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ON THE DEVELOPMENT OF OSTEOPOROSIS

EXPERIMENTAL STUDIES IN THE ADULT RAT

BY
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The paper on bone glycosaminoglycans written together with Dr. Lars Vejlens, Institute of Pathology (Head: Prof. B. Engfeldt, M.D.), University of Uppsala, Uppsala, Sweden.

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PREFACE

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INTRODUCTION

DEFINITION

"The definition of osteoporosis is the least controversial aspect of an otherwise highly controversial subject" (*Rose* 1967). According to the generally accepted definition, osteoporosis is a condition resulting from a widespread lack of bone with no detectable chemical abnormality in the bone that remains (*Albright and Reifenstein* 1948, *Nordin* 1961, *Lafferty et al.* 1964, *Dent and Watson* 1966, *Rose* 1967). Although this definition has never been seriously challenged, there are reports according to which it would not hold true. Thus, osteoporotic bone has been found to show cellular differences from the normal, *i.e.* an increased frequency of devitalized osteocytes and empty lacunae (*Urist et al.* 1963) and disturbed bone cell metabolism as measured *in vitro (Nichols Jr. and Flanagan* 1966). A slight but definite reduction in the calcium content and the Ca:P ratio of osteoporotic bone has been reported by *Birkenhaeger-Frenkel et al.* (1961). Results of studies on the composition of microscopic bone structures from human material of varying ages indicate changes in the contents of calcium, phosphorus and nitrogen measured as percentage by weight (*Strandh and Norlén* 1965). Changes in organic bone matrix have been reported from studies with the aid of X-ray diffraction and electron microscopy (*Little et al.* 1962). In osteoporotic subjects decreased hydroxyproline contents have been found in bone specimens from the iliac crest (*Birkenhaeger-Frenkel* 1966). Biochemical studies (*Cassucio et al.* 1962) indicate a decrease with age in protein nitrogen and polysaccharides and changes in the glucosamine/galactosamine ratio in fractions of vertebral bone tissue extracted according to *Dische et al.* (1958). The qualitative changes in bone matrix observed by *Little et al.* (1962) and *Cassucio et al.* (1962) have been related to the osteoporotic process. The significance of this as a pathophysiological mechanism remains to be determined, however.

CLINICAL OSTEOPOROSIS

Osteoporosis is not a single disease entity, but is a condition produced by many different factors (*Frost* 1963). Despite the varying aetiology the end result — osteoporosis — is now a well recognized condition and justifies its description by a single term (*Dent and Watson* 1966). In the following table the main causes have been listed according to *Rose* (1967):

THE MAIN CAUSES OF OSTEOPOROSIS

1. Endocrine disturbances
   - Hypogonadism
   - Cushing's syndrome
   - Cortisone administration
   - Acromegaly
   - Thyrotoxicosis
2. Nutritional disturbances
   - Scurvy
   - Calcium deficiency
   - Protein deficiency
3. Idiopathic
   - Juvenile
   - Pregnancy
   - Postmenopausal
   - Senile
4. Inherited
   - Osteogenesis imperfecta
5. Immobilization

In the numerous attempts made in the past to find the aetiological agents which could provide the clues to the pathogenesis of idiopathic osteoporosis, endocrine and nutritional disturbances have been extensively studied in this respect.

Of the endocrine disturbances, thyrotoxicosis is considered by some authors to give rise to osteitis fibrosa rather than osteoporosis (*Follis* 1953, *Rose* 1967). In acromegaly the predominant bone disorder does not seem to be osteoporosis but a changed distribution of spongious and compact bone in the skeleton (*Jesserer* 1963).

In hypogonadism osteoporosis may occur both in males and females (*Albright et al.* 1942) and the hypothesis that oestrogen deficiency is the cause of human osteoporosis was advanced by *Albright and Reifenstein* (1948). This hypothesis was extended later to include the possibility that senile and other forms of osteoporosis are caused by an imbalance between "anabolic" or gonadal hormones and "anti-anabolic" or adrenal corticoid hormones (*Reifenstein* 1957, *Urist and Vincent* 1960). The role of the menopause in the occurrence of osteoporosis has been emphasized by radiological observations of a fall in bone density corresponding in time to the menopause (*Jasani et al.* 1965, *Meema et al.* 1965, *Meema* 1966, *Nordin et al.* 1966). However, only a low degree of correlation between age at the menopause and age at the onset of symptomatic osteoporosis was found by *Saville* (1967) in a study of women who had had a surgical and women with a natural menopause. The menopause would then be only one of different factors of importance for the occurrence of osteoporosis. The original concept of *Albright et al.* (1940) that involutional osteoporosis is due to osteoblastic failure consequent to a deficiency of gonadal hormones, has been made untenable by results of more recent investigations with refined morphological techniques and of calcium balance and radiocalcium kinetic studies. It has been concluded from results of these studies that new bone formation is commonly normal in postmenopausal and senile osteoporosis (*Heaney and Whedon* 1958, *Nordin* 1960) and that osteoblastic activity does not diminish with age (*Jowsey* 1960, *Frost and Villanueva* 1961). Instead, bone resorption appears to be markedly increased in osteoporosis (*Frost* 1961, *Riggs et al.* 1964).

Hypercorticoidism of endogenous origin is often associated with osteoporosis (*Albright and Reifenstein* 1948, *Follis* 1951, *Iannaccone et al.* 1960, *Storey* 1963). In prolonged cortisone therapy osteoporosis is a serious complication (*Bunim et al.* 1955, *Reifenstein* 1958, *Rosenberg* 1958); however, there are reports suggesting that the contribution of other factors may be necessary for the development of corticosteroid osteoporosis. Thus, 85 per cent of the patients of comparable ages and skeletal conditions do not develop osteoporosis on comparable steroid therapy (*Schlesinger et al.* 1961). It has not been possible to relate the incidence of fractures to the dose of steroids or to the duration of treatment (*McConkey et al.* 1962). In patients with allergic diseases treated with steroids, only a small effect on the skeleton has been reported (*Krokowski and Stresemann* 1967).

Of the nutritional disturbances, scurvy leads to radiological signs of osteoporosis in adults and has recently been studied in Johannesburg Bantu (*Seftel et al.* 1966). However, the clinical picture in these subjects is confused by the presence of hepatic siderosis and other factors (*Dent and Watson* 1966).

Protein deficiency has been alleged to cause osteoporosis in humans. However, in the majority of subjects the bone lesion appears to be both osteoporosis and osteomalacia due to lack of vitamins as well as other deficiences (*Jesserer* 1963).

The role of pure calcium deficiency in the pathogenesis of osteoporosis is controversial. *Albright and Reifenstein* (1948) and *Rose* (1967), among others, have considered that specific calcium deficiency is not of primary aetiological importance, while other authors (*Whedon* 1959, *Nordin* 1960, *Fraser* 1962) have emphasized the possible role of dietary calcium in the pathogenesis. Although some dietary surveys have shown a normal calcium consumption in osteoporotic persons (*Urist* 1960, *Smith and Frame* 1965) and little relation between mild osteoporosis and calcium intake (*Nordin* 1964), other studies have shown a lower mean calcium intake in osteoporotic than in normal persons (*Nordin* 1961, *Lutwak* 1964) and that severe osteoporosis only seems to occur in individuals on a low calcium intake (*Nordin* 1964). However, man has a considerable ability to adapt to restricted calcium intake by enhanced intestinal absorption or by decreased urinary excretion (*Malm* 1958) and therefore any further loss of bone mineral might be prevented unless a failure of adaptation to the low calcium level occurs. Some persons are, however, unable to adapt to calcium restriction and in these subjects a protracted negative calcium balance results (*Malm* 1958). The nature of this adaptation mechanism is unknown. It seems, however, that osteoporotic patients are less able than normal subjects to reduce their urinary calcium (*Bhandarkar and Nordin* 1962) and calcium excretion appears to be independent of the intake in osteoporosis (*Nordin* 1961). The observed calcium requirement appears to be increased in osteoporosis (*Nordin* 1962).

During the adult life span there is a gradual decrease in bone mass which occurs more or less physiologically in all normal people. Thus, there is a steady loss of apparent density of whole bones from 20 years on in both sexes, and in both white and negro races (*Trotter et al.* 1960). These observations were confirmed by *Lindahl and Lindgren* (1962), who found a parallel decrease in bone density in the upper end of the tibia and the vertebral column with increasing age. The radiographic density of bone removed at autopsy has also been reported to decrease steadily after the age of 20 years in both sexes (*Caldwell* 1962). In agreement with these findings are those of *Arnold* (1964), who showed a steady decrease from the age of 30 years in the weight of ash per unit volume of bone removed at autopsy from the lumbar vertebral cortex. From 20 years on there is, in both sexes, a progressive increase in resorption spaces in the cortical bone (*Atkinson* 1965). These observations might indicate

a physiological progressive loss of bone in all normal people with increasing age, probably due to increased bone resorption. Of importance for this process is certainly the more sedatory life of old people than at younger ages. Osteoporosis due to immobilization is well known. It is not considered, however, that a more sedatory life is the prime aetiological factor in the pathogenesis, rather it might be secondary to symptomatic osteoporosis and induce an additional loss of bone mineral.

From the above findings *Rose* (1967) drew the conclusion that osteoporosis is a condition towards which all people progress gradually, beginning soon after the age of 20 years. However, according to *Dent and Watson* (1966), symptomatic osteoporosis is caused by the action of an additional process which accelerates this gradual loss of bone, resulting in greatly increased negative calcium balances of the order of 200—400 mg per day until the process limits itself spontaneously. This additional process, which is of uncertain origin, thus causes acute attacks of osteoporosis and these can be observed in the young, the adult and also the senile patient.(*Dent* 1968). *Urist* (1959) attributed osteoporosis to the absence of a purported "anti-osteoporosis factor". The loss of bone mass in normal aging is obviously much slower than in osteoporosis (*Urist* 1959).

In an attempt to incorporate the numerous both unrelated and contradictory observations in osteoporotic patients into a unified concept of osteoporosis, *Heaney* (1965) related the osteoporotic process to calcium metabolism and to the demands placed upon the homeostasis of the extracellular fluid calcium. According to this concept dietary calcium deficiency and the intestinal malabsorption syndromes may produce osteoporosis simply by presenting the organism with less mineral than is needed for minimal losses; osteoporosis results because of homeostatic efficiency. Relative or absolute deficiency of gonadal hormones would lead to a negative calcium balance because of increased bone resorption, not so much because these hormones decrease resorption *per se*, but because they appear to moderate the resorptive response of osteoclasts to homeostatic stimuli, and in their absence bone becomes hyperresponsive to parathyroid hormone (*Heaney* 1965).

ANIMAL EXPERIMENTS

Calcium deficiency.

There are several reports indicating that osteoporosis can be induced in animals by dietary calcium deficiency. Attempts to produce rickets in animals by feeding

low calcium diets were made in the middle of the last century. In 1885 *Pommer* described for the first time the characteristic features of rickets. In a review article he concluded that low calcium diets caused osteoporosis, not rickets (*Pommer* 1925). In a later review of the subject in 1948, *Follis* noted that rickets had been produced by low calcium diets but that no descriptions were available for animals fed diets containing optimal amounts of vitamin D. More recently, *Nordin* (1960), in a review, intended to show that osteoporosis could be produced in animals by a negative calcium balance, usually induced by low calcium diets. However, only young growing animals were studied in these experiments. There were only two reports of the histological appearance of the bones resulting from a diet deficient solely in calcium, where adequate amounts of vitamin D were provided (*Crawford et al.* 1957, *Harrison and Fraser* 1960).

While young rats maintained on low calcium diets with adequate amounts of vitamin D have been reported to develop osteoporosis (*Crawford et al.* 1957, *Harrison and Fraser* 1960), there are also reports that young growing rats fed a similar diet develop skeletal changes typical of rickets (*Park et al.* 1922, *Hess* 1929, *Carttar et al.* 1950, *Engfeldt et al.* 1962, *Gershon-Cohen et al.* 1962, *McClendon and Blaustein* 1965). The experiments of *Gershon-Cohen et al.* (1962) suggest that low calcium diets produce rachitic lesions in the young growing rat but that when the animals attain an adult age the condition is transformed into osteoporosis. Weaned rats maintained on low calcium diets for a certain period of time show signs of rickets, but this changes later into osteoporosis without an alteration of diet (*McClendon and Blaustein* 1965). It is, therefore, considered erroneous to relate the bone lesions induced in young growing rats to osteoporosis, as has been done earlier by *Crawford et al.* (1957), *Harrison and Fraser* (1960) and *Fraser* (1962). Moreover, calcium deficiency in young animals interferes with skeletal growth because a normal calcium intake is necessary for the growth of all tissues, including bone, regardless of whether other conditions necessary for calcification are present or not (*Carttar et al.* 1950, *Toothill and Hosking* 1968).

The adult rat is considered to be resistent to the development of osteoporosis by restricted calcium intake because of its effective adaptation to a low calcium level reported in calcium balance studies (*Nicolaysen et al.* 1953). There are, however, no earlier reports confined to studies on the effect of prolonged calcium deficiency on the skeleton of the adult rat, or the pathomechanisms responsible for the skeletal changes induced by such treatment.

Studies with bone-seeking isotopes are inconclusive with regard to the skeletal reaction of experimental animals to prolonged calcium restriction. Thus, a greater retention than normal of a dose of strontium was reported by *Harrison and Fraser* (1960), while *Copp and Suiker* (1962) found a reduced retention of Ca^{45} in young rats after dietary calcium restriction. In calcium-deficient adult rats the data obtained indicated normal Ca^{45} retention and increased bone resorption, as compared with normal controls (*Copp and Suiker* 1962). In this study the bone tissue was not studied further, however.

In adult cats fed a low calcium meat diet, *i.e.* calcium 0.007 and phosphorus 0.18 per cent on a wet weight basis, radiological evidence of osteoporosis was found after 10 weeks (*Jowsey and Gershon-Cohen* 1964). The bone changes induced were described as osteoporosis. However, the appearance of resorption cavities in the cortical bone and the observed increase in number of newly formed osteones together with the described parathyroid hyperplasia, suggest that the observed bone lesion could have been osteomalacia or osteitis fibrosa rather than osteoporosis. This is supported by the fact that young, growing dogs, receiving a low calcium diet with vitamin D supplements, develop erosion and resorption of bone with increase in the amount of fibrous tissue associated with the bones (*Campbell and Douglas* 1965). Similar observations have also been reported in mature dogs maintained on a low calcium, high phosphorus diet. In this study the condition was called "nutritional hyperparathyroidism" (*Saville and Krook* 1968). In a recent study on adult cats fed the low calcium, high phosphorus meat diet described above, an evident but transient osteomalacia was reported at 5 months on the low calcium diet, while after 13 months the condition had changed into osteoporosis. Also in these animals secondary hyperparathyroidism occurred (*Jowsey and Raisz* 1968). These reports indicate that the reaction of the skeleton to prolonged calcium restriction is still poorly understood and that a possible relationship between calcium deficiency and development of osteoporosis in adult mammals needs further investigation.

Treatment with corticosteroids.

Animal experiments have also been used for the study of osteoporosis induced by hormonal disturbances. Thus, osteoporosis has been reported in young rabbits after cortisone treatment (*Sissons and Hadfield* 1955, *Storey* 1957 and 1960). The similarity of these lesions to those observed in Cushing's disease was pointed out by *Sissons* (1956). From histological observations it was deduced that the

effect of cortisone is inhibitory to new bone formation and that the bone rarefaction is due to unopposed continuance of the normal osteolytic processes (*Sissons and Hadfield* 1955, *Sissons* 1956). More recently it has been concluded from morphological studies that the cortisone osteoporosis in rabbits occurs, not only from a cessation of bone formation, but primarily from a direct degrading effect which causes a massive resorption of bone (*deValderrama and Munuera* 1966).

The results reported from studies on the effect of cortisone on the skeleton of young growing rats are not conclusive. Observed dense metaphyseal bone was believed to be due to delayed bone resorption (*Follis* 1951). Later it was reported that osteoporosis occurred only when the cortisone-treated young rats were fed a diet containing a normal or lowered amount of calcium and phosphorus (*Storey* 1960). Osteoporosis has also been reported in growing male rats after prolonged cortisone therapy (*Kahn and Skoryna* 1959).

In growing male rats, osteoporosis has been claimed to result from a combination of bilateral adrenalectomy, orchidectomy and cortisone administration (*Caldwell* 1962). Administration of oestradiol cured the osteoporotic changes in these animals despite continued treatment with cortisone, while administration of testosterone resulted in only very slight improvement. These results suggest that this radiographically observed osteoporosis might have been caused by an excess of corticosteroids in the absence of oestradiol. However, since young animals were used, the interference of the treatment with normal skeletal growth and development makes it difficult to interpret the results obtained.

In adult cortisone-treated rats, the collagen content of the whole femur has been reported to be unchanged, while the hexosamine content was decreased. This led to the suggestion that a decrease of mucopolysaccharides precedes the events which give rise to osteoporosis in Cushing's disease (*Sobel and Marmorston* 1954). Although changes in the hexosamine content of the whole femur must reasonably be considered as non-representative of the mucopolysaccharides of the bone tissue itself, this observation provides considerable interest, but needs further investigation.

The well known effect of corticosteroids upon the calcium metabolism must, further, be taken into consideration in studies concerning the pathogenesis of cortisone-osteoporosis (*Heaney* 1965). The skeletal retention of bone-seeking isotopes in young growing rats has been reported to be inhibited by the administration of corticosteroids (*Clark et al.* 1959, *Milhaud et al.* 1960, *Bohr and Dawids* 1964). Calcium excretion has consistently been found to be increased. The results reported concerning the effect on intestinal calcium absorption are

not quite conclusive, however. The administration of hydrocortisone to young rats has been considered to inhibit the uptake by bone of Ca^{45}, which was also found to be decreased in nephrectomized rats; this led to the suggestion that the limiting factor may lie in the synthesis of chondroitin sulphate (*Clark et al* 1959). No reports have been found regarding the effect of combined oophorectomy and prednisolone administration on mature bone tissue.

In the majority of the experimental studies reviewed, young growing animals have been used and an interference between the well known growth inhibitory effect of corticosteroids and the skeletal reaction to the treatment cannot, therefore, be eliminated. The effect of such treatment upon the adult skeleton in which calcification and growth are completed awaits further investigation, especially with regard to the development of osteoporosis.

SUMMARIZING REMARKS

1. Although osteoporosis is a condition with a clear and generally accepted definition, there are reports indicating that this definition can no longer be considered adequate. Thus, cellular differences between osteoporotic and normal bone have been observed by morphological and *in vitro* metabolic studies. Changes in the bone minerals have been reported from macro- and microchemical studies. Finally, changes in organic bone matrix have been found by physical and biochemical methods.

2. In the numerous attempts made to elucidate the aetiological factors connected with the pathogenesis of osteoporosis, nutritional and hormonal disturbances have been studied and considered to cause the condition. The opinions expressed in this respect are controversial.

3. During the normal aging of the adult skeleton there is a slow, steady loss of bone, probably due to a slight increase in bone resorption.

4. The opinion has been held that this steady loss of bone during normal aging is the prime aetiological factor for the pathogenesis of osteoporosis.

5. Another opinion is that clinical osteoporosis is caused by certain aetiological factors responsible for an acceleration of the normal rate of bone loss for a limited period of time.

6. Although these additional agents are still unknown, nutritional and hormonal disturbances must be taken into consideration. The effects on the adult skeleton of prolonged calcium restriction and an imbalance between gonadal and adrenal corticoid hormones need further investigation.

7. In the pathogenesis of osteoporosis, the concept of deficient new bone formation and osteoblastic failure has been abandoned. Instead, increased bone resorption is considered to be the predominant feature in most of the osteoporoses.

8. In a unified concept of osteoporosis, disturbed calcium homeostasis is considered to be the main pathophysiological mechanism responsible for induction of different osteoporoses. This theory needs further proof, however.

9. In numerous experiments, different bone lesions have been reported by maintaining animals on low calcium diets for certain periods of time. The reaction of the adult skeleton to prolonged calcium restriction is, however, still poorly understood.

10. In rabbits, osteoporosis can be produced by cortisone treatment. In growing rats, it occurs only with normal or low intakes of calcium and phosphorus during cortisone treatment. In growing animals the inhibitory effect of corticosteroids on growth interferes with the skeletal reaction to the treatment. This interference must be taken into consideration, especially when its relation to the development of osteoporosis is concerned.

11. In adult rats, there are no reports of osteoporosis produced by corticosteroids. While no effect has been found on the hydroxyproline content of the whole bone, the suggested decrease in the concentration of mucopolysaccarides has been related to the development of osteoporosis. This needs to be investigated further.

12. In growing male rats, roentgenological osteoporosis develops after combined bilateral adrenalectomy, orchidectomy and cortisone administration. Contrary to the experience in clinical osteoporosis, the skeletal lesions induced by this treatment could be cured by oestradiol. Further studies regarding the reaction of the adult skeletal tissue to hormonal imbalance, *e.g.* excess corticosteroids and lack of gonadal hormones, would seem justified, therefore.

13. Studies on the effect of these nutritional and hormonal disturbances upon calcium homeostasis in the adult animal appear to be of interest in the pathogenesis of osteoporosis.

THE PRESENT INVESTIGATION

Concerning the pathogenesis of human osteoporosis, prolonged dietary calcium deficiency and imbalance between gonadal and adrenal corticoid hormones have received the greatest interest. The lack of coherent information on the effect of these disturbances upon the adult skeletal tissue with regard to the development of the condition, motivated the present investigation. Most previous experimental studies have been performed on young animals, whereas osteoporosis is characteristically a disorder of old age. While in clinical osteoporosis most of our present knowledge concerns the different features of the osteoporotic skeleton and not the development of the condition, in interpreting most earlier studies of experimental osteoporosis the interference of the induced disturbances with normal skeletal growth and development must be considered.

CHOICE OF EXPERIMENTAL ANIMAL

The adult laboratory rat was used in the present investigation mainly because in this species the minimal calcium need for equilibrium is well defined for different ages. Furthermore, in this species the adaptation to restricted calcium intake has been extensively studied by calcium balance techniques. In principle, the results of these studies agree with the observations made in humans (*Nicolaysen et al.* 1953). The laboratory rat is, further, less liable to uncontrolled variation than man. While in the rat the relation of different, induced disturbances to the development of osteoporosis can be studied under well defined experimental conditions, in man such possibilities are either restricted or non-existent. The fact that complete closure of some of the epiphyseal zones does not occur in the rat, does not necessarily mean that there is continued, active skeletal growth in the adult animal. Thus, it has been reported that in the adult rat the epiphyseal cartilages are inactive (*El-Maraghi et al.* 1965). During the first 6 months of life calcification of bone is rapid and remains

steady thereafter (*Henry and Kon* 1953). Moreover, the uptake of radioactive calcium in bones of rats diminishes rapidly from the ages of 3 to 6 months and then decreases only little with age, in accordance with measurements of growth rate determined by repeated labelling with tetracycline (*Bohr* 1968). Only rats stated to be 11½ to 12½ months old at the start of the experiment were used in the present investigation, except for the animals of one special control group, where older rats were used. To ensure that the skeleton was fully mineralized at the start of the experiment, all rats had been bred on an optimum intake of calcium and phosphorus, *i.e.* 1.4 per cent Ca and 1.2 per cent P, and adequate amounts of vitamin D.

PURPOSE OF THE INVESTIGATION

The aim of the present investigation was to determine in adult rats whether or not disturbances induced by a) prolonged calcium restriction and b) oophorectomy combined with prolonged prednisolone administration, resulted in changes of the mature bone tissue and calcium metabolism which could be related to the development of osteoporosis according to the definition of *Albright and Reifenstein* (1948). Further, the studies were planned with the purpose of following the sequence of changes in various parameters which could be related to the development of the disorder.

The results reported herewith will concern the following specific relationships:

I. The effect of prolonged calcium restriction on the skeletal tissue of adult male rats.

II. The effect of prolonged calcium restriction on the blood calcium level and the distribution of Ca^{45} between blood and bone in adult male rats.

III. The effect of combined oophorectomy and prolonged prednisolone administration on the skeletal tissue of adult rats.

IV. The effect of combined oophorectomy and prolonged prednisolone administration on the blood calcium level and the distribution of Ca^{45} between blood and bone in adult rats.

V. The glycosaminoglycans of compact bone tissue in experimental osteoporosis induced by prednisolone treatment of oophorectomized rats or by calcium restriction.

References, see p. 191.

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Studies on the Development of Experimental Osteoporosis

I. The Effect of Prolonged Calcium Restriction on the Skeletal Tissue of Adult Male Rats.

by

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INTRODUCTION

The onset of generalized osteoporosis in humans has been related to a low intake or malabsorption of calcium (*Nordin* 1960, 1961, 1962, *Nordin et al.* 1964, *Spencer et al.* 1964). It has also been stated that in the generalized osteoporoses, the bone mass is reduced because of "the calcium needs of the organism" (*Heaney* 1965). In human volunteers it has been shown by *Malm* (1958) that a negative calcium balance can be induced by changing from a normal to a low calcium intake. In the great majority of the subjects, adaptation took place and the balance became less negative. In a few subjects, however, there was a failure to adapt to the low calcium intake even after long periods of observation. In these subjects, the persisting negative calcium balance eventually resulted in osteoporosis.

The relationship between calcium retention and body stores of calcium has been studied extensively in the rat. In young, growing animals a previous low intake of calcium increases the retention of calcium in a subsequent period on a high calcium diet (*Fairbanks and Mitchell* 1936, *Rottensten* 1938, *Nicolaysen* 1943, *Henry and Kon* 1947 and 1953). In old rats also, the absorption of calcium has been found to be definitely increased after prolonged calcium restriction (*Henry and Kon* 1953, *Nicolaysen et al.* 1953). However, experiments by *Henry et al.* (1960) indicated that old rats fed a low calcium diet need a minimum of 4 months to adapt themselves to the low calcium intake and that this period of adaptation is longer for old than for young rats. In old rats, a slow reduction of the absorption efficiency was observed by *Kane et al.* (1949), while no change in the calcium metabolism with age was indicated by the findings of *Henry and Kon* (1953) and *Nicolaysen* (1956). *Hansard and Crowder* (1957) found that the true digestibility of dietary calcium by rats declined from 41 % at 48 to 72 weeks of age to 24 % at 106 weeks, while the requirement for maintenance approximately doubled. It has also been reported that aged rats (22 to 32 months compared to 10 to 12 months) are subject to a greater loss of

calcium from the body but that they are able to maintain calcium equilibrium by increasing the uptake of dietary calcium from the intestine (*Hironaka et al.* 1960).

Despite numerous studies on the adaptation by the rat to a restricted calcium intake, the reaction of the skeleton to long-term calcium deficiency is still poorly understood. Young growing rats have been reported to develop osteoporosis on a diet deficient in calcium but adequate in vitamin D (*Crawford et al.* 1957, *Harrison and Fraser* 1960). However, it has also been reported that growing rats fed a similar diet deficient only in calcium develop skeletal changes typical of rickets (*Park et al.* 1922, *Hess* 1929, *Engfeldt et al.* 1962). The experiments of *Gershon-Cohen et al.* (1962) suggest that a low calcium diet results in rachitic lesions in the young growing rat but that in adults the condition is transformed into osteoporosis. A weaned rat placed on a low calcium diet shows signs of rickets for a short time only. The rickets changes thereafter into osteoporosis without a change of diet (*McClendon and Blaustein* 1965).

The mature rat is generally considered to be resistent to the development of osteoporosis on receiving a low calcium diet because of its effective adaptation to a low calcium intake. It has been stated that a marked adaptation, viz. a distinctly increased efficiency of calcium absorption which compensates fully for any previous loss, will occur only when the old rats have suffered a substantial loss of calcium from the skeleton. This delayed reaction might be expressed as a considerable period of latency. In principle, this finding agrees with observations in man (*Nicolaysen et al.* 1953). However, there appears to have been no previous investigation confined to a study of the effect of prolonged calcium deficiency on the skeleton of the adult rat and the pathomechanisms responsible for the skeletal changes induced by such treatment. It was considered that such an investigation would provide valuable information concerning the relationship between calcium deficiency and the development of osteoporosis, since the mechanisms involved in the adaptation to a restricted calcium intake seem to be similar for rats and humans.

The present investigation was undertaken with the aim of studying the response of the mature skeletal tissue to prolonged calcium restriction and of following the development of possible skeletal changes during different periods of maintained low calcium intake. This report concerns the composition and the histology of the skeletal tissue in one year old male rats maintained on a low calcium diet for periods of time varying from 1 week up to 1 year.

EXPERIMENTAL

A total number of 95 adult male rats of the Sprague-Dawley strain[1] were divided into two experimental series. Sixty animals comprised a short-term and thirty-five animals a long-term series. Apart from the duration of the test diet, both series were treated identically. All animals, except the controls of the long-term series (see page 21), were stated to 12 to 12 1/2 months old at the start of the experiment. The mean initial body weights of the animals of the two series were 499.0 ± 5.2 and 501.8 ± 9.2 grams, respectively. All animals had been bred on a standard laboratory ration containing, on a dry weight basis, 1.4 per cent Ca and 1.2 per cent P and adequate amounts of vitamin D.

Experimental animals.

All the experimental animals were fed the low calcium diet of General Biochemical Inc., Chagrin Falls, Ohio. This diet has the following composition (GBI Bulletin D-15):

Ingredients	*Composition*
Whole Dried Egg (ether extracted)	27.0 %
Sucrose	10.0
Lard	10.0
Dried Yeast	7.0
Cod Liver Oil[2]	2.0
Starch	40.0
Salt mix	4.0
Salt mix	*Gms/100 lbs.*
Ferric citrate	29.462
Magnesium chloride	855.122
Manganous sulfate	.414
Potassium alum	.190
Potassium chloride	355.491
Potassium citrate	371.976
Potassium iodide	.085
Potassium sulfate	37.620
Sodium chloride	164.430
Sodium fluoride	1.048

[1] Anticimex Co., Stockholm.

[2] Vitamin D_3 120-600 I.U./100 g diet, which was protected against daylight.

Chemical analyses showed that the diet contained 0.09 per cent Ca and 0.55 per cent P[1]. The Ca:P ratio was 1:6.1. The diet was supplied with an adequate amount of vitamin D. All animals were given their diet and deionized water *ab libitum*. The daily calcium intake was estimated at 18-20 mgs per animal. Groups of 5 rats each were kept on this regimen for different periods of time; in the short-term series ten groups for 1 to 16 weeks, and in the long-term series six groups for 6 to 12 months. In the short-term series, another group of 5 animals was fed the above described test diet supplemented with 4 % $CaCO_3$, this diet thus containing a total amount of 1.7 per cent Ca and 0.55 per cent P. The Ca:P ratio was 1:0.4. This diet and tap water were given *ab libitum* for 16 weeks and the daily calcium intake of these animals was estimated at 340—360 mgs.

Control animals.

In the short-term series, one group of 5 rats received a normal laboratory ration[2] containing 1.0 per cent Ca and 0.75 per cent P for 16 weeks. The Ca:P ratio was 1:0.75. Diet and tap water were given *ab libitum*. The daily calcium intake of the rats belonging to this group was estimated at 200—220 mgs. In the long-term series, one control group of 5 animals was fed the normal laboratory ration and tap water for 4 weeks in order to accustom the animals to the laboratory routine. These animals were stated to be approximately 20 months old when delivered to the laboratory.

Without access to direct sunlight, all the animals were housed five to each cage at a temperature of 18—20°C. The cages were large enough to allow the animals to move about freely. No coprophagy was observed. The series were planned so that all the animals within each series were sacrificed at the same time. This was performed by bleeding them to death from the femoral artery under ether anaesthesia. After sacrifice the animal bodies were kept in the deep freezer at – 20°C pending the subsequent preparations.

Body weight.

In all animals the initial and the final body weight was recorded. In addition, all the animals of the short-term series were weighed weekly.

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[1] Here and in the following text the figures calculated on a dry weight basis are given.

[2] Ewos Co., Södertälje, Sweden.

Radiographs.

At the start of the experiment each animal was examined radiologically under ether anaesthesia and at the end of the experiment this examination was repeated. Each rat was placed with the spinal column and the extremities fixed directly on an X-ray film and the same exposure conditions, film and developer were used throughout.

Measurement of the cortical thickness in the mid-shaft of the femur.

This examination was performed on all animals of the long-term series. The whole femur was dissected free from soft tissues and periosteum and its length from the top of the greater trochanter to the most distal point of the femoral condyles was measured with a caliper. A fine bandsaw was then used to divide the bone in the middle, ± 0.5 mm, of the diaphysis, perpendicularly to the long axis of the diaphysis. With the aid of a caliper the internal and external transverse diameters were measured at the four cut surfaces obtained from the two divided femora of each animal. The cortical thickness at this point was calculated as the difference between the external and internal diameters and with an accuracy of 0.05 mm. Average values were used. This figure was divided by the mean value for the external transverse diameter of the shaft at the same level and this fraction was multiplied by 100. In this way the "femur score" was calculated according to *Barnett and Nordin* (1960).

Ash determinations.

The ash content of the whole tibia, including bone marrow and articular cartilages, was determined in both experimental series as follows: After careful dissection from soft tissues and periosteum, the bone was placed in a weighing container and the wet weight was immediately recorded in a *Mettler* balance with an accuracy of 0.05 mg. All bones within each series were thereupon ashed simultaneously in a muffle furnace at 700°C for 16 hours and the weight of the ashes was then recorded. The ash content was expressed as per cent of the wet weight. In the short-term series, the ash contents of both tibiae were determined and average values recorded. As no statistically significant difference between the ash contents of the right and the left tibiae was found, only the right tibia was used in the long-term series.

Hydroxyproline determinations.

The hydroxyproline content of the whole third metacarpal bone, including bone marrow and articular cartilages, was determined in both series by the method of *Neuman and Logan* (1950). The bone was carefully freed of soft tissues and periosteum. All bones within each series were dried simultaneously at 48° C for 24 hours and were then weighed in weighing containers. Protein hydrolysates were prepared by autoclaving the whole bone with 0.5 ml of 6 N hydrochloric acid in sealed tubes for 10 hours at 100° C. A hydrolysis curve showed maximum hydroxyproline values after 6 hours and there was no decrease even after 21 hours' hydrolysis. Duplicate determinations were performed throughout and average values were used. The coefficient of variation for these analyses was 2.0 per cent.

The gross bone density of the humerus.

The density of the whole bone, including bone marrow and articular cartilage, was determined according to *Robinson and Elliott* (1957). The right humerus of the animals of the two series was carefully dissected free from surrounding soft tissues and periosteum. All bones within each series were soaked simultaneously in deionized water at 4° C for 16 hours. Each bone was then suspended on a fine wire attached to the condyles and weighed while submerged in water at room temperature. This was the weight of the hydrated whole bone submerged in water. All bones of each series were then dried simultaneously at 105° C for 16 hours and, using weighing containers, the air-dried weight was recorded. The density of the dried whole bone was calculated by dividing the dry weight of the bone by the weight of water displaced by the dry bone volume.

Preparation of undecalcified sections.

The two proximal tail vertebrae of each animal of the long-term series and one metacarpal bone from some of the animals of the short-term series were freed of most of the surrounding soft tissues and then fixed and dehydrated in 6 changes of 96 per cent alcohol for 6 days. The specimens were then placed in unpolymerized washed methyl methacrylate with the addition of a catalyst, prepared as described by *Jowsey et al.* (1965). The specimens were embedded in glass bottles with semipolymerized monomer for one week and polymerization was then completed in an oven maintained at 37° C. The glass bottle was then

broken and the excess methacrylate was trimmed off with a bandsaw. For the subsequent examinations of the vertebrae, the plastic blocks were sawn into slices approximately 0.5 mm thick and exactly perpendicular to the long axis of the vertebra, while the metacarpal bones were ground directly as described below.

Tetracycline labelling.

All animals of the long-term series were "labelled" with chlortetracycline (Aureomycin®, Lederle) in two doses of 20 mg per kg body weight administered by intraperitoneal injection 10 and 20 days before sacrifice. Approximately 60 μ thick undecalcified methacrylate-embedded cross sections from the second tail vertebra were mounted under coverslips in fluorescence-free balsam (Permount) and examined in a Zeiss fluorescence microscope fitted with an UV light source (High Pressure Vacuum lamp HBO 200, exciter filters UG 2 and UG 5, barrier filters 65 and 41).

Quantitation of the amount of vertebral bone.

Quantitation of the amount of bone present in a standard area was performed in the long-term series as follows: Undecalcified sections embedded in methacrylate from the first tail vertebra were ground, first on an oscillating grinder, then by hand, to a thickness of approximately 50 μ. Only sections containing most of the transverse processes were studied; these were examined unstained with the aid of an integrating eye-piece (Zeiss I) as described by *Wagner* (1965). The number of vertebral bone "hits" of 200 points was determined with the point network exactly in the centre of the section at a linear magnification of 30 x; the accuracy was found to be more than 95 per cent.

Microradiographs.

Microradiographs were prepared from the animals of the long-term series as follows: Undecalcified cross sections embedded in methacrylate from the second tail vertebra were ground on a Knuth-Rotor grinder to a thickness of approximately 60 μ, Silicon-Carbide papers, Nos. 220 and 600, and water as a lubricant, being used. The sections were then placed in direct contact with a fine-grain emulsion (Kodak MR-plates) and were radiographed in a Philips

PW 1009 apparatus provided with a copper anode and a nickel window, the X-ray tube operating with 20 kV and 20 mA. After an exposure time of 15 minutes the plates were developed for five minutes in Kodak D 158, washed and fixed in a conventional acid fixative. Longitudinal sections of metacarpal bones in the short-term series were ground first on an oscillating grinder, then by hand, and microradiographs were prepared as described above.

Preparation of decalcified sections.

The fourth metacarpal bone of all the rats of the short-term series and the third tail vertebra of those of the long-term series were fixed in 10 per cent neutral formaldehyde for 24 hours and then decalcified for 12-14 days in a mixture of equal parts of monosodium citrate (20 %) and formic acid (44 %). The specimens were then immersed in 5 per cent disodium sulphate for twenty-four hours, washed with water for twenty-four hours and dehydrated by successively immersing in 70, 95 per cent and absolute alcohol (2 changes). Finally, the specimens were immersed first in methyl benzoate and then in xylol, and embedded in paraffin. Five μ thick longitudinal sections of the metacarpal bones and cross sections of the vertebrae were cut and stained with Ehrlich's haematoxylin and eosin and, in addition, with azocarmine G. Sections from the vertebrae were also stained with an 0.5 per cent solution of toluidine blue in phthalate at pH 4.4 as described by *Bélanger and Hartnett* (1960).

Statistical treatment of data.

For the computation, an analysis of variance with one-way lay out according to *Scheffé* (1961) was used. The hypothesis of no difference between groups was tested with a 5 per cent significance level. Significant contrasts between groups were tested at the same significance level according to *Scheffé* (1961). For computation of the data obtained from the one group fed the test diet enriched with calcium, Student's t-test was used according to *Brownlee* (1965) and with a significance level of 5 per cent[1].

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[1] In the following text the term "significant" will stand for "statistically significant".

RESULTS

Six deaths occurred among the whole material, five among the experimental animals in the long-term series and one in the short-term series, all probably due to advanced age. These animals were excluded. The remaining animals were in good health.

Body weights (Fig. 1, Table 1 a, b).

The rats fed the normal laboratory ration increased their body weight by almost 10 per cent during the first 7 weeks of observation. From then on and until the 16th week, the body weight remained unchanged. In the animals fed the low calcium diet the body weight increased by almost 30 per cent during the first 7 weeks. Subsequently, and up to the end of the 16th week, no significant change was noted. The animals fed the low calcium test diet supplemented with

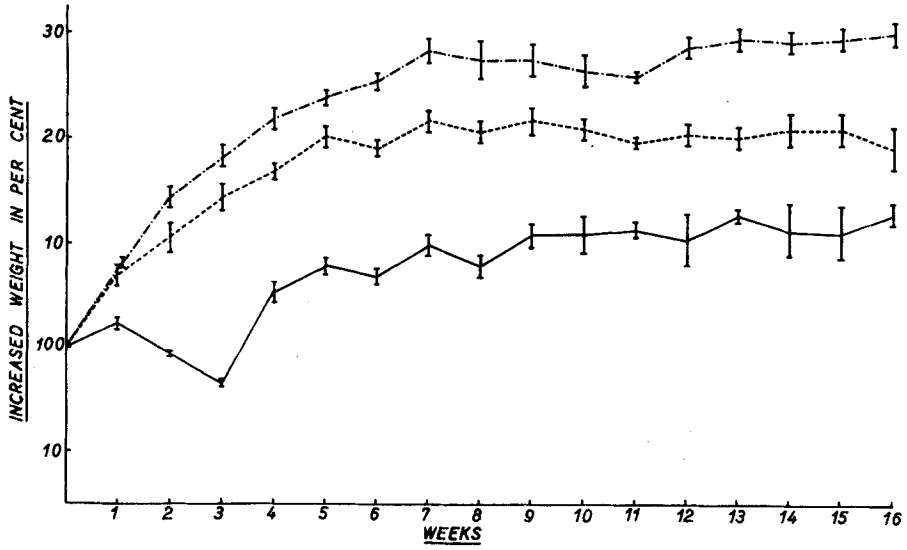


Fig. 1. Body weight change in per cent of the initial weight in the three groups of rats fed the normal laboratory ration (uninterrupted line), the low calcium diet (broken-dotted line) and the calcium-enriched diet (broken line) for 16 weeks. The bars represent the standard error of the mean.

