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THE INFLUENCE OF HORMONAL TREATMENT AND ORCHIECTOMY, OOPHORECTOMY AND THYROIDECTOMY ON EXPERIMENTAL FRACTURES

A QUANTITATIVE P^{32}-AUTORADIOGRAPHIC, ROENTGENOLOGIC AND TISSUE-ANALYTIC STUDY

BY

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I INTRODUCTION

Recent research has shown that endocrine factors play an important role among the biological agents affecting the regeneration of bone. Growth hormone in itself and particularly in combination with thyrotropin has been demonstrated to stimulate osteogenesis and to have a beneficial effect on the rate of bone repair, while cortisone exerts an inhibitory effect (15, 17, 19, 32). A short time ago, the osseous anabolic effect of growth hormone together with thyrotropin was also established in human fractures in which the consolidation tendency was poor (16). In these references, the theoretical aspects of hormonal effects on bone repair and on the calcium and phosphorus metabolism have been considered in more detail. On the whole, the significance of endocrine factors in fracture healing is receiving increasing attention (39, 40).

In contrast to the wide-spread administration of anabolic steroid hormones in the hope of promoting bone repair and shortening the patients' time of confinement, the results of investigation concerning the effect of such hormones are rather scanty and controversial, and the explanation they furnish of the matter is unsatisfactory. The results obtained in experimental studies with different animals are also at variance and little convincing.

In some experimental studies androgen hormone had no effect on the rate of fracture healing (1), while other authorities report that it was favourably influenced by sex hormones (3, 11, 12, 24). Recently, adequate dosage of norandrosterone propionate has been found to further the healing of fractures and to produce a positive nitrogen

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balance, whereas massive doses caused retardation (25). The development of callus in animals has been observed to be more rapid when oestrogen was administered (13), while according to other results (29) the effect was doubtful, and in another study (7) oestrogen slightly retarded the formation of callus. Furthermore, inhibition of growth and increased endosteal formation of bone in the rat has been reported (36). Upon ovariectomy, the callus formation as well as the breaking strength of the bones is stated to decrease (22). Thyroxine has been found to accelerate and cortisone (desoxycorticosterone) to depress the osteogenesis in experimental fractures (10).

In the way of methods, the studies have often been based on mere descriptive observations. Hardly any use has been made of quantitative methods in investigations concerning the effect of hormones or of hormonal deficiency.

In the present attempt to clarify the effects of endocrine factors, the writer endeavoured to establish optimum conditions for systematic, quantitative elucidation of the subject. The following approaches were enlisted to this end: autoradiography with phosphorus-32, roentgenographic studies including planimetry of x-rays, and histoquantitative analysis. The effect of androgen (19-norandrostenolone phenyl propionate), oestrogen (oestradiol monobenzoate) and thyroxine (DL-β-(3,5-diiodo-4-[3',5'-diiodo-4'-hydroxyphenoxy]-phenyl)-α-aminopropionic acid) was investigated by administering the hormone in question, and by removal of the testes, ovaries and thyroid gland, respectively. In addition to the thyroxine and thyroidectomy experiments also the effect of replacement therapy was studied by administering thyroxine to thyroidectomized animals.

II MATERIAL AND METHODS

Experimental Animals and Groups

White rats of 150—210 g body weight were used in a total number of 220 animals including the controls, namely, 145 males and 75 females. All animals were given the same kind of food and were kept in uniform laboratory conditions.

A closed complete fracture of the right tibia was caused to each rat and left to heal without fixation employing a previously established technique (15). Starting with the beginning of fracture healing, part of the animals were given intramuscular injections of the substances mentioned in the introduction (p. 6), while others were subjected to removal of endocrine glands at the occasion when the fracture was produced. A third part of the material served as controls, which were given physiological saline injections. The animals were sacrificed after one to five weeks and subsequently treated as outlined in the next paragraph.

Two deaths occurred within a few days of the gland removal. These animals are not included in the series.

The results recorded in the present studies were considered within the following groups:

Androgen Group — Dosage 5 mg every second day; sacrificed 7, 14, 21, 28 and 35 days after the fracture, five animals at each time for roentgenographic and histological studies. Seven animals served the autoradiographic studies. Their sacrificing dates coincided with the above-mentioned times. The group thus consists of 32 male animals.

Oestrogen Group — Dosage 3 mg every second day; five animals each sacrificed at the above-mentioned times. Seven rats for autoradiographic studies. Total 32 female rats.

Thyroxine Group — Dosage 0.3 mg every second day, times of sacrificing as above. Autoradiographic studies: seven rats. Altogether 32 male rats.

Orchiectomy Group — Sacrificed as above. Autoradiographic studies: seven rats. Total 32 rats.

Oophorectomy Group — Sacrificed as above. Autoradiographic studies: seven rats. Total 32 rats.

Thyroidectomy Group — Sacrificing times as above, 2 animals at each time, plus seven rats for autoradiographic studies. Total 17 male rats.

Thyroidectomy Plus Thyroxine Group — Thyroxine dosage subsequent to thyroidectomy, 0.3 mg every second day; sacrificed at the abovementioned times, 3 animals at each time, total 15 male rats. No autoradiographic studies were carried out with this treatment.

Control Group — Sacrificing times as above, 2 males and 2 females at each time. Autoradiographic studies: eight rats. Total number of animals: 18 male and 10 female rats.

Methods

In each group the average weight of the animals at the moment of sacrificing was determined for the different times of sacrificing as a check on the experimental conditions. Nothing of special bearing on the matter under investigation was revealed by these values and they are not reported in the results.

In the quantitative autoradiographic studies each animal was given, four hours prior to sacrificing, P^{32} radiophosphorus (N. V. Philips-Duphar, Amsterdam, Holland) by intraperitoneal injection in a quantity equivalent to 1 microcurie per 1 g body weight. Undecalcified specimens of the fractured bones were embedded in methylmetacrylate. After grinding down to 100 μ thickness, contact autoradiographs were made of them on Ilford Ilfex Fine Granular film. All autoradiographs were made on one piece of film, together with a reference scale produced with a step wedge and x-rays. Four densitometer readings were taken of each autoradiograph, namely, of (1) the highest density in the callus area, (2) the epiphyseal line, (3) the diffuse deposition in the diaphysis, and (4) at the end of the bone fragment (Fig. 1). The apparatus was an indicating densitometer by the Macbeth Corp., New York, N.Y., U.S.A., fitted with a diaphragm having an aperture 0.5 mm in diameter. The readings were corrected with reference to the step scale.

Of the autoradiographs taken at the early and late stage of the bone

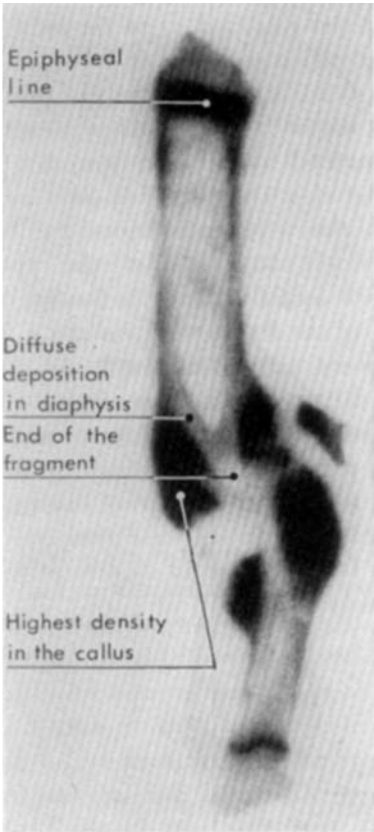


Fig. 1. Autoradiograph (enlarged) produced from a longitudinal section through the fractured tibia, illustrating the quantitative local variations of radio-phosphorus deposition and the selection of points of densitometric measurement.

repair process in each experimental group reproductions are included in this paper as Figs. 3, 6 and 8 which serve to illustrate the topographic locations where radio-phosphorus deposition occurred.

