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Formation of New Bone in Osteolathyrism

An Autoradiographic Investigation, Using S^{35} and H^3 Thymidine

By

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With 4 Figures in the Text

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Intoxication with the lathyrus factor causes extensive damage to the mesenchymal tissue. In the long bones of the limbs, especially the humerus and the femur, rapidly growing osteophytes arise at the sites of muscle attachment and produce grotesque deformations. Bergquist and Hulth showed that these osteophytes are formed as comparatively straight spiculae in the originally very irregular proliferations of pre-osteoblasts. These spiculae are formed around parallel capillaries, which are directed toward the surface of the corticalis. Thus they are similar to the periosteal callus which forms after fractures. It may be of interest to use autoradiography to investigate the synthesis of chondroitin sulphuric acid (S^{35}) in the matrix and also to try and follow, by H^3-thymidine labelling, the cell divisions of pre-osteoblasts and the conversion of these cells into osteoblasts, osteocytes and osteoclasts.

By means of H^3 thymidine we obtain a labelling which exclusively reflects the DNA synthesis in the cell nuclei. Young considers that the pre-osteoblasts (= osteoprogenitor cells) lose their capacity for cell division when they are "modulated" to form the specialized osteoblasts. In animals killed an hour after the thymidine injection he could not find any osteoclasts or osteocytes which incorporated the isotope, and labelled osteoblasts were rare. The DNA synthesis and its result, mitosis, occur chiefly in cells with a less specialized morphology.

In this investigation I allowed different intervals to elapse between the injection of the thymidine or the S^{35} and the killing of the animal, in order to try and ascertain the time which osteoclasts and osteocytes take for labelling and to what extent S^{35} is metabolized by the different cell types.

Material and Methods

Two series of young rats, weighing about 50 g each, were used (about 50 animals in all). Both series received 0.1% amino-acetonitrile (AAN) in their drinking water. Five controls in each group were given ordinary water without AAN. The animals were killed with ether (two experimental animals and one control at a time) at different times from 1 to 19 days from the beginning of the experiment. The first series of rats received S^{35} in the form of $Na_2S^{35}O_4$ from the Amersham Radiochemical Centre in a dose of 5 microcuries per g body weight and for each interval after the beginning of the experiment two of the animals received S^{35} 4 days before death and two 2 hours before death (cf. Engfeldt and Westerborn).

H^3 thymidine was given to half the animals 2 hours before death in a dose of $^1/_2$ microcurie per g body weight. The remaining animals received this dose at the following times before death: 3 days (6), 7 days (1) or 10 days (2). The controls were given the dose 2 hours or 3 days before death.

After the animals had been killed and an X-ray photograph taken, the humerus and femur were dissected out on both sides. They were fixed in formalin, decalcified in EDTA solution and, after being embedded in paraffin, cut into sections 5 μ thick. Most of the preparations

were pre-stained with hematoxylin before stripping film was laid on them. Some of the S^{35} preparations were left unstained. The film on the preparations was exposed for 4 weeks (S^{35}) and 6 weeks (H^3 thymidine). In addition the usual hematoxylin sections were made in series with the autoradiographic sections.

Results

Between the 3rd and 4th day after the beginning of the experiment a diffuse carpet of cells is developed in the periosteum and this forms from the surface of the corticalis — at first without affecting it — spiculae which continue to grow. By degrees there is resorption of bone basally among the spiculae through the osteoclasts, which are to be found in great numbers at the edge of the capillaries, which are widened into sinusoids. In front of the spiculae there is throughout the diffuse carpet of osteoprogenitor cells, separated from the musculature by a fibrous layer (Fig. 1b).

Autoradiography with S^{35}. S^{35} which is given 2 hours before death produces a wholly diffuse labelling of the area of the osteoprogenitor cells, while the area of bone formation is not labelled. These preosteoblasts are obviously able to metabolize the sulphur to chondroitin sulphuric acid. However, it is impossible to determine in this dense carpet of cells whether or not the S^{35} occupies an intracellular position from the start, as in the chondrocytes (ENGFELDT et al.), although certain areas in the preparation indicate such an intracellular processing of the sulphur (see Fig. 1a). When S^{35} is given 4 days before death, we find, as Fig. 1c shows, that the labelling is almost exclusively confined to the matrix of the bone trabeculae in the zone which was being formed when the S^{35} was administered. This difference between the autoradiographs when S^{35} was given 2 hours or 4 days before death was the same whether the intoxication with AAN was of short or of long duration.

