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A Study of Newly Formed Bone in Lathyritic Rats

Microradiographic, Tetracycline Labelling, and Microangiographic Techniques

By

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With 5 Figures in the Text

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Osteolathyrism was first induced experimentally by Geiger and co-workers in 1933 by feeding Lathyrus odoratus peas (sweet peas) to rats. The toxic substance in these peas is beta-(N-gamma-L-glutamyl)-amino-proprionitrile, but other aminonitriles are also found to be capable of giving rise to osteolathyrism. The most active of these is amino-acetonitrile (AAN).

Different skeletal deformities occur in osteolathyrism; exostoses at some muscle insertions on the long bones are among the characteristic features of the condition. The exostoses form at the muscle insertions from the inner periosteal layer. This is the result of lively osteoblastic proliferation at those sites. New bone is then laid down there. Experiments by Hamre and Yeager demonstrated that exostoses failed to appear on the shaft of long bones where muscle tension had been eliminated by section of various muscles. Severe changes also develop in the epiphysial cartilage with cellular and matrical changes and a tendency to epiphysiolysis. In puppies given a single dose of AAN, Engfeldt et al. found disturbances in the epiphysial cytomorphosis and matrical changes, after as little as 12 and 24 hours, respectively. Amato and Bombelli studied the lesions of the epiphysial cartilage microangiographically and observed that the minor blood vessels were seriously affected and probably contribute quite largely to the disorganisation and lack of calcification.

In 1957 Milch et al. discovered intravital labelling of newformed bone by tetracycline compounds. Tetracycline is accessible to study owing to its fluorescent properties on radiation with ultra-violet light. In experimental animals and man, tetracycline affords largely the same information as autoradiography with calcium or calcium-like isotopes (Harris et al., and Hulth et al.).

The aim of the present study was to study in growing rats fed with AAN the characteristic deformation of the long bones by means of tetracycline labelling, and to attempt to relate the findings to histologic and microradiographic observations. The investigation also included microangiographic examination, chiefly to study the early vascularization in newly formed bone.

Material and Methods

The experiments were performed on 60 rats of both sexes aged around 3 weeks and weighing between 40 and 65 g. All were fed on rat food cakes with free access to water. Eleven animals served as controls, and the remaining 49 received water containing 1 g of AAN per litre. The rats fed with AAN and the controls were sacrificed in groups of three and one, respectively, 4 to 49 days after the start of the experiments. All the animals were given Terramycin® (Pfizer)[1], 50 mg per kilogram of body weight intra-abdominally, 48 hours

[1] We are greatly indebted to the manufacturers for supplying the Terramycin.

before death. The vascular system of 18 rats was filled with diluted India Ink, 1:4 via a catheter in the abdominal aorta. During the course of 2 hours, approximately 20 ml of the India Ink mixture were injected into each animal in doses of 2 ml. Immediately after this procedure and plain roentgen examination, each animal was placed in the deep freeze at a temperature of -20^0 C. When the experimental series was completed, the animals were thawed in 10 per cent formalin. Twenty-four hours later, one humerus was removed for histologic examination from the first 30 animals, and the femora and tibiae from all the animals. The latter bones were fixed for another day in formalin and subsequently dehydrated in alcohol, transferred to unpolymerized methyl methacrylate, and after a further 24 hours to methyl methacrylate. The resulting plastic block was sawn into flat plates 1 mm thick of either longitudinal or transverse sections of the bones. The sections were then ground down to a thickness of 70 to 100 micra. Some of the sections were examined microradiographically, using a Philips diffraction unit equipped with an X-ray tube utilizing a Cu anode as radiation source. The sections were placed in close contact with a fine-grain photographic emulsion and exposed to X-rays generated at 24 kV. After processing, the pictures thus obtained were enlarged by photomicroradiography.

When the microradiographic examination had been done, all the ground sections — including those not studied microradiographically — were mounted in non-fluorescent balsam (Permount). The study of the tetracycline fluorescence was done with a conventional microscope with the large Zeiss fluorescence mercury lamp with a UG 1 or UG 5 filter (Zeiss).

The specimens selected for histologic examination were decalcified in formic acid, embedded in paraffin, cut into 10-micron thick sections, and stained with haematoxylin-eosin.

