An injectable material consisting of calcium sulphate mixed with hydroxyapatite was investigated as a possible alternative to autograft in the restoration of bone defects. The material was studied both in vitro in simulated body fluid (SBF) and in vivo when implanted in rat muscles and into the proximal tibiae of rabbits. Variation in the strength and weight of the material during ageing in SBF was measured. Tissue response, material resorption and bone ingrowth were studied in the animal models.

A good tissue response was observed in both the rat muscles and rabbit tibiae without inflammatory reactions or the presence of fibrous tissue. Ageing in SBF showed that during the first week carbonated hydroxyapatite precipitated on the surfaces of the material and this may enhance bone ingrowth.

Calcium sulphate has been used as a substitute for bone-graft in filling bone defects since 1892. Peltier cited the work of Dreesmann of the Trendelenburg clinic who observed complete healing in two out of three lesions with the bone defects which had been grafted with calcium sulphate. The early results were variable, presumably because of the inconsistent crystalline structure, purity and quality of the calcium sulphate. In the 1960s and 1970s Peltier and Peltier and Jones published extensive clinical data on the use of calcium sulphate to fill bone defects and concluded that calcium sulphate was a substitute which enhanced the formation of bone and, for selected indications, gave results which were comparable with autograft. Implantation of calcium sulphate into bone or soft tissue did not produce a foreign-body reaction and, by itself, did not induce bone. The formation of new bone occurred only when periosteum or bone was also present. Peltier further concluded that the presence of calcium sulphate in a wound did not inhibit the formation of bone and that it was removed from the site of implantation irrespective of whether or not new bone formation occurred. Infection in wounds containing calcium sulphate was not complicated by sequestration of the material; it either drained out or was absorbed. In the 1990s the use of calcium sulphate was gradually substituted by calcium phosphates, mainly hydroxyapatite (HA), for bone-grafting. The main reason for this change was the rapid resorption and low strength of calcium sulphate.

Hydroxyapatite (HA) is a biocompatible material with osteoconductive properties. It has been shown that the presence of HA particles promotes early bone ingrowth in a titanium-bone-chamber model. However, bone-graft substitutes consisting solely of particles or granules are mechanically weak and the particles may migrate from the site of the implant before ingrowth of new bone tissue secures them in place. By combining HA particles and calcium sulphate our aim was to obtain an injectable osteoconductive material which would give initial containment and stability.

Vitamin E is an antioxidant and a radical scavenger which may enhance the healing of fractures and stimulate bone ingrowth. We added it to the calcium sulphate-hydroxyapatite (CS-HA) material in order to increase the injectability, to create porosity-enhancing bone ingrowth and to stimulate the healing process in the defect.

We have studied resorption and the mechanical strength of calcium sulphate with HA, with or without vitamin E, in simulated body fluid (SBF) and in muscle pockets in rats. The biocompatibility and bone ingrowth of calcium sulphate, with and without HA and vitamin E, were also investigated using a rabbit bone harvest chamber (BHC) model.

Materials and Methods

Preparation of materials. α-Calcium sulphate hemihydrate (CSH) powder (CaSO₄ 1/2H₂O) (Bo Ehrlander AB,
Gothenburg, Sweden) was mixed with 40 wt% sintered HA powder (Ca_{10}(PO_{4})_{6}(OH)_{2}) (Sichuan University, Chengdu, China) with a median particle size of 5 μm. The HA powder had a Ca/P ratio of 1.67 and a specific surface area of 3.31 m² g⁻¹ in which the pore volume and the pore size were 0.012 cm³ g⁻¹ and 15.56 nm, respectively. An accelerator, calcium sulphate dihydrate (CaSO₄.2H₂O), made from the CSH was added at 0.4 wt% to the total powder mix which was then divided in two parts and 5 wt% of vitamin E added to one. The injectable materials were prepared by mixing the powders with distilled water at a liquid-to-powder ratio (L/P) of 0.25 ml g⁻¹ in closed mixing containers (Optivac, scandiMed AB, Sjöbo, Sweden). The pastes obtained were then injected into Teflon moulds in which they set. After one day the samples were removed from the moulds.

