THE REPAIR OF EXPERIMENTAL DEFECTS IN RABBIT SKULLS
Observations after Implantation with Decalcified, Deproteinised and Deep Frozen Homogenous Whole Bone

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Implants of homogenous and especially heterogenous bone are more slowly incorporated than autografts and may be rejected. Bone has been treated in various ways in attempts to reduce the incidence of implant rejection while still retaining such osteogenic properties as it may have, thereby improving its rate and degree of incorporation. Usually pieces of bone have been either decalcified or macerated to remove the protein but there are conflicting reports about the relative efficacy of these implants.

In 1957 Ray and Holloway published the results of experiments on the repair of defects in rat skulls using homo-implants of frozen whole bone, decalcified bone and deproteinised bone. They found the best substitute for fresh autogenous bone implants to be decalcified bone, whereas none of twelve defects implanted with deproteinised bone showed more than slight healing. Other authors, however, have reported the success of deproteinised implants. Maatz, Lentz and Graf (1954) obtained successful incorporation of deproteinised implants in bone defects in dogs. Hancox, Owen and Singleton (1961) found deproteinised heterogenous implants to be well incorporated into experimental bone defects in sheep. Because there might be a species variation in the response to implants of deproteinised bone, the experiments of Ray and Holloway have been repeated, but using the rabbit. The healing of standard bone defects without any implant has also been studied as a control.

METHODS
Preparation of material—Homotransplants (one rabbit to another) were used throughout the experiments. Under aseptic conditions the ilium of a freshly killed adult rabbit was removed and trimmed free from muscular attachments and peristemum. Immediately before implantation each implant was cut up into a fine mixture of cortical and cancellous bone. Whole bone implants were preserved for from one to ninety-one days at -20 degrees Centigrade before use.

Decalcified implants were made from bone which had been preserved for from eighty-eight to ninety-three days at -20 degrees Centigrade. They were extracted for eight hours by ethylenediamine (95 per cent in water) in a Soxhlet extractor, for at least forty cycles, and further extracted with distilled water for another eight hours to remove the ethylenediamine. The implants were then used from one to sixty-four days later.

Decalcified implants were made after preservation at -20 degrees Centigrade for from thirty-eight to eighty-eight days by decalcification in ethylenediamine tetra-acetic acid (EDTA) at pH 7 for thirteen to nineteen days. No residual calcium was seen on radiographic examination. They were then rinsed in isotonic saline and used after preservation from one to ten days at -20 degrees Centigrade.

Operation—Eighteen adult rabbits weighing 1·3 to 3 kilograms were anaesthetised with intraperitoneal Nembutal. The calvarium was exposed, the epicranium was incised on each side and a circular defect five millimetres in diameter was made in each parietal bone with an electrically driven low speed dental burr. Drilling was intermittent and the bone was sponged with isotonic saline to prevent heat necrosis. The dura was exposed but not perforated. Bleeding was stopped and each defect to be implanted was loosely packed with finely divided fragments of prepared bone. The epicranium and skin were sutured. The two defects were implanted with different materials so that, to some extent, each animal provided its own control or contrast. Of the thirty-six skull defects, twelve were implanted with whole bone, nine with decalcified bone and nine with deproteinised bone; six defects were left with no implant.

Examination of results—Each animal was killed six weeks after operation and the vault of the skull was sawn off. This was radiographed and then fixed in 4 per cent formaldehyde. After decalcification
in EDTA each vault was divided along the sagittal suture and each of the two halves was trimmed to form a block of tissue surrounding the defect. Each block was then bisected along a sagittal line passing through the centre of the burl hole. The two fragments containing the halves of each defect were sectioned, one in a vertical, the other in a horizontal plane. Serial sections 5 μ thick were made and at least twenty-four sections from each, representing the whole thickness of the defect, were studied after staining with haematoxylin and eosin.

Thirty-four defects in seventeen rabbits were considered: one rabbit developed osteomyelitis at the site of each defect (one was implanted with decalcified bone, the other with whole bone), and was not used.

