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AN EVALUATION OF THE USE OF STRONTIUM^{85} FOR THE ASSESSMENT OF EXPERIMENTAL BONE GRAFTS

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Studies of bone grafts have been made in experimental animals for reasons ranging from the ascertaining of the cellular changes during osteogenesis, and the nature of osteogenic induction, to the search for the "ideal" bone bank material. One of the major drawbacks to studies of bone graft behaviour has, however, been the lack of an objective and relatively simple means of assessing the degree of osteogenesis occurring in the graft. This paper reports the evaluation of a method which fulfills these criteria and allows the rapid assessment of large numbers of experimental grafts.

MATERIALS AND METHODS

Animals used in this study were inbred strain AS Wistar Rats. Grafts used were fresh or preserved cancellous bone taken from the ilium of isohistogenic rats. These grafts contained both cortical surfaces and were approximately 6×6 mm square. Fresh grafts were implanted with the contained bone marrow, but were scraped free of adherent muscle and as much periosteum as possible.

Freeze-dried grafts were prepared from iliac bone using a Chemlab freeze drying unit and were used after 7–10 days storage at +4° C. Frozen bone was stored at —20° C for up to 3 weeks. Decalcified freeze-dried bone was prepared by treating iliac bone with 0.6 N HCl for 2 days in a shaker at +4° C. The bone was then washed six times with sterile 0.9 per cent saline, followed by six washes in sterile distilled water. Freeze drying was then carried out and the dried material stored at 4° C for up to 2 weeks. No attempt to remove bone marrow was made with any of these grafts.

Grafts were implanted into a pocket made in the skin of the back of the recipient between the dermis and panniculus carnosis muscle. The skin incisions were closed by means of Michel skin clips. Each rat received from two to four grafts.

Isotope Technique

The isotope used in these experiments was Strontium chloride (Sr^{85}) (Radiochemical Centre, SOS. IP.) which was injected intravenously, or by the intra-

peritoneal route, at a dose of 8 μC/100 g body weight. Both routes were equally effective.

For evaluation of the grafts the rats were killed with chloroform after removal of 1 ml blood. Bone grafts were recovered, dissected free of connective tissue, weighed and fixed in neutral formol saline. Both ilia were removed from each recipient and weighed. A section of cortical bone from one femur was also taken and weighed after the marrow had been washed out. Blood samples, grafts in fixative and the cancellous and cortical bone samples were then placed in a Nuclear Enterprises NE 8312 Auto γ and β counter and the activity of the contained Sr^{85} counted. Background readings were subtracted from the total counts given by the bone and the grafts and these figures were then adjusted to give counts per minute per milligram of tissue. An osteogenic index could then be calculated as follows:

$$\frac{\text{ct/min/mg of graft}}{\text{ct/min/mg of ilium}}$$

Grafts, once they had been counted were then decalcified and processed for histological examination so that assessment made by use of the isotope might be checked microscopically.

RESULTS

Experiment 1—Time between administration of isotope and sacrifice

Uptake of bone-seeking isotopes occurs by a number of processes such as simple exchange and diffusion, or active incorporation into bone mineral during new bone formation (Bauer et al. 1958). Only the latter process is, however, of any value in assessing osteogenic activity of bone. Experiments have therefore been carried out in order to determine the optimal interval between isotope administration and examination so that the Sr^{85} measured would mainly be that incorporated into bone mineral and not merely present by virtue of exchange.

Seven groups, each consisting of four 3-month-old rats, were injected i.v. with Sr^{85}. Groups were killed after 4, 24, 48, 72 hours and 4, 5 and 8 days. Radioactivity present in each of the ilia was estimated. Each piece of bone was then placed in 20 ml of a 1 per cent solution of $CaCl_2$ in 0.9 per cent saline and agitated continuously for 24 hours at + 4° C. The bone was blotted dry and its radioactivity recounted. The bones were then returned to fresh $CaCl_2$ solution for a further 24 hours agitation. The Sr^{85} in the bones was then counted again. Thus each piece of bone was eluted for 48 hours. Preliminary studies had indicated that by 48 hours a plateau has been reached in the elution

Table 1. The uptake and incorporation of Strontium^{85} in the skeleton of rats at various times after injection of the isotope.