**Weight loss and compressive strength during 10 days in SBF.** We prepared 12 cylindrical specimens, 13 mm in diameter and 26 mm in height, six of which contained vitamin E (CS-HA-vitE) and the other six did not (CS-HA). All 12 samples were immersed in 20 ml of SBF at pH 7.4 and 37°C in polyethylene pots with covers. One specimen was placed in each pot so that it was completely covered by the SBF. The specimens were taken from the pots and the suspended weight was recorded in distilled water after 1, 3, 7 and 10 days. The SBF was changed every third day. At 1, 5 and 10 days two specimens of each material were compressed until failure using a universal testing machine (MTS-Bionix 858, Eden Prairie, Minnesota). The compression speed was 1 mm/min and the test was performed in air at room temperature ±20°C with respect to room temperature. Non-soaked samples were used as a control group.

**Dissolution in vitro versus in vivo.** Six cylindrical specimens (Ø = 4 mm, h = 8 mm) of the CS-HA without vitamin E were immersed for a week in SBF to study the dissolution in vitro. They were stored in polyethylene pots with covers at 37°C at a volume-to-surface-area ratio of 100 mm² mm⁻². The SBF was changed at four and six days. After seven days in the solution, the samples were removed from the pots and their size measured.

The dissolution in vivo was measured using a rat model. Sprague-Dawley rats weighing around 200 g were allowed to acclimatise for one week before the operations. All the rats were anaesthetised with peritoneal injections of 0.5 to 0.6 ml of a solution containing 1 ml of pentobarbital (60 mg ml⁻¹), 2 ml of diazepam (65 mg.ml⁻¹) and 1 ml of saline (0.15 M). Three different materials were tested: a) calcium sulphate (CS), b) calcium sulphate + 40 wt% HA (CS-HA) and c) calcium sulphate + 40 wt% HA + 5 wt% vitamin E (CS-HA-vitE). They were sterilised by gamma irradiation. Preset cylindrical rods of materials were manufactured. One rod of each material was inserted into three separate muscle pouches in the abdomen of each rat. CS was in the right pouch, CS-HA in the upper left pouch and CS-HA-vitE in the lower left pouch. There was a distance of at least 6 mm between the implants. Nine rats were used for each time period. The animals were killed by the peritoneal injection of an overdose of pentobarbital at one and four weeks. After harvesting the rods, the volume of the materials remaining in the muscle pouches were measured using Vernier callipers. Statistical analysis of the results was performed using one-way ANOVA.

**Observations by environmental scanning electron microscopy (ESEM).** Observations of the surface of the material were performed on CSH-HA by studying the samples used for in vitro dissolution before (dry) and after one day in SBF. Observations of the microstructure in the bulk of CS-HA and CS-HA-vitE were made by studying the fracture surfaces obtained by compression testing of both materials. The samples used had been immersed in SBF for ten days. All microscopic observations were performed using an environmental scanning electron microscope (ESEM; Electroscan 2020, Boston, Massachusetts) with a thermoelectric cooling stage which allowed variations in temperature of ±20°C with respect to room temperature.