The consolidation of each fracture was assessed, using a scale 0-1-2-3 (0 — Poor callus; 1 — Slight callus; 2 — Fair callus; 3 — Solid, firm callus), by visual examination of x-rays taken after sacrificing and considering also the simultaneous findings on palpation. The ratings were averaged for each group and time of sacrificing.

Paper prints at magnification 5× were made of the x-rays and the area of the callus visible in them was determined, using a planimeter.

Means were taken of the recorded areas (in mm^2 of the original x-ray) for each group and sacrificing date.

For histological examination and analysis of the callus components by the line sampling method, decalcified preparations derived from the fracture sites were stained by the Pentachrom I and II methods (26).

As regards the detailed technique of the line sampling procedure, reference is made to the writer's previous publication (15) and to its theoretical foundation (34, 35). In brief, the method implies that the fracture specimens are sectioned longitudinally through the centre of the medullary cavity of the fractured bone and a line is drawn from the end of the bone fragment at right angles to the shaft. The intercepts of this line representing different tissue components of the callus (new bone, vascularization, hyaline cartilage, fibrous cartilage, fibrous tissue, blood clot) are then recorded and the aggregated percentage of each component is plotted to produce a diagram illustrating conditions at the different stages of healing. A special microprojector is used in this work. The method has also been applied in studies of fracture healing during anticoagulant treatment (31).

III RESULTS

Controls

Phosphorus^{32} Deposition

In an arbitrary relative scale based on the densitometer readings, the amount of P^{32} deposited in the callus area appearing densest in the autoradiographs of the controls was equivalent to a value of about 100 during the observation period between 7 and 21 days, declining to 40 at 35 days (Fig. 2a). The mineral deposition on the epiphyseal plate seems to follow the course of that in the callus, being consistently 25 to 50% lower. The values recorded for the end of the fragment and for the diffuse deposition in the diaphysis were both very nearly constant throughout the period of observation; that of the end of the bone fragment being about 40% lower. Compared to the callus, the diffuse diaphyseal deposition varied between one-tenth and one-third.

Consolidation and Size of Callus

Early callus formation was noted in the animals of the control group 14 days after the fracture. The degree of consolidation assessed as described on p. 9, increased at a fairly uniform rate and was nearly complete (rating 3) in all animals at 35 days. The average rating of 1.5 would have been reached slightly before the 21st day (Fig. 4a).

According to planimetry of x-rays, the size of the callus increased from virtually zero at 7 days nearly linearly to about 12 mm^2 on the average on the 35th day (Fig. 4b).

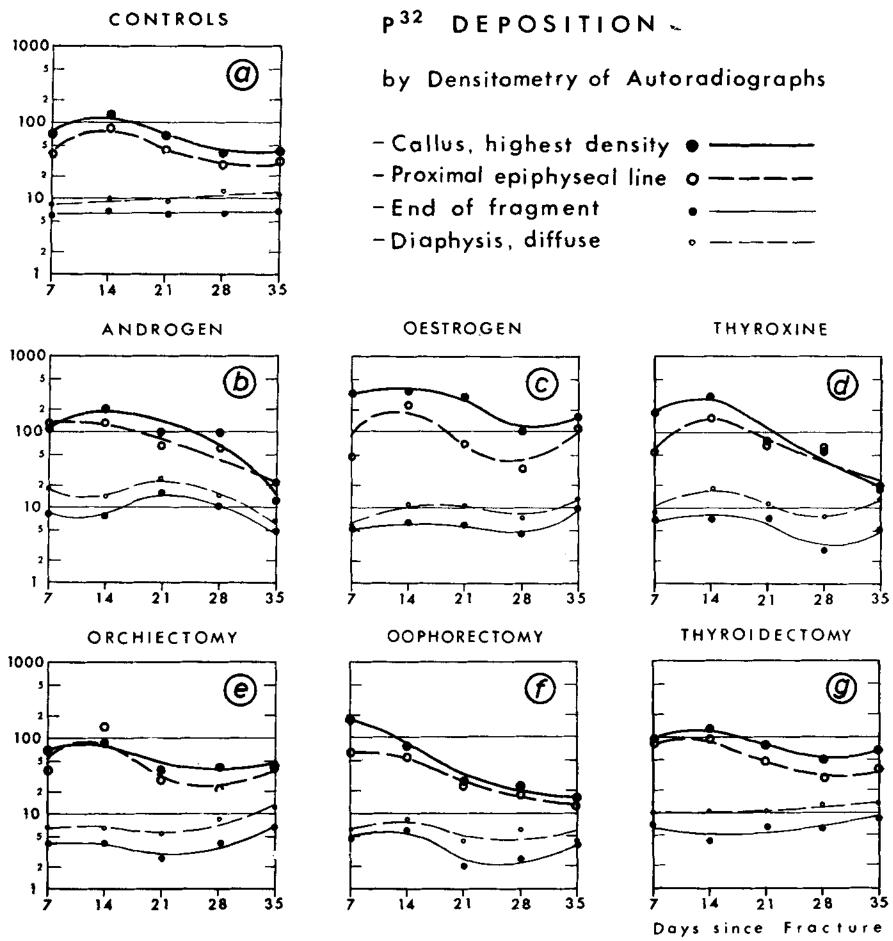


Fig. 2. Results of densitometric evaluation of autoradiographs (cf. Fig. 1) in the control and test series, reflecting the strength of phosphorus activity at specified sites in the callus and fractured bone during progress of the repair process.

Ordinate values: Relative amount of P^{32} accumulation within four hours of tracer injection.

Histological Studies

Histological Findings. Study of the microsccpic slides revealed a similar general course of the fracture healing in male and female controls, which did not differ from the previously described changes (15).

7 days after the fracture, the area around the ends of the fragments is dominated by strong cell proliferation in fibrous connective tissue. The periosteum is thickened and the space between the outer surface of the fragment and the periosteal fibrous layer is filled with immature fibrous tissue. In the homogeneous structure occurs basophilously staining hyaline cartilage, while osteoblasts are seen close to the diaphysis, part of them already cemented to the new bone that has been formed in the area between periosteum and outer diaphyseal surface. Many of the osteocytes at the ends of the fragments close to the fracture site are anuclear, and the fragments are surrounded by a blood clot.

At 14 days the fragments are still surrounded by tissue rich in fibres and containing cells. The formation of new bone between periosteum and bone has increased; also endosteal formation of bone is observed. The area of new bone formation does not extend to the ends of the fragments.

21 days after the fracture a subperiosteal calcified callus is seen to have formed between the fragments and it is clearly demarcated from the connective tissue, which is rich in cells. The new bone appears to be produced from hyaline cartilage, but also from fibrous cartilage. At the same time cartilaginous cells dissolve, and the cavities formed in this manner become epithelium-lined vascular spaces.

At 28 and 35 days, trabecular formation of new bone is predominant, bridging the gap between the fragments both periosteally and endosteally. The lacunae at the ends of the fragments are empty even yet, and there are signs of resorption of bone.

Line Sampling. The following trends are observable in the relative distribution of the different callus components on measurement by the histoquantitative line sampling procedure (Figs. 5 and 4c).

At the initial stage of the repair process, ~~at~~ seven days, the callus mainly consisted of fibrous tissue (70—80%). This tissue gradually decreased in the course of healing and ultimately vanished. At 14 days, fibrous cartilage had appeared and also hyaline cartilage, the first-mentioned constituting a considerable portion of the callus at this stage. From the third week onward, new bone and concomitant vascularization are seen as new components; both increased in parallel, while the immature tissue components diminished. Thus, in the final stage, the two kinds of tissue made up the matured callus, together with a small quantity (less than 5%) of hyaline tissue.

