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THE REVERSIBILITY OF DISUSE OSTEOPOROSIS

EXPERIMENTAL STUDIES IN THE ADULT RAT

BY
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INTRODUCTION

Loss of the activity of an extremity is followed by atrophy of its muscle as well as its articular cartilage and bone components. The latter is usually called bone atrophy of disuse, or localized or disuse osteoporosis.

In animal experiments disuse of an extremity has been brought about mainly in four different ways, viz. a) *denervation* (Nasse 1880, Grey and Carr 1915, Allison and Brooks 1921, Armstrong 1946, Gillespie 1954, Geiser and Trueta 1958, Landry and Fleisch 1964, Kharmosh and Saville 1965, Semb 1966, Gedalia 1966, Klein et al. 1968, Sevastikoglou and Larsson 1972 b), b) *amputation* (Hulth and Olerud 1962, Eriksson and Olerud 1966, Sevastikoglou and Larsson 1972 a) or bone resection and disarticulation (Allison and Brooks 1921, Klein et al. 1968), c) *resection of the Achilles tendon* (Brandes 1913, Rieder 1936, Geiser and Trueta 1958, Landry and Fleisch 1964), and d) *immobilization with a plaster of Paris cast* (Grey and Carr 1915, Allison and Brooks 1921, Scaglietti and Casuccio 1936, Rieder 1936, Landoff 1942, Geiser and Trueta 1958, Semb 1966, Haike et al. 1967, Sevastikoglou and Mattsson 1972 a). Other methods of inducing experimental atrophy of disuse in an extremity have also been described, e.g. fixation of the extremity by suturing it subcutaneously in a thoracic pouch (Müller 1924), wiring of the calcaneus to the tibia (Müller 1924), and immobilization of the knee joint by splint fixation between the femur and tibia (Evans et al. 1960, Thaxter et al. 1965).

Extensive reviews of earlier investigations are to be found in the works of Landoff (1942), Geiser and Trueta 1958, and Semb (1966), among others.

Bone atrophy of disuse represents a metabolic disturbance of the skeleton common in traumatologic patients. The localized osteoporosis affecting the immobilized extremity can be but slight, and almost undetectable by plain radiographic examination, but it can also be pronounced, then giving rise to radiographic signs and clinical symtoms as a result of the disturbed calcium metabolism. In some special cases it develops as a part of Sudeck´s atrophy.

It is well known that the degree of and the time interval for development of bone atrophy of disuse vary from patient to patient. It is also known that the extent of the skeletal changes depends on different factors, such as the age of the patient, the length and the form of the immobilization and the type of trauma, among others.

Studies concerning the nature of the skeletal changes induced by immobilization, their development and their aetiology are numerous. The character of the muscular affection caused by immobilization has also been studied fairly extensively. However, very little is known as to whether these changes are reversible after remobilization or not. Indeed there are very few reports based on clinical observations (Nilsson 1966, Sevastikoglou et al. 1969) and experimental data (Geiser and Trueta 1958, Haike et al. 1967) regarding the post-remobilization fate of the skeletal and muscular changes due to immobilization. In a series of investigations carried out in this laboratory (Sevastikoglou et al. 1972), it was observed that in below-knee amputated adult rats the femur of the amputated leg developed severe localized osteoporosis, which, however, showed signs of regression towards the end of the observation period (Sevastikoglou and Larsson 1972 a). The same evidence of regression was provided when immobilization was brought about by a plaster cast (Sevastikoglou and Mattsson 1972 a) and also by denervation of the extremity (Sevastikoglou and Larsson 1972 b).

The observation mentioned above and the fact that there is surprisingly little information regarding the process of reversibility of the changes induced in an extremity by inactivity were considered to motivate a systemic investigation. The present work was therefore carried out in order to study the effect of remobilization on the muscle and bone components of an extremity which had been previously immobilized with a plaster cast for 16 weeks. Remobilization was achieved simply by removing the plaster cast and allowing the animals to walk freely around in their cages until they were killed at different time intervals from 1 to 48 weeks from the start of remobilization, these comprising the observation periods. In order to ensure that the results obtained were due to the remobilization *per se*, two parallel series consisting of normal untreated and further immobilized animals, respectively, were examined in an identical way. Age factors and any trend to spontaneous restitution in spite of continued immobilization could thereby be excluded.

Rats were chosen as the experimental animals for this investigation mainly because it has been stated earlier that their calcium metabolism is similar to that of man (Nicolaysen and Eeg-Larsen 1953, Bronner and Lamaire 1969). Furthermore, apart from several reasons for the choice of rats in similar experiments reported earlier (Larsson 1969) these animals are easily handled and are resistent to trauma and infection. There are some disadvantages in the use of rats, however. Thus, in studies concerning their activities under varying experimental conditions difficulties are encountered due to the fact that they are nocturnal in their activity habits, as pointed out earlier (Goff et al. 1957). The rats used in this work were male adult animals which at the beginning of the observation period were approximately one year old and had thus reached about one third of their life span, viz. 2.5 to 3 years. It has been stated earlier that in experimental studies regarding disturbances of the skeletal metabolism adult animals should be used to eliminate the interference of skeletal growth with the skeletal response to the treatment (Larsson 1969 a). In the rat, calcification of bone is rapid during the first 6 months of life and remains steady thereafter (Henry and Kon 1953).

The present investigation was undertaken with the purpose of obtaining information regarding the following main questions

- to what extent does immobilization give rise to reversible changes concerning body weight, walking pattern, joint movement and muscular weight?

- to what extent are the induced loss of bone, the changes in wet weight and hydrated gross bone density reversible, and is it possible by reactivation to stimulate the atrophied adult rat bone to increased new bone formation?

- to what extent do changes in the collagen and mineral contents as well as in the calcium metabolism occur during the process of reversibility?

Information regarding these questions related to the "Reversibility of Disuse Osteoporosis" in the adult rat was provided by the following separate studies:

I. General observations.

II. Quantitative studies of the skeletal mass and bone formation.

III. Studies on bone composition.

IV. Studies on the chemical composition of the bones.

V. Studies of calcium metabolism.

A list of references to this part of the work is to be found at the end of this book.

Definition: The term *variable* used in this work refers to the difference between the right leg and the left leg for the factor under study. The right leg constituted the remobilized hind limb in the R series, the continuously immobilized hind limb in the C series and the right normal hind limb in the N series. The left hind leg constituted the untreated control leg in the R and C series and the contralateral normal hind leg in the N series.
The time relationship for these variables for each experimental series was described in the form of *regression curves* of second degree, i.e. an expression $Y = a + bt + ct^2$ was adapted for each variable Y, where the constants a, b and c were estimated from the sample. For the specific variables the regression curves and the observed mean of the variables at each observed time point were presented in a diagram.
Particular interest was devoted to the task of comparing the time relationship for the variable Y between the different experimental series.
For a specific time point t_0 a regression curve is determined by the *location* $Y_{0_i} = a_i + b_i t_0 + c_i t_0^2$ and the *slope* $Y'_{0_i} = b_i + 2c_i t_0$ (i = N, R or C).

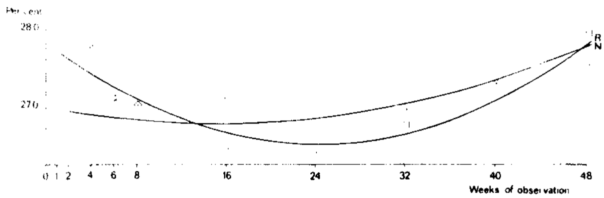
The hypothesis H_0: $Y_{0_i} = Y_{0_j}$ and $Y'_{0_i} = Y'_{0_j}$

H_1: $Y_{0_i} \neq Y_{0_j}$ and/or $Y'_{0_i} \neq Y'_{0_j}$ $\quad i \neq j$ $\quad i,j = N,R,C$

was tested for each combination of i and j and for t_0 taking on values corresponding to every second week in the interval 1-48 weeks.
The tests were performed at the 5% significance level ($p < 0.05$), i.e. the probability of falsely rejecting the null-hypothesis H_0 is less than 0.05.
For a pair of experimental series these tests resulted in a partitioning of the t-axis into regions where the null-hypothesis was rejected, i.e. regions where the two regression curves under study could be separated regarding location- and/or slope-differences.
The *theoretical derivation* of the test above is made under the assumption that the two-dimensional random variable (Y_{0_i}, Y'_{0_i})

is normally distributed, its mean vector and covariance-matrix being estimated from the sample of experimental serie i (i=N,R,C). The testvariable used then turns out to be distributed as Hotelling's T^2. For more detailed derivation see Morrison (1967).

Example. In the figure below the regression curves corresponding to the R and N series are shown. The test procedure



resulted in a rejection interval 0 - 18 weeks. This rejection was probably due to difference in slope alone from the 8th week, and due to a combination of slope and location differences for small t-values. For t-values beyond the 18th week the two slopes seem similar and the difference in location is not great enough to cause rejection on its own.

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THE REVERSIBILITY OF DISUSE OSTEOPOROSIS

I. General Observations in the Adult Rat [1].

by

Sten Mattsson

The effect of inactivity of an extremity on its muscular and bone components has been well established in earlier investigations, both in man and in animals. Very little is known, however, about the fate of the muscular and the bone affection after reactivation. Immobilization with a plaster cast provides a possibility of studying the degree of reversibility of the changes that have developed, when the cast is removed and the extremity remobilized. This opportunity has been taken in some earlier studies on animals (Geiser and Trueta 1958, Haike et al. 1967) and in man (Nilsson 1966). However, there are no previous reports regarding the reversibility of localized changes due to inactivity, based on observations made in a prospective investigation in experimental animals. Earlier studies on rats have provided very little information concerning the normal and the abnormal walking pattern, the range of joint movement and the degree of restitution of the atrophied muscular component of the extremity after remobilization of the animals.

(1) This investigation has been aided by grant nr 17X-69-06 from the Swedish Medical Research Council and by the Faculty of Medicine, University of Umeå, Sweden.

The aim of the present investigation was to study in adult rats:

a. the effect of different states of immobilization and remobilization on the body weight,

b. the walking pattern and the range of joint movement in the hind limb in normal animals,

c. their behaviour and pattern of movement during immobilization of one hind limb with a plaster cast,

d. their behaviour and pattern of movement after removal of the cast and

e. the degree of restitution, if any, of the impaired joint movement and of the atrophied muscles of the immobilized extremity after remobilization.

MATERIALS

Animals used for the studies

Male albino Sprague-Dawley rats[1] were used. All animals were 8 to 10 months old at the beginning. They were maintained under identical standard laboratory conditions throughout the experiment. The rats were bred on a standard laboratory diet[2] containing, on a dry weight basis: 0.8 per cent calcium, 0.6 per cent phosphorus, 22.5 per cent protein and adequate amounts of vitamin D, i.e. about 15 IU D_2/10 gms diet. Food and tap water were provided *ad libitum*. Rabbit cages were used, with 5 rats to each cage, thus providing adequate space for each animal to move around freely.

Altogether 250 rats were allocated into 3 series as follows.

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1) Anticimex Co., Stockholm
2) The diet was provided by Ewos AB, Södertälje, Sweden

Experimental animals

R series: This series comprised 100 animals divided into 10 equal groups. Their mean initial body weight was 505 $\pm$ 25 gms. The right hind leg of these rats was immobilized with a plaster cast, as described below, for 16 weeks. After this period the animals were *remobilized* by removing the plaster cast. The rats from each group were killed 1, 2, 4, 6, 8, 16, 24, 32, 40 and 48 weeks from the first day of remobilization.

Control animals

a. *N series:* This series comprised 50 animals divided into 5 equal groups. Their mean initial body weight was 511 $\pm$ 27 gms. These rats were left *untreated* and served as normal controls. They were allowed to move around freely in their cages both during the initial period of 16 weeks and subsequently until they were killed 2, 6, 16, 32 or 48 weeks thereafter.

b. *C series:* This series comprised 100 rats divided into 10 equal groups. Their mean initial body weight was 511 $\pm$ 27 gms. These rats were immobilized initially for 16 weeks in the same way as those of the R series, but in this series the immobilization was then *continued* until the animals were killed at intervals between 1 and 48 weeks, corresponding to those of the R series.

METHODS

Technique of immobilization

The right hind leg was padded with a strip of polyvinyl and then immobilized by application of a plaster cast around the pelvis and abdomen. The whole extremity was thus immobilized with the hip joint in a neutral position as described below, the knee joint at 35^{o} flexion and the ankle - talocrural and subtalar - joint at about 45^{o} dorsiflexion from the right angle to the longitudinal axis of the tibia. The plaster cast was changed at least every second week.

When applying the cast special care was taken to leave the left hip and leg completely free for normal movements. The cast was applied without anaesthesia, with the rat fixed in a simple cage constructed for the purpose.

Killing of the animals

The animals were killed by exsanguination from the abdominal aorta under ether anaesthesia.

The positions of the joints and walking pattern of the hind legs of the normal rat

Six rats of series N were radiographed *in vivo* to study the resting posture of the hind legs.

Clinical observations of the walking pattern of the three series of rats were made continuously during the course of the experiment. Recording by means of slow motion and ordinary cinematography was performed on 1 occasion in 2 animals of series R, remobilized for 2 days, and in another 2 remobilized for 3 weeks. Furthermore the motion pattern of 2 animals from series C and 2 normal animals was also studied by cinematography.

Preparation of the hind legs

Both hind legs from 3 rats out of each group in all three series were used for the investigations described in this paper. The limbs were disarticulated at the hip joint immediately after killing of the animal. The muscles around the hip were detached from the pelvic skeleton. The limb specimens were then stored at -20°C pending subsequent studies. After thawing the specimens at room temperature examination and removal of the muscles was carried out on a table covered with wet filter paper in order to keep them moist.

Articular mobility

A. In 3 rats from the N series, the passive movement in the joints of the hind limbs was recorded by means of a square rule, while they were still alive, without anaesthesia.

B. In 3 rats of each group of series R, N and C the range of movement of the hip joint was studied in connection with the disarticulation of the joint immediately after the animal was killed. At the time of disarticulation the muscles were soft and relaxed and there were no signs of *rigor mortis*.

C. The full articular mobility of the knee and ankle joints was measured on the thawed limbs from 3 rats by means of a square rule. The joints and muscles were distended gently during the examination, without applying any excess force.

Weight of muscle

The gastrocnemius-soleus and the anterior tibial muscles were thoroughly dissected free from surrounding tissues and were placed immediately in a closed damp container of known weight. The specimens were then weighed on a Mettler balance, with an accuracy of 0.05 mg.

Statistical methods

The regression analysis and null hypothesis testing, which have been described previously were used (Mattsson 1972). The term variable refers to the difference between the values for the right and the left leg of the factor under study.
Chi-2-test was used for comparisons of the mortality rates between the three series of animals.
Student's t-test was used for comparisons of the body weight of the animals at different time points.
The following significance grading was used:

non-significant	$p > 0.05$
almost significant	$0.01 < p < 0.05$
significant	$0.001 < p < 0.01$
highly significant	$p < 0.001$

RESULTS

The mortality of the total 250 animals was 10 per cent. Ten, 1 and 15 deaths occurred among the rats of series R, N and C, respectively. The mortality rate for the C series was almost significantly higher than that for the N series. On the other hand, there was no significant difference in mortality between series R and N.

The surviving rats as a whole sustained the treatment well. The immobilized extremity was usually depilated and in some cases there were superficial ulcerations of the skin at the groin. Ulcerations of the skin were also observed in the prominent part of the heel, but there was no pronounced oedema of the leg or the paw.

Body weight

During the initial period of immobilization (16 weeks) the rats of the N series increased their body weight by about 50 gms, i.e. approximately 10 per cent. During the same period of time the rats of series R and C decreased in weight by a mean of 95 $\pm$ 10 and 84 $\pm$ 12 gms, respectively, i.e. approximately 18 per cent.

The body weight reduction in the R and C series is significant.

On remobilization the body weight of the rats from the R series increased rapidly during the first 4 weeks by about 70 gms. The maximum mean weight, 603 $\pm$ 33 gms, was reached on the 24th week of observation and thereafter slowly decreased, to 534 $\pm$ 37 gms on the 48th week (Fig. 1).

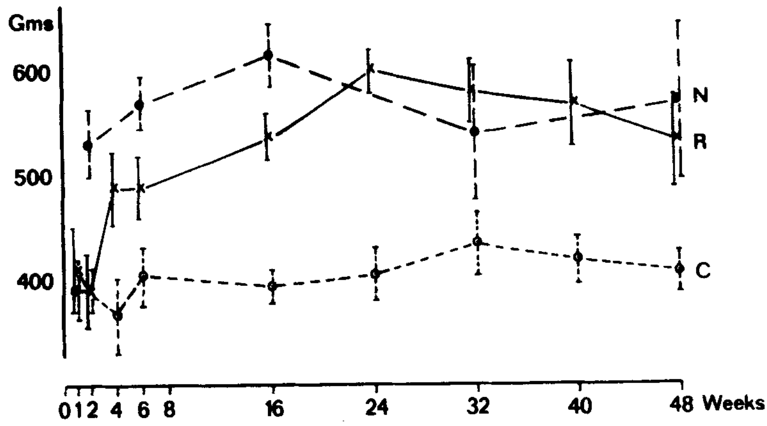


Fig. 1. Final body weight of the rats of series R, N and C.

The body weight of the rats of series C remained fairly constant at about 400 gms during the 48 weeks of continued immobilization, indicating that a steady state level had been attained after the initial 16-week long immobilization period.

The rats of series N increased their body weight to a maximum of 616 ± 38 gms by the 16th week of observation. Thereafter a slight decrease was noted (Fig. 1).

The positions of the joints and walking pattern of the hind legs of the normal rat

When a normal rat stands resting on a horizontal plane it keeps the hind legs in approximately the neutral position shown in Fig. 2.

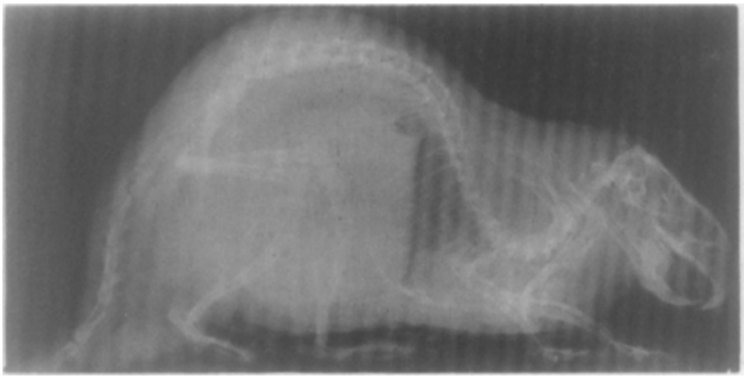


Fig. 2. Radiograph of a normal rat in the resting position.

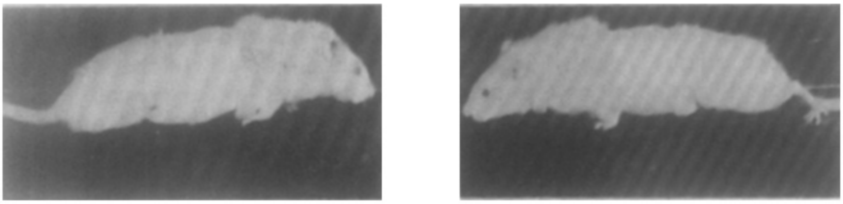
Thus, with the hip joint in the neutral position, the angle between the femur and the horizontal plane ranges from 10° ventrally to 10° dorsally. The pelvis and lower part of the lumbar spine form an angle of between 45° and 60° with the horizontal plane, depending on how much the rat has gathered itself together.

The angle of the knee joint ranges from 80° - 45° of flexion.

The foot is kept in 10° - 25° dorsiflexion at the ankle (talocrural plus subtalar joints) measured from the right angle in relation to the longitudinal axis of the tibia.

When walking, the rat has a somewhat rocking gait. The main part of the movement takes place in the hip joint, the femur being extended dorsally about 45° - 110° from the resting position. The range of active movement in the knee joint is rather restricted, to a range of 80° - 45° of flexion, i.e. the same as in the resting posture. The ankle is moved to a range between approximately 25° dorsiflexion to 10° plantar flexion. The faster the rat walks, the greater the dorsal extension at the hip joint and the plantar flexion at the ankle (cf. Figs. 3-6).

The walking pattern of the immobilized rat



Figs. 3 and 4. Immobilized rat. The right hind leg is completely immobilized under the plaster cast. The left leg moves freely.

During the period of immobilization in plaster cast the rats of the R and C series moved around with some difficulty in their cages, using only three legs. Otherwise they seemed to be quite unaffected. They did not haul their hind part on the ground when walking (Figs. 3 and 4).