RESULTS

In interpreting the radiological findings it was assumed that all radio-opaque material within the margins of the non-implanted and decalcified bone-implanted defects represented newly

<table>
<thead>
<tr>
<th>Type of implant</th>
<th>Very slight</th>
<th>Moderate</th>
<th>Good</th>
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<tbody>
<tr>
<td>No implant</td>
<td>0</td>
<td>6</td>
<td>0</td>
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<tr>
<td>Whole bone</td>
<td>0</td>
<td>4</td>
<td>7</td>
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<tr>
<td>Decalcified bone</td>
<td>5</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Deproteinised bone</td>
<td>2</td>
<td>7</td>
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Very slight: Less than one-third of the defect filled with new bone.
Moderate: From one-third to two-thirds of the defect filled with new bone.
Good: Over two-thirds of the defect filled with new bone.

In no defect did repair appear complete.

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<tr>
<th>Type of implant</th>
<th>Very slight</th>
<th>Incorporation</th>
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<td></td>
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<td>Moderate</td>
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<td>Whole bone</td>
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<td>Decalcified bone</td>
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Grading was made upon the proportion of implant fragments surrounded by newly formed appositional bone and upon the amount of such appositional bone.
fragments. In some specimens the appositional growth was very slight. Multinucleated giant cells (osteoclasts) or a lymphocytic infiltrate, and sometimes both, were seen around some implanted fragments in all the implanted defects, but to a varying degree; and some absorption of the implants was apparent in every instance. The degree of incorporation of the implants, assessed histologically, is shown in Table II.

**No implant (six defects)**—These defects became filled with a loose fibrous connective tissue into which grew bone. Radiographs (Fig. 1) showed that new bone grew in to fill approximately the peripheral third of the defect in two cases and about two-thirds of the defect in the remaining four cases. Histological examination confirmed these findings. There is, therefore, in the rabbit skull a considerable power of spontaneous bone regeneration though not sufficient to restore a five-millimetre defect in six weeks.

**Whole bone (eleven defects)**—Histological examination showed that in no case was the entire defect bridged with mature bone. In four defects, graded radiologically as showing good repair, there was only moderate incorporation of the implants, with appositional bone formation limited to the peripheral parts of the defects. Radiology thus exaggerates the appearance of incorporation of these implants (Fig. 1).

Fibro-fatty connective tissue filled the defects and surrounded the implants. New bone had formed marginally and grown along the implanted fragments towards the centre (Fig. 3). Some fragments surrounded by multinucleated giant cells and lymphocytes were undergoing absorption (Fig. 4). Few osteoclasts were to be seen in those cases in which there was better incorporation of the implant. All eleven defects, however, showed overall a mixture of incorporation and of absorption of the implanted bone fragments.

The amount of new bone formed in the defects cannot be measured accurately but some comparison can be made with those defects which were not implanted and the assessment in Table II is based on this. In only three cases did it appear that the implant had promoted the healing process, whereas in one, and in certain areas of several others, the implants appeared to have delayed repair.

**Decalcified bone (eight defects)**—The histological findings in the defects filled with decalcified bone could be assessed radiologically (Fig. 2). Unlike the whole bone implants, the decalcified implants in general excited little host reaction (Fig. 5). The defects were filled with, and the implants were surrounded by, connective tissue which in many areas was largely adipose tissue. A well developed inflammatory response with a lymphocytic infiltrate around some
Figure 3—A defect implanted with whole bone, showing new bone in apposition to the implanted fragments. (Haematoxylin and eosin, × 125.) Figure 4—A defect implanted with whole bone. Osteocytes and lymphocytes surround the implanted fragments. In other areas appositional bone is seen, as in Figure 3. (Haematoxylin and eosin, × 500.)