Androgen and Orchiectomy

ANDROGEN

Phosphorus^{32} Deposition

The levels of P^{32} deposition noted in the callus in the androgen group at different times were mostly 40—50% above those in the controls except for the late stage, at which the deposition fell below one-half of the control value (Fig. 2b). No such abrupt decline is seen in the values recorded for the epiphyseal line, which otherwise mostly remained below the values of the callus by about the same amount as in the controls.

The deposition values relative to the end of the bone fragment and to the diffuse area of the diaphysis are somewhat elevated from the controls except for the late stage, at which they fell below the controls, too.

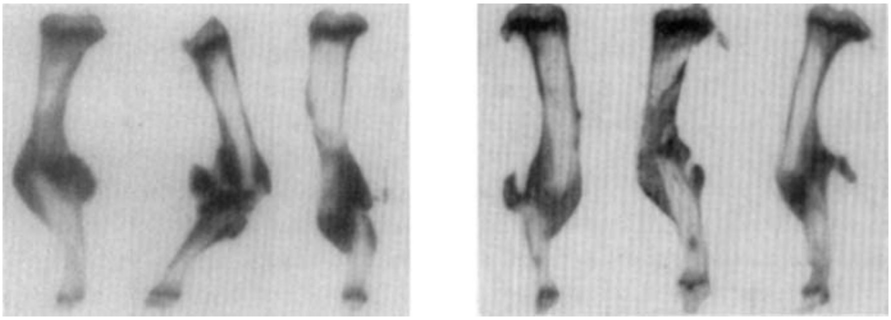
Consolidation and Size of Callus

At 14 days, examination of the x-rays revealed distinct presence of callus, and the average degree of consolidation was better than 1.5 according to the 0—3 scale. This indicates that the process was about one week ahead of that in the controls. At 28 days the degree was close to 3, but at the end of the period of observation (after 35 days' androgen treatment) poorer consolidation (equivalent to average rating 2.0) was once more recorded (Fig. 4a).

The size of callus, determined by planimetry, differed slightly from zero already at 7 days and increased at a high, subsequently somewhat slower rate to about 15 mm^2 on the average at 35 days (25% higher than in the controls) (Fig. 4b). It should be noted that the results of planimetry refer to the area of a longitudinal section of the callus. If the measurements were evaluated in terms of actual mass of callus tissue, the latter parts of all curves would be raised and their initial portions suppressed.

Histological Studies

Histological Findings. Throughout the tests with animals under androgen hormone treatment, the callus had progressed quite notably further in its development than in the controls at the corresponding



Control Androgen Orchiectomy
7 days

Control Androgen Orchiectomy
35 days

Fig. 3. Autoradiographs of longitudinal sections through the fractured tibia at the early stage of the bone repair process (7 days, on the left) and at its final stage (35 days, on the right) in the control, androgen and orchiectomy groups.

times. Considering the entire repair process, the observation is made that the differentiation of cells and callus tissue, which is characterized by osteoblast seams in the proliferating osteogenetic layer of the periosteum, and the formation of new bone were markedly accelerated furnishing evidence of earlier maturation.

At seven days, the callus has great size and the histological picture is governed by powerful formation of hyaline cartilage. New bone extends from deep in the area between periosteum and outer diaphyseal surface up to the ends of the fragments. It is accompanied by profuse vascularization, and incipient endosteal formation of new bone is also seen. Fibrous tissue and fibrous cartilage occur scantily in the área around the fragments, as well as residues of the blood clot. The regenerative process shows no quantitative changes in respect of the callus components, but closer study on the cellular level reveals that the cells of the mature cartilage tissue are hypertrophic and activated. On comparison with the controls, the histogenetic development of the callus can be said to have reached the same stage of maturity as that of the controls one week later, at 14 days.

At 14 days, the periosteal formation of new bone is seen to have strengthened, and there are bony trabeculae between the outer side of the cortical layer and the periosteum. At 21 days, the fragments are enveloped in a fairly continuous trabecular callus.

At a distance from the ends of the fragments, where the local condi-

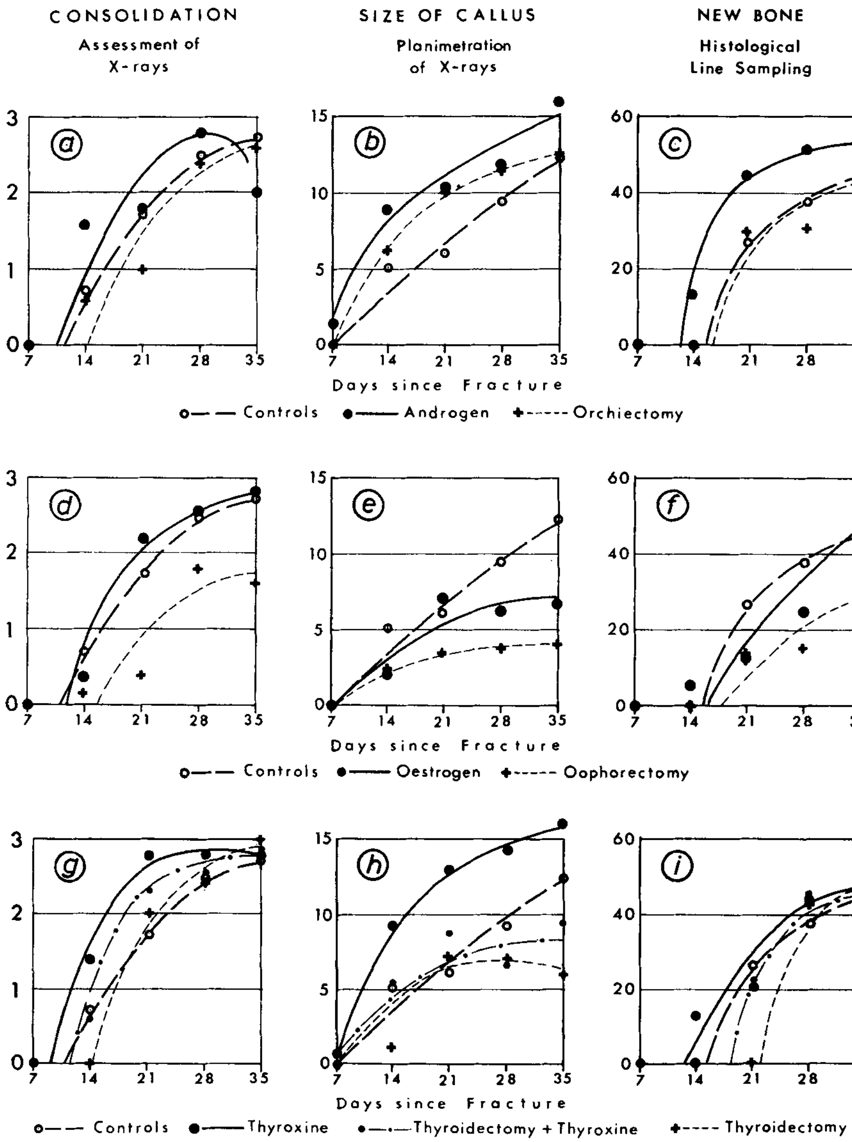


Fig. 4. Results of studies elucidating consolidation, development of callus and rate of formation of new bone in the control and test groups.

Diagrams on the left — Degree of consolidation revealed by x-rays and clinical palpation. *Ordinates:* Mean of ratings (maximum 3.0), cf. p. 9.

Diagrams in the centre — Area of callus as seen in x-rays, means of five animals. *Ordinates:* Area in original x-ray, in mm^2.

Diagrams on the right — Amount of newly formed bone relative to total callus tissue, according to histoquantitative analysis (cf. Figs. 5, 7 and 9). *Ordinates:* Percentage of new bone areas, referred to total length of transect.

tions of circulation are favourable, formation of new bone seems to take place directly by osteoblast activity. In peripheral locations, in the area around the ends of the fragments, where there is posttraumatic vascular ischaemia, the formation of new bone is by the intermediate phase of hyaline cartilage and also by transformation of fibrous cartilage into calcified cartilage and newly formed bone.