The walking pattern of the remobilized rat

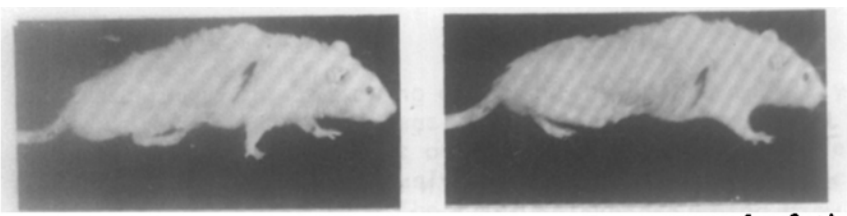


Fig. 5a and b. **Remobilized rat 2 days after removal of the** plaster cast.

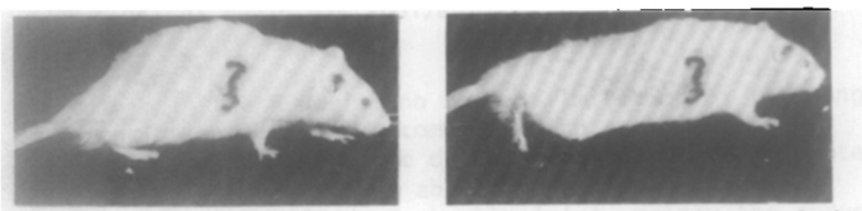


Fig. 6a and b. Remobilized rat 3 weeks after removal of the plaster cast.

In both instances the right hind leg is kept in a non weight-bearing posture (Figs. 5a and 6a) and in a weight-bearing posture (Figs. 5b and 6b).

From the time of removal of the plaster cast the rats were allowed to walk around freely in their cages. There was a clear difference in the weight-bearing and active movements of the right remobilized leg as compared with the left non-immobilized control extremity. Two days after remobilization the remobilized hind leg was only lightly pressed against the floor when the rat walked slowly (Fig. 5a and b). Some more pressure force was applied when it moved around faster. The walking movements took place almost exclusively at the hip joint while the active movements of the ankle were still restricted.

Three weeks after removal of the plaster cast full weight was borne on the remobilized leg (Fig. 6a and b). There were no signs of dysfunction of the hip or knee joints, but in many cases there was still some restriction of the active movement of the ankle (cf. Fig. 4 and 6b).

Joint movement

a. *In living rats* from the N series no marked variations were noted concerning the passive movement in the different joints. Neither was any difference observed in the range of movement between the joints of the right and left hind leg. The results of these examinations are given in Table 1.

Table 1. Passive joint movement of the hind leg of the normal rat.

Joint	Direction of movement	Range of movement (1)
Hip in relation to the horizontal plane with the pelvis at an angle of about 60°	Extension dorsally	110 - 115°
	Flexion ventrally	10°
	Abduction	60°
	Inward rotation	about 45°
	Outward rotation	about 45°
Knee open angle between longitudinal axes of femur and tibia	Extension	120 - 125°
	Flexion	20°
Ankle (talar plus subtalar) measured from a right angle in relation to the longitudinal axis of the tibia	Dorsiflexion	65°
	Plantar flexion	50°

(1). Mean values of 6 examined joints.

b. *Post mortem* the following observations on the mobility in the joints of the hind legs were made: The pronounced limitation of joint movement of the immobilized extremities in series R and C was obviously diminished when the muscle attachments were released. The rigidity due to capsular shrinkage seemed thus to be of less importance for the restricted joint movement.

Hip joint: In the R series no marked limitation of the range of extension or flexion as compared with the N series was observed at any point of the observation period. A moderate restriction of the range of abduction was noted, however. In series C the mobility of the hip joint, mainly the abduction, decreased progressively towards the end of the observation period. In no case was ankylosis observed in the hip joint. In the N series no signs of limited movement in the hip joint were noted during the period of observation.

Knee joint: No appreciable changes of the knee joint movement of the non-immobilized extremities were observed during the period of observation (Fig. 7 b). Furthermore, there was a normal range of knee flexion during the course of the experiment in both the immobilized and the non-immobilized extremities. In contrast, there was appreciable restriction of the extension in the R and C series (Fig. 7 a and c). Thus the knee joint extension regression curves showed significantly different courses in the animals from each of the three series (Fig. 8). The restricted range of extension of the knee joint due to the initial immobilization did not increase later despite remobilization. The mean values of the knee joint movement in the rats of the C series (Fig. 7 c) and the regression curves of the N and C series suggest that continuous immobilization resulted in further restriction of the mobility of the knee joint.

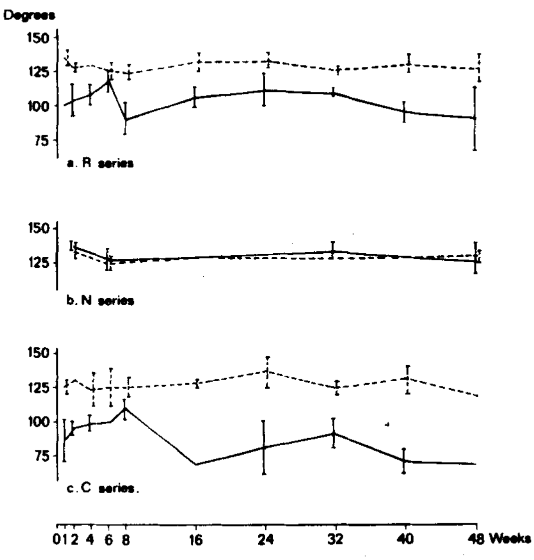


Fig. 7 a-c. Mean values of the range of passive extension in the right (——) and left (- - -) knee joints in series R, N and C.

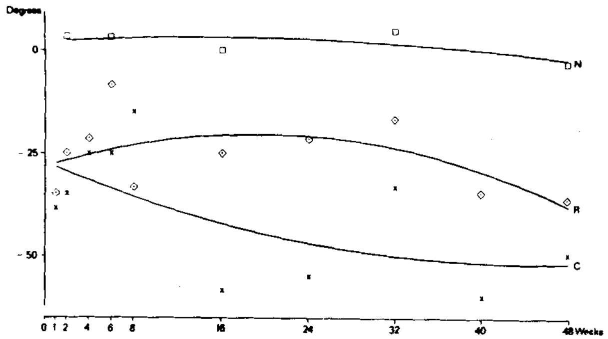


Fig. 8. Range of extension of the knee joint. Regression curves and mean variables for series R (◇), N (□) and C (x).

Ankle joint: During the period of observation there were no appreciable differences in passive ankle joint mobility from that of the non-immobilized extremities (Fig. 9 a-c). The regression curves for the R and C series differ significantly from that of the N series throughout the observation period (Fig. 10). Between the R and N series only a minor difference in slope and very small distance variation existed. These observations indicate that there was no evident increase in mobility of the ankle joint during the 48 weeks of remobilization. The regression curves of the R and C series differ significantly after the 18th week of observation. Anchylosis of the continued immobilized ankle joint occured towards the end of the experiment.

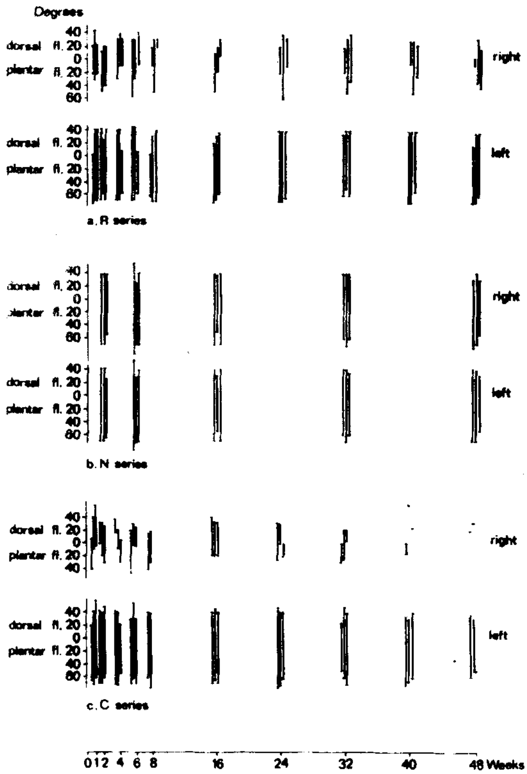


Fig. 9 a-c. Range of movement of the ankle joints of series R, N and C.

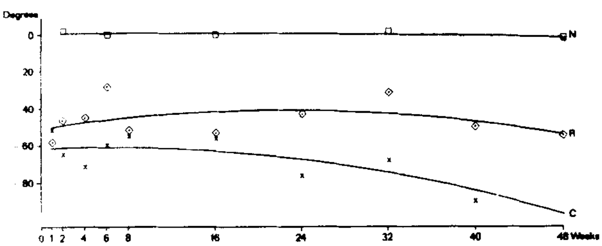


Fig. 10. Range of movement of the ankle joint. Regression curves and variables of R (◇), N (□) and C (x) series.

Muscle weight changes

Gastrocnemius + soleus muscles: The course of the muscle weight of the left control limb of series R with time agreed to a large extent with that of the corresponding limb of series N (Fig. 11 a and b). The observed reduction of the muscle weight by about 25 per cent between the 16th and 48th weeks in both the N and R series was considered to be due to the advanced age of the rats. Whether the remobilization *per se* led to an increase in the muscle weight of the right remobilized limb is hard to say, since the initial increase in muscle weight of the left control limb seemed to be of the same magnitude. Continued immobilization did not appear to give rise to further atrophy of the muscles in series C (Fig. 11 c). Regression analysis confirmed that there were no signs of reversibility due to remobilization *per se*.

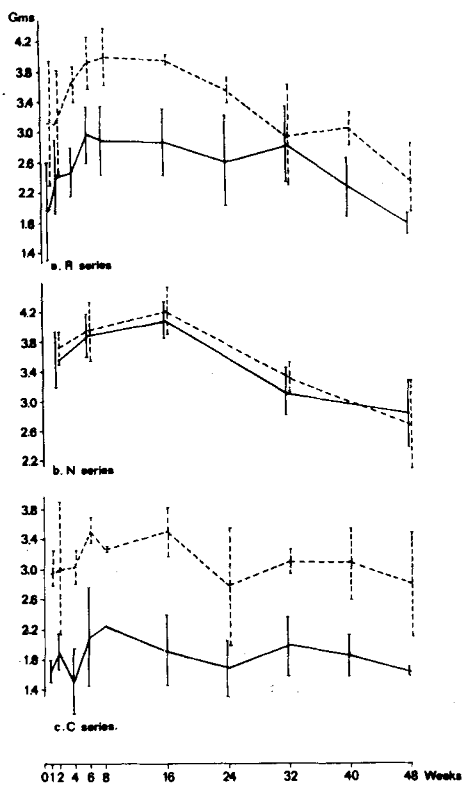


Fig. 11 a-c. Mean values of the weight of the right (——) and left (- - -) gastrocnemius plus soleus muscles in series R, N and C.

Anterior tibial muscle: The results were almost identical to those for the combined gastrocnemius and soleus muscles.

DISCUSSION

Differences in the mortality rate observed between the rats from the three series suggest that the treatment itself and not the advanced age of the animals was the main lethal cause. The changes in body weight observed during the course of the experiment support this assumption further. Thus the body weight of the immobilized rats decreased by about 18 per cent during the initial period of plaster fixation. No further decrease of the body weight was recorded in the continuously immobilized rats. A similar decrease in body weight was observed also in an earlier experiment (Sevastikoglou and Mattsson 1972 a). When the animals in the R series had had their plaster removed and were allowed to move about freely, their total body weight increased rapidly again and by about the 20th week reached the same level as the body weight of the animals of series N. This observation indicates that the loss in body weight of the rats from series R was treatment dependent and reversible.

The period of the initial immobilization - 16 weeks - was long in consideration of the life span of the rat. This period was, however, chosen intentionally because of the observation made earlier that in rats immobilized in an identical way bone atrophy reached a steady state level after 16 weeks. A trend of spontaneous restitution despite continued immobilization was observed later (Sevastikoglou and Mattsson 1972 a). This observation necessitated a control series of continuously immobilized animals. Further, the advanced initial age of the animals and the prolonged observation period in this experiment necessitated also another control series of untreated animals of the same age as the experimental ones. This series of normal rats was to serve as controls to find out if there were any, age-dependent variation. The results of the present studies showed that some observed changes which occured from the middle of the observation period in the normal untreated rats were obviously age-dependent. At this point the animals had reached only approximately half their life span, viz. 2.5 to 3 years. Thus the use of dual control series and the observed age-dependent changes have been proved of utmost importance for the interpretation of the obtained results throughout this investigation.

Plaster fixation was used as the method of immobilization because it allows remobilization of the extremity at will. Further, by fixation with a plaster cast the whole limb is subjected to a total and equal degree of inactivity. Plaster cast fixation has also been used in previous immobilization-remobilization experiments in guinea-pigs (Haike et al. 1967).

The observation has been made here that when walking, the normal rat moves mainly by dorsiflexion of the hip joint and plantar flexion of the ankle. The simultaneous extension of the knee joint is fairly limited. This observation is contradictory to the statement that the rat, when walking around in its cage, makes use of a range of movement of about 90° in the knee joint (Hall 1964).

The residual passive movement in the hip and knee joints observed *post mortem* in the rats from the R series, though restricted, appeared to be of a sufficiently large range to allow normal walking if utilized actively. Clinical studies and cinematographic recording of the walking pattern confirmed this assumption. Remobilization of knee joints after splint fixation for 16 weeks in rats has also been reported to fail to restore completely the range of movement (Evans et al. 1960).

Observations in rabbits the one extremity of which was immobilized in a subcutaneous thoracic pouch or by wiring of the calcaneus to the tibia (Müller 1924), in dogs in which one knee joint was immobilized in a plaster cast (Scaglietti and Casuccio 1936) and in adult rats in which one knee joint was immobilized by a splint (Evans et al. 1960), suggest that it is the muscular rather than the capsular structures that are mainly responsible for the limitation of movement after immobilization. These observations have been confirmed by the findings in the present study.

SUMMARY

In adult male Sprague-Dawley rats one hind leg was immobilized in a plaster cast for 16 weeks. The rats were then remobilized by removal of the cast and were allowed to walk around freely until they were killed in groups from 1 to 48 weeks after the time of remobilization. Normal as well as continuously immobilized animals served as controls.

On walking the hind legs of the normal rat were found to be moved mainly by dorsiflexion of the hip joints and plantar flexion of the ankles. A fairly small range of extension occurred simultaneously in the knee joints.

The initial immobilization resulted in a significant decrease of the body weight of the animals. After remobilization the body weight increased rapidly again to normal.

The initial period of immobilization also resulted in an altered walking pattern, a decreased range of joint movement in the immobilized extremity and a decrease in weight of its muscles.

On remobilization of the experimental animals the gait was rapidly restored practically to normal. On the other hand the restricted mobility of the joints as well as the decrease in muscle weight were found to be irreversible.

Continued immobilization of the extremity did not result in further decrease of the body weight or of the muscle mass of the extremity. On the other hand the movement of the joints was still more restricted during the period of prolonged immobilization.

An age-dependent reduction of the muscle weight by approximately 25 per cent without a corresponding decrease in body weight was noted from the middle of the observation period when the rats had reached half their life span, both in the normal and the experimental animals.

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THE REVERSIBILITY OF DISUSE OSTEOPOROSIS

II. Quantitative Studies of the Skeletal Mass and Bone Formation in the Adult Rat[1)]

by

Sten Mattsson

Clinical as well as experimental investigations concerning the local and general response to inactivity during the development of disuse osteoporosis have been reviewed previously (Mattsson 1972). It seems, however, that no investigations have been carried out earlier for studying on a morphological basis, the possibility of restoration of skeletal changes induced by inactivity of an extremity after reactivation has been introduced.

Conventional radiographic methods used for evaluating the degree of mineralization of the skeleton are rather crude (Kellgren 1956, Virtama and Helelä 1969). New radiographic

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(Barnett and Nordin 1960, Virtama and Helelä 1969, Garn 1970) and densitometric methods (Cameron and Sorenson 1963, Mack 1965, Nilsson 1966, Sorenson and Cameron 1967) have therefore been developed to provide more reliable information regarding the mineralization of the skeleton *in vivo*. The observation that tetracycline is deposited rapidly and exclusively on bone surfaces undergoing active mineralization (Milch et al. 1957) has been utilized for chemical quantitative determination of bone formation in specimens from rats during the development of disuse osteoporosis (Landry and Fleisch 1964). A quantitative microradiographic technique for estimation of bone formation and resorption has also been described (Jowsey et al. 1965).

Each of the methods mentioned above is afflicted with certain limitations depending mainly on subjective judgement, difficulties in preparing exactly comparable bone specimens and large variations of the values due to biological differences. It is, however, possible to demonstrate changes in degree and rate of mineralization appearing under different experimental conditions, even when a relatively small number of specimens is examined at each time interval over a long period of observation, if the results of studies based on several methods show a similar trend.

The aim of the present investigation was:

a. to study, on a morphological basis, quantitative changes regarding the amount of bone and the bone formation activity during the process of reversibility of bone atrophy of disuse, produced in one hind leg of adult rats by immobilization with a plaster cast followed by remobilization for different time intervals.

b. to compare the results obtained with those of corresponding studies in normal and continuously immobilized control rats.

MATERIALS

The materials used in these studies were provided from animals used in an earlier investigation (Mattsson 1972 a). They were adult male Sprague-Dawley rats aged 8 to 10 months.

Experimental animals

In the so called *R series* (remobilized) the right hind limb was immobilized by a plaster cast for 16 weeks. The cast was then removed and the animals were allowed to walk around freely. Ten groups were observed for 1 to 48 weeks after remobilization before the animals were killed.

Control animals

a. The *N series* (normal, untreated) comprised 5 groups of rats which did not undergo any form of immobilization and were observed for 16 plus 2 to 48 weeks.

b. In the *C series* (continuously immobilized), comprising 10 groups, the rats were immobilized continuously for 16 plus 1 to 48 weeks and then killed.

The time of observation concerning all three series was calculated from the time of remobilization of series R.

METHODS

Tetracycline labelling in vivo

Three animals from each group of the three series were "labelled" with two doses of 10 mg oxytetracycline (Terramycin, Pfizer) administered by intraperitoneal injection 2 weeks and 1 week before the rats were killed.

Femoral and tibial length

After the bones had been freed of soft tissues the lengths of the femur and tibia were measured from the most proximal to the most distal parts of the bone. The measurement was performed with a caliper, accuracy 0.05 mm. Three animals from each group of series R, N and C were examined.

Radiography

The dissected femurs and tibiae from three animals of each group of the three series were examined radiographically on a frontal and lateral projection. The bones were fixed directly on the x-ray film (Agfa Structurix D 4). The same conditions of exposure (50 MAS, i.e. 60 KV, 250 m A, 0.20 sec and 100 cm film-focus distance) and development of the films were used throughout.

Evaluation of radiographs

The radiographs were studied by "blind" examination twice at x 2 magnification. The radiopacity of the femur and tibia was estimated by a subjective assessment under simultaneous comparison with a radiograph of bones of series N from the 2nd week of observation. The term osteopenia is defined as "too little calcified bone" (Bauer et al. 1958). The degree of osteopenia was classified as:

uncertain (0): cortex of normal regular thickness, dense network of trabeculae and normal radiopacity of the whole bone, with no certain difference from the normal control bones.

moderate (1): cortex of normal regular thickness, decreased radiopacity of trabeculae in the metaphysis and epiphysis, and normal radiopacity in the rest of the bone.

pronounced (2): thickness of cortex regularly or irregularly diminished, with increased diameter of the medullary canal, thin and sometimes few trabeculae, decreased radiopacity of the whole bone, and in some cases spots of sclerosis due to decalcification of the neighbouring bone.

Femur and tibia scores

The femur and tibia scores were determined by the method of Barnett and Nordin (1960), except that the external (ED) and internal (ID) transverse diameters were measured at the midshaft and not at the thickest point of the cortex because of the irregular shape of the rat bones. Measurements were made with a Peak scale lens at x 7 magnification, with an accuracy of 0.1 mm. The sum of the "medial" and "lateral" cortices (ED minus ID) (Fig. 1) was divided by ED and the fraction was multiplied by 100. In this way the femur and tibia scores were calculated for 3 animals of each group from series R, N and C. Separate calculations were made for the bones from the right and left leg. A decreased score indicates reduced cortical thickness and hence increased cortical bone resorption.

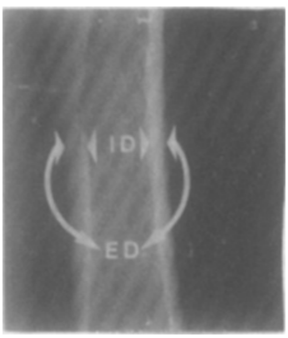


Fig. 1. The external (ED) and internal (ID) transverse diameters of the femoral midshaft.

