,

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