At 28 and 35 days the periosteal and endosteal formation of bone has become still stronger. At the same time, resorption is seen to start at the ends of the bone fragments. It was evident on comparison with the controls that at this stage no longer any such differences in maturity of the callus can be demonstrated histologically as were strikingly manifest at the early stage of healing. In other words, the untreated animals have by then largely caught up with the head-start of those under androgen hormone treatment. This is borne out by the disappearance of immature tissue components from the histological picture presented by both groups.

Line Sampling. (Fig. 5.) Analysis by the line sampling method with respect to the different callus components revealed that the callus of the animals under androgen hormone treatment contained, at an early stage of repair (at 7 days), hyaline and fibrous cartilage and fibrous tissue. The last-mentioned was present in small quantity (about 25%) and it became less and disappeared already before the end of the fourth week. In the course of progressing repair, fibrous and hyaline tissue were replaced by formation of new bone and by its attendant vascularization already at an early stage — from the second week onward. In particular, the new bone formation is seen to take place at an accelerated rate during the early half (first three weeks) of the healing period (see also Fig. 4c), While the callus of the controls contained more than 50% immature tissue components at this stage, their percentage in the animals under androgen hormone treatment was less than 10%. Towards the end of the repair process, the differences in tissue component distribution between the two groups tend to level out. The powerful predominance of mature components demonstrates that the differentation has been speeded up by the effect of androgen hormone especially at an early stage.

ORCHIECTOMY

Phosphorus^{32} Deposition

The results of autoradiography describing the deposition of radiophosphorus in the callus area and on the epiphyseal line in the orchiectomy group are rather similar with those of the controls. The diffuse deposition and that at the end of the fragment are seen to be lowered at the early stage of repair and most notably about the end of the third week (to one-half), reverting to control level by the 35th day (Fig. 2e).

Consolidation and Size of Callus

The orchiectomized animals displayed at 14 days a fair roentgenologically observable callus approximating that in the control group. On the whole, however, the progress of consolidation seemed to be slightly retarded in comparison with the controls (Fig. 4a). At the final stage (35 days) the callus seen in the x-ray, though indicating satisfactory consolidation, was less dense. The structure of the bone on the whole suggested osteoporosis.

The development of callus size (Fig. 4b) is intermediate between that in the androgen-treated animals and in the controls.

Histological Studies

Histological Findings. In the orchiectomized animals, the fresh callus at seven days is seen to be made up by tissue which is comparatively rich in cells. The cells are smaller and the interstitial matter is fibrillar in character. In closer vicinity of the subperiosteal area, the cells increase in size and the matrix becomes homogenous. Close to the outer surface of the fragments, formation of new bone can be seen in a narrow zone, but it stops far from the fractured end of the host bone, which is enclosed in a blood clot.

At 14 days, there is increased formation of new bone as in the androgen group at seven days, but stopping short of the ends of the fragments. The peripheral area contains ample immature cartilage tissue and fibrous tissue. The hyaline tissue cells are frequently anuclear, degenerated, and the impression is that although cartilage cells are plentiful, their normal differentiation and calcification is delayed.

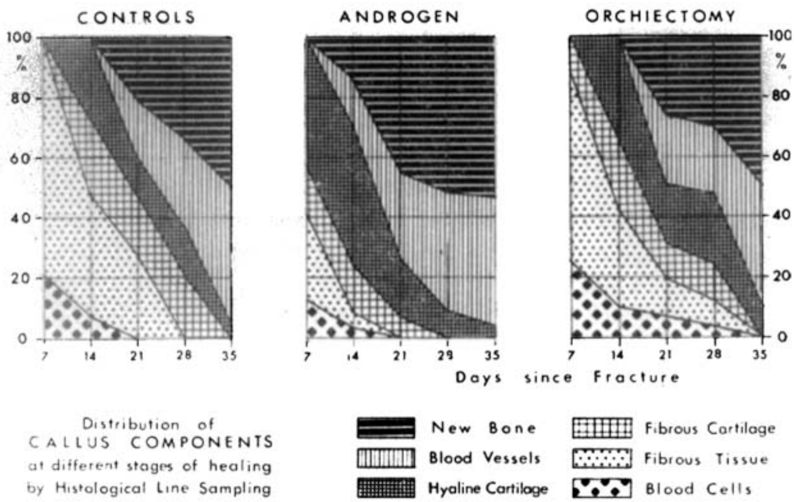


Fig. 5. Histoquantitative tissue analysis diagrams, showing the per cent. composition of the callus at different times after the fracture in the control, androgen and orchiectomy groups.

At 21 days, the callus has reached considerable size and the formation of bone has increased from what it was one week earlier. The calcified cartilage has likewise increased, but there is still immature cellular tissue left. It is worth while to observe that, compared to conditions under androgen hormone treatment, the trabeculae of new bone are irregular and thin and present osteoporotic appearance. Immature tissue persists in semblance of a seam in the periphery, close to the raised periosteum.

At 28 days, the callus has decreased in size and its percentage of new bone tissue has increased. There is still fibrous tissue and fibrous cartilage between the bone fragments.

At 35 days, the formation of new bone is seen to have increased both periosteally and endosteally. In the middle between the fragments an area with immature callus, extending up to the periosteum, is noted. These observations suggest that the regeneration of bone has been slightly disturbed at the initial stage of the repair process.

Line Sampling. (Figs. 5 and 4c.) Histoquantitative study of the specimens derived from orchiectomized animals revealed that at the early stage of healing the callus in the fracture area was mainly composed of fibrous tissue. This tissue, and likewise the blood clot, persisted throughout the repair process, becoming less and finally disappearing only at its very end. The contribution of fibrous cartilage and hyaline cartilage to the callus was fairly constant from the beginning and throughout the process and the composition of the callus shows little deviation from that in the controls in this respect. Formation of new bone and vascularization of the callus started after the second week and progressed at a fairly uniform rate to a final value virtually equal to that in the control and androgen groups.

Oestrogen and Oophorectomy

OESTROGEN

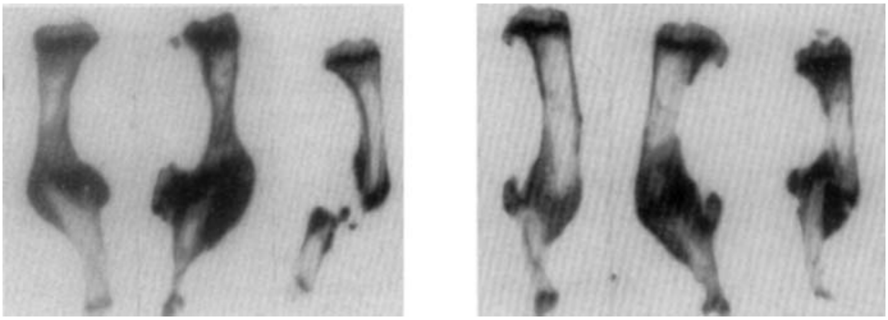
Phosphorus[32] Deposition

The autoradiographs of the specimens in the oestrogen group (Fig. 2c) reveal the most striking departure from the controls in that the deposition in the callus area was about four times that in the control group throughout the repair process. The deposition on the epiphyseal plate was not as greatly elevated. The other two curves are approximately at control level.

Consolidation and Size of Callus

Incipient callus formation is seen in the x-rays of the oestrogen-treated animals at 14 days, after which the degree of consolidation progressed very rapidly, above the rating 2.0 already at 21 days and coming close to 3 at the end of the period of observation (Fig. 4d). In the x-rays taken at this stage distinct sclerosis can be observed in the area around the ends of the fragments. There are no signs of bone condensation occurring in the marrow canal.