Preparation of undecalcified sections

Four bone sections (Fig. 2) were prepared from each hind leg of 2 to 3 animals of each group of series R, N and C. They were sawn out by means of a fine band-saw. The specimens consisted of section a (intertrochanteric), section b (diaphyseal perpendicular to the femoral midshaft), section c (sagittal from the medial part of the lateral femoral condyle) and section d (sagittal from the medial part of the lateral tibial condyle).

The bone sections were embedded in methyl methacrylate and then ground on a Knuth-Rotor grinder to a thickness of between 60 and 90 µ.

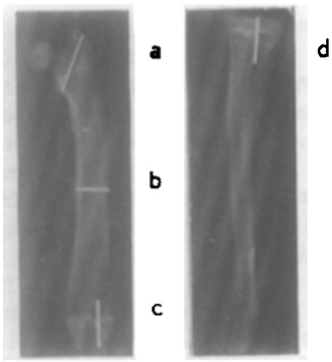


Fig. 2. A schematic illustration of how the specimens were taken.

Microradiographs

The ground sections were placed in direct contact with Kodak spectroscopic plates, type 649 - 0. Radiography was performed with a Siemens micrography tube, type AGW 2 ö, with a tungsten target and a 0.1 mm thick beryllium window. The tube was energized with 15 kV.

Quantitation of tetracycline labelling

For examination of tetracycline induced fluorescence, the sections were mounted under coverslips in fluorescence-free balsam (Eukit[1]) and examined in a Zeiss fluorescence microscope fitted with an UV light source (High Pressure Vacuum Lamp Osram HBO 200 W), with a filter combination of exciter filters UG 2 and UG 5 and barrier filters 65 and 41.

1) O. Kindler, Freiburg, West Germany.

In the following text the marginal line of a bone surface area in a cross section is called a *surface length.*

Three different methods of quantitation of bone formation were used.

a. One method was based on studies described by Jowsey et al. (1965). In this the microradiographs of the intertrochanteric (section a, Fig. 2) and epiphyseal condylar (section c, Fig. 2) bone sections of the femur were photographed at either x 25 or x 43 magnification (Figs. 3 and 4). All tetracycline labelled bone within the intertrochanteric (section a, Fig. 2) and epiphyseal condylar (section c, Fig. 2) areas was marked with coloured chalk on the surface lines of the photographs. The total surface lengths as well as the tetracycline labelled parts were measured with a map measurer, the distance being recorded in centimeters. Thereafter the percentage tetracycline labelled surface length of the total was calculated.

On the intertrochanteric sections (Fig. 3 and section a, Fig. 2) the percentage tetracycline labelled surface length was calculated only on the trabecular surface areas. On the epiphyseal condylar sections (Fig. 4 and section c, Fig. 2) this percentage was calculated on both the trabecular surfaces and the epiphyseal surfaces of the subchondral bone plate (Fig. 4:A) and epiphyseal scar (Fig. 4:B).

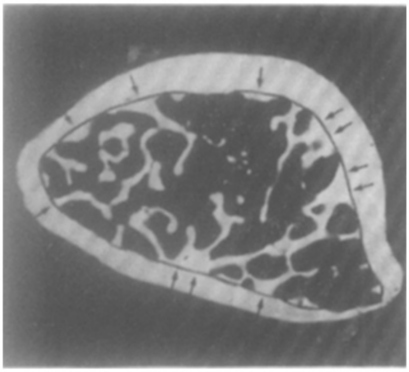


Fig. 3. Microradiograph of intertrochanteric section (section a, Fig. 2). Note the way of delineating the trabecular from the cortical bone surface area. (x 7·5).

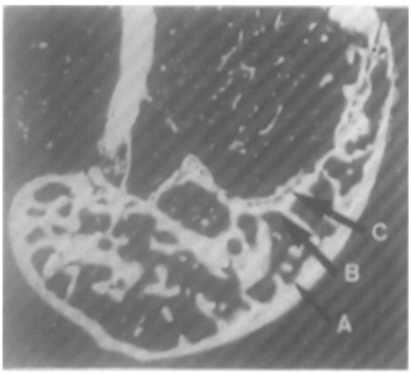


Fig. 4. Microradiograph of epiphyseal condylar section (section c, Fig. 2) A = epiphyseal surface of the subchondral bone plate, B = epiphyseal surface of the epiphyseal scar, and C = epiphyseal scar (x 7·5).

b. The diaphyseal bone sections (section b, Fig. 2) of the femoral midshaft were photographed at x 8 magnification in the fluorescence microscope and reproductions of the slides were drawn to a total magnification of x 85 with the aid of a diavisor apparatus. At the same time the tetracycline labelled surface lengths were marked with coloured chalk and were then calculated as percentage of the total surface lengths.

c. The epiphyseal condylar sections (section d, Fig. 2) of the tibia were judged qualitatively in the fluorescence microscope (Figs. 11-13), using the following criteria: extent of tetracycline labelling in relation to inactive bone surface lengths, width of fluorescent bands and distance between the bands of double labelled surface lengths.

Quantitation of bone surface areas

The percentage bone surface area in a given total area was calculated on the drawings and photographs with the aid of a planimeter as follows:

a. Total bone surface area per total diaphyseal area of the femoral midshaft (section b, Fig. 2).

b. Bone surface area of the intertrochanteric sections (Fig. 3 and section a, Fig. 2); this was calculated in three ways:

   i. Total bone surface area per total intertrochanteric area.

   ii. Trabecular bone surface area per total endosteal area. The latter could be estimated by delineating the inner surface line of the cortex from the bases of the trabeculae in a fairly standardized manner (Fig. 3).

   iii. Cortical bone surface area per total intertrochanteric area, which could be calculated by subtracting the area inside the "endosteal line" from the total intertrochanteric area.

c. Total bone surface area per total epiphyseal condylar bone (Fig. 4 and section c, Fig. 2). The bone surface was demarcated proximally by the epiphyseal scar (Fig. 4:C).

Statistical analysis

Analysis of variance was performed in order to test the femur and tibia scores and the quantitation of tetracycline labelled bone surface lengths and of bone surface areas.
Student's t-test was used for comparison of the femoral and tibial lengths, and the external diameter of the midshaft of the femur and tibia between the different series.

RESULTS

Femoral and tibial length

The results of the study of the bone length (Table 1) showed that the right femur and tibia of series N were approximately 1 mm or 2 per cent longer than the corresponding bones of series R and C, the difference being significant. A measurable difference in length between corresponding right and left bones from the same animal was observed only occasionally, and therefore only the length of the right bones was recorded.

Table 1. The lengths of the femur and tibia, and significance levels of the differences in the right bones between the different series.

	Series			p		
	R	N	C	R vs N	R vs C	C vs N
Right femur						
No.	30	14	27	<0.01	ns	<0.01
Mean (mm)	42.8	43.7	42.8			
Range (mm)	41.4-44.9	41.9-45.6	41.1-45.0			
S.D.	0.8	1.0	0.9			
Right tibia						
No.	30	14	27	<0.05	ns	<0.01
Mean (mm)	45.0	45.9	44.6			
Range (mm)	42.3-49.0	42.7-48.2	42.4-47.8			
S.D.	1.3	1.4	1.2			

External diameter of the femur and the tibia

Statistical analysis of the external diameters (ED) of the femur and tibia of the right and left side, respectively, of series R, N and C showed that there were no mutual significant differences between the three series. Thus there was no evidence of atrophy of the subperiosteal bone due to the treatment.

Evaluation of radiographs

The bones from the rats of the N series had a normal radiographic appearance (Fig. 5) up to about the 16th week of observation. Signs of "pronounced osteopenia", as defined above, were present in practically all the bones from the examined rats of this series after the 32nd week of observation (Fig. 6). Several bones from the treated extremities of the rats of series R and C showed signs of "moderate osteopenia" (Fig. 7) as early as at the first weeks of observation. The degree of osteopenia and the number of osteopenic bones increased with progressing observation time and "pronounced osteopenia" was seen in almost all the examined bones from the 32nd week onwards (Table 3). The osteopenia resulting from immobilization seemed thus to be unaffected by remobilization. The "pronounced osteopenia" present in the animals from all three series towards the end of the observation period seemed, on the other hand, to be age-dependent.

Fig. 5

Fig. 6

Fig. 7

Fig. 5. Normal femurs and tibiae at the 2nd week of observation. The cortical bone is thick and regular and there is normal radiopacity of the whole bone.

Fig. 6. Normal femurs and tibiae at the 32nd week of observation. The cortical bone is decreased in thickness and irregular, and cancellization and reduced opacity as well as spots of sclerosis are indicative of "pronounced osteopenia".

Fig. 7. Remobilized (right) and control (left) femurs and tibiae, 4th week of observation. There is an obvious decrease of the regularity and thickness of the cortical bone as compared with the bones of Fig. 5. The difference is somewhat more pronounced in the bones from the treated right extremity.

Table 3. Degree of osteopenia of the bones evaluated from the radiographs. 0 = uncertain, 1 = moderate, 2 = pronounced. The first, the second and the third figure in each of the 4 columns relates to an individual bone from the same animal.

Series	Weeks of observation	Grade of osteopenia			
		Femurs		Tibiae	
		Right	Left	Right	Left
R (remobilized)					
	01	1:2:2	0:0:1	2:2:2	0:0:1
	02	2:2:2	0:1:2	2:2:2	0:0:1
	04	2:2:2	1:1:1	2:2:2	1:1:1
	06	2:2:2	0:1:1	2:2:2	1:1:1
	08	1:1:2	0:0:1	1:1:2	0:0:2
	16	1:2:2	1:1:2	2:2:2	1:1:1
	24	2:2:2	1:1:2	2:2:2	0:1:1
	32	2:2:2	1:1:2	2:2:2	1:1:2
	40	2:2:2	1:1:2	2:2:2	1:1:1
	48	2:2:2	2:2:2	2:2:2	1:2:2
N (normal)					
	02	0:0:0	0:0:0	0:0:1	0:0:1
	06	0:0:0	0:0:0	0:0:0	0:0:0
	16	0:1:2	0:1:1	0:1:2	0:1:2
	32	2:2:2	2:2:2	1:2:2	1:2:2
	48	2:2:2	2:2:2	1:1:1	1:1:1
C (continuously immobilized)	01	2:2:2	0:0:1	2:2:2	0:1:2
	02	1:2:2	0:1:1	1:2:2	0:0:0
	04	1:2:2	1:1:2	1:2:2	1:2:2
	06	0:1:2	0:0:0	0:1:2	0:0:0
	08	2:2:2	0:0:2	1:2:2	0:1:2
	16	2:2:2	1:1:1	2:2:2	1:1:2
	24	2:2:2	1:2:2	2:2:2	1:2:2
	32	2:2:2	2:2:2	2:2:2	1:2:2
	40	2:2:2	1:1:2	2:2:2	0:1:2
	48	2:2	2:2	2:2	2:2

Femur and tibia scores

The values obtained of the femur score are plotted individually against time in Fig. 8 and the mean values of the femur and tibia scores for the right and left bones are given in Table 4. The mean values were analysed statistically with no regard to the time of observation or hence to the period of treatment or the age of the animals. The aim was to find out whether there was any significant degree of reversibility or not in the bones from the R series as a whole.

Table 4. Right and left femur and tibia scores in series R, N and C.

	Right leg		Left leg	
	Femur score	Tibia score	Femur score	Tibia score
R				
No.	30	30	30	30
Mean	27	38	30	44
Range	20-33	31-49	22-39	34-50
S.D.	4	4	5	4
N				
No.	15	15	15	15
Mean	29	42	29	43
Range	20-40	31-49	20-42	28-48
S.D.	7	6	8	5
C				
No.	29	28	29	28
Mean	25	35	30	43
Range	18-32	24-47	19-39	33-53
S.D.	4	5	5	5
p				
R vs N	ns	< 0.05	ns	ns
R vs C	ns	ns	ns	ns
C vs N	< 0.05	< 0.001	ns	ns

The score of the remobilized tibia was significantly lower than that of the tibia of the N series. The femur score of the remobilized extremity showed no significant difference from the corresponding value for the N and C series. Thus, neither of the bones of the remobilized extremity showed indications that remobilization had led to increased thickness of the cortical bone. The scores obtained from the bones of the treated leg of the C series showed that continued immobilization resulted in a significant decrease of the cortical thickness as compared with the corresponding values from the N series.

When the individual score values of the femurs were plotted against time it was found that in the R and, especially, the C series the scores of the right femurs were almost consistently lower than those of the left femurs (Fig. 8). There was a clear tendency to decreasing scores after about the 8th week of observation. This was valid for all series, both for the bones from the right and for those from the left leg. The decreasing femur scores were obviously associated with the increasing age of the animals; in other words they were age-dependent. These observations were, however, not applicable to the tibiae.

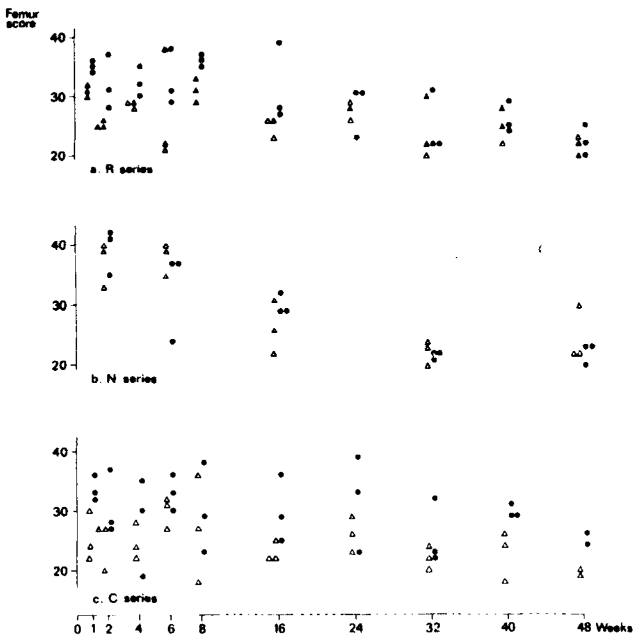


Fig. 8. Femur score for the right (Δ) and left (●) leg of series R, N and C during the period of observation.

Quantitation of bone surface areas

Table 5. Bone surface area in per cent of the total area in different sections from the right (r) and left (l) femurs of the R, N and C series.

	Percentage bone surface areas of the femoral sections				
	Epiphyseal (section c, Fig. 2)	Diaphyseal midshaft (section b, Fig. 2)	Intertrochanteric (section a, Fig. 2)		
			Total bone area of total intertrochanteric surface area	Trabecular bone area of "endosteal" surface area	Cortical bone area of total intertrochanteric surface area
R_r					
No.	24	19	12	14	14
Mean	49	53	48	25	31
Range	29-63	43-58	43-55	15-34	24-43
S.D.	8	4	4	6	5
N_r					
No.	8	7	5	5	5
Mean	59	57	59	32	40
Range	45-68	53-68	54-68	27-39	36-48
S.D.	7	5	5	6	5
C_r					
No.	8	5	5	5	6
Mean	43	50	46	20	31
Range	31-52	37-57	42-49	10-25	24-35
S.D.	8	8	3	6	4
p					
R_r vs N_r	< 0.05	ns	< 0.001	ns	< 0.01
R_r vs C_r	ns	ns	ns	ns	ns
C_r vs N_r	< 0.001	ns	< 0.001	< 0.05	< 0.05
R_l					
No.	22	18	15	17	16
Mean	55	56	55	31	35
Range	41-68	45-66	43-65	15-39	24-47
S.D.	8	4	7	7	6
N_l					
No.	7	5	6	6	6
Mean	58	59	54	29	36
Range	49-71	53-67	45-58	25-34	29-43
S.D.	7	5	5	4	4
C_l					
No.	7	5	5	5	5
Mean	54	56	53	27	36
Range	36-69	48-63	46-60	18-34	24-50
S.D.	13	5	6	6	9
p					
R_l vs N_l	ns	ns	ns	ns	ns
R_l vs C_l	ns	ns	ns	ns	ns
C_l vs N_l	ns	ns	ns	ns	ns

Despite great care in sawing out of the sections some inter-trochanteric sections (section a, Fig. 2) had to be discarded owing to a too high position towards the calcar femorale or to a too low position towards the diaphysis of the bone. A few broken sections also had to be discarded. This explains the different numbers of observations presented in Table 5.

The individual percentage bone surface areas obtained from the bone sections of the right leg in the R series, when considered together for the whole experimental period, were significantly lower than the corresponding values obtained from the N series, despite remobilization. This was valid for sections a and c, (Fig. 2) but not for sections b, which did not differ significantly between the two series. Neither were significant differences observed between the R and C series, or in bone sections from the left leg between the three series (Table 5).

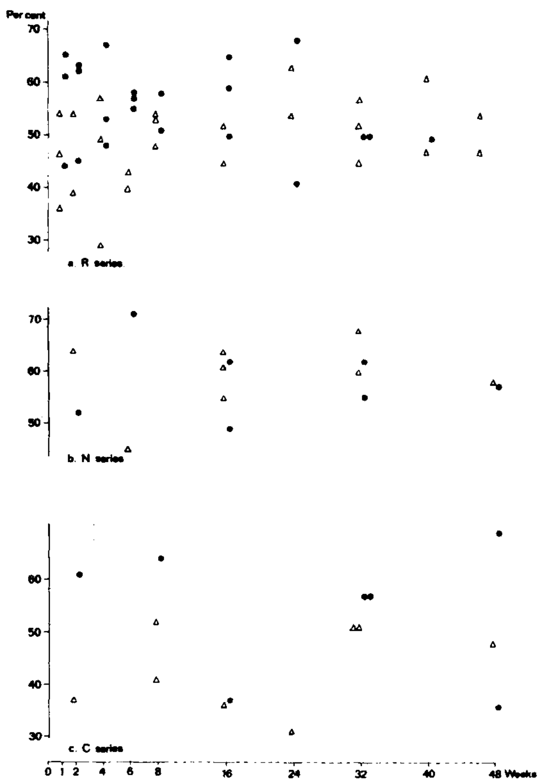


Fig. 9. Bone surface areas in per cent of the total epiphyseal area of the right (Δ) and left (●) epiphyseal condylar sections (section c, Fig. 2).

In Fig. 9 the individual values of the bone surface areas from the distal femoral epiphysis (section c, Fig. 2) are plotted against time. The distribution of the values is very similar to those of other bone sections, which are therefore not presented here. Those sections also showed no indications of reduced bone atrophy following remobilization of the extremity.

Thus, the studies showed that remobilization did not result in any marked increase of the initially reduced percentage bone surface area. The values obtained from the rats of the C series indicated that a steady state level had been reached already at the beginning of the observation period. No appreciable variations occurred later. However, in this case, as in the N series, the different sections available for study were few.

Quantitation of tetracycline labelling

Differences in total percentage fluorescent surface lengths in the different sections (sections a, b and c, Fig. 2) from the right and left femur were tested statistically between the R, N and C series. The differences that existed were not significant. In Fig. 10 the individual values of the percentage tetracycline labelled surface lengths in the distal femoral epiphysis (section c, Fig. 2) are plotted against time.

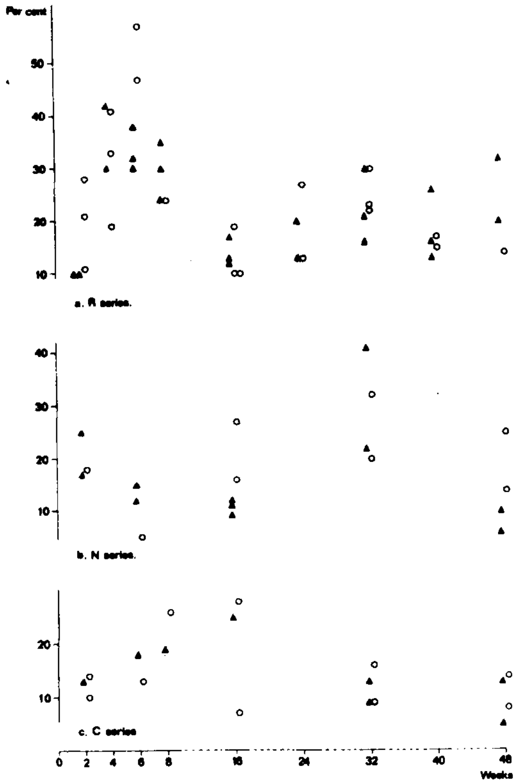


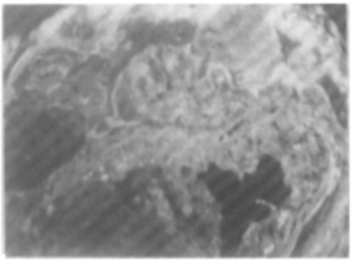
Fig. 10. Tetracycline labelled surface lengths in per cent of the total bone surface lengths from the epiphyseal sections (section c, Fig. 2) of the right (Δ) and left (O) femurs. R, N and C series.