Results

The weight curves in the rats given AAN and the controls differed widely. The AAN-group gained little or no weight, which may in part be attributable to AAN-induced changes around the teeth making eating difficult. In rats given AAN for one week, plain roentgen examination revealed cloudy thickening of the cortical layers of the humeri and femora, and of the pelvic bones along the innominate line (Fig. 1 a). All the rats given AAN for two weeks showed distinct osteophytes on the long bones. In the group treated with AAN for seven weeks, the long bones were greatly deformed (Fig 1 b). The humerus and femur were the bones most markedly affected. Bone deposition also occurred on the fibula and radius. The spine showed kyphoscoliosis and gibbus deformation.

Histologic finding. The feature most highly characteristic of osteolathyrism is the lively osteoblastic proliferation in the deeper periosteal layers at certain muscle insertions on the humerus and femur. This is very quick and so lively that the superficial periosteal layers bulge at these sites. In our specimens, we also found less marked proliferation at places where there were no muscle insertions. In histologic specimens from animals fed AAN for seven days, new bone formation could be detected among the tumor-like cellular proliferation. This bordered on the cortical layer and had a very irregular appearance. In animals given AAN for a longer period, both the cellular proliferation and the laying down of new bone had increased. The cortical layer was intact in rats given a short course of AAN, but in those treated for longer periods there was pronounced rarefaction with widening of the vascular channels in the bone and absorption on the endosteal side. As the deposition of bone increased, regressive changes occurred at the bases of these deposits, and large cavities developed and in some instances merged with the original medullary cavity to form a common space. In places among the bone trabeculae, rounded basophilic formations appeared — apparently corresponding to Selye's basophilic globules.

Findings of fluorescence microscopy and microradiography. In specimens from animals given AAN for four days, the first change to be detected was radiation of vessels from the cortical layer in a centrifugal manner towards the periosteum bulging out from the cortex — that is, vessels radiating through the zone

Fig. 1 a and b. *Roentgenograms.* a Rat given AAN for one week. Cloudy thickening of the cortical layer on the femora and humeri and on the pelvic bones along the innominate line. b Rat given AAN for seven weeks. Very marked deformation of the long bones. Exostoses chiefly on the femora and humeri. Epiphysiolysis proximally in the humerus and tibia and in the distal femur. Thickness of the pelvic bones increased. Spine deformed.

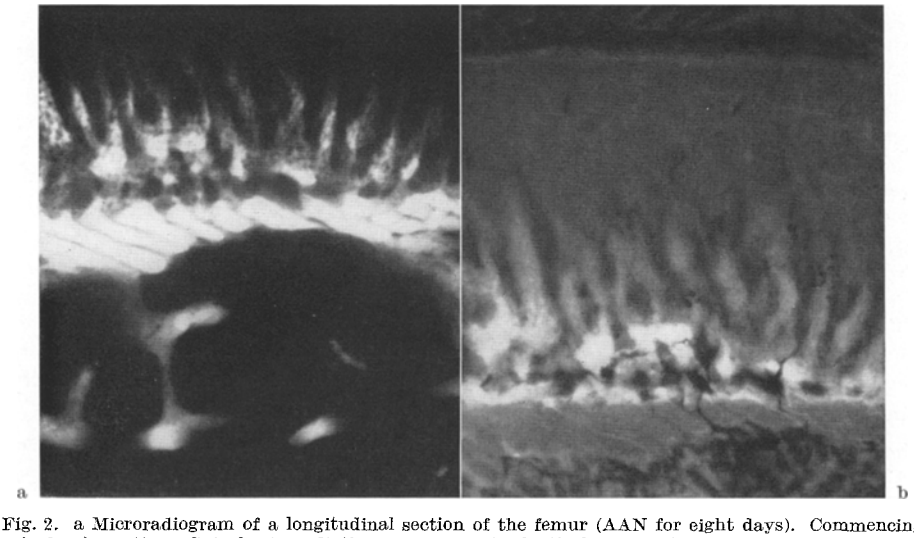

Fig. 2. a Microradiogram of a longitudinal section of the femur (AAN for eight days). Commencing spicular formation. Spicules in radiating arrangement. Cortical layer not changed. Pale zones have heavier mineralization. Magnification × 20. b Same specimen as in Fig. 2 a examined in the fluorescence microscope. Bright zones show the strongest Terramycin fluorescence (bases of the spicules). Spicule tips only slightly fluorescent. Periosteum thickened and bulging. Vessels filled with India Ink penetrate into the periosteum from surrounding muscle. Vessels perceptible also between the spicules. Magnification × 20

of cellular proliferation (see above). In specimens from rats given AAN for eight days, fluorescent bone spicules were also seen between the radiating vessels. These spicules growing between the vessels were fairly regularly arranged. The regularity of the vascular and the secondary spicular formation contrasted against