The growth of the callus in size (Fig. 4e) according to the results of planimetric study started after the 7th day but proceeded at slow rate so that the area recorded at 35 days was no more than 7 mm^2 on the average, i.e., only 60% of the corresponding figure in the control group.



Control Oestrogen Oophorectomy
7 days

Control Oestrogen Oophorectomy
35 days

Fig. 6. Autoradiographs of longitudinal sections through the fractured tibia at the early stage of the bone repair process (7 days, on the left) and at its final stage (35 days, on the right) in the control, oestrogen and oophorectomy groups.

Histological Studies

Histological Findings. At 7 days, the callus of the animals under oestrogen hormone treatment consisted mainly of fibrous tissue and fibrous cartilage. In the periosteal area, close to the outer side of the diaphysis, incipient formation of new bone is observed, which remains far from the end of the fragment. Hyaline cartilage can be seen, and the ends of the fragments are surrounded by a blood clot.

At 14 days, somewhat increased formation of new bone is noted, while the immature callus components are still ample in amount. There is endosteal bone formation between the fragments, but no signs of sclerosis of the marrow are present. Osteoblasts and osteoclasts are rather scanty. The bone marrow is activated.

At 21 days, the development of the callus has progressed from the status one week before, although its appearance suggests that normal cell differentiation is disturbed and there are hyaline cartilage islets. The formation of new bone, starting deep in the subperiosteal area, extends far into the periphery and into the space around the ends of the fragments. This area contains as characteristic components hyaline and calcified cartilage between the fragments.

At 28 days, markedly increased formation of new bone is seen, and the size of the callus is fairly small. Its peripheral part contains immature cartilage and fibrous tissue, and at the points where bone formation has taken place the bone trabeculae are conspiciously thick and condensed, indicating greater abundance of newly formed bone. Osteoblasts are ample, but the osteoclasts are far between. The cartilage cells are small, often anuclear, and the boundary between trabecular bone and immature cartilage tissue is diffuse. In several areas the formation of new bone takes place by the intermediate step of fibrous tissue.

At 35 days, osseus consolidation has ensued between the fragments, both subperiosteally and endosteally. The vascularization is rich, and in the area close to the fragments a residue of hyaline cartilage forms a V-shaped zone.

Line Sampling. (Figs. 7 and 4f.) In the animals under oestrogen hormone treatment, the early callus is seen to contain fibrous tissue, fibrous cartilage and a blood clot. Fibrous tissue and fibrous cartilage both persisted in parallel during the course of the repair process until they had disappeared at the final stage when bony consolidation between the fragments had taken place, consistent with the maturation of the callus in the control experiments. New bone, and vascularization in its company, barely appeared in the second week and both increased at accelerated rate during the later stages. At the end of the observation period, at 35 days, the mature callus was composed of new bone (at about 50%) and of vascular space filled with new blood vessels.

OOPHORECTOMY

Phosphorus^{32} Deposition

Starting somewhat above control level at 7 days, the deposition in the callus is seen to decline throughout the period of observation, its 35-day value being about one-half that found in the controls (Fig. 2f). Likewise, the epiphyseal line values are quite far below the controls, although it should be pointed out that the plots referring to the callus area are remarkably close to them at all times except 7 days. The diffuse diaphyseal deposition as well as that at the end of the fragment was notably lowered.

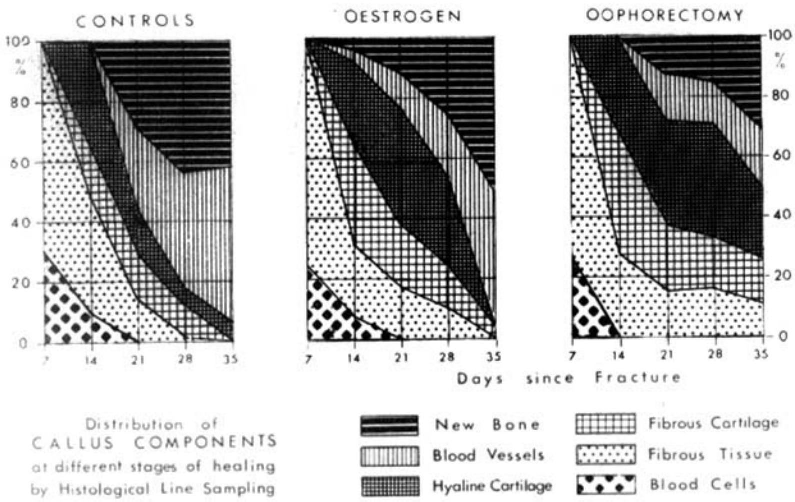


Fig. 7. Histoquantitative tissue analysis diagrams, showing the per cent. composition of the callus at different times after the fracture in the control, oestrogen and oophorectomy groups.

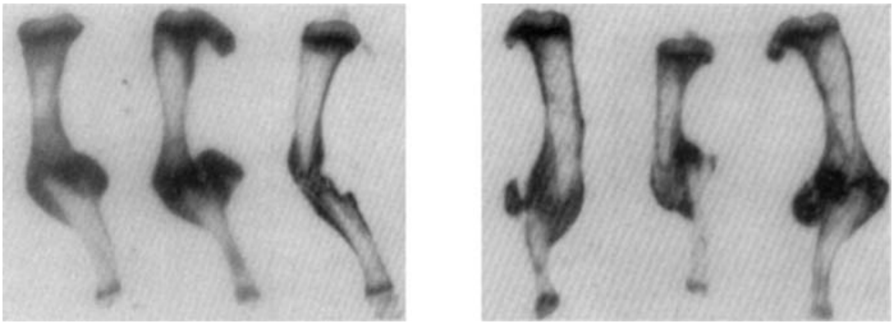
Consolidation and Size of Callus

In the oophorectomy group hardly any callus can be seen in the x-rays relating to 14 days and consolidation was quite poor even at the close of the third week. The average degree of consolidation never reached the level of 2.0 (Fig. 4d). The late x-rays clearly reveal osteoporotic changes and the fragments have undergone severe dislocation in several instances.

The planimetric size of the callus began to rise from zero after the first week, but the slow ascent of the curve is short-lived so that the area at the end of the period of observation remains as low as 4 mm^2 (Fig. 4e).

Histological Studies

Histological Findings. The young callus at 7 days is made up of profuse fibrous tissue rich in cells and of fibrous cartilage. Most nearly adjacent to the outer surface of the diaphysis there is slight new bone formation and vascularization; bone-producing osteoblasts are seen in considerable number. Here the early formation of bone takes place directly from fibrous cartilage and there is no hyaline cartilage.



Control Thyroxine Thyroidectomy
7 days

Control Thyroxine Thyroidectomy
35 days

Fig. 8. Autoradiographs of longitudinal sections through the fractured tibia at the early stage of the bone repair process (7 days, on the left) and at its final stage (35 days, on the right) in the control, thyroxine and thyroidectomy groups.

At 14 days, the callus still contains fibrous tissue in ample amount and immature cartilage, but also hyaline cartilage. The gap between the fragments is even now filled with fibrous tissue and immature cartilage. The formation of bone in the subperiosteal area has increased, and there is hyaline cartilage adjacent to the ends of the fragments. The status is histologically evidently delayed in comparison to the control group.

At 21 days, increased formation of new bone is noted, and the callus is fairly small. There is still fibrous tissue and fibrous and hyaline cartilage in the area around the ends of the fragments.

At 28 and 35 days, the formation of new bone has further increased. The trabecular formations are regular and accompanied by some vascularization in the callus, which is still quite small in size. Incipient resorption is seen at the ends of the fragments. At 35 days there still occurs a zone with fibrous tissue and fibrous and hyaline cartilage between the fragments.

Line sampling. (Figs. 7 and 4f.) The composition of the fracture callus found in the animals subjected to oophorectomy is different from all those treated in the foregoing in the respect that all types of tissue components except the blood clot persist up to the final stage in the callus development.