Autoradiography with H^3 Thymidine. On the *3rd day after the beginning of the experiment* an increased number of labelled cells were observed in the periosteum and naturally a large quantity of labelled hematopoetic cells in the marrow spaces. The latter cells seemed equally numerous in the controls. After 5 days' supply of AAN, the proliferation had increased further and here and there incipient bone formation was seen. Only the osteoprogenitor cells were labelled, not the fully formed osteocytes in the bone islets from animals labelled 2 hours before death.

In preparations from animals with more advanced osteolathyrism, labelled 2 hours before death, there was a high degree of labelling of osteoprogenitor cells in the proliferation zone and between the bone spiculae right down to their bases (see Fig. 2a from the proliferation zone). In all these cell nuclei the number of grains was approximately 50 and more with this short-term labelling, which seems to be at least the normal number in the course of DNA synthesis before mitosis in these cells. In many places there was also a similar labelling, of cells which, considering their location, were apparently osteoblasts. These cells were closely connected with the newly formed bone (Fig. 2b). On the other hand, no osteocytes or osteoclasts were labelled.

On examination *3 days after the administration of H^3 thymidine* there were still large numbers of osteoprogenitor cells. The number of grains in these, however, was usually less than before — about 20—25 — indicating that at least one cell

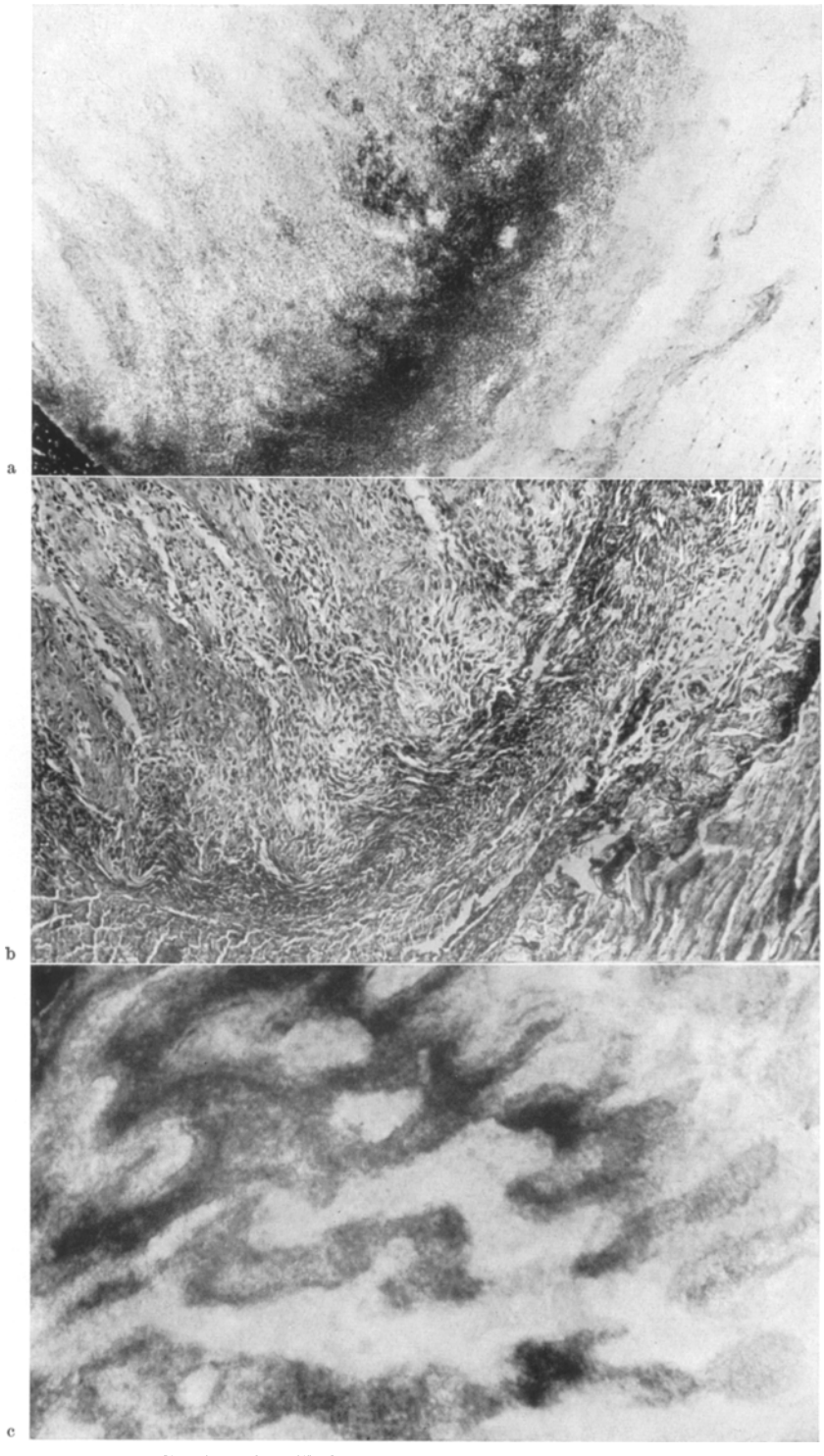
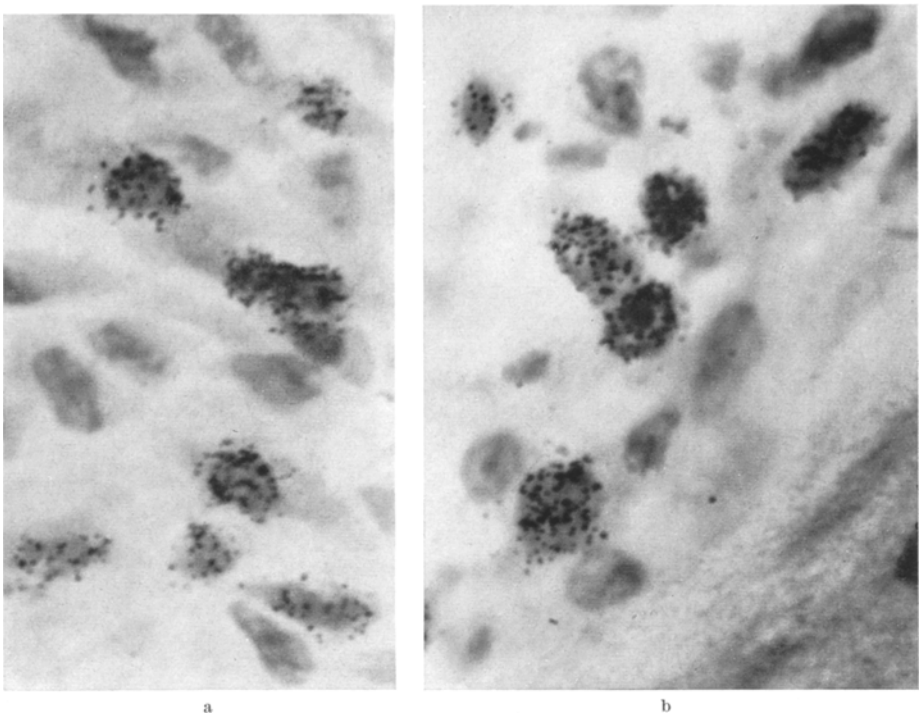


Fig. 1. a and c are radioautographs with S^{35} administered 2 hours and 4 days before death resp. The microphotograph b shows the bone spiculae of an osteophyte to the left and in front of them the zone of pre-osteoblasts. At the top in the right part the muscle is seen. At short-labelling the radiosulphate is mainly situated in the zone of pre-osteoblasts (in the middle of the picture) probably intracellularly (a). 4 days after administration the radiosulphate is seen in the matrix of the new formed bone trabeculae (c)



a b

Fig. 2a and b. Radioautographs with H^3 thymidine 2 hours after injection. Both the pre-osteoblasts (a) and some of the osteoblasts (b) are labelled