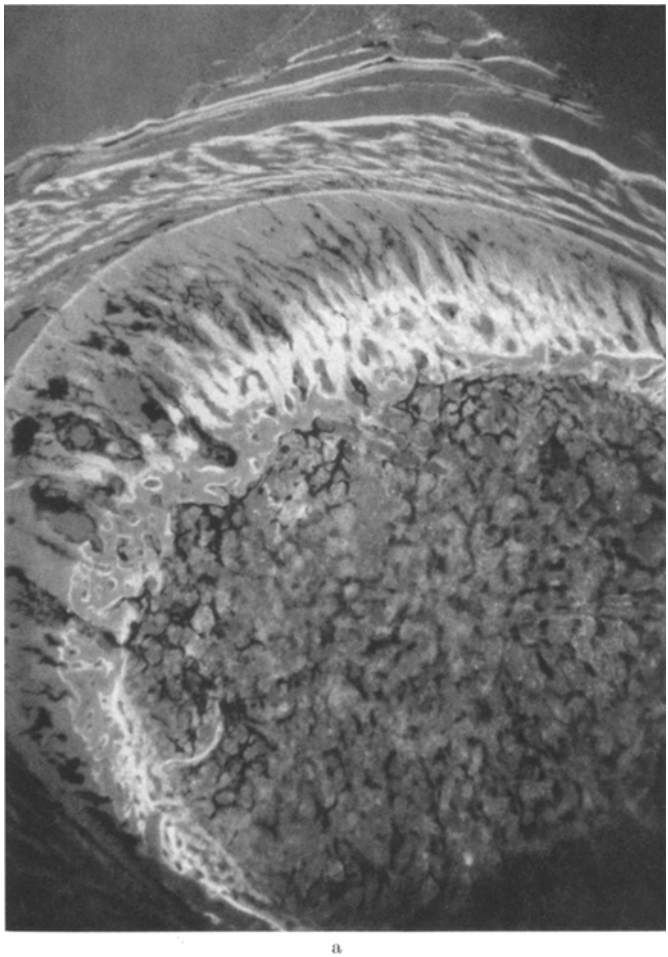

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Fig. 3a and b. *Fluorescence photomicrographs.* a Transverse section of the femur (AAN for eight days). Periosteal thickening. Radiating vessels filled with India Ink and fluorescent bone spicules. Fluorescence also perceptible in the cortical layer. Magnification × 20 (reduced to $^3/_4$).

the irregularity of the almost tumour-like cellular proliferation seen in the histologic specimens. The tetracycline produced strong fluorescence perceptible from the bases of the spicules at the cortex out towards their tips (Fig. 2b).

In specimens in which spicules were only starting to form, no fluorescence other than the normal periosteal or endosteal was seen in the cortical layer (Fig. 2b). As the spicules grew, the fluorescence in the cortical layer increased — suggesting that it was undergoing transformation (Fig. 3a). The newly formed bone tissue, as also the underlying transformed cortical layer, was highly vascularized. The vessels, which radiated out towards the raised periosteum, appeared

to be in direct continuity with both medullar and muscle vessels (Fig. 3b). Large absorption cavities gradually formed in the cortical layer and were filled with bundles of vessels containing India Ink. In rats given AAN for longer periods, the cortex was in places thinner and the medullary cavity wider.