**Biocompatibility test.** This was performed using six, adult, lop-eared rabbits (weight 4.2 to 4.9 kg). They were all older than six months and had closed tibial epiphyses on radiographs. BHCs (Fig. 1) were implanted bilaterally in the proximal tibiae of each rabbit. The BHC is a titanium cylinder which contains a core with a groove of size 1 x 1 x 5 mm facing the bottom of the cylinder. This is coaxial with holes in the outer cylinder, providing a continuous canal through the entire device for tissue ingrowth. When the core is removed, the tissue inside the bone ingrowth canal is uncovered and can be harvested without disturbing the surrounding bone. The chamber can be used for repeated harvesting. Formation of bone in the chamber has been shown to be sensitive to various forms of disturbance (e.g. particulate biomaterials, demineralised bone matrix and soluble factors). Two groups of materials were implanted in pairs in the BHCs in each rabbit. In the first group pure calcium sulphate (CS) was compared with CS-HA and in the second group CS-HA was compared with CS-HA-vitE. Each material was injected into the canal of the core in the BHC. Once the material had set, after approximately four minutes,
the core was inserted into the chamber. In the first group, CS was implanted on one side and CS-HA on the other side of the same rabbit. After harvesting the sides were changed for the next implantation period. The same design was used for the second group (CS-HA v CS-HA-vitE).

Tissue was harvested at three and six weeks. The harvested tissue was fixed in 10% buffered formalin, decalcified in Pereny’s solution, dehydrated in increasing concentrations of ethanol solutions followed by xylene and hot paraffin substitution, embedded in paraffin blocks, cut into sections 6 µm thick and stained with haematoxylin and eosin. Histological and histomorphometric examinations were performed on the cut sections.

The type, amount and location of bone and fibrous tissue were noted, as well as the presence of any foreign-body reaction or acute or chronic inflammation. The distance of bone ingrowth at each end of the BHC canal and the amounts of formation of new bone were measured by an imaging system (analySIS; Soft Imaging System, Münster, Germany) connected to a microscope (Olympus BX 50; LRI Instrument, Tokyo, Japan). The rate of bone ingrowth was determined by dividing the distance of ingrowth by the total length of the canal of the BHC (5 mm). The values were normalised in respect of the HA content for each material. For the materials containing 40% HA, only 60% of the bone harvest canal was available for bone ingrowth since the HA was not resorbable.

Statistical analysis was performed using Student’s paired t-test for comparison of two materials at the same time point, while an unpaired t-test was performed for comparisons at different times.

Results

Weight loss and compressive strength. The weight obtained for the two materials, with and without vitamin E, was measured in distilled water after 1, 3, 7 and 10 days of immersion in SBF. The CS-HA-vitE specimens had a lower density than the CS-HA specimens. At day 1, six samples for each material were weighed while for the other times two samples were weighed. The results showed slight increases in weight between one and three days in SBF. These increases were 2.0% and 1.7% for CS-HA and CS-HA-vitE, respectively. They continued until day 7 when the gains in weight were 2.9% for CS-HA and 2.8% for CS-HA-vitE. Then the weight decreased for both materials between day 7 and day 10, but there was still a gain in weight for both materials in respect of day 1. At day 10 the gain was 1.1% for CS-HA and 1.0% for CS-HA-vitE. No statistical analysis was performed since only two samples were weighed at each time point, but the difference was not likely to be significant.

We showed a large difference in strength between non-soaked and wet samples. Non-soaked samples of both materials had a compressive strength of 27 MPa while wet samples had a strength of 17 MPa for CS-HA and 12 MPa for CS-HA-vitE. Wetting of the materials halved their strength. During ten days of immersion in SBF, a small decrease in strength was detected. At ten days, CS-HA had a strength of 13 MPa while that of CS-HA-vitE was 9 MPa. It was notable that the specimen had changed in shape during the study since the original cylindrical shape had become conical. It should be noted that the strength was calculated by using the cross-sectional area, measured at the ends of the ends of the specimens, which remained after the soaking.

Dissolution in vitro versus in vivo. After one week of dissolution in vitro, the six CS-HA samples had a mean size of 50.3 (2.9 sd) mm³, which is the same as a mean reduction in size of 46.7 (3.0)%). The dissolution occurred in a layer-by-layer process. During the soaking in SBF thin layers of the materials sloughed off the samples and accumulated on the bottom of the pots. Similar observations were made for the dissolution in vivo.