Groups	Initial body weight, grams	S.E.M.	Final body weight, grams	S.E.M.	Difference in % of the initial body weight	S.E.M.
Normal laboratory ration for 16 weeks	454.0	7.6	502.0	16.9	13.5	1.0
„ „ „ long-term series	453.0	13.5	466.3	13.0	3.0	0.4
Low calcium diet for 1 week	503.4	11.8	521.4	12.6	3.6	0.1
„ „ „ „ 2 weeks	510.2	9.2	536.2	10.5	5.1	0.5
„ „ „ „ 3 „	470.3	5.1	510.9	12.6	8.6	1.8
„ „ „ „ 4 „	482.3	4.6	522.6	10.2	8.5	1.3
„ „ „ „ 5 „	532.4	9.3	562.4	11.6	5.6	0.8
„ „ „ „ 8 „	504.7	6.6	544.1	8.5	7.8	1.6
„ „ „ „ 10 „	529.6	11.8	578.6	9.3	9.4	1.6
„ „ „ „ 12 „	529.8	18.8	608.1	23.7	14.8	0.9
„ „ „ „ 14 „	476.7	13.6	595.3	10.8	25.1	2.5
„ „ „ „ 16 „	469.0	14.1	610.5	15.4	30.1	0.9
„ „ „ „ 6 months	501.3	11.5	510.0	13.0	1.8	1.6
„ „ „ „ 7 „	577.9	8.1	567.2	21.0	- 1.4	2.5
„ „ „ „ 8 „	534.4	7.6	550.5	22.4	3.8	3.2
„ „ „ „ 9 „	522.6	32.6	525.9	24.1	1.8	6.7
„ „ „ „ 10 „	452.8	9.3	540.4	27.6	20.7	3.7
„ „ „ „ 12 „	484.7	18.7	562.2	51.5	15.3	7.1
Calcium-enriched diet for 16 weeks	482.6	13.4	576.9	22.6	19.2	2.0

Table 1 a. The initial and final body weights of the rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.
(S.E.M. = standard error of the mean).

calcium showed an increase in their initial body weight by approximately 20 per cent during the first 5 weeks; from that time onwards and until the end of the 16th week it remained unchanged (Fig. 1). All rats given the test diet showed an appreciable increase in their subcutaneous and peritoneal fat tissue. This was more pronounced in the animals fed the low calcium diet than in those for which this diet was enriched with calcium. The final body weight showed no significant difference in the groups of rats fed the low calcium diet for 5 weeks up to 1 year (Table 1 a, b).

Weeks	Normal laboratory ration Body weight change %	S.E.M.	Low calcium diet Body weight change %	S.E.M.	Calcium-enriched diet Body weight change %	S.E.M.
1	2.5	0.63	8.4	0.64	7.1	1.13
2	– 1.3	0.32	14.4	1.11	10.7	1.40
3	– 3.4	0.67	18.6	1.13	14.5	1.36
4	5.5	1.11	21.9	0.99	17.2	0.88
5	7.9	0.80	24.2	0.69	20.2	0.97
6	7.0	0.70	25.6	0.83	19.2	0.75
7	9.9	1.18	28.4	1.31	21.7	1.08
8	7.7	0.99	27.6	1.75	20.6	1.04
9	10.7	1.74	27.7	1.49	21.7	1.36
10	11.0	1.85	26.6	1.63	21.1	0.94
11	11.4	0.71	26.1	0.60	19.8	0.61
12	10.5	2.72	28.7	1.13	20.4	1.01
13	13.0	0.64	29.4	1.04	20.3	0.96
14	11.6	2.77	29.2	1.04	21.0	1.50
15	11.3	2.83	29.5	0.94	20.8	1.48
16	13.4	0.97	30.1	0.92	19.2	2.00
	$F = 9.91$ sign.		$F = 29.54$ sign.		$F = 12.12$ sign.	
	$F_{0,95}$ (15.61) = 1.85		$F_{0,95}$ (15.64) = 1.84			

Table 1 b. Body weight change in per cent of the initial weight in the three groups of rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet, for 16 weeks.

Femur length.

The femur length in the long-term series showed no significant difference between the control group and the groups of animals fed the low calcium diet.

Radiographs.

Radiological examination revealed that the epiphyseal zones of the long bones were closed in all animals. In some animals, distributed at random among the whole material, the remnants of the epiphyseal zones were recognized as a sclerotic line. In none of the animals maintained on a low calcium diet for 1 to 16 weeks was any notable change observed on radiographs taken at the start and at the end of the experimental period. On the other hand, all animals fed the low calcium diet for 6 months and longer showed a slight but definite reduction of the cortical thickness of the tubular bones and the pelvis and decreased density of the trabecular pattern in the metaphyses and in the tail vertebrae.

The cortical thickness in the mid-shaft of the femur (Table 2).

Direct measurement of the cortical thickness was performed in the long-term series and revealed significantly lower values for the "femur score" in all the six groups of rats fed the low calcium diet for 6 to 12 months compared with the control group. The lower values for the cortical thickness corresponded to higher values for the internal transverse diameter, while the external diameter showed no significant difference.

The ash content of the whole tibia (Fig. 2, Table 3).

The ash contents of the tibiae of the rats fed the low calcium diet for 1 to 12 weeks showed no significant difference from those of the rats fed the normal laboratory ration. Significantly lower values, decreasing with time, were obtained in the eight groups of rats kept on a low calcium intake for 14 weeks to one year. The decrease amounted to 6.2 % after 16 weeks of calcium restriction and 13.3 % after 12 months. The group of rats fed the diet enriched with calcium for 16 weeks showed slightly higher values than those of the group fed the normal laboratory ration for 16 weeks. This difference was not significant,

Groups	No. of animals	External diameter, mm	S.E.M.	Internal diameter, mm	S.E.M.	Femur score	S.E.M.
Normal laboratory ration	5	5.10	0.03	2.69	0.02	47.7	0.29
Low calcium diet for 6 months	4	5.11	0.07	3.29	0.19	35.7	3.51
„ „ „ „ 7 „	4	5.41	0.02	4.01	0.10	25.9	2.11
„ „ „ „ 8 „	3	5.13	0.09	3.40	0.16	33.7	3.57
„ „ „ „ 9 „	5	5.21	0.10	3.66	0.22	30.0	3.06
„ „ „ „ 10 „	5	5.20	0.09	3.59	0.17	30.8	3.51
„ „ „ „ 12 „	4	5.06	0.13	3.63	0.15	28.3	3.48
		F = 1.81 n.s.		F = 7.04 sign.		F = 6.54 sign.	
				$F_{0.95}$ (6.23) = 2.58			

Table 2. The external and internal transverse diameter at the mid-shaft of the femur and the "femur score" in rats fed the normal laboratory ration and the low calcium diet.

however. Similar values were obtained for the control group of the short-term and the long-term series.

The hydroxyproline content of the whole metacarpal bone (Fig. 3, Table 4).

The hydroxyproline content of the whole metacarpal bone of the rats fed the low calcium diet for 1 to 10 weeks, showed no significant differences compared with those of the group fed the normal laboratory ration. Significantly lower

Groups	No. of animals	% ash	S.E.M.	
Normal laboratory ration	10	48.2	0.31	
Low calcium diet for 1 week	4	48.0	0.48	
„ „ „ „ 2 weeks	5	47.7	0.25	
„ „ „ „ 3 „	5	47.4	0.46	
„ „ „ „ 4 „	5	45.5	0.52	
„ „ „ „ 5 „	5	47.1	1.00	
„ „ „ „ 8 „	5	46.6	0.68	
„ „ „ „ 10 „	5	47.8	0.20	
„ „ „ „ 12 „	5	47.6	0.42	
„ „ „ „ 14 „	5	45.3	0.28	sign.
„ „ „ „ 16 „	5	45.2	0.43	
„ „ „ „ 6 months	4	44.9	0.96	
„ „ „ „ 7 „	4	44.5	0.17	
„ „ „ „ 8 „	3	44.4	0.61	
„ „ „ „ 9 „	5	43.6	0.82	
„ „ „ „ 10 „	5	43.3	1.08	
„ „ „ „ 12 „	4	41.8	1.80	
		$F = 7.89$ sign.		
		$F_{0.95}\ (16.67) = 1.82$		
Calcium-enriched diet for 16 weeks	5	48.8	0.45	n.s.

Table 3. The percentage ash content of the whole tibia in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

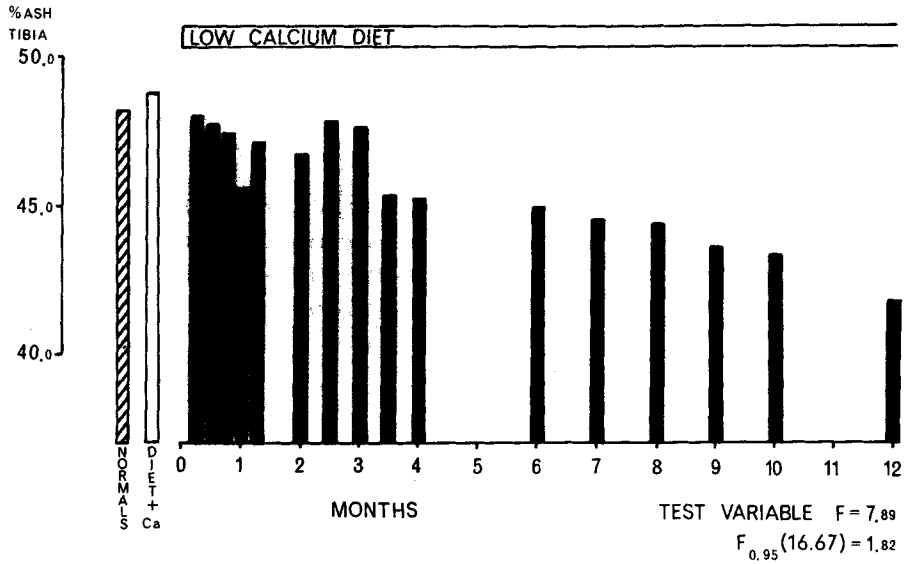


Fig. 2. The percentage ash content of the tibia in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

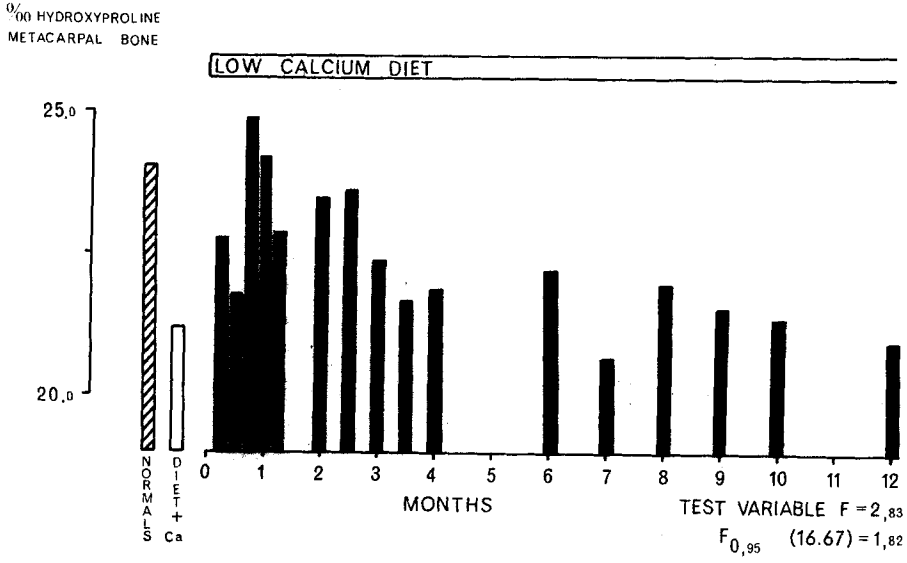


Fig. 3. The promille hydroxyproline content of the metacarpal bone in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

values were obtained in the nine groups of rats fed the low calcium diet for 12 weeks to 12 months than in the normal controls. The group of rats fed the calcium-enriched diet for 16 weeks showed slightly lower values than the group fed the normal laboratory ration. This difference was significant. Comparison between the two control groups showed no significant difference.

Groups	No. of animals	‰ hydroxy-proline	S.E.M.	
Normal laboratory ration	10	24.1	0.39	
Low calcium diet for 1 week	4	22.8	0.71	
„ „ „ „ 2 weeks	5	21.8	0.43	
„ „ „ „ 3 „	5	24.9	1.11	
„ „ „ „ 4 „	5	24.2	1.06	
„ „ „ „ 5 „	5	22.9	0.64	
„ „ „ „ 8 „	5	23.5	0.93	
„ „ „ „ 10 „	5	23.7	0.81	
„ „ „ „ 12 „	5	22.4	0.72	
„ „ „ „ 14 „	5	21.7	0.66	
„ „ „ „ 16 „	5	21.9	0.84	
„ „ „ „ 6 months	4	22.3	0.52	
„ „ „ „ 7 „	4	20.7	0.56	sign.
„ „ „ „ 8 „	3	22.0	0.75	
„ „ „ „ 9 „	5	21.6	0.76	
„ „ „ „ 10 „	5	21.4	0.51	
„ „ „ „ 12 „	4	21.0	0.69	
		$F = 2.83$ sign.		
		$F_{0.95}\ (16.67) = 1.82$		
Calcium-enriched diet for 16 weeks	5	21.2	0.86	sign.

Table 4. The promille hydroxyproline content of the metacarpal bone in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

The gross bone density of the humerus (Fig. 4, Table 5).

The values obtained from the rats fed the low calcium diet for 1 to 16 weeks showed no consistent changes with time during the period of restricted calcium intake, but only a small intergroup variation, and the statistical analysis revealed no significant differences. The six groups of rats kept on a restricted calcium intake for 6 to 12 months showed significantly lower values than the normal controls. The group of rats fed the calcium-enriched diet for 16 weeks showed slightly higher values than the groups fed the normal laboratory ration. This difference was significant. Similar values were obtained for the two control groups.

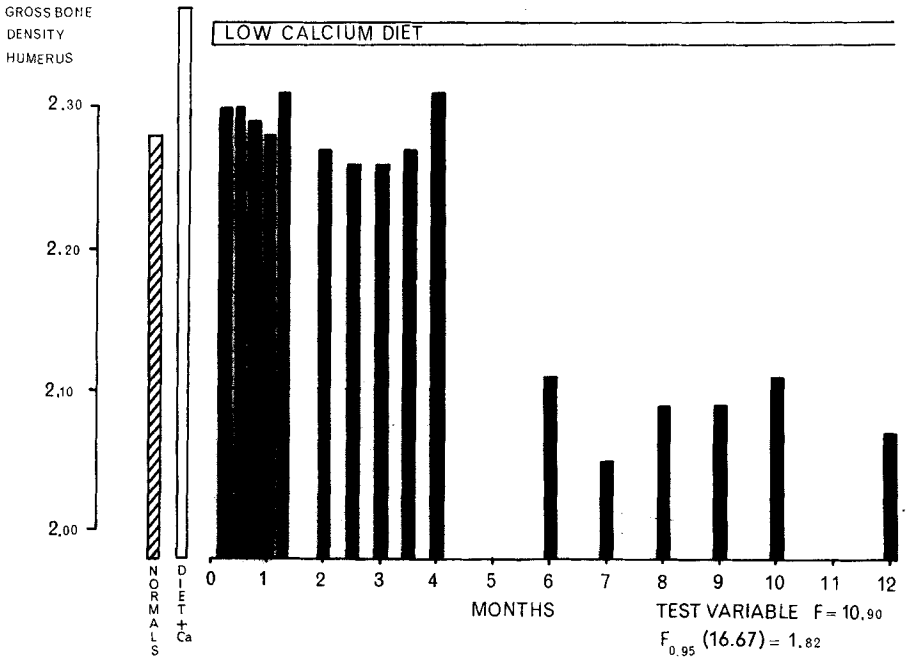


Fig. 4. The gross bone density of the humerus in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

Groups	No. of animals	Density	S.E.M.	
Normal laboratory ration	10	2.28	0.016	
Low calcium diet for 1 week	4	2.30	0.023	
„ „ „ „ 2 weeks	5	2.30	0.032	
„ „ „ „ 3 „	5	2.29	0.039	
„ „ „ „ 4 „	5	2.28	0.025	
„ „ „ „ 5 „	5	2.31	0.023	
„ „ „ „ 8 „	5	2.27	0.022	
„ „ „ „ 10 „	5	2.26	0.016	
„ „ „ „ 12 „	5	2.26	0.017	
„ „ „ „ 14 „	5	2.27	0.005	
„ „ „ „ 16 „	5	2.31	0.015	
„ „ „ „ 6 months	4	2.11	0.013	sign.
„ „ „ „ 7 „	4	2.05	0.048	
„ „ „ „ 8 „	3	2.09	0.038	
„ „ „ „ 9 „	5	2.09	0.039	
„ „ „ „ 10 „	5	2.11	0.046	
„ „ „ „ 12 „	4	2.07	0.049	
		$F = 10.90$ sign.		
		$F_{0.95}\ (16.67) = 1.82$		
Calcium-enriched diet for 16 weeks	5	2.37	0.014	sign.

Table 5. The gross bone density of the humerus in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

The percentage of vertebral bone "hits" (Table 6).

This was examined in the long-term series. The six groups of animals maintained on a low calcium diet for 6 to 12 months showed significantly lower values than the control group.

Microradiography.

Microradiographs of undecalcified vertebral cross sections showed that in the normal control rats the cortical and trabecular bone was quite evenly mineralized

Groups	No. of animals	% hits	S.E.M.	
Normal laboratory ration	5	46.9	1.68	
Low calcium diet for 6 months	4	36.2	1.92	sign.
„ „ „ „ 7 „	4	32.5	1.32	
„ „ „ „ 8 „	3	35.2	0.44	
„ „ „ „ 9 „	5	36.7	2.80	
„ „ „ „ 10 „	5	37.7	0.88	
„ „ „ „ 12 „	4	31.5	4.04	
		F = 5.46 sign.		
		$F_{0.95}$ (6.23) = 2.53		

Table 6. The vertebral bone mass, calculated as percentage bone "hits", in rats fed the normal laboratory ration and the low calcium diet.

(Fig. 5). The bone had a non-lamellar (woven) appearance as described by *Enlow* (1963). No secondary osteones or resorption cavities were observed. In the animals maintained on a low calcium intake for 6 to 12 months, the microradiographs had a similar appearance to those in the control group, with the exception that the bone trabeculae were considerably thinner (Figs. 6 and 7). There was no difference in the degree of mineralization, and both the cortical and the trabecular bone were as evenly mineralized as in the control animals. No resorption cavities were found. Microradiographs from longitudinal sections of the metacarpal bone in the short-term series showed that the bone was evenly mineralized and that the epiphyseal zones were closed.

Histological observations.

In the short-term series decalcified, longitudinal sections from the metacarpal bone of each animal were stained with haematoxylin-eosin and azocarmine G, respectively. In all the animals the epiphyseal zones were closed. The sections stained with haematoxylin-eosin showed that in the control group the diaphysis contained mostly small osteocytes. Osteocytes with enlarged spherical lacunae were located approximately midway between the border of the central marrow cavity and the periosteum. Occasionally, the lacunae in that area were empty

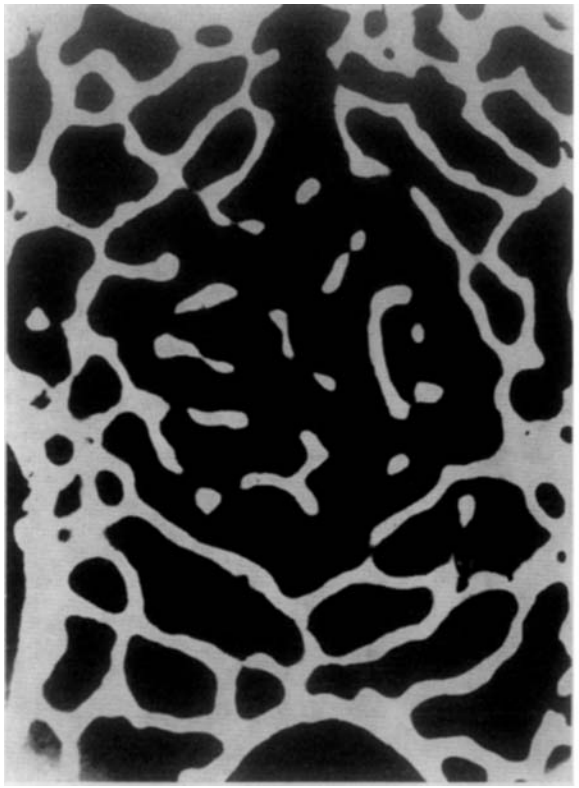


Fig. 5. Microradiograph of a cross section through the second tail vertebra of a normal rat. The bone is evenly mineralized. (x 25).

Fig. 6. Microradiograph of a cross section through a corresponding part of the second tail vertebra of a rat fed the low calcium diet for 12 months. (x 25).

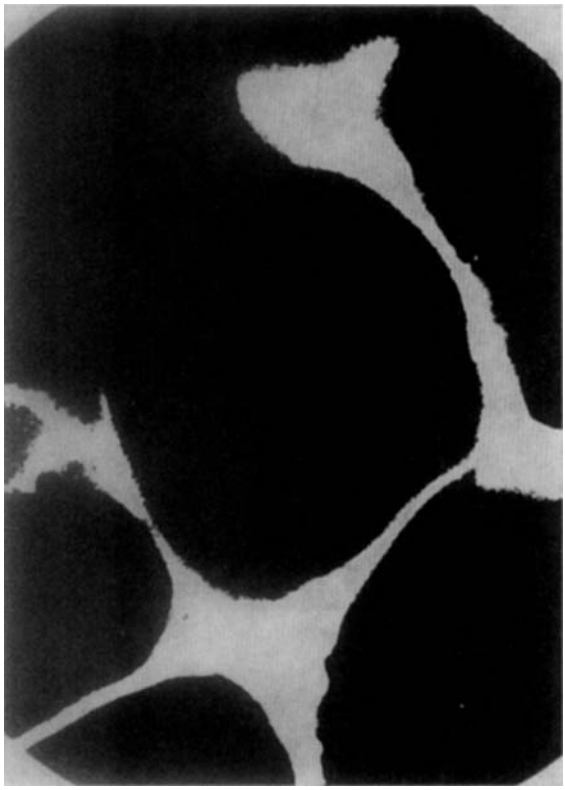


Fig. 7. A detail of the microradiograph shown in fig. 6. The bone is evenly mineralized and there is no difference from normal in the degree of mineralization of the remaining bone tissue. (x 100).

of cells and had become filled with an amorphous substance. The immediate border of the osteocyte lacunae and the processes of the osteocytes were slightly basophilic. In the metaphyseal bone trabeculae, slightly basophilic areas were seen. These areas were located either centrally or peripherally in a trabecula, and frequently extended to its surface. Occasionally, small scattered, irregular areas which were slightly basophilic were observed in the cortex.

Histological sections from rats fed the low calcium diet for 1 to 16 weeks showed a similar appearance to those from the normal control animals. In all sections, osteoblasts and osteoclasts were scanty. In fact, no osteoclasts were observed either in the normal controls or in the animals fed the low calcium diet. The osteocytes had the same appearance as in the control animals, and

no change in the staining properties was observed. The slightly basophilic areas did not differ from those in the control animals. The osteoid tissue was very scanty in the normal animals. No increase in the amount of osteoid tissue was observed in the calcium-deficient animals. Neither was any difference observed in the amount of fibrous tissue associated with bone.

In the long-term series, decalcified cross sections of the vertebrae were stained with toluidine blue at pH 4.4 and, in addition, with haematoxylin-eosin and azocarmine G. In the different animals, sections were cut from parts of the vertebra chosen at random. In each animal, all the sections were cut from the same part of the vertebra, allowing adjacent sections to be examined after different staining procedures. The cortical bone contained mostly small osteocytes, but larger osteocytes were also found, especially midway between the marrow cavity and the periosteum. The trabecular bone also contained small osteocytes and, occasionally, slightly larger ones. The cytoplasm, the immediate border of

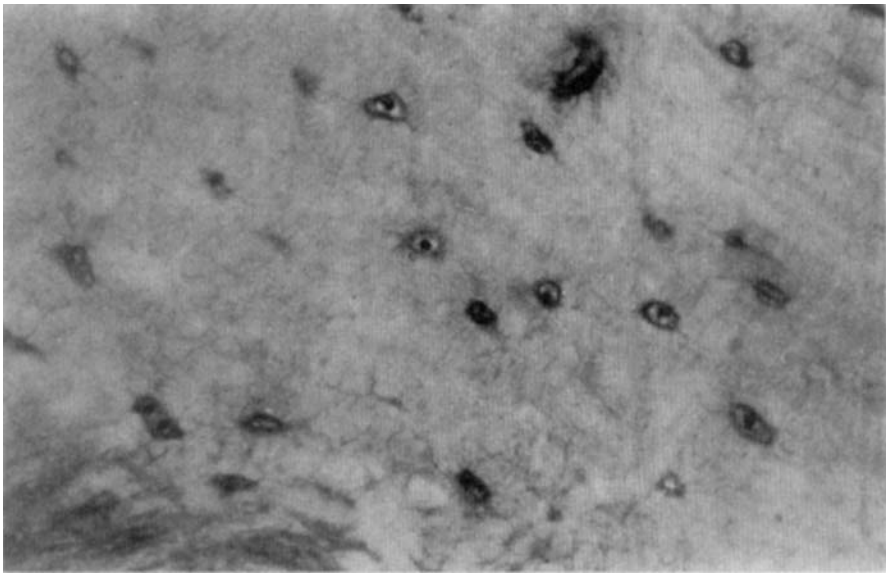


Fig. 8. Photomicrograph of vertebral cortical bone from a normal adult rat. A variety of osteocytes and also empty lacunae can be seen. Haematoxylin-eosin. (x500).

Fig. 9. Photomicrograph of vertebral bone from a normal rat, showing slightly basophilic areas. Haematoxylin-eosin. (x125).

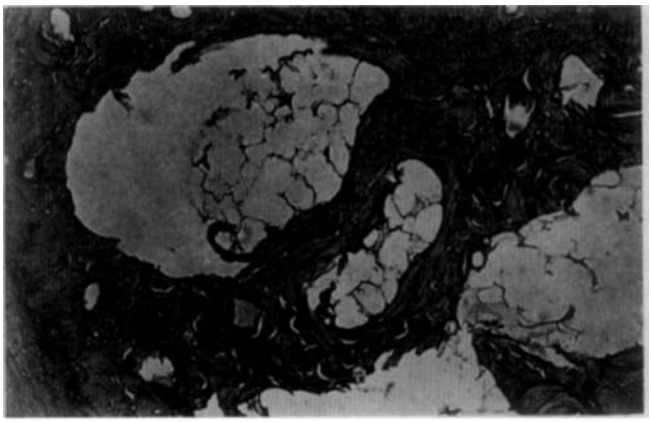


Fig. 10. Photomicrograph of an adjacent section of the vertebral bone shown in fig. 9. Between the dark-staining collagen bundles light areas con be observed. Azocarmine G. (x125.)

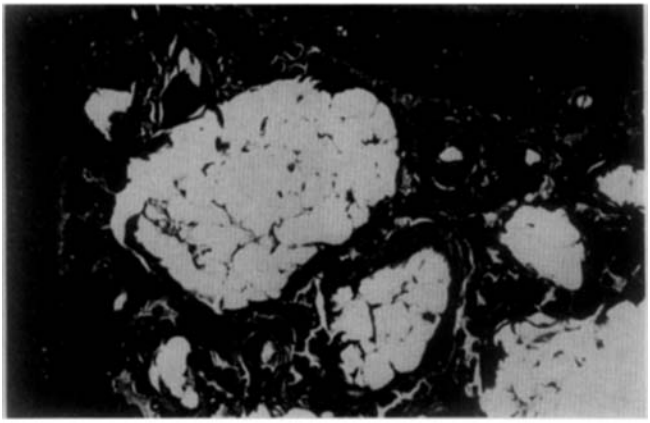
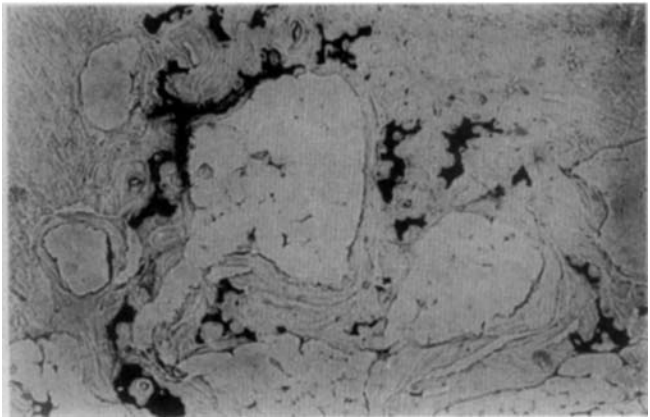


Fig. 11. Photomicrograph of an adjacent section of the vertebral bone shown in fig. 9 and 10. Areas, which stained slightly basophilic with haematoxylin-eosin and light, or not at all, with azocarmine G, show an evident metachromasia. Toluidine blue at pH 4.4 (x125).



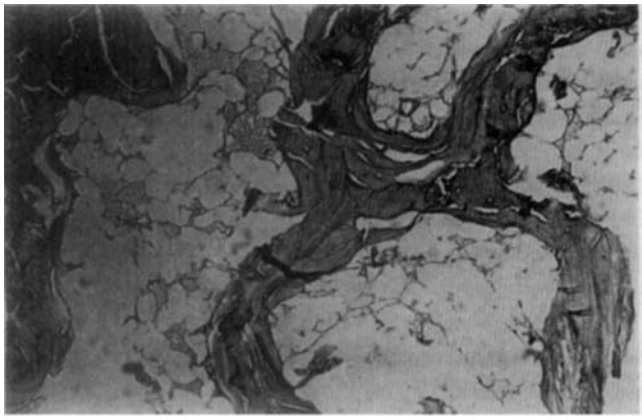


Fig. 12. Photomicrograph of vertebral bone from a rat fed the low calcium diet for 9 months. Bone trabeculae with slightly basophilic areas. Haematoxylin-eosin. (x125).

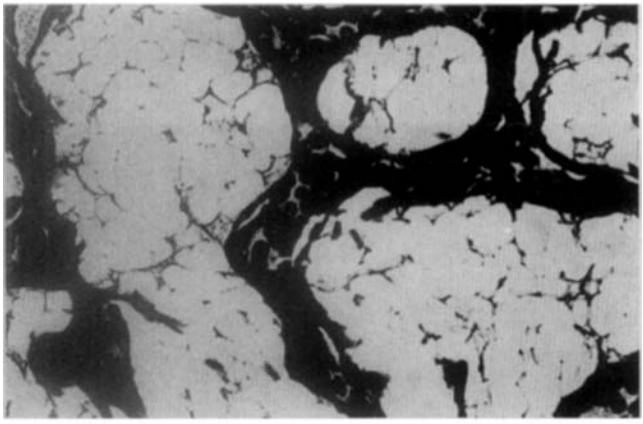


Fig. 13. Photomicrograph of an adjacent section of the vertebral bone shown in fig. 12. Small areas staining only light, or not at all, located between the dark-staining collagen bundles. Azocarmine G. (x125).

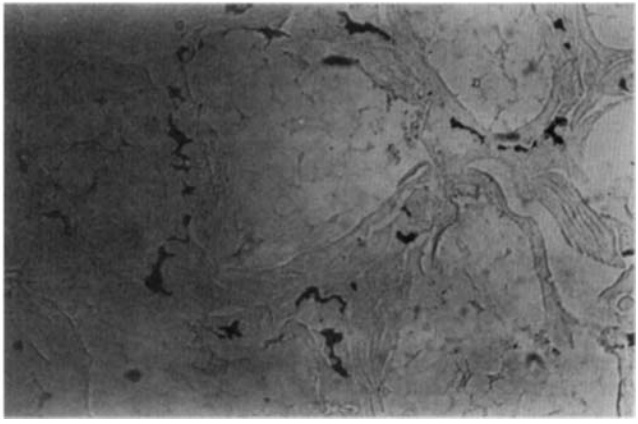


Fig. 14. Photomicrograph of an adjacent section of the vertebral bone shown in fig. 12 and 13. The areas described in fig. 12 and 13 show an intense metachromasia. No difference in the staining properties of these areas was observed between normal rats and rats fed the low calcium diet for 6 to 12 months. Toluidine blue at pH 4.4. (x125).

the osteocyte lacunae, and the processes of the osteocytes were slightly basophilic with haematoxylin-eosin, and metachromatic with toluidine blue. Lacunae empty of cells were occasionally found, especially in the cortical bone (Fig. 8). Some of these lacunae were filled with an amorphous substance which was metachromatic with toluidine blue stain. The osteocytes and the lacunae had a similar appearance in normal and experimental animals. The frequency of osteoblasts and osteoclasts was very low in the normal control animals, and no increase was found in the calcium-deficient, osteoporotic animals. The sections stained with haematoxylin-eosin or azocarmine G showed that the amount of osteoid tissue in the control animals was very small. No increase of the osteoid tissue was observed in the animals maintained on a prolonged low calcium diet. As in the tubular bone, areas which showed slightly basophilic staining with haematoxylin-eosin were also found in the vertebral sections (Fig. 9). These

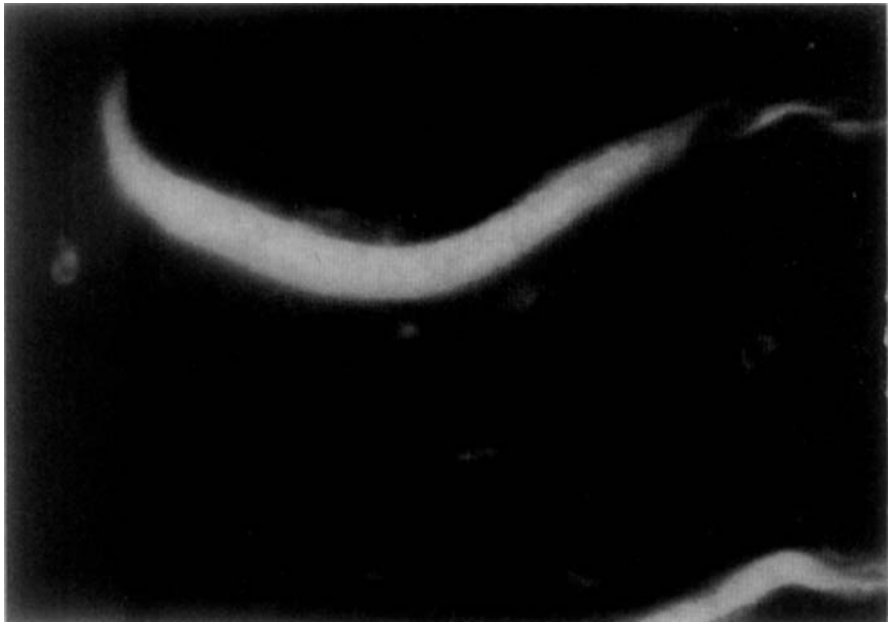


Fig. 15. Fluorophotomicrograph showing labelling of a vertebral bone trabecula in a normal rat. Despite the time interval of 10 days between the two administered doses of chlortetracyline there is only one fluorescent line, indicating a relatively low rate of new bone formation. (x400).

areas were located in the trabecular bone and were observed most frequently in the metaphyseal regions. Nearer the mid-portion of the vertebra, the basophilic areas decreased in frequency. The basophilic areas were located in the spaces intervening between parallel-fibered bone (Fig. 10), and were metachromatic with toluidine blue (Fig. 11). No difference in the staining properties of these areas was observed between normal animals and osteoporotic animals fed the low calcium diet for 6 to 12 months (Figs. 12—14).

Fluorescence microscopy.

In the normal animals, fluorescence of newly formed bone was observed mainly on the trabecular surfaces (Fig. 15), and on the surfaces of bone canals. Only parts of the trabecular surfaces were fluorescent, while others were not. Despite the time interval of 10 days between the two administered doses there was, as a rule, only one fluorescent line to be observed. Occasionally, double

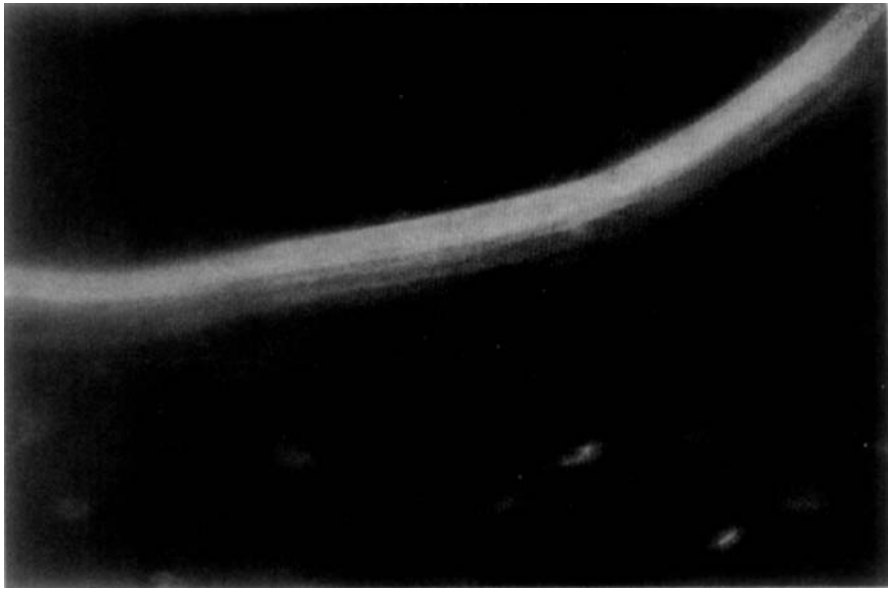


Fig. 16. Fluorophotomicrograph showing labelling of a vertebral bone trabecula in a rat fed the low calcium diet for 9 months. The labelling is similar to that in the normal rat. (x400).

fluorescent lines were found. The distance between the lines was quite short, indicating a relatively low rate of new bone formation.

In the osteoporotic animals fed the low calcium diet for 6 to 12 months, the tetracycline fluorescent micrographs had a similar appearance to those in the normal control animals (Fig. 16). No difference in the degree of trabecular labelling was observed. Only occasionally were double fluorescent lines found and the distance between the lines was not apparently different from that in the control animals. The bone canals showed the same degree of labelling as in the normal rats. Both in the normal and in the calcium-deficient rats, the fluorescent uptake was highest in the metaphyseal regions of the vertebra.

DISCUSSION

The present study is concerned with the reaction of the well-mineralized skeleton of one-year old, fully grown rats to a restricted calcium intake. The preliminary feeding induces different degrees of saturation of the calcium stores (*Fairbanks and Mitchell* 1936) and a normal calcium intake is necessary for the growth of all the tissues, including bone, whether other conditions necessary for calcification are present or not (*Carttar et al.* 1950, *Toothill and Hosking* 1968). The animals used in the present investigation had been bred on an optimum intake of calcium, phosphorus and vitamin D and rats with a mature skeleton were used in order to eliminate the interference of skeletal growth with the skeletal response of the treatment. The fact that the body weight of the control animals remained almost unchanged during the period of observation, in combination with the absence of any difference in length of the bones, strongly suggests that the skeletal growth of the animals used was completed at the start of the experiment. This is further supported by the observation that the uptake of radioactive calcium in bones of rats diminishes rapidly from the age of 3 to 6 months and then decreases very little with age in accordance with the growth rate (*Bohr* 1968). By histological examination it has been found that the epiphyseal cartilages are inactive in aged rats (*El-Maraghi et al.* 1965). This observation was confirmed by the examinations performed in the present study.

Old rats need approximately 0.5 per cent calcium in the diet to maintain calcium equilibrium (*Henry and Kon* 1947). In the present investigation, the

rats kept on a low calcium intake were given a diet containing 0.09 per cent calcium and adequate amounts of phosphorus and vitamin D. The prevailing experimental conditions were, therefore, sufficient to bring the animals into a negative calcium balance. In a negative calcium balance, the difference between calcium intake and output is evidently taken from the skeleton. In the ten groups of animals maintained on a low calcium intake for 1 to 16 weeks, no change was observed in the roentgenological appearance of the skeleton and the density of the whole bone. Neither the hydroxyproline content nor the ash content of whole bones showed any significant changes during the first 12 and 14 weeks of calcium restriction. Not until after 14 weeks on a restricted calcium intake was a definite reduction in bone mass, *i.e.* a reduction of both the mineral and organic components as determined by the measurement of the contents of ash and hydroxyproline of whole bones, observed in the old rats used in the present study. This finding supports the observations by *Henry et al.* (1960) that old rats kept on a similar low calcium diet remain in negative calcium balance for at least 4 months. In the present study a reduction of both the trabecular and the cortical bone, resulting in a decrease in the density of the whole bone, was found on X-ray examination, by direct measurement of the cortical thickness in the mid-shaft of the femur, *i.e.* "femur score", and in the percentage of vertebral bone "hits". The reduction in the ash and the hydroxyproline contents of whole bones indicates a concomitant reduction of both the mineral and the collagen component of the skeletal tissue. Thus, during the long-term negative calcium balance induced by a restricted calcium intake in this experiment, mobilization of skeletal calcium occurred with a concomitant breakdown of organic bone matrix.