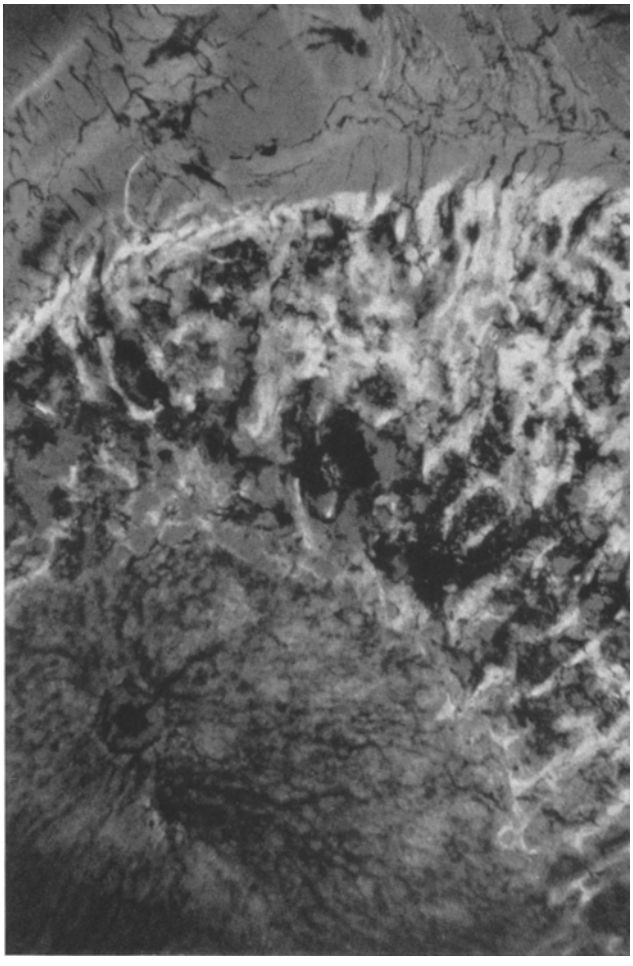

Fig. 3 b. Transverse section of the femur (AAN for 18 days). Numerous vessels filled with India Ink are seen in the newly formed fluorescent bone and in the cortical layer, which is undergoing transformation. The vessels appear to emanate from the muscle surrounding the periosteum and to issue into a central vein in the medullary cavity. Magnification × 20 (reduced to $^3/_4$)

The *microradiographic examination* showed the bone freshly laid down in the form of spicules to have a lighter degree of mineralization than the cortex (Fig. 2a). The mineralization was also heavier at the bases of the spicules than at their tips. In specimens from animals given AAN for two to three weeks, the regularity of the trabecular arrangement disappeared more and more; the trabeculae were transformed and assumed varying pattern. Further, pronounced rarefaction of some trabeculae was seen. Towards the periphery, a new cortical layer usually appeared with longitudinal trabeculae which, in animals given AAN for three

weeks, showed heavy mineralization. In some places new spicules were seen to form outside the secondary cortex, and in specimens from animals given AAN for even longer periods a third cortical layer was seen at some sites (Fig. 4).

On comparing the microradiographic and fluorescence microscopic appearances of the different specimens it was noted that heavily mineralized skeletal regions were only weakly fluorescent, while lightly mineralized parts — that is, freshly laid down — showed strong fluorescence (Figs. 2a and b).

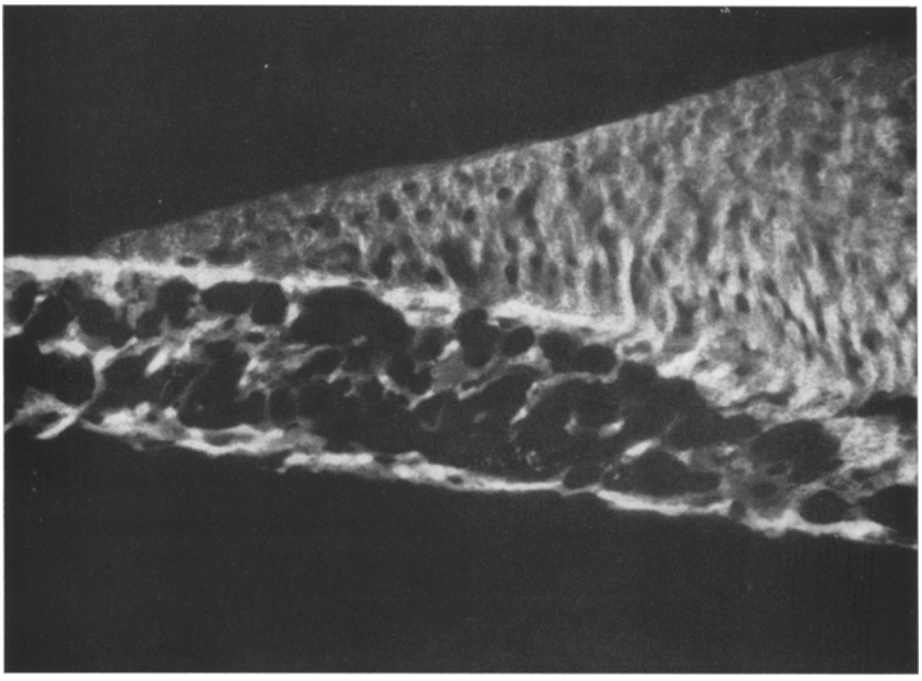

Fig. 4. *Microradiogram.* Longitudinal section of the humerus from a rat given AAN for 21 days. Primary and secondary cortices. Newly formed bone tissue outside the latter. Magnification × 20 (reduced to $^9/_{10}$)