In the rat muscles after one week slight swelling was seen in the tissues surrounding the material which were starting to fall apart. The surfaces of the implanted rods had dissolved forming a slurry around the hard core of the rod. This remaining hard core had a volume of 31.7 (3.1) mm³ for CS, which was significantly larger than those of the CS-HA and the CS-HA-vitE samples which were, 6.1 (1.5) mm³ and 9.1 (2.0) mm³, respectively (p < 0.0001) (Fig. 2).

After four weeks the tissue which surrounded the material was macroscopically normal. The CS was almost com-
pletely resorbed. The CS-HA and CS-HA-vitE were still present with slurry surrounding the rods which fell apart when touched.

**Observations in ESEM.** Of the samples dissolved in vitro after one day in SBF there was a thin layer of precipitate on the surface which consisted of small apatite crystals forming characteristic ball-formed precipitates. It adhered loosely to the sample and sloughed off in sheets when touched. Under this layer, HA particles were seen in the material. In most of the samples observed, the layer of precipitates had been sloughed off and only the HA particles were found at the surface.

The surfaces of the fracture showed that CS-HA-vitE contained large pores of approximately 50 µm in diameter in addition to the microporosity, while CS-HA was more compact. In some cases, these larger pores even formed channels in the material (Fig. 3).

**Biocompatibility test.** The ingrowth in the BHC showed formation of trabecular bone at three and six weeks in both groups. For the first group, CS v CS-HA, after three weeks in the chamber containing CS, the material was seen to be resorbed from the two entrances to the centre followed by tissue ingrowth. The bone ingrowth rate was 23% of the canal length at three weeks. The border between the material and ingrowing tissue showed no clear foreign-body reaction or acute or chronic inflammation. The ingrowing tissue contained normal trabecular bone. No fibrous tissue reaction was observed at the surface of the materials. At six weeks, most of the CS had been resorbed and connective tissue filled the canal. The amount of newly formed bone was larger than at three weeks. The rate of bone ingrowth was calculated to be 52% at six weeks. The remodelling of the new bone was ongoing. In the chambers containing CS-HA, part of the calcium sulphate was resorbed after three and six weeks, but HA particles were still present. The normalised bone ingrowth rates for CS-HA were 13% at three weeks and 25% at six weeks. No inflammatory reaction was seen. The HA particles were surrounded by new bone. The bone ingrowth distance for CS was 0.57 (0.14) mm at three weeks and 1.31 (0.37) mm at six weeks (Fig. 4). The difference in bone ingrowth between the two time periods was significant (p = 0.0010). The bone ingrowth distance for CS-HA was 0.19 (0.12) mm at three weeks and 0.38 (0.17) mm at six weeks (p = 0.0517; Fig. 4). There was a significant difference in bone ingrowth distance between CS and CS-
HA. The p values were 0.0007 and 0.0041 at three and six weeks, respectively.

For the second group, CS-HA versus CS-HA-vitE (Fig. 5), for CS-HA the bone ingrowth distance was 0.19 (0.12) mm at three weeks and 0.62 (0.52) mm at six weeks and for CS-HA-vitE 0.14 (0.14) mm and 0.48 (0.22) mm, respectively. There was no significant difference in both ingrowth distances between CS-HA and CS-HA-vitE (p = 0.4283 at three weeks and p = 0.5464 at six weeks) (Fig. 5). The normalised bone ingrowth rates for CS-HA-vitE were 9% at three weeks and 32% at six weeks.

Discussion

The difference in weight with time of CS-HA and CS-HA-vitE may be due to the lower density of the latter because of the porosity caused by the addition of vitamin E. Both materials gained weight during the first seven days rather than the expected weight loss, but with the calcium-based material in SBF there was also the creation of calcium phosphate precipitates on the surface of the material. These precipitates were formed by the reaction between released calcium ions from the samples and phosphate ions in the solution. The layer of precipitates was only loosely adherent and sloughed off in sheets with time and/or when the SBF was changed. As the sheets fell off the surface, the specimen lost weight. The same observations were made by Spinelli, Ricci and Parsons10 who studied the mass loss of pure calcium sulphate aged in SBF or water for 25 days. The reaction between calcium and phosphate proceeded until no more calcium sulphate was left, as long as the SBF was changed so that new phosphate ions were available. The Ca/P ratio of the precipitates corresponded to carbonated hydroxyapatite, which is similar to bone mineral and is reported to stimulate the bone ingrowth in vivo. Precipitation of this bone-like apatite has also been observed on the surface of chemically-treated titanium plates when immersed in SBF.11 A more rapid bone contact was obtained on these surfaces compared with samples without the apatite layer.12