Time after injection of Strontium^{85}	No. of samples	Uptake by ilium (ct/min/mg) (±S.E.)	% isotope remaining after elution* (±S.E.)	Incorporation by ilium† (ct/min/mg)
4 hours	6	717.2 (±16.35)	57.0 (±0.67)	509.9
24 hours	8	816.6 (±27.80)	78.6 (±0.70)	697.4
48 hours	8	832.4 (±18.64)	85.4 (±0.34)	740.0
72 hours	8	858.4 (±45.10)	91.0 (±0.61)	810.3
96 hours	8	707.6 (±18.92)	92.4 (±0.51)	680.0
5 days	6	719.6 (±31.39)	95.0 (±0.30)	692.2
8 days	8	610.1 (±13.10)	–	–

* Per cent of original Sr^{85} which could be eluted over a period of 48 hours *in vitro* by CaCl.

† These data are derived from the uptake shown in column 3 corrected by per cent shown in column 4.

curve and a negligible amount of radioactivity is elutable during the third 24 hour period (48–72 h).

Strontium^{85} was rapidly taken up *in vivo* by the iliac bone during the first 4 hours after injection (Table 1, col. 3), and uptake continued to increase over the next 68 hours. The radioactivity in the bone then fell.

When the bones were eluted with $CaCl_2$ solution, the Sr^{85} was readily lost from those removed from rats killed 4 hours after injection and almost 50 per cent of the total activity was eluted (Table 1, col. 4). It was not until 3–4 days after injection that the amount of Sr^{85} which was firmly bound in the bone and consequently not eluted reached 90 per cent of the total radioactivity. It can be assumed that this "non-elutable" strontium mainly represents that which had been incorporated during new bone formation. The uptake data can be corrected by the elution data to give a value for strontium incorporation due to accretion in the skeleton (Table 1, col. 4). The peak of incorporated strontium was found to occur at 3 days. Thereafter a fall in Sr^{85} content occurred which was presumably due to resorption. On the basis of these data 3½ days was chosen as the interval between administration of the strontium and sacrifice in later studies.

Experiment 2

The previous experiment indicated that the readily exchangeable strontium formed only a small percentage of the total radioactivity in

Table 2. The uptake of Strontium^{85} and histological osteogenesis in autografts of fresh, preserved and decalcified bone grafts, 4 weeks after grafting.

Type of graft	No. of rats	Strontium index (± S.E.)	Histological evidence of new bone
Fresh	12	1.45 (±0.14)	++++
Frozen	12	0.19 (±0.01)	—
Freeze-dried	12	0.18 (±0.01)	—
Decalcified	12	0.07 (0.002)	±

Table 3. An illustration of the effect of recipient age on Strontium^{85} uptake by graft and skeleton, and on the osteogenic index.

Age of recipient at start of experiment	Sr^{85} activity (ct/min/mg) in: (± S.E.)						Osteogenic index (± S.E.)		
	Graft			Ilium					
	1 wk	2 wks	3 wks	1 wk	2 wks	3 wks	1 wk	2 wks	3 wks
7 months	612.6 (±43.7)	1895.9 (±112.6)	1295.4 (±115.8)	523.6 (±16.8)	429.0 (±18.3)	413.2 (±19.2)	1.16 (±0.08)	4.45 (±0.4)	3.13 (±0.2)
3 months	404.8 (±43.2)	1367.8 (±130.5)	1204.6 (±69.0)	783.8 (±14.7)	673.5 (±16.4)	630.4 (±16.4)	0.52 (±0.05)	2.04 (±0.2)	1.84 (±0.11)