It should be observed that these values represent the total labelling after injection of tetracycline 2 plus 1 weeks before the time of observation. Despite the large variation of the values and the small number of observations there was a clear disposition of the values from the R series at two different levels. Thus, high values were obtained initially, from the 3rd to the 7th week after remobilization, recorded at the 4th, 6th and 8th weeks of observation. The values lay at the lower level after the 8th week. No appreciable trends were observed among the values from the N and C series. Identical distributions of values were derived from other sections (sections a and b, Fig. 2), which are therefore not presented here.

Qualitative estimation of tetracycline labelling

Studies of the fluorescent surface lengths in epiphyseal tibial sections (section d, Fig. 2) gave results largely similar to those of studies of the quantitative tetracycline labelling in sections from the distal femoral epiphysis (section c, Fig. 2). Thus, high tetracycline labelling in the form of abundant fluorescent surface lengths was observed in specimens from the R series between the 3rd and 7th week after remobilization. Similar heavy labelling was observed only occasionally in specimens from the C and N series. Particularly in sections from the C series the fluorescent bands were often very thin and the distances between double labellings were extremely small (Figs. 11-13). Reproducible measurements between the double fluorescent bands were impossible to make in the examined sections from the femoral and tibial epiphyses.

Fig.
11 a.



b.

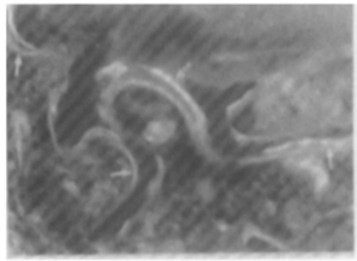
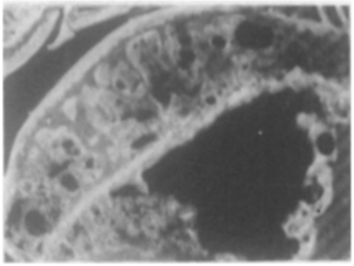


Fig.
12 a.



b.

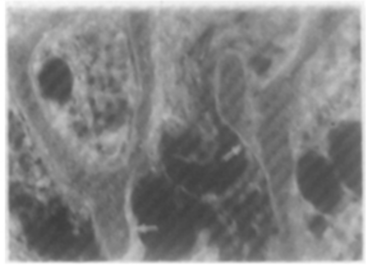
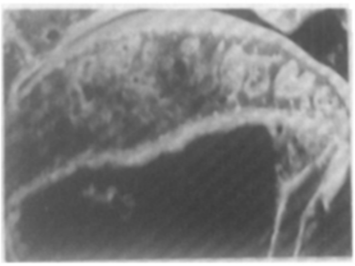
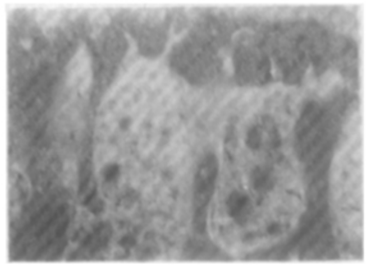


Fig.
13 a.



b.



Figs. 11-13. Fluorescent and inactive bone surface lengths in sections from tibial epiphyses (section d, Fig. 2) a x 16, b x 70.

Fig. 11. Remobilized right tibia. Fourth week of observation. Note the fluorescent bands and the double bands.

Fig. 12. Normal right tibia. Sixteenth week of observation. Note the narrow fluorescent bands.

Fig. 13. Continued immobilized tibia. Second week of observation. Note the small extension of narrow fluorescent bands.

DISCUSSION

As mentioned earlier, adult rats were intentionally used in this investigation to eliminate the interference of skeletal growth with the skeletal response to the treatment. (Larsson 1969 a). However, the studies of the length of the femur and the tibia from the right hind leg of the rats of the three series showed that the bones from the remobilized and continuously immobilized animals were significantly shorter than the corresponding bones from the untreated control rats. At the beginning of the experiment the rats were 8 to 10 months old and their mean body weight was about 500 gms. Larsson (1969 a) using one year old rats of the same strain, also with a mean initial body weight of about 500 gms, and maintained under identical laboratory conditions as the animals of this investigation found no significant differences in femoral length during the period of observation of up to one year. It is known that the epiphyseal cartilage zones of the rat do not close completely, but it has been reported that in the adult rat open epiphyseal cartilage plates are inactive (El-Maraghi et al. 1965) and that bone calcification remains constant after the first six months of life (Henry and Kon 1953). The conclusions drawn from these observations are a) that the length of the femur and the tibia of the normal rats increased during the 16 weeks corresponding to the 16 weeks of initial immobilization of the rats from the remobilized and continued immobilized series; b) that immobilization resulted in arrest of the longitudinal growth of the bone in the rats of the latter two series; c) that during the period of observation, viz. from the start of remobilization of the experimental rats up to the end of the experiment, the growth of all the rats had ceased; and d) that the open epiphyseal cartilage plates are active up to about the end of the first year, at least in the rats of the strain used in this laboratory.

Information regarding the amount of bone was gathered by evaluation of plain radiographs and calculation of the femur and tibia scores as well as measurement of the percentage bone surface area in microradiographs from bone sections. Each of these methods is obviously afflicted with limitations. However, together they gave uniform and apparently accurate information on existing differences. For quantitation of the percentage tetracycline labelled surface lengths a modification of a method used earlier by Jowsey and co-workers (1965) was applied.

As mentioned earlier (Mattsson 1972 a), on remobilization after 16 weeks of previous immobilization of one hind limb, the rat began to bear weight on the limb within a few days. After 3 weeks it walked again in a fairly unhindered manner. It is known that disuse is associated with bone atrophy, and increased use with bone hypertrophy (Albright and Reifenstein 1948, Heaney 1962). Bone is stressed by muscle action as well as by the pressure of gravity (Wyse and Pattee 1954, Geiser and Trueta 1958). It seems, therefore, that from these points of view there were good prerequisites for restoration of bone atrophy on remobilizing the rats in this experiment.

It has been stated earlier that if the duration of immobilization in man is so prolonged that the trabeculae of the cancellous bone partly disappear, restitution takes place by "hypertrophic atrophy", i.e. thickening of the remaining trabeculae (Willert 1966). As already mentioned, the present results did not provide evidence of any measurable increase of the bone mass despite the increase in new bone formation activity recorded for a short period.

The results of studies regarding the reversibility of localized bone atrophy of disuse are largely divergent. Thus, it has been reported that in rabbits 4 weeks of remobilization following a 4 week period of immobilization of the one hind leg by a plaster cast resulted in measurable restitution of both the trabecular and the cortical bone of the calcaneus as observed radiographically (Geiser and Trueta 1958). It has also been reported that in guinea pigs immobilization of one hind leg with a plaster cast for periods no longer than 5 weeks was followed by restoration of the breaking strength and the elasticity of the tibia to normal when the plaster cast was removed for 12 weeks (Haike et al. 1967). Densitometric studies in patients with fractures of the tibia and varying periods of immobilization revealed that the mineral content of the femoral condyle remained decreased as long as 10 years after the injury. A tendency to restoration of the lost mineral during the first 7 years was, however, observed in male patients. In most of these cases post-traumatic osteopenia was obvious on the radiographs during the first year following the fracture but completely normal radiographs were obtained later (Nilsson 1966). On the other hand, it has also been reported that the osteopenia is visible in radiographs as long as 9 years after trauma (Steinbach 1964) or below-knee amputation (Sevastikoglou et al. 1969). The results of the present investigation support the view that at least in adult rats the reversibility of bone atrophy induced by prolonged immobilization of one hind leg is extremely low, if any.

Age-dependent osteopenia was revealed on radiographs and by measurements of the femur score from the middle of the observation period. The rats were then 19 months old or approximately in the middle of their life span, viz. 2,5 to 3 years (Farris and Griffith 1963). Similar observations regarding the muscles of the extremity were made earlier (Mattsson 1972 a) and together with the present findings stress further the importance of age-dependent factors for evaluation of the results from immobilization experiments.

The observations regarding the rate of new bone formation in the continuously immobilized rats are in agreement with previous reports that 20 weeks after nerve resection of one hind limb in rats weighing 210 gms the tetracycline labelling determined chemically in the tibia and femur of the treated leg dropped to "subnormal values" as compared with the bones of the untreated control leg (Landry and Fleisch 1964).

The results reported above support earlier observations (Mattsson 1972 a) suggesting that prolonged immobilization of an extremity leads to irreversible changes which remain uninfluenced by the duration of remobilization. They are, further, in agreement with other results reported elsewhere, concerning studies of the bone composition and the calcium metabolism (Mattsson 1972 a, c and e).

SUMMARY

The amount of bone and the intensity of bone formation were studied in the femur and tibia of adult rats in which one hind leg, after initial immobilization with a plaster cast, was subsequently remobilized for 1 to 48 weeks. Normal untreated animals as well as continuously immobilized animals served as controls.

Studies of the skeletal mass were based on evaluation of plain radiographs, calculation of the femur and tibia scores and measurements of the percentage bone surface area in several sections of the bones. For evaluation of the intensity of bone formation quantitation of percentage tetracycline labelled surface lengths was used.

Evidence of increased new bone formation was obtained between the 3rd and 7th weeks after removal of the plaster cast, in the bones from both the remobilized and the contralateral extremities. In continuously immobilized and untreated normal controls there were no marked variations of the bone formation rate during the period of observation. Despite the early increase of bone formation there was no evidence of increased bone mass as a result of the remobilization, indicating that the bone atrophy which developed during the initial immobilization was largely irreversible.

Age-dependent osteopenia was recorded on radiographs in all the three series during the latter part of the observation period.

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THE REVERSIBILITY OF DISUSE OSTEOPOROSIS

III. Studies on Bone Composition in the Adult Rat[1)]

by

Sten Mattsson

Ever since Allison and Brooks (1921) studied the composition of bone in dogs in which one extremity had been immobilized by section of the brachial plexus, excision of the upper end of the humerus or with a plaster of Paris cast, it has been known that disuse of an extremity leads to loss of wet weight, and gross density of its bones as well as a reduction of their ash and organic content. Further, it has been shown that the bone matrix is lost in equal proportion to the mineral component (Allison and Brooks 1921, Nordin 1961, Klein et al. 1968). A number of studies concerning these problems have been reported (e.g. Armstrong 1946, Slack 1954, Gillespie 1954, Kharmosh and Saville 1965, Gedalia 1966, Klein et al. 1968, Sevastikoglou et al. 1972). These investigations have dealt with the bone composition during the acute stage of the development of bone atrophy and the results show good agreement as far as comparison is allowed

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by the different species, the ages of the animals, the method of immobilizing the extremity, the duration of immobilization and the way in which the results are presented.

Few investigations have been made, either experimentally or in humans, on the composition of bone tissue after the chronic stage of disuse atrophy has been reached. Very little is known, further, regarding the composition of bone during the process of reversibility of osteoporosis of disuse. Earlier radiographic studies in rabbits (Geiser and Trueta 1958), as well as densitometric studies of the femoral condyles in patients after fracture of the tibia (Nilsson 1966) give information of the mineral mass but not of the composition of the cortical and cancellous bone. The study of strength and elasticity of bone from remobilized guinea pigs reported by Haike et al. (1967) provides, on the other hand, an index of the total bone mass. Furthermore, the data reported in previous studies are limited quantitatively owing to a lack of sensitive methods of recording small changes in bone mass.

The purpose of the present investigation was to study:

a) the effect of remobilization after 16 weeks of immobilization on the changes in composition concerning the wet weight and the hydrated density of the whole femur and tibia as well as the percentage ash content of their cortical bone;

b) the changes in composition of the bone in the chronic stage of disuse atrophy;

c) the changes in composition of bone during the ageing process in normal untreated animals;

d) the extent to which contralateral untreated bones in immobilization-remobilization experiments can be regarded as normal.

MATERIALS

The materials for the present study were provided from the same animals as used in an earlier investigation (Mattsson 1972 a).

Experimental animals

In the so called *R series* (remobilized) the right hind limb of adult rats was first immobilized in a plaster cast for 16 weeks. The cast was then removed and the limb remobilized. Ten groups of animals were observed for 1 to 48 weeks after removal of the plaster cast before being killed.

Control animals

a. The *N series* (normal, untreated) consisted of 5 groups which did not undergo any form of immobilization; they were observed for 16 plus 2 to 48 weeks, and served as normal controls for the rats of the R series.

b. The rats of the *C series* (continuously immobilized) were immobilized continuously for 16 plus 1 to 48 weeks before being killed. They were divided into 10 groups and served as immobilized controls.

The period of remobilization of series R, 1 to 48 weeks, comprised the actual observation period for all the three series.

Five to 7 rats from each group of each of the three series were examined. For the 48-week groups, however, only 4 animals of the R series and 3 of the C series were still alive and available for the present study.

METHODS

Preparation of material

After the animals had been killed the femur and the tibia from both hind legs of all rats were thoroughly dissected free from soft tissues and periosteum. The fibula was detached from the tibia and discarded.

Wet weight determination

The dissected bones were immediately weighed on a Mettler balance, with an accuracy of 0.05 mg, and then frozen and kept at -20°C pending further study.

Hydrated gross bone density determination

The frozen bone was placed in deionized water at 4°C for 24 hours. It was then weighed first in air and then in a container with deionized water, being suspended on a fine wire. The difference in weights represented the volume of the bone. The hydrated density of the intact bone could thus be calculated by dividing the value obtained from the wet weight determination in air by the volume of the bone.

Preparation of cortical bone powder

The most proximal and distal parts of each bone, including the articular cartilage, were removed by means of a fine band-saw. The remaining part of the bone was crushed and thoroughly freed from blood and marrow. The cleaned cortical bone fragments were defatted and dehydrated in five changes of acetone for three days and were then allowed to dry at room temperature. The specimens were pulverized in an Intermediate model Wiley Laboratory Mill provided with a 60-mesh sieve. The bone powder was stored in small stoppered plastic tubes at room temperature until used.

Organic and ash contents

Roughly 100 mgms of defatted dehydrated cortical bone powder were weighed on a Mettler balance. The powder was then ashed in a muffle furnace at 700°C for 16 hours and weighed according to standardized methods used in this laboratory. The percentage content of defatted organic material could then be calculated by subtraction of the ash (inorganic) component from the powder weight.

Statistical methods

In addition to regression analysis and null hypothesis testing, which have been described previously (Mattsson 1972), the following statistical methods were used.
The mean variables of the R vs N, R vs C and C vs N series were subjected to t-testing at the different time points. The term variable refers to the difference between the values for the right and the left leg of the factor under study. The null hypothesis (H_o) meant that there were no differences between the variables at the observed time points in question. The same significance grading as in a previous paper was used (Mattsson 1972 a).

By means of analysis of variance, the mean value for the right and left tibia, respectively, of the R, N and C series was studied with regard to statistically significant variations during the observation period 1 - 48 weeks.

The mean values of the left control bone of the R and the left normal bone of the N series with time were also described in the form of regression curves of second degree. Hypothesis testing between the series regarding differences in slope and/or distance was carried out.
In the N series Student's t-test was used for comparisons between the right and left leg, for pairing observations at the different time points.

RESULTS

I. Wet weight

1. Femur

A. Differences in wet weight between the three series

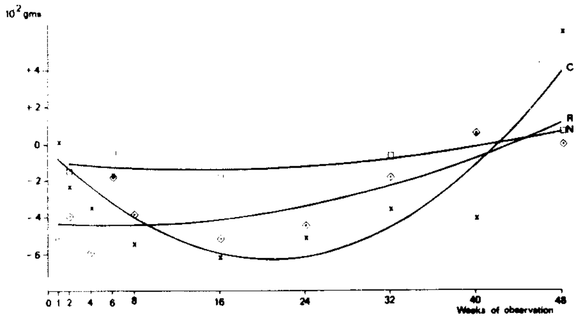

Fig. 1. Differences between wet weights of the femurs. Regression curves and mean variables. R (◈), N (□) and C (x) series.

R versus N series: The regression curves differ significantly up to the end of the 26th week of observation, which is considered to be due largely to distance. The mean variables differ almost significantly at the 16th week.

R versus C series: The regression curves differ significantly except between the 20th and 34th weeks of observation. The mean variables are almost significantly different at the end of the first week. The mean variable for the C series at the 48th week is based on observations from only 3 animals and none of the other mean variables of this series approximate those of the R or N series at the end of the observation period. The steep rise in the regression curve of the C series towards the end must, therefore, be regarded as uncertain.

C versus N series: The regression curves follow significantly different courses throughout the observation period. The mean variables show a significant difference at the 48th week. The same reservation for the C series as mentioned above is valid.

It was concluded from these results that during the first half of the observation period there were no signs of reduction of the differences between the R and N series. Thus up to the middle of the observation period there was no indication that remobilization *per se* had resulted in restitution of the wet weight of the femur. The question whether the later reduction of the differences was due to restitution or to other factors, or to both, had to be analysed further.

B. Differences in wet weight within each of the three series

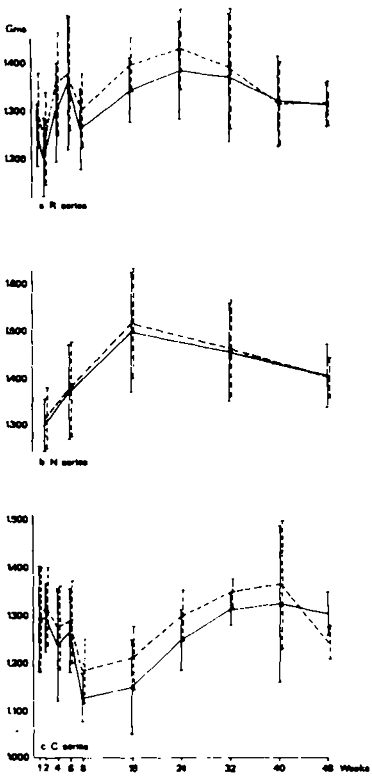

Fig. 2 a-c. Wet weight of the right (——) and left (---) femur in the R, N and C series.

No significant differences are found between the two femurs in the N series during the observation period (Fig. 2b). From the 2nd to the 16th week of observation there is an almost significant and a significant increase of the wet weight of the right and left femur, respectively, by about 15 per cent. The later apparent decrease is not significant.
Statistical analysis of each femur of the R and C series shows no significant differences, on paired testing, between the different groups for either femur (Fig. 2 a and c).
The courses for the wet weight with time of the R and N series (Fig. 2 a and b) suggest, rather, that the reduction of their wet weight differences (Fig. 1) during the later half of the observation period is due essentially to age factors.

C. <u>Comparison of wet weight between the left control femur of the R series and the left normal femur of the N series</u>

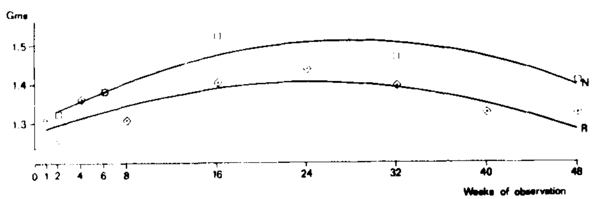

Fig. 3. Wet weight of the left femur of the R (◇) and N (□) series during the observation period. Regression curves and mean values.

The regression curves differ significantly throughout the observation period (Fig. 3). This difference is assessed to be due mainly to distance, with lower values for the femur of the R series. The wet weight of the latter cannot therefore be regarded as normal.

2. Tibia

A. Differences in wet weight between the three series (Fig. 4)

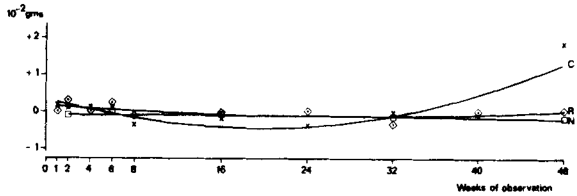

Fig. 4. Differences between wet weights of the tibiae. Regression curves and mean variables of series R (◇), N (□) and C (x).

R versus N series: The regression curves show no significant differences during the period of observation. Both curves run close to the zero line. The mean variables are almost significantly different between the two series only at the 2nd and 6th weeks; at these time points the values of the R series lie at a higher level than those of the N series.

R versus C series: The regression curves follow significantly different courses throughout the observation period. The difference in distance is small up to the 32nd week of observation. The mean variables show no significant differences.

C versus R series: The regression curves follow significantly different courses except between the 12th and 18th weeks. The difference is considered to be due only to differences in slope except during the last part of the observation period. The mean variables show no significant differences between the series.

The analysis of the wet weight differences of the tibia gave no information on the possible effect of remobilization of the tibia, since the courses of the R and N series did not differ significantly and, further, the wet weight of the right, remobilized tibia was not lower than that of the left control tibia of the R series until, at the earliest, the 8th week of remobilization.