At the initial stage, the contribution of fibrous tissue was strongly predominant; its quantity decreased to about one third of this by the end of the second week. The fibrous and hyaline cartilage components were otherwise dominant throughout the repair process, whereas new bone, its formation starting after 14 days, was poorly represented all the time. The same is true for the vascularization of the callus. Judging by the distribution of the callus components, the relative predominance of immature tissues commenced at an early stage and continued throughout the healing process. This indicates continuous disturbance of the differentiation and maturation of the callus tissue.

Thyroxine and Thyroidectomy

THYROXINE

Phosphorus^{32} Deposition

Compared to the values recorded for the controls, the deposition data relating to the callus area in the specimens from thyroxine-treated animals agree fairly well with those found in the androgen group (Fig. 2d). The uptake at 7 days seems to be somewhat higher and so is the lowered value at 35 days. The densities measured on the epiphyseal line are rather strongly scattered but seem to be consistent with the androgen findings. The end-of-fragment and diffuse diaphyseal deposition curves are rather similar as in the control group.

Consolidation and Size of Callus

Consolidation was seen to be well under way at 14 days in the thyroxine group, with a consolidation close to the rating 1.5 (Fig. 4g). Already one week later the rating was as close to the maximum 3.0 as it rose in this group and as high as in the androgen group at its best. The dip at 35 days observed in the androgen group is absent here. Some sclerosis in the fracture area is evident in the x-rays.

The course of the callus size graph closely follows that in the androgen group during an early part of the period of observation, but its ascent continues at a good rate even when that of the androgen callus slows down. The ultimate value at 35 days is therefore slightly higher (Fig. 4h).

Histological Studies

Histological Findings. At 7 days, the animals treated with thyroxine presented a callus of considerable size, containing mainly fibrous tissue and fibrous cartilage, some hyaline cartilage and a scanty amount of new bone. Osteoblasts are seen in fair amount. The predominant feature in the callus is marked cell proliferation. The hyaline cartilage cells are hypertrophic, activated.

At 14 days, the formation of new bone is lively, starting in the subperiosteal area and extending up to the end of the fragments. Furthermore, profuse formation of hyaline cartilage, and little fibrous cartilage and fibrous tissue, is seen. The bone trabeculae are regular in form and rather thick; they are produced by osteoblastic activity. Peripherally, in smaller extent, cartilage tissue becomes ossified. The maturation of the callus has progressed farther than at the corresponding time in the controls.

At 21 days, the quantity of new bone is seen to have increased, and there are bone trabeculae between the fragments both endosteally and periosteally. Moreover, hyaline cartilage in the process of becoming calcified is noted. The findings suggest accelerated maturation of the callus and tendency of early bony repair.

At 28 and 35 days, a strong connection between the fragments has become established, resulting in a mature osseous callus.

Line Sampling. (Figs. 9 and 4i.) Histoquantitative analysis of the fracture callus produced under the effect of thyroxine reveals increased rate of repair from the earliest stage of examination onward. At seven days, the callus was composed of fibrous tissue, fibrous and hyaline cartilage and a blood clot. The relative quantity of fibrous tissue and fibrous cartilage at 14 days is scanty, whereas there is plenty of mature hyaline cartilage (about 40%). The latter was replaced, in the course of healing, by new bone and its accompanying vascularization, which occur from the earliest stage onward. New bone is observed earlier than in the controls. At 35 days, the callus consisted of vascularized, strong new bone containing less than 5% hyaline cartilage.

THYROIDECTOMY

Phosphorus^{32} Deposition

The radiophosphorus uptake in the intensely blackened area of the callus and at the epiphyseal plate in the thyroidectomy group is very

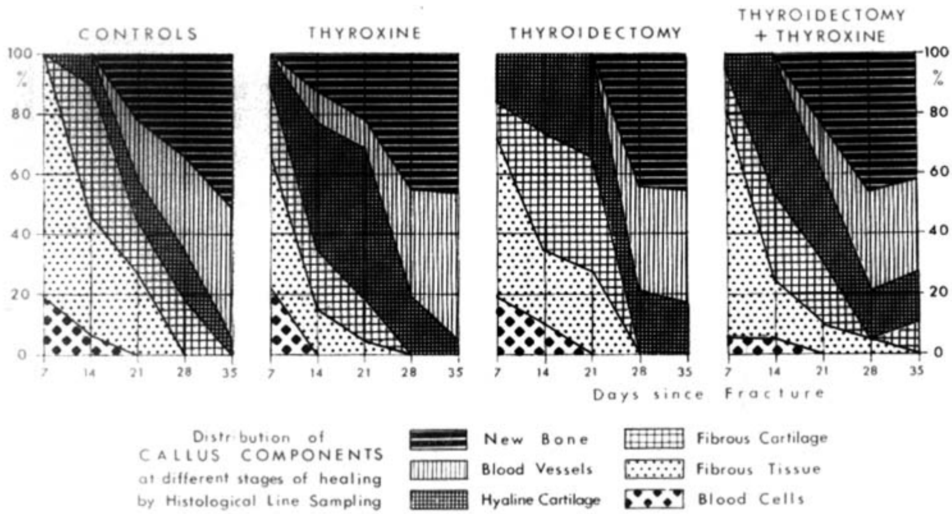


Fig. 9. Histoquantitative tissue analysis diagrams, showing the per cent. composition of the callus at different times after the fracture in the control, thyroxine, thyroidectomy and thyroidectomy plus thyroxine groups.

nearly coincident with the control values both in respect of character of the graph and level. The same is true for the other two recorded quantities (Fig. 2g).

Consolidation and Size of Callus

None of the x-rays of thyroidectomized animals taken at 7 and 14 days revealed traces of callus, but at 21 days the average degree of consolidation had reached the considerable value of 2.0. It continued to rise even further, up to 3.0 at 35 days (Fig. 4g).

The average planimetric size ascends little above 7 mm^2; this level was already reached on the 21st day after the fracture (Fig. 4h).

Histological Studies

Histological Findings. At 7 days, the callus is seen to contain great amounts of immature tissue components — fibrous tissue and fibrous cartilage. The hyaline cartilage cells are small, and the formation of new bone has its site deep under the detached periosteum, close to the outer surface of the diaphysis. The blood clot persists at the end of the fragments.

At 14 days, the components of the callus are still fibrous tissue and fibrous cartilage, and some quantity of hyaline cartilage. The formation of new bone is poor throughout. The development of the callus is clearly retarded.

At 21 days, the callus still contains much fibrous tissue, and there is no sign of new bone between the fragments.

At 28 and 35 days, the immature tissues have subsided and been replaced by a callus with bone trabeculae bridging the gap between the fragments. The trabeculae are rather thin and irregularly arranged; the callus is fairly large. There is some cartilage tissue left.

Line Sampling. (Figs. 9 and 4i.) The line sampling measurements reveal that the early callus is composed of fibrous tissue and fibrous and hyaline cartilage. At 21 days these components were present in fairly equal amounts, while fibrous tissue and fibrous cartilage disappeared by the time of 28 days. At 35 days there was still hyaline tissue left. A striking feature is the late appearance of new bone in the picture presented by the callus. New bone does not occur before the fourth week; subsequently, at the final stage, the callus was made up of new bone, new blood vessels and nearly 20% of hyaline cartilage.

THYROIDECTOMY PLUS THYROXINE

Consolidation and Size of Callus

Examination of the x-rays taken at 14 days in the group in which replacement thyroxine treatment was given to thyroidectomized rats revealed a slight amount of callus (Fig. 4g). During the next week quite rapid progress of consolidation brought its average degree up above 2.0. The ultimate status did not differ from that in the controls or in the thyroxine group; the density of the bone was as in the controls.

In respect of callus size according to planimetric study, the initial course of the graph obtained in the thyroidectomy group seems to be reproduced here up to the time of 21 days (Fig. 4h). Subsequently there is some further rise, while the 35-day value remains half-way between the thyroidectomy and control plots.