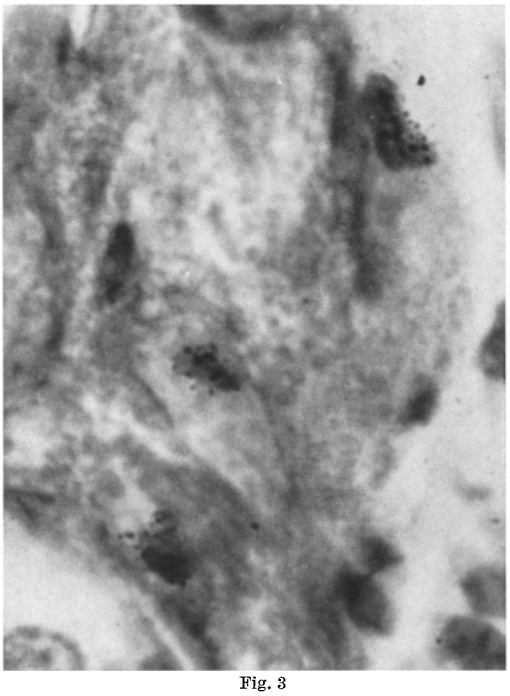


Fig. 3

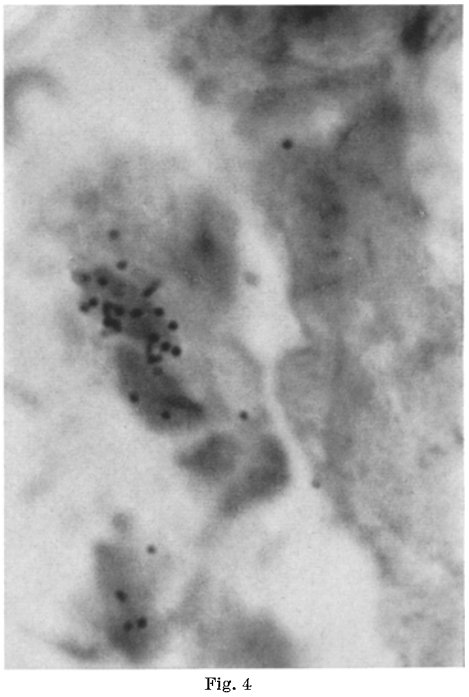


Fig. 4

Fig. 3. Radioautographs with H^3 thymidine. 3 days after administration the osteocytes of the new formed trabeculae are labelled

Fig. 4. Radioautograph with H^3 thymidine with a labelling time of three days. Here a multinuclear cell, apparently an osteoclast, is labelled

division had taken place. There were now plenty of labelled osteoblasts and even some labelled osteocytes enclosed in lacunae (Fig. 3). The number of grains in these varied but remained between 25 and 50, indicating that not all of them had undergone cell division after the pre-osteoblast phase. In preparations from animals labelled 7 days and 10 days before death, there were fewer, labelled, free, osteoprogenitor cells, but there were still some. There were large numbers of labelled osteocytes in the bone trabeculae. As regarded osteoclasts, there were none labelled in the 2-hour preparations, nor in those with the later labelling times of 7 days and 10 days. On the other hand, a small number could be found in the 3-day preparations (Fig. 4). On the histological preparations from cases of advanced lathyrism there could be observed different phases of osteoclast formation, which seemed to take place *via* osteoprogenitor cells, which combine to form these polynuclear cells.

Discussion

Autoradiographs, using S^{35} and H^3 thymidine, were made from rats with osteolathyrism, the isotopes being administered either 2 hours or 3 or more days before death. It could then be seen that the radio-sulphate taken up into the osteogenic field in short-term labelling, i.e. in or around the osteoprogenitor cells, was situated 4 days later in a belt right across the fully formed bone trabeculae in the bone matrix. The radio-sulphate is probably first taken up intracellularly in the pre-osteoblasts, before being distributed in the matrix round the finished osteocytes, which is in agreement with earlier investigations (ENGFELDT et al. or KENNEDY et al.).

