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FRACTURE CALLUS AND MAST CELLS IN RATS WITH CALCIUM AND VITAMIN D DEFICIENCY

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The effect of calcium and vitamin D deficiency on bone metabolism is not fully understood. There are several reports, however, indicating that osteoporosis can be induced experimentally by dietary calcium deficiency. Nordin (1960) showed that osteoporosis is produced in young animals by diets low in calcium. Larsson (1969) demonstrated the effects of prolonged lack of calcium on the skeletal tissue and on the blood calcium level of adult rats. It is also known that vitamin D deficiency and a diet low in phosphorus in young animals leads to pronounced bone changes comparable to those seen in human rickets. Hjertquist and his co-workers (1970) noted differences in the chemical composition of cortical diaphyseal bone in rats during vitamin D deficiency. An osteopenic condition of the bone can be induced in young laboratory animals by the administration of Shohl's diet "E" (Shohl et al. 1936), which is deficient both in calcium and in vitamin D. This diet lowers the calcium level in the blood and in bone ash, but does not affect the amount of phosphorus in the serum. Accordingly, a condition of osteomalacia and osteoporosis with secondary ostitis fibrosa is produced.

Soon after the fracture an early callus, resembling granular tissue, begins to develop. During the first stages of this process fibrocartilage is formed between and around the fracture surfaces. Cells from the periosteum, the endosteum, from the canals of Havers, and the connective tissue around the bone seem to play an active part in the formation of the early callus (McLean & Urist 1968). Fibroblasts and mast cells from the connective tissue in the callus are also important dur-

ing the regeneration process (Bassett 1962, Lindholm et al. 1967). The inner layers of the periosteum contain proliferating osteoblasts, which are activated when the fracture occurs and start building up an outer or periosteal callus and forming new bone. There are also indications that new bone can develop endosteally directly from one fragment to another (Schenk & Willenegger 1969). When long bones are fractured, the growth of callus and new bone may thus take place in several different ways.

The process of callus formation has been the subject of recent studies; Koskinen (1965) and Slätis & Rokkanen (1965) have considered histological, histoquantitative and radiological aspects, whereas Aho (1966) has done work with the electron microscope. Microradiography was used by Nilsonne (1959) to study the mineral phase of fracture repair. Tetracycline fluorescence combined with microangiography and microradiography (Hult & Olerud 1964, Slätis & Rokkanen 1967) and labelling with radioactive isotopes (Koskinen 1965, McDonald et al. 1957, Solheim 1965) have also been tried. The callus mast cell has been investigated by, among others, Lindholm and his co-workers (1967, 1969). A connection between mast cell degranulation and calcium precipitation has also been found (Selye 1967). The nature of this relationship is still obscure. Recently, parathyroid hormone has been used experimentally to stimulate mast cell accumulation in bone (Rockoff & Armstrong Jr. 1970). In osteoporosis and in hyperparathyroidism, an increased number of mast cells in the bone marrow has been found (Frame & Nixon 1968). In the present work, callus formation and mast cells in rats with calcium and vitamin D deficiency and in normal rats were studied by mast cell counts, microradiography, tetracycline fluorescence and autoradiography with 35 S isotope.

MATERIAL AND METHODS

Male Wistar rats aged 21 days and weighing about 35 g were reared as follows: One group of 25 rats was fed a standard laboratory diet (Mankans mouse and rat food, Oriola Ltd.) and water *ad libitum* to serve as controls; in another group of 30 rats calcium and vitamin D deficiency was produced by feeding Shohl's diet "E" consisting of 79 per cent corn meal, 20 per cent glutene, 1 per cent sodium chloride and potassium phosphate equal to 1 per cent of phosphorus (Shohl & Wolbach 1936).