The bone composition of the rats in the group fed the test diet enriched with calcium for 16 weeks suggests an increase in the degree of mineralization compared with that of the animals fed the normal laboratory ration for the same period of time. Thus, there was a significant increase in gross bone density compared with that of the normal controls. The observed increased ash content, together with a decrease in the percentage hydroxyproline content of whole bones, suggests an increased degree of mineralization of the bone collagen. This is further supported by the decreased retention of intraperitoneally administered Ca^{45} found in the same animals in a subsequent study (*Larsson* 1969).

It has been suggested from observations made in calcium balance studies on old rats that a considerable calcium deprivation by 5 per cent or more of the total body calcium is followed by a distinct, compensatory increase of the calcium absorption which continues with high efficiency until the total loss

in the calcium deprivation period has been compensated (*Nicolaysen et al.* 1953). In the present investigation, the maximum decrease in the ash content of the whole bone amounted to 13.3 per cent and was found in the group of rats maintained on a low calcium intake for 12 months. After the 14th week of restricted calcium intake there was a successive reduction in the ash content of the tibia. This finding is not consistent with a delayed increase in calcium absorption and calcium equilibrium despite continuous low calcium intake. A further reduction in bone mass would not have been expected otherwise in the groups of rats kept on the low calcium diet for the longer periods of time, *i.e.* 10 to 12 months. It is, thus, of great interest that, contrary to the suggestion of *Nicolaysen et al.* (1953), no tendency to a restitution of the bone tissue lost was observed in the groups of rats maintained on prolonged calcium restriction. The continuous loss of bone mineral observed in the present investigation indicates that the old rats failed to adapt to the restricted calcium intake during the experimental period of one year. Since the variability in calcium absorption studies is considerable, with a reported coefficient of variation of approximately 25 (*Nicolaysen et al.* 1953), a slight continuous calcium loss from the skeleton would be difficult to determine with such techniques.

With the tetracycline double-labelling procedure no difference in the amount of bone surfaces showing new bone formation was observed in the normal control animals and in the osteoporotic rats fed the low calcium diet for 6 to 12 months. Neither was any difference in the degree of mineralization noted on microradiographs of undecalcified vertebral bone sections. There were, further, no cellular differences from the normal in decalcified, histological sections of bone in the different experimental animals. It is concluded therefore, that the bone changes induced in the present investigation are in accordance with those of typical osteoporosis as described by *Collins* (1966).

In many earlier animal experiments purporting to demonstrate the effect of calcium deprivation, inanition and therefore protein-calorie deficiency also occurred (see *Platt et al.* 1961, *El-Maraghi et al.* 1965). This dual aetiology was eliminated in the present experiment in which the animals were kept on a high-calorie low calcium diet. The possibility of bone atrophy due to inactivity was also excluded.

Various morphological changes of the bone tissue, *i.e.* basophilia of the bone trabeculae, fibrosis and increased clasmatocytic activity, have been reported to be characteristic of osteoporosis in rats (*McClendon and Blaustein* 1965). However, such changes appear to be more typical of osteitis fibrosa than of osteoporosis, and were not observed in the present investigation. Although areas

showing basophilia with haematoxylin-eosin are normally present in the metaphyseal bone in the rat, some investigators have recognized the association of similar areas with resorption (*Ruth* 1954, 1961). Also in the old rats used in the present investigation, basophilic areas were found in the bone trabeculae of the metaphysis of the vertebrae. Examination of decalcified sections stained with haematoxylin-eosin, azocarmine G or toluidine blue at pH 4.4 indicated that these areas consisted of intercellular substance probably containing acid mucopolysaccharides extending from the epiphysis into the bone trabeculae. The staining properties of these areas were similar in osteoporotic animals and normal controls. No changes were observed which could be related to the osteoporotic process. Intensified staining of mucopolysaccharides has been reported in areas where bone removal is taking place (*Heller-Steinberg* 1951, *Engel* 1952). While in those experiments young growing animals were studied under conditions of induced rapid bone resorption, nu such changes were observed in the adult animals used in the present investigation.

Osteolysis, *i.e.* osteocytic resorption of cells surrounded by matrix that contains mucopolysaccharides and induces toluidine blue metachromasia has been observed after the institution of low calcium or high phosphorus diets (*Bélanger* 1965). Osteoclastic resorption has been considered to be of far less importance than osteolysis in both normal bone remodelling and in accelerated resorption as, *e.g.* in hyperparathyroidism (*Bélanger et al.* 1963, *Bélanger and Robichon* 1964, *Gries* 1966, *Brown et al.* 1966). In the present investigation no increase in metachromasia around osteocytes was observed in the calcium-deficient osteoporotic animals, compared with the normal controls. The appearance of increased osteolysis is probably dependent upon the degree of calcium deficiency induced.

While young rats are reported to develop skeletal changes typical of rickets upon specific calcium deficiency (*Park et al.* 1922, *Hess* 1929, *Carttar et al.* 1950, *Engfeldt et al.* 1962, *Gershon-Cohen et al.* 1962, *McClendon and Blaustein* 1965), no signs of rickets or osteomalacia were found at any stage of calcium deficiency by the histological, microradiographical and tetracycline labelling studies performed in the present investigation. Osteoporotic bone changes reported in young calcium-deficient rats by *Crawford et al.* (1957) and *Harrison and Fraser* (1960) are apparently late changes secondary to calcium-deficiency rickets and disturbed skeletal growth (*Gershon-Cohen et al.* 1962, *McClendon and Blaustein* 1965). No conclusions can be drawn from those experiments regarding the maintenance of the mature skeleton and the development of osteoporosis, which characteristically is a disease of the adult individual.

In lactating female rats a reduction of the bone tissue has been induced by instituting a low calcium diet (*Tomlin et al.* 1953, *McClendon and Blaustein* 1965). These changes were described as osteoporosis and could be reversed by the administration of tricalcium phosphate (*McClendon and Blaustein* 1965). However, the morphological criteria of osteoporosis used suggest that the condition induced was osteitis fibrosa rather than osteoporosis.

In 6-month old male rats fed for 20 weeks with a low calcium diet containing 0.26 per cent calcium and 0.45 per cent phosphorus, a significant reduction in bone mass and in weight of ash from whole bone was recorded (*Ferguson and Hartles* 1966). Decalcified sections and microradiographs of undecalcified sections from the femur showed no differences from the normal, except that the shaft appeared thinner. These results are confirmed by those of the present investigation. However, as only one observation period was used in the experiment by *Ferguson and Hartles* (1966), no information was obtained concerning the development of the bone reduction. Other investigators (*El-Maraghi et al.* 1965) found little, if any, effect on the weight of ash/cm^3 in the femur in 2-year old rats maintained on a low calcium diet, *i.e.* 0.11 per cent calcium and 0.55 per cent phosphorus for 12 weeks. This observation is in agreement with the present results showing that approximately 14 to 16 weeks are required for the development of an evident reduction in bone mass of adult rats fed a similar diet. The statement by *El-Maraghi et al.* (1965) that a dietary calcium concentration of 0.11 per cent would be adequate for maintenance in old rats, thus appears to be not valid.

Osteoporosis has also been reported in adult cats fed a low calcium, high phosphorus meat diet, *i.e.* calcium 0.007 and phosphorus 0.18 per cent on a wet weight basis (*Jowsey and Gershon-Cohen* 1964). After 10 weeks on this diet radiologic evidence of decreased mineral density was found and microradiographs of undecalcified bone sections showed a reduction in the width of bone trabeculae and the appearance of resorption cavities in the cortical bone. The increased resorption was accompanied by an increase in number of newly formed osteones. Contrary to the experience in clinical osteoporosis, the reduction in bone mass was reversed by feeding a high-calcium diet. In that experiment and in an extended similar study (*Jowsey and Raisz* 1968) the induced bone changes were related to observed secondary hyperparathyroidism. Cats fed the low calcium meat diet for 5 months showed definite osteomalacia. In the present study no such changes were observed during the development of the osteoporosis. The appearance of osteomalacia is probably dependent upon degree of calcium deficiency and the dietary calcium:phosphorus ratio. The osteomalacia reported

by *Jowsey and Raisz* (1968) was apparently transient, since in cats fed the low calcium diet for 13 months the osteoid borders, although highly increased in frequency, were of normal width. The decrease in resorption but not in formation observed in such animals treated with a high calcium diet for 1 month suggested that the bone changes induced by the low calcium diet could be reversed. In adult dogs maintained on a low calcium, high phosphorus diet, bone lesions as in osteitis fibrosa have recently been reported (*Saville and Krook* 1968). Histological examination of decalcified bone sections showed the presence of numerous osteoblasts with the appearance of resorption cavities and, further, osteoblasts and osteoid tissue covering the surfaces of cancellous bone. Despite the definitely increased amount of osteoid tissue the calcium accretion rate by bone was normal (*Saville and Krook* 1968). This condition, suggesting qualitative changes in the bone tissue, was called "nutritional hyperparathyroidism".

Although prolonged deficiency or malabsorption of calcium has been related to the onset of osteoporosis in humans (*Nordin* 1960, 1961, 1962, 1964), and calcium therapy is widely used in the treatment of the condition, the relation of calcium deficiency to osteoporosis is not clear (*Dent and Watson* 1966). Of primary importance in this connection is the observation by *Malm* (1958) that a negative calcium balance prevails in human volunteers for a relatively long time after the institution of a low calcium intake. In a review of the literature it has been stated that osteoporosis can be produced in adult animals by a negative calcium balance, usually induced by low calcium diets (*Nordin* 1960). However, it was only young growing animals that were studied in these experiments. It is now established that the reaction of the growing skeleton is quite different to that of the mature skeleton. In young rats the bone lesions correspond to those in typical rickets. When there is a rapid reduction in bone mass due to pronounced calcium deficiency, osteitis fibrosa or osteomalacia will occur both in growing and in mature animals. In the present investigation, the development of osteoporosis is described in mature rats placed on a low calcium diet for varying periods of time from the age of one year. Results of calcium balance studies indicate that the general principle of the adaptation of calcium absorption in old rats can be applied also to other species (*Nicolaysen et al.* 1953). The results of the present investigation suggest, therefore, that prolonged calcium restriction might contribute to the onset of symptomatic osteoporosis in adult humans, also.

SUMMARY

One-year-old fully grown rats were maintained on a low calcium diet with normal contents of phosphorus and vitamin D for periods varying from 1 week up to 1 year. The effect of the treatment upon the skeletal tissue was studied by radiographical, microradiographical and histological methods. Quantitation of the amount of bone tissue was performed in special bones by the determination of the "femur score" and the vertebral bone "hits". In special whole bones, the contents of ash and hydroxyproline and the gross bone density were examined. The uptake and distribution of chlortetracycline administered by intraperitoneal injection 10 and 20 days before sacrifice, was studied in vertebral bone sections.

In the adult rats used, the skeletal growth was completed at the start of the experiment. By radiologiccal examination performed at the start and at the end of each experimental period, the occurrence of a slight but definite reduction of the cortical thickness of the tubular bones and a decreased density of the trabecular pattern in the metaphyseal bone was found in all animals fed the low calcium diet for 6 months and longer. These osteoporotic rats showed significantly lower values for the "femur score" and the vertebral bone "hits" than for the normal controls. The ash content of the tibia and the hydroxyproline content of the metacarpal bone showed significantly decreasing values during the observation period. The six groups of rats kept on a restricted calcium intake for 6 to 12 months showed significantly lower values for the gross bone density of the humerus, on comparison with those of the normal controls. On microradiographical examination, a reduction in the number of vertebral bone trabeculae was observed in the rats fed the low calcium diet for 6 months and longer. The remaining bone trabeculae were considerably thinner than normal, but no change in degree of mineralization was observed, the bone being as evenly mineralized as in the normal rats. In these osteoporotic rats the tetracycline fluorescent micrographs had a similar appearance to those in normal rats. Thus, no apparent difference in the amount of bone surfaces showing fluorescence of newly formed bone was observed. On histological examination of decalcified sections stained with haematoxylin-

eosin, no osteoclasts were observed either in the normal or in the osteoporotic animals. Sections of vertebral bone stained with toluidine blue at pH 4.4 showed, in the metaphyseal bone, areas with evident metachromasia. No difference in the staining properties of these areas was found between normal and osteoporotic animals.

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Studies on the Development of Experimental Osteoporosis

II. The Effect of Prolonged Calcium Restriction on the Blood Calcium Level and the Distribution of Ca^{45} between Blood and Bone in Adult Male Rats

by

Sven-Erik Larsson

INTRODUCTION

In human osteoporosis, disturbances in calcium metabolism and the efficiency of calcium homeostasis in maintaining extracellular fluid calcium has been considered to be of major pathophysiological importance for the reduction of bone mass (*Heaney* 1965), in contradistinction to the concept of a primary defect ("accelerated ageing of bone") in the bone tissue itself (*Urist* 1962). Studies in osteoporotic patients with bone-seeking isotopes have contributed little to our understanding of the osteoporotic process (*Nordin* 1962). Normal skeletal accretion of bone-seeking isotopes or stable strontium is generally reported (*Heaney and Whedon* 1958, *Dow and Stanbury* 1960, *Fraser et al.* 1960, *Nordin* 1960, *Dymling* 1964), but both reduced accretion (*Bauer et al.* 1957, *Eisenberg and Gordan* 1961, *Dymling* 1962, *Bronner et al.* 1963, *Lafferty et al.* 1964) and increased (*Nordin* 1959) have also been observed. However, a reduced accretion by the skeleton in osteoporosis may be relative, due to reduced bone mass. Usually, clinical osteoporosis takes several years to develop until the disease can be diagnosed, and from results of isotope studies in the osteoporotic patient it is difficult, therefore, to draw any conclusions concerning the pathomechanisms involved during this prolonged process of bone reduction.

Available data obtained from studies with bone-seeking isotopes are inconclusive with regard to the skeletal reaction to prolonged calcium restriction in experimental animals. A greater retention than normal of a dose of strontium has been reported in calcium-deficient young rats (*Harrison and Fraser* 1960). However, since the calcium-deficient animals failed to grow or to gain weight at the same rate as the control animals, it is difficult to interpret the results obtained. These findings were not confirmed by *Copp and Suiker* (1962), who observed a reduced retention of Ca^{45} in young rats after dietary calcium restriction. In calcium-deficient adult rats their data indicated normal Ca^{45} retention and increased bone resorption. In calcium-deficient pregnant rats, the percentage of orally administered Ca^{45} retained has been reported to be 3.5 to 4.0 times that retained by control animals (*Bawden and Osborne* 1962).

The adaptation to a reduced calcium intake has been extensively investigated by balance studies in the rat (*Henry and Kon* 1947, 1953, *Barucha and McCay* 1954, *Nicolaysen* 1943, 1956, *Henry et al.* 1960). Old rats need a minimum of 4 months to adapt themselves to a low calcium intake. This period of adaptation

is longer for old than for young rats (*Henry et al.* 1960). According to *Nicolaysen et al.* (1953) a marked adaptation, *i.e.* a distinctly increased efficiency of calcium absorption, will occur in old rats only when they have suffered a substantial loss of calcium from the skeleton; in principle, this finding agrees with observations in man. It has been considered that the increased efficiency of calcium absorption would compensate fully for any previous loss (*Nicolaysen et al.* 1953). However, in a previous study (*Larsson* 1969 a), the development of a significant reduction in bone mass was found in adult male rats maintained on a prolonged low calcium intake for varying periods of up to one year. Histological examination revealed that the bone changes induced in these animals fulfilled the criteria for osteoporosis described by *Collins* (1966).

The present investigation was undertaken in order to elucidate the pathomechanisms involved during the development of this type of osteoporosis. The study presented here concerns the effect of prolonged calcium restriction in adult male rats on the blood calcium level and the distribution of Ca^{45} between blood and bone after intraperitoneal administration of the isotope.

EXPERIMENTAL

The same experimental material was used as in the previous investigation (*Larsson* 1969 a). A total of 95 adult male rats of the Sprague-Dawley strain[1] were divided into two experimental series. Sixty animals comprised a short-term, and 35 a long-term series. Apart from the duration of the test diets, both series were treated identically. All animals, except the controls of the long-term series (see below), were stated to be 12 to 12 1/2 months old at the start of the experiment. The mean initial body weight of the animals of the two series was 499.0 ± 5.2 and 501.8 ± 9.2 grams, respectively. The animals had been bred on a standard laboratory ration containing 1.4 per cent Ca and 1.2 per cent P* and adequate amounts of vitamin D.

Experimental animals.

All the experimental animals were fed the low calcium test diet of General Biochemical Inc., Chagrin Falls, Ohio. The ingredients and composition of this diet have been described in a previous investigation (*Larsson* 1969 a). Chemical

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[1]) Anticimex Co., Stockholm.

*) Here and in the following text the figures based on dry weight are given.

analyses showed that the diet contained 0.09 per cent Ca and 0.55 per cent P. The Ca:P ratio was 1:6.1. The diet was supplied with an adequate amount of vitamin D. All animals received diet and deionized water *ad libitum*. The daily calcium intake was estimated at 18—20 mgs per animal. Groups of 5 rats each were kept on this regimen for different periods of time; in the short-term series ten groups for 1 to 16 weeks, in the long-term series six groups for 6 to 12 months.

Another group of 5 animals was fed the test diet described above supplemented with 4 per cent $CaCO_3$, *i.e.* this diet contained a total amount of 1.7 per cent Ca and 0.55 per cent P. The Ca:P ratio was 1:0.4. This diet and tap water were given *ad libitum* for 16 weeks, and the daily calcium intake of these animals was estimated at 340—360 mgs.

Control animals.

In the short-term series, one group of 5 rats received a normal laboratory ration[1] containing 1.0 per cent Ca and 0.75 per cent P for 16 weeks. The Ca:P ratio was 1: 0.75. Diet and tap water were given *ad libitum*. The daily calcium intake was estimated at 200—220 mgs. In the long-term series, one group of 5 animals was fed the normal laboratory ration and tap water for 4 weeks to accustom the animals to the laboratory routine. These animals were stated to be approximately 20 months old when delivered to the laboratory.

All the animals were housed five to each cage without access to direct sunlight and at a temperature of 18—20° C. The animals moved about freely in the cages. No coprophagy was observed. The series were planned in such a way that all the animals within each series were sacrificed at the same time. In all animals the initial and final body weights were recorded. In addition, all the animals of the short-term series were weighed weekly.

Isotope administration.

Seventy-two hours before sacrifice, each animal was injected intraperitoneally with 20 microcuries of Ca^{45} * in 1.0 ml physiological saline solution from a single batch of $Ca^{45}Cl_2$ for each series.

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[1]) Ewos Co., Södertälje, Sweden.

*) Calcium chloride in aqueous solution 2—5 c/g Ca, C.E.S.2, The Radiochemical Centre, Amersham, England.

Plasma sampling.

Blood was collected from all animals at the time of sacrifice, which was performed by bleeding the animal to death from the femoral artery under ether anaesthesia. In addition, from the animals in the long-term series 0.3 to 0.4 ml tail blood was collected at intervals of 12, 24, 36 and 48 hours following the administration of the isotope. The collected blood was centrifuged immediately at 3,000 r.p.m. for 15 minutes, and the supernatant plasma was pipetted into acid washed test tubes and kept at – 20°C pending subsequent analyses.

Bone sampling.

In the animals of the long-term series the right fore paw was removed under light ether anaesthesia 24 hours and the left one 48 hours after the Ca^{45} administration. The specimens were then kept at – 20°C for subsequent determination of the Ca^{45} activity of the second metacarpal bone. The animals were relatively unaffected by the operations. After sacrifice, both tibiae of the animals of the short-term series, and the right tibia and the right fifth metatarsal bone of those of the long-term series were taken for isotope studies.

Preparation of material for Ca^{45}-activity determinations.

A. *Whole bone.* The following bones were carefully freed of surrounding soft tissues and periosteum: a) both tibiae in the animals of the short-term series, but only the right one in the long-term series; b) in the animals of the long-term series, in addition the right and the left second metacarpal bone and the right fifth metatarsal bone were used.

B. *Pure cortical bone tissue.* In the animals of the long-term series the tubular bones, except the right tibia and humerus, were carefully dissected free from soft tissues and periosteum. The ends of the bones and the articular cartilage were removed, using a bandsaw. The diaphyses were split into small bone fragments which were carefully cleaned of all bone marrow, no solvents being used. The bone fragments were defatted and dehydrated in 5 changes of acetone for 3 days. This material was finally air-dried and then pulverized, using an Intermediate model Wiley Laboratory Mill provided with a 60-mesh sieve. The whole bones and approximately 75 mgs of cortical bone powder of all specimens of each series were ashed simultaneously in a muffle furnace at 700°C for 16 hours. The exact weight of the ash was determined with the aid of a Mettler balance and with an accuracy of 0.05 mg. The ash of the whole tibia

was dissolved in 5.0 ml of 1.2 N hydrochloric acid. When the ash was completely dissolved, deionized water was added to the solution to a final volume of 100 ml. An aliquot of 2.0 ml was pipetted into a 25 ml polyethylene counting vial which was then filled with a gel scintillator composed of 2.5-Diphenyloxazole (PPO) 1.0 g, 1.4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene (POPOP) 0.3 g, Naphtalane 100.0 g, Dioxane 1 litre and thixotropic gel powder (CAB-O-SIL) to saturation. The ash of the small bones and the ash of the cortical bone powder were dissolved directly in 2.0 ml of 1.2 N hydrochloric acid in the counting vials, and counting was performed after addition of the scintillator.

C. *Plasma.* The Ca^{45} activity was determined directly, using 1.0 ml of plasma collected at sacrifice and about 0.2 ml of plasma obtained from the blood collected from the tail at different intervals after the administration of the isotope. This procedure was considered suitable for the purpose of the present study (see *Budy* 1963) and was recommended by *Kumar* (1966). The same scintillator was used as for the bone ash solutions.

ANALYTICAL METHODS

Determination of the Ca^{45} activity[1] was carried out in a Packard Tri-Carb Liquid Scintillation Spectrometer[2]. This counting was performed at + 3°C simultaneously for all the specimens within each series. The samples were usually counted 3 × 5 minutes each, at least 10,000 impulses being recorded. The background was of the magnitude of 25 to 30 c.p.m. The coefficient of variation for the quotient between the two energy channels used was 2.8 to 3.2 per cent. The stability of the spectrometer was tested as follows: One Ca^{45} sample was counted 60 times during a period of about 15 hours. The coefficient of variation was 1.0 per cent, *i.e.* the stability of the apparatus was good. Corrections were made for background and decay. Corrections for self-absorption were not considered necessary because of the constancy of counting efficiency.

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[1]) The method for determination of the Ca^{45} activity as used in the Orthop. Res. labor., Presbyterian-St Luke's Hosp., Chicago, Ill., has been applied in this investigation.

[2]) Model 314 EX-2, Packard Instruments Co., La Grange, Ill., USA.

The calcium content of cortical bone powder was analysed by atomic absorption spectrophotometry with the aid of a Perkin-Elmer Model 303 Instrument. About 50 mgs of bone powder were ashed as described above. The ash was weighed on a Mettler balance and then dissolved in 10 ml of 1.2 N hydrochloric acid. After dilution with deionized water to the desired calcium concentration, a 5 per cent lanthanum solution in 25 % (v/v) HCl was added so that the final calcium concentration was within an optimum working range of 1 to 10 ppm and with 0.5 per cent lanthanum[1]. In this way the calcium:lanthanum ratio was 1: 1000, as recommended by *Willis* (1961) for suppression of the interference by phosphorus within the solution. The added calcium recovery in these bone ash solutions was found to be 98 per cent. Duplicate determinations were performed throughout. The coefficient of variation for these analyses was less than 1 per cent.

The calcium concentration of plasma obtained from the blood collected at sacrifice was determined by flame photometry, using an Eppendorf's photometer. Duplicate determinations were always performed. The error of the method was less than 1 per cent (*Johansson* 1966).

Treatment of isotope data. The Ca^{45} activity of bone was expressed as counts per minute per milligram of ash. No significant difference was found in the calcium contents of ash from pure cortical bone tissue of the normal and of the calcium-deficient animals; the mean calcium concentration was 38.09 $\pm$ 0.20 per cent. Therefore, the expression counts per minute per milligram of ash is equivalent to the specific activity, and the factor 0.3809 was used when the bone accretion rates were calculated (see below). The Ca^{45} activity of plasma at 72 hours after the isotope injection was expressed as counts per minute per milligram of calcium. The plasma Ca^{45} activity curves (Fig. 3) were also based upon values expressed as counts per minute per milligram of calcium. Because of the necessity of collecting as little blood as possible at the different periods after the Ca^{45} administration, plasma calcium determinations were performed only on blood samples obtained at sacrifice.

The calcium accretion rates for the whole tibia (mg calcium/hour/mg calcium of the tibia) were calculated according to *Bauer et al.* (1955). The mean values within each group were used throughout.

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[1]) Perkin-Elmer Review, May, 1966.

Statistical treatment of data. The analytical data obtained from the rats in each group were subjected to analysis of variance with one-way lay-out according to *Scheffé* (1961). The hypothesis of no difference between groups was tested at the 5 per cent significance level. When a significant result was obtained, data for one or more groups were combined in order to find significant contrasts. These were tested according to *Scheffé* (1961). The hypothesis of no difference between the data of the group of rats fed the calcium-enriched diet, and those of the normal control group in the short-term series was tested with Student's t-test as described by *Brownlee* (1965). A 5 per cent significance level was used. In the present study the term "significant" stands for "statistically significant" as found with the tests described above.

RESULTS

Six deaths occurred among the whole material, five among the experimental animals in the long-term series and one in the short-term series, all probably due to advanced age. These animals were excluded. The remaining animals were in good health. The body weights have been reported in a previous study (*Larsson* 1969 a) on the same material.

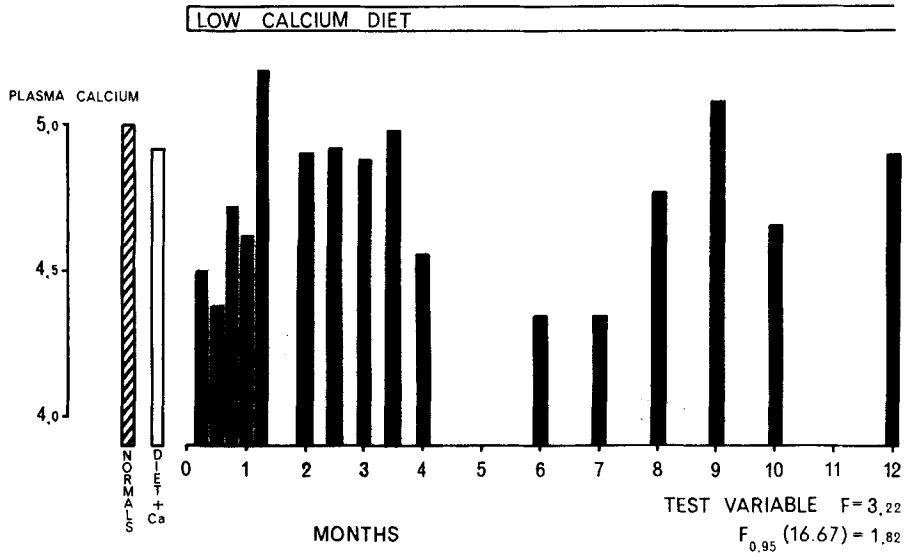

Fig. 1. Plasma calcium in rats fed the normal laboratory ration, the calcium-enriched diet and the low calcium diet.

Plasma calcium (*Fig.* 1, *Table* 1).

There was no significant difference in plasma calcium between the normal control animals of the short-term and the long-term series. The animals fed the calcium-enriched diet for 16 weeks showed similar values to those of the control animals. In the rats maintained on the low calcium diet for 1, 2, 3 and 4 weeks subnormal values were noted. This fall in plasma calcium was, however, not significant. In the five groups of rats maintained on a low calcium diet for 5 to 14 weeks normal plasma calcium values were observed. Subnormal values were again noted in the animals fed the low calcium diet for 4, 6 and 7 months. Neither was this fall in plasma calcium significant. In the four groups

Groups	No. of animals	Plasma calcium mEq./l	S.E.M.	
Normal laboratory ration	10	5.0	0.06	
Low calcium diet for 1 week	4	4.5	0.12	N.S.
„ „ „ „ 2 weeks	5	4.4	0.25	
„ „ „ „ 3 „	5	4.7	0.17	
„ „ „ „ 4 „	5	4.6	0.20	
„ „ „ „ 5 „	5	5.2	0.07	
„ „ „ „ 8 „	5	4.9	0.14	
„ „ „ „ 10 „	5	4.9	0.10	
„ „ „ „ 12 „	5	4.9	0.23	
„ „ „ „ 14 „	5	5.0	0.10	
„ „ „ „ 16 „	5	4.6	0.21	N.S.
„ „ „ „ 6 months	4	4.3	0.13	
„ „ „ „ 7 „	4	4.3	0.12	
„ „ „ „ 8 „	3	4.8	0.14	
„ „ „ „ 9 „	5	5.1	0.04	
„ „ „ „ 10 „	5	4.7	0.09	
„ „ „ „ 12 „	4	4.9	0.11	
		$F = 3.22$ sign.		
		$F_{0.95}\ (16.67) = 1.82$		
Calcium-enriched diet for 16 weeks	5	4.9	0.06	N.S.

Table 1. Plasma calcium in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

of animals maintained on the low calcium diet for 8 to 12 months, the plasma calcium showed no significant change. The reductions in plasma calcium amounted to approximately 12 per cent in the groups of rats kept on the low calcium diet for 2 weeks and 6 and 7 months.

The plasma Ca^{45} specific activity at 72 hours after the Ca^{45} injection (Fig 2, Table 2).

The values of the two control groups fed the normal laboratory ration showed no significant difference. The group of rats kept on a low calcium intake for

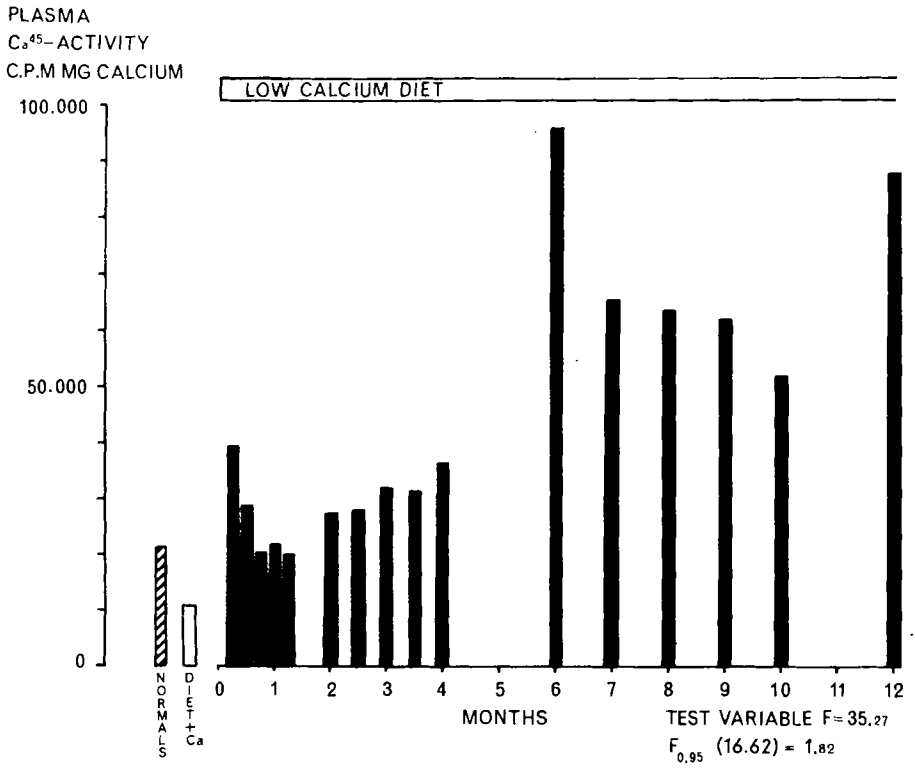

Fig. 2. Ca^{45} activity in plasma 72 hours after the intraperitoneal administration of 20 microcuries of Ca^{45} per animal in rats fed the normal laboratory ration, the calcium-enriched diet and the low calcium diet.

Groups	No. of animals	Plasma Ca^{45} C.P.M./mg calcium	S.E.M.	
Normal laboratory ration	10	21576	1060	
Low calcium diet for 1 week	4	39486	4458	sign.
„ „ „ „ 2 weeks	5	29200	2375	
„ „ „ „ 3 „	5	20507	2843	
„ „ „ „ 4 „	5	22022	2936	
„ „ „ „ 5 „	5	20048	1325	
„ „ „ „ 8 „	5	27670	1948	
„ „ „ „ 10 „	5	27880	2367	
„ „ „ „ 12 „	5	32190	1670	
„ „ „ „ 14 „	5	31428	1694	
„ „ „ „ 16 „	5	36705	3932	
„ „ „ „ 6 months	3	96054	4947	sign.
„ „ „ „ 7 „	4	65638	2471	
„ „ „ „ 8 „	3	63640	10454	
„ „ „ „ 9 „	4	62257	8623	
„ „ „ „ 10 „	3	51886	7233	
„ „ „ „ 12 „	3	87780	6714	
		$F = 35.27$ sign.		
		$F_{0.95}\ (16.62) = 1.82$		
Calcium-enriched diet for 16 weeks	5	12224	631	sign.

Table 2. Plasma Ca^{45} specific activity 72 hours after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

1 week showed significantly increased values compared with those of the rats fed the normal laboratory ration. The animals fed the low calcium diet for 3 to 5 weeks showed approximately the same values as those of the normal controls. Significantly higher values were noted in animals maintained on a restricted calcium intake for 8 weeks to 1 year. This difference was most pronounced in the groups treated for 6 to 12 months. The rats fed the calcium-enriched diet for 16 weeks showed significantly lower values compared with those of the normal controls.

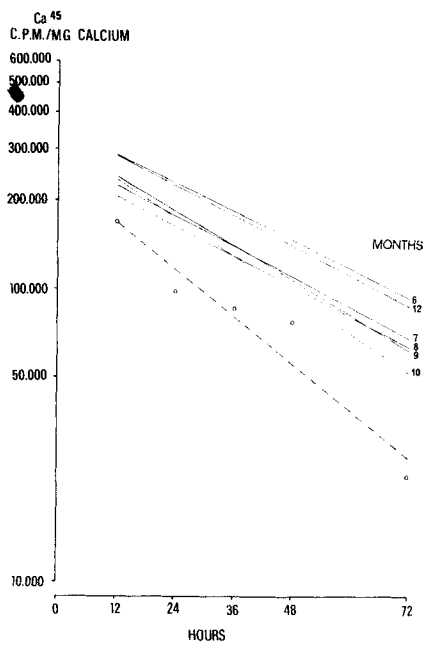

Fig. 3. Ca^{45} specific activity in plasma 12, 24, 36, 48 and 72 hours after the isotope administration in rats fed the normal laboratory ration (broken line) and rats fed the low calcium diet for 6 to 12 months (uninterrupted lines). Each point is the mean value of the control group. Logarithmic graph.

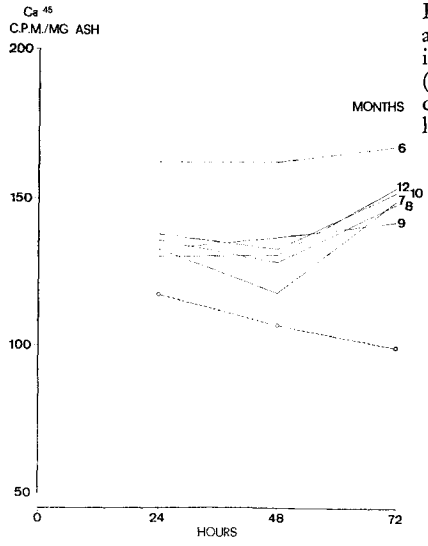

Fig. 4. Ca^{45} activity of small bones 24, 48 and 72 hours after the isotope administration in rats fed the normal laboratory ration (broken line) and rats fed the low calcium diet for 6 to 12 months (uninterrupted lines).

Groups	No. of animals	24 hours after Ca^{45} inj. C.P.M./mg ash M.-C. II dx.	S.E.M.	48 hours after Ca^{45} inj. C.P.M./mg ash M.-C. II sin.	S.E.M.	72 hours after Ca^{45} inj. C.P.M./mg ash M.-T.V dx.	S.E.M.
Normal laboratory ration	5	117.5	5.9	107.0	7.1	99.4	2.7
Low calcium diet for 6 months	3	162.5	13.8	162.6	8.5	177.9	16.2
„ „ „ „ 7 „	4	132.8	5.1	118.2	5.7	148.9	8.6
„ „ „ „ 8 „	3	136.0	8.9	128.4	5.3	148.0	9.0
„ „ „ „ 9 „	5	132.9	6.1	136.6	11.2	142.2	8.4
„ „ „ „ 10 „	4	137.7	4.4	132.7	9.6	152.3	8.4
„ „ „ „ 12 „	3	130.5	9.7	130.9	3.4	153.5	6.6
		F = 3.13 sign.		F = 3.89 sign.		F = 8.33 sign.	
				$F_{0.95}$ (6.20) = 2.60			

Table 3. Bone Ca^{45} activity at different times after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in rats fed the normal laboratory ration and the low calcium diet. (M.-C. II dx.=right 2nd metacarpal bone, M.-C. II sin.=left 2nd metacarpal bone, M.-T. V dx.=right 5th metatarsal bone).

The plasma Ca^{45} activity at different periods after the Ca^{45} injection was examined in the long-term series (Fig. 3). All groups maintained on prolonged calcium restriction showed consistently higher values than those of the control group. The Ca^{45} activity showed throughout a decrease with time after the injection, which was more rapid for the control group than for the experimental groups.

The Ca^{45} activity of small bones at different periods after the Ca^{45} injection was also examined in the long-term series (Fig. 4, Table 3). In all experimental groups consistently higher values were obtained than in the control group. The

Groups	No. of animals	Ca^{45} activity C.P.M./mg ash	S.E.M.	
Normal laboratory ration	9	260.9	5.8	
Low calcium diet for 1 week	4	276.0	16.4	
„ „ „ „ 2 weeks	5	317.1	18.7	
„ „ „ „ 3 „	5	520.7	5.7	
„ „ „ „ 4 „	5	504.5	21.3	
„ „ „ „ 5 „	5	412.9	15.4	
„ „ „ „ 8 „	5	369.7	15.0	
„ „ „ „ 10 „	5	388.4	10.1	
„ „ „ „ 12 „	5	400.9	15.4	
„ „ „ „ 14 „	5	402.7	13.3	
„ „ „ „ 16 „	5	406.3	11.9	sign.
„ „ „ „ 6 months	4	370.5	41.6	
„ „ „ „ 7 „	4	401.1	20.5	
„ „ „ „ 8 „	3	375.0	18.2	
„ „ „ „ 9 „	5	371.8	18.9	
„ „ „ „ 10 „	4	405.0	31.5	
„ „ „ „ 12 „	3	334.1	16.2	
		$F = 16.89$ sign.		
		$F_{0.95}$ (16.64) $= 1.82$		
Calcium-enriched diet for 16 weeks	4	188.6	20.0	sign.

Table 4. The Ca^{45} uptake of the tibia (c.p.m./mg ash) 72 hours after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in rats fed the normal laboratory ration, the low calcium diet and the calcium-enriched diet.

values were significantly higher at all periods after the Ca^{45} injection in all the experimental groups and showed a slight increase with time after the isotope injection. In contrast, the values obtained from the rats of the control group showed a slight decrease, progressing with time after the injection of the isotope.

The Ca^{45} activity of the tibia 72 hours after the Ca^{45} injection (Fig. 5, Table 4). The values of the two groups of rats fed the normal laboratory ration showed no significant difference.

In the rats fed the low calcium diet for 1 to 4 weeks, successively higher values were found compared with those of the normal controls, with a maximum increase by approximately 100 per cent in the 3rd and 4th weeks. In the

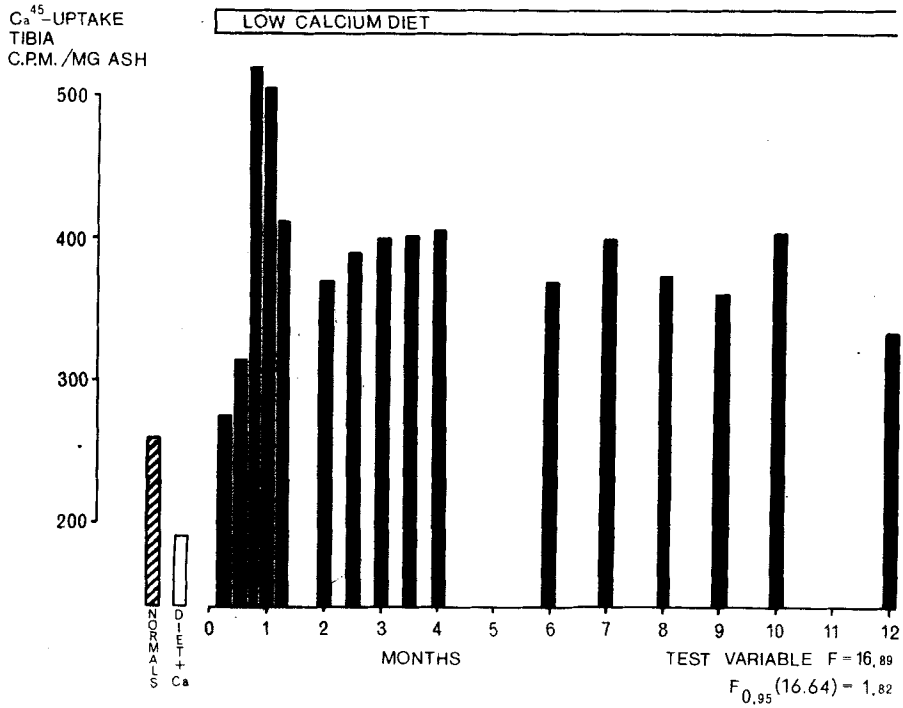

Fig. 5. The uptake of Ca^{45} in the tibia 72 hours after the intraperitoneal administration of 20 microcuries of Ca^{45} in rats fed the normal laboratory ration, the calcium-enriched diet and the low calcium diet.

following 5 to 16-week groups, the Ca^{45} uptake was increased by approximately 60 per cent. The increased Ca^{45} uptake observed between the 3rd and the 16th week of treatment was statistically significant. The six groups of rats kept on a restricted calcium intake for 6 to 12 months showed a consistently increased isotope uptake by the tibia by approximately 40 per cent of that of the control group. When the combined values of all these groups were compared with the control group, the increase was found to be significant. The animals fed the calcium-enriched diet for 16 weeks showed a significantly decreased Ca^{45} uptake compared with that of the control animals.