The compressive strength decreased when the materials became wet. Calcium sulphate consists of needle-shaped crystals which interlock with each other and contribute to the strength of the material.13 When wet the friction between the crystals decreases, making it easier for the crystals to slide against each other and therefore decreasing the strength.14 CS-HA was stronger than CS-HA-vitE at all test points except when dry. The higher strength was due to the lower porosity. Only one sample of each material was tested in dry conditions, giving only an indication of the strength. The pores formed in CS-HA-vitE were due to the vitamin E which is a viscous fluid which tends to form droplets inside the material. Interestingly, the droplets were not always round and created channels inside the CS-HA-vitE. The advantage of the pores is that they may enhance the ingrowth of new bone tissue into the material. Vitamin E, which is an antioxidant, has also been shown to stimulate bone ingrowth5 and to improve the healing of a fracture gap.4

When comparing dissolution in vitro with that in vivo it was shown that for both methods the dissolution of the materials occurred in a layer-by-layer process. Sheets of the materials sloughed off and the size of the samples decreased quickly. The dissolution occurred faster in vivo than in vitro. In the latter it was passive, i.e., it only took place by ion exchange, but in vivo it was active, which meant that, in addition to the ion exchange, there were cells which were actively involved in the dissolution of the material. Moreover, the movement of the muscles around the pouch may have helped removal of the material. The differences in dissolution seen in the three materials implanted in rat muscles remain unexplained.

The in vivo dissolution basically demonstrated that CS, CS-HA and CS-HA-vitE showed a good tissue response. CS dissolved more slowly during the first week, compared with CS-HA and CS-HA-vitE, but was totally resorbed in four weeks. Similar results have been reported by Bell15 who implanted CS into muscles of adult dogs. He reported total absorption at 4.7 weeks. The CS-HA samples, however, lost their cylindrical shape rapidly when implanted. Parts of the material sloughed off and formed a slurry around the remaining rods. The reason for this may be that when CS is dissolved, the remaining HA particles cannot retain the contained structure. Once the material starts to fall apart body fluids and cells penetrate the materials and cause further dissolution.

In an empty BHC, bone tissue grows into the entire canal of the chamber in three weeks.9 When materials are implanted in the canal the speed of bone ingrowth is dependent on the rate of resorption of the material. Cured CS is a dense material and therefore the bone tissue grows on to the material. However, once CS is resorbed the new bone can easily grow into the canal. CS was replaced by bone faster than CS-HA and CS-HA-vitE. It has been shown that in larger defects CS resorbs too quickly, leaving an ‘unfilled space’. In materials containing HA, after CS was resorbed, the remaining HA particles remained in place and slowed down the ingrowth of new bone. The manner of bone formation is that new bone grows around the HA particles and completely integrates them into the bone tissue. Vitamin E did not show any significant effect on the bone ingrowth. The material did not cause a foreign-body reaction. There was close contact between the material and bone tissue without fibrous tissue or inflammatory reaction. Long-term studies are needed to show whether the HA may be resorbed.

The most interesting finding was that trabecular bone grew around the HA particles and completely embedded them in bone tissue. Similar observations were made by Wang et al3 and Sato, Koshino and Saito,16 reported that in rabbit tibiae after 24 weeks HA particles were surrounded by thick trabecular bone.
No benefits have been received but a commercial party will be involved in the future.

References