B. Differences in wet weight within each of the three series

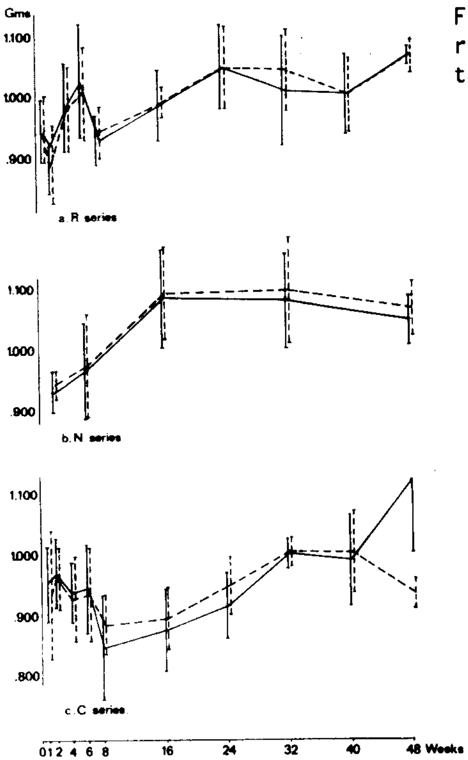

Fig. 5 a-c. Wet weight of the right (——) and left (---) tibia in the R, N and C series.

No significant differences in wet weight are found between the right and left tibiae of the N series (Fig. 5 b). The mean wet weight values for each of the tibiae of the N series show significant increases by approximately 15 per cent up to the 16th week of observation. Thereafter no significant changes are seen.

For the tibia of the right remobilized leg of the R series no significant differences are noted on paired testing between the different groups (Fig. 5 a). On the other hand, in the left control tibia of series R there is a significant increase in wet weight between the 2nd and 16th weeks. A similar significant increase exists in the corresponding tibia of the N series.

In the C series paired testing shows no significant variations between the groups for the right or left tibia (Fig. 5 c).

C. Comparison of wet weight between the left control tibia of the R series and the left normal tibia of the N series

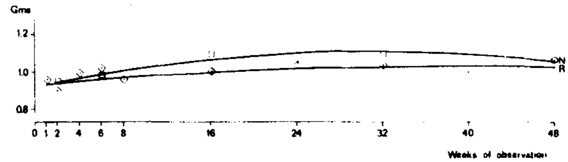

Fig. 6. Wet weight of the left tibia of the R and N series during the observation period. Regression curves and mean values. (◇) R series, (□) N series.

The regression curves differ significantly throughout the observation period. The wet weight of the left control tibia of the R series cannot, therefore, be regarded as normal.

II. Hydrated gross bone density

I. Femur

A. Differences in hydrated gross bone density between the three series (Fig. 7)

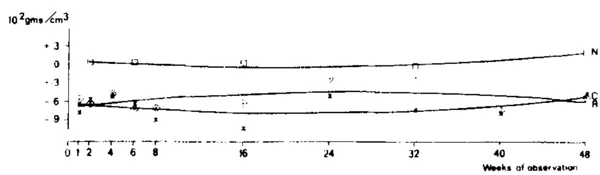

Fig. 7. Hydrated gross bone density of the femurs. Regression curves and mean variables of series R (◇), N (□) and C (x).

R versus N series: The courses of the regression curves differ significantly throughout the observation period. The mean variables are significantly different between the two series at all time points except at the 32nd week ($p<0.01$, $p<0.01$, $p<0.001$, $p>0.05$ and $p<0.05$, respectively).

R versus C series: The regression curves differ significantly throughout the observation period, probably due exclusively to differences in slope. The mean variables are almost significantly different at the 16th and 32nd weeks of observation but not at the 24th week or at the other time points.

C versus N series: The regression curves differ significantly throughout the observation period. The mean variables are significantly different at all time points ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$ and $p<0.05$, respectively).

To summarize, the differences of the gross bone density followed different time courses for all three series. No definite decrease in distance took place between the R and N series or between the C and N series. Thus, there were no signs of restitution of the gross bone density as a result of remobilization of the femur, nor of spontaneous restitution during continued immobilization.

B. Differences in hydrated gross bone density within each of the three series

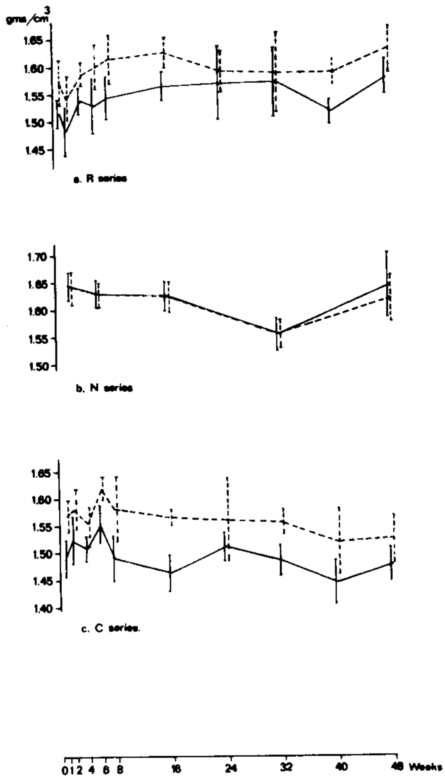

Fig. 8 a-c. Hydrated gross bone density of the right (——) and left (---) femur of the R, N and C series.

N series: No significant differences are seen between the values of the two femurs (Fig. 8 b). On paired testing of the mean values for the 2nd and 32nd weeks a highly significant decrease is noted at the latter time point for both the right and left femur. Subsequently, up to the 48th week, there is an increase which is significant for the right femur and almost significant for the left femur.

R and C series: Paired testing reveals no significant differences apart from an almost significant decrease of the gross density of the continuously immobilized femur of the C series between the 6th and 16th weeks of observation. This is considered to be of no importance (Fig. 8 a and c).

C. Comparison of hydrated gross bone density between the left control femur of the R series and the left normal femur of the N series

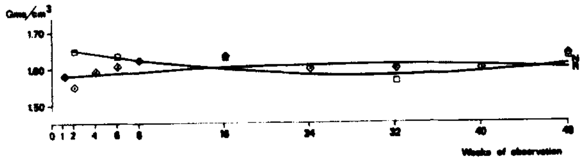

Fig. 9. Hydrated gross bone density of the left femurs of the R (◈) and N (□) series. Regression curves and mean values.

The regression curves are significantly different both from the 2nd to the 26th weeks and from the 46th to 48th weeks (Fig. 9). The gross bone density of the left control femur of the R series cannot, therefore, be regarded as normal.

2. Tibia

A. Differences in hydrated gross bone density between the three series (Fig. 10)

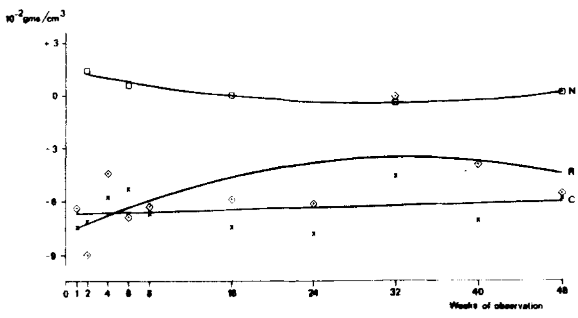

Fig. 10. Hydrated gross bone density of the tibiae. Regression curves and mean variables of series R (◈), N (□) and C (x).

R versus N series: The regression curves have significantly different courses throughout the observation period. The mean variables differ significantly between the series at the 2nd, 6th and 16th weeks ($p<0.001$, $p<0.001$, $p<0.001$). There is no significant difference at the 32nd and 48th weeks.

R versus C series: There are no significant differences either between the regression curves or the mean variables.

C versus N series: The regression curves are significantly different throughout the observation period. The mean variables differ significantly at the 2nd, 6th and 16th weeks ($p<0.01$, $p<0.01$, $p<0.001$) but at the 32nd and 48th weeks the differences are not significant.

It was concluded that no statistically evident restitution of the hydrated gross bone density took place as a result of remobilization *per se*, in the right, previously immobilized tibia of the R series, when the results were related to those of the N and C series. The fact that the mean variables showed no significant differences towards the end of the observation period might be due to the small number of observations at these time points and, therefore, did not necessarily mean that there was a definite equilibration between the series.

B. Hydrated gross bone density within each of the three series

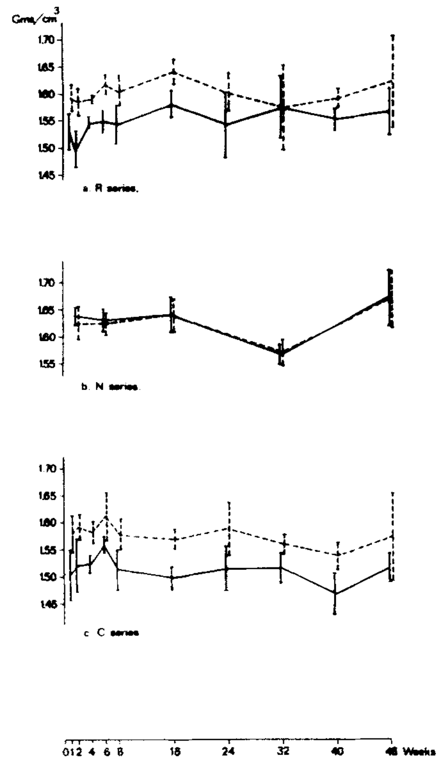

Fig. 11 a-c. Hydrated gross bone density of the right (——) and left (---) tibia of the R, N and C series.

N series: There are no significant differences between the right and left tibiae (Fig. 11 b). As in the femur, there is a reduction of the bone density at the 32nd week of observation. For the right tibia the decrease between the 2nd and 32nd weeks is significant. For the left tibia the apparent decrease is not significant between these two time points but it is significant between the 16th and 32nd weeks. From that time point up to the 48th week the increase is significant for both tibiae.

R and C series: There is a slight increase which is almost significant in the right remobilized tibia of the R series between the 2nd and 16th weeks. This is considered to have no definite importance (Fig. 11 a and c).

To summarize, the remobilization of the right femur and tibia of the R series gave no definite increase of the bone density. The course for the tibiae of the C series appeared to be stable. In the N series the bone density of both the femur and tibia was significantly decreased at the 32nd week of observation, but the importance of this finding is uncertain, as a significant increase took place after this time point.

C. Comparison of the hydrated gross bone density between the left control tibia of the R series and the left normal tibia of the N series (Fig. 12)

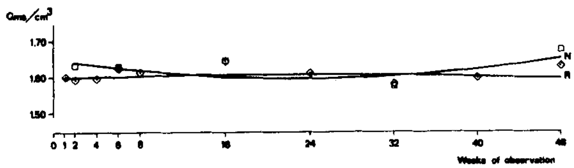

Fig. 12. Hydrated gross bone density of the left tibiae of the R (◇) and N (□) series during the observation period. Regression curves and mean values.

The regression curves differ significantly between the 2nd and 10th weeks of observation. The left control tibia of the R series cannot, therefore, be regarded as normal.

III. Percentage ash content of the cortical bone powder

1. Femur

A. Differences in percentage ash content between the three series (Fig. 13)

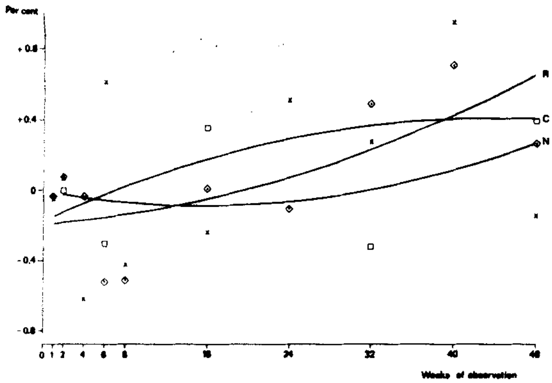

Fig. 13. Percentage ash contents of the femurs. Regression curves and mean variables. R (◇), N (□) and C (x) series.

R versus N, R versus C and C versus N series: There are no significant differences either between the regression curves or the mean variables. Thus neither remobilization nor continued immobilization resulted in any definite changes in the percentage ash content.

B. Differences in percentage ash content within each of the three series

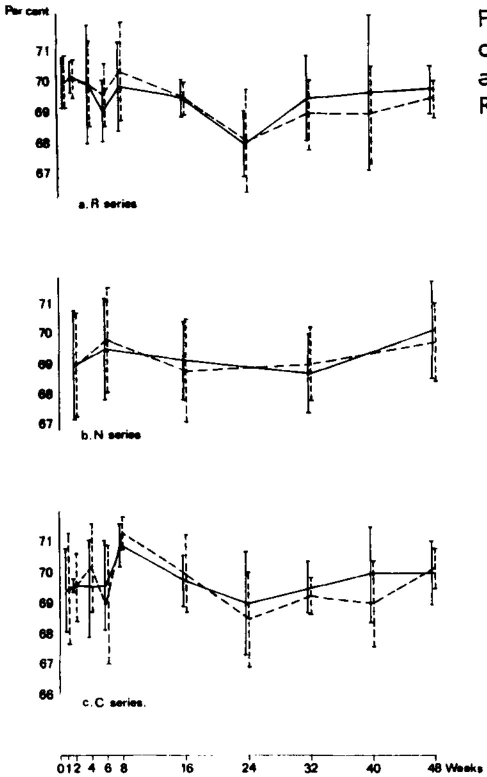

Fig. 14 a-c. Percentage ash content of the right (——) and left (---) femur of series R, N and C.

No significant difference is found between the two femurs of the N series, on paired testing (Fig. 14 b). Neither is there any significant change in the percentage ash content during the course of the observation period, in either of the femurs from the three series (Fig. 14 a-c).

C. Comparison of percentage ash content between the left control femur of the R series and the left normal femur of the N series

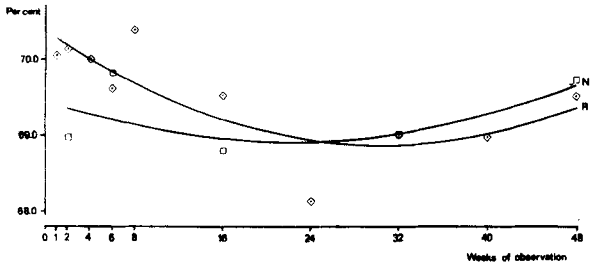

Fig. 15. Percentage ash content of the left femur of the R (◇) and N (□) series during the observation period. Regression curves and mean values.

The courses of the regression curves show no significant differences (Fig. 15).

Thus, there were no significant changes in the percentage ash content of the dried cortical bone of the femur in the various comparisons between or within series R, N and C during the period of observation.

2. Tibia

A. Differences in percentage ash content between the three series (Fig. 16)

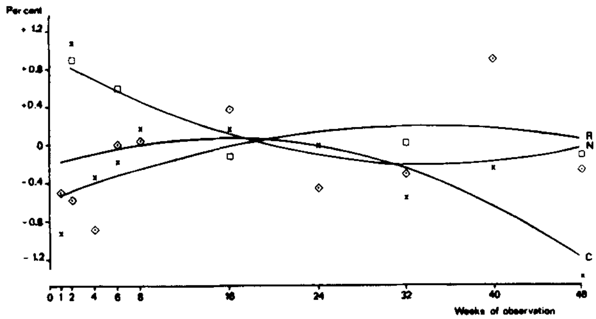

Fig. 16. Percentage ash content of the tibiae. Regression curves and mean variables. R (◇), N (□) and C (x) series.

R versus N series: The courses of the regression curves differ significantly up to the end of the 20th week of observation. The curve of the N series has an initially descending slope. From the end of the 16th week it runs close to the zero line. The mean variables show an almost significant difference between the series at the 2nd week of observation, but otherwise no significant differences.

R versus C series and C versus N series: There are no significant differences between either the regression curves or the mean variables.

It is hardly possible to draw any definite conclusions concerning the initial difference between the regression curves of the R and N series, nor concerning the almost significant difference between the mean variables at the 2nd observation week. It must be noted, however, that the curve of the N series deviates largely from the zero line in the first part of the observation period.

B. <u>Differences in percentage ash content within each of the three series</u> (Fig. 17)

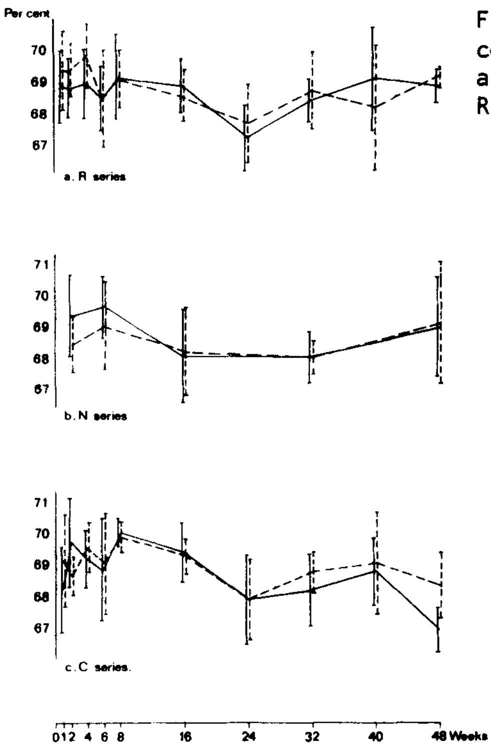

Fig. 17 a-c. Percentage ash content of the right (——) and left (---) tibia of the R, N and C series.

The values for the right and left tibiae of the N series show no significant differences on paired testing (Fig. 17 b). Relatively wide ranges are found in the values of the individual groups (Fig. 17 a-c). The variations in percentage ash content of both the right and left tibiae of the R and N series and the left tibia of the C series are not statistically significant. The percentage ash content of the tibia of the right, continuously immobilized limb of the C series shows an almost significant variation during the course of the observation period. This is considered to be of no importance.

The difference between the two tibiae of the N series compared with that between the two tibiae of the R series showed significant deviations on regression analysis (Fig. 16). From the variations of the percentage ash content of each of the individual bones, however, the differences were considered to lie within the limits of the error of the method, and were not therefore regarded as true.

In conclusion, it was considered that there was no difference in the percentage ash content of the cortical bone powder between the tibiae of the three series during the observation period.

C. <u>Comparison of percentage ash content between the left control tibia of the R series and the left normal tibia of the N series</u>

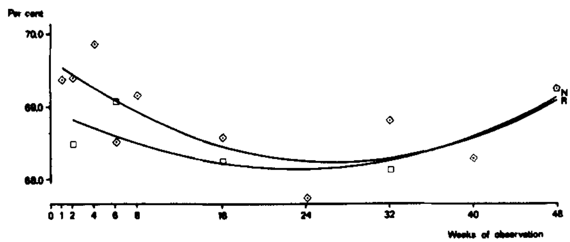

Fig. 18. Percentage ash content of the left tibia of the R (◇) and N (□) series during the observation period. Regression curves and mean values.

The differences in percentage ash content between the left tibia of the R and N series are not significant at any part of the observation period (Fig. 18).

DISCUSSION

Earlier reports on the reversibility of bone atrophy of disuse are based on studies that have not directly concerned the composition of the bones. Thus, it has been found that if the leg of a guinea pig is immobilized by a plaster cast for more than 4 weeks the strength and elasticity of the tibia become reduced and that if the leg is remobilized these changes remain irreversible regardless of the duration of remobilization (Haike et al. 1967). The decreased breaking strength of the tubular bones after immobilization of one extremity in dogs is due to reduced bone mass, as demonstrated in studies on microscopic structures from immobilized bones (Semb 1966).

Densitometric studies of the femoral condyles in patients with healed tibial fractures have, further, shown a decreased mineral content as compared with the femur of the non-fractured contralateral leg, even when more than one decade had elapsed since the injury (Nilsson 1966). The results of the present studies are in agreement with these reports in so far as the investigations can be compared. Thus, it was found that in the rats of the R series (remobilized) the hydrated gross bone density of the remobilized femur and tibia remained at a significantly lower level and showed a different course as compared with the normal bones of the N series (non-treated). The same observation was valid for the wet weight of the remobilized femur. On the other hand, the percentage ash content of the cortical bone powder from the remobilized bones did not differ significantly from that of the corresponding bones from the extremities of the rats from the N series. The observations were considered to indicate that changes in bone composition induced by the initial immobilization in the rats of this investigation remained irreversible despite prolonged remobilization. This conclusion is supported by earlier observations based on quantitative determinations of the skeletal mass and the degree of new bone formation (Mattsson 1972 b) and on calcium metabolic studies (Mattsson 1972 e) carried out on the same rats as used in the present investigation.

The results from the studies of the composition of the bones from the normal, untreated rats of the N series showed that the weights of the whole femur and tibia increased significantly during the first third of the observation period. Subsequently it remained constant or showed evidence of a slight decrease which was, however, not significant. On the other hand, the hydrated density of the whole bones remained fairly constant throughout the time of observation, except that a significant decrease occurred at the 32nd week. This decrease is unexplained as it was followed by an increase again at the 48th week. These results suggest that an increase in the volume of the bones occurred until at least the 16th week of observation, and that there was no real increase in wet weight when the latter was expressed per net bone volume. Finally, it should be noted that the percentage ash content of the dried defatted cortical bone remained almost constant throughout the time of observation.