After 4–5 weeks a moderately dislocated fracture of the right tibia and fibula was produced by manual compression under ether anaesthesia in all the rats. After fracturing, the general condition of the animals was followed closely and

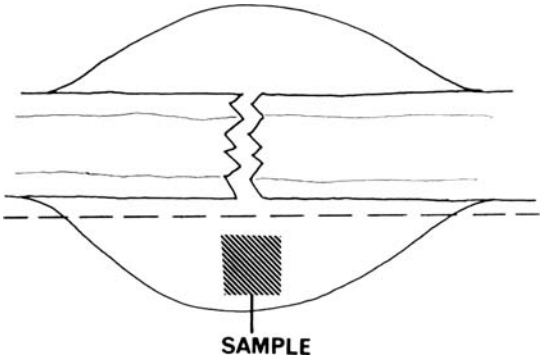


Figure 1. Schematic drawing of fracture callus showing the site of the sample used for histoquantitation and autoradiography.

weight gains and crown-rump lengths were noted each week. The healing of the fractures, with special regard to the size and stability of the callus, was also studied. The rats were sacrificed 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 15, 19, 21, 26, 28, 35 and 42 days after the fracture, their hind limbs X-rayed and the site of the fracture dissected.

From 25 normal and 30 rats with calcium and vitamin D deficiency, samples of the fracture callus were taken according to Figure 1 for a semi-quantitative mast cell count in the following manner: The samples were fixed in 4 per cent alkaline lead acetate in water for 48 hours, embedded in paraffin wax and cut in 10 μm sections. Every second section was mounted and stained with 1 per cent toluidine blue in water. From these sections the mast cells of the fibrous callus tissue were counted in 2 mm^2 (30.2 microscopic fields at 450 $\times$ magnification). The number of mast cells per mm^3 was then calculated using the formula of Floderus (1944):

$$x = n \frac{1000}{a + b + 2h}$$

n = number of nuclei per mm^2,
a = thickness of the section, in this case 10 μm,
b = diameter of the nuclei, in this case 5 μm,
h = diameter of the smallest nuclear segment found in the section, in this case 0.5 μm.

In the combined autoradiographic, microradiographic and tetracycline fluorescence investigation, 10 normal and 12 rats with calcium and vitamin D deficiency were used. They were injected 48 hours prior to sacrifice with 4 μ Ci/g body weight of 35 S in 0.5 per cent sodium sulphate as a carrier and 50 mg of oxytetracycline (Terramycine, Pfizer, Belgium) intramuscularly. The rats were sacrificed weekly after fracturing and the fracture site dissected.

Samples for micro-autoradiographs were taken from the callus lump as shown in Figure 1, were fixed in lead acetate, and the sections prepared as described above. The mounted sections were coated with Kodak AR–10 stripping film, ex-

posed for 21 days at 4°C and developed in Kodak D 19 developer according to the instructions of the manufacturers. The micro-autoradiographs were stained with Unna's polychrome blue and were mounted and examined with a high power microscope. An estimate of the amounts of 35 S-labelled substances in the mast cells of the fibrous callus during the different stages of healing was obtained by counting the silver grains over five typical mast cells in each preparation.

The rest of the callus lump with the fractured bones was then fixed in alcohol, dehydrated and embedded in methyl methacrylate. By grinding and polishing, sections of 100 μm thickness were made from each fracture and microradiographed using 22 kV, 8 mA for 30 min at a distance of 30 cm on Kodak High Resolution plates. The same sections were then used to make contact autoradiographs by pressing them against Ilford Ilfex X-ray film. The exposure time was four weeks and the films were developed in Kodak D 19 developer.

The distribution of tetracycline fluorescence in the methylmethacrylate ground and polished sections was studied in two different ways. First the sections were viewed and photographed on ordinary black-and-white film under a Hanovia (Slough, England) ultraviolet lamp. Then they were inspected in detail with a low power microscope with an Osram MBO lamp. The primary blue-violet transmitting filter was No. BG 123 mm and the secondary filter No. K–530. Blood bone marrow and blood chemistry data were also collected, but they are not published in this report.