Figure 5—A defect implanted with decalcified bone. Implanted fragments with no appositional new bone are lying in fatty connective tissue free from osteoclasts or inflammatory cells. (Haematoxylin and eosin, × 30.) Figure 6—A defect implanted with decalcified bone. This implant shows abundant appositional new bone. (Haematoxylin and eosin, × 125.)
implant fragments was present in only one of the defects. Osteoclasts were scanty in every specimen. In the five cases showing slight incorporation the decalcified bone fragments lay in fatty connective tissue and were surrounded by a single layer of flattened cells resembling inactive osteoblasts, but there was no seam of new bone or osteoid. In only three defects was there much new bone and this had been laid down in apposition to the implanted fragments (Fig. 6). The histological assessment of repair is shown in Table II. The overall effect of the decalcified implants was certainly not to promote bony repair and, as shown in five cases, they may well have delayed this process.

**Deproteinised bone (nine defects)—** Three defects showed good incorporation in new bone histologically, whereas none was classified as showing good repair radiologically (Fig. 2).

![Figure 7](image1.jpg)  
**Figure 7**—A defect implanted with deproteinised bone. New bone surrounds the implant fragments which also contain some newly formed Haversian systems. (Haematoxylin and eosin, × 50.)

![Figure 8](image2.jpg)  
**Figure 8**—A defect implanted with deproteinised bone. The implant is being absorbed and replaced by bone. (Haematoxylin and eosin, × 500.)

Histologically these sections presented a characteristic appearance. Little remained of the implants—which had been deproteinised before implantation and then decalcified during routine preparation for histological examination. The fragments appeared in the sections as tissue-free spaces containing only some amorphous eosinophilic coagulum. The skull defects were filled by highly cellular connective tissue more like that seen in the defects implanted with whole bone rather than in those implanted with decalcified bone.

Clumps of osteoclasts and a few lymphocytes were seen around some implanted fragments. Osteolysis was sometimes present in areas showing much new ossification in the host tissues and in this respect these implants resembled neither whole bone nor decalcified implants. Replacement of implant fragments by new bone was much more in evidence than in either of the other materials studied.

In general the deproteinised implants provoked a vigorous cellular response in which absorption of the implant and new bone formation occurred together (Figs. 7 and 8). Three transplants provoked very little new bone formation and may have impaired healing, but the other six implants may have assisted it.

**DISCUSSION**

The readiness with which bone will form in a variety of pathological conditions and the tendency of experimental fractures and bone defects to heal spontaneously make the
interpretation of the reaction to various "bone graft" materials very difficult. False conclusions can be drawn from limited histological analysis. In all of the present experiments there were some areas where new bone had been laid down in apposition to implanted fragments but this cannot be regarded as necessarily indicating bone induction by the graft, since in non-implanted defects considerable healing by new bone took place in the six weeks of the experiment. In those implanted defects in which healing was least it would be misleading to regard a little appositional bone formation at the periphery of the defect as indicating induction of osteogenesis by the implant, since the total new bone formed might be less than that expected had no implant been placed in the defect at all. In much published work no controls with non-implanted defects have been taken into account, and claims as to efficacy of various bone implant materials are open to criticism.

The term "bone graft" is misleading. In fresh autogenous implants a few superficial cells of the transplant may survive, proliferate and take a part in the incorporation of the transplant, but apart from this the function of the transferred bone is not to proliferate itself as a true graft, but to promote bone formation by the recipient, and in some cases to act as a temporary splint while this occurs. The term "bone implant" is more accurate.

A useful bone implant must stimulate new bone formation and should eventually be absorbed. Of the three materials studied in the present investigation none proved to be ideal. With each of the three materials there were some cases in which, by comparison with non-implanted defects, the implants seemed even to be impeding repair, but there were also others in which repair had reached a more advanced stage than that found in the non-implanted defects. It seems likely that these latter implants stimulated osteogenesis, but this cannot be proved. A method is needed whereby the amount of new bone formed in experimentally implanted defects can be assessed quantitatively.