From previous investigations on the composition of normal bone it is known that hydrated cortical bone specimens with the same density also contain similar amounts of mineral, ash and organic matter (Robinson and Elliot 1957). Since mineral is the largest component of bone by weight, bone density and weight measurements largely depend on changes in the mineral content. When bone atrophy develops, bone tissue is replaced by water, fat and presumably protein (Klein et al. 1968).

The results of the present investigation are somewhat contradictory to earlier observations. Thus, it has been reported that there is a decrease in the water content of human and dog bone with age and a corresponding increase in ash content, while the organic content does not decrease with calcification, but water is displaced by bone mineral (Vogt and Tønsager 1949, Robinson and Elliot 1957, Mueller et al. 1966). On the other hand the occurrence of a peak in mineralization of human bone at the age of about 50 years has also been described; the quantity of bone per unit volume was then decreased (Jowsey et al. 1965, Mueller et al. 1966, Eichler 1970). However, differences in composition of the ageing bone may be species bound (Robinson and Elliot 1957).

The present observation that there were no definite age-dependent changes in composition of the whole femur and tibia from the rats of series N is seemingly in contradiction to the marked signs of cancellization and decreased radiopacity merging with sclerotic spots which was found in an earlier study to be associated with the increasing age of the rats (Mattsson 1972 b). Similar studies on rats under varying experimental conditions have shown, however, that despite structural changes connected with ageing the composition of the bone remains constant (Stevens and Ray 1962).

In several experiments on localized bone atrophy of disuse carried out in this laboratory evidence has been provided that the treatment itself affects the skeletal metabolism of the rats in a general way, and the question as to whether the untreated left hind leg can be regarded as normal or not has been discussed (Sevastikoglou et al. 1972). The results of the present studies indicated that the wet weight and the hydrated density of the whole femur and tibia of the untreated left leg from the rats of the R series differed significantly from the corresponding values from the legs of the animals of the N series. These observations led to the conclusion that the untreated left hind limb in immobilization-remobilization experiments cannot be regarded as normal. In earlier studies on the development of disuse osteoporosis the results obtained have usually been expressed as percentage difference of the corresponding values from the untreated contralateral limb of the same animal (Gillespie 1954, Slack 1954, Landry and Fleisch 1964, Kharmosh and Saville 1965). When the values from the bones of the untreated extremity were compared with the corresponding values from one group of normal animals there was good agreement between the results (Haike et al. 1967, Klein et al. 1968). It has also been reported that in man the mineral content of the femur of the uninjured limb is not appreciably affected after a fracture of the contralateral tibia (Nilsson 1966). In the light of the results of the present investigation it is obvious that the animals used as controls in experiments such as these should not only be normal but also of the same age as the experimental animals. The accuracy of the methods used for recording the skeletal changes, as well as species- and agedependent factors and the duration of inactivity might also influence the interpretation of the results from investigations of this kind.

The studies concerning the wet weight and the hydrated bone density of the femur and the tibia from the continuously immobilized rats provided no evidence of spontaneous regression of the induced bone atrophy, either within the rats of this series or in comparison with the corresponding values from the rats of the N series. The slight increase in wet weight of the bones observed towards the end of the observation period was not significant. It has been found earlier that the bone ash ratio (gm/cc) of whole bones from denervated rabbit limbs showed signs of spontaneous restitution after the 8th to 10th week post surgery (Kharmosh and Saville 1965). Similar indications of spontaneous regression of the bone atrophy induced in adult rats by different types of inactivity of their extremities have also been observed earlier (Sevastikoglou et al. 1972). The divergent results between the studies mentioned above and the present studies might be due partly to the fact that in the former the conclusions were drawn from occasional observations. The sequence of events was followed thoroughly in the present study. The use of different methods of recording and different statistical comparisons might also explain this divergence to some extent.

SUMMARY

After induction of bone atrophy of disuse by immobilization of the right hind leg of adult male rats with a plaster cast for 16 weeks, the following observations were made with regard to composition of the bone:

After remobilization of the extremity for 1 to 48 weeks the hydrated gross bone density of the femur and tibia remained reduced as compared with that of the normal control rats.
The wet weight of the femur differed significantly in its time course from that of the normal control animals until the results were considered to be influenced by age changes. In the tibia no reduction of the wet weight resulted from the foregoing immobilization.
Both after remobilization and on continued immobilization the percentage ash and organic contents of the defatted dehydrated cortical bone powder remained normal.

On continued immobilization no significant changes in bone composition occurred that could be considered to indicate spon taneous restitution of the induced osteoporotic changes.

The wet weight of the normal femur and tibia from the animals of the untreated series increased significantly during the first third of the observation period, i.e. until the rats were about 1 1/2 years old. Subsequently it remained almost constant. No simultaneous increase in the hydrated gross bone density occurred, however.

The composition of the bones from the untreated contralateral extremity of the remobilized animals was found to be affected by the treatment in such a way that they could not be regarded as normal.

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THE REVERSIBILITY OF DISUSE OSTEOPOROSIS

IV. Studies on the Chemical Composition of the Bones in the Adult Rat[1]

by

Sten Mattsson

Disuse osteoporosis or bone atrophy of disuse is commonly defined as a condition with decreased bone mass per volume without qualitative changes of the remaining bone (Allison and Brooks 1921, McLean and Urist 1960, Nordin 1961, Klein et al. 1968, Sevastikoglou et al. 1972). In this respect the results of biochemical studies of localized osteoporosis, however, are not in complete agreement. Thus, changes in the calcium content of the bone in disuse osteoporosis have been described by Semb (1966), and changes in the bone matrix (collagen and/or ground substance) by Little (1962) and Mishima (1964). On the other hand, it has also been reported that no definite biochemical changes can be registered in disuse osteoporosis (Landoff 1942, Sevastikoglou et al. 1972). Studies of generalized osteoporosis in humans have also given contradictory results as regards biochemical changes of the bone (Little 1962, Casuccio

1) This investigation has been aided by grant nr 17X-69-07 from the Swedish Medical Research Council and by the Faculty of Medicine, University of Umeå, Sweden

et al. 1962, Birkenhäger-Frenkel 1966), while such changes have been denied from studies in rats (Larsson and Vejlens 1969).

As no chemical studies concerning the reversibility of bone atrophy of disuse have been reported previously, the present investigation was undertaken with the purpose of elucidating:

a) the collagen (hydroxyproline) and mineral (calcium) contents of cortical bone during the process of reversibility of induced localized bone atrophy.

b) the collagen and mineral contents of cortical bone during immobilization of the extremity when the atrophy has reached a chronic stage.

c) the reliability of considering the contralateral control extremity as normal.

d) age-dependent changes of the hydroxyproline and calcium contents of the normal cortical bone.

MATERIALS

The animals for the present studies were provided from the same materials as those used in earlier investigations (Mattsson 1972 a and c).

Experimental animals

In the so called *R series* (remobilized), which comprised 10 groups of adult rats, the right hind limb was first immobilized by a plaster cast for 16 weeks, after which time the cast was removed and the leg thus remobilized for periods varying between 1 and 48 weeks before the animals were killed.

Control animals

The *N series* (normal, untreated) comprised 5 groups of rats which did not undergo any form of immobilization. They were observed for 16 plus 2 to 48 weeks.

The rats of the *C series* (continuously immobilized), comprising 10 groups, were immobilized continuously for 16 plus 1 to 48 weeks and then killed.

Five to 7 rats from each experimental group of the R, N and C series were studied. From the 48-week groups, however, only 4 animals of the R series and 3 animals of the C series were still alive and available for the present study.

The time of remobilization of series R, 1 to 48 weeks, constituted the observation period for all the three series.

METHODS

Preparation of the cortical bone powder

A detailed description of the preparation of the femur and tibia and the further preparation of dehydrated defatted diaphyseal cortical bone powder has been given in an earlier report (Mattsson 1972 c).

Organic content of the cortical bone powder

The method of determination of the ash content is described in a previous publication (Mattsson 1972 c). The organic content, i.e. the difference between the dry defatted cortical bone powder and its ash content, was calculated as percentage of the former.

Determination of hydroxyproline

The method of Neuman and Logan (1950) was used. Samples of 34 ± 3 mgs of the cortical bone powder were hydrolyzed in 6 N hydrochloric acid for 16 hours in sealed glass tubes. Hydrolysis curves showed identical values after 6 hours and subsequently up to 21 hours. The mean value of duplicate determination on each sample was used.
The coefficient of variation for the analysis was 2.0 per cent. The hydroxyproline content was calculated as per cent both of the cortical bone powder and its organic content.

Determination of calcium

Bone powder was ashed for 16 hours at 700°C. Approximately 20 mgs of the ash were weighed on a Mettler balance. The ash was dissolved in 5.0 ml of 1.2 N hydrochloric acid. Deionized water was added up to 100 ml. One ml of the solution was added to 9 ml of a 1 % lanthanum chloride solution. The calcium : lanthanum ratio was 1:1000, as recommended for suppression of the interference by phosphorus in the solution (Willis 1961). Standards containing 100 γ/ml were used for comparison. Analysis was performed by spectrophotometry, using an SP 90 Atomic absorption spectrophotometer. Duplicate determinations were performed. The coefficient of variation for the analysis was 1 per cent. The calcium content was calculated in per cent both of the cortical bone powder and its ash content.

Statistical methods

The same statistical methods were used as in previous papers, i.e. regression analysis with null hypothesis testing, analysis of variance and student's t-test for pairing observations. The term variable refers to the difference between the values for the right and the left leg of the factor under study (Mattsson 1972, 1972 a and 1972 c).

RESULTS

I. Hydroxyproline content

1. Femur

A. Differences in hydroxyproline content between the three series

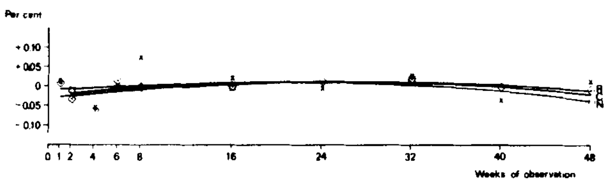

Fig. 1. Percentage hydroxyproline content per cortical bone powder. Regression curves and mean variables of the R (◇), N (□) and C (x) series.

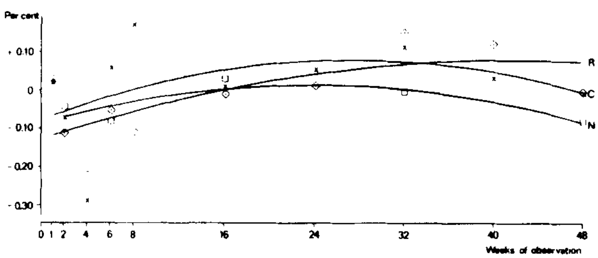

Fig. 2. Percentage hydroxyproline content per organic content of the cortical bone powder. Regression curves and mean variables of the R (◇), N (□) and C (x) series.

The three regression curves (R versus N, R versus C and C versus N series) showed no mutual significant differences either when the percentage hydroxyproline content was expressed per cortical bone powder or per its organic content (Figs. 1 and 2).

Neither were any significant differences found between the series on testing of the mean variables for different time intervals against each other.

B. Percentage hydroxyproline content per cortical bone powder within each of the three series

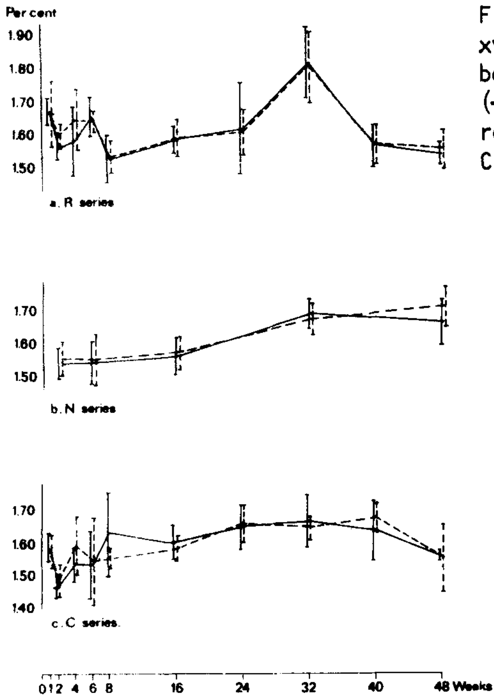

Fig. 3 a-c. Percentage hydroxyproline of the cortical bone powder of the right (——) and left (---) femurs, respectively, of the R, N and C series.

In Fig. 3 the mean values for the individual femurs in the three series are shown diagrammatically. No significant difference was found between the two femurs of the N series during the observation period. On the other hand the hydroxyproline content per cortical bone powder showed a highly significant increase in both the right and left femur of this series from the start of the observation period up to its end.

No statistical analysis of the individual femurs of the R and C series was performed as regards the hydroxyproline content, since such an analysis was considered to lie beyond the scope of this investigation.

C. Comparison of percentage hydroxyproline content between the left control femur of the R series and the left normal femur of the N series

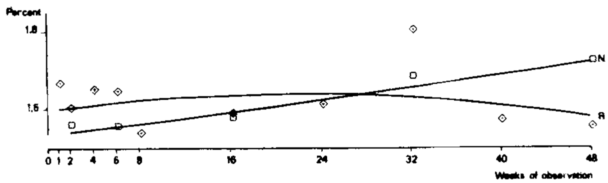

Fig. 4 a.

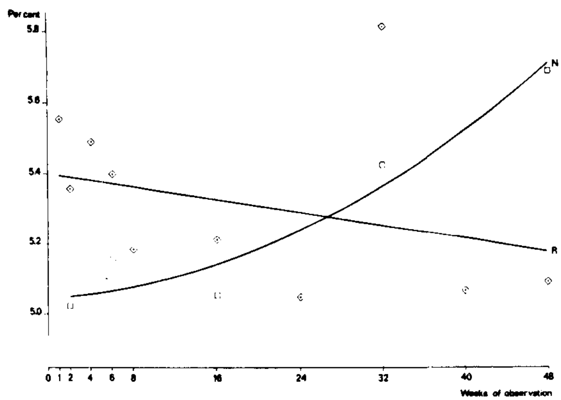

Fig. 4 b.

Figs. 4 a-b. Percentage hydroxyproline content a) per cortical bone powder and b) per organic content, of the left femurs of the R (◇) and N (□) series. Regression curves and mean values.

The regression curve of the mean values of the R series differed significantly from that of the N series throughout the period of observation, both on calculation per cortical bone powder (Fig. 4 a) and per organic content of the bone powder (Fig. 4 b). The hydroxyproline content of the control bone of the R series was thus influenced by the treatment of the animal and could not be regarded as normal.

2. Tibia

A. Differences in hydroxyproline content between the three series

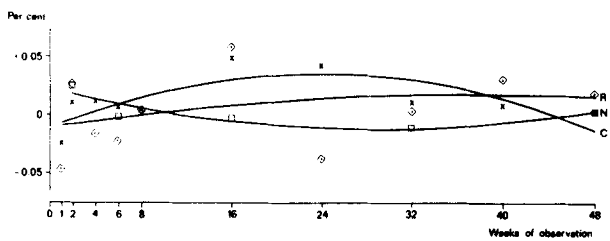

Fig. 5. Percentage hydroxyproline content per cortical bone powder. Regression curves and mean variables. R (◇), N (□) and C (x) series.

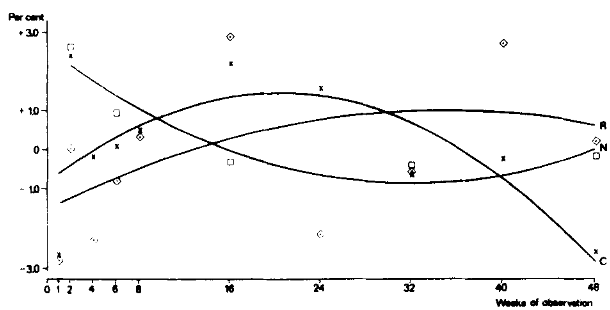

Fig. 6. Percentage hydroxyproline content per organic content of the cortical bone powder. Regression curves and mean variables. R (◇), N (□) and C (x) series.

Comparison of the regression curves between the three series (R versus N, R versus C and C versus N series) showed no significant differences when the hydroxyproline content was calculated per cortical bone powder (Fig. 5). When it was expressed per organic content of the cortical bone powder, on the other hand, a significant difference was found between the R and N series up to the 20th week of observation and between the C and N series up to the 10th week (Fig. 6). No significant differences were observed between the mean variables of the three series on paired testing at the given time points.

B. Percentage hydroxyproline content per cortical bone powder within each of the three series

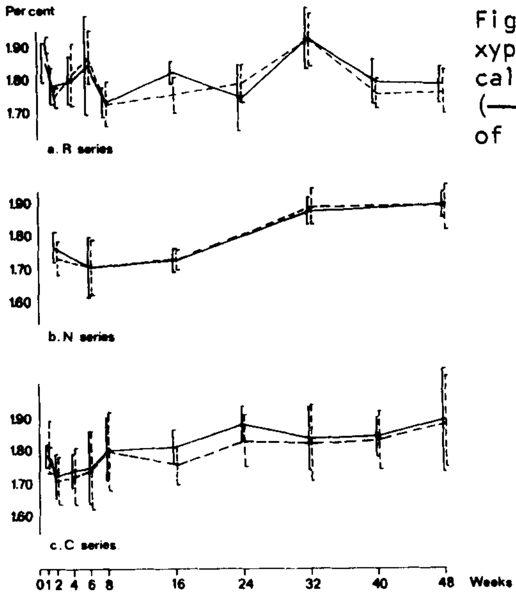

Fig. 7 a-c. Percentage hydroxyproline content per cortical bone powder of the right (——) and left (---) tibia of the R, N and C series.

In Fig. 7 the mean values of the percentage hydroxyproline content of the cortical bone powder for the individual tibiae are presented. No significant differences were found between the two tibiae of the N series (Fig. 7 b). On the other hand, the hydroxyproline content of both tibiae increased highly significantly during the 48 weeks of observation.

The hydroxyproline content per cortical bone powder of the normal tibia was about 11 per cent higher than that of the normal femur. This difference is significant.

C. Comparison of percentage hydroxyproline content between the left control tibia of the R series and the left normal tibia of the N series

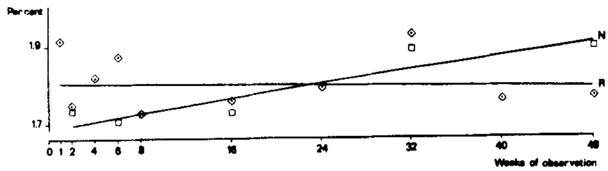

Fig. 8 a.

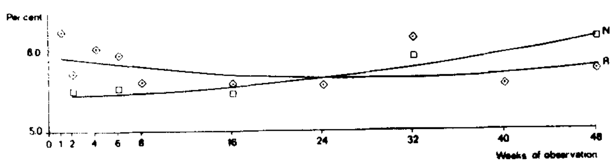

Fig. 8 b.

Figs. 8 a-b. Percentage hydroxyproline content a) per cortical bone powder and b) per organic content, of the left tibiae of the R (◈) and N (□) series. Regression curves and mean values.

When the hydroxyproline content was expressed in per cent of the cortical bone powder (Fig. 8 a), significant differences were found between the two regression curves throughout the observation period; for the hydroxyproline content expressed per organic content (Fig. 8 b), on the other hand, the curves were significantly different from the 2nd to the 36th weeks of observation. From this it may be concluded that as in the case of the femur the hydroxyproline content of the left control tibia of the R series is not to be regarded as normal.

II. Calcium content

1. Femur

A. Differences in percentage calcium content between the three series

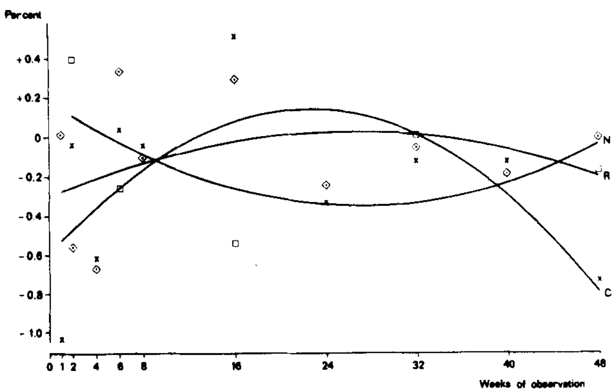

Fig. 9. Percentage calcium content per ashed cortical bone powder. Regression curves and mean variables. R (◇), N (□) and C (x) series.