RESULTS

The rats with calcium and vitamin D deficiency were in poor general condition, with sluggish movements and matted fell, in contrast to the naturally fed controls. Comparison of the weight gain and crown-rump length in the two groups shows (Figure 2) that the diet deficient in calcium and vitamin D inhibited growth to a large extent. Fracture healing progressed very slowly in the deficient rats, the callus lump was not palpable until eight days after the fracture, and the healing process was not completed even in 42 days. The new bone was fragile and, clinically speaking, the fracture was in a state of delayed union (Table 1). In the series of X-ray photographs a marked difference in the degree and rate of mineralization was noted. The rats with calcium and vitamin D deficiency showed generally a much smaller callus and undermineralized bones (Figure 3).

In the sections with toluidine blue, fibrous callus mast cells could be demonstrated in both types of rats during the entire period of fracture healing. Mast cells from normal rats were strongly granulated, but in the animals deficient in calcium and vitamin D these cells mostly had a degranulated appearance and only a few cells carried sparse granules.

In the callus of normal rats the mast cell count reached a maximum

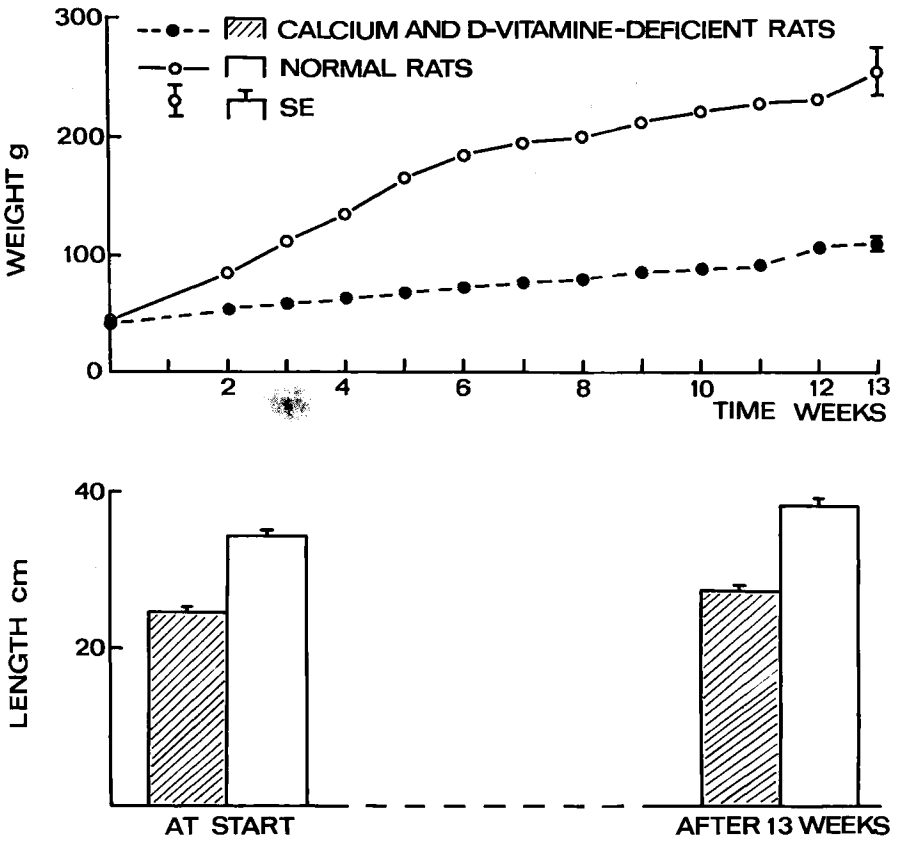


Figure 2. Body weight and crown-rump length of normal rats versus rats with calcium and vitamin D deficiency.

Table 1. Progress of fracture healing in normal versus calcium and D-vitamin deficient rats.