In contrast to the very active appositional periosteal osteogenesis at muscle insertions, the endochondral growth was slight. The epiphysial cartilage quickly became disorganized (after less than one week of AAN feeding), the chondrocytes increased greatly in number, fissures appeared in the cartilage, and epiphysiolysis frequently developed at the boundary with the metaphysis. This latter disorder was seen at the distal femoral and proximal humeral epiphyses in most animals given AAN for more than two weeks. Even in instances in which there was no epiphysiolysis, the normal longitudinal trabeculation from the epiphysial cartilage was replaced by a greatly irregular bone formation. The normally highly regular vascular pattern was also wholly disorganized (Figs. 5a to d).

Fig. 5. a Fluorescence photomicrograph. Longitudinal section of the proximal tibial epiphysis from a rat given AAN for eight days. Epiphysis greatly thickened. Metaphysial bone trabeculae of irregular arrangement, fairly strongly and uniformly fluorescent. Metaphysial vessels highly irregular. Magnification × 20. b Microradiogram. Same section as in Fig. 5a. The epiphysial thickening and irregularity of the metaphysial trabeculae are even more distinctly visualized than in Fig. 5a. Magnification × 20. c Fluorescence photomicrograph. Normal tibial epiphysis from a rat. Longitudinal section. The bone trabeculae in the epiphysis regularly arranged, as also the vessels filled with India Ink. Fluorescence strongest far distally in the metaphysis. Magnification × 20. d Microradiogram. Same section as in Fig. 5c. Magnification × 20