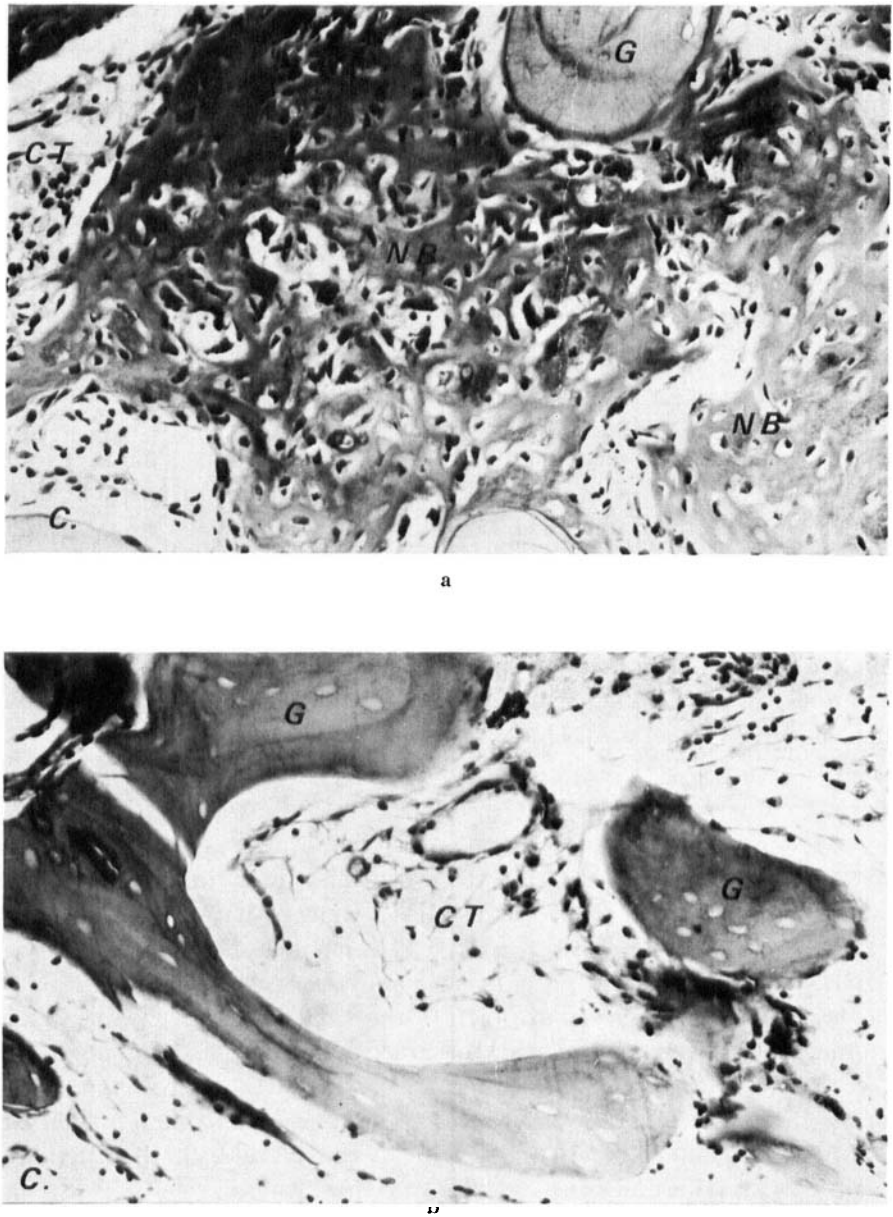

Figure 1. Photomicrographs showing a) cellular population and new bone formation in fresh iliac bone isografts after 4 weeks and b) typical appearance of a graft of frozen bone. N.B. = New Bone. CT = connective tissue. G = Old graft. (H & E + Alcian Blue, × 250).

the bone when a period of 3½ days was allowed to elapse between injection of isotope and the estimation. This experiment does not, however, indicate the extent of exchangeable strontium held in the deeper parts of the bone. "Deep exchange" may be a slow process. In order to estimate the importance of slow deep exchange, rats were grafted with living bone autografts, freeze-dried and frozen autografts and decalcified autografts. After 4 weeks the experiment was terminated, the isotope having been injected 3½ days before sacrifice. Sr^{85} uptake was estimated for each graft and expressed relative to skeletal activity, as an osteogenic index.

The results are shown in Table 2. Histologically new bone was abundant in the fresh autografts (Figure 1 a) whilst no new bone was seen in either frozen or freeze-dried grafts (Figure 1 b). Decalcified grafts contained a very small amount of new bone. The index of strontium uptake was high in the case of the fresh grafts but low in the case of calcium-free grafts. Despite the absence of new bone formation in frozen and freeze-dried grafts there was some strontium retention. This retention, which may be due to the slow "deep exchange" process, however, was small and represented only 18–19 per cent of the skeletal uptake. The fact that negligible strontium was retained in the calcium-free grafts also suggests that the "deep exchange" process does occur in intact grafts but its magnitude is not great.