Treatment	Days of first appearance				
	Metachromasia in ground substance	Cartilage, confirmed histologically	Bone, confirmed histologically	Callus lump palpable	Consolidation studied by manipulation and X-ray
Control diet	2	2	5	4	21
Calcium and D-vitamin deficient diet	3	5	5	8	42

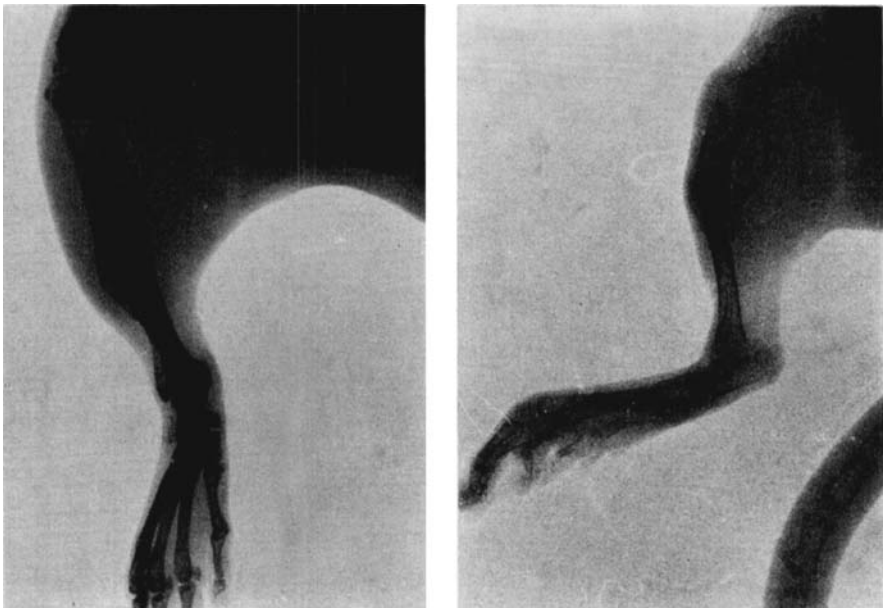


Figure 3. Radiographs of healing bones 35 days after the fracture. The normal rat (left) exhibits a consolidated fracture with prominent callus, in contrast to the unmineralized callus with no consolidation of the fracture seen in the deficient animal (right). A difference in the dimensions of the leg bones is also evident.

of 4000 cells per mm^3 two weeks after the fracture, i.e. immediately before the start of gross mineralization. In the rats with calcium and vitamin D deficiency the mast cell count remained at 2–300 cells per mm^3, and not until the 35th day did it reach about 1900 cells per mm^3. At this stage the fracture was not yet stable. The rise in the mast cell count was subsequently followed by mineralization. The mast cell counts in the fracture callus of the normal rats and those with calcium and vitamin D deficiency are shown in Figure 4 as a function of the time after the fracture.

Isotope 35 S is taken up by the mast cells of the callus during all stages of fracture repair (Figure 4). Silver grain counts as an indication of 35 S-labelled substances correlated with the total number of mast cells in normal callus. No rise was observed in the 35 S uptake by the mast cells of the animals with calcium and vitamin D deficiency despite finally rising cell counts (Figure 5).

The results obtained by microradiography, 35 S autoradiography and tetracycline fluorescence from series of methacrylate sections of