The Ca^{45} activity of cortical bone 72 hours after the Ca^{45} injection (*Table* 5).

The Ca^{45} uptake was further examined in cortical bone powder obtained from the rats of the long-term series. In the rats maintained on the low calcium diet for 6 to 9 months, the values obtained were approximately 20 per cent higher than in the normal control group. The two groups of animals fed the low calcium diet for 10 and 12 months showed only slightly higher values than those of the control group.

Groups	No. of animals	Ca^{45} activity C.P.M./mg ash	S.E.M.
Normal laboratory ration	5	64.6	2.7
Low calcium diet for 6 months	4	86.7	7.7
„ „ „ „ 7 „	4	86.3	3.6
„ „ „ „ 8 „	3	73.9	2.5
„ „ „ „ 9 „	5	74.0	3.0
„ „ „ „ 10 „	4	65.8	4.6
„ „ „ „ 12 „	3	69.7	5.6
		$F = 4.23$ sign.	
		$F_{0.95}(6.21) = 2.58$	

Table 5. The Ca^{45} uptake of compact bone powder (c.p.m./mg ash) 72 hours after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in rats fed the normal laboratory ration and the low calcium diet.

The calcium accretion rate for the tibia (*Table* 6).

The accretion rate (mg calcium/hour/mg calcium of the tibia) was examined in the rats of the long-term series. In the group of rats fed the low calcium diet for 6 months, the mean accretion rate was lower than in the control group. The four groups of animals maintained on a low calcium intake for 7 to 10 months showed approximately the same mean values as for the control group. Finally, a lower accretion rate was found in the group of rats kept on a restricted calcium intake for 12 months, compared with that in the control group.

Groups	ACCRETION RATE (mg Ca/hour/mg Ca of the tibia)
Normal laboratory ration	11.34×10^{-5}
Low calcium diet for 6 months	6.57×10^{-5}
„ „ „ „ 7 „	10.89×10^{-5}
„ „ „ „ 8 „	10.30×10^{-5}
„ „ „ „ 9 „	10.09×10^{-5}
„ „ „ „ 10 „	10.50×10^{-5}
„ „ „ „ 12 „	7.69×10^{-5}

Table 6. The mean calcium accretion rate for the tibia in rats fed the normal laboratory ration and the low calcium diet.

DISCUSSION

Material from the same animals was studied in a previous investigation, and it was then found that there was no significant difference in femur length between normal and experimental animals and that the skeletal growth and calcification were completed in these rats. The degree of mineralization studied by microradiography of undecalcified sections of vertebral bone was the same in normal and osteoporotic animals (*Larsson* 1969 a).

Although there is usually no abnormality in blood biochemistry in clinical osteoporosis, disturbances in calcium metabolism and the efficiency of calcium homeostasis in maintaining extracellular fluid calcium have been considered as the primary pathomechanism for the development of osteoporosis in humans (*Heaney* 1965). In this respect, it was considered of great interest to follow the changes in the blood calcium level and the distribution of Ca^{45} between blood and bone during the development of osteoporosis induced in these adult rats by prolonged calcium restriction. It has been reported earlier that old rats need a minimum of 0.5 per cent calcium in the diet for equilibrium (*Henry and Kon* 1947). By feeding a diet containing 0.13 per cent calcium and 0.38 per cent phosphorus, old rats remain in negative calcium balance for at least 4 months (*Henry et al.* 1960). In the present study adult male rats were maintained on a similar low calcium diet containing 0.09 per cent calcium and 0.55 per cent phosphorus with adequate amounts of vitamin D for periods varying from 1 week up to 12 months. Because of this restriction of the calcium intake, the animals treated for 14 to 16 weeks and longer developed significant skeletal changes characteristic of osteoporosis (*Larsson* 1969 a).

It was found in the present study that during the development of osteoporosis slightly subnormal blood calcium values appeared not only initially but also at later periods of time, with normalized values meanwhile. Corresponding to the transient initial fall in blood calcium induced in the present investigation by changing from a normal to a low calcium intake, subnormal blood calcium has been reported in healthy humans also after instituting a low calcium diet (*Nordin* 1964, *MacFadyen et al.* 1965, *Nordin et al.* 1965). This fall of the blood calcium level is certainly due to the large stresses upon the mechanisms involved in blood calcium regulation during a period of negative calcium balance. The hypocalcemia of calcium deprivation in humans is believed to be "transient" and very small changes in blood calcium concentration are considered to be corrected by bone mineral without the intervention of the parathyroid glands, due to the existence of a genuine steady state between bone mineral and tissue fluid (*Nordin et al.* 1965). However, the results of the present study indicate that subnormal blood calcium values occur at different periods of time during prolonged calcium restriction and that these changes are relatively slowly corrected by bone mineral (see below). The falls in the blood calcium level recorded in the present investigation were, further, of such a magnitude that stimulated parathyroid activity might have been involved for the normalization of the blood calcium concentration.

Under normal circumstances the well-documented constancy of blood calcium

(*Copp et al.* 1960) is exquisitely well regulated by means of a negative feedback (*McLean and Urist* 1955). Normally, the strain of rats used in the present study shows a markedly constant blood calcium. During an 8-week period, 8 adult normal male rats showed a blood calcium concentration of 4.8 – 4.9 mEq/l and the standard error of the mean with the same analytical method used in the present investigation ranged from 0.02 – 0.06 (*Johansson* 1966). Eventual seasonal variations as reported by *Biering* (1950) were eliminated in the present study since all blood samples were collected and analyses were performed on all animals within each experimental series at the same time. Neither was there any significant difference in the blood calcium level of the control groups of each series. In short-term, infusion experiments on dogs, an attempt was made to evaluate quantitatively the effectiveness of negative feedback in regulating blood calcium. The findings in those experiments support the view that the constancy of blood calcium is not due to an extremely efficient homeostatic regulation but must result in part from the relatively small stresses, positive or negative calcium loads, which are normally placed upon the system (*Hausmann and Riggs* 1966). The recurrent decreases in the blood calcium level observed at different periods of time in the present investigation, and which were followed by normalization, were probably due to the large stresses induced by the prolonged calcium restriction upon the mechanisms involved in blood calcium regulation.

Low calcium diets with a high phosphorus content have been reported to result in a fall of the blood calcium in different species, *e.g.* adult dogs (*Saville and Krook* 1968), adult cats (*Jowsey and Raisz* 1968) and adult rats (*Engfeldt et al.* 1954). In the present investigation it was found that the restriction of calcium alone with an adequate supply of phosphorus and vitamin D also causes a reduction of the blood calcium level. It is well known that there is a relationship between dietary levels of calcium and phosphorus and the parathyroids, and further, between high blood phosphorus concentration and depressed blood calcium, although the exact mechanism of these effects is not clear (*Nordin* 1961). However, decreased blood calcium has been reported in different species after institution of low calcium diets with very varying Ca: P ratios, such as *e.g.* 1: 45 (*Jowsey and Raisz* 1968), 1:16 (*Engfeldt et al.* 1954), 1:10 (*Saville and Krook* 1968), 1: 6.1 (the present investigation) and 1: 1.5 (*Campbell and Douglas* 1965). With regard to the relation between reduced calcium intake and the development of osteoporosis in humans, it is of interest that a slight fall in the blood calcium level occurs at different periods of time in adult rats during prolonged calcium restriction when the diet is normal in phosphorus and vitamin D. It is likely that this also occurs when the low

calcium intake is combined with various levels of dietary phosphorus. Changes in calcium metabolism and in the mechanisms involved in calcium homeostasis might then be expected in prolonged calcium restriction in aged humans, also.

The described changes in the blood calcium level and the distribution of Ca^{45} between blood and bone 72 hours after the intraperitoneal administration of the isotope (Fig. 6), provide special interest, especially with regard to the development of osteoporosis. Thus, the normal distribution of Ca^{45} rapidly changed upon restriction of the calcium intake. After only 1 week of calcium deficiency the rats showed an increase by approximately 100 per cent in the Ca^{45} specific activity in the blood and a slightly increased Ca^{45} activity in bone, indicating increased calcium retention. This was probably the result of decreased endogenous Ca^{45} and to a minor degree decreased urinary Ca^{45} secondary to the observed fall in blood calcium. The adaptation of the rat to a reduced calcium intake is brought about by increased absorption of calcium, and results in a decrease of the endogenous calcium (*Nicolaysen et al.* 1953, *Henry et al.* 1960). Also in humans adaptation to a reduced calcium intake is effected by a more efficient absorption (*Malm* 1958, *Thorangkul et al.* 1959).

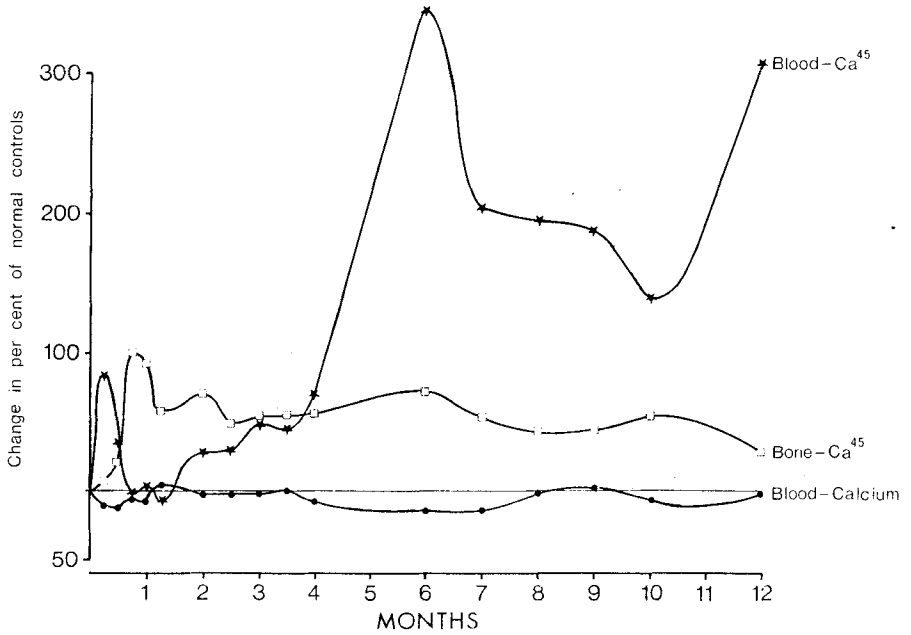

Fig. 6. For explanation, see text above.

After this initial phase, there was a further rapid change in the distribution of Ca^{45}. Despite a continued subnormal blood calcium concentration there was a decrease to the normal level of the specific activity of Ca^{45} in the blood during the 3rd week of maintained calcium restriction. At the same time there was a shift to a corresponding increase, by approximately 100 per cent, in the deposition of Ca^{45} into bone mineral. The same distribution of Ca^{45} was observed during the 4th and 5th weeks of calcium restriction. These findings indicate indirectly that the subsequent normalization of blood calcium after the hypocalcemic period was brought about by increased bone resorption and mobilization of skeletal calcium to the blood. The observed increase in the deposition of Ca^{45} into bone mineral appears to be secondary to the still more augmented mobilization of bone calcium.

During the 5th week of calcium deficiency the blood calcium returned to normal and subsequently remained at a normal level until the 14th week. Initially in this period, there was another shift in the distribution of Ca^{45} between blood and bone. Thus, at the 8th week of calcium restriction the Ca^{45} activity of both blood and bone had reached a level 50 to 60 per cent above that of the normal controls. Thereafter, this distribution of Ca^{45} remained unchanged until the 16th week.

These results clearly demonstrate a continued increased level of calcium retention during negative calcium balance induced by the maintained calcium restriction. In contrast, the group of rats fed the test diet enriched with calcium showed significantly decreased Ca^{45} activity in both blood and bone in comparison with the normal controls. The fact that all animals received the same dose of Ca^{45}, regardless of their body weight, does influence the present results but only to quite a small extent. The higher final body weight of the experimental animals compared with the controls was due to an increase in adipose tissues in the former. With such an increase in adipose tissues the percentage total body water is reduced (*Ljunggren et al.* 1957). This would imply that the experimental animals would have been afforded more Ca^{45} compared with that of the normal controls if the administered dose had been related to body weight. The results of the present study confirm earlier observations that animals accustomed to a high calcium intake must adjust their absorptive mechanism to a lower supply of calcium (*Carlsson* 1951, *Hansard et al.* 1951, *Nicolaysen et al.* 1953, *Henry et al.* 1960). It must be pointed out that this situation is quite different from that when the intake of calcium is low throughout life. In these circumstances rats bred on a low calcium diet remain in calcium equilibrium at levels which would lead to negative calcium balance in rats

bred on a higher or normal calcium diet (*Henry and Kon* 1953, *Nicolaysen* 1956). A similar adaptation is also well documented for humans (for ref. see *Henry and Kon* 1953, *Malm* 1958).

With the continuation of the restricted calcium intake, a slightly subnormal blood calcium was again observed in the rats kept on the low calcium diet for 16 weeks and 6 and 7 months. At the 8th month of calcium restriction a normalization of the blood calcium concentration occurred and thereafter it remained normal until the 12th month. A shift in the Ca^{45} distribution indicated that a new dynamic balance was induced in these animals by the prolonged calcium deficiency. Thus, the Ca^{45} specific activity in blood showed an increase by 100 to 200 per cent, while the Ca^{45} activity in bone was increased by only about 40 per cent. In these animals, the contribution of structural changes in cancellous bone to the balance and distribution of calcium must be considered. With the development of osteoporosis there is a loss of cancellous bone and of cortical bone from the endosteal surfaces as described in the first part of the present investigation (*Larsson* 1969 a). The amount of bone surfaces avaliable for intensive calcium deposition, *i.e.* surface accretion, is reduced. There was, further, no apparent change in the amount of bone surfaces showing new bone formation, as observed with the tetracycline double labelling technique. This demonstrates the importance of always considering these circumstances in dynamic calcium studies of normal and osteoporotic individuals. Except for a change in the distribution of Ca^{45} between blood and bone due to the observed reduction of cancellous bone, an accelerated calcium absorption, *i.e.* a further decrease in endogenous Ca^{45}, as suggested by *Nicolaysen et al.* (1953) and *Henry et al.* (1960), would also contribute to the highly elevated Ca^{45} specific activity in blood observed in the osteoporotic rats. By this regulation of the calcium distribution and by increased mobilization of bone calcium, the blood calcium concentration can be sustained in prolonged negative calcium balance.

The sequence of events reported here demonstrates that in induced negative calcium balance the efficiency of calcium homeostasis in maintaining extracellular fluid calcium is the primary pathomechanism for the development of calcium deficiency osteoporosis.

The Ca^{45} activity of pooled cortical bone powder 72 hours after administration of the isotope was found to be less than 1/4 of that of the whole tibia in the normal animals. This relationship is of about the same order of magnitude as the corresponding ratio of tibia shaft to whole tibia found in normal young rats by *Bauer and Carlsson* (1955). It is well established that the calcium metabolism is more intensive in the ends than in the shaft of long bones, and

in trabecular compared with cortical bone tissue. As for the whole tibia the Ca^{45} activity of the cortical bone powder in the present study was significantly higher for the long-term calcium-deficient rats than for the normal controls. In the 6 groups of rats fed the low calcium diet for 6 to 12 months the mean Ca^{45} uptake by the cortical bone was only 19 per cent higher, while the mean uptake by the whole tibia was 41 per cent higher than in the normal controls. Contributory factors to these differences of 19 to 41 per cent might be the structural changes in cancellous bone described above and, further, the higher Ca^{45} activity of the bone marrow blood in the osteoporotic animals compared with the normal controls.

The Ca^{45} uptake by the small bones at different time intervals after the administration of the isotope showed a significant increase progressing with time after the isotope injection in the groups of rats maintained on prolonged calcium restriction, while there was a slight tendency to a decrease in the normal control group. This difference could be explained by the consistent high Ca^{45} activity in the blood in the calcium-deficient groups, which would afford the bone more Ca^{45} to be built in, compared with the normal controls.

In clinical osteoporosis, the bone accretion rate, or mineralization rate, is usually reported to be normal (*Heaney and Whedon* 1958, *Fraser et al.* 1960, *Dow and Stanbury* 1960, *Dymling* 1964, *Nordin et al.* 1964), although both reduced accretion (*Bauer et al.* 1957, *Eisenberg and Gordan* 1961, *Dymling* 1962, *Bronner et al.* 1963, *Lafferty et al.* 1964) and increased accretion (*Nordin* 1959) have also been observed. It was therefore considered of interest to estimate the calcium accretion rate in the osteoporotic animals of the present study. Estimations were performed on the whole tibia (mg calcium/hour/mg calcium of the tibia) of the rats of the long-term series. In the group of rats maintained on a low calcium diet for 6 months, a lower mean accretion rate was found than in the control group, whereas in the groups kept on a restricted calcium intake for 7, 8, 9 and 10 months approximately the same values were found as in the normal control group. Finally, the group of animals fed the low calcium diet for 12 months also showed a decreased accretion rate. In 4-month old rats fed a low calcium diet (calcium 0.037 per cent, phosphorus 0.45 per cent) for 6 months, a normal accretion rate was found while the resorption was increased (*Copp and Suiker* 1962). These results are in agreement with those of the present study. However, in the study of *Copp and Suiker* (1962) the effect of the treatment upon the skeletal tissue was not investigated further. The findings concerning the rate of calcium accretion in the present investigation indicate that the skeleton rendered osteoporotic by prolonged

calcium restriction retains calcium to approximately the same extent as the normal skeleton, although a reduced accretion was found in some groups of rats. These results are in good agreement with those generally reported for human osteoporosis. However, when the rate of calcium accretion by bone in osteoporotic individuals is compared with that in normals, differences in bone mass must be considered. Furthermore, the higher Ca^{45} activity in the bone marrow blood of the osteoporotic animals in the present study compared with that of the normal controls will also influence the obtained values. It must be pointed out, moreover, that "bone accretion" cannot be equated with bone formation, which has been done in many earlier studies. In the present investigation calcium accretion is interpreted as a measure only of the retention of calcium by the whole bone, and not of the formation of new bone. In a previous study (*Larsson* 1969 a), no appreciable difference from the normal was found in the amount of bone surfaces showing new bone formation, as estimated by the tetracycline labelling method. Normal female rats of the same strain and age as the normal male rats of the present study showed a lower frequency of tetracycline labelled surfaces of the vertebral bone (*Larsson* 1969 b) and also a lower bone Ca^{45} retention (*Larsson* 1969 c). These findings might indicate some relationship between accretion and bone formation. Nevertheless, it is well known that the amount of tracer localized as "diffuse uptake" in the bone tissue is both high and variable and this usually makes such a correlation difficult to establish (*Rowland* 1960).

In the pathogenesis of generalized osteoporosis in humans, a low intake or malabsorption of calcium has been considered to play an important role (*Nordin* 1960, 1961, 1962, 1964), as well as the efficiency of calcium homeostasis (*Heaney* 1965). After the institution of a low calcium diet in human volunteers, a negative calcium balance has been observed for relatively long periods of time (*Malm* 1958). However, there is no evidence to prove that calcium deficiency really gives rise to osteoporosis in humans. The adaptation to a low calcium intake has been studied extensively in old rats by calcium balance techniques (*Henry and Kon* 1947, 1953, *Barucha and McCay* 1954, *Nicolaysen* 1943, 1955, *Henry et al.* 1960). In humans, principally the same adaptory mechanisms are responsible as in the rat (*Nicolaysen et al.* 1953). In a previous investigation (*Larsson* 1969 a) the development of osteoporosis was observed in old rats after prolonged calcium restriction. The findings presented here show that significant changes in the calcium dynamics occur during this process of bone reduction. It is suggested that this course of events proceeds in different phases in the adaptation to a low calcium intake. Initially and also later, the

blood calcium level is subnormal due to the low calcium intake. Because of the efficiency of calcium homeostasis, the blood calcium level is normalized. The Ca^{45} data presented here indicate indirectly that this normalization of the blood calcium level is brought about by increased bone resorption, mobilizing skeletal calcium to the blood at the expense of the bone mass.

SUMMARY

One-year-old fully grown rats were maintained on a low calcium diet with normal contents of phosphorus and vitamin D for periods varying from 1 week up to 1 year. Seventy-two hours before sacrifice, 20 microcuries of Ca^{45} were administered to each animal by intraperitoneal injection, and the Ca^{45} activity in plasma and bone was compared with that of normal controls and, in addition, with that of animals fed the test diet enriched with calcium.

The plasma Ca^{45} activity 72 hours after the isotope injection showed increased values by 100 per cent in the group of rats fed the low calcium diet for 1 week when compared with that of the normal controls. The groups of animals kept on a restricted calcium intake for 3 and 4 weeks showed the same values as the controls. In these experimental groups there was a significant increase by 100 per cent in the deposition of Ca^{45} into the skeleton, while there was a concomitant, slight fall in the plasma calcium. In the groups of rats fed the low calcium diet for 8 to 14 weeks, the plasma calcium was normal, and in these groups there was an increase in the plasma and bone Ca^{45} activity by 50 to 60 per cent. In the groups of animals kept on the low calcium intake for 4 to 7 months, the plasma calcium was again slightly reduced. These groups, and those treated for 9 to 12 months, showed an increase in the plasma Ca^{45} activity by 100 to 200 per cent, whereas the Ca^{45} activity in bone was increased by 40 per cent. The groups treated for 8 to 12 months showed normalized plasma calcium. In the group of rats fed the diet enriched with calcium, the plasma Ca^{45} activity was 40 per cent lower and the bone Ca^{45} activity 60 per cent lower than in the control group. The plasma calcium showed normal values.

The calculated calcium accretion rate for the tibia (mg calcium/hour/mg calcium of the tibia) was described in rats fed the low calcium diet for 6 and

12 months, while in rats treated for 7, 8, 9 and 10 months the values were approximately the same as in the control group.

The results show that a slight initial fall in plasma calcium occurs on instituting a low calcium diet to adult rats, and also at later stages subnormal plasma calcium values can be observed. The findings concerning Ca^{45} indicate indirectly that the normalization of plasma calcium is brought about by increased mobilization of skeletal calcium to the blood. This results in a reduction of bone mass and the development of osteoporosis.

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Studies on the Development of Experimental Osteoporosis

III. The Effect of Combined Oophorectomy and Prolonged Prednisolone Administration on the Skeletal Tissue of Adult Rats

by

Sven-Erik Larsson

INTRODUCTION

The hypothesis that oestrogen deficiency is the cause of human osteoporosis was advanced by *Albright and Reifenstein* (1948). This hypothesis was modified to include the possibility that senile as well as other forms of osteoporosis are caused by a state of lack of balance between anabolic or gonadal hormones and anti-anabolic or adrenal corticoid hormones (*Reifenstein* 1957). Hypercorticoidism of endogenous origin in humans is often associated with osteoporosis (*Follis* 1951). Osteoporosis is, moreover, one of the most serious complications in prolonged cortisone therapy (*Bunim et al.* 1955, *Rosenberg* 1958).

Osteoporosis has been induced experimentally in young rabbits by cortisone administration (*Sissons and Hadfield* 1955, *Storey* 1957). *Sissons* (1956) pointed out the similarity of these lesions to those observed in Cushing's disease. As far as the rabbit is concerned, it has been concluded that the effect of cortisone is inhibitory to new bone formation and that bone rarefaction is due to the unopposed continuance of the normal osteolytic processes (*Sissons and Hadfield* 1955, *Sissons* 1956). From histological observations it has also been deduced that cortisone osteoporosis results, not only from a cessation of bone formation, but primarily from a direct degrading effect which causes a massive resorption of bone (*deValderrama and Munuera* 1965).

Unlike the rabbit, the young growing rat does not always develop osteoporosis after cortisone administration. Instead, dense metaphyseal bone has been observed. This was believed to be due to delayed bone resorption (*Follis* 1951). However, when the dietary Ca and P are lowered or the ratio altered during cortisone administration, also young rats develop osteoporosis similar to that seen in rabbits treated with cortisone. In cortisone-treated rats maintained on a diet containing 1.5 per cent Ca and 1.0 per cent P, increased density of the metaphyseal bone occurs. At a normal Ca:P level, *i.e.* 1.0 per cent Ca and 0.6 per cent P, or a reduced level, osteoporosis develops. This difference in the reaction of the bone has been believed to be associated with the well developed adaptive calcium metabolism of young rats (*Storey* 1960).

Growing male rats subjected to bilateral adrenalectomy and orchidectomy become more sensitive to cortisone. In spite of a high calcium intake, cortisone

treatment results after 100 days in radiographically evident osteoporosis. Oestradiol cures the osteoporosis in these animals despite continuous cortisone administration, while only very slight improvement occurs in rats given testosterone (*Caldwell* 1962). The curative effect of oestrogens has also been observed in birds rendered severely osteoporotic with large doses of ACTH or cortisone (*Urist* 1958). In young rats, but not in other animals, oestrogens inhibit bone resorption (*Budy et al.* 1952, *Lindquist et al.* 1960).

The inhibitory effect of excess cortisone upon normal skeletal growth is well known. In the experiments performed on rabbits, young growing animals were used and, in addition to the osteoporotic changes, inhibited bone growth was observed (*Sissons and Hadfield* 1955, *Storey* 1957, *deValderrama and Munuera* 1965). This inhibitory effect is also well documented for young rats (*Follis* 1951, *Laron et al.* 1958, *Kowalewski* 1962, *Stanisavljevic et al.* 1962, *Hulth and Olerud* 1963, *Tapp* 1966). As far as the young growing animal is concerned, both interference with normal skeletal growth and a possible eventual effect on the calcium metabolism must be taken into consideration. In view of the inhibitory effect of cortisone on the formation of connective tissues (*Asboe-Hansen* 1961), inhibited synthesis of bone matrix is, furthermore, to be expected.

The present investigation was undertaken in order to examine the effect of combined oophorectomy and prednisolone administration on the mature skeleton of one-year old, fully grown rats and to study the eventual development of skeletal changes during the progress of treatment. The aim of this study was to determine whether this hormonal disturbance does, in fact, give rise to bone reduction of the mature skeleton of the adult rat as might be expected from the results concerning growing rats in the experiments of *Caldwell* (1962).

EXPERIMENTAL

Animals.

A total of 55 adult female rats of the Sprague-Dawley strain[1] were used. All animals were stated to be 11 1/2 to 12 months old at the start of the experiment and their mean initial body weight was 348.5 ± 2.7 grams. The animals were divided at random into ten experimental groups and one control group of 5

[1] Anticimex Co., Stockholm, Sweden.

rats each. The experimental animals were oophorectomized under ether anaesthesia via a midline abdominal incision under sterile conditions. The identification of the first ovaries removed was verified by histological examination. All the animals recovered very quickly after the operation. No postoperative complications were observed. From the third day after oophorectomy each animal received 2.5 mg prednisolone[1] daily, dissolved in their drinking water, which was supplied intermittently in such a way that the prednisolone was consumed within 1—2 hours. The periods of prednisolone treatment varied from 1 to 16 weeks. The *control* group consisted of *normal* rats and was observed for 16 weeks. These animals were not oophorectomized and received no prednisolone but were otherwise treated in the same way as the experimental animals.

The animals were housed individually in screen-bottomed wire cages in a separate room without access to direct sunlight and with a room temperature of 18—20°C. All animals received the same laboratory ration[2] and tap water in unrestricted quantities, except for the intermittent supply of drinking water in the experimental animals during administration of prednisolone, as mentioned above. Chemical analysis of the diet given showed that it contained a total of 1.0 per cent calcium and 0.55 per cent phosphorus. The quantity of food consumed was estimated for one animal of each group during a period of 5 days, starting at the 7th day before sacrifice, by recording daily the weight of the pellets given to each animal and the weight of the food not consumed. No antibiotics were given. The series were planned so that all the animals were sacrificed at the same time. This was performed by bleeding them to death from the femoral artery under ether anaesthesia.

Methods.

The methods used are essentially the same as described in greater detail in a previous paper (*Larsson* 1969 a).

Body weight and weight of the adrenals.

All the animals were weighed weekly. From all animals the adrenal glands were removed after sacrifice. The glands were dissected free from all surrounding fat tissue, they were immediately placed in weighing containers and their wet weight was recorded on a Mettler balance with an accuracy of 0.05 mg.

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[1] Precortalon Aquosum®; kindly supplied by Pharmacia Ltd., Uppsala, Sweden.
[2] Ewos Co., Södertälje, Sweden.

Radiography.

The skeleton of each animal was examined radiographically under light ether anaesthesia at the beginning and end of the experimental period. Each rat was placed with the spinal column and the extremities fixed directly on an X-ray film. The same exposure conditions, film and developer were used throughout.

Measurement of the cortical thickness of the mid-shaft of the femur.

This was performed after division of the bone at the middle of the diaphysis, (± 0.5 mm), perpendicular to its long axis. The internal and external transverse diameters were carefully measured with the aid of a vernier caliper at the four cut surfaces of the two divided femora of each animal. Mean values were used. The cortical thickness at this point was calculated as the difference between the external and the internal diameter and with an accuracy of 0.05 mm. This figure was divided by the value for the external transverse diameter of the shaft at the same level and this fraction was multiplied by 100. In this way the femoral score was calculated according to *Barnett and Nordin* (1960).

Ash determinations.

The ash content of the whole, right tibia including the bone marrow and articular cartilages was determined as described previously (*Larsson* 1969 a), and was expressed as per cent of the wet weight.

Hydroxyproline determinations.

The hydroxyproline content of the whole third metacarpal bone including the bone marrow and the articular cartilages was determined by the method of *Neuman and Logan* (1950). All bones within the whole series were dried simultaneously at 48°C for 24 hours and were then weighed in weighing containers. Protein hydrolysates were prepared by autoclaving the whole bone with 0.5 ml of 6 N hydrochloric acid in sealed tubes for 10 hours at 100°C. Duplicate determinations were performed throughout, and the mean value was used. The coefficient of variation for these analyses was 2.0 per cent.

Density of the dried humerus.

The density of the dried, whole bone including the bone marrow and articular cartilages was determined according to the method of *Robinson and Elliott* (1957).

Preparation of undecalcified sections.

The first tail vertebra of all the rats was freed of most of the surrounding soft tissues, and then fixed and dehydrated in 6 changes of 96 per cent alcohol for 6 days. The specimens were embedded in methacrylate as described earlier (*Larsson* 1969 a). Using a fine bandsaw, the trimmed plastic blocks were cut into sections approximately 0.5 mm thick and exactly perpendicularly to the long axis of the vertebra.

Quantitation of the amount of vertebral bone.

Quantitative determination of bone present in a standard area was performed as follows: Undecalcified, methacrylate-embedded sections from the first tail vertebra were ground, first on an oscillating grinder and then by hand, to a thickness of approximately 50 μ. Only that section which contained most of the transverse processes was examined, unstained, with the aid of an integrating eyepiece (Zeiss I) as described by *Wagner* (1965). The number of vertebral bone "hits" of 200 points was determined with the point network exactly in the centre of the section, at a linear magnification of 30 ×.

Microradiography.

Of all the animals, undecalcified methacrylate-embedded cross sections from the second tail vertebra were ground on a Knuth-Rotor grinder to a thickness of approximately 80 μ with water as a lubricant and using Silicone-Carbide papers Nos. 220 and 600. The sections were radiographed on Kodak MR-plates in a Philips PW 1009 apparatus. The procedure was the same as described previously (*Larsson* 1969 a).

Tetracycline labelling.

All animals were "labelled" with chlortetracycline (Aureomycin®, Lederle) in 2 doses of 20 mg per kg body weight administered by intraperitoneal injection 10 and 20 days before sacrifice. Approximately 60 μ thick undecalcified, methacrylate-embedded cross sections from the second tail vertebra were mounted under coverslips in fluorescence-free balsam (Permount) and were examined in a fluorescence microscope (Zeiss) fitted with an UV High Pressure Vacuum lamp HBO 200 with exciter filters UG 2 and UG 5 and barrier filters 65 and 41.

Preparation of decalcified sections.

The third tail vertebra of all animals was fixed in 10 per cent neutral formalin for 24 hours and then decalcified for 12—14 days in a mixture of equal parts of 20 % monosodium citrate and 44 % formic acid. The specimens were then immersed in 5 per cent disodium sulphate for 24 hours, washed with water for 24 hours and dehydrated by successive immersion in 70 per cent, 95 per cent and absolute alcohol (2 changes). The specimens were then immersed first in methyl benzoate, and then in xylol, and were finally embedded in paraffin. Sections 5 μ thick were cut and were stained with Ehrlich's haematoxylin and eosin and, in addition, azocarmine G. Adjacent sections were also stained with an 0.5 per cent solution of toluidine blue in phthalate at pH 4.4 as described by *Bélanger and Hartnett* (1960).

Preparation of histological sections of the adrenals.

The adrenal glands from the normal controls and the animals treated for 14 and 16 weeks were fixed in 10 per cent neutral formalin, embedded in paraffin and cut into sections 5 μ thick. Representative sections were stained with Ehrlich's haematoxylin and eosin.

Statistical treatment of data.

The analytical data obtained from the rats in each group were subjected to analysis of variance with one-way lay-out according to *Scheffé* (1961). The hypothesis of no difference between groups was tested at the 5 per cent significance level. When a significant result was obtained, data of one or more groups were combined in order to find significant contrasts. These were tested according to *Scheffé* (1961). In the present study the term "significant" stands for "statistically significant" as found with the tests described above.

RESULTS

All animals survived the treatment except one. This animal was sacrificed because of a parotid tumour and was excluded. No infections or any other intercurrent diseases occurred. The normal control animals showed a slight increase in body weight by 5 to 7 per cent during the first 6 weeks. Subsequently,

Groups	No. of animals	Initial body weight, grams	S.E.M.	No. of animals	Final body weight, grams	S.E.M.
Normal controls	5	335.8	6.0	5	312.4	4.2
Ooph. + prednisolone for 1 week	5	359.5	7.2	4	285.6	5.3
„ „ „ 2 weeks	5	354.6	10.4	5	285.6	9.7
„ „ „ 3 „	5	359.3	7.0	5	271.5	8.7
„ „ „ 4 „	5	338.5	7.3	5	289.5	13.6
„ „ „ 5 „	5	360.2	9.7	4	283.0	13.0
„ „ „ 8 „	5	358.2	7.2	5	270.0	10.4
„ „ „ 10 „	5	338.8	5.2	4	265.4	5.6
„ „ „ 12 „	5	343.4	5.2	4	257.3	8.6
„ „ „ 14 „	5	327.4	7.3	5	263.8	14.7
„ „ „ 16 „	5	362.2	11.6	5	252.9	8.9
		$F = 2.43$ sign.			$F = 2.95$ sign.	
		$F_{0.95}(10.44) = 2.05$			$F_{0.95}(10.40) = 2.08$	

Table 1 a. The initial and final body weights of normal control rats and oophorectomized rats treated with prednisolone for different periods of time (S.E.M. = standard error of the mean).

until the end of the 16th week, the body weight remained unchanged. All the experimental animals showed a considerable loss of body weight (Table 1 a, b, Fig. 1). During the first 3 weeks of prednisolone treatment there was a decrease in body weight by approximately 20 per cent, after which there was a further decrease by 10 per cent of the initial value. The estimated food consumption was found to be slightly less than normal during the first 2 weeks of prednisolone treatment. Subsequently, there was no appreciable change in food intake in relation to body weight. Considerable depletion of the adipose and muscle tissues and atrophy of the skin occurred in all corticosteroid-treated animals. Otherwise the animals were in good health and moved about freely in their cages.

Weeks	Normal controls %	S.E.M.	Oophorectomy + Prednisolone %	S.E.M.
1	4.0	0.90	– 13.6	1.90
2	4.4	1.39	– 17.9	2.19
3	– 0.5	2.22	– 19.4	1.67
4	– 3.0	1.62	– 20.4	1.96
5	2.9	1.70	– 21.5	1.73
6	5.1	2.29	– 19.7	1.87
7	8.3	1.39	– 19.7	1.41
8	7.9	1.51	– 20.2	1.74
9	7.8	2.43	– 18.7	1.77
10	7.3	1.54	– 21.8	1.70
11	5.9	2.01	– 24.2	1.71
12	7.4	1.23	– 27.6	2.06
13	7.2	1.16	– 26.8	1.46
14	6.1	0.58	– 27.0	1.42
15	5.9	1.35	– 28.3	2.00
16	5.4	1.32	– 30.1	2.08
	F = 3.71 sign.		F = 6.27 sign.	
	$F_{0.95}$ (15.64) = 1.84			

Table 1 b. The percentage change in body weight of the initial value in normal control rats and in oophorectomized rats treated with prednisolone for 16 weeks.

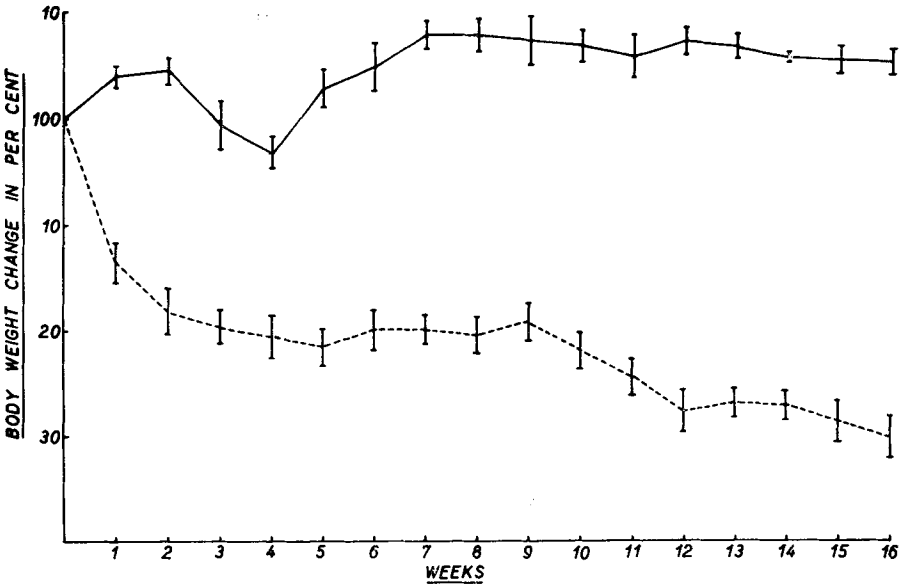

Fig. 1. Body weight change in per cent of the initial weight in normal control rats (whole line) and in oophorectomized rats treated with prednisolone for 16 weeks (broken line). The bars represent the standard error of the mean.

The length of the femur showed no significant difference between the normal control group and the groups of oophorectomized rats treated with prednisolone for 1 to 16 weeks.

Histological examination of the ovaries removed from ten animals revealed both primary and secondary follicles in all specimens.

Radiography.

The radiograms of the skeleton showed closed epiphyseal zones in all animals at the start of the experimental period. On comparison of the radiograms obtained at the start and at the end of the experimental period, no noteworthy changes in the calcification of the skeleton were observed in any of the animals.

Cortical thickness at the mid-shaft of the femur (Table 2).

In the ten groups of oophorectomized rats treated with prednisolone for 1 to 16 weeks, consistently lower mean values were found when compared with that of the normal control group. In the groups treated for 8 and 16 weeks, respectively, the calculated femur score was significantly lower than that of the control group. The mean values of the nine groups of rats treated for 2 to 16 weeks were approximately 10 to 20 per cent lower than that of the control group.

Groups	No. of animals	Femur score	S.E.M.
Normal controls	5	43.5	2.02
Ooph. + prednisolone for 1 week	5	39.9	2.13
„ „ „ 2 weeks	5	37.0	1.27
„ „ „ 3 „	5	36.6	1.56
„ „ „ 4 „	5	35.4	0.73
„ „ „ 5 „	5	37.3	1.41
„ „ „ 8 „	5	34.3	0.54
„ „ „ 10 „	5	38.4	1.03
„ „ „ 12 „	4	35.8	0.79
„ „ „ 14 „	5	37.7	0.71
„ „ „ 16 „	5	33.3	1.76
		$F = 4.07$ sign.	
		$F_{0.95}\ (10.43) = 2.06$	

Table 2. The "femur score" in normal control rats and in oophorectomized rats treated with prednisolone for different periods of time.

Percentage of vertebral bone "hits" (Table 3).

Compared with the control group, consistently lower mean values were found in all experimental groups. The three groups of rats treated for 12 to 16 weeks showed mean values 15 to 18 per cent lower than that of the normal controls. This difference was not significant, however.