Despite these difficulties, a dominant pattern of reaction emerged in each experimental group and some comparisons could be made. Decalcified implants tended to become embedded in a fatty connective tissue in which little bone was formed. Collections of inflammatory cells were few and osteoclasts were rare. Deproteinised implants often became surrounded by a cellular connective tissue in which much appositional new bone was formed but at the same time scattered osteoclasts were often seen, and the absorption of implanted fragments and their replacement by new bone were prominent features. The reaction to these two implant materials thus differs markedly from that occurring in rats reported by Ray and Holloway (1957). They found that negligible new bone was formed in defects implanted with deproteinised bone, whereas considerable new bone formed around decalcified fragments. They considered that, provided mechanical factors were not of importance, the best substitute for a fresh autogenous bone implant is the organic matrix of bone devoid of its inorganic salt. It is not easy to explain these different results except as a species difference between rabbits and rats.

Ray and Holloway (1957), in interpreting their results, considered that successful replacement of an implant depended on the readiness with which it could be invaded by vascular elements and they believed that the presence of inorganic salts within the implant appeared to impede the process. In the present experiment all three forms of implant material were well vascularised. At the normal epiphyses of long bones there is a continual process of vascular invasion and absorption of calcified cartilage matrix and its replacement by bone. A mineralised matrix does not here impair vascularisation and replacement by bone, and, by analogy, the mere presence of mineral salts is not likely to impair incorporation of a bone graft. The explanation for the failure of the deproteinised implants performed by Ray and Holloway must lie elsewhere. In man, implants of deproteinised bone are said by Hurley, Zeier and Stinchfield (1960) to have been tolerated and eventually incorporated in bone; but Sharrard and Collins (1961) have also reported the successful use of decalcified bone grafts in producing spinal fusion in scoliotic children.
It is indeed remarkable that bone may be treated in a variety of ways and yet, when implanted into defects in living bone, still become incorporated in new-formed bone. Chalmers and Sissons (1959) investigated the fates of fresh autografts, fresh homografts, frozen homografts, autoclaved homografts, freeze-dried homografts, deproteinised bone, decalcified homografts and freeze-dried irradiated homografts when implanted into femoral defects in dogs. They found that, in general, all were incorporated in the same way. All these preparations appeared to share, therefore, a common property which would induce bone formation. Some authors, however, have doubted the ability of any form of implant to induce bone formation by the recipient. Siffert (1955) considered that transplants served primarily as a passive scaffolding along which new bone might grow. Jarry and Uhthoff (1960) considered that what has been regarded as the osteogenetic activity of transplants was simply because of the reaction caused by the preparation of the bed for the implant. These opinions emphasise the need for the application of methods whereby quantitative assessment may be made of new bone formation in experimental defects after implantation with various "bone graft" materials. In the present investigation, by comparison of implanted with non-implanted defects it appeared possible that each of the implant materials studied had, in some cases, accelerated the rate of repair. An osteogenic effect on the part of any one implant, however, remains unproven.

**SUMMARY**

1. Experimental defects in the cranial vaults of young adult rabbits were implanted with decalcified, deproteinised and deep frozen homogenous whole bone. The experiments were similar to those of Ray and Holloway (1957) except that these workers used rats as the experimental animals. In addition, six control defects were made and not implanted.
2. All animals were killed six weeks after operation and thirty-four defects were studied by radiology and by histology.
3. All implants became surrounded by connective tissue and in all cases some new bone formed in apposition to implanted fragments. The degree of incorporation of the implants in new bone varied widely, not only between the three implanted groups, but also within each group. In general, new bone formation was greatest in defects implanted with deproteinised and whole bone, least in defects implanted with decalcified bone.
4. The fate of bone implants and the extent to which they can be said to induce osteogenesis are discussed.

I am grateful to Professor D. H. Collins for his advice and encouragement, to Mrs Pauline Glossop for technical assistance and to Mr T. L. Platts for help with the photography. The work was made possible by a grant by the Empire Rheumatism Council.

**REFERENCES**