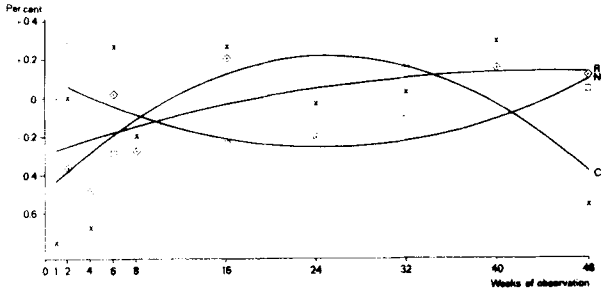

Fig. 10. Percentage calcium content per cortical bone powder. Regression curves and mean variables. R (◇), N (□) and C (x) series.

Neither the regression curves nor the mean variables showed any significant differences between the three series (R versus N, R versus C and C versus N series) during the observation period when the percentage calcium content was expressed a) per ashed cortical bone powder or b) per cortical bone powder (Figs. 9 and 10).

B. Percentage calcium content per ashed cortical bone powder within each of the three series

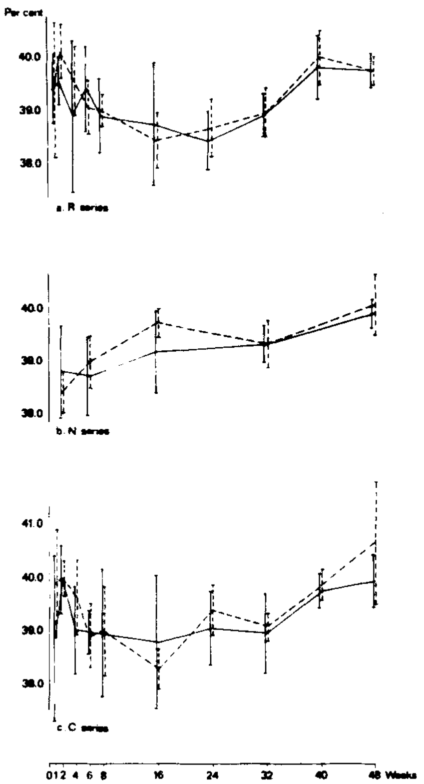

Fig. 11 a-c. Percentage calcium content per cortical bone ash of the right (——) and left (---) femurs of series R, N and C.

In the N series there were no significant differences of the percentage calcium content per ashed cortical bone powder between the mean values for the two femurs at the different determination points (Fig. 11 b). The calcium content increased in both femurs in this series, on the other hand, throughout the observation period - almost significantly in the right femur and highly significantly in the left.

No statistical analysis was performed concerning the individual femurs of the R and C series (Figs. 11 a and c) since such an analysis was considered to lie outside the purpose of this investigation.

C. <u>Comparison of percentage calcium content between the left control femur of the R series and the left normal femur of the N series</u>

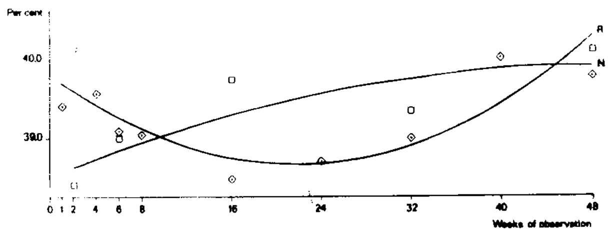

Fig. 12 a.

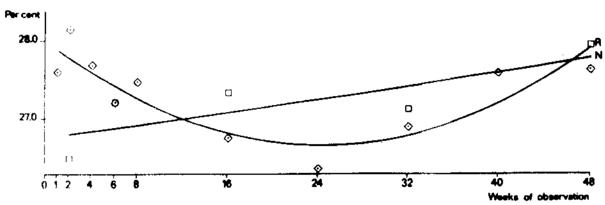

Fig. 12 b.

Figs. 12 a and b. Percentage calcium content a) per ashed cortical bone powder and b) per cortical bone powder, of the left femurs of the R (◇) and N (□) series. Regression curves and mean values.

The regression curves differ significantly throughout the observation period both when the calcium content is expressed as per cent of the ash content and as per cent of the cortical bone powder (Fig. 12 a-b). This means that the calcium content of the left control femur of the R series cannot be regarded as normal.

2. Tibia

A. Differences in calcium content between the three series

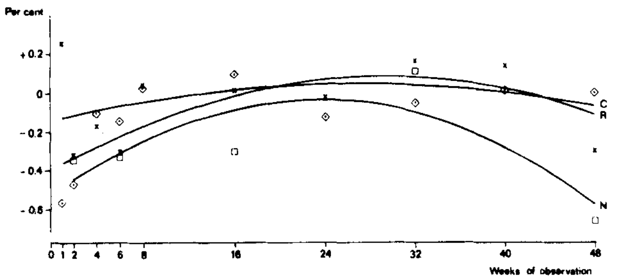

Fig. 13. Percentage calcium content per ashed cortical bone powder. Regression curves and mean variables. R (◇), N (□) and C (x) series.

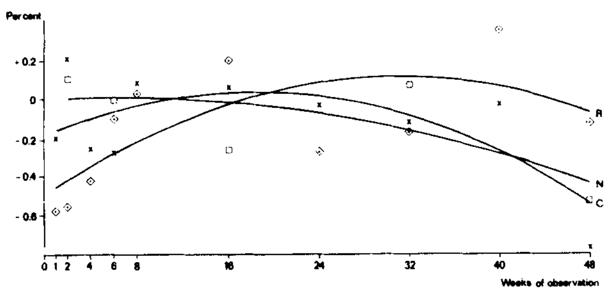

Fig. 14. Percentage calcium content per cortical bone powder. Regression curves and mean variables. R (◇), N (□) and C (x) series.

The results of the comparisons between the three series (R versus N, R versus C and C versus N series) are in complete agreement with those of the femur, i.e. there are no significant differences between the regression curves or the mean variables of the percentage calcium content of the tibiae expressed either per ashed cortical bone powder (Fig. 13) or per cortical bone powder (Fig. 14).

B. <u>Percentage calcium content of ashed cortical bone powder within each of the three series</u>

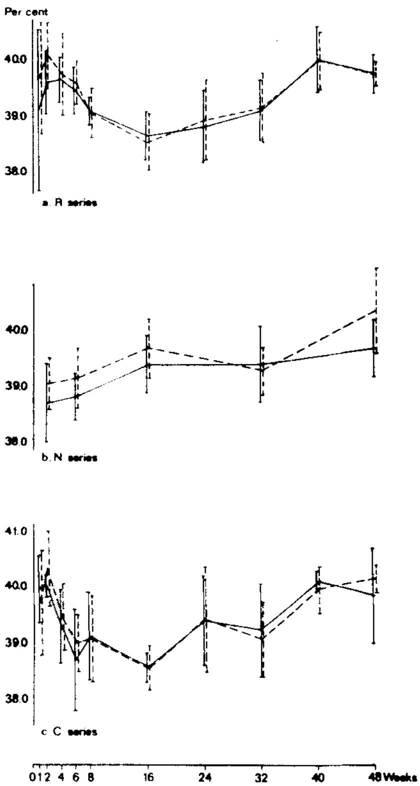

Fig. 15 a-c. Percentage calcium content of the ashed cortical bone powder in the right (——) and left (---) tibiae of series R, N and C.

In the N series no significant differences were obtained between the calcium content per cortical bone ash of the right and left tibiae at the different determination points. During the observation period there was an almost significant increase of the calcium content of the right tibia and a significant increase in the left tibia (Fig. 15 b).

No statistical analysis was performed for the individual tibiae of the R and C series as it was considered to lie beyond the scope of this study (Fig. 15 a and c).

In contrast to the hydroxyproline content, no appreciable difference in calcium content was found between the normal tibia and femur of the N series.

C. <u>Comparison of percentage calcium content between the left control tibia of the R series and the left normal tibia of the N series</u>

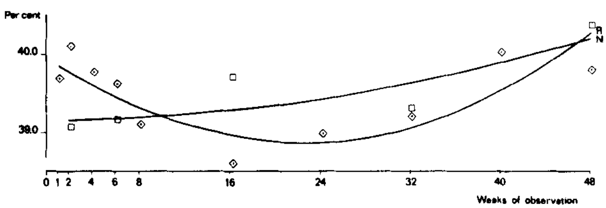

Fig. 16 a.

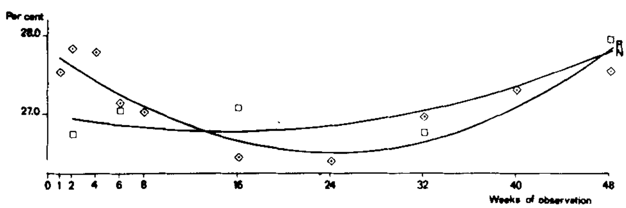

Fig. 16 b.

Figs. 16 a and b. Percentage calcium content a) per ashed cortical bone powder and b) per cortical bone powder, of the left tibiae of the R (◇) and N (□) series. Regression curves and mean values.

The regression curves differ significantly throughout the observation period when the calcium content is expressed per bone ash (Fig. 16 a) and up to the 18th week of observation when it is expressed per cortical bone powder (Fig. 16 b). Therefore, the percentage calcium content of the left control tibia of the R series cannot be considered as normal.

DISCUSSION

On hydrolysis, collagen yields about 14 per cent of hydroxyproline, an amino acid considered specific for collagen (Neuman and Logan 1950). In studies concerning the chemical composition of bone, hydroxyproline determinations are commonly used as an index of collagen concentration. Determinations of the calcium content of the bone are, on the other hand, used as an index of its mineral content.

The results of the present studies concerning the hydroxyproline and calcium contents of cortical bone powder derived from the previously immobilized as well as the contralateral untreated femur and tibia, revealed that neither remobilization nor continued immobilization *per se* altered the collagen and mineral contents of the bones as compared with those of bones from normal rats.

Some significant differences in the hydroxyproline content of the tibiae observed between the R (remobilized) and the N (non-treated) series as well as the C (continuously immobilized) and N series were attributed to biological variations and/or methodological errors (cf Mattsson 1972 c) and were, therefore, ascribed no importance.

The present results are in agreement with earlier observations that no difference exists in the calcium, hydroxyproline, hexosamine and glycosaminoglycans contents of the cortical bone powder from extremities made inactive either by immobilization with a plaster cast or below-knee amputation (Sevastikoglou and Mattsson 1972 a, Sevastikoglou and Larsson 1972 a). Landoff (1942) observed that during the acute stage of development of bone atrophy after immobilization of the one hind leg of rabbits with a plaster cast, the organic and the mineral parts of the bone were broken down almost in parallel without signs of decalcification. Similar results were obtained in dogs whose one fore-leg had been immobilized by nerve section or elbow disarticulation (Klein et al. 1968). It is evident that in these studies as well as in the present investigation the skeletal changes induced by immobilization had the characteristic of osteoporosis. In contrast to these reports are the observations that in the course of bone atrophy induced in dogs by immobilization of the one lower extremity by a plaster cast or section of the sciatic nerve, the resorption of bone mineral occurred earlier than the destruction of bone matrix (Mishima 1964). Significantly decreased concentrations of calcium, phosphorus and nitrogen have also been reported in periosteal lamellar bone from immobilized extremities of rabbits and dogs while no similar changes occurred in the endosteal bone. Inability to measure the net bone volume was considered as a reason for the observed changes in the periosteal bone specimens (Semb 1966). Degradation of the individual collagen bundles has been stated to occur in rabbit bones made osteoporotic by immobilization of the one hind leg with a plaster cast (Little et al. 1962). There may be various explanations for these discrepant reports. Thus, methodological differences, differences in the material studied, and unobserved age changes might be responsible. The fact that many reports are based on percentage differences calculated on the basis of the control bone from the untreated extremity of the same individual might also be responsible. As reported earlier, such control material cannot be regarded as normal (Mattsson 1972 c).

In the present study it was noted that both the percentage calcium content per ashed cortical bone and the percentage hydroxyproline content per dried defatted cortical bone of the femur and tibia increased progressively and significantly in normal adult rats during the period of observation, suggesting that the chemical composition of the bone sustained age-dependent changes. No explanation for this relationship can be advanced at present.

The observation made here that in normal rats the percentage hydroxyproline of the cortical bone powder of the tibia lay about 11 per cent - and significantly - higher than that of the femur is worth to be noted. This observation has been made also in earlier parallel studies (Sevastikoglou et al. 1972).

From a chemical viewpoint the control bone from the untreated extremity of the experimental animals cannot be regarded as normal. Thus the hydroxyproline and calcium contents from the untreated control bone of the R series differed from those of the corresponding bone of the N series. This observation supports the conclusions drawn from the studies concerning the composition of the bones in a previous study (Mattsson 1972 c).

The main conclusion reached from the chemical studies is that neither reactivation nor continued immobilization in a chronic stage of disuse atrophy of the cortical bone leads to a change in the collagen (hydroxyproline) or mineral (calcium) contents with time.

SUMMARY

One hind leg of adult male rats was immobilized in a plaster cast for 16 weeks. The animals were then remobilized by removal of the cast and killed after observation periods of 1 to 48 weeks. Normal untreated as well as continuously immobilized rats served as controls. Studies of the chemical composition of the bone revealed that

- the hydroxyproline and calcium contents of the cortical bone powder are not changed by remobilization and neither do they alter during the chronic stage of bone atrophy of disuse.

- the hydroxyproline content of dehydrated defatted cortical bone in the normal rat femur lies about 11 per cent - and significantly - lower than in the tibia.

- the hydroxyproline and calcium contents of the contralateral untreated femur and tibia are not unaffected by the treatment, and these bones cannot, therefore, be regarded as normal.

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THE REVERSIBILITY OF DISUSE OSTEOPOROSIS

V. Studies of Calcium Metabolism in the Adult Rat [1)]

by

Sten Mattsson

Despite extensive investigations of the bone calcium metabolism in humans and animals during recent decades, there are still several questions unanswered. Thus very little is known so far about changes in the metabolic state of bone under varying conditions of health and disease, variations in the calcium exchange reactions, the mechanisms involved in strict bone formation and long-term mineralization, or the changes in the calcium metabolism during the life span.

Many investigations have been carried out with regard to the calcium metabolism both during the development and during the chronic phase of bone atrophy of disuse (Deitrich et al. 1948, Wyse and Pattee 1954, Whedon and Shorr 1957, Whedon 1960, Heaney 1962, Jowsey et al. 1965, Kharmosh and Saville 1965, Sevastikoglou et al. 1972). By means of tracer studies in humans it has been shown that the bone formation rate is higher than normal during the development of bone atrophy in immobilized individuals, and that it is normal or subnormal during its chronic phase (Heaney 1962).

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1) This investigation has been aided by grant nr 17X-69-08 from the Swedish Medical Research Council and by the Faculty of Medicine, University of Umeå, Sweden

The reversibility of the skeletal changes in bone atrophy of disuse has been studied to only a very minor extent previously. Thus the state of mineralization of the bone in patients with fractures has been investigated by densitometric methods (Nilsson 1966), and radiographic studies have been carried out in amputees (Sevastikoglou et al. 1969). However, few experimental studies have been made regarding the reversibility of skeletal changes after remobilization of a previously immobilized extremity (Geiser and Trueta 1958, Haike et al. 1967), and none at all concerning the calcium metabolism in this context.

The aim of the present study was to investigate:

a) the calcium metabolism during the reversibility of skeletal changes, produced in one hind leg of adult rats by immobilization with a plaster cast followed by remobilization, and
b) to compare the results obtained with those of corresponding metabolic studies in normal and continuously immobilized rats examined in an identical way at different time intervals.

MATERIALS

Materials for the present studies were provided from the animals used in an earlier investigation (Mattsson 1972 a).

Experimental animals

In the so called *R series* (remobilized), which comprised 10 groups, the right hind limb of adult rats was first immobilized by a plaster cast for 16 weeks, after which time the cast was removed and the leg was thus remobilized for periods varying between 1 and 48 weeks before the animals were killed.

Control animals

The *N series* (normal, untreated) comprised 5 groups of rats which did not undergo any form of immobilization and were observed for 16 plus 2 to 48 weeks.

The rats of the *C series* (continuously immobilized), comprising 10 groups, were immobilized continuously for 16 plus 1 to 48 weeks and then killed.

Five to 7 rats from each group of the R, N and C series were examined. However, from the 48-week groups only 4 animals of the R series and 3 animals of the C series were still alive and available for the present study.

The period of remobilization of series R constituted the observation time for all the three series of animals.

METHODS

Isotope

The isotope used was Ca^{45}, i.e. calcium chloride in aqueous solution 2 - 10 mCi/mg Ca[1]. Three different batches were used during the course of the experiment.
Administration: 15 µCi Ca^{45} in 1.0 ml physiological saline was injected intraperitoneally into each animal 72 hours before killing.

Preparation of the cortical bone powder

A detailed description of the preparation of the femur and tibia and the further preparation of dehydrated defatted diaphyseal cortical bone powder has been given in an earlier paper (Mattsson 1972 c).

Bone sampling

About 100 mgs of cortical bone powder were ashed at 700°C for 16 hours in a muffle furnace. The ash was put into a counting polyethylene vial and weighed on a Mettler balance with an accuracy of 0.05 mg. It was dissolved in 2.0 ml of 1.2 N HCl for 24 hours. Ten ml of the gel scintillator used

1) C.E.S.2, The Radiochemical Centre, Amersham, England.

in this laboratory (Larsson 1969 b) was then added and the vial was stored at -20°C pending scintillation counting.

Blood serum sampling

Under light ether anaesthesia blood was collected by cutting off a piece of the tail of each rat 3, 7, 24 and 48 hours after the isotope injection. Usually 20 - 25 drops or approximately 1 ml of blood could be collected on each occasion. At 72 hours the animals were killed by exsanguination from the abdominal aorta and all blood available was collected. The serum calcium content was determined in 72-hour samples. For determination of the serum Ca^{45} activity samples obtained 3, 7, 24, 48 and 72 hours after the isotope injection were used; a known amount of serum was pipetted into counting polyethylene vials and 12 ml of gel scintillator were added. The samples were then kept at -20°C until counting was performed.

Methods of analysis of the Ca^{45} activity

The determinations were made in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320. All samples from each batch were counted simultaneously at +3°C. The first vials were always recounted to check the stability of the specimens and apparatus. The variation between the first and second recording from the same vials was less than 4 per cent. Depending on the activity of the samples counting was performed for 1 to 10 min for different batches. Correction was made for the background activity which was at least ten times lower than the activity of the samples. Samples of the injected solution were also counted. The results were computed and were expressed as counts/min/mg bone ash and counts/min/mg Ca of serum, respectively. They were further calculated per activity of the injected dose.

Serum calcium determination

Duplicate fluorometric determinations were made with a Technicon Auto Analyzer and a Turner filter fluorometer, i.e. the routine method used in the central laboratory of this hospital.

Statistical methods

The same statistical methods as in previous studies were used, i.e. regression analysis with null hypothesis testing, analysis of variance and student's t-test for pairing observations. The term variable refers to the difference between the values for the right and the left leg of the factor under study (Mattsson 1972, 1972 a and 1972 c).

RESULTS

I. Ca^{45} activity of cortical bone powder

1. Femur

A. Difference in bone specific activity between the three series

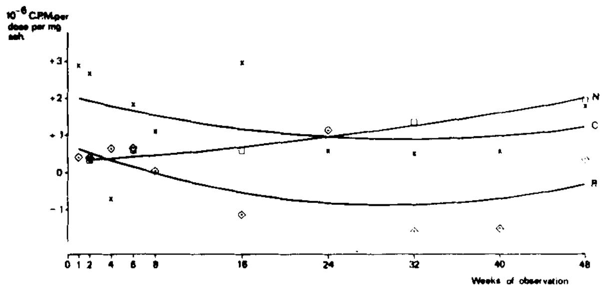

Fig. 1. Specific activity of the femur. Regression curves and mean variables of R (◈), N (□) and C (x) series.

R versus N series: The regression curves differ significantly from the 24th week of observation, probably mostly due to distance (Fig. 1). The mean variables show no significant differences on testing against each other at the time points studied.

R versus C series and C versus N series: No significant differences were revealed either between the regression curves or between the mean variables.

B. Specific activity of the femurs within each of the three series

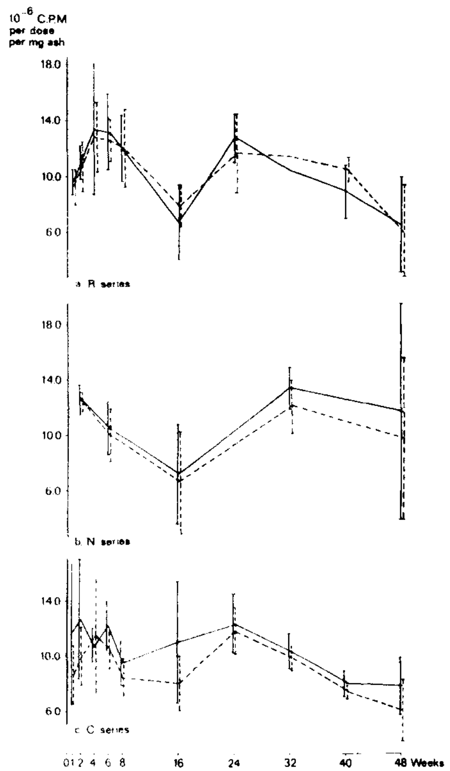

Fig. 2 a-c. Specific activity of the right (——) and left (---) femur of series R, N and C.