Groups	No. of animals	% Hits	S.E.M.
Normal controls	5	40.1	1.03
Ooph. + prednisolone for 1 week	4	34.0	3.87
„ „ „ 2 weeks	5	36.2	2.26
„ „ „ 3 „	5	32.9	2.75
„ „ „ 4 „	5	38.1	1.79
„ „ „ 5 „	5	35.0	1.97
„ „ „ 8 „	5	40.4	1.59
„ „ „ 10 „	5	37.6	1.55
„ „ „ 12 „	4	32.3	3.20
„ „ „ 14 „	5	33.9	1.78
„ „ „ 16 „	5	34.9	2.27
		$F = 1.55$ n.s.	
		$F_{0.95}(10.42) = 2.06$	

Table 3. The percentage of vertebral bone "hits" in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Groups	No. of animals	% Ash	S.E.M.	
Normal controls	5	50.4	0.39	
Ooph. + prednisolone for 1 week	5	47.7	0.41	
„ „ „ 2 weeks	5	47.8	0.51	
„ „ „ 3 „	5	49.5	0.66	
„ „ „ 4 „	5	48.4	0.46	
„ „ „ 5 „	5	49.4	0.68	
„ „ „ 8 „	5	48.0	0.63	
„ „ „ 10 „	5	49.7	0.69	
„ „ „ 12 „	4	48.9	0.41	
„ „ „ 14 „	5	47.4	0.60	sign.
„ „ „ 16 „	5	47.2	0.51	
		$F = 3.64$ sign.		
		$F_{0.95}(10.43) = 2.06$		

Table 4. The percentage ash content of the whole tibia in normal control rats and in oophorectomized rats treated with prednisolone for different periods of time.

Ash content of the whole tibia (Table 4).

The two groups of oophorectomized rats treated with prednisolone for 1 and 2 weeks showed values approximately 5 per cent lower than those of the normal control group. This difference was not significant, however. Only slightly decreased values were found in the six groups of rats treated with prednisolone for 3 to 12 weeks. The two groups of rats treated with prednisolone for 14 and 16 weeks showed values more than 6 per cent lower than those of the control group. This difference was significant.

Hydroxyproline content of the metacarpal bone (Table 5).

All the experimental groups showed mean values which were consistently lower than that of the control group. These differences were not significant, however.

Groups	No. of animals	‰ Hydroxy-proline	S.E.M.
Normal controls	5	26.1	0.38
Ooph. + prednisolone for 1 week	5	25.6	0.60
„ „ „ 2 weeks	5	24.7	1.02
„ „ „ 3 „	5	24.5	0.67
„ „ „ 4 „	5	25.3	0.56
„ „ „ 5 „	5	24.5	0.62
„ „ „ 8 „	5	25.0	0.57
„ „ „ 10 „	5	25.5	0.44
„ „ „ 12 „	4	25.5	0.86
„ „ „ 14 „	5	24.5	0.42
„ „ „ 16 „	5	25.0	0.39
		$F = 0.73$ n.s.	
		$F_{0.95}(10.43) = 2.06$	

Table 5. The promille hydroxyproline content of the metacarpal bone in normal control rats and in oophorectomized rats treated with prednisolone for different periods of time.

Density of the dried humerus (Table 6).

All the experimental groups except the group treated for 2 weeks showed mean values which were consistently lower than that of the control group. These differences were not significant, however.

Groups	No. of animals	Density	S.E.M.
Normal controls	5	2.34	0.040
Ooph. + prednisolone for 1 week	5	2.23	0.069
„ „ „ 2 weeks	5	2.33	0.092
„ „ „ 3 „	5	2.20	0.106
„ „ „ 4 „	5	2.18	0.022
„ „ „ 5 „	5	2.08	0.075
„ „ „ 8 „	5	2.16	0.109
„ „ „ 10 „	5	2.24	0.111
„ „ „ 12 „	4	2.16	0.028
„ „ „ 14 „	5	2.10	0.039
„ „ „ 16 „	5	2.14	0.104
		$F = 1.09$ n.s.	
		$F_{0.95}(10.43) = 2.06$	

Table 6. The gross bone density of the humerus in normal control rats and in oophorectomized rats treated with prednisolone for different periods of time.

Microradiography.

The microradiographs of undecalcified vertebral cross sections from the animals in the control group showed that both the cortical and the trabecular bone were evenly mineralized (Fig. 2). In this nonlamellar (woven) bone there were no secondary osteones and no resorption cavities. In all experimental animals, microradiography showed that the cortical and the trabecular bone had the

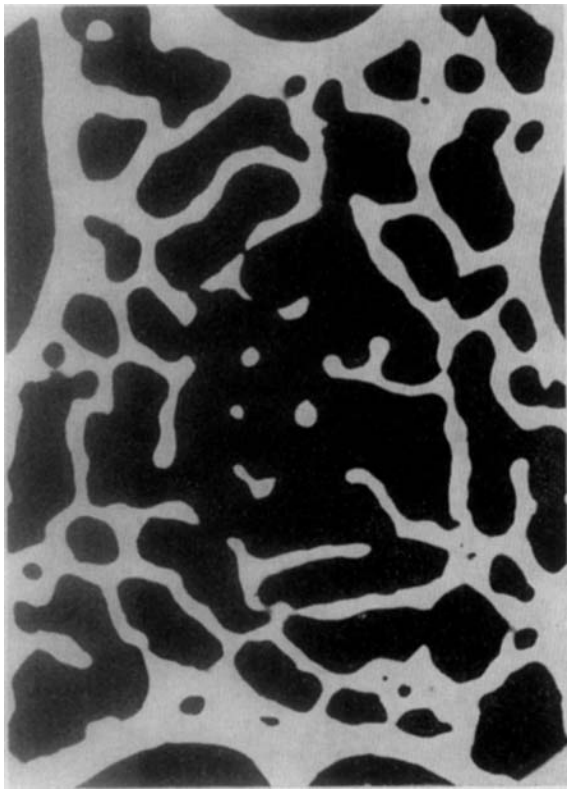

Fig. 2. Microradiograph of a cross section through the second tail vertebra of a normal rat. (x25).

same degree of mineralization and were as evenly mineralized as in the control animals. In the experimental animals the bone trabeculae appeared slightly thinner than in the normal controls, but otherwise no differences were found (Fig. 3). No resorption cavities were seen.

Histological observations.

In all animals decalcified cross sections of the third tail vertebra were stained with toluidine blue at pH 4.4 and, in addition, with haematoxylin-eosin and azocarmine G. The sections were cut from parts of the vertebra chosen at random in the different animals. In each animal, however, all the sections were

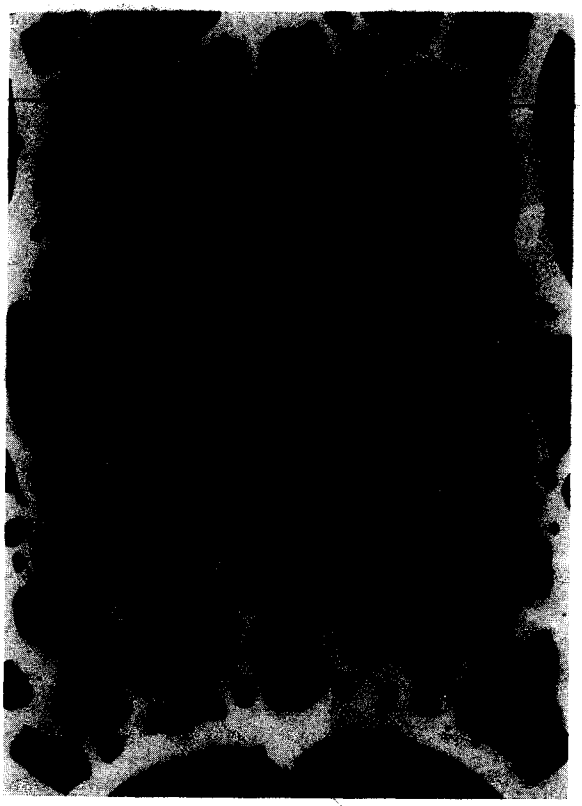

Fig. 3. Microradiograph of a cross section through a corresponding part of the second tail vertebra of a rat after combined oophorectomy and prednisolone administration for 16 weeks. The bone trabeculae are slightly thinner than normal. (x25).

cut from the same part of the vertebra, allowing adjacent sections to be examined after the three different staining proceduces.

In both the cortical and the trabecular bone of the normal animals, osteocytes with enlarged, almost spherical lacunae were frequently found. These osteocytes had often retracted to the lacunar wall and showed a pyknotic nucleus (Fig. 4). Lacunae which were empty of cells were regularly found, especially in the cortical bone mid-way between the marrow cavity and the periosteum but often in the cortical bone layers nearest to the cavity and also in the bone trabeculae. These lacunae and the canaliculi stained violet with haematoxylin-eosin and were metachromatic with toluidine blue. The immediate border of the osteocyte lacunae and the canaliculi stained violet with haematoxylin-eosin and were metachromatic with toluidine blue. This was often found also in lacunae empty of cells. No osteoid tissue was observed, nor osteoblasts or osteoclasts.

Distinct areas showing slight basophilic staining with haematoxylin-eosin were regularly found in the trabecular bone of the metaphyseal parts of the vertebra (Fig. 5). Nearer the midportion of the vertebra, the basophilic areas decreased in frequency and were not seen in the cortical bone. They stained only faintly blue with azocarmine G and were located in the spaces intervening between parallel-fibered bone staining deep blue (Fig. 6). These areas showing basophilic staining with haematoxylin-eosin gave evident metachromasia with toluidine blue (Fig. 7).

In the experimental animals the same histological observations were made as in the normal controls. The osteocytes, the lacunae, the capsule of the lacunae and the canaliculi had the same appearance and showed the same staining properties in the prednisolone-treated, oophorectomized animals as in the controls. No difference in the frequency of hypertrophic osteocytes and lacunae empty of cells was observed. Neither were osteoid tissue, osteoblasts or osteoclasts

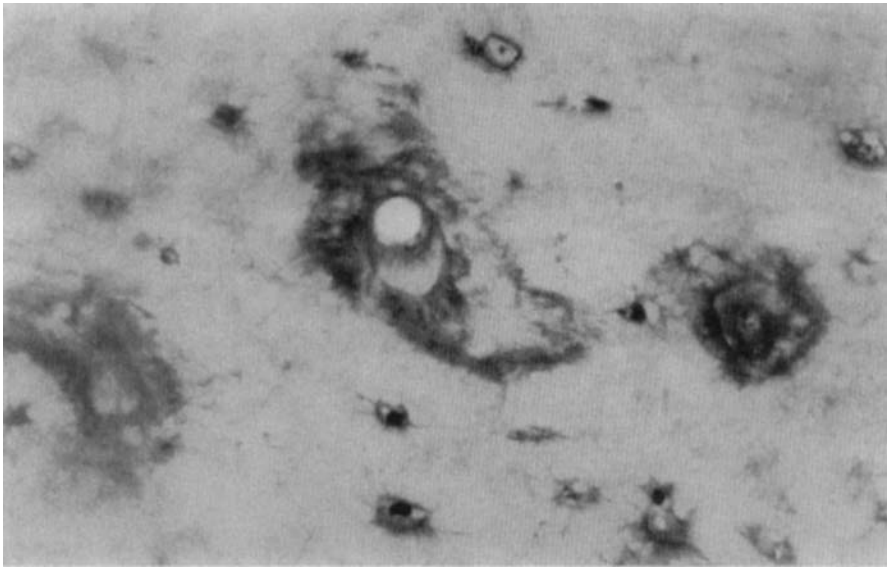

Fig. 4. Photomicrograph of cortical bone from a vertebra of a normal rat. Osteocytes with enlarged lacunae can be seen. There are also empty lacunae. Haematoxylin-eosin. (x500).

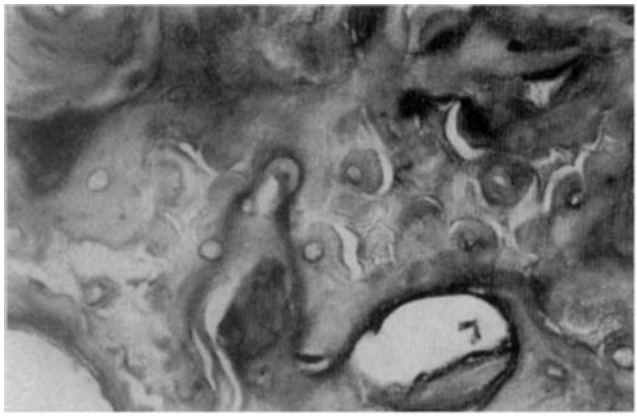

Fig. 5. Photomicrograph of vertebral bone from a normal rat, showing basophilic areas. Haematoxylin-eosin. (x500).

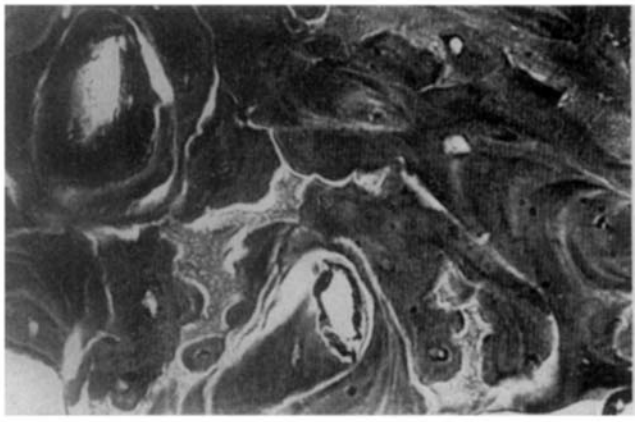

Fig. 6. Photomicrograph of an adjacent section of the vertebral bone shown in Fig. 5. The basophilic areas are light and located between the dark collagen bundles. Azocarmine G (x500).

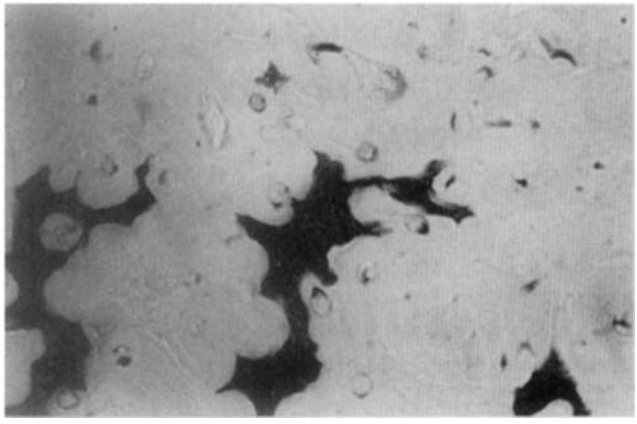

Fig. 7. Photomicrograph of an adjacent section of the vertebral bone shown in Figs. 5 and 6. The basophilic areas show evident metachromasia. Toluidine blue at pH 4.4. (x500).

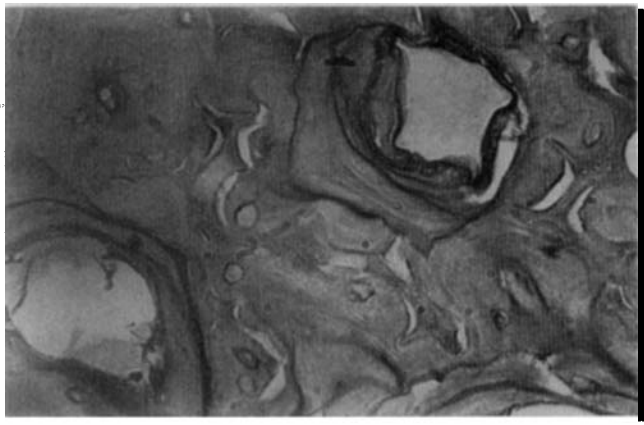

Fig. 8. Photomicrograph of vertebral bone from a rat treated with combined oophorectomy and prednisolone administration for 16 weeks. Basophilic areas can be observed. Haematoxylin-eosin. (x500).

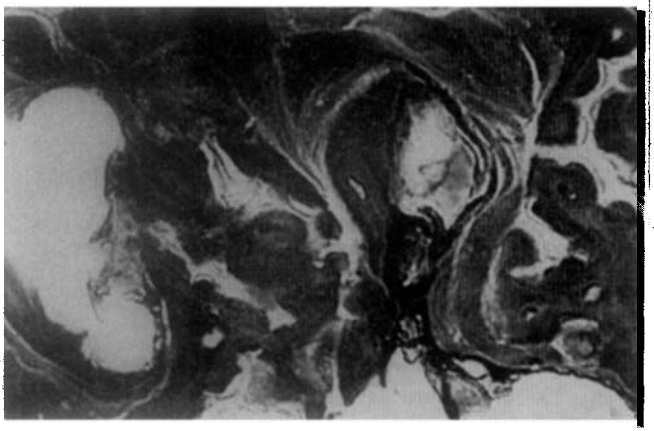

Fig. 9. Photomicrograph of an adjacent section of the vertebral bone shown in Fig. 8. The basophilic areas, located between the dark collagen bundles, are stained only lightly, or not at all. Azocarmine G. (x500).

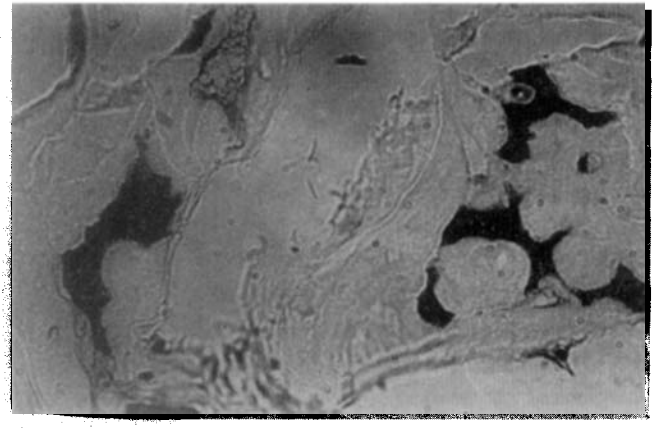

Fig. 10. Photomicrograph of an adjacent section of the vertebral bone shown in Figs. 8 and 9. The basophilic areas show evident metachromasia. There was no difference from the normal in the degree of metachromasia after treatment with combined oophorectomy and prednisolone administration for up to 16 weeks. Toluidine blue at pH 4.4. (x500).

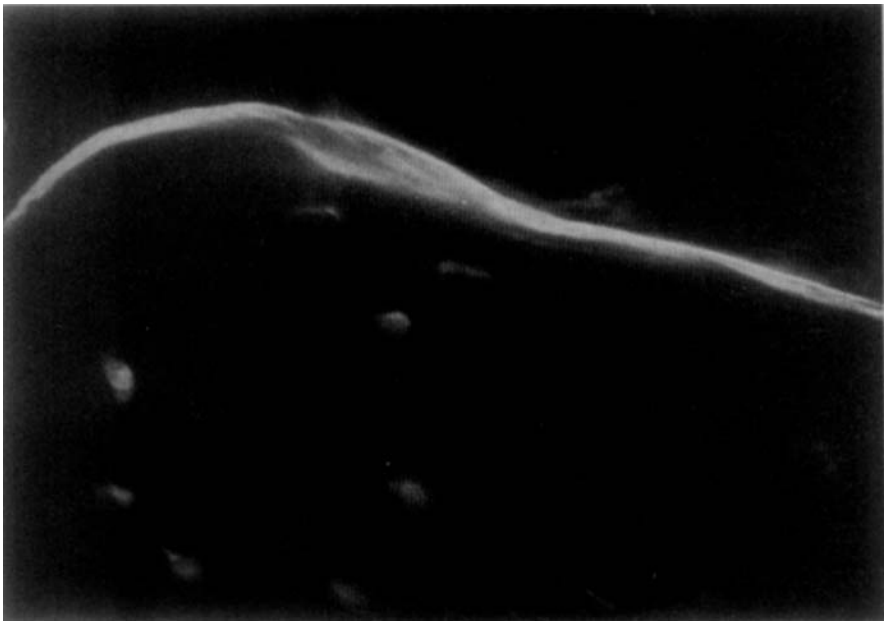

Fig. 11. Fluorophotomicrograph of undecalficied section of vertebral bone from a normal rat. Despite the time interval of 10 days between the 2 administered doses of chlortetracycline, only one narrow fluorescent line can be seen. (x400).

observed in the treated animals. The areas showing metachromasia with toluidine blue exhibited the same staining properties in corticosteroid-treated as in normal animals (Figs. 8—10).

Fluorescence microscopy.

In the normal animals, fluorescence of newly formed bone was observed in only very few parts of the trabecular bone surfaces and on the surfaces of bone canals (Fig. 11). Despite the time interval of 10 days between the two administered doses, only one narrow fluorescent line was found. These observations indicate that the rate of new bone formation in these old rats is very low. In the experimental animals, the fluorescent micrographs (Fig. 12) were similar to those in the normal controls.

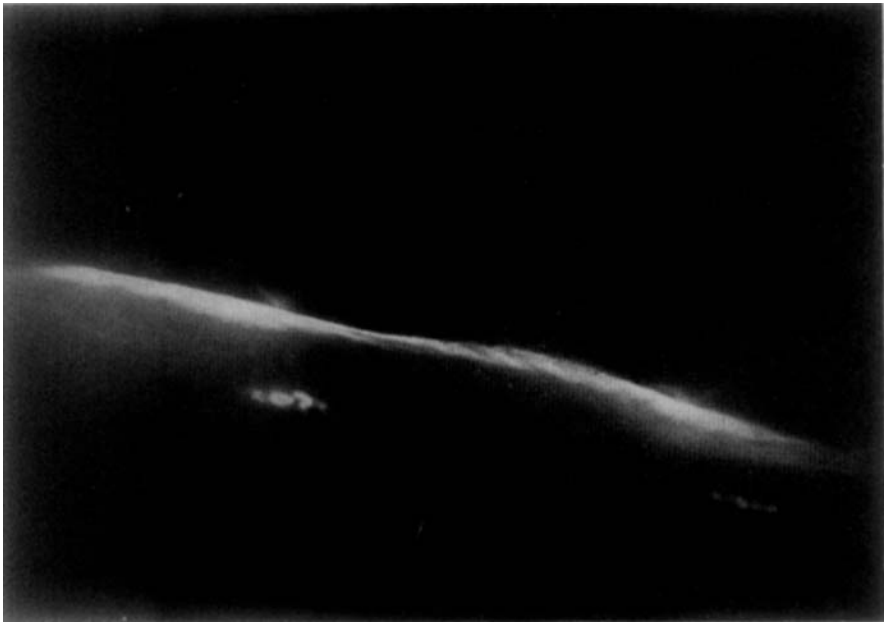

Fig. 12. Fluorophotomicrograph of undecalcified section of vertebral bone from a rat treated with combined oophorectomy and prednisolone administration for 16 weeks. There is only a thin fluorescent line after the 2 labellings with chlortetracycline. (x400).

Weight and histology of the adrenals (Table 7).

All the ten groups of oophorectomized rats treated with prednisolone for 1 to 16 weeks showed a decrease in the weight of the adrenals. In the five groups of rats treated for 8 to 16 weeks, the decrease was significant. This also held when the weight of the adrenals was correlated to the body weight.

Histological examination showed pronounced atrophy of the zona fasciculata in the corticosteroid-treated animals.

DISCUSSION

This study is concerned with the reaction of the well mineralized skeleton of one-year old fully grown female rats to combined oophorectomy and prednisolone treatment for different periods of time. It has been observed earlier that after

Groups	No. of animals	Adrenal weight, mg	S.E.M.	No. of animals	Adrenal weight/body weight (x10^{-5})	S.E.M.
Normal controls	5	51.8	4.09	5	16.6	1.26
Ooph. + prednisolone for 1 week	4	36.2	4.90	4	12.6	1.48
„ „ „ 2 weeks	5	43.4	4.32	5	15.3	1.54
„ „ „ 3 „	5	42.7	5.77	5	15.8	2.19
„ „ „ 4 „	5	41.0	6.15	5	14.1	1.97
„ „ „ 5 „	4	38.5	5.29	4	13.8	2.14
„ „ „ 8 „	5	19.1	1.19	5	7.1	0.44
„ „ „ 10 „	4	17.1	4.14	4	6.4	1.46
„ „ „ 12 „	4	18.7	3.39	4	7.2	1.08
„ „ „ 14 „	4	15.8	0.81	4	5.9	0.54
„ „ „ 16 „	4	19.6	0.92	4	8.0	0.44
		F = 9.57 sign.			F = 7.92 sign.	
			$F_{0.95}$ (10.38) = 2.10			

Table 7. Weight of the adrenals (mg) and weight of the adrenals/body weight (x10—5) in normal control rats and in oophorectomized rats treated with prednisolone for different periods of time.

a certain age female rats attain a constant body weight (*Singer et al.* 1952). This was found also in the present study, in which the body weight of the normal control rats remained unchanged after about the 6th week of the experiment. The radiological examination showed, further, that the skeletal growth was complete in all animals at the beginning of the experiment. No significant difference in femur length was found between normal and experimental animals.

In the present study, oophorectomy was performed in order to reduce the endogenous oestrogens which are known to have a calcium-retaining effect, in contradistinction to the calcium-wasting effect of corticosteroids (*Gordan et al.* 1967). In addition to considerable depletion of adipose and muscle tissues and atrophy of the skin, the effect of the treatment was manifested by pronounced atrophy of the adrenal cortex, especially the zona fasciculata. Rats treated with cortisone for 21 days show a selective depletion of lipid in the zona fasciculata and the zona reticularis, whereas the zona glomerulosa becomes hypertrophied and full of lipid (*Wexler* 1963). This inactivity of the adrenal cortex induced by cortisone administration would mean a decrease not only of endogenous cortisone from the zona fasciculata but also of oestrogens and androgens from the zona reticularis. Furthermore, histological examination of the ovaries removed in the present experiment revealed both primary and secondary follicles. These circumstances indicate that the experimental conditions of the present study induced the hormonal lack of balance which has been considered to cause senile osteoporosis in humans (*Reifenstein* 1957).

In an earlier experiment designed to study the effect on the skeleton of a hormonal disturbance similar to that in osteoporotic humans as reported by *Reifenstein* (1957), young male rats were deprived of endogenous adrenal corticosteroid and gonadal steroid hormones by total adrenalectomy and gonadectomy, and were injected daily with cortisone acetate in a dose of 7 mg per kg body weight (*Caldwell* 1962). There was radiographic evidence of osteoporosis after 100 days of cortisone treatment, and after 160 days the ratio femoral calcium/wet weight showed a decrease by approximately 25 per cent. In the adult oophorectomized rats studied in the present investigation no radiographic signs of osteoporosis were observed at any period of the prednisolone treatment. The ash content of the whole tibia showed a slight decrease initially, but no consistent change was observed subsequently in the rats treated for different periods of up to 12 weeks. Thereafter, a significant decrease by 6 to 7 per cent was noted in the two groups of rats treated for 14 and 16 weeks, respectively. Histological examination of decalcified sections and microradiographs of undecalcified vertebral sections showed no differences from the normal. The

loss of bone minerals observed in the rats treated for 14 and 16 weeks therefore corresponds to osteoporosis as described by *Collins* (1966). This is supported by the fact that in a subsequent study (*Larsson and Vejlens* 1969) on the same material no significant change in the ash content of the compact bone was found in these animals. The tendency to a decrease of the hydroxyproline content of the whole metacarpal bone observed in the present study also suggests a reduction of skeletal tissue. The same tendency to slightly decreased values was found with regard to the cortical thickness of the mid-shaft of the femur, the percentage of vertebral bone "hits" and the density of the whole humerus. Except for the "femur score" no significant differences were found with these methods which might have been due to greater methodological variations than for the ash determinations.

In view of the considerable depletion of other tissues, such as adipose and muscle tissues and the skin, the skeletal tissue of the adult rat must be regarded as fairly stable to the action of combined oophorectomy and excess corticosteroids. From results obtained in adult rats after cortisone treatment alone for up to 8 weeks, the same conclusion concerning bone collagen was reached by *Sobel and Marmorston* (1954). These findings are in contradistinction to results concerning the young growing skeleton of the rat (*Storey* 1960, *Caldwell* 1962) and of the rabbit (*Sissons and Hadfield* 1957, *Storey* 1957, *deValderrama and Munuera* 1965). In rabbits, also, the effect of corticosteroids on the adult skeleton is less pronounced than in young animals (*Storey* 1961).

It seems as if the hormonal disturbance induced in the experiment of *Caldwell* (1962) had a more pronounced effect on the skeletal tissue than that of the present study. However, in *Caldwell's* experiment young growing rats were used, and the results of the two studies are therefore not comparable. Thus, combined adrenalectomy, orchidectomy and cortisone treatment caused a pronounced interference with the normal skeletal growth and development. Also in other experimental studies where young animals have been used, the reported osteoporotic bone lesions induced by excess corticosteroids seem to have been mainly the result of interference with normal skeletal development. In the present investigation on fully grown animals the hormonal disturbance exerted its effect on the maintenance of the mature skeletal tissue, which is the essential problem in connection with the development of osteoporosis in humans.

It has been proposed that senile osteoporosis develops as a result of inhibited protein synthesis of the bone matrix (*Reifenstein* 1957). There is experimental evidence that cortisone primarily reduces the synthesis of bone collagen and does not increase its degradation in the rat (*Smith and Allison* 1965). Results

obtained in studies of the metabolism of bone collagen following intraperitoneal injection of glycine-2-C^{14} in the rat, indicate that a major proportion of bone collagen is synthesized at an early age and that the rate of metabolism of collagen in bone is an inverse function of age (*Kao et al.* 1965). This means that the reaction of bone collagen to excess cortisone is more pronounced in young than in adult individuals. Besides this inhibitory effect upon collagen synthesis, the well-documented calcium-wasting effect of corticosteroids (*Gordan et al.* 1967) may also be more pronounced in the growing skeleton, not only because of the high rates of bone apposition and resorption in the young animal but also because of more extensive bone calcium exchange in the young than in the mature skeleton (*Leblond et al.* 1950, *Tomlin et al.* 1953). In humans, adrenal steroids have been reported to produce osteoporotic bone lesions in children more frequently than in adults and generally after treatment on lower doses and in less time (*Schlesinger et al.* 1961).

It has been found in experiments on young growing rats that calcium intake and changes in calcium metabolism may be of primary importance for the development of osteoporosis following corticosteroid therapy (*Storey* 1960). Young rabbits treated with cortisone and fed a low calcium diet develop osteoporosis associated with extensive osteoclastic activity and secondary hyperparathyroidism (*Storey* 1961). The experiment of *Clark and Roth* (1961) suggests that hydrocortisone decreases the reabsorption of calcium by the renal tubules in the adult rat. This calcium-wasting effect was also demonstrated in a study on the same material as used in the present experiment (*Larsson* 1969 b). The data obtained indicated increased mobilization of skeletal calcium for maintenance of the blood calcium level. There was no consistent change in the calcium accretion rate of the bone. The decrease in bone mass found in the present study therefore represents a considerable calcium loss and corresponds well with that observed in one-year old male rats fed a low calcium diet (0.09 per cent calcium) for the same periods of time (*Larsson* 1969 a). In that experiment, also, a significant reduction of the ash content of the whole tibia by 6 to 7 per cent was noted after 14 and 16 weeks on a low calcium diet. These findings suggest that changes in calcium metabolism and the efficiency of calcium homeostasis may be of major importance for the slight bone mass reduction observed after combined oophorectomy and prednisolone administration in the present study. It is possible that a more significant reduction in bone mass might occur on extension of the duration of prednisolone treatment. However, it is conceivable that reduction in caloric intake might then occur, and this alone can give rise to osteoporosis (*El-Maraghi et al.* 1965). In the present

investigation the experimental animals treated with prednisolone for 2 weeks and more showed no apparent difference in food consumption in relation to body weight, compared with that of the normal controls. It is therefore hardly likely that a reduced calcium intake would be responsible for the bone reduction observed after 14 and 16 weeks of treatment.

Microscopic examination of decalcified sections of the vertebral bone from the normal adult female rats showed a high frequency of retracting osteocytes. Enlarged spherical lacunae, entirely empty or containing a pyknotic nucleus, were regularly found, especially in the cortical bone. These changes were unspecific and were found to the same extent in normal as in prednisolone-treated oophorectomized animals. Similar morphological changes have also been observed in aged humans and have been ascribed to physiologic aging of bone (*Sherman and Selakowitch* 1957). In patients with severe osteoporosis these changes are more pronounced (*Urist et al.* 1963). The morphologic appearance of the bone observed in the present study did not seem to be related to the reaction of the bone tissue to the treatment given.

In histological sections of vertebral bone tissue no osteoblasts or osteoclasts were found either in the normal or in the experimental rats. A small amount of bone surface showing tetracycline labelling was the only sign of low osteoblastic activity. No change was observed in the corticosteroid-treated oophorectomized animals, in contrast to observations on growing animals in which excess corticosteroids regularly suppress osteoblast formation (*Stanisavljevic et al.* 1962, *Hulth and Olerud* 1963). In addition, on administration of high doses of cortisone acetate highly increased osteoclast formation has been observed in young rabbits (*deValderrama and Munuera* 1965). In humans with hypercorticoidism the reported bone changes are not quite conclusive. Thus, rarefaction of vertebral bone trabeculae with seemingly adequate numbers of osteoblasts and no evidence of osteoclastic resorption was reported by *Follis* (1951). Decreased formation and resorption of bone was reported by *Storey* (1963). Other investigators have deduced from histological (*Sissons* 1956) and microradiographic (*Jowsey et al.* 1965) studies that osteoporosis in human hypercorticoidism results from impaired new bone formation in the presence of normal or increased bone resorption. Recently, an elevated number of active osteoblasts and a high uptake of tetracycline have been observed in human hypercorticoidism, and in severe cases high osteoclastic activity (*Birkenhäger et al.* 1967). While in young rats excess cortisone exerts a pronounced effect on the kinetics of the bone cell populations (*Simmons and Kunin* 1967), there was no such effect in the old rats used in the present study.

Histological examination of decalcified vertebral cross sections showed a regular frequency of distinct areas giving evident metachromasia with toluidine blue at pH 4.4 and located in spaces intervening between parallel-fibered bone in the metaphyseal bone trabeculae and metaphyseal cortex. These metachromatic areas contained only a small amount of collagen stainable with azocarmine G. These areas seem to consist of intercellular acid mucopolysaccharides extending from the epiphyseal region inwards to the metaphysis, and no such areas were observed in the diaphyseal cortex. In the experimental animals no change in staining properties of these areas was found. It is well known that excess of corticosteroids decreases the synthesis of acid mucopolysaccharides in connective tissues and that the incorporation of S^{35}-sulphate into chondroitin sulphate of costal cartilage in rabbits is diminished after corticosteroid treatment (*Boström and Odeblad* 1954, *Whitehouse and Boström* 1961). In one-year old rats treated with cortisone acetate in doses of 15 to 18 mg/kg for up to 8 weeks, a decrease in the hexosamine contents of the whole femur was found, in contrast to unchanged collagen contents, and it was suggested that a decrease of mucopolysaccharides precedes the events which lead to the development of osteoporosis in Cushing's disease (*Sobel and Marmorston* 1954). The changes observed may be unspecific with regard to the mucopolysaccharides of the bone tissue itself, and no support for this theory can be obtained from the observations made in the present study. This point is discussed further in a biochemical investigation (*Larsson and Vejlens* 1969).

The results of the present study show that the skeletal tissue of one-year old rats is relatively stable to the action of combined oophorectomy and prednisolone administered for up to 16 weeks. Despite a pronounced decrease in body weight there was only a slight but significant decrease of the bone tissue in animals treated for 14 and 16 weeks. This may, however, correspond to a considerable calcium loss in view of the effect of corticosteroids on calcium metabolism and calcium homeostasis. This is discussed further in a following study (*Larsson* 1969 b).

SUMMARY

In one-year old rats the effect on the skeletal tissue of combined oophorectomy and prednisolone administration (2.5 mg/animal/day, Precortalon aq®) for 1 to 16 weeks was studied. This treatment caused considerable depletion of adipose and muscle tissues, a total decrease in the body weight by 30 per cent and

significant atrophy of the adrenal cortex (zona fasciculata). In spite of the extensive loss in body weight induced, the effect of the treatment on the skeletal tissue was moderate. There were no roentgenological signs of osteoporosis. In the groups of oophorectomized rats treated with prednisolone for 14 and 16 weeks a significant reduction of the ash content of the tibia was found, while the groups of rats treated for shorter periods of time showed slightly, but insignificantly lower values than those of the normal controls. The cortical thickness of the mid-shaft of the femur, calculated as the "femur score" also showed significantly decreased values. There was also a slight, though not significant, decrease in the percentage of vertebral bone "hits", the hydroxyproline content of the metacarpal bone and the density of the humerus. Microradiographs of undecalcified vertebral bone sections showed no changes in the degree of mineralization. In the normal controls, histological examination of decalcified sections revealed a high frequency of retracting osteocytes in enlarged lacunae, and lacunae which were empty of cells were regularly found. Osteoblasts and osteoclasts were not seen, and there was only a very small amount of vertebral bone surface showing tetracycline labelling. Areas containing intercellular substance (acid mucopolysaccharides) giving metachromasia in toluidine blue were observed in the trabeculae of the vertebral bone. In the experimental animals, the bone tissue showed the same morphological appearance as in the normal controls. The areas containing acid mucopolysaccharides showed the same staining properties as in the normal animals. No notable difference in tetracycline labelling was found. The observed tendency to reduced bone mass is discussed with regard to the development of osteoporosis.

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Studies on the Development of Experimental Osteoporosis

IV. The Effect of Combined Oophorectomy and Prolonged Prednisolone Administration on the Blood Calcium Level and the Distribution of Ca^{45} between Blood and Bone in Adult Rats

by

Sven-Erik Larsson

INTRODUCTION

Treatment with glucocorticoids causes both a negative calcium balance and loss of bone in adult humans (*Albright and Reifenstein* 1948). The pathomechanisms involved during the development of the corticosteroid-induced osteoporosis are still poorly understood, however. The antianabolic effect of glucocorticoids on connective tissues is well known and it has been proposed from histological observations that the decrease in the amount of bone tissue in humans with Cushing's syndrome is the result of inhibited new bone formation (*Sissons* 1956). It has also been pointed out from quantitative histological studies of rib sections that bone formation is profoundly depressed in corticoid treated patients and that bone resorption also appears to be depressed but to a lesser degree than the formation (*Villanueva et al.* 1966). With the aid of quantitative microradiography of sections from the iliac crest, decreased bone formation and increased resorption have been demonstrated both in Cushing's syndrome and after corticosteroid therapy (*Jowsey et al.* 1965). The results obtained with the aid of bone-seeking isotopes or stable strontium are not quite conclusive with regard to the effect of corticosteroids on the skeletal tissue. Measurements of the bone mineralization rate by a variety of techniques have failed to find any consistent difference in this respect between normal and steroid-treated individuals (*Nordin et al.* 1963, *Nordin et al.* 1964). Studies by means of stable strontium of a large number of patients taking synthetic steroids have revealed an increase in urinary excretion and bone accretion rate; however, in three cases of Cushing's syndrome small pool sizes and low turnover rates were found (*Gordan and Eisenberg* 1963). From strontium kinetic studies on corticoid treated osteoporotic patients it has been concluded that the bone accretion is normal or even increased and that the eventual decrease in bone mass is caused by accelerated bone resorption (*Eisenberg* 1966).

Osteoporosis similar to that in humans has been induced experimentally in young rabbits by excess cortisone (*Sissons and Hadfield* 1955, *Storey* 1957). The conclusion was drawn from histological studies that the bone rarefaction is the result of inhibited bone formation with an unopposed continuance of bone resorption (*Sissons and Hadfield* 1955, *Sissons* 1956). It has also been deduced from observations based on histological studies in young rabbits that cortisone osteoporosis results not only from a cessation of bone formation, but primarily from a direct degrading effect which causes a massive resorption of bone (*deValderrama and Munuera* 1966).

In young rats, cortisone osteoporosis similar to that in rabbits has been reported by *Storey* (1960). It was shown that bone rarefaction occurred only when the rats were fed a diet in which the calcium and phosphorus contents were normal, *i.e.* 1.0 and 0.6 per cent, respectively, or lowered; on a high calcium-phosphorus intake there was an increase in the density of the metaphyseal bone. This difference in the reaction of the bone to cortisone was believed to be associated with the well developed adaptive calcium metabolism of the rat. It has been known for many years that corticoids exert considerable influence on the calcium metabolism. The skeletal retention of bone-seeking isotopes has been claimed to be inhibited by the administration of corticoids to rats (*Clark et al.* 1959, *Milhaud et al.* 1960, *Bohr and Dawids* 1964). Calcium excretion has been found consistently to be increased, but there is disagreement concerning the effect on intestinal calcium absorption. Young male rats subjected to bilateral adrenalectomy and orchidectomy are more sensitive to cortisone and develop osteoporosis despite a high calcium intake (*Caldwell* 1962). Oestradiol cured the osteoporosis in these animals despite continuous cortisone administration, whereas only very slight improvement was noted in animals given testosterone.

The present study is a continuance of a previous investigation (*Larsson* 1969 a) and was undertaken in order to obtain some information regarding the pathomechanism underlying the observed significant reduction of bone mineral caused by the hormonal imbalance induced in adult rats by oophorectomy combined with prolonged prednisolone administration. The data to be reported here concern the effect on the blood calcium level, the distribution of Ca^{45} between blood and bone and the calcium accretion rate of the bone in the same material of oophorectomized rats kept on prednisolone treatment for different periods from 1 up to 16 weeks.