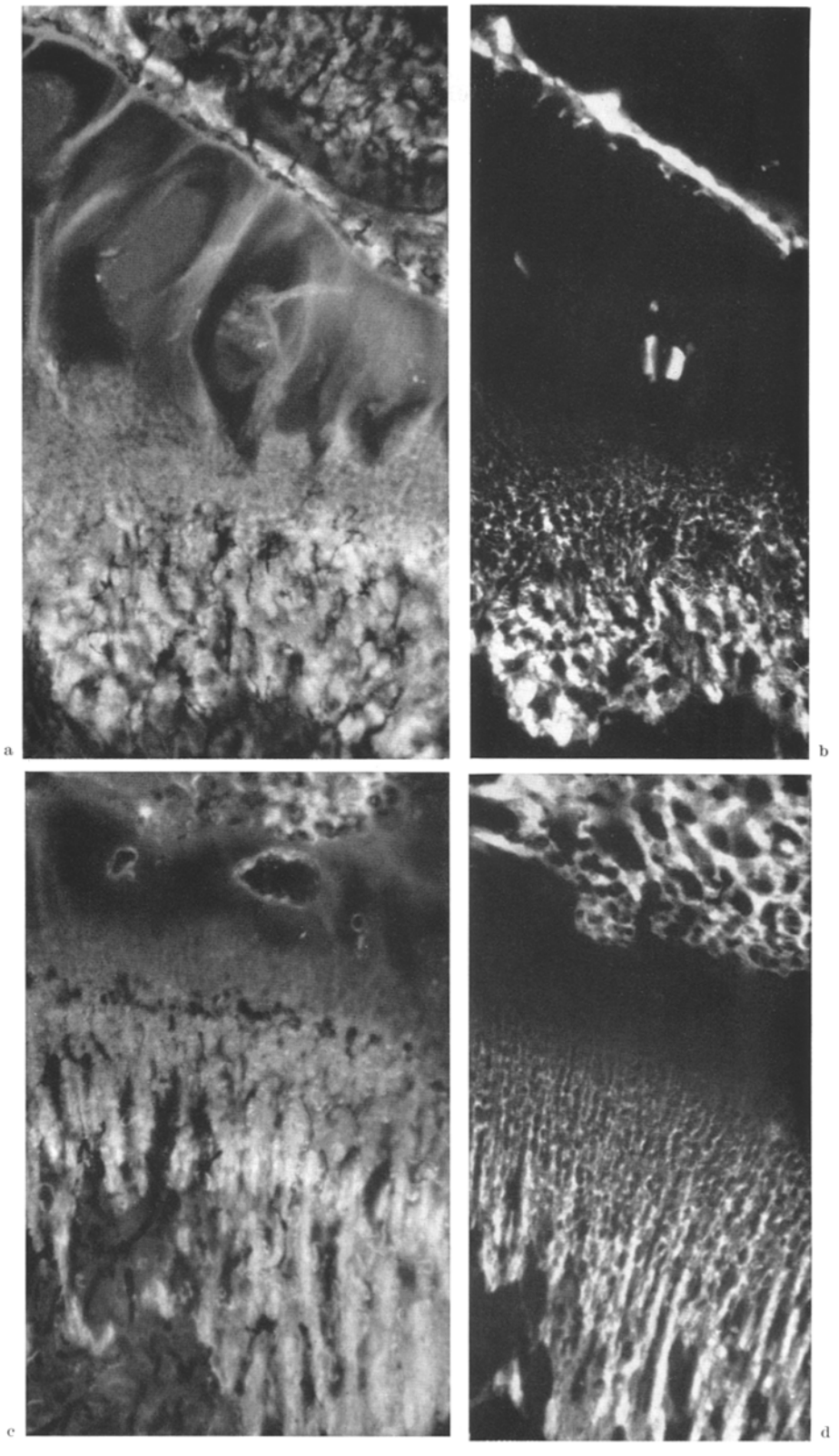

Fig. 5 (caption see p. 200)

Discussion

Exostoses induced by AAN-feeding in rats were studied by means of histologic, microradiographic, and tetracycline fluorescence microscopic examination, in combination with India Ink microangiography. The roentgen films of rats given AAN for somewhat longer than one week revealed distinct bone hyperplasia on the femur and humerus. Bone deposition was also seen on the proximal third of the radius, on the fibula and on the pelvic bones. Earlier studies have shown that exostoses appear chiefly at the sites of muscle insertions and are modified or fail to appear if the muscles are sectioned. In the present investigation no deformation of exostosis type was observed on, for instance, the patella or tarsal bones where there are strong tendon insertions. It is, then, not solely mechanical traction together with AAN intoxication which leads to bone deposition. In our experiments, bone was gradually deposited generally on the femur and humerus, both around and along the whole length of the shafts. It would seem that the conditions are particularly favourable to the development of AAN-induced bone deposition at places where muscles are inserted directly on to a bone, possibly owing to the fact that the collateral circulation there is particularly good between muscle and medullary vessels. Our study lends support to this assumption.

Notwithstanding the initial great irregularity of the osteoblastic proliferation in the deeper periosteal layers, the primary new bone formation consisted in regular, radiating spicules which gradually increased in length between the likewise radiating vessels. These vessels appeared to be in direct continuity with those of the medullary cavity and cortex, but were also in communication with the vessels of the surrounding muscles. The microangiograms gave no clear indication as to the direction of the blood flow in these vessels — that is, whether the radiating vessels originate in ones normally present in the periosteum or the cortex. But in late, strongly developed exostoses, the exosteal vessels appeared to originate in arteries in surrounding muscle and to issue into a large central vein in the medullary cavity (Fig. 3b). The slightness of the initial changes in the cortex resulting from the new periosteal bone formation also suggests that the blood supply came from the periphery — as otherwise one would expect more cortical absorption. However, the cortical layer gradually underwent rarefaction and in places disappeared at the same time as the vascularization of the cortex increased. Notwithstanding the presence of numerous bone spicules growing from its surface, the entire thickness of the cortex was at first wholly devoid of fluorescence. This is suggestive of metabolic inactivity. On the other hand, the spicules were strongly fluorescent owing to their high mineral metabolism. Microradiograms of these spicules showed light mineralization as compared with the cortical layer. In specimens from animals given AAN for longer periods, the rule seemed to be that poorly mineralized zones showed the strongest fluorescence. This agrees with the observations of isotope labelling. The degree of labelling with both tetracycline and isotopes is proportionate to the water phase in different bone regions. The more abundantly a region of bone is hydrated, the more it is available to substances in the blood. The heavy tetracycline labelling seen in exostoses induced by AAN feeding may also be dependent upon the particularly plentiful vascularization of such bone.