Histological Studies

Histological Findings. In the experiments involving both thyroidectomy and treatment with thyroxine, the callus present at seven days contains fibrous tissue, fibrous and hyaline cartilage and in the subperiosteal area, formation of new bone, which does not reach the end of the fragment. There is a fair number of osteoblasts. A blood clot surrounds the fragments.

At 14 days, the callus displays considerable formation of hyaline cartilage; there is some fibrous cartilage and fibrous tissue between the fragments, the apposition of which is good. The formation of new bone has progressed quite far, though not up to the end of the fragment. The maturation of the callus has progressed farther than at the equivalent time in the thyroidectomized animals; histologically, the status is even superior to that seen in the controls.

At 21 days, the formation of new bone has increased and the bone extends to the fracture area, bridging the gap. Hyaline cartilage is left in the peripheral area, and a zone of fibrous cartilage and fibrous tissue is seen between the fragments.

At 28 and 35 days, a fairly large osseous callus is seen periosteally and endosteally. There is a little fibrous tissue and hyaline cartilage in the space between the fragments.

Line Sampling. (Figs. 9 and 4i.) Histoquantitative study revealed the callus at seven days to consist mainly of fibrous tissue; some fibrous cartilage and incipient formation of hyaline cartilage are encountered in addition. The relative quantity of immature tissue components is slightly less than in the controls, indicating somewhat accelerated maturation. On the other hand the quantity of hyaline cartilage is somewhat greater throughout the period of observation. The formation of new bone is equivalent to that in the development of callus in the controls. At the final stage the quantity of new bone is slightly less, as is that of new blood vessels.

The persistent presence of hyaline cartilage and its fairly constant percentage throughout the period of observation is similar to the corresponding phenomenon in the thyroidectomy group, while it was seen in even stronger degree in the oophorectomized animals.

IV DISCUSSION

The influence of endocrines on the healing of fractures and on bone repair in general has long constituted an enthusiastically pursued subject of orthopaedic research. Convincing proof has been found of an osseous anabolic effect exerted by certain hormones. Thus, in experimental fracture, vigorous osteogenesis is called forth by growth hormone; its characteristic features include a rather specific reaction in the form of activated cells in the hyaline cartilage tissue, to which a role has been assigned in the induction systems for osteogenesis (19, 20, 37). The uptake of radioactive phosphorus reveals increased activity in the area around the bone fragments (15).

Thyrotropin produces rapid maturation in the differentiation of the callus tissue, and this hormone boosts the action of growth hormone during the bone repair process (15). This synergetic effect of the thyrotropic hormone was shown by re-establishment of growth in body length with the aid of growth hormone after hypophysectomy and its marked augmentation when thyroxine was administered (2). Thyroxine also stimulates the preosteoblasts in enchondral ossification (33).

The present findings are in support of such favourable effect of thyroid hormone on the regeneration of bone tissue, as evidenced by more rapid maturation of the callus, starting at the earliest stage of repair, and by increased formation of new bone and vascularization. Bony consolidation also occurred sooner, and autoradiography showed increased mineralization of the bone during the first half of the repair period.

On the other hand cortisone has a delaying effect on all processes in bone repair (5, 6). The cartilage tissue fails to undergo normal maturation. The course of the process is characterized by profuse occurrence of fibrous tissue and a tendency of delayed osseous union, which is also borne out by lower mineralization seen in autoradiographic studies with P^{32} (6, 15).

As regards the effects of thyroidectomy, there obviously is no simple explanation to account for the observations. However, at the early stage of healing clearly delayed differentiation is noted in the callus, which is rich in hyaline tissue and also contains immature tissue components in ample amount. This may be due to a non-specific effect on the metabolism resulting from the lack of thyroid secretion or rather, as certain facts might be taken to indicate, to lowered pituitary function causing reflectory reduction of the growth hormone output. One of the first signs seen after removal of the thyroid gland is degranulation of acidophils in the hypophysis, the presumptive source of growth hormone (2). In keeping with the above-mentioned observations is the slow consolidation noted in the present study in the thyroidectomized animals. The phosphorus deposition in the callus area, however, instead of going down as could be expected, remained closely at the level of the controls and was about one third of that recorded in the experiments with thyroxine in their initial stage.

The immediate effect of thyroidectomy on the development of the callus tissue is also a point worth discussing although the thyroid hormone is known to be relatively long-lived and the full effect of its deprivation might not develop immediately, whereas growth hormone is reported to have a short survival time and would be expected to disappear promptly after hypophysectomy (23, 30). This might partly account for the apparent slow response in phosphorus uptake at the early stage of repair.

The problem of restoring the thyroid hormone function by replacement therapy after thyroidectomy has been studied in respect of the growth of bone tissue. It has been shown that thyroxine injections stimulate active growth in thyroidectomized rats and concurrently the pituitary function is restored (2). The phenomenon was consistently demonstrable from the histological point of view in the present study, as well as it was evident in the assessments of bony consolidation and in the planimetric callus size data. The degree of maturity of the callus at the different stages of repair was approximately equivalent to that in the control group.

Oestrogenic hormones have been profoundly studied in their effects with birds (23). Experiments with mice, using C^{14}-labelled oestrone, indicate selective deposition of the hormone in the endosteum (9, 23). The entire marrow cavity may fill with new bone. Oestrogen has also been found to exert an influence on the bone growth apparatus and to participate in endochondral regeneration (9). Combined autoradiography and kinetic analysis (4) has been used to demonstrate inhibition of resorption by oestrogen, to which the endosteal bone formation is attributed rather than to growth-promoting function (36).

In the present study no mineral deposition in the medullary cavity was observed. Instead, the accumulation of P^{32} in the callus was strikingly elevated under oestrogen treatment, indicating its increased mineral content. The histological examination revealed high density and thickness of the bone trabeculae, while the osteoclasts were rather few. Roentgenographically, sclerosis could be seen at the ends of the fragments and the callus was small. These peculiarities became more pronounced with continued oestrogen therapy. The observations made in respect of this group are consistent with the concept that the ossification-promoting effect of oestrogen involves reduced resorption of bone as a particular characteristic of its calcifying action.

No such over-mineralization as in the oestrogen group was evident in the oophorectomized rats. On the contrary the autoradiographs revealed a strong decline of the deposition during the progress of repair. Assessment of the x-rays disclosed delayed consolidation between the fragments, and the callus size determined by planimetry of the x-rays was minimal, in fact rather consistently only one-half of that in the oestrogen group at the corresponding times. No readily obvious cause for this phenomenon suggests itself on the strength of existing knowledge.

The effects elicited by androgen administration in experimental fractures are commonly attributed to the proteo-anabolic action which this hormone evokes in the protein matrix of the bone and which leads to calcification (11). In the present study the influence of androgen manifested itself in rapid maturation of the callus characterized by early formation of new bone and vascularization. The callus, which was large on the strength of the planimetric studies, displayed elevated P^{32} deposition, though with a marked decreasing tendency towards the end of the observation period. It may also be noted that the assessment of consolidation at 35 days gave a poorer rating than in the control series and also inferior to that recorded in the androgen group at 28 days. This might be speculatively associated with previously reported similar effects of relatively high androgen dosage (1).

The densitometric measurements of autoradiographs, while serving the purpose of elucidating the course of fracture repair under the effect of endocrine agents or their deficiency, enable also observations to be made concerning the accumulation of the labelled mineral substance in the epiphyseal plate, at the ends of the bone fragments and in diffuse distribution in the diaphysis at a distance from the fragment end.

The high densities appearing in the autoradiographs as areas of intense blackening have long been associated with new-bone mineralization (8, 14, 15). In contrast, the diffuse darkening is thought to be connected mainly with the portion of the bone consisting of exchangeable matter.