Experiment 3—Effect of age of recipient on Osteogenic Index

An important consideration with this technique was found to be the age of the recipient animal. It was found that incorporation of strontium by the rat skeleton decreases sharply with increasing age after 4½ months (Figure 2). This phenomenon may introduce two artefacts into the quantitation of uptake by grafts. Firstly, as the older host will take up less strontium into the skeleton there will be more isotope available for uptake by the graft: this may lead to small differences in graft uptake in young and old recipients. Secondly, as the osteogenic index is related to the skeletal rate of new bone formation the ratio calculated in old hosts will be considerably higher than in young hosts. These effects are illustrated in Table 3.

DISCUSSION

Strontium^{85}, being a γ-emitting isotope is amenable to external counting and is, therefore, a potentially useful tracer for the assessment of

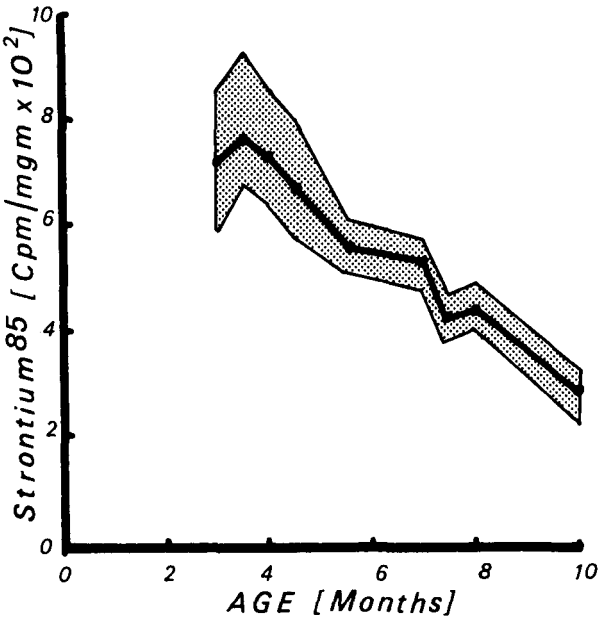

Figure 2. The mean incorporation of Sr^{85} into iliac bone of rats of different ages. Stippled region = standard deviation.

new bone formation *in vivo*. It has in fact been used by a number of workers for the assessment of malignancies affecting the skeleton (Bauer & Wendeberg 1959, Gerson et al. 1972, Kofman et al. 1963, Parsons et al. 1969), and for the study of fracture repair and inflammatory and reactive lesions in the skeleton (Bauer & Wendeberg 1959, Dymling & Wendeberg 1961).

Radioisotopes of strontium have been widely used in studies of calcium metabolism on the assumption that strontium and calcium follow similar metabolic pathways (Bauer et al. 1955, Samachson & Spencer 1961). There are, however, some differences between the handling of these two elements in the body. Thus calcium is better absorbed than strontium from the gut and is also excreted more slowly (Harrison et al. 1966). The former difference is irrelevant in the context of the present study in which the strontium is administered parenterally.

Strontium is taken up into the skeleton in two ways. The first is by diffusion and simple exchange with calcium in mature bone, while the second is by active incorporation into the mineral of newly formed bone (Bauer et al. 1958).In studies of osteogenic sarcomata it has been

shown that high levels of uptake Sr^{85} generally occurred in areas of new bone formation and not in areas of established bone (Charkes et al. 1966, Gerson et al. 1972).

In order to measure new bone formation it is essential to allow a sufficient length of time to elapse between administration of the isotope and the assessment of radioactivity in order to enable the passively exchanged isotope to become diluted. This leaves the incorporated isotope as the major countable activity. The first experiment reported above showed that this time interval, in the rat, should be three to four days. Bauer & Wendeberg (1959), using human subjects, found a higher ratio of counts in areas of new bone formation, compared with the normal contralateral bone, when the counts were made 4 or 7 days after injection of isotope than when the assessment was made two days after injection (see their case 4). Sr^{85} has been used by Goldberg & Lance (1972) in a study of bone grafts in rabbits but only 48 hours was allowed to elapse between administration of the isotope and sacrifice. Thus in this study it is likely that a significant amount of passively exchanged isotope was still present in the graft.