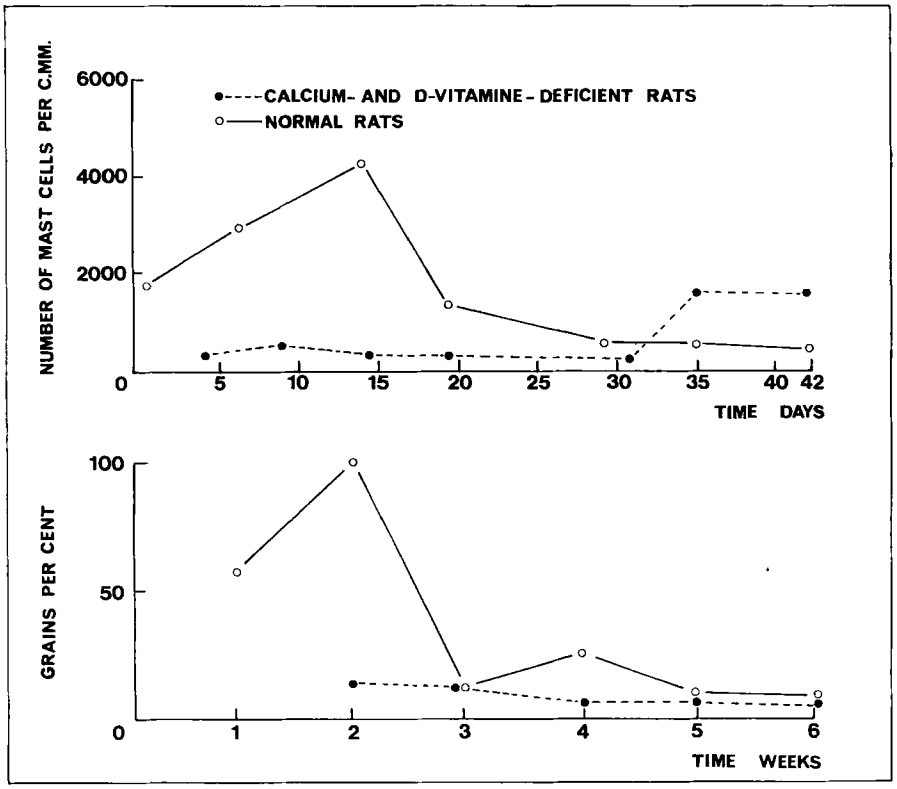


Figure 4. The number of mast cells in fracture callus and their uptake of 35 S as a function of the time since the fracture. Isotope uptake was estimated by grain counts and is shown in per cent of the highest number noted.

the entire fracture sites are summarized in Figure 6. In evaluating these photographs it must be noted that roentgen density results from calcium crystals in bone and callus, while tetracycline tends to accumulate in tissue subsaturated with calcium ions just before crystallization starts.

35 S is mainly found in sulphated mucopolysaccharides of the fibrocartilaginous tissues characteristic of the organic phase in fracture repair. Fracture healing is much slower in rats with calcium and vitamin D deficiency which have plenty of fibrocartilaginous tissue components in the callus as late as 5–6 weeks after the fracture.

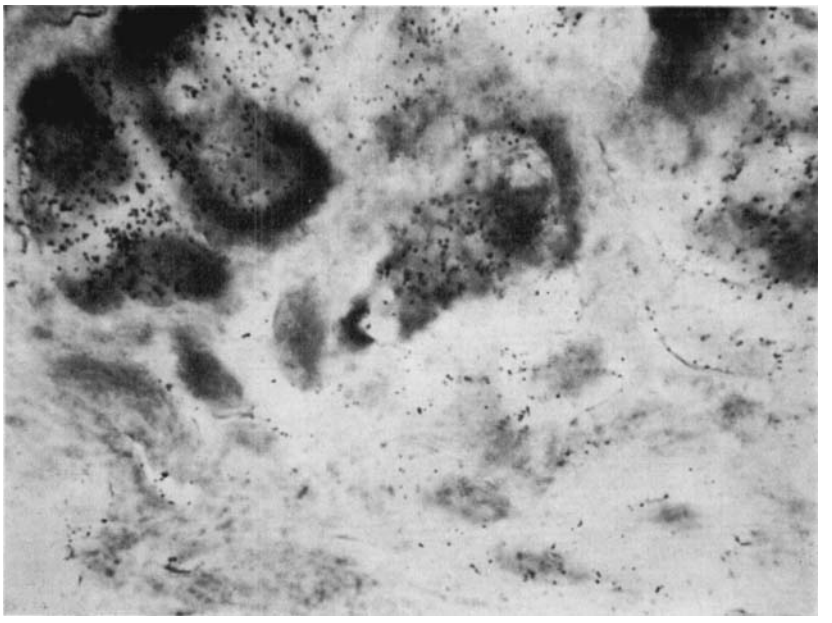


Figure 5. (Left).

Figure 5. Microautoradiographs of mast cells in fibrous callus tissue 15 days after the fracture. In normal rats (left) the mast cells are granulated and show heavy uptake of 35 S isotope. The animals with calcium and vitamin D deficiency (right) have degranulated mast cells with almost no uptake of isotope. (800 ×, Unna's polychrome blue.)