The densitometrically measured values referring to the strongest blackening in the callus region on one hand and to the epiphyseal plate on the other as well as those found for the end of the fragment and for the diffuse deposition in the diaphysis seem to be very closely correlated and from their relations some inferences may be possible with regard to the mineralization during progress of bone repair.

The values showing the uptake of phosphorus in the callus, though varying within considerably wide limits in the different hormone administration and ectomy groups, are invariably higher than the corresponding data found for the epiphyseal plate. Both varied in the same manner during the observation period. Variations of largely similar character have been reported in studies concerning the normal healing of fractures (21, 38). The deposition at the end of the fragment was smallest of all in every set of four values and quite far below the deposition in the callus. These values are slightly but constantly exceeded by the diffuse deposition in the diaphysis. This indicates that the osteones of the devitalized end of the bone are inactive and unable to initiate the regeneration.

If the present results are evaluated from a general point of view, it must be noted that no claim of their general validity can be made without further consideration, nor is it safe to extrapolate from them directly to the effects of endocrines in human bone repair. However, it has been clearly demonstrated that these hormones, which one has not considered to possess targets with obvious bearing on bone (27, 28), as exemplified particularly by androgen with its effect on the nitrogen balance, exert an influence which enables the biological phenomenon involved in bone repair to be controlled to a certain extent. Further research may be able to show the value of these agents as aids in the treatment of certain kinds of cases in which it is desired to simulate the regeneration of bone. Opportunities for such studies are presented by numerous diseases of the bone with endocrine aetiology and by bone injuries occurring in association with them.

V SUMMARY

Investigations concerning the repair of experimental fractures of the tibia under hormonal treatment or hormonal deficiencies were carried out with the aid of densitometric measurement of autoradiographs, augmented by roentgenological observations, both visual and planimetric, and by histological and histoquantitative studies.

The quantitative autoradiograph evaluating method, by which data were obtained on the relative strengths of the radio-phosphorus deposition occurring at different times in the callus area, at the epiphyseal line, at the end of the bone fragment and diffusely in the diaphysis, proved to be instructive, furnishing information on the amount and development of mineralization under the different experimental conditions during the progress of fracture repair. With few exceptions, the P^{32} uptake correlated fairly well with the results of the roentgenographic and histoquantitative studies.

The highest density in the autoradiographs was regularly seen in the callus area, the values representing the isotope accumulation at the epiphyseal line being lower by about 30%. The diffuse deposition in the diaphysis and the uptake at the end of the fragment were in a lower order of magnitude, about one-fifth of the first-mentioned values. The mineralization at the end of the fragment was consistently lower than that indicated by the diffuse diaphyseal deposition.

In the callus area a marked increase in radiophosphorus uptake (40—50%) was noted at the early stage of bone regeneration under the effect of androgen and also of thyroxine treatment. Largely similar results were recorded in both respective experimental groups as regards mineralization, and the uptake also declined in both groups to the control level or somewhat below at the end of the observation period.

The action of oestrogen revealed itself in a most striking deposition of radio-phosphorus exceeding that in the controls by a factor of about 4. In contrast to all other experimental groups, moreover, the uptake remained at an elevated level throughout the repair process. It is suggested that the phenomenon is due to calcifying action of the hormone, enhanced by reduced resorption of bone.

Orchiectomy and thyroidectomy were found to have fairly slight influence on the bone repair process. The values indicating phosphorus activity at the fracture site coincided with the control level. On removal of the thyroid gland, however, the results of histoquantitative tissue analysis give evidence of delay at the early stage of repair in the cell differentiation involved in the maturation of the newly formed bone.

Upon oophorectomy, a falling tendency of the phosphorus deposition in the callus prevailed throughout the period of observation. The value recorded at the final stage of the repair process was only about one-half of the control level.

In the discussion the contribution of endocrines as biological factors influencing the regeneration of bone is considered. The need of further research is stressed by which an accurate idea might be formed of the beneficial effects in man that can be derived from hormones.

VI ZUSAMMENFASSUNG

Es sind Versuche betreffs der Knochenheilung bei experimenteller Tibiafraktur unter Hormonbehandlung bzw. Hormonmangel angestellt worden, wobei densitometrische Messungen an Autoradiogrammen durch sowohl visuelle als auch planimetrische röntgenologische Beobachtungen sowie histologische und histoquantitative Untersuchungen ergänzt wurden.

Die angewandte quantitative Methode zur Auswertung der Autoradiogramme, mittels welcher Angaben über die relative Stärke der zu verschiedenen Zeiten im Bereich des Kallus, an der Epiphysengrenze, am Ende des Knochenfragments sowie diffus in der Diaphyse stattfindenden Deposition von radioaktivem Phosphor gewonnen wurden, erwies sich als instruktiv, indem sie Aufschluss über Betrag und Entwicklung der Mineralisation unter den verschiedenen Experimentalverhältnissen im Verlauf des Reparaturprozesses lieferte. Mit wenigen Ausnahmen wies der P^{32}-Niederschlag recht gute Korrelation mit den Ergebnissen der zusätzlichen röntgenographischen und histoquantitativen Studien auf.

Die grösste Dichte in den Autoradiogrammen wurde regelmässig im Bereich des Kallus wahrgenommen, während die Werte für die Anreicherung der Isotope an der Epiphysengrenze um etwa 30% niedriger lagen. Der diffuse Niederschlag in der Diaphyse und die Aufnahme am Ende des Fragments waren geringerer Grössenordnung, etwa ein Fünftel der erstgenannten Werte. Die Aktivität der Mineralisation am Fragmentende war durchgehend niedriger als die durch die diaphysäre Deposition angezeigte.

Im Kallusbereich wurde eine merkliche Steigerung der Phosphoraufnahme (um 40—50%) im zeitigen Stadium der Knochenregeneration unter Einfluss von Androgen und auch bei Thyroxintherapie beobachtet. In beiden Versuchsgruppen wurden weitgehend übereinstimmende Ergebnisse bezüglich der Mineralisation verzeichnet; desgleichen ging die

Phosphoraufnahme in beiden Gruppen am Ende der Beobachtungsperiode auf Kontrollhöhe oder etwas weiter herab.

Die Wirkung von Oestrogen äusserte sich in einer sehr auffallenden Deposition von radioaktivem Phosphor, die Kontrollwerte etwa vierfach übertreffend. Im Gegensatz zu allen übrigen Versuchsgruppen hielt sich ferner hier die Aufnahme während der gesamten Dauer des Reparaturprozesses auf gesteigerter Höhe. Es wird geltend gemacht, dass die Erscheinung auf calzifierender Wirkung des Hormons, durch herabgesetzte Knochenresorption unterstützt, beruhen kann.

Bei Orchiektomie und Oophorektomie wurde recht wenig Einfluss auf den Knochenreparaturprozess festgestellt. Die Werte, welche die Phosphoraktivität am Frakturort anzeigen, fielen mit dem Spiegel bei den Kontrollen zusammen. Die Ergebnisse der histoquantitativen Gewebeanalyse nach Entfernung der Schilddrüse zeugen allerdings von einem Verzug der beim Reifen des neugebildeten Knochengewebes wesentlichen Zelldifferenzierung im zeitigen Stadium des Reparaturvorgangs.

Nach ausgeführter Oophorektomie bestand eine fallende Tendenz der Phosphordeposition während der gesamten Beobachtungsperiode. Im Endstadium des Reparaturprozesses wurde ein Wert verzeichnet, der nur der halben Höhe bei den Kontrollen gleichkommt.

In der Besprechung wird der Beitrag der endokrinen Drüsen und ihrer Sekretionen als biologische Faktoren mit Einfluss auf die Knochenregeneration berührt. Die Notwendigkeit weiterer Forschungsarbeit wird betont, um einen zutreffenden Begriff von den günstigen Wirkungen zu erhalten, die durch Hormone im Menschen erzielt werden können.

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