MATERIAL AND METHODS

MATERIAL

Fifty-five adult female rats of the Sprague-Dawley strain[1] were used. All animals were stated to be 11 1/2 to 12 months old at the start of the experiment and had a mean initial body weight of 349.5 ± 2.7 grams. The animals were divided at random into ten experimental groups and one control group of 5 rats each. The experimental animals were oophorectomized via a midline

[1] Anticimex Co., Stockholm.

abdominal incision under ether anaesthesia. All the animals recovered very quickly after the operation. From the third day after oophorectomy each animal received 2.5 mg prednisolone[1] daily, dissolved in their drinking water. This was supplied intermittently in such a way that the prednisolone was consumed during the following 1 — 2 hours. The periods of prednisolone treatment varied from 1 up to 16 weeks. The *control* group consisted of *normal* rats and was observed for 16 weeks. The experimental and the control animals received the same ordinary laboratory food ration[2] and tap water *ad libitum,* although, as mentioned above, the experimental animals were given their drinking water more intermittently. Chemical analyses showed that the total calcium and phosphorus in this diet amount to 1.0 and 0.55 per cent, respectively. All animals were housed in individual cages in a separate room and were not exposed to sunlight. The animals could move around freely in their cages.

INVESTIGATIONAL PROCEDURES

A detailed description of the procedures used has been given in a previous paper (*Larsson* 1969 b). The body weight of each animal was recorded weekly. The series was planned so that all the animals were sacrificed at the same time. Seventy-two hours before sacrifice 20 microcuries of Ca^{45}* in 1.0 ml physiological saline solution were injected intraperitoneally in each animal. All administered solutions were prepared from a single batch of $Ca^{45}Cl_2$. At intervals of 6, 24 and 48 hours after the isotope injection 0.4 to 0.5 ml tail blood was collected and after 72 hours the animals were sacrificed by bleeding to death from the femoral artery under ether anaesthesia. The blood samples obtained at 6 and 48 hours after the Ca^{45} injection were taken from three animals of each group and at 24 hours from the remaining two. The collected blood was centrifuged immediately at 3,000 r.p.m. for 15 minutes and the plasma transferred into acid washed test tubes and kept at – 20°C for later determinations of Ca^{45} activity. The whole animal bodies were kept at – 20°C pending the subsequent bone preparations.

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[1] Precortalon Aquosum®, kindly supplied by Pharmacia Co., Uppsala.

[2] Ewos Co., Södertälje.

* Calcium chloride in aqueous solution 2—5 c/g Ca, C.E.S. 2, The Radiochemical Centre, Amersham, England.

Determination of the Ca^{45} activity of the bone.

The whole right tibia including the bone marrow and the articular cartilages was carefully freed of surrounding soft tissues and periosteum. All bones were ashed simultaneously in a muffle furnace at 700°C for 16 hours, after which the ash weight was recorded with the aid of a Mettler balance and with an accuracy of 0.05 mg. All ashed bones were then dissolved in 5.0 ml of 1.2 N HCl in 100 ml volumetric flasks. When the ash was completely dissolved, the flasks were filled to the mark with deionized water. An aliquot of 2.0 ml from the final solution was transferred into a 25 ml polyethylene counting vial which was filled with a gel scintillator composed of 2.5 -Diphenyloxazole (PPO) 1.0 g, 1.4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene (POPOP) 0.3 g, Naphtalane 100.0 g, Dioxan 1 litre and thixotrophic gel powder (CAB-O-SIL) to saturation.

Determination of the Ca^{45} uptake of pure compact bone tissue.

Acetone-dried bone powder was prepared from the tubular bones as described elsewhere (*Larsson* 1969 b). Approximately 100 mgs of bone powder were ashed at 700°C in a muffle furnace for 16 hours. The exact ash weight was then recorded, after which the ash was dissolved in 2.0 ml of 1.2 N HCl directly in the counting vials. After addition of the scintillator, counting was performed

Ca^{45} activity of plasma.

This was determined directly, using 1.0 ml of plasma collected at sacrifice and about 0.2 ml of plasma obtained from the tail blood collected at different intervals after the administration of the isotope. This procedure was considered suitable for the purpose of the present study (see *Budy* 1963) and has been recommended by *Kumar* (1966). The same scintillator was used as for the bone ash solutions.

Calcium determination of the plasma.

This was performed by flame photometry, using an Ependorf photometer, on plasma collected at sacrifice. Duplicate determinations were always performed. The error of the method was found to be less than 1 per cent.

Calcium content of the cortical bone powder.

This was determined by atomic absorption spectrophotometry using an automated Perkin-Elmer Model 303 Instrument as described elsewhere (*Larsson* 1969 b).

For suppression of the interference by phosphorus, lanthanum chloride was added to the solutions as recommended by *Willis* (1961). The added calcium recovery in these bone ash solutions was found to be 98 per cent. Duplicate determinations were performed throughout. The coefficient of variation for these analyses was less than 1 per cent.

Ca^{45} activity determinations.

These were performed with the aid of a Packard Tri-Carb Liquid Scintillation Spectrometer[1]. Corrections were made for background and decay. It was not considered necessary to introduce any corrections for self-absorption because of the constancy of counting efficiency.

Treatment of isotope data.

The Ca^{45} activity of bone was expressed as counts per minute per milligram of ash. No significant difference was found in the calcium content of ash obtained from pure cortical bone tissue of the normal and the treated animals; the mean calcium content was 38.09 ± 0.20 per cent. Therefore, the expression c.p.m. per milligram of ash is equivalent to the specific activity and the factor 0.3809 was used when the bone accretion rates were calculated (see below). The Ca^{45} activity of plasma at 72 hours after the isotope injection was expressed as c.p.m. per milligram of calcium. The plasma Ca^{45} activity curves (Fig. 2) were also based upon values expressed as c.p.m. per milligram of calcium. Because of the necessity for collecting as little blood as possible at the different periods after the administration of Ca^{45}, calcium determinations were only performed on blood samples obtained at sacrifice.

The accretion rates for the whole tibia, expressed as mg calcium/hour/mg calcium of the tibia, were calculated according to *Bauer et al.* (1955). The mean values within each group were used throughout.

Statistical treatment of data.

The analytical data obtained from the rats in each group were subjected to analysis of variance with one-way lay-out according to *Scheffé* (1961). The hypothesis of no difference between groups was tested at the 5 per cent

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[1] Model 314 EX-2, Packard Instrument Company, LaGrange, Ill. USA.

significance level. When a significant result was obtained, data of one or more groups were combined in order to find significant contrasts. These were tested according to *Scheffé* (1961). In the following the term "significant" will stand for "statistically significant" as found with the tests described above.

RESULTS

All animals survived except one, which was excluded. The normal control rats showed a slight initial increase in body weight but after 6 weeks the body weight remained at a constant level of 5 to 7 per cent above the initial value. The group of oophorectomized animals maintained on prednisolone treatment for 16 weeks showed a total loss of body weight by approximately 30 per cent of the initial value. A considerable depletion of the adipose and muscle tissues was observed in all experimental animals, but otherwise the animals were in good condition. No infections or any other intercurrent diseases were observed.

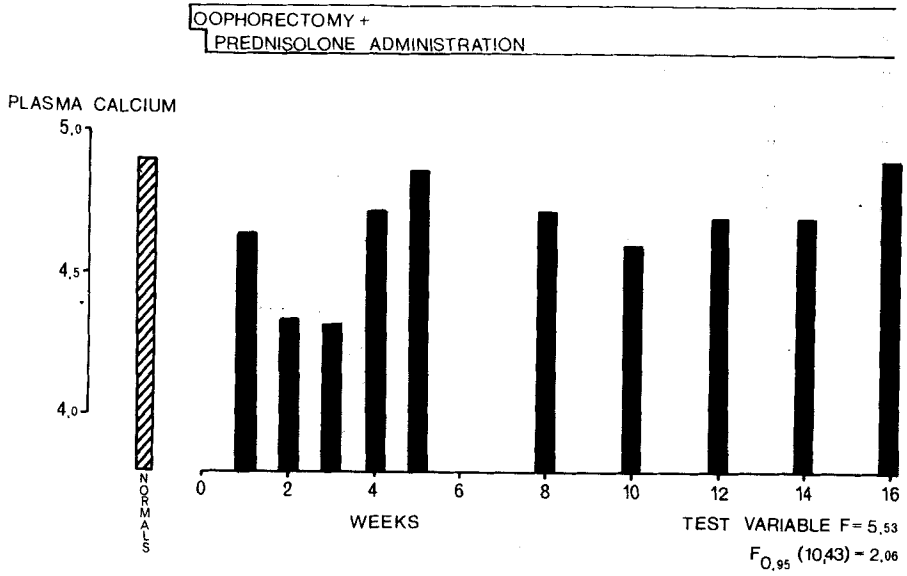

Fig. 1. Plasma calcium in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Plasma calcium (Fig. 1, Table 1).

The values of the oophorectomized rats treated with prednisolone for 1, 2, 3 and 4 weeks were consistently lower than those of the control animals. In the groups of rats treated for 2 and 3 weeks the fall in plasma calcium was significant and amounted to approximately 12 per cent. The group of animals treated for 5 weeks showed normal values but, again, subnormal values were recorded in the four groups of oophorectomized rats maintained on prednisolone treatment for 8, 10, 12 and 14 weeks. This decrease was not significant, however. Finally, normal plasma calcium values were found in the group of animals treated for 16 weeks.

Groups	No. of animals	Plasma calcium mEq./l	S.E.M.	
Normal controls	5	4.9	0.07	
Ooph. + prednisolone for 1 week	5	4.6	0.10	
„ „ „ 2 weeks	5	4.3	0.08	sign.
„ „ „ 3 „	5	4.3	0.03	sign.
„ „ „ 4 „	5	4.7	0.08	
„ „ „ 5 „	5	4.9	0.08	
„ „ „ 8 „	5	4.7	0.02	
„ „ „ 10 „	5	4.6	0.09	
„ „ „ 12 „	4	4.7	0.04	
„ „ „ 14 „	5	4.7	0.14	
„ „ „ 16 „	5	4.9	0.05	
		$F = 5.53$ sign.		
		$F_{0.95}(10.43) = 2.06$		

Table. 1. Plasma calcium in normal rats and oophorectomized rats treated with prednisolone for different periods of time (S.E.M.=standard error of the mean).

Plasma Ca^{45} activity at different periods after the Ca^{45} injection (Fig. 2).

In all groups of oophorectomized rats treated with prednisolone for different periods of time there was a more rapid fall in plasma Ca^{45} activity than in

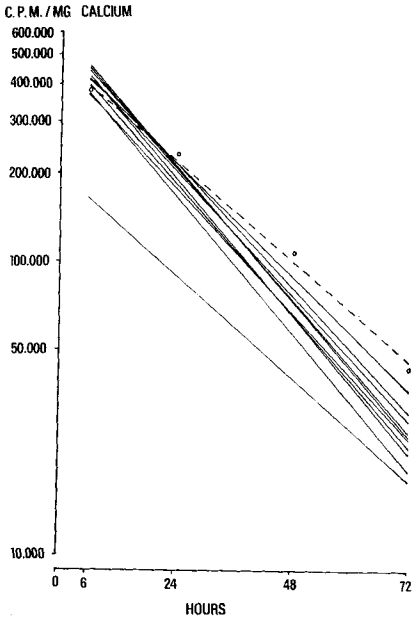

Fig. 2. Plasma Ca^{45} specific activity, corrected for body weight, at 6, 24, 48 and 72 hours after the isotope injection in normal control rats (broken line) and in oophorectomized rats treated with prednisolone for different periods of time (whole line). Each dot represent the mean value for the control group.

the normal control group. At 24, 48 and 72 hours after the administration of the isotope the plasma Ca^{45} activity showed consistently lower values in the experimental animals than in the normal controls.

Plasma Ca^{45} specific activity at 72 hours after the Ca^{45} injection (Fig. 3, Table 2).

In comparison with the normal control group, lower values were obtained in the group of oophorectomized rats treated with prednisolone for 1 week. This difference was not significant, however. The two groups of animals treated for 2 and 3 weeks showed approximately the same values as the controls. In all the seven groups of oophorectomized rats kept on prednisolone treatment for 4 to 16 weeks, consistently decreased values were noted. In these groups the plasma Ca^{45} activity was approximately 40 per cent lower than in the normal control group. When the combined values for these groups were tested against those of the normal control group the difference was found to be significant.

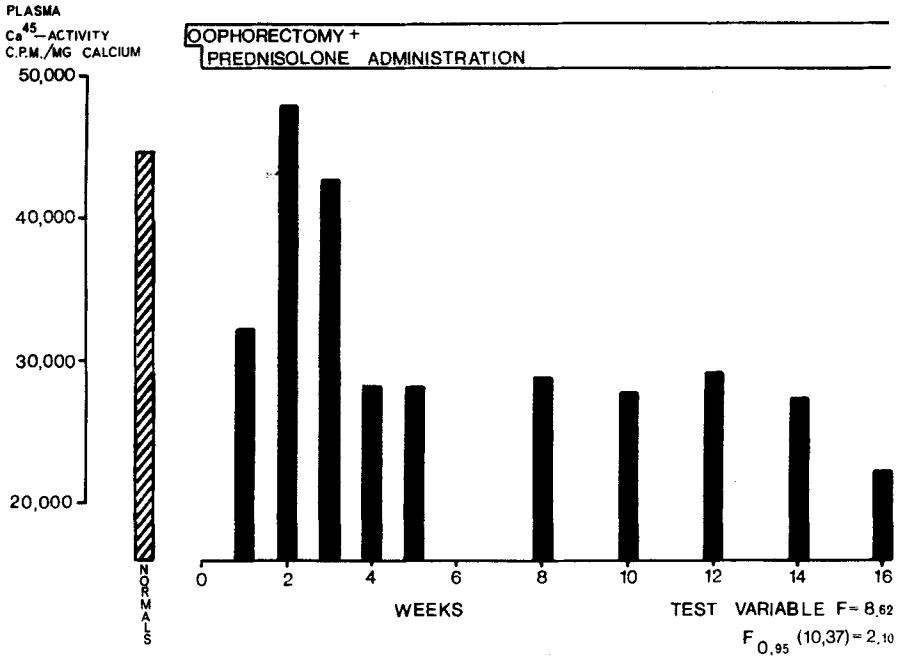

Fig. 3. Plasma Ca^{45} specific activity 72 hours after the intraperitoneal administration of 20 microcuries of Ca^{45} per animal in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Groups	No. of animals	Plasma Ca^{45} c.p.m./mg calcium	S.E.M.	
Normal controls	5	44597	2037	
Ooph. + prednisolone for 1 week	3	32189	2457	
„ „ „ 2 weeks	4	47847	5556	
„ „ „ 3 „	4	42621	5436	
„ „ „ 4 „	5	28202	3125	
„ „ „ 5 „	3	28156	208	
„ „ „ 8 „	5	28856	2124	
„ „ „ 10 „	5	27839	1188	Sign.
„ „ „ 12 „	4	29253	2233	
„ „ „ 14 „	5	27379	1497	
„ „ „ 16 „	5	22326	1821	
		$F = 8.62$ sign.		
		$F_{0.95}(10.37) = 2.10$		

Table 2. Plasma Ca^{45} specific activity 72 hours after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Ca^{45} activity of the tibia 72 hours after the Ca^{45} injection (Fig. 4, Table 3).

Slightly higher values were obtained in the groups of oophorectomized rats treated with prednisolone for 1 and 2 weeks compared with those of the normal controls. This difference was not significant, however. In the group of oophorectomized rats treated for 3 weeks the values obtained showed no difference from those of the normal controls. In the groups of oophorectomized rats treated for 4 to 16 weeks, successively lower bone Ca^{45} activity values were noted, decreasing with the duration of prednisolone treatment. When the combined values of the five groups of rats treated for 8 to 16 weeks were tested against those of the normal controls and the four groups of rats treated for 1 to 4 weeks, the difference was found to be significant. In the groups of rats treated for 14 to 16 weeks the values were approximately 30 per cent lower than those of the normal controls.

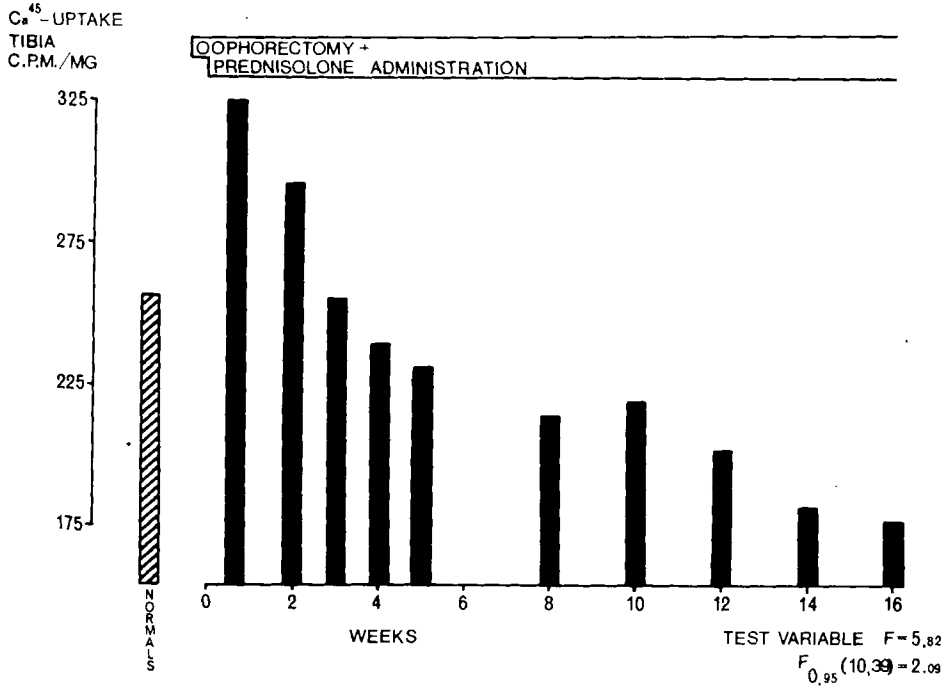

Fig. 4. The uptake of Ca^{45} by the tibia 72 hours after the intraperitoneal administration of 20 microcuries of Ca^{45} per animal in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Groups	No. of animals	Ca^{45} activity c.p.m./mg ash	S.E.M.	
Normal controls	5	257.4	11.7	
Ooph. + prednisolone for 1 week	5	325.4	33.5	
„ „ „ 2 weeks	4	295.9	31.4	
„ „ „ 3 „	5	256.0	7.3	
„ „ „ 4 „	5	240.2	20.2	
„ „ „ 5 „	3	232.0	20.5	Sign.
„ „ „ 8 „	5	215.0	14.8	
„ „ „ 10 „	4	219.5	12.3	
„ „ „ 12 „	4	202.9	5.0	
„ „ „ 14 „	5	183.1	11.6	
„ „ „ 16 „	5	177.9	18.9	
		F = 5.82 sign.		
		$F_{0.95}$ (10.39) = 2.09		

Table 3. The Ca^{45} uptake of the tibia (c.p.m./mg ash) 72 hours after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Groups	No. of animals	Ca^{45} activity c.p.m./mg ash	S.E.M.	
Normal controls	4	44.6	4.0	
Ooph. + prednisolone for 1 week	3	45.7	10.3	
„ „ „ 2 weeks	4	46.0	5.9	
„ „ „ 3 „	5	44.2	1.9	
„ „ „ 4 „	5	36.0	2.1	
„ „ „ 5 „	3	33.1	1.4	Sign.
„ „ „ 8 „	5	31.2	1.7	
„ „ „ 10 „	4	35.7	2.6	
„ „ „ 12 „	4	26.2	2.5	
„ „ „ 14 „	5	29.8	1.5	
„ „ „ 16 „	5	28.0	2.4	
		F = 4.68 sign.		
		$F_{0.95}$ (10.36) = 2.11		

Table 4. The Ca^{45} uptake of compact bone (c.p.m./mg ash) 72 hours after the intraperitoneal injection of 20 microcuries of Ca^{45} per animal in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Ca^{45} activity of compact bone powder 72 hours after the Ca^{45} injection (Table 4). Approximately the same values were obtained for the three groups of oophorectomized animals treated with prednisolone for 1, 2 and 3 weeks as for the normal control group. In the seven groups of rats treated for 4 to 16 weeks consistently lower values were obtained, with a maximum decrease in the groups treated for 12, 14 and 16 weeks. When the combined values of the five groups of rats treated for 8 to 16 weeks were tested against those of the normal controls and the four groups of rats treated for 1 to 4 weeks, the difference was found to be significant.

Groups	Accretion rate (mg Ca/hour/mg Ca of the tibia)
Normal controls	5.63×10^{-5}
Ooph. + prednisolone for 1 week	7.52×10^{-5}
„ „ „ 2 weeks	5.93×10^{-5}
„ „ „ 3 „	5.08×10^{-5}
„ „ „ 4 „	5.14×10^{-5}
„ „ „ 5 „	5.33×10^{-5}
„ „ „ 8 „	5.98×10^{-5}
„ „ „ 10 „	5.01×10^{-5}
„ „ „ 12 „	5.59×10^{-5}
„ „ „ 14 „	4.18×10^{-5}
„ „ „ 16 „	8.20×10^{-5}

Table 5. The mean calcium accretion rate for the tibia in normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Calcium accretion rate for the tibia (Table 5).
This was calculated as mg calcium/hour/mg calcium of the tibia. In the groups of oophorectomized rats treated with prednisolone for 1 and 2 weeks, higher mean values were obtained than in the normal control group. The six groups of rats treated for 3 to 12 weeks showed approximately the same values as that of the normal control group. In the group of rats treated for 14 weeks, a lower accretion rate was found, while the group of rats treated for 16 weeks showed a higher accretion rate compared with the control group.

DISCUSSION

Corticosteroid administration to young animals always interferes with the normal bone growth (*Follis* 1951, *Sissons and Hadfield* 1955, *Laron et al.* 1958, *Kowalewski* 1962, *Stanisavljevic et al.* 1962, *Hulth and Olerud* 1963, *Tapp* 1966, *Simmons and Kunin* 1967). When young, still growing animals are used in studies concerning the effects of corticosteroids on the bone mineralization rate the growth inhibitory effect of the treatment must be taken into consideration. No such interference could occur in the present study since fully grown, one year old rats were used. Only a very slight initial body weight increase occurred in the normal control rats and after 6 weeks of observation the body weight remained unchanged. It has been established earlier that in mature female rats no further skeletal growth occurs after a constant body weight has been attained (*Singer et al.* 1952). The pronounced decrease in body weight recorded in the experimental animals was caused by depletion of the adipose and muscle tissues due to the treatment. In a previous study (*Larsson* 1969 a) the effect on the skeletal tissue was examined in the material used in the present investigation. Measurements of the femur length showed no significant differences between normal and corticosteroid treated animals and the epiphyseal zones were closed. After the combined treatment with oophorectomy and maintained prednisolone administration for 14 and 16 weeks there was a significant decrease in the ash content of the whole tibia by 6 to 7 per cent, which represents a considerable loss of calcium. Microradiographs of undecalcified vertebral sections showed that the bone tissue was well mineralized, and there was no difference in the degree of mineralization observed on microradiographs from normal and experimental animals. It was of interest, therefore, to examine the changes in calcium metabolism occurring during the process of bone reduction in these adult rats, especially since earlier work has been confined almost exclusively to the effect of corticosteroids on young growing animals where interference with skeletal growth has to be considered.

The significant fall in blood calcium observed in the groups of oophorectomized rats treated with prednisolone for 2 and 3 weeks is of especial interest. It has been reported earlier that cortisone administration does not reduce the blood calcium level or affect hypercalcemia induced by vitamin D in young rats (*Williams et al.* 1962). In dogs prednisolone treatment results in a significant increase in the blood calcium by 7 per cent (*Garrett et al.* 1964). This is in disagreement with observations in humans that in cases of adrenal insufficiency

there is a tendency towards hypercalcemia (*Walser et al.* 1963) and that cortisone tends to reverse the hypercalcemia accompanying different diseases such as sarcoidosis and hypervitaminosis D (*Dent 1956, Connor et al.* 1956). Adrenalectomy, further, gives rise to increased concentrations of blood calcium in dogs (*Rogoff and Stewart* 1928), rabbits (*Kisch* 1924) and rats (*Rohdenburg and Krehbiel* 1925). It has been stated that hydrocortisone has no effect on the blood calcium of patients or animals with functioning parathyroids (*Stoerk et al.* 1963), but lowers the blood calcium level in hypoparathyroid patients (*Leifer and Hollander* 1953) and in parathyroidectomized animals of several species (*Mirvish and Bosman* 1929, *Stoerk and Arison* 1961). It has also been shown that 3.5 times more parathyroid extract is needed to maintain normal blood calcium concentrations in hydrocortisone-injected parathyroidectomized rats than in controls subjected to parathyroidectomy alone. The steroid had no effect on the blood calcium of normal animals (*Stoerk et al.* 1963). In contrast, it has been reported that an excess of cortisone reduces the normal blood calcium level in young rats (*Laron et al.* 1958, *Simmons and Kunin* 1967). This has also been observed in rabbits (*Pincus et al.* 1951). The fall in the blood calcium level observed in the present study indicates that considerable changes in the calcium metabolism are induced in the adult rat by combined oophorectomy and prednisolone administration. After normalization following the significant decrease during the initial 2 and 3 weeks, slightly subnormal values were again noted between the 8th and the 14th week of treatment, followed by further normalization.

In maintaining the blood calcium at a normal level the bone tissue has an important function as a mineral reservoir (*Copp and Shim* 1963). The results reported by *Stoerk et al.* (1963) suggest that stimulated parathyroid function might be involved in the maintenance of the blood calcium level during cortisone administration. This would give rise to increased mobilization of skeletal calcium to the blood, and these investigators suggest that this mechanism might be responsible for the cortisone induced osteoporosis. However, in a study of cortisone-treated, still growing, male rats no signs of increased parathyroid activity were observed (*Hansson* 1967). The initial decrease in blood calcium observed in the present study was of such an order of size that stimulated parathyroid function might well have been involved. The calcium-wasting effect of corticoids is well known (*Gordan et al.* 1967). The calcium-retaining effect of oestradiol has been demonstrated by *Caldwell* (1962) in growing male rats rendered osteoporotic by combined bilateral adrenalectomy, orchidectomy and cortisone treatment. Despite continuous cortisone administration oestradiol

cured the osteoporotic bone changes in these animals. In the rat, but not in other species, oestrogens have been reported to inhibit bone resorption (*Budy et al.* 1952, *Lindquist et al.* 1960). In the present study the significant fall in the blood calcium level was induced by the combination of oophorectomy, which resulted in a reduced production of endogenous oestrogens, and daily administration of prednisolone. This caused a significant decrease in the mineral content of the whole tibia after treatment for 14 and 16 weeks (*Larsson* 1969 a). The contradictory results obtained in earlier studies concerning the effect of excess corticosteroids on the blood calcium level might be explained by the present findings indicating that during the progress of the treatment, periods of decreased blood calcium values are followed by periods of normal values. Furthermore, the combination of oophorectomy and prednisolone treatment might have a more pronounced blood calcium lowering effect than corticosteroid treatment alone.

The plasma Ca^{45} activity curves indicate an increased elimination rate of intraperitoneally administered Ca^{45} from the blood in the experimental groups in comparison with that of the normal control group. On examination of the Ca^{45} activity values of the plasma and the tibia at 72 hours after the isotope injection, it is seen that the group of rats treated for 1 week showed decreased plasma Ca^{45} activity and slightly increased Ca^{45} activity in the bone. The rats treated for 2 weeks showed approximately normal plasma Ca^{45} activity but slightly increased bone Ca^{45} activity and the group treated for 3 weeks showed approximately the same values for both the plasma and bone Ca^{45} activity as those of the normal control group. The groups of rats treated for 4 to 16 weeks showed concomitant decreases in the plasma and bone Ca^{45} activity values in spite of the fact that with consideration of their body weights these animals were injected with a higher dose of Ca^{45} than the normal controls. The Ca^{45} activity of compact bone tissue showed the same decrease as that of the whole tibia, although the uptake of Ca^{45} by the compact bone only amounted to less than one fifth of that of the whole tibia. These data indicate that prednisolone administration to oophorectomized mature rats results in increased calcium excretion and decreased calcium retention, progressing with the duration of prednisolone treatment. The decrease observed in the blood calcium level seems to be secondary to poor or retarded adaptation to the increased calcium excretion.

It has been reported that corticosteroids increase the excretion of administered bone-seeking isotopes in young rats (*Clark et al.* 1959, *Milhaud et al.* 1960, *Bohr and Dawids* 1964, *Clark and Smith* 1964). In young growing animals this might be due to decreased reabsorption of calcium by the renal tubules, as

reported by *Clark and Roth* (1961), and to an inhibitory effect of the corticosteroids on normal bone growth, resulting in decreased bone mineralization (see below). In dogs, prednisolone increases the amount of intravenously administered Ca^{45} eliminated in the faeces and urine (*Collins et al.* 1962) and in man, cortisone and ACTH increase the calcium excretion in both the urine and faeces (*Luft and Sjögren* 1951). The tubular reabsorption of calcium is reduced in humans treated with corticosteroids, possibly due to an anti-anabolic effect on the cells of the tubules (*Laake* 1960). The effect of adrenal cortical steroids on intestinal calcium absorption in the rat has been a subject of disagreement. According to *Milhaud et al.* (1960) corticosteroids decrease calcium absorption. However, no effect of hydrocortisone on the over-all gastro-intestinal calcium absorption was found in the rat by *Clark and Smith* (1964).

Administration of hydrocortisone to rats has been reported to inhibit the uptake of Ca^{45} by bone, which was also found to be decreased in nephrectomized rats, and it was suggested that the limiting factor may lie in the synthesis of chondroitin sulphate (*Clark et al.* 1959). Similar conclusions were drawn from the observation that the retention by the rat of injected radioactive strontium and calcium measured over the total body and over the tail was decreased after the administration of dexamethasone (*Bohr and Dawids* 1964). The specific activity of the blood was not determined, but because the specific activity of the urine was increased while the urinary output of calcium was normal it was suggested that the decreased retention was due to a reduction in the amount of bone available for mineralization. In both these studies, interference by the corticosteroids with normal growth might well explain the results obtained, especially in the experiment of *Clark et al.* (1959) where male rats weighing between 160 and 250 gms were used. *Bohr and Dawids* (1964) used female rats weighing about 200 gms and in these animals cortisone administration caused a relatively small, though definite decrease in the retention of bone-seeking isotopes.

In the present study, the calculated mean accretion rate for the tibia was found to be increased in the groups of oophorectomized animals treated with prednisolone for 1 and 2 weeks, while in the groups of rats treated for 3 to 12 weeks approximately normal values were found. The groups treated for 14 and 16 weeks showed fluctuating values. These experimental results are in agreement with those obtained after administration of various corticoids to normal human volunteers, in whom the bone accretion rate did not fall but remained normal or even increased slightly (*Gordan and Eisenberg* 1963). The proposals made by *Clark et al.* (1959) and by *Bohr and Dawids* (1964) may be

valid with regard to young, still growing animals. However, in the skeleton of the adult rat in which calcification and growth are completed, the accretion rate was normal or even increased after combined oophorectomy and corticosteroid administration. This implies that the induced hormonal imbalance did not exert any appreciable inhibitory effect on the bone mineralization rate. Evidence was obtained in this investigation to indicate that the decreased skeletal uptake of intraperitoneally administered Ca^{45} is the result of increased elimination of Ca^{45} from the blood due to increased excretion brought about by the treatment. The data presented here strongly suggest that the slight reduction of the bone tissue found in a previous study (*Larsson* 1969 a) was caused by increased bone resorption which would mobilize skeletal calcium for maintenance of the blood calcium level. With extension of the periods of prednisolone treatment, a more pronounced osteoporosis would develop, due to the continuous calcium loss.

SUMMARY

Ten groups of 5 fully grown, one-year old female rats were oophorectomized and then treated with prednisolone for periods varying from 1 up to 16 weeks. One control group of 5 normal rats was kept on the same normal laboratory ration for 16 weeks. A total loss of body weight by approximately 30 per cent was observed in the group of oophorectomized rats treated with prednisolone for 16 weeks. In the normal control rats the body weight showed a slight initial increase but thereafter it remained almost constant throughout the observation period of 16 weeks.

The plasma calcium showed a significant fall in the groups of rats treated for 2 and 3 weeks. In the group treated for 5 weeks, normal values were noted. In rats treated for 8 to 14 weeks subnormal plasma calcium values were observed, while in the group of rats treated for 16 weeks the values were again normal.

There was a higher elimination rate of intraperitoneally administered Ca^{45} from the blood in the experimental groups in comparison with that of the control group. The Ca^{45} specific activity of plasma at 72 hours after the isotope injection was significantly decreased in the group of rats treated for 1 week, while there was a concomitant tendency to an increase in the Ca^{45} activity of bone. In the group of rats treated for 3 weeks the Ca^{45} activity of both plasma and bone showed values similar to those of the normal group.

All the seven groups of rats treated for 4 to 16 weeks showed consistently decreased values for both the plasma and bone Ca^{45} activity.

The Ca^{45} activity of compact bone powder was appreciably lower than that of the whole tibia. In the three groups of rats treated for 1, 2 and 3 weeks the former activity showed approximately the same values as those of the normal control group. In the seven groups of rats treated for 4 to 16 weeks, consistently decreased values were obtained, with a maximum reduction in the groups of animals treated for 12, 14 and 16 weeks.

The calculated calcium accretion rate for the tibia, expressed as mg calcium/hour/mg calcium of the tibia, showed increased mean values in the groups of rats treated for 1 and 2 weeks, compared with that of the normal control group. The six groups of oophorectomized rats treated with prednisolone for 3 to 12 weeks showed approximately the same mean values as that of the normal control group. In the group treated for 14 weeks a lower mean accretion rate was found, while it was higher in the group of animals treated for 16 weeks compared to that of the control group.

The presented data show that the hormonal imbalance induced in adult rats by combined treatment with oophorectomy and prolonged prednisolone administration evidently results in increased calcium deprivation because of increased excretion progressing with the duration of prednisolone treatment. There was an increased elimination from the blood of intraperitoneally administered Ca^{45} and a corresponding decrease in the uptake of Ca^{45} by bone. Except for an initial increase, the calcium accretion rate by bone was approximately the same in the experimental and control rats. These data indicate indirectly that the significant reduction of bone tissue observed in the same material and reported elsewhere was caused by increased bone resorption which would mobilize skeletal calcium to the blood to normalize the observed significant fall in the blood calcium level.

ACKNOWLEDGEMENTS

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The Glycosaminoglycans* of Compact Bone Tissue in Experimental Osteoporosis Induced by Prednisolone Treatment of Oophorectomized Rats or by Calcium Restriction**

by

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* The nomenclature of Balasz and Jeanloz (1965) has been followed.

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Abbreviations: CPC, cetylpyridinium chloride; CP, cetylpyridinium; EDTA, disodium ethylenediamine tetraacetic acid.

INTRODUCTION

Very little is known about the function of the bone glycosaminoglycans. It has been suggested that chondroitin sulphate plays a role in the mineralization stage during bone formation (*Glimcher* 1959, *Sobel et al.* 1960), but opinions are divergent.

The formation of glycosaminoglycans of connective tissues appears to be under endocrine control (for ref. see *Brunish* 1966). An inhibitory effect of corticosteroids on the synthesis of glycosaminoglycans in different connective tissues has been demonstrated both *in vitro* and *in vivo*. In view of the decrease observed in S^{35}-sulphate incorporation into the chondroitin sulphate of rib cartilage (*Boström and Odeblad* 1954, *Whitehouse and Boström* 1961), some influence also on bone tissue glycosaminoglycans could be anticipated. However, the effect of excess corticosteroids on the glycosaminoglycans in adult bone tissue does not appear to have been studied. From the finding of a decrease in the ratio of total hexosamine to collagen in the *whole* femur of one year old rats it has been suggested that corticosteroids reduce the content of glycosaminoglycans in bone, which would precede the events leading to the development of osteoporosis (*Sobel and Marmorston* 1954). However, these results cannot be interpreted as an effect on bone tissue glycosaminoglycans alone, since the articular cartilage of the femur, with its high content of glycosaminoglycans, was also included in the specimens. Furthermore, the total hexosamine content does not reflect the glycosaminoglycan content alone.

In human osteoporosis, changes in bone matrix have been reported from studies with the aid of X-ray diffraction and electron microscopy (*Little et al.* 1962). Decreased hydroxyproline contents have been found in bone specimens from the iliac crest in osteoporotic subjects (*Birkenhaeger-Frenkel* 1966). With biochemical methods, *Casuccio et al.* (1962) noted a decrease with age in protein nitrogen and hexosamines, and changes in the glucosamine/galactosamine ratio in several fractions of vertebral bone tissue extracted according to the method of *Dische et al.* (1958). The significance of these findings in relation to the osteoporotic process and to a possible role of glycosaminoglycans in this connection is far from clear.

The present investigation was undertaken in order to obtain information

regarding the quantitative and qualitative relationships of the glycosaminoglycans in the compact bone tissue of adult rats during the development of two types of experimental osteoporosis, induced by (1) oophorectomy combined with prednisolone treatment, or (2) prolonged calcium restriction. The material was analysed mainly by methods based on the solubility properties of the glycosaminoglycan complexes with cetylpyridinium. By adapting the technique of *Antonopoulos et al.* (1964), information was obtained on the distribution of the chondroitin sulphate with regard to molecular size. The isolated chondroitin sulphate was further characterized by physical and chemical methods. In addition, the concentrations in compact bone tissue of glycosaminoglycans, hexosamines, hydroxyproline, ash and calcium were determined in normal conditions and during the development of these two different types of osteoporosis.

MATERIAL

1. Compact bone tissue of normal and prednisolone-treated oophorectomized adult rats.

Fifty-five adult female rats of the Sprague-Dawley strain[1] were used. All the animals were 11 1/2 to 12 months old at the start of the experiment and had a mean initial body weight of 348.5 ± 2.7 grams. The material was divided at random into ten experimental groups and one control group with 5 rats in each. The experimental animals were first oophorectomized via a midline abdominal incision, under ether anaesthesia. All these animals recovered very quickly after the operation. From the third day after the oophorectomy each animal was given 2.5 mg prednisolone[2] daily, dissolved in their drinking water, which was supplied intermittently in such a way that the prednisolone was consumed during the following 1—2 hours. All animals were housed in individual cages. The periods of treatment varied from 1 to 16 weeks. The control group consisted of 5 normal rats and was observed for 16 weeks. The experimental and the control animals received the same ordinary laboratory food ration[3] and tap water. All animals except one survived through the

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[1] Anticimex Co., Stockholm.

[2] Precortalon Aquosum®, Organon, kindly supplied by Pharmacia Co., Uppsala.

[3] Ewos Co., Södertälje.

period of observation. There were no signs of infections or any other intercurrent diseases. The experimental animals lost about one third of their initial body weight. The femur length was the same as in the normal animals, which confirmed that no interference with skeletal growth had occurred.

The series was planned so that all the animals were sacrificed at the same time. This was performed by bleeding them to death from the femoral artery under ether anaesthesia. Immediately after sacrifice the animals were placed in a deep freezer at $-20°C$ pending the preparations of the long tubular bones. The bones were carefully dissected free from soft tissues and periosteum. The epiphyses, including the cartilage, were removed, using a bandsaw. The diaphyses were split into small bone fragments which were carefully cleaned from bone marrow with no use of solvents. The bone fragments were further defatted and dehydrated with 5 changes of acetone for 3 days. The material was finally air dried and then pulverized in an Intermediate model Wiley Laboratory Mill provided with a 60-mesh sieve. The defatted, dry bone powder obtained was stored in containers with snap caps pending the subsequent analyses.

2. *Compact bone tissue of normal and calcium-deficient adult male rats.*
Forty-one adult male rats of the Sprague-Dawley strain[1] were used. The experimental animals were 12 to 12 1/2 months old at the start of the treatment and were divided into seven groups. The control group consisted of five 21-month old rats. The mean initial body weight of all rats was 505.2 ± 8.2 grams. All animals had been bred on a diet with an optimal intake of calcium, phosphorus and vitamin D. The experimental animals received a calcium deficient diet[2] containing 0.09 per cent Ca, 0.55 per cent P and 2.0 per cent cod-liver oil. The animals of the experimental groups were supplied with diet and deionized water *ad libitum* for 3 weeks and 6, 7, 8, 9, 10 and 12 months, respectively. The control group received a normal laboratory ration[3] containing 1.0 per cent Ca and 0.75 per cent P. All animals were kept in a sunless room. Five deaths occurred among the experimental animals, probably related to high age. Three of these animals, which were frozen immediately after death after 4—6 months on a calcium deficient diet, were included in the study, however. The bones were prepared and the bone powder was produced as described above.

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[1] Anticimex Co., Stockholm.

[2] Bulletin D-15, General Biochemical Inc., Chagrin Falls, Ohio, USA.

[3] Ewos Co., Södertälje.

METHODS

Hexosamine was determined by the Elson and Morgan reaction as described by *Antonopoulos et al.* (1964), except that a 1:2 scaled down procedure was used in most cases.

Aminosugar was characterized by the Dowex 50 H^+ column chromatographic procedure of *Antonopoulos* (1966).

Uronic acid was determined by the carbazol method of *Dische* (1947).

Sulphate was determined by the benzidine method as described by *Antonopoulos* (1962).