Young has shown that *in the main* the osteoprogenitor cells are capable of division but not the osteocytes or osteoblasts. In rapidly growing bone, however, he found labelled osteoblasts with short-term labelling with thymidine, though comparatively rarely. From this he concluded that, as soon as the specialized osteoblast is formed, it loses its capacity for division. This change in the mother cell results in an increase in nuclear and cytoplasmic RNA and means that the cell gains the capacity to produce specific proteins (especially collagen), which the pre-osteoblast cannot do. He considers that this transformation is reversible and therefore calls the process "modulation". OWEN et al., on the other hand, found that about 4% of the osteoblasts were labelled 1 hour after the injection. In osteolathyrism in the particularly rapidly growing bone of young rats there were some labelled osteoblasts 2 hours after the thymidine had been administered. It is also possible to find large numbers of osteoprogenitor cells labelled 10 days after the injection of thymidine, which is also in agreement with the findings of OWEN et al. OWEN et al. were able to show that thymidine can be taken up by bone-forming cells without concomitant cell division. Thus, for example, these authors found both osteoblasts and osteocytes with full labelling, indicating that they had not undergone any cell division. The uptake of thymidine in highly differentiated cells is considered by PELC to be dependent on a renewal of DNA which has a connection with the cell function but not with mitosis, but it is not known what this connection is. In any case, the cell morphogenesis in new bone formation in osteolathyrism does not differ from that in other kinds of rapid new-bone formation, as regards the behaviour of radiosulphate and H^3-thymidine.

As regards the formation of osteoclasts, YOUNG takes the view that these are mainly formed from pre-osteoblasts without any intermediate phase, i. e. *via* some other cell, such as an osteocyte or osteoblast. TONNA et al. considered it most likely that osteoclasts are formed as a result of the fusion of osteoblasts. They observed labelling in osteoclasts 36 hours after the injection of thymidine, and labelled osteoclasts had disappeared ten days after the injection of isotope. This is in close agreement with my findings of thymidine-labelled osteoclasts three days after the injection of the isotope. The osteoclasts were not labelled if a longer period was allowed to elapse between the injection of the isotope and death, which may indicate that they are non-dividing cells and have a relatively short life.

Summary

The author has investigated the formation of new bone in the periosteum of rats under amino-acetonitrile intoxication, using autoradiography with S^{35} and H^3 thymidine. In short-term labelling with radio-sulphate the isotope is found in the diffuse carpet of cells consisting of pre-osteoblasts, but if a longer period is allowed to elapse between the sulphate administration and death, the sulphate appears in the matrix of the fully formed bone trabeculae. If H^3-thymidine is administrered two hours before death, mainly pre-osteoblasts and blood-forming cells (in the bone marrow) are found to be labelled, but a small number of osteoblasts is also labelled. Three days after the thymidine injection there is labelling of pre-osteoblasts, osteoblasts, osteocytes and osteoclasts. If a still longer time is allowed to elapse between the injection of the isotope and death, it is not possible to find any labelled osteoclasts but, on the other hand, the other cells are still labelled, even some pre-osteoblasts.

Die Neubildung von Knochen bei Osteolathyrismus

Eine autoradiographische Untersuchung mittels S^{35}- und H^3-Thymidin bei Lathyrismus

Zusammenfassung

Am 3.—4. Versuchstag findet sich am Rattenfemur periostal eine diffuse Wucherung der Präosteoblasten. Kurz vor dem Tode injiziertes S^{35} wird von den Präosteoblasten aufgenommen. Bei längerem Intervall zwischen Injektion von S^{35} und Tod erscheint das markierte Sulfat in der Knochenmatrix und in den neugebildeten Knochenbälkchen. 2 Std vor dem Tode injiziertes H^3-Thymidin findet sich vorwiegend in den Präosteoblasten und in den blutbildenden Zellen des Knochenmarks, während von den Osteoblasten nur eine Minderzahl markiert ist. 3 Tage nach Thymidininjektion sind sowohl Präosteoblasten wie Osteoblasten, Osteocyten und Osteoclasten markiert. Bei noch längerem Intervall sind keine markierten Osteoclasten mehr nachweisbar, dagegen von den übrigen Zellen immer noch einige wenige, selbst einige Präosteoblasten, markiert.

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