DISCUSSION

The content of hexosamines in fracture callus increases during the early stage of fracture healing (Bolognani 1961). An abundance of glycosaminoglycans, hyaluronic acid and chondroitin sulphates is typical during this stage. The synthesis of collagen starts later in the process of repair (Antonopoulus et al. 1965, Solheim 1965). Solheim & Fransson (1966) found chondroitin sulphate to be the main constituent of the callus on the seventh day after the fracture.

Metachromatically staining tissues rich in glycosaminoglycans and especially chondroitin sulphates readily incorporate 35 S (Balazs 1965). Accumulation of S 35 at the fracture site during the early phase of healing was noted by McDonald et al. (1957) and Singh & Upuda (1964). On the contrary, no uptake was observed by Duthie & Barker (1955). Active 35 S uptake by the chondroblasts of fracture

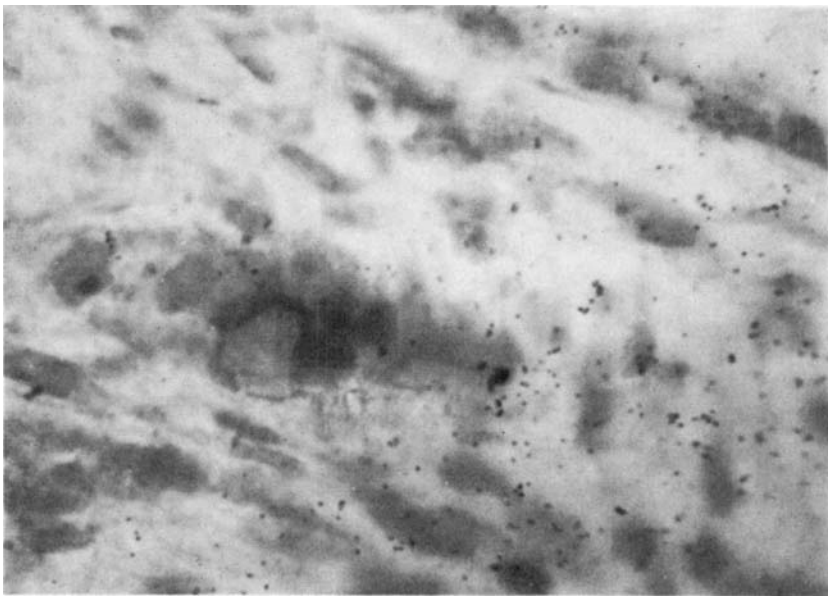
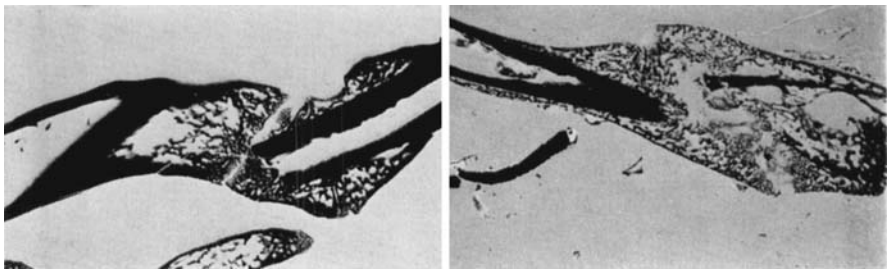


Figure 5. (Right).

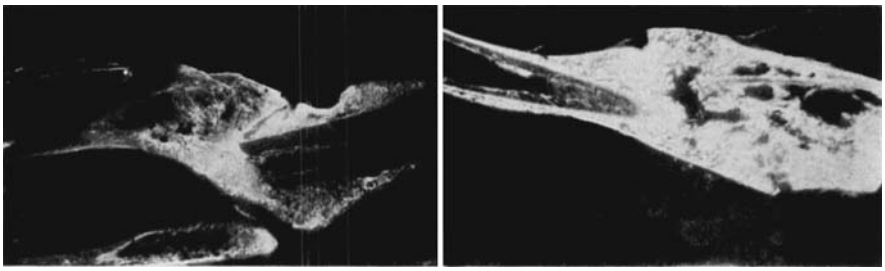
callus was demonstrated by Singh et al. (1961). The isotope is probably incorporated in glycosaminoglycans synthetized by these cells (Howell & Carlsson 1964). Sulphated glycosaminoglycans in the ground substance are thought to be related to mineralization of bone (Balazs & Rogers 1965), and it has been observed that synthesis of chondroitin sulfate takes place in immediate contact with calcifiable material (Belanger 1954). According to Solheim (1965), a depolymerization of glycosaminoglycans probably occurs before calcification starts.