Calcium in bone powder was determined by atomic absorption spectrophotometry, using a Perkin-Elmer Model 303 Instrument. About 50 mg of bone powder was ashed at 700°C in a muffle furnace for 16 hours. The ash was weighed on a Mettler balance with an accuracy of 0.05 mg and the percentage ash content calculated. The ash was then dissolved in 10 ml of 1.2 N hydrochloric acid. After dilution to the desired calcium concentration a 5 % lanthanum solution in 25 % (v/v) HCl was added so that the final calcium concentration was within the optimum working range of 1 to 10 ppm and with 0.5 % lanthanum (see Perkin-Elmer Rev. May 1966). In this way the calcium: lanthanum ratio was 1: 1000, as recommended by *Willis* (1961) for depression of the phosphorus interference. The added calcium recovery in these bone ash solutions was found to be 98 per cent. Duplicate determinations were performed throughout. The coefficient of variation for these analyses was less than 1 per cent.

Hydroxyproline was determined by the method of *Neuman and Logan* (1950). The coefficient of variation for these determinations was 2 per cent.

Susceptibility to testicular hyaluronidase[1] was tested by the procedure described by *Solheim* (1965).

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[1] Obtained by the courtesy of Dr. B. Högberg, AB Leo, Hälsingborg, Sweden.

Infrared spectrophotometry[1] was carried out using a Unicam SP 200 spectrophotometer. The KBr pellet technique was used.

Determination of total hexosamine concentration.

Hexosamine determination (see above) was performed after hydrolysis of 5 mg bone powder in 1 ml of 6 N HCl for 8 hours. After evaporation of the acid, the residue was dissolved in 0.5 ml of water. The coefficient of variation was 3 per cent. (The residue sometimes contained fine black-brown particles. Therefore, in some series of determinations 20 mg powder was hydrolysed instead, the residue was dissolved in 2 ml of water and after filtration through a paper[2] the hexosamine was determined on 0.5 ml aliquots. The values were the same as when filtration was omitted).

For determination of hexosamine in tissues, *Boas* (1953) recommended an ion exchange step to eliminate non-specific chromogens. In the case of bone hydrolyzed as described above, this step can be omitted as no such chromogens were found.

Determination of the concentration of CPC-precipitable glycosaminoglycans.

In a 10 ml volume flask about 45 mg of bone powder was digested at 65°C in about 9 ml of a digestion mixture containing EDTA (pH adjusted to 7 with NaOH), cysteine HCl and recrystallized papain[3] suspension to concentrations of 0.075 M, 0.005 M and 1—2 per cent, respectively. After 3—6 hours, when a clear solution was obtained, the volume was brought to the mark with water and the contents thoroughly mixed. Three aliquots of 3.0 ml each were pipetted into test tubes, 4 ml of a half per cent (w/v) aqueous CPC[4] solution was added to each and the precipitate allowed to settle over night. The precipitate was recovered by centrifugation and meticulous decanting and after hydrolysis and evaporation hexosamine determinations were made.

Determinations on aliquots from a digest of larger quantity showed a coefficient of variation of 3 per cent.

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[1] Kindly performed by Dr. L.-Å. Fransson, Department of Physiological Chemistry, University of Lund, Lund, Sweden.

[2] Munktell's No. OOH-S1-80-90.

[3] Worthington, 18.8 mg protein per ml.

[4] CPC, special grade, AB RECIP, Stockholm, Sweden.

In test experiments, when different amounts (20-50 mg) of bone powder were digested it was found that the amount of hexosamine containing material precipitated from 2 or 3 ml of digest was directly proportional to the amount of bone powder digested (Fig. 1).

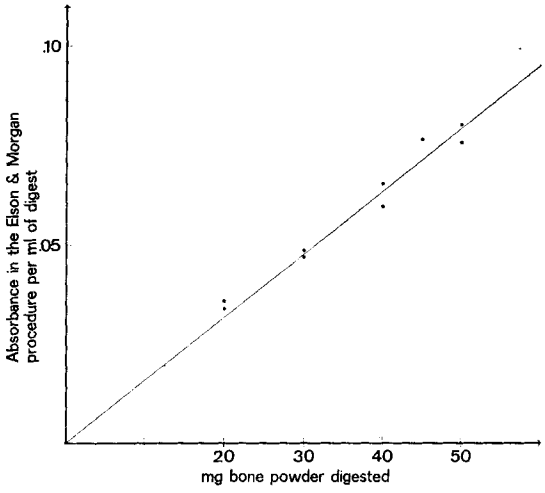

Fig. 1.
Amount of hexosamine (expressed as absorbance) in CPC-precipitate per ml of digest; various amounts of bone powder were digested. Each point represents the mean of one 2 ml and two 3 ml aliquots from one digest (except the 45 mg digest, where the mean of one 2 ml and one 3 ml aliquot was used).

Microfractionation of glycosaminoglycans.

About 750 mg of bone powder, pooled from all animals within each group, was digested at 65°C in 50 ml of a digestion mixture containing EDTA, cysteine HCl and papain suspension to concentrations of 0.2 M, 0.005 M and 1 pro mille, respectively, for 3—4 hours. After dilution with 8 volumes of water, one volume of a 1 per cent aqueous solution of CPC was added and the resulting precipitate allowed to settle over night. The precipitate was collected by centrifugation in a 100 ml glass tube, washed with redistilled water and dried for about half an hour in a desiccator. 400 μl of a 60 per cent (v/v) aqueous solution of n-propanol was added and the precipitate brought into solution by stirring with a glass rod. A fine-disperse residue remained undissolved and was centrifuged down. From the clear supernatant 50 μl aliquots were transferred by a constriction pipette to CPC-cellulose microcolumns and were fractionated according to the method of *Antonopoulos et al.* (1964) with the following eluants: 1 per cent CPC, 0.30 M NaCl, $MgCl_2$ solutions with step-wise increasing concentrations from 0.30 to 0.75 M and finally 6 M HCl. The salt solutions contained 0.05 per cent CPC.

The fine-disperse material, which could not be dissolved in the aqueous propanol, was found in test experiments to contain only a few per cent of the whole hexosamine content of the solution. When supernatant propanol aliquots were fractionated at the same time as aliquots with undissolved matter stirred up, the resulting solubility profiles did not differ noteworthily, and recoveries in both cases were about 90 per cent. Centrifugation was preferred in the final procedure because of a tendency to clogging in the columns when uncentrifuged solutions were used.

Isolation and fractionation of bone glycosaminoglycans on the macroscale.

The procedure used by *Hjertquist and Vejlens* (1968) was followed essentially. Digestion was performed as described above, with 50 ml of digestion mixture per g (3—15) bone powder. Eight volumes of water and one volume of a 1 per cent CPC solution were added, and after 48 hours the precipitate formed was collected on glass filter paper[1] in a Büchner funnel. It was subsequently dissolved in 65 per cent aqueous propanol. (The paper was minced in the propanol solution which was then sucked out through a new glass filter until the minced paper was dry). To the clear solution 3 volumes of ethanol were added and the sodium salts of glycosaminoglycans were precipitated by adding a few ml of saturated sodium acetate aqueous solution. The precipitated material was recovered by centrifugation and dried with ethanol and ether. The powder was dissolved in water and small amounts of undissolved matter were spun down and discarded. To the supernatant (3—4 ml) as much of a 2 M NaCl solution was added so as to obtain a final concentration of 0.02 M NaCl, after the addition of approximately two volumes of a 1 per cent CPC solution. On the following day the precipitated complexes were recovered by centrifugation, washed, dried for half an hour in a desiccator and dissolved in 60 per cent propanol. A small amount of substance remained undissolved. This was suspended and the solution placed on top of a CPC-cellulose column, prepared according to *Antonopoulos et al.* (1961). Dimensions of columns: 1 x 25 cm for 25—40 mg dried polysaccharide, 1 x 15 cm for 10 mg. Elution was performed with 1 per cent CPC, 0.3 M NaCl, 0.3 M $MgCl_2$ and 0.83 M $MgCl_2$ and finally in some cases 1.0 M $MgCl_2$. All salt solutions contained 0.05 per cent CPC. The collection of fractions, detection of peaks (by CP-complex precipitation) and

[1] Whatman, GF 81.

recovery of sodium salts of the glycosaminoglycans were performed as described by *Antonopoulos et al.* (1965).

Statistical methods.

The analytical data obtained from the rats in each group were subjected to analysis of variance with one-way lay-out according to *Scheffé* (1961). The hypothesis of no difference between groups was tested at the 5 per cent significance level. When a significant result was obtained, data of one or more groups were combined in order to find significant contrasts. These were tested according to *Scheffé* (1961).

RESULTS

Contents of ash, calcium and hydroxyproline.

The major components of the defatted, dried bone powder, *i.e.* mineral and collagen, were examined by determinations of the ash, calcium and hydroxyproline contents. In order to reduce the effects of small methodological variations from one occasion to another, all samples at each investigation were treated at the same time whenever possible (*e.g.* ashing procedure, weighing, calcium determinations and hydrolysis), otherwise the samples were taken at random for the analyses.

In the corticosteroid experiment the ash and calcium contents showed slightly lower values in the three groups of animals treated for 1 to 3 weeks than in the normal control group (Table 1). When the values for these groups combined were tested against those of the normal control group the differences were statistically significant; in the case of calcium, however, only when the groups treated for 2 and 3 weeks were combined. In the seven groups of rats treated for 4 to 16 weeks no significant changes were found, however. The hydroxyproline contents for treated groups showed slightly fluctuating values as compared with those of the control group, but the variations were not statistically significant. In the calcium-deficiency experiment no significant changes were noted in the concentrations of ash, calcium or hydroxyproline (Table 2).

Groups	No. of animals	Ash %	S.E.M.	Calcium %	S.E.M.	Hydroxy-proline ‰	S.E.M.
Normal controls	5	65.51	0.43	25.59	0.38	16.4	0.51
Ooph + prednisolone for 1 week	5	63.21	0.57	24.08	0.28	17.1	0.58
„ „ „ 2 weeks	5	62.97	0.56	23.56	0.30	17.2	0.32
„ „ „ 3 „	5	62.13	0.44	23.54	0.25	16.2	0.44
„ „ „ 4 „	5	65.57	0.76	24.71	0.37	17.1	0.44
„ „ „ 5 „	5	66.26	0.56	24.90	0.20	16.1	0.39
„ „ „ 8 „	5	67.05	0.50	24.73	0.32	16.0	0.35
„ „ „ 10 „	5	65.60	0.25	24.95	0.24	16.2	0.30
„ „ „ 12 „	4	65.73	0.15	25.12	0.26	16.2	0.59
„ „ „ 14 „	5	65.67	0.30	24.88	0.14	17.1	0.22
„ „ „ 16 „	5	65.61	0.41	25.01	0.24	16.5	0.13
		$F = 10.12$ sign.		$F = 5.34$ sign.		$F = 1.38$ N.S.	
				$F_{0.95}\ (10.43) = 2.06$			

Table 1. The concentrations of ash, calcium and hydroxyproline in compact bone powder from normal control rats and oophorectomized rats treated with prednisolone for different periods of time. (S.E.M.=standard error of the mean).

Groups	No. of animals	Ash %	S.E.M.	Calcium %	S.E.M.	Hydroxy-proline ‰	S.E.M.
Normal laboratory ration	5	63.13	0.40	24.28	0.23	16.4	0.23
Low calcium diet for 3 weeks	6	63.48	0.19	23.98	0.30	16.8	0.17
„ „ „ „ 4-6 months	3	63.92	0.39	24.58	0.49	16.1	0.34
„ „ „ „ 6 „	4	63.37	0.29	24.62	0.20	16.1	0.23
„ „ „ „ 7 „	4	63.03	0.66	24.39	0.77	16.0	0.28
„ „ „ „ 8 „	3	62.55	0.38	23.56	0.25	16.2	0.36
„ „ „ „ 9 „	5	63.82	0.33	23.88	0.15	16.3	0.37
„ „ „ „ 10 „	5	63.12	0.26	24.08	0.32	16.4	0.13
„ „ „ „ 12 „	4	63.71	0.83	24.45	0.77	16.4	0.38
		F = 0.87 N.S.		F = 0.59 N.S.		F = 0.93 N.S.	
				$F_{0.95}$ (8.30) = 2.27			

Table 2. The concentrations of ash, calcium and hydroxyproline in compact bone powder from rats fed a normal laboratory ration and rats fed a low calcium diet for different periods of time.

Groups	No. of animals	Total hexosamines ‰	S.E.M.	Glycosamino-glycans ‰	S.E.M.
Normal controls	5	1.48	0.02	0.39	0.009
Ooph + prednisolone for 1 week	5	1.51	0.01	0.40	0.018
„ „ „ 2 weeks	5	1.56	0.03	0.41	0.015
„ „ „ 3 „	5	1.49	0.00	0.41	0.023
„ „ „ 4 „	5	1.46	0.01	0.41	0.012
„ „ „ 5 „	5	1.49	0.03	0.40	0.017
„ „ „ 8 „	5	1.46	0.03	0.40	0.015
„ „ „ 10 „	5	1.41	0.00	0.41	0.020
„ „ „ 12 „	4	1.46	0.03	0.38	0.007
„ „ „ 14 „	5	1.48	0.02	0.40	0.009
„ „ „ 16 „	5	1.43	0.01	0.40	0.014
		F = 3.16 sign.		F = 0.49 N.S.	
		$F_{0.95}$ (10.43) = 2.07			

Table 3. The concentrations of total hexosamines and CPC-precipitable glycosaminoglycans (as hexosamines) in compact bone powder from normal control rats and oophorectomized rats treated with prednisolone for different periods of time.

Groups	No. of animals	Total hexosamines ‰	S.E.M.	Glycosamino-glycans ‰	S.E.M.
Normal laboratory ration	5	1.55	0.02	0.46	0.008
Low calcium diet for 3 weeks	6	1.48	0.01	0.43	0.006
„ „ „ „ 4-6 months	3	1.49	0.07	0.44	0.023
„ „ „ „ 6 „	4	1.53	0.05	0.46	0.017
„ „ „ „ 7 „	4	1.50	0.02	0.42	0.017
„ „ „ „ 8 „	3	1.49	0.01	0.41	0.026
„ „ „ „ 9 „	5	1.53	0.01	0.45	0.012
„ „ „ „ 10 „	5	1.55	0.04	0.42	0.018
„ „ „ „ 12 „	4	1.48	0.01	0.42	0.024
		F = 0.87 N.S.		F = 1.20 N.S.	
			$F_{0.95}$ (8.30) = 2.27		

Table 4. The concentrations of total hexosamines and CPC-precipitable glycosaminoglycans (as hexosamines) in compact bone powder from rats fed a normal laboratory ration and rats fed a low calcium diet for different periods of time.

Total hexosamine concentration.

The determinations of the total hexosamine concentration were performed on bone powder from the individual rats. About twenty 20 mg samples or fifty 5 mg samples of bone powder were weighed and analysed simultaneously. In the calcium-deficiency experiment the 5 mg sample analysis was used throughout. Duplicate analyses were performed in approximately one third of the material. In the corticosteroid experiment the two groups of rats treated for 1 and 2 weeks showed slightly increased values (Table 3). This increase was not statistically significant, however. The eight groups of animals treated with prednisolone for 3 to 16 weeks showed no consistent change which could be correlated to the duration of treatment but only a small, insignificant intergroup variation.

In the calcium-deficiency experiment the values of the eight experimental groups did not differ significantly from those of the control group (Table 4).

Concentration of glycosaminoglycans.

The glycosaminoglycan concentration determinations were also performed on bone powder from individual animals. Nine samples, randomly selected within each of the experimental series, were digested and precipitated at the same time. Hydrolysis and the remaining steps of the procedure were carried out on 9 or 18 samples simultaneously. The results are presented in Tables 3 and 4.

No statistically significant differences were found between the various groups within each experimental series. No change with the duration of treatment was noted. The hexosamines emanating from glycosaminoglycans corresponded to less than a third of the total hexosamines.

Microfractionation of bone glycosaminoglycans.

In this case 750 mg of pooled bone powder consisting of equal parts from each of the rats in separate experimental and control groups were used. Three columns were run for each group. The recovery was found to be between 90 and 110 per cent.

The fractions eluted with 1 per cent CPC and 6 M HCl only occasionally contained traces of hexosamine.

The 0.3 M NaCl fraction constituted about 6 per cent of the sum of the eluted hexosamines and no differences were noted between the control and treated groups.

The sum of the $MgCl_2$ fractions (the chondroitin sulphate fraction) constituted about 94 per cent of the eluted hexosamines. The means of the values of the $MgCl_2$ fractions from the three columns were used for constructing solubility profiles. In every fractionation experiment two different groups were investigated at the same time and thus tested against each other as shown in Table 5.

Table 5. Groups tested pair-wise with the microfractionation procedure.

ST 10 w ST 3 w	ST 8 w ST 14 w	CaD 6 m CaD 10 m	SC ST 16 w
CaC CaD 12 m	CaD 8 m ST 2 w	CaC ST 2 w	CaC CaD 3 w

w = weeks m = months ST = steroid treatment CaD = calcium deficiency SC = control group, steroid experiment CaC = control group, calcium deficiency experiment

Two of the groups (ST 2 w, CaC) were tested 2 or 3 times against different groups. As the two solubility profiles emanating from microfractionations performed simultaneously were always nearly identical, it is concluded that there were no differences in the solubility properties of the CP-chondroitin sulphate from the different groups. When profiles from fractionations performed on different occasions were compared, deviations were noted, increasing with time and eventually becoming larger than expected on the basis of the reproducibility found previously. An increasing parallel shift to the right of the CP-chondroitin sulphate solubility curves was noted. Control fractionations showed that this phenomenon was due to a change with time of the solubility properties of the $MgCl_2$ solutions, which was not reflected in the titrated Cl^2-ion concentrations. This phenomenon might possibly have been caused in some way by the storage of salt solutions in small (10 ml) sealed glass bottles with rubber stoppers (the intention was to minimize evaporation), as such effects have not been seen in other studies when the salt solutions have been stored instead in larger, plastic bottles with screw caps.

Table 6. Chemical and physical characteristics of the various glycosaminoglycan fractions isolated from rat cortical bone.

Fraction	Amount isolated	Per cent of dry weight: Hexosamine	Hexuronic acid	Sulphate	Hexuronic acid[1] / Hexosamine	Sulphate[1] / Hexosamine	Sulphate[2] / Hexuronic acid	Per cent of hexosamine: galactosamine / glucosamine	Susceptibility to hyaluronidase	Infrared pattern in accordance with
0.83 M $MgCl_2$ female controls	3.5 mg	22.5	30.0	14.9	1.23	1.23	1.00	100 / 0	+	Chondroitin -4-sulphate
0.83 M $MgCl_2$ prednisolone treated	10.0 mg	22.7	30.3	15.3	1.23	1.26	1.02	100 / 0	+	Chondroitin -4-sulphate
0.83 M $MgCl_2$ male controls	7.0 mg	18.6	23.6	12.0	1.17	1.20	1.03	100 / 0	+	Chondroitin -4-sulphate
0.83 M $MgCl_2$ Ca-deficiency	12.0 mg	21.8	29.9	14.4	1.27	1.23	0.97	100 / 0	+	Chondroitin -4-sulphate
0.30 M $MgCl_2$ female and male controls pooled	5.0 mg	3	5	not analysed	1.7	—	—	85 / 15	not tested	—
0.30 M $MgCl_2$ prednisolone treated	4.0 mg	7	6	„	0.9	—	—	70 / 30	„	—
0.30 M $MgCl_2$ Ca-deficiency	4.0 mg	4	6	„	1.5	—	—	75 / 25	„	—
0.3 M NaCl prednisolone and Ca-deficiency pooled	4.0 mg	2	2	„	1.0	—	—	18 / 82	„	—

[1] Molar ratios with hexosamine = 1.00
[2] Molar ratios with hexuronic acid = 1.00

Isolation and characterization of chondroitin sulphate.

This was performed on a pool of bone powder from each of the following groups: male control group (calcium-deficiency experiment), female control group (corticosteroid experiment), calcium-deficient groups (from 6 to 12 months on low calcium intake) and prednisolone-treated oophorectomized groups (8 to 16 weeks of treatment). The two latter pools comprised 15 g of bone powder each, while that of the control group in the calcium-deficiency experiment comprised 9 g and in the corticosteroid experiment 3 g. The crude isolated glycosaminoglycans amounted to approximately 40 mg from each of the pools of the treated groups, and 20 and 10 mg, respectively, from those of the control groups. In every column separation peaks were eluted in the following three salt solutions used: 0.3 M NaCl, 0.3 M $MgCl_2$ and 0.83 M $MgCl_2$; none in 1 % CPC or 1 M $MgCl_2$. The fractions eluted with 0.3 M NaCl and obtained from the experimental groups in the two series were combined. The precipitated material from the corresponding fractions of the control groups was too small for analysis. The fractions eluted with 0.3 M $MgCl_2$ which were recovered from the control groups were combined. The amounts isolated as Na salts and the results of chemical and physical characterization of the fractions are given in Table 6. For all the fractions eluted with 0.83 M $MgCl_2$ the results are in accordance with chondroitin-4-sulphate with a similar degree of sulphation. The analytical results of the 0.3 M NaCl fractions and the 0.3 M $MgCl_2$ fractions are not wholly conclusive. It is considered, however, that the glycosaminoglycans in these fractions constituted a mixture of hyaluronic acid and low molecular and/or low sulphated chondroitin sulphate, the former predominating in the NaCl fractions and the latter in the 0.3 M $MgCl_2$ fractions. Corresponding fractions isolated from cortical bone from dogs (*Hjertquist and Vejlens* 1966, 1968) have been shown to contain hyaluronic acid and a mixture of hyaluronic acid and chondroitin sulphate, respectively.

DISCUSSION

Isolation of glycosaminoglycans from tissues by means of papain digestion in the presence of EDTA, followed by precipitation of the liberated glycosaminoglycans with cetylpyridinium chloride, is now a well established technique. As shown by *Hjertquist and Vejlens* (1968) this procedure can also be used for

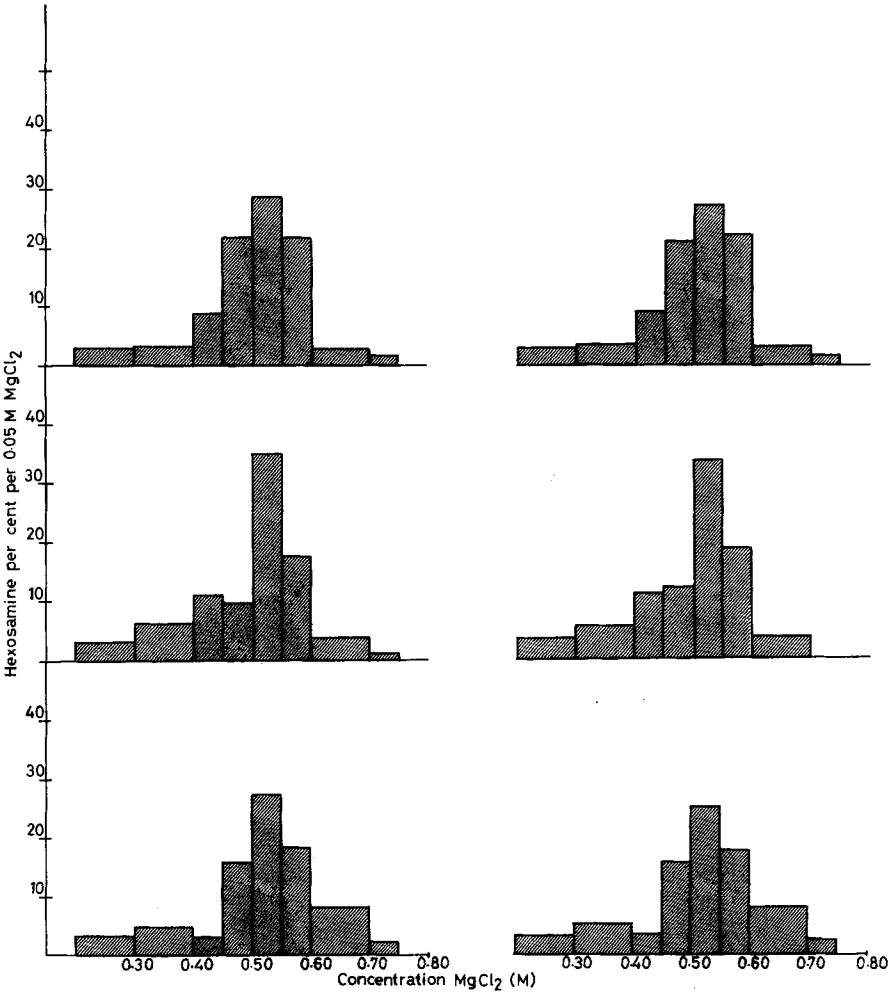

Fig. 2. Some representative solubility profiles of the CP-chondroitin sulphate. Amount of hexosamine expressed as percentage of total hexosamines eluted with $MgCl_2$ solvents. Upper profiles: left SC, right ST 16 w. Middle profiles: left SC, right CaD 12 m. Lower profiles: left ST 2 w, right CaD 8 m. (For notation, see Table 5.). Note the almost identical appearance of solubility profiles on the same horizontal line, which are from microfractionations performed in parallel.

bone, provided that the EDTA content of the digestion mixture is high enough to chelate the bone calcium. In this latter study chondroitin sulphate amounting to 0.14—0.18 per cent on the basis of total dry weight was isolated from compact dog bone. The amount of chondroitin sulphate isolated from compact rat bone in the present study was only 0.07—0.08 per cent, on the same basis. These lower values can partly be explained by the higher mineral content of rat bone as compared with dog bone. Further, a higher percentage loss of chondroitin sulphate during the isolation and purification procedures in the present study is likely, because of the smaller amount of initial material available than was used in the investigation by *Hjertquist and Vejlens* (1968).

Other investigations have shown hat chondroitin-4-sulphate is the predominating glycosaminoglycan of compact bone tissue from ox (*Meyer et al.* 1956), dog (*Hjertquist and Vejlens* 1968), rabbit and homo (*Vejlens*, unpublished). Also in the rat bone studied in the present investigation the main glycosaminoglycan was a sulphated galactosaminoglycan with characteristics of chondroitin-4-sulphate.

The original procedure for microfractionation of CP-glycosaminoglycans described by *Antonopoulos et al.* (1964) was developed on cartilage and allowed a simultaneous quantitative and qualitative analysis. Because of the low content of glycosaminoglycans and the high mineral content of the bone tissue examined in the present study, quantitative analyses were performed separately from the microfractionation. The quantitative determination of the bone glycosaminoglycans as CPC-precipitable components of a papain digest is a relatively simple procedure. It appears less tedious than the method described by *Bollet* (1958) in which no proteolysis is used, protamine is the precipitating agent and dialysis steps are included. In the present investigation the amount of bone powder used for the quantitative analyses of the glycosaminoglycans was approximately 50 mg, which allowed triple hexosamine determinations from one digest. If required, a single determination can be performed on only 10—12 mg of bone powder. In that case the digestion, precipitation and the Elson and Morgan reaction are carried out in the same test tube.

Only a minor proportion of the total hexosamines in compact bone tissue originates from acid glycosaminoglycans — in the rat bone analysed in the present study less than a third. The major part seems to be associated with glycoproteins (*Herring* 1964, *Burckard et al.* 1966).

In the present study the microfractionation procedure of *Antonopoulos et al.* (1964) was adapted to allow relatively small amounts of bone powder to be analysed for the construction of solubility profiles of CP-chondroitin sulphate.

In order to facilitate the handling of the obtained CP-glycosaminoglycan complexes for subsequent solubilization in a small volume of aqueous propanol, somewhat more bone powder (750 mg) was used than was necessary to run three columns. Smaller amounts can be used; the lower limit for only one column is in the range of 50—100 mg of bone powder.

The composition of the bone powder with regard to the relation of mineral/collagen was essentially unchanged in each of the experimental series. This supports the view that the rarefaction of the bones observed in previous studies of the same rats (*Larsson* 1969 a, b) after longer periods of treatment was, in fact, consistent with osteoporosis. The only exception was the slightly lower mineral content per weight of the bone powders from oophorectomized rats treated with prednisolone for 1—3 weeks. They did not, however, exhibit the corresponding increase of hydroxyproline which would be expected if an initial phase of osteomalacia was involved. That the lower ash and calcium values mentioned may have originated from a methodological error cannot thus be excluded. Furthermore, no demineralization of bone tissue was found in rabbits treated with dexamethasone for 3 weeks (*Vejlens*, unpublished).

The effect of corticosteroids on the metabolism of glycosaminoglycans has been studied in different tissues both *in vitro* and *in vivo*. Using S^{35}-labelled sodium sulphate as the tracer substance *Layton* (1951 a, b) noted a decrease in the activity of "bound sulphate" in different tissues after the administration of cortisone. The production of glycosaminoglycans by connective tissue cells in tissue culture becomes decreased in the presence of methylprednisolone (*Castor* 1962). Rats given cortisone *in vivo* show a decreased uptake of radioactive-labelled sulphate and/or acetate by hyaluronate and chondroitin sulphate isolated from the skin (*Schiller and Dorfman* 1957). The incorporation of S^{35}-labelled sodium sulphate in the sulphate groups of chondroitin sulphate of calf cartilage becomes decreased both *in vitro* and *in vivo* (*Boström and Odeblad* 1953, *Whitehouse and Boström* 1961). In recent years results have been presented suggesting an inhibitory effect of corticosteroids also on the degradation of glycosaminoglycans in rabbit costal cartilage (*Kaplan and Fisher* 1964). Low concentrations of hydrocortisone have no significant inhibitory effect on the synthesis of chondroitin sulphate by cartilaginous embryonic chick tibiotarsi in organ culture, but have a marked effect on the distribution of the glycosaminoglycan between the tibiotarsus and the culture medium, suggesting inhibited degradation (*Schryver* 1965). As far as the mature bone tissue is concerned nothing has been reported concerning the effect of corticosteroids on the glycosaminoglycans.

In previous studies (*Larsson* 1969 c, d), data obtained with the aid of Ca^{45} indicated increased mobilization of skeletal calcium for maintenance of the blood calcium level both after combined oophorectomy and prednisolone administration and after prolonged calcium restriction. The results of the present investigation show that during this process of prolonged increased bone resorption the remaining compact bone tissue is quite unchanged with regard to its major components and also to the glycosaminoglycans, even after combined oophorectomy and prednisolone treatment for 16 weeks or maintained calcium restriction for up to 1 year. Apart from an unchanged concentration, there is no change in the molecular weight distribution of the bone chondroitin sulphate, as indicated by unchanged solubility profiles at a similar grade of sulphation.

The lack of an apparent corticosteroid effect on the glycosaminoglycans of mature bone tissue is further supported by results obtained in a short-term study of almost fully mature rabbits treated for 3 weeks with other glucocorticosteroids *i.e.* dexamethasone, 0.5 mg/kg body weight daily, or cortisone, 3 mg/kg daily. The concentrations of total hexosamine, glycosaminoglycans, hydroxyproline and calcium did not differ from those of control animals, and neither did the solubility profiles of the CP-complexes of chondroitin sulphate (*Vejlens,* unpublished).

These findings suggest that the treatments employed did not cause any changes of the glycosaminoglycans in the bone, in any case not in the already formed bone tissue. Although the bone formation rate in old rats is rather low, there is some new bone formation, which can be observed histologically as a relatively small amount of bone surface showing tetracycline uptake (*Larsson* 1969 a, b). The amount of glycosaminoglycans emanating from bone tissue laid down during the shorter periods of treatment may be too small to influence the analytical results. Whether this also holds true for longer periods of treatment is more difficult to say. However, it is not unlikely that the major portion of the glycosaminoglycans of compact bone tissue is synthesized during the period of skeletal growth and that the total turnover of glycosaminoglycans in the normal mature skeleton is very low, analogous to that of bone collagen as reported by *Kao et al.* (1965). Therefore, in the present study an effect of the treatment on glycosaminoglycans of bone tissue during formation cannot be fully excluded, especially in the case of steroid administration. On the other hand, the observed similarities of these two types of osteoporosis may support the possibility of a common, more essential factor in the pathogenesis, *e.g.* enhanced bone resorption because of a need of calcium mobilization. Further studies along these lines, particularly concerning the turnover of bone tissue

glycosaminoglycans, should provide more knowledge about osteoporotic conditions of these kinds.

SUMMARY

The composition of compact bone from adult rats during the development of experimental osteoporosis was studied. Groups of male rats subjected to calcium restriction for various periods up to 12 months and female, oophorectomized rats treated with prednisolone for up to 16 weeks were used. Adult, normal rats of corresponding sexes served as controls.

The dried, pulverized bone was analysed for contents of mineral (ash, calcium), collagen (hydroxyproline), total hexosamine and glycosaminoglycans (as hexosamine of cetylpyridinium-precipitable polysaccharides). The relation of mineral/collagen remained mainly unchanged by the treatments, thus confirming that the rarefaction of the bones of animals treated for longer periods, noted in previous studies, was genuine osteoporosis. The concentrations of total hexosamines and of hexosamines originating from glycosaminoglycans were not affected by the treatments. Of the latter, more than 90 per cent could be accounted for by chondroitin sulphate (see below), and they amounted to less than a third of the total hexosamines.

The glycosaminoglycans of the bone tissue both from normal and experimental animals were isolated and fractionated on the macroscale using the cetylpyridinium precipitation method. The main component was identified as chondroitin-4-sulphate. A smaller part was presumably hyaluronic acid.

Smaller amounts of glycosaminoglycans from bone pooled within experimental groups were also isolated as cetylpyridinium complexes, applied in propanol solution on microcolumns and fractionated. The results indicated that no change in the molecular size pattern of chondroitin sulphate occurred with the different forms of treatment.

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GENERAL DISCUSSION

Any attempt to give a survey of the enormous amount of literature concerning clinical osteoporosis meets necessarily with a profusion of contradictory theories of its aetiology (*Dent* 1968). In normal aging, there is a gradual steady loss of bone beginning soon after the age of 20 years. This has been considered by some authors to be the main cause of human osteoporosis (*Rose* 1967). However, there are, most likely, additional factors accelerating bone loss and giving rise to symptomatic osteoporosis in juvenile, adult and also senile patients (*Dent and Watson* 1966). Further investigations regarding such factors are therefore important both from the clinical and theoretical point of view. In this respect, the reaction of the mature bone tissue to nutritional and hormonal disturbances is of special interest. In contrast to the multitude of incoherent data obtained in extensive clinical studies on patients with advanced osteoporosis, little information has been provided concerning bone loss in experimental animals. Much of the research and interpretation of data has been influenced by the extensive literature on rickets in rats. However, studies of skeletal growth in young animals do not seem to be relevant to the problems associated with the maintenance of the adult skeleton (*Hegsted* 1967). Also concerning induced hormonal disturbances, young animals have been studied almost exclusively. Therefore, the present investigation was undertaken with the purpose of determining whether or not disturbances induced by a) prolonged calcium restriction and b) oophorectomy combined with prolonged prednisolone administration, resulted in changes of the mature bone tissue and calcium metabolism which could be related to the development of osteoporosis according to the definition of *Albright and Reifenstein* (1948).

A. Calcium deficiency.

Although restricted calcium intake in adult men results in a negative calcium balance persisting for relatively long periods of time (*Malm* 1958), there is no evidence that a reduced calcium intake can, in fact, give rise to osteoporosis in humans. It was stated by *Nordin* (1960) that osteoporosis could be induced in adult animals by low calcium diets. However, the experimental studies

reported concern almost exclusively young growing animals and have not yielded coherent results.

The adult rat is generally considered to be resistent to the development of osteoporosis due to its effective adaptation to a low calcium intake, as reported in calcium balance studies (*Nicolaysen* 1960). However, the variability in calcium absorption studies is considerable (*Nicolaysen et al.* 1953), and a prolonged, slight loss of calcium from the skeleton would be difficult to determine with calcium balance techniques. The general principle for the adaptation of calcium absorption to reduced calcium intake in old rats is similar to that in aged humans (*Nicolaysen et al.* 1953). The adult rat needs 0.5 per cent calcium in the diet for calcium equilibrium (*Henry and Kon* 1947) and has been reported to remain in negative calcium balance for at least 4 months on restricted calcium intake (*Henry et al.* 1960). Therefore, the reaction of the skeletal tissue and calcium homeostasis to reduced calcium intake in the adult rat provides special interest with regard to the development of osteoporosis.

The effect of instituting a low calcium diet with normal contents of phosphorus and vitamin D on the bone tissue and calcium metabolism has been studied from different points of view in the present investigation on adult rats. A short preliminary communication of a part of this study was given by *Larsson and Sevastikoglou* (1966). The studies were planned with the purpose of following the sequence of changes in various parameters which could be related to the development of osteoporosis.

It has been suggested from results of calcium balance studies on old rats that a marked adaptation of calcium absorption will occur only when the animals have suffered a substantial loss of calcium from the skeleton. This distinctly increased efficiency of calcium absorption would compensate fully for any previous loss (*Nicolaysen et al.* 1953). However, the results obtained in those studies were not quite conclusive. Thus, rats fed a low calcium diet, *i.e.* with 0.04 per cent calcium, showed a successive increase in calcium absorption, while rats given a moderately low calcium diet, *i.e.* 0.25 per cent, did not show this reaction even after several months of calcium underfeeding (*Nicolaysen et al.* 1953).

In two-year old rats fed for 12 weeks with a diet containing only 0.11 per cent calcium and 0.55 per cent phosphorus, little, if any, effect was found in the weight of ash/cm^3 in the whole femur (*El-Maraghi et al.* 1965). In the present investigation adult rats were maintained on an almost similar low calcium diet, *i.e.* 0.09 per cent calcium and 0.55 per cent phosphorus, with adequate

amounts of vitamin D. It was found, in accordance with the results reported by *El-Maraghi et al.* (1965), that a relatively long period of calcium restriction in adult rats does not result in any notable skeletal changes. However, in the present study an evident reduction of bone mass was observed after 14 to 16 weeks of calcium deficiency. These results support those obtained in calcium adaptation studies by *Nicolaysen et al.* (1953) and *Henry et al.* (1960), that adult rats remain in negative calcium balance for a relatively long period of time after the institution of a low calcium diet. The conclusion by *El-Maraghi et al.* (1965) that a dietary calcium concentration of 0.11 per cent would probably be adequate for maintenance of the calcium balance in old rats therefore appears to be not valid. Furthermore, it was found in the present investigation that on prolonged calcium restriction there was a successive decrease in the ash content of the whole tibia by 6.2 per cent after 16 weeks and 13.3 per cent after 12 months of calcium deficiency. These results are not in agreement with the opinion of *Nicolaysen et al.* (1953) that a highly increased efficiency of calcium absorption would compensate for any previous loss in a calcium deprivation period. The findings of the present study are more in line with the observations made by the same authors that in adult rats on a moderately calcium deficient diet there is no such compensatory increase of the calcium absorption.

Corresponding to the reduction of the ash content of the tibia in the present study there was a decrease in the hydroxyproline content of the metacarpal bone. This indicates that there was a concomitant breakdown of minerals and organic bone matrix. Further, there was a decrease in the density of the humerus. Thus, a widespread loss of bone mass was observed, comprising both cancellous bone, recorded as a decrease in the number of vertebral bone "hits", and compact bone due to endosteal resorption resulting in the observed reduction in cortical bone thickness.

Histological examination revealed no signs of osteomalacia or osteitis fibrosa. No osteoclasts were observed either in the normal or in the experimental animals. In human osteoporosis bone resorption is known to occur without any obvious formation of osteoclasts (*Urist* 1958). Since there is no correlation between the number of osteoclasts and the ability of the bone, under parathyroid stimulation, to supply calcium to the extracellular fluid (*Talmage* 1967), osteoporosis could possibly develop without any notable formation of osteoclasts. The vertebral bone of the osteoporotic animals was evenly mineralized, as observed on micro-radiographs of undecalcified sections, and showed no apparent difference from

the normal in the amount of bone surface labelled with tetracycline. These observations suggest that a slight continuous loss of bone calcium with a concomitant breakdown of organic matrix occurs on the bone surfaces in the adult rat during prolonged calcium restriction. This long-term process results in osteoporosis.

In a unified concept of the pathomechanisms in human osteoporosis, the long-term process of bone reduction has been related to disturbances in calcium metabolism and the efficiency of calcium homeostasis in maintaining the extracellular fluid calcium *(Heaney* 1965). Since there are no data demonstrating such a process it was considered of interest to examine the blood calcium level and the distribution of Ca^{45} between blood and bone during the development of the described calcium deficiency osteoporosis.