The periodicity in the values for different time intervals is large and no tendency to an increase or decrease of the values for the individual bones is noted (Fig. 2 a-c). Statistical analysis revealed that the observed variations of the right remobilized femur of the R series and the right continuously immobilized femur of the C series were not significant at any part of the observation period (Figs. 2 a and c). On the other hand almost significant variations existed within the values of the right normal femur of the N series and the left femur in all three series during the course of observation (Figs. 2 a-c). These differences are, however, considered to be of no importance since the diagrams do not show any definite trend of increase or decrease of the Ca^{45} uptake with time.

C. <u>Comparison of specific activity between the left control femur of the R series and the left normal femur of the N series</u>

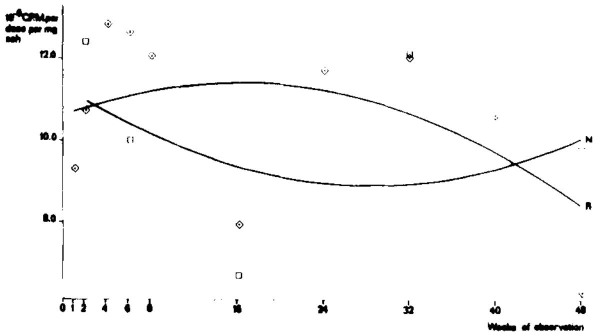

Fig. 3. Specific activity of the left femur of the R (◇) and N (□) series. Regression curves and mean values.

Apparent differences between the regression curves are statistically non-significant (Fig. 3).

2. Tibia

A. Difference in specific activity between the three series

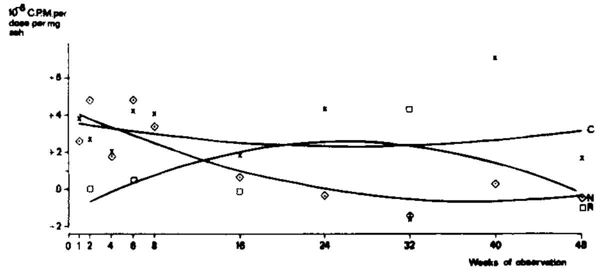

Fig. 4. Specific activity of the tibia. Regression curves and mean variables of R (◇), N (□) and C (×) series.

R versus N series: The regression curves differ significantly throughout the observation period, probably mostly by slope except during the first weeks (Fig. 4). Testing of the mean variables between the two series reveals significant differences at the 2nd and 6th weeks of observation ($p<0.001$ and $p<0.001$, respectively).

R versus C series: There are no significant differences between the regression curves. The mean variables differ almost significantly at the 24th week of observation, with a lower Ca^{45} uptake in series R than in series C.

C versus N series: The regression curves do not differ significantly. There is an almost significant difference between the mean variables at the 6th week with a lower Ca^{45} uptake in series N.

B. Specific activity of the tibiae within each of the three series

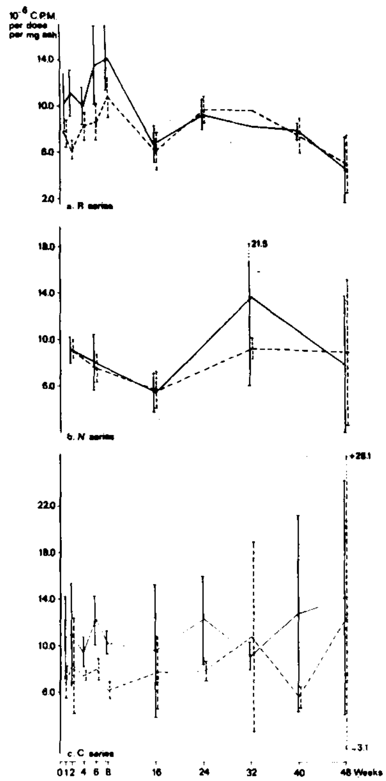

Fig. 5 a-c. Specific activity of the right (——) and left (---) tibia of series R, N and C.

Also for each of the tibiae, the periodicity in the Ca^{45} specific activity is large throughout the observation period (Fig. 5 a-c). Almost significant differences within the values of the left control bone of the R series and the right normal bone of the N series, during the period of the observation are present but these differences are considered to be without importance. Otherwise, no significant differences are found. The apparent increase in Ca^{45} uptake of the remobilized tibia of series R at the 6th and 8th weeks of observation is not statistically significant either.

C. Comparison of the specific activity between the left control tibia of the R series and the left normal tibia of the N series

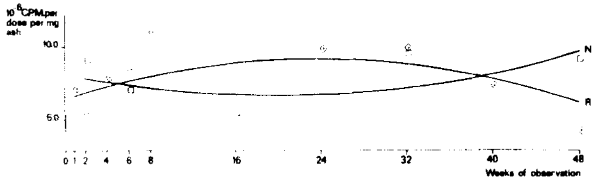

Fig. 6. Specific activity of the left tibia of the R (◇) and N (□) series. Regression curves and mean values.

The regression curves show significantly different courses from the 28th week until the end of the observation period (Fig. 6).

To summarize the observations of the R versus N series show that the Ca^{45} specific activity differences follow significantly different courses throughout the observation period in the case of the tibia and during its latter half for the femur. The tibia also shows significantly higher values for the variables in the R as compared with the N series up to the end of the 6th week of observation, indicating a high uptake in the remobilized tibia during that period. The regression curves for the femurs of series R and N probably differ most in distance, which means that the bone specific activity of the R series lies at a lower level during the time period in question. No difference between the variables of the two series is obtained, however.

It is evident from the course of the Ca^{45} uptake that in this respect the left control tibia in the R series cannot be regarded as normal. For the left femurs, on the other hand, no significant difference is found between the courses for the R and N series.

II. Serum calcium specific activity

The mean values of the serum specific activity in the three series, plotted logarithmically against time, are shown in Fig. 7 a-c.

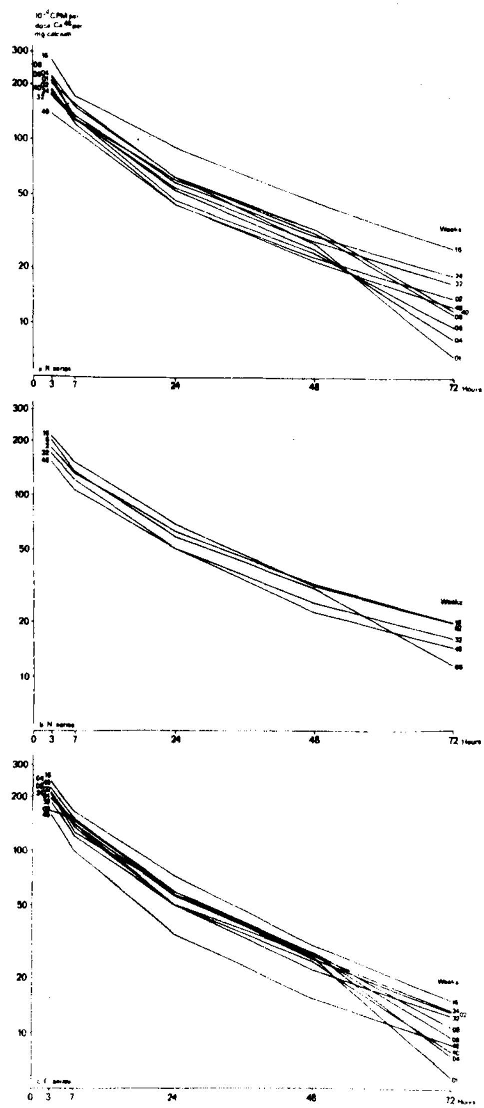

Fig. 7 a-c. Mean values of the Ca^{45} activity 3, 7, 24, 48 and 72 hours after administration of the isotope. R, N and C series. Logarithmic graph.

The drop in serum specific activity is rapid during the first hours after administration of the isotope. However, there is still an appreciable amount of Ca^{45} in the serum 24 hours later. On the whole, the differences between the diagrams of the three series appear to be negligible.

III. <u>Serum calcium concentration</u> (Fig. 8)

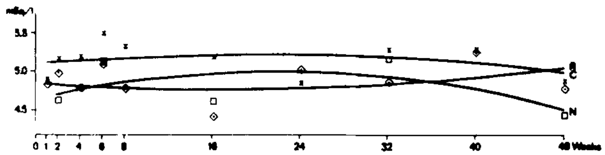

Fig. 8. Serum calcium concentration. Regression curves and mean values for series R (◇), N (□) and C (x).

R versus N series: The regression curves do not show any mutual significant differences during the period of observation. The distances between the mean values at the time points studied are not significant except at week 32, when the distance is almost significant.

R versus C series: The regression curves are significantly different until a few weeks before the end of the observation period, when they intersect. The mean values differ almost significantly at the 4th, 6th and 32nd weeks of observation and significantly at the 16th week.

C versus N series: There are significant differences between the regression curves up to the 14th week of observation but not after this time. The mean values differ almost significantly at the 2nd, 16th and 48th weeks of observation.

Thus the serum calcium concentration of the R series appears to be normal during the observation period. The initial differences between the R and N series, on the one hand, and the C series, on the other, not only in the course of their regression curves but also in the form of significantly higher mean values of the C series, indicate that the serum calcium concentration of the C series lies at a higher level than that of the two other series during the first third of the observation period.

DISCUSSION

The use of radioisotopes as an index of calcium accretion was first considered impossible because of the chemical exchange reactions (Sachs 1953). Evidence was then presented showing that bone seeking isotopes are valuable for quantitative evaluation of the skeletal accretion, resorption, and exchange reactions (Bauer et al. 1955). It was stated, further, that tracers are retained in bone as a result of formation in the strictest sense of the word, long-term exchange and secondary mineralization (Ito et al. 1957, Heaney 1962, Jowsey et al. 1965), and also that $calcium^{45}$ and $calcium^{40}$ are deposited in bone tissue in the same proportions as they occur in blood at the same time (Bauer et al. 1955). However, objections have been raised against the justification of the assumption that a single rate process governs isotope fixation (Neuman and Neuman 1958) and that bone formation and resorption in different pathological states can be estimated from isotope kinetics (Rasmussen and Tenenhouse 1967).

The results of the present investigation provide evidence that after 16 weeks of immobilization the remobilization of the extremity *per se* results in an increase of the calcium uptake of the cortical bone tissue from the tibia but not from the femur. This increase is significant for a few weeks after remobilization. The observations seem to indicate that during the early period of remobilization of the extremity there is increased bone formation in the tibia while that of the femur remains mainly unchanged. This conclusion is valid if the process of long-term exchange can be considered negligible. However, it has been suggested that long-term exchange and secondary mineralization are quantitatively too important to be ignored (Jowsey et al. 1965, Rowland 1967).

An increased bone formation rate in the tibia during a short initial phase of remobilization is supported by the results of earlier studies where new bone formation was evaluated by tetracycline labelling (Mattsson 1972 b). In that investigation, however, there was evidence of increased bone formation activity during the first weeks of remobilization not only in the tibia but also in the remobilized femur, as well as in the bones of the contralateral untreated but apparently not unaffected extremity. The two methods of evaluation of the bone formation activity used - Ca^{45} and tetracycline labelling - do not give exactly comparable information. The uptake of radioisotope should be interpreted as physiological behaviour of the bone (Armstrong 1967), and any tracer study shows mainly the flow of the labelled atom from blood to bone (Jowsey et al. 1965, Rowland 1967). Furthermore, lowly mineralized structures may aquire and retain more tracer calcium than other regions (Rowland 1967). On the other hand the tetracycline labels exclusively newly forming bone (Milch et al. 1957), but very low concentrations may also be bound to areas that are not sites of mineralization of new bone matrix (Harris et al. 1962).

The results of the present studies provide further support for the conclusion drawn from earlier investigations that immobilization of an extremity induces skeletal changes that remain irreversible despite prolonged remobilization (Mattsson 1972 b and c).

In studies on poliomyelitic patients (Heaney 1962) and denervated extremities of both rabbits (Kharmosh and Saville 1965) and adult rats (Sevastikoglou and Larsson 1972 b), the Ca^{45} specific activity of bone has been reported to be increased during the acute phase of disuse osteoporosis, suggesting a high rate of bone formation. However, during the chronic phase of immobilization of paralytic patients the bone formation rate as measured by means of radiocalcium was found to be normal or subnormal (Heaney 1962). Decreased Ca^{45} uptake of bone from about the 10th week of inactivation was also found in the studies mentioned above in rabbits and adult rats. This is in agreement with the finding in the present study that the bone Ca^{45} specific activity in the animals of series C (continuously immobilized) did not differ significantly from that in the normal animals. A chronic stable metabolic stage of the immobilized skeletal parts of the extremity was obviously reached in these rats.

In the normal adult control rats of series N of the present investigation no noteworthy difference in the rate of disappearance of Ca^{45} from the blood was found which could be attributed to the ageing of the animals, despite the fact that their age increased from one to two years during the observation period. This is somewhat contradictory to earlier observations that adult normal rats show a slower fall of serum specific activity than young animals (Bauer et al. 1955). The rate of disappearance of Ca^{45} from the blood of three normal dogs of different ages has also been reported to be most rapid in the youngest and least rapid in the oldest dog (Jowsey et al. 1965). The differences between the results of the present and these earlier studies are probably due to the fact that comparison between young growing, middle-aged, and senile animals, was made in the earlier studies while the present observations were based on animals which had reached the middle of their life span at the beginning of the experiment. After that time there was probably a constant ratio of disappearance of Ca^{45} from the blood of the rat.

It has been reported earlier that the serum calcium level during the development of disuse osteoporosis tended to increase in four healthy young men immobilized in plaster casts from waist to toes for 6 to 7 weeks (Deitrick et al. 1948). The same finding has been reported from studies in growing rats the one hind leg of which was inactivated by nerve section over a period of 8 weeks (Gedalia et al. 1966), and in adult rats the one hind leg of which was immobilized with a plaster cast for 18 weeks (Sevastikoglou and Mattsson 1972 b). However, a normal serum calcium level has also been noted in patients with a post-traumatic paraplegia or with poliomyelitic paralysis in the acute and chronic stages of the disease (Wyse and Pattee 1954, Whedon and Shorr 1957, Whedon 1960, Heaney 1962). There might be several explanations for the discrepancy between the results for apparently similar clinical and experimental conditions. Species-bound and age-dependent factors, different lengths and causes of inactivity, and possibly a periodicity in the development of the calcium metabolic disturbances might be some of the reasons for disagreement.

SUMMARY

The calcium metabolism was studied in adult male rats the one hind leg of which was immobilized for 16 weeks with a plaster cast. The cast was then removed and the rats allowed to move freely until they were killed at different intervals from 1 - 48 weeks after the beginning of remobilization. Normal and continuously immobilized rats were also studied as controls for a similar length of time. Fifteen microcuries of Ca^{45} were injected intraperitoneally into each animal 72 hours before it was killed. The serum calcium level and the bone and serum specific activities were determined. After remobilization there was an increased uptake of the isotope in the cortical bone of the remobilized tibia during a short initial period as compared with that of the corresponding normal tibia. No such differences were found with respect to the femur. The specific activities of the tibia and femur followed different time courses.

During continued immobilization the bone specific activity was not significantly different from that of the normal animals. There was no appearent difference in the rate of disappearance of the isotope from the serum of the rats of the three series which could be attributed to the different treatment or to the age of the animals.

The contralateral untreated tibia of the remobilized animals could not be considered normal as regards bone specific activity. This was not valid, however, for the femur of the untreated leg.

The serum calcium concentration was significantly increased in the continuously immobilized animals during the first part of the observation period, and was normal in the remobilized animals.

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SUMMARIZING REMARKS AND CONCLUSIONS

Our knowledge regarding the fate of the osteoporotic changes due to disuse of an extremity after reactivation of the limb is extremely limited. Earlier studies in this laboratory largely confirmed previous findings concerning the development of bone atrophy of disuse. However, a tendency to a slight spontaneous regression of the changes was also observed after a prolonged period of immobilization in adult rats (Sevastikoglou et al. 1972). These were the two main reasons for planning and carrying out the present investigation.

In the foregoing studies of this investigation an attempt has been made to collect as much information as possible regarding the reversibility, if any, of bone atrophy and to some extent also of muscular atrophy due to disuse of an extremity. Adult male albino rats were used for the purpose of this investigation. During an initial period of 16 weeks one hind leg was immobilized with a plaster cast. The leg was remobilized later by removal of the cast. Normal untreated animals as well as continuously immobilized rats served as controls during the observation period of 1 to 48 weeks after remobilization of the experimental animals.

I. In the following the observations made in the different studies concerning the main questions presented in the introduction of this work are summarized and some conclusions are drawn:

a. During the period of immobilization the animals walked around on three legs in a fairly unhindered manner and were largely unaffected by the treatment. However, their body weight was reduced by 18 per cent, but this decrease was soon regained after remobilization. Simultaneously a reduction of the muscle mass of the immobilized leg was recorded which, however, remained irreversible after reactivation. The range of passive joint movement also became irreversibly restricted, especially in the ankle and knee.

b. During the initial period of immobilization clear signs of disuse osteoporosis had developed. The bone mass of the immobilized femur and tibia was decreased, as confirmed by determinations of the hydrated density of the whole bones, the percentage bone area in different sections of the femur, measurement of the femur and tibia score and radiographic evaluation of the osteopenia.

c. Continued immobilization of the control animals did not lead to additional changes except for still further restriction of the joint movements.

d. On remobilization the animals began to bear weight on the reactivated extremity. In a few weeks a normal walking pattern was observed clinically except for restricted mobility of the remobilized ankle.

e. Increased anabolic activity of the examined bones from both the remobilized and the control hind leg was observed immediately after remobilization. This was confirmed by determining the activity of new bone formation by tetracycline labelling and the retention of Ca^{45} in the cortical bone. The former was found to be increased in the femur and tibia of both hind legs during the first weeks after remobilization. The latter was increased only in the remobilized tibia during the same period of time. At about the 8th week of remobilization the increased bone formation activity slowed down to normal levels again. The process of increased bone formation was thus of short duration.

f. Despite the initial increase in new bone formation no signs of an increase of the reduced bone mass were noted after reactivation of the animals. The percentage bone of total surface areas, as well as the hydrated density, remained decreased after remobilization of the previously immobilized femur and tibia. Neither did the radiographic evaluation nor the measurement of the femur and tibia score provide evidence of reversibility of the induced osteoporosis.

g. The collagen (hydroxyproline) and mineral (calcium) contents of the cortical bone tissue of both the treated series of rats did not show any alterations as compared with the normal untreated animals. It was thus evident that the skeletal changes had the general characteristics of osteoporosis.

h. Except for the changes in Ca^{45} specific activity of bone described above, no alterations in the calcium metabolism were recorded after the remobilization. An increased serum calcium level was found during the early period of the chronic stage of continued immobilization.

II. Besides the results summarized above concerning the main objectives of the investigation, some other observations were considered to be of importance:

a. The experimental arrangement of this investigation, permitting comparison between the treated and the control animals in more than one respect, showed that at least in long-term experiments of immobilization-remobilization the untreated contralateral leg cannot be considered as normal and therefore cannot be used as a control for assessment of changes occurring in the ipsilateral treated leg.

b. The hydroxyproline per dried defatted cortical bone powder in the tibia was found to be significantly higher, about 11 per cent, than that of the femur. This observation was valid for the bones of both legs from the rats of the untreated series.

c. The length of the femur and tibia of the normal animals was found to be about one mm, or two per cent, longer than the treated bones of the remobilized and continuously immobilized series. This provided evidence that the skeletal growth of at least these rats continued up to the 9th to 13th months of age. The skeletal growth of the treated rats was, furthermore, affected by the initial immobilization.

d. Age-dependent changes such as decrease in muscle weight and osteopenia were recorded in the untreated and the remobilized animals from about the middle of the observation period, i.e. when the rats were about 1 1/2 years old. Osteopenia was evidenced only by the bone appearance in the radiographs and the score of the femur.

The results of the present investigation have thus provided evidence that there is practically no reversibility of the skeletal and even the muscular changes induced by prolonged immobilization of the one hind leg of adult rats. Since the changes observed obviously concern the properties and cellular activity of the skeletal tissue and cannot be considered to be species bound, the observations made here in the rat are probably valid for other species also.

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