The mast cell synthesizes and/or stores glycosaminoglycans (Asboe-Hansen 1964, Jorpes et al. 1937). Mast cells from the peritoneal fluid of the rat have been found to contain chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and heparin (Horsfield 1966). Callus mast cell count studies in accelerated, normal and delayed fracture healing in rats have shown that maximum values as a rule precede the mineral phase and consolidation of the fracture (Lindholm et al. 1969). The isotope 35 S is also taken up by the callus mast cells, especially during the early phase of fracture repair (Lindholm et al. 1971). The mast cell also seems to be metabolically related to calcification; Selye (1965) showed experimentally that mast cell degranula-

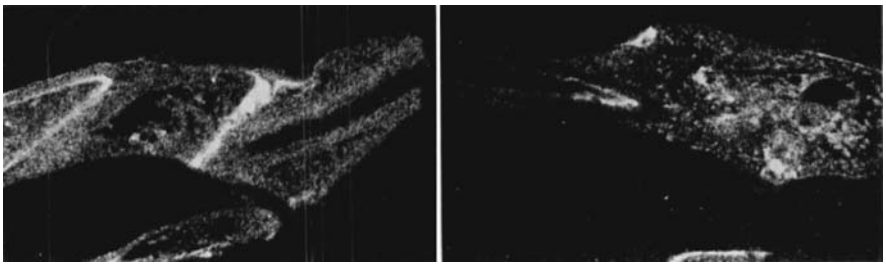
Normal rats. *Calcium and D-vitamin deficient rats*



Microradiography



Tetracycline fluorescence



35 *S autoradiography*

Figure 6. Microradiographs, tetracycline fluorescence and autoradiographs from sections of fracture sites in normal rats (left) versus rats with calcium and vitamin D deficiency (right). The normal rat was sacrificed 35 days, the deficient animal 42 days after fracture. The microradiographs show in the normal rat a remodelling fracture with dense trabeculated bone and only a narrow interfragmentary zone. The deficient animal still has a large fracture gap and partly trabeculated bone. The tetracycline fluorescence coincides in the normal rat with the endosteal zone, but in the deficient animal includes all parts of the callus lumps. The 35 S uptake in the normal rat is also restricted to the endosteal zone, but appears to be more widely dispersed in the osteopenic animal.

tion causes calcium precipitates in the surrounding tissues under certain conditions. This has led to the hypothesis that callus mast cell products act as carriers of calcium during the mineral phase of bone repair (Lindholm et al. 1969).

Calcium and vitamin D deficiency has several effects on bone metabolism (Larsson 1969, Sevastikoglou et al. 1970). A diet deficient in calcium and vitamin D (calcium:phosphorus ratio 1:16) causes osteopenic changes in the bone matrix and marrow of long bones and increases the number of mast cells, especially in the endosteum in contact with osteoid tissue (Urist & McLean 1957). On the whole, several types of cells are involved in the formation of new bone and in preparing the ground substance for the deposition of minerals (Weidman 1963).

Clues to the role of the mast cell in relation to mineral exchange and induction of bone remain to be traced at the electron microscopy and histochemical levels through studies concerned with its manifold intracellular syntheses and complicated granule-extrusion mechanism.

SUMMARY

Rats with calcium and vitamin D deficiency were examined physically, roentgenologically, by microradiography and by tetracycline fluorescence for early fracture callus. The number of mast cells and their uptake of 35 S was noted.

In calcium and vitamin D deficiency bone repair was delayed and large masses of fibrocartilage remained as components of callus as late as five weeks after the fracture. The callus mast cells were found to be degranulated, and they did not take up 35 S-labelled substances as did the mast cells in the callus of the control animals before consolidation of the fractures.

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