The method described above affords a relatively simple and objective means of assessing the state of new bone formation in bone grafts. The Osteogenic Index used in this paper was devised in order to relate the strontium bound in the graft to the rate of new bone formation in the general skeleton on a weight basis. By relating the isotope uptake of the graft to that by the host's skeleton the osteogenic index therefore enables a comparison to be made between osteogenesis at the site of the graft and that in the host skeleton. It is also possible to correct for any discrepancies in the injected dose between individuals. The technique would also be suitable for the monitoring of bone graft progress in the human subject using external counting or gamma scanning techniques.

The above data emphasise that in designing experiments careful consideration must be given to the age of the recipient. This is particularly important for long-term experiments. Skeletal osteogenesis in rats declines rapidly over the age of 5 months to 10 months. Animals between these ages should therefore be avoided as far as possible; the grafts if placed in them should not be compared with those in younger hosts, as considerably higher osteogenic indices can be found in the former.

The histological appearance of the fresh bone grafts was con-

sistent with their high strontium^{85} uptake. All of these isografts showed a profusion of new bone in the intertrabecular spaces and all but two gave Sr^{85} uptake indices considerably in excess of 1.0. In contrast no preserved graft contained any histologically detectable new bone, and none gave uptake index in excess of 0.26. Uptake levels in these grafts, however, never fell below an index of 0.13. Most freeze-dried decalcified grafts took up much lower amounts of strontium and most contained only very small amounts of new bone. These observations indicate that the strontium uptake by the frozen and freeze-dried grafts, measured after 3½ days, was due to remaining passively exchanged isotope rather than active incorporation. As this passively exchanged isotope is retained, it may be suggested that isotope reaching the deep mineral of the graft is removed again by exchange much more slowly than that exchanged into the more superficial mineral. The elution experiment described above also suggests that 3–4 days after injection of isotope about 6 per cent of the total Sr^{85} activity in the bone can still be ascribable to isotope in the exchange pool. From the above data it is clear that the amount of strontium retained in these grafts by this deep exchange is small in comparison to the amount incorporated during osteogenesis in fresh grafts. However, it should be borne in mind when evaluating grafts with low osteogenesis, and an index of below 0.30 should be regarded as negative for calcium-containing grafts.

The association of high levels of strontium uptake with grafts in which considerable new bone is present is strong evidence that the greatest part of the strontium detected in these grafts is in fact in the newly formed bone. As Sr^{85} is a gamma-emitting isotope it is unfortunately not possible to obtain direct evidence of this association by means of high resolution autoradiography. The gross forms of autoradiography employed by others using this isotope are of no value at the histological level (Gerson et al. 1972).

Methods previously used for the assessment of new bone formation in bone grafts require manipulation of the graft to render it suitable for either histological examination or biochemical analysis. The histological methods have been adapted to afford a crude score of osteogenesis (Burwell 1966, Chalmers et al. 1960, Salama et al. 1973). They are, however, time consuming and tedious, because in order to be truly representative they require the cutting of serial sections; furthermore they are subjective, and at best only semiquantitative. Fischein & Urist (1972) have used the biochemical determination of alkaline

phosphatase as an index of osteogenesis. This technique is, however, also lengthy and requires a homogenate to be prepared from the graft which is thus not available for a histological check. Furthermore it is possible that other cells within the graft, which are not involved in osteogenesis, may interfere. For example, neutrophil granulocytes in the inflammatory state usually contain elevated levels of alkaline phosphatase (Leonard et al. 1958, Wulf 1963).

SUMMARY

A method is described which permits a simple objective and quantitative assessment of new bone formation in experimental bone grafts. This method is based upon the incorporation of the γ-emitting isotope, strontium^{85}, into new bone within a graft. This isotope is easily counted using a crystal scintillation system.

Various factors have been found to be important for the application of this method. It has been found that the interval between administration of isotope and assessment of the radioactivity within the graft is important in order to avoid the masking of incorporation during new bone formation by the uptake of strontium into old bone by exchange processes. Comparison of strontium uptake in grafts of dead bone, lacking osteogenesis, with that in decalcified material indicates the existence of a slow exchange process, probably involving the deep mineral of the graft. This slow exchange, however, would account for only a small amount of the strontium retained in fresh grafts in which new bone formation is abundant. The age of the recipients must also be standardized for comparative studies.

This method, as well as being useful for quantitative studies of bone grafts in experimental animals, may also have an application for the clinical assessment of bone grafts in the human.

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