In contrast to the periosteal osteogenesis, endochondral growth appeared to be more or less retarded. In some specimens from animals given AAN for about two weeks, a network of bone trabeculae of irregular growth were seen in the metaphysis instead of the normal straight ones. In instances in which epiphysiolysis had occurred, no new bone formation whatever was seen. The cause of this defective endochondral growth is the strongly toxic action of AAN on the function of the cartilage cells.

The manner in which the aminonitriles induce the many changes is not understood. Certain studies have suggested that enzymatic damage is responsible (AMATO et al., and KUHLMAN). However, the results of the different investigations in the literature do not always agree as regards the nature of the enzymatic damage. Whatever that may be, it seems in the first place to affect the synthesis of collagen (SMILEY, 1962). Collagen is formed, certainly, but its maturation is retarded so that no cross-linkages occur between the fibres. This results in an excessive amount of acid-soluble collagen as compared with insoluble collagen. The problem is what induces the *proliferation* of the collagen-producing *osteoblasts* in the deep periosteal layers at the sites of certain muscle insertions. According to HAMRE et al. who used adult rats in their experiments, the proliferation starts as early as a few hours after the administration of aminonitrile, so that it must be regarded as a *primary effect of the aminonitrile intoxication*. Osteogenesis started on the seventh to eight day, which agrees with the results of the present study. In growing rats, as used in our experiments, the proliferation is more diffusely distributed over the femur and humerus, but the earliest exostoses appear at the sites of muscle insertions. In adult rats, on the other hand, the proliferation seems to be strictly limited to the muscle insertions. The periosteum there appears to have a special tendency to react. The lathyrus factor and the muscle factor must coexist if exostoses are to develop. The former is necessary to the cellular proliferation and the latter probably promotes osteogenic differentiation by mechanical action.

Summary

Histologic, micro-radiographic, and combined tetracycline labelling and India Ink microangiographic techniques were used to study newly formed bone in exostoses induced by amino-acetonitrile (AAN) in growing rats. The initial change was diffuse cellular proliferation in the deep periosteal layers, most lively at the sites of broad muscle insertions—especially on the femur and humerus. When one week had elapsed, exostoses started to develop; at the start these had the appearance of radiating bone spicules. The vascular pattern in the exostoses was also radiating, the vessels appearing to originate in periosteal arteries and issue into a central vein in the medullary cavity. The tetracycline fluorescence was strong in the spicules, but not more marked than is normal in the cortical layer. However, the cortical layer gradually underwent transformation and absorption, during which processes the fluorescence became very strong.

Tetracycline-induced fluorescence in combination with India Ink microangiography appears to be very helpful to the experimental study of the relation between vascularity and new bone formation or bone absorption.

Knochenneubildung bei Ratten mit Lathyrismus

Mikroradiographie, Markierung mit Tetracyclin, Mikroangioradiographie

Zusammenfassung

Zum Studium der Exostosen, welche sich bei mit Amino-Acetonitril behandelten jungen Ratten entwickeln, werden histologische, mikroradiographische Untersuchungsmethoden, kombiniert mit Tetrazyklin-Markierung und Gefäßinjektionen mit chinesischer Tusche, angewendet. Die initialen Veränderungen bestehen in einer zelldichten Proliferation der tiefen Periostschichten, besonders an den Stellen breiter Muskelinsertionen an Femur und Humerus. Nach 1 Woche entwickeln sich die ersten radiären Spiculae im Wechsel mit radiären Gefäßen, welche von periostalen Arterien abzuzweigen scheinen und ihr Blut in die zentrale Markvene ableiten. Die neugebildeten Spiculae zeigen eine deutliche Tetrazyklin-Fluorescenz entsprechend derjenigen in der normalen Corticalis. Im weiteren Versuchsverlauf wird das Osteophyt teils abgebaut, teils umgebaut. Dieser Prozeß ist mit einer zunehmenden Tetrazyklin-Fluorescenz des Osteophyten verbunden. Die Kombination einer Tetrazyklin-Markierung mit Tuscheinjektionen der Gefäße scheint eine brauchbare Methode zu sein, um die Beziehungen zwischen Vascularisation und Knochenneubildung zu überprüfen.

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For further references, see Doerr 1960, and Engfeldt et al. 1960.

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