It was found in the present investigation that during the development of calcium deficiency osteoporosis the blood calcium values were slightly subnormal initially and also at later periods of time, with normal values in between. The hypocalcemia secondary to overfeeding of phosphorus is well known (*Liegeois and Derivaus* 1951). In the present study the dietary phosphorus was normal, according to *Henry and Kon* (1947), and the hypocalcemia therefore seems to have been caused by the negative calcium balance induced by the lowered calcium intake. The initial transient fall in the blood calcium concentration found in the present investigation corresponds well with that found in short-term experiments in healthy adult humans after the institution of a low calcium diet (*Nordin* 1964, *MacFadyen et al.* 1965). The hypocalcemia of calcium deprivation in humans is believed to be "transient" and very small changes in blood calcium concentration are considered to be corrected by bone mineral without the intervention of the parathyroid glands, due to the existence of genuine steady state between bone mineral and tissue fluid (*Nordin et al.* 1965). During the development of osteoporosis caused by the prolonged negative calcium balance induced in the present investigation, slightly subnormal blood calcium values appeared at various periods of time and seemed to be relatively slowly corrected by bone mineral. While under normal circumstances the well-documented constancy of blood calcium (*Copp et al.* 1960) is exquisitely well regulated by means of a negative feedback (*McLean and Urist* 1955), a slightly subnormal blood calcium concentration is seen in prolonged calcium restriction due to the large stresses upon the mechanisms involved in blood calcium regulation. This circumstance does not seem to be in line with the proposal made by *Nordin* (1961) that primary depression of ionic calcium, as produced

by a low calcium diet, would cause undersaturation of tissue fluid and immediate shedding of bone mineral with restoration of the blood calcium level. Instead, stimulated parathyroid activity might well be involved during the normalization of the blood calcium concentration, since a decrease by less than 5 per cent in the blood calcium level may provide an adequate stimulus for increased production of parathyroid hormone (*Copp* 1963). The possible role played by the parathyroid glands for the development of the osteoporosis described in the present investigation will be the subject of future studies.

The distribution of Ca^{45} between blood and bone at 72 hours after the intraperitoneal administration of the isotope is of special interest with regard to the development of osteoporosis. According to *Bauer et al.* (1961), 72 hours after the Ca^{45} injection more than 90 per cent of the observed Ca^{45} activity of the bone has been incorporated into the nonexchangeable fraction of bone salt through accretion. The normal distribution of Ca^{45} rapidly changed upon restriction of the calcium intake in the present study. After only 1 week of calcium deficiency there was an increase by approximately 100 per cent in the blood Ca^{45} activity, indicating increased calcium retention. Since the adaptation of the rat to restricted calcium intake is brought about by increased absorption of calcium (*Nicolaysen et al.* 1953, *Henry et al.* 1960), the increased blood Ca^{45} specific activity was due mainly to decreased endogenous Ca^{45}. To a minor degree, a decrease in urinary Ca^{45} secondary to the observed slight fall in blood calcium might contribute to the increase in the blood Ca^{45}. Also in humans, adaptation to reduced calcium intake is effected by more efficient absorption (*Malm* 1958, *Thorangkul et al.* 1959).

After this initial phase, there was an increase by approximately 100 per cent in the deposition of Ca^{45} into bone mineral on the 3rd week of continuous calcium restriction, and despite a persistent subnormal blood calcium concentration there was a decrease to the normal level of the blood Ca^{45} specific activity. The same distribution of Ca^{45} prevailed also on the 4th and 5th weeks of calcium restriction. The subsequent normalization of the blood calcium concentration after the hypocalcemic period must then have been brought about by increased mobilization of skeletal calcium to the blood. The observed increased deposition of Ca^{45} into bone mineral appears to be secondary to the still more increased mobilization of bone calcium.

After the blood calcium concentration had returned to normal on the 5th week of calcium restriction there was another shift in the distribution of Ca^{45} between blood and bone. Thus, on the 8th week of calcium deficiency the Ca^{45}

activity of both blood and bone had reached a level 50 to 60 per cent above that of the normal controls. This distribution of Ca^{45} remained unchanged until the 16th week of maintained calcium restriction, and during this period the blood calcium was normal.

These data clearly demonstrate a persistent increase in calcium retention during prolonged negative calcium balance, induced by continuous calcium restriction. In contrast, rats fed the test diet enriched with calcium showed significantly decreased Ca^{45} activity of both blood and bone in comparison with the normal controls. The results confirm earlier observations (*Hansard et al.* 1951, *Nicolaysen et al.* 1953, *Carlsson* 1953, *Henry et al.* 1960) that animals accustomed to a high calcium intake must adjust their absorptive mechanism to a lowered supply of calcium.

With continuation of the restricted calcium intake and the development of osteoporosis, slightly subnormal blood calcium values were again observed in rats kept on the low calcium diet for 16 weeks and 6 and 7 months. Subsequently, between 8 to 12 months of calcium restriction, the blood calcium was normal During the hypocalcemic period another shift in the Ca^{45} distribution indicated that a new dynamic balance was induced in these animals by the prolonged calcium deficiency. Thus, the blood Ca^{45} specific activity showed an increase by 100 to 200 per cent, while the Ca^{45} activity of bone was increased by only about 40 per cent. In these animals, the contribution of structural changes in cancellous bone to the balance and distribution of calcium must be considered. Due to the loss of cancellous bone and of cortical bone from the endosteal surfaces, the amount of bone surface available for intense calcium deposition, *i.e.* surface accretion, is reduced. There was no apparent change in the amount of fluorescent bone surface as observed with the tetracycline labelling method. These circumstances would therefore contribute to the highly increased blood Ca^{45} specific activity observed in these osteoporotic animals. Further acceleration of calcium absorption, as suggested by *Nicolaysen et al.* (1953) and *Henry et al.* (1960), would have a similar effect.

This dynamic sequence of events found in the present investigation demonstrates that with a negative calcium balance induced by prolonged calcium restriction the efficiency of calcium homeostasis in maintaining extracellular fluid calcium is the primary pathomechanism for the development of this type of osteoporosis. During the loss of bone mineral there are shifts in the calcium dynamic balance at different periods of time, associated with the occurrence of a slightly subnormal blood calcium concentration.

In clinical osteoporosis the bone accretion rate, or mineralization rate, is usually reported to be normal (*Heaney and Whedon* 1958, *Fraser et al.* 1960, *Dow and Stanbury* 1960, *Dymling* 1964, *Nordin et al.* 1964). However, both a decreased accretion rate (*Bauer et al.* 1957, *Eisenberg and Gordan* 1961, *Lafferty et al.* 1964) and an increased (*Nordin* 1959) have also been found. It was therefore considered of interest to estimate the accretion rate in the normal and osteoporotic animals of the present investigation. In 4 groups of rats fed the low calcium diet for 7, 8, 9 and 10 months the estimated mean accretion rate values for the *whole* tibia (mg calcium/hour/mg calcium of the tibia) were similar to that of the normal control group. In 2 groups of rats fed the low calcium diet for 6 and 12 months, respectively, lower values were obtained as compared with that of the control group. These data are in agreement with those reported in human osteoporosis. A normal accretion rate was also reported by *Copp and Suiker* (1962) for 4-month old rats fed a low calcium diet, *i.e.* 0.037 per cent calcium and 0.43 per cent phosphorus for 6 months. In this latter experiment the effect of the treatment on the skeletal tissue was not studied further. When the calcium accretion rate by bone in osteoporotic individuals is compared with that in normals, differences in bone mass must be considered. Furthermore, in the present study the higher Ca^{45} activity of bone marrow blood of the osteoporotic animals compared with that of the normal controls also has some influence on the values obtained. These circumstances make it difficult to draw any conclusions regarding the calcium accretion rate by the bone tissue *per se*. The data obtained in the present study only suggest that there is no appreciable difference from the normal in the retention of calcium by the *whole* tibia and probably by the *whole* skeleton of the osteoporotic rats. It is pointed out that calcium accretion by bone cannot be equated with bone formation, since the amount of tracer localized as "diffuse uptake" in the bone tissue is too high and variable to allow such a correlation (*Rowland* 1960).

Lactating female rats have been reported to develop osteoporosis on feeding with a low calcium diet (*Tomlin et al.* 1953, *McClendon and Blaustein* 1965). Those changes could be reversed by the administration of tricalcium phosphate (*McClendon and Blaustein* 1965). However, the morphological criteria of osteoporosis used, *i.e.* basophilia of the bone trabeculae, fibrosis and increased clasmatocytic activity, suggest that the bone lesions induced might have been osteitis fibrosa rather than osteoporosis. Kittens fed a low calcium, high phosphorus diet develop osteitis fibrosa (*Greaves et al.* 1958). This condition is also well known in young cats kept on a low calcium, high phosphorus meat

diet (*Scott* 1957, 1959, *Roberts and Scott* 1961) and has been shown to be caused by nutritional secondary hyperparathyroidism (*Krook et al.* 1963). While young cats develop osteitis fibrosa, adult cats fed a similar low calcium, high phosphorus meat diet, *i.e.* 0.007 per cent calcium and 0.18 per cent phosphorus on a wet weight basis, have been reported to develop osteoporosis (*Jowsey and Gershon-Cohen* 1964). In these adult cats there was an increased bone resorption accompanied by an increase in number of newly formed osteones. Contrary to the experience in clinical osteoporosis, the reduction in bone mass was reversed by feeding a high calcium diet. In that experiment and in an extended similar study (*Jowsey and Raisz* 1968), the induced bone changes were related to observed secondary hyperparathyroidism. However, in the latter experiment cats fed the low calcium meat diet for 5 months showed a definite osteomalacia, while cats maintained on this diet for 13 months were reported to have developed osteoporosis, since the osteoid borders, although highly increased in frequency, were of normal width. The decrease in bone resorption but not in formation observed in such osteoporotic animals after treatment with a high calcium diet for 1 month suggested that the bone changes induced by the low calcium diet could be reversed. This condition differs from that in human osteoporosis by the markedly increased bone formation; osteoporosis in man being characterized by an increase in resorption alone, with the bone formation remaining relatively normal. Furthermore, young dogs receiving a low calcium diet develop skeletal changes typical of osteitis fibrosa (*Campbell and Douglas* 1965), as also do mature dogs maintained on a low calcium, high phosphorus diet (*Saville and Krook* 1968). Horses fed either a low calcium, high phosphorus diet (*Krook and Lowe* 1964) or optimal calcium but excessive amounts of phosphorus (*Gries* 1966) develop osteitis fibrosa due to nutritional secondary hyperparathyroidism. This condition has also been reported in swine fed diets unbalanced in calcium and phosphorus (*Brown et al.* 1966). According to *Jowsey* (1968), growing animals fed a diet low in calcium and high in phosphorus easily develop a frank secondary hyperparathyroidism with depressed serum phosphate and eventually depressed serum calcium, osteitis fibrosa and a gross hyperplasia of the parathyroids, while in the adult animals the same calcium deficient diet produces no biochemical changes of this kind but symptoms similar to osteoporosis. These findings do not seem to be in accordance with results reported for mature dogs fed a low calcium, high phosphorus diet (*Saville and Krook* 1968). In these animals there was a decrease in gross bone density, and histological examination of decalcified bone sections showed the presence of numerous osteoclasts with the appearance of resorption cavities and, further, osteoblasts

and osteoid tissue covering the surfaces of cancellous bone. Despite the definitely increased amount of osteoid tissue, the calcium accretion rate by bone was normal. This might suggest qualitative changes in the bone tissue due to the observed secondary hyperparathyroidism. The opinion has been held that "excessive parathyroid hormone prevents the mineralization of osteoid since the glucoproteins do not undergo depolymerization, which necessarily must precede mineralization" (*Krook et al.* 1963). On the other hand, it has been proposed that the parathyroid hormone caused depolymerization of the ground substance in bone (*Engel* 1952, *Engel et al.* 1953). Further, in accelerated bone resorption as in secondary hyperparathyroidism, osteolysis, *i.e.* osteocytic resorption of cells surrounded by matrix containing acid glycosaminoglycans, is considered to play an important role (*Bélanger* 1965).

In the processes of calcification and decalcification the protein-polysaccharides are probably of importance as controlling factors, since at the concentration in which they are found in the vicinity of calcifying sites they would certainly affect the distribution and movement of calcium and phosphate in those regions. The present concepts of the role of the ground substance in calcification has been discussed in a recent extensive review (*Bowness* 1968). Arguments were presented that acid glycosaminoglycans of ground substance possess properties and occur in situations which would enable them to exert an influence on both the rate and the extent of calcification and decalcification. With regard to human osteoporosis, it is of interest that fractions of bone glycosaminoglycans extracted according to *Dische et al.* (1958) have been reported to be influenced by both aging and osteoporosis. A versene-soluble component, rich in galactosamine, was reduced 50 per cent, and the galactosamine/glucosamine ratio was decreased in aged or osteoporotic subjects (*Cassucio et al.* 1962). Since these changes have been related to the osteoporotic process it was considered of interest to examine the glycosaminoglycans of compact bone tissue during the development of calcium deficiency osteoporosis in the present investigation. From the findings described above, changes in the ground substance of bone could be expected in a condition of protracted increased bone resorption. No experimental studies appear to have been reported previously concerning the relationship of the bone glycosaminoglycans to such a long-term process of bone reduction. Preliminary reports of this work have been presented by *Larsson and Vejlens* (1967, 1968).

In normal animals and in animals examined at different stages of calcium deficiency in the present study no changes were found in the contents of ash,

calcium or hydroxyproline of the compact bone tissue. Further, there were no changes in the concentrations of total hexosamines and acid glycosaminoglycans. In acutely increased bone resorption induced by administration of parathyroid extract, the mobilization of bone calcium has been related to metabolic changes in organic bone matrix (for references see *McLean* 1956, *Geschwind* 1961). No such changes were found during the long-term bone reduction in the present investigation. From the finding of a decrease in bone matrix hexosamine in rats treated with parathyroid extract for varying periods of up to 3 days it has been suggested that the dissolution of organic bone matrix and calcium mobilization are closely correlated effects of parathyroid hormone (*Johnston et al.* 1961). However, in dogs treated for 3, 4 or 5 days with parathyroid extract no changes have been found in either the concentrations of hexosamines and glycosaminoglycans of the compact bone tissue or the molecular weight distribution of the chondroitin sulphate (*Hjertquist and Vejlens* 1968). Administration of parathyroid hormone for 3 days to guinea pigs has been reported to cause a significant decrease in the concentration of calcium, but no change in concentration of hydroxyproline, DNA or uronic acid of the compact bone. A small but not significant decrease in the bone hexosamine concentration occurred (*Bollet et al.* 1963). It was suggested that mobilization of mineral from bone occurs without a concomitant decrease in the concentration of matrix constituents. The discrepancies of the results might be due to various factors such as differences in species, age of the animals, periods of treatment and doses of administered parathyroid extract. During the prolonged process of bone reduction resulting in calcium deficiency osteoporosis in the present investigation there were no changes in the concentrations of minerals, collagen (determined as hydroxyproline), hexosamines or glycosaminoglycans of the compact bone tissue.

It was found in the present investigation that in the compact bone tissue of the adult rat, the hexosamines corresponding to acid glycosaminoglycans constituted approximately one third of the total hexosamine concentration. The main component of the acid glycosaminoglycans was found to be chondroitin-4-sulphate, which was shown by *Meyer et al.* (1956) to be the predominating glycosaminoglycan of bovine compact bone tissue. Apart from an unchanged concentration of the bone chondroitin sulphate there was no change with the development of osteoporosis in its molecular weight distribution, as indicated by recorded unchanged solubility profiles and grade of sulphation. This indicates that there was no depolymerization of the glycosaminoglycans of the compact bone tissue during the development of osteoporosis induced by prolonged calcium restriction.

It is concluded that prolonged calcium restriction in adult rats results in a slight continuous loss of bone calcium due to the efficiency of calcium homeostasis in maintaining the blood calcium concentration at the expense of the bone mass. From histological, microradiographic and tetracycline-labelling studies, and biochemical analysis of the remaining compact bone tissue, evidence was obtained indicating that the bone changes induced do in fact correspond to osteoporosis according to the definition of *Albright and Reifenstein* (1948).

B. Combined oophorectomy and prednisolone administration.

The role of corticosteroid hormones in the homeostasis of the adult skeleton is not clear, in contradistinction to their well documented inhibitory effect upon normal skeletal growth and development. Although it has been pointed out in a review by *Silberberg and Silberberg* in 1956 that adult animals should be used in studies of the relationship between steroid hormones and osteoporosis, most available data are still derived from experiments on young animals. The interference of the administered hormones with normal bone growth makes it difficult to make any inferences from such experiments with regard to the maintenance of the mature skeleton and the development of osteoporosis in the adult individual. The osteoporotic bone changes reported in young growing rats after treatment with corticosteroids for various periods of time (*Kahn and Skoryna* 1959, *Storey* 1960, *Borberg and Lücker* 1964) and in young rats first subjected to bilateral adrenalectomy and orchidectomy (*Caldwell* 1962), seem to be related to the anti-anabolic effect of corticosteroids upon the normal development of the skeleton. Anabolic hormones were found to prevent the osteoporosis reported by *Borberg and Lücker* (1964) and oestrogens cured the osteoporosis reported by *Caldwell* (1962) despite continuous cortisone administration. This curative effect might also be due to the fact that in young rats, oestrogens inhibit bone resorption (*Budy et al.* 1952, *Lindquist* 1960). Young mice, also, have been reported to develop osteoporotic bone changes upon cortisone treatment and these can also be prevented with oestrogens (*Glickman and Shklar* 1955).

It is well known that corticosteroids induce cytologic changes in the young growing skeleton, while in the mature bone this effect is less evident. In young rabbits there is a reduction in number of osteoblasts and new bone formation due to diminished cell division, while osteoclast formation and bone resorption continue (*Hadfield* 1955, *Storey* 1957, *deValderrama and Munuera* 1965). This effect on the bone cell populations results in a massive resorption of

bone in young rabbits, while old animals respond only to a slight degree (*Storey* 1961). Studies in young rats have provided evidence suggesting that in this species the cellular response to excess corticosteroids is dependent also on calcium intake and the ratio of calcium to phosphorus in the diet. Thus, when the diet contains high, balanced amounts of calcium and phosphorus, cortisone administration results in dense metaphyseal bone due to inhibition of both the formation and resorption processes (*Follis* 1951, *Storey* 1960). With a normal, decreased or unbalanced Ca/P intake, the response of the metaphyseal bone is the same as in rabbits (*Sissons and Hadfield* 1956, *Storey* 1960, *Simmons and Kunin* 1967).

Since bone actively participates in homeostatic processes in the body, the well recognized effect of adrenal cortical hormones upon calcium metabolism might also influence the bone tissue. In young growing animals the inhibitory effect of corticosteroids on growth makes it difficult to evaluate the hormonal influence on calcium metabolism and bone mineralization *per se*. In a recent study on young rats the calcium metabolism was studied with the aid of Ca^{45}, and the growth rate was determined by repeated labellings with tetracycline. It was found that during treatment with corticosteroids the calcium accretion rate showed a reduction corresponding to the inhibition of growth (*Bohr* 1968).

It is evident from the findings discussed above that the maturity and age of the skeletal tissue are of decisive importance for the effect exerted by excess corticosteroid hormones. In the present investigation one-year old rats which had passed the period of active growth were used. Histological examination of vertebral bone of the control rats revealed morphological changes which could be attributed to normal chronologic or physiologic aging. Morphological changes due to physiologic aging of bone have been described in aged humans (*Sherman and Selakovitch* 1957) and are considered to be more pronounced in patients with severe osteoporosis (*Urist et al.* 1963). In the present study the morphological appearance of the bone tissue was similar in the prednisolone-treated oophorectomized rats and the normal controls. In the vertebral bone tissue no osteoclasts at all were observed, either in the normal controls or in the oophorectomized rats treated with prednisolone for varying periods up to 16 weeks. A small amount of bone surface showing tetracycline uptake was the only sign of low osteoblastic activity. Despite the time interval of 10 days between the two administered doses, only a narrow fluorescent line was seen in the normal rats. A similar low degree of tetracycline labelling was also observed in the oophorectomized, prednisolone-treated rats.

In the present study there was a significant decrease by 6 to 7 per cent in the ash content of the whole tibia after treatment with oophorectomy combined with administration of prednisolone for 14 or 16 weeks. There was a slight but insignificant decrease in the hydroxyproline content of the whole metacarpal bone, the gross bone density of the humerus and the percentage of vertebral bone "hits". On direct measurement, a significant reduction was observed in the cortical thickness of the mid-shaft of the femur, probably caused by increased endosteal bone resorption. On microradiographs of undecalcified vertebral sections the bone tissue was evenly mineralized in both normal and experimental animals. These findings suggest that, in adult rats, combined treatment with oophorectomy and prednisolone administration for prolonged periods of time, *i.e.* at least 14 or 16 weeks, results in moderate osteoporosis.

Being a part of the connective tissue, bone is also affected by the influence of steroid hormones on the formation and development of this tissue. The greater effect observed in young growing rats in the experiment by *Caldwell* (1962) might be due to the well known anti-anabolic action of cortisone on collagen synthesis as reported by *Smith and Allison* (1965), while in the present study this interference would probably be rather small since bone collagen in rats is synthesized predominantly at an early age (*Kao et al.* 1965). Besides an inhibitory effect on the synthesis of bone collagen, a direct degrading effect upon bone collagen similar to that on dermal collagen as reported by *Houck and Patel* (1965) and *Kowalewski* (1966) must be considered. However, there are other reports suggesting only an anti-anabolic action of corticosteroids on collagen synthesis but no effect on the catabolism of collagen (*Kivirikko et al.* 1965, *Smith and Allison* 1965). The metabolism of bone collagen in relation to the development of experimental osteoporosis will be a subject of future study.

In view of the above discussion, it is of interest that the significant decrease in ash content of the whole tibia in the groups of oophorectomized rats treated with prednisolone for 14 or 16 weeks, corresponds to that previously observed in male rats after specific calcium restriction for the same periods of time. Thus, in the former the decrease amounted to 6.1 per cent, and in the latter to 6.2 per cent. This suggests that changes in calcium metabolism induced by this hormonal disturbance might be of special interest with regard to the development of this type of osteoporosis. In this respect, the effect on the blood calcium level, the distribution of intraperitoneally administered Ca^{45} between blood and bone, and on the calcium accretion by bone would provide further

information. A study of these effects has been reported, in part, previously (*Larsson* 1968).

It was found in the present investigation that combined treatment with oophorectomy and prednisolone administration resulted in a slight initial fall in the blood calcium concentration, followed by a return to normal values, corresponding to the observations after the institution of a low calcium diet in adult male rats. Thus, with both these different disturbances slightly subnormal blood calcium values were noted in groups of rats treated for 1, 2, 3 and 4 weeks. Also at later periods of time slightly subnormal blood calcium values were recorded. While no effect of cortisone on the blood calcium level was found in young rats by *Williams et al.* (1962) and an increase was observed after prednisolone administration to dogs (*Garrett et al.* 1964), there are other reports indicating that cortisone lowers the blood calcium in young rats (*Laron et al.* 1958, *Simmons and Kunin* 1967) and in rabbits (*Pincus et al.* 1951). These contradictory results might be due to such factors as the age of the experimental animals, the calcium intake and duration and the dose of corticosteroid hormone administered. From the observation that hydrocortisone reduces the blood calcium in patients with hypoparathyroidism (*Leifer and Hollander* 1953) and in parathyroidectomized animals of different species (*Mirvish and Bosman* 1929, *Stoerk and Arison* 1961), it has been suggested that failure to observe the same effect in normal animals was due to secondary hyperparathyroidism (*Stoerk et al.* 1963). From the findings in young rats that the hypocalcemia following parathyroidectomy is aggravated by hydrocortisone and that 3.5 times more parathyroid extract is needed to sustain normal blood calcium concentrations in the presence of hydrocortisone, it has been suggested that the development of cortisone osteoporosis might be due to secondary hyperparathyroidism and increased bone resorption (*Stoerk et al.* 1963). However, in a recent study of cortisone-treated, young rats a slight decrease in blood calcium was recorded but there were no morphological signs of increased parathyroid activity (*Hansson* 1967). Since the animals were fed a high calcium diet it is possible that this prevented the development of secondary hyperparathyroidism. A recent preliminary study of the parathyroids in cortisone-treated adult rabbits gave no conclusive results as to the effect of cortisone alone or cortisone plus excess calcium (*Adams and Jowsey* 1967). Thus, the role played by the parathyroids in the development of cortisone osteoporosis remains to be determined. The effect of the hormonal disturbance induced by combined oophorectomy and prolonged prednisolone administration on the parathyroid function will be a subject of future study.

In the present study there was an increased elimination from the blood of intraperitoneally administered Ca^{45} after oophorectomy combined with prednisolone administration for varying periods of time. Furthermore, a change from the normal was observed in the distribution of Ca^{45} between blood and bone examined 72 hours after isotope injection. After an initial phase, combined treatment with oophorectomy and prednisolone administration for 4 to 16 weeks resulted in a concomitant decrease in the Ca^{45} activity of blood and bone. These findings indicate that in the adult rat the hormonal disturbance induced by combined oophorectomy and prolonged prednisolone administration results in an increased loss of calcium persisting throughout the period of treatment. The slight decrease observed in the blood calcium concentration seems to be secondary to poor or retarded adaptation to this calcium wasting effect.

Corticosteroid administration has been reported to increase the excretion of injected bone-seeking isotopes in young rats (*Clark et al.* 1959, *Milhaud et al.* 1960, *Bohr and Dawids* 1964, *Clark and Smith* 1964). In young growing animals this might be due to decreased reabsorption of calcium by the renal tubules as reported by *Clark and Roth* (1961) but also to an inhibitory effect of the corticosteroids on normal bone growth resulting in decreased bone mineralization as demonstrated by *Bohr* (1968). The reports regarding the effect of corticosteroids on intestinal calcium absorption in the rat are not fully conclusive. Thus, a decreased calcium absorption was reported by *Milhaud et al.* (1960) while no effect on the over-all gastro-intestinal calcium absorption was found by *Clark and Smith* (1964). In dogs, prednisolone increases the amount of intravenously administered Ca^{45} eliminated in the faeces and urine (*Collins et al.* 1962), and in humans cortisone and ACTH increase the calcium excretion in both faeces and urine (*Luft and Sjögren* 1951). The reabsorption of calcium by the renal tubules has been reported to be reduced in humans treated with corticosteroids, possibly due to an anti-anabolic effect of the hormone on the tubular cells (*Laake* 1960).

In young growing rats excess corticosteroids have been reported to inhibit the uptake of bone-seeking isotopes by bone (*Clark et al.* 1959, *Bohr and Dawids* 1964) and it has been suggested that the limiting factor may lie in the synthesis of chondroitin sulphate (*Clark et al.* 1959). In the adult, fully grown rats used in the present investigation the estimated mean calcium accretion rate for the tibia was found to be increased in the two groups of oophorectomized rats treated with prednisolone for 1 and 2 weeks, while in six groups of animals treated for 3 to 12 weeks approximately normal values were found. Two groups of rats treated for 14 and 16 weeks showed fluctuating values. These results

indicate that in the adult rat the hormonal disturbance induced by combined oophorectomy and prolonged prednisolone administration causes no apparent decrease in the bone mineralization rate. The findings by *Clark et al.* (1959) and *Bohr and Dawids* (1964) are in line with the observation that in growing rats excess corticosteroids reduce the calcium accretion rate in correspondence with the inhibition of growth (*Bohr* 1968). The results of the present study are in agreement with those reported in adult healthy humans (*Gordan and Eisenberg* 1963) and in humans treated with corticosteroids (*Nordin et al.* 1963, *Nordin et al.* 1964). Normal or even increased bone accretion has been reported in osteoporotic patients treated with corticosteroids and it has been suggested that the eventual decrease in bone mass caused by excess corticosteroids might be the result of accelerated bone resorption (*Eisenberg* 1966). The calcium-wasting effect of combined oophorectomy and prednisolone administration in adult rats demonstrated in the present investigation indicates that the considerable loss of bone mineral observed after treatment for 14 or 16 weeks was brought about by increased bone resorption which would mobilize bone calcium for maintenance of the blood calcium concentration. With extension of the periods of prednisolone treatment a more pronounced osteoporosis would develop, due to the continuous calcium wasting.

On histological examination of the vertebral bone in the present study, areas showing basophilic staining with haematoxylin-eosin were observed in the metaphyseal bone trabeculae. In sections stained with azocarmine G these areas exhibited only light or no staining and were found to be located in spaces intervening between parallel-fibered bone. The evident metachromasia with toluidine blue at pH 4.4 indicated that these areas of intercellular substance probably consisted of acid glycosaminoglycans extending into the trabecular bone as remnants from the epiphyseal cartilage, according to *Dziewiatkowski et al.* (1957) and *Campo and Tourtellotte* (1967). There was no change in metachromasia after the combined treatment with oophorectomy and prednisolone administration. From the findings in cortisone-treated one-year old rats of a decrease in the hexosamine content of the whole femur but no change in the collagen content, it has been suggested that a decrease of glycosaminoglycans precedes the events leading to the development of osteoporosis in Cushing's disease (*Sobel and Marmorston* 1954). However, this observation may be unspecific with regard to the glycosaminoglycans of the bone tissue itself and no support for this theory is obtained from the findings of the present study.

Changes in bone glycosaminoglycans in fractions of vertebral bone tissue extracted according to *Dische et al.* (1958) have been reported in normal human

aging and more abruptly with osteoporosis (*Cassucio et al.* 1962). The significance of these findings in relation to the osteoporotic process remains to be determined. As reported previously in the present investigation, neither quantitative nor qualitative changes of the bone glycosaminoglycans were found during the development of calcium deficiency osteoporosis. In view of the hormonal influence on the metabolism of glycosaminoglycans in other connective tissues (*Dziewiatkowski* 1964, *Priest* 1967), hormonal disturbances could be expected to influence also the glycosaminoglycans of the bone tissue. However, no reports appear to have been made previously regarding the effect of excess corticosteroids on the glycosaminoglycans of mature bone tissue.

As already mentioned, there was a significant decrease in the ash content of the tibia in rats treated by oophorectomy combined with prednisolone administration for 14 or 16 weeks. On chemical analyses, no significant changes in the concentrations of ash, calcium or hydroxyproline in the compact bone tissue were found in these animals. These findings indicate that the loss of minerals from the whole bone corresponds to osteoporosis. Furthermore, no changes were found in the concentrations of hexosamines or acid glycosaminoglycans in the compact bone tissue. The main glycosaminoglycan was a sulphated galactosaminoglycan with infrared characteristics compatible with chondroitin-4-sulphate. In previous experiments corticosteroids have been reported to decrease the concentration of hexosamines in other connective tissues without reducing the collagen content (*Sobel et al.* 1958). However, the suggestion of *Sobel and Marmorston* (1954) that a decrease in bone glycosaminoglycans might precede the events which lead to the development of osteoporosis in Cushing's disease, seems to be refuted by the results obtained in the present investigation. With regard to the metabolism of acid glycosaminoglycans in other connective tissues, there is now good evidence that the turnover of the acid glycosaminoglycans and their corresponding polysaccharide-protein complexes is fairly rapid, at least in comparison with the turnover of collagen (*Boström* 1968). The turnover rate of glycosaminoglycans is related to the age of the individual and varies in different tissues. In the adult rat, the biological half-life of chondroitin sulphate in skin has been estimated to be 8 to 11 days (*Boström and Gardell* 1953, *Schiller et al.* 1956) and in cartilage 16 days (*Boström and Gardell* 1953). The incorporation of S^{35} into the glycosaminoglycan fraction of shaft bones of rats of different ages has been studied by *Takemitsu* (1961). In rats 7 days old, the specific activity of the radioactive sulphur in the glycosaminoglycan fraction reached a peak 18 hours after the intraperitoneal administration of $Na_2S^{35}O_4$, and the half-life time was 6.7 days. In older animals, there was

a gradual shift until the peak was reached at 48 hours, the specific activity of sulphur decreased, and the half-life time became shorter. Although the metabolic turnover of the bone glycosaminoglycans *in toto* probably decreases with increasing age, the combined treatment with oophorectomy and prednisolone administration for varying periods up to 16 weeks could be expected to have some effect on the formation of the bone glycosaminoglycans in the old rats used in the present study. After corticosteroid treatment, a decrease in incorporation of S^{35}-sulphate into chondroitin sulphate of bovine cartilage *in vitro* has been reported (*Boström and Odeblad* 1954, *Whitehouse and Boström* 1961) However, a possible inhibitory effect on the bone glycosaminoglycans might have been too small to influence the analytical, quantitative results obtained in the present investigation. On the other hand, diminution of both the formation and breakdown of the chondroitin sulphate caused by the excess corticosteroids, as reported by *Schiller and Dorfman* (1957) in a study on rat skin, is also possible. From the findings of an increase of hyaluronate in the vitreous humor and an increase of keraton sulphate in costal cartilage of rabbits treated with prednisolone, it has been suggested that corticosteroids have an overall inhibitory effect on the degradation of these glycosaminoglycans (*Kaplan and Fisher* 1964). A similar effect has also been reported in a study on the synthesis of chondroitin sulphate by cartilaginous embryonic chick tibiotarsi in organ culture (*Schryver* 1965).

Apart from an unchanged concentration, there was no alteration in the molecular weight distribution of the bone chondroitin sulphate, as indicated by unchanged solubility profiles and sulphation grade, after combined treatment with oophorectomy and prednisolone administration in the present study. This indicates that there was no depolymerization of the glycosaminoglycans of the compact bone tissue during the development of the osteoporosis induced by this hormonal disturbance.

To summarize, in the adult rat both prolonged calcium restriction and hormonal disturbance induced by combined oophorectomy and prolonged prednisolone administration lead to a significant reduction of the skeletal tissue, fulfilling the criteria of osteoporosis given by *Albright and Reifenstein* (1948). During this prolonged process of increased bone resorption, changes in calcium metabolism and the efficiency of calcium homeostasis in maintaining extracellular fluid calcium appear to constitute the primary pathomechanism for the development of the disorder. Neither quantitative nor qualitative changes were found in the glycosaminoglycans of the remaining compact bone tissue during this long-term process of increased bone resorption.

GENERAL SUMMARY AND CONCLUDING REMARKS

1. The aim of the investigation was to determine in the adult rat whether or not disturbances induced experimentally by a) prolonged calcium restriction and b) oophorectomy combined with prolonged prednisolone administration, resulted in changes of the mature bone tissue and calcium metabolism which could be related to the development of osteoporosis according to the definition of *Albright and Reifenstein* (1948). Further, the studies were planned with the purpose of following the sequence of changes in various parameters which could be related to the development of the disorder. An attempt was made to investigate the possible relationship of changed bone mass to alterations in calcium metabolism under these two conditions. Further, the possible effect of these different disturbances on the compact bone tissue was studied with special regard to the glycosaminoglycans.

2. Introductory remarks are made on certain aspects of clinical osteoporosis with special reference to the pathogenesis.

3. A critical survey is made of the literature concerning experimental osteoporosis induced by calcium deficiency or corticosteroid administration.

4. The effect of prolonged calcium restriction on the skeletal tissue was studied in adult male rats. The ash content of the tibia showed values decreasing significantly with time. After 16 weeks there was a decrease by 6.2 per cent and after 12 months by 13.3 per cent, indicating a continuous slight loss of bone minerals throughout the experimental period. The hydroxyproline content of the metacarpal bone showed also a significant decrease, indicating a concomitant breakdown of organic bone matrix. Radiographic examination showed reduced cortical thickness of the tubular bones and decreased density of the trabecular pattern in the metaphyseal bone after calcium restriction for 6 months and longer. These animals showed significantly lower values for the "femur score" and the vertebral bone "hits" than the normal controls. No change in mineralization of the vertebral bone was observed on examination

of microradiographs of undecalcified sections. Tetracycline fluorescent micrographs showed no difference from the normal. These findings indicate that the rarefaction of bone represented osteoporosis. No osteoclasts were observed in normal or experimental rats. In the metaphyseal bone, areas were observed which were metachromatic with toluidine blue at pH 4.4. No change in metachromasia was observed in osteoporotic animals. Rats fed the test diet enriched with calcium for 16 weeks showed a tendency to increased ash content, decreased hydroxyproline content and increased density of whole bones compared with normal controls, which might suggest a possible increase in degree of calcification.

5. The effect of prolonged calcium restriction on the blood calcium level and the distribution of Ca^{45} between blood and bone was studied in adult male rats. The blood calcium was slightly subnormal 1 to 4 weeks after commencement of calcium restriction. The blood Ca^{45} specific activity 72 hours after the isotope injection showed an increase by 100 per cent after 1 week of calcium restriction, indicating increased calcium retention. Animals treated for 3 or 4 weeks showed similar values to the normal controls, but increased deposition of Ca^{45} into the skeleton by 100 per cent despite subnormal blood calcium. On the 8th to 14th week of calcium deficiency, normal blood calcium values and increased Ca^{45} activity in blood and bone by 50 to 60 per cent were recorded, indicating continued increase in the retention of calcium. Rats treated for 4 to 7 months showed subnormal blood calcium, while in animals fed the diet for 8 to 12 months, normal values were found. Osteoporotic animals kept on the low calcium diet for 6 to 12 months showed highly increased blood Ca^{45} activity, by approximately 200 per cent, while the uptake of Ca^{45} by bone was increased by 40 per cent. In animals fed the diet enriched with calcium, the blood Ca^{45} activity was 40 per cent and bone Ca^{45} activity 60 per cent lower than in controls, indicating decreased retention of calcium. The mean calcium accretion rate for the tibia (mg calcium/hour/mg calcium of the tibia) was decreased in rats fed the low calcium diet for 6 and 12 months, while rats treated for 7, 8, 9 and 10 months showed approximately the same values as the control group. From the Ca^{45} data obtained it was deduced that the maintenance of the blood calcium level was brought about by increased mobilization of skeletal calcium to the blood. During the loss of bone minerals there were shifts in the calcium dynamic balance at different periods of time, associated with the occurrence of a slightly subnormal blood calcium concentration.

6. The composition of compact bone from adult rats during the development of calcium deficiency osteoporosis was studied. The glycosaminoglycans were analysed using the cetylpyridinium precipitation method. No significant changes were recorded in the concentrations of ash, calcium, hydroxyproline, total hexosamines or hexosamines originating from the glycosaminoglycans. The unchanged relation of mineral/collagen confirmed that the induced bone rarefaction was genuine osteoporosis, according to the definition of *Albright and Reifenstein* (1948). The main glycosaminoglycan was chondroitin-4-sulphate and a smaller part presumably hyaluronic acid. The unchanged solubility properties of the chondroitin sulphate—cetylpyridinium complexes and unchanged sulphation grade indicated that no change in the molecular size pattern of chondroitin sulphate occurred during the development of the osteoporosis. Of the hexosamines originating from glycosaminoglycans, more than 90 per cent could be accounted for by chondroitin sulphate, and they amounted to less than a third of the total hexosamines.

7. The effect of combined oophorectomy and prolonged prednisolone administration on the skeletal tissue was studied in adult rats. The excess of corticosteroids caused considerable depletion of adipose and muscle tissues and significant atrophy of the adrenal cortex (zona fasciculata). During the observation period of 16 weeks no radiographic signs of osteoporosis were seen. A significant reduction by 6.1 per cent in the ash content of the tibia was recorded after 14 and 16 weeks of treatment. The "femur score" showed significantly decreased values, while there was only a slight though not significant decrease in the percentage of vertebral bone "hits", the hydroxyproline content of metacarpal bone, and the density of the humerus. Microradiographic examination of undecalcified vertebral bone sections showed no changes in degree of mineralization. Only a small amount of bone surface showing tetracycline labelling was observed both in normal and experimental animals. Histological examination of decalcified sections of vertebral bone from normal and experimental animals showed morphological changes which could be attributed to physiologic aging of bone. In the metaphyseal bone of the vertebrae, areas were observed which stained metachromatically with toluidine blue at pH 4.4. No change in metachromasia was observed in oophorectomized rats treated with prednisolone for different periods of time.

8. The effect of combined oophorectomy and prolonged prednisolone administration on the blood calcium level and the distribution of Ca^{45} between

blood and bone was studied in adult rats. The blood calcium showed subnormal values in rats treated for 1 to 4 weeks, while normal values were recorded after 5 weeks. Slightly subnormal values were observed in rats treated for 8 to 14 weeks and, finally, normal values after 16 weeks. The rate of elimination of intraperitoneally administered Ca^{45} from the blood was increased in all experimental groups compared with that of the normal control group. Rats treated for 4 to 16 weeks showed consistently decreased values for both the blood and bone Ca^{45} activity recorded 72 hours after the isotope injection. The uptake of Ca^{45} by compact bone amounted to less than one fifth of that of the whole tibia and showed also a decrease in rats treated for 4 to 16 weeks. The mean calcium accretion rate of the tibia (mg calcium/hour/mg calcium of the tibia) was found to be increased in the two groups of oophorectomized rats treated with prednisolone for 1 and 2 weeks, while in six groups of animals treated for 3 to 12 weeks approximately normal values were found. Two groups of rats treated for 14 and 16 weeks showed fluctuating values. It was deduced from the Ca^{45} data obtained that the blood calcium was sustained by increased mobilization of skeletal calcium, resulting in the loss of bone minerals observed in a previous study.

9. The composition of compact bone was studied in normal adult rats and oophorectomized rats treated with prednisolone for varying periods up to 16 weeks. While in previous studies a significant reduction by 6 to 7 per cent in the mineral content of the whole tibia had been found in rats treated for 14 or 16 weeks, there was no change in the relation of mineral/collagen in the compact bone of these animals, thus confirming that the bone changes noted represented osteoporosis. The concentrations of total hexosamines and of hexosamines originating from glycosaminoglycans were not affected by the combined treatment with oophorectomy and prednisolone administration. Of the hexosamines emanating from glycosaminoglycans, more than 90 per cent could be accounted for by chondroitin sulphate, and they amounted to less than a third of the total hexosamines. The main glycosaminoglycan was identified as chondroitin-4-sulphate. A smaller part was presumably hyaluronic acid. Results of microfractionation studies of glycosaminoglycan complexes with cetylpyridinium indicated that no change in the molecular size pattern of chondroitin sulphate occurred with the treatment.

10. The hormonal disturbance induced by combined oophorectomy and prednisolone administration resulted in a loss of bone minerals corresponding

to that observed after pure calcium restriction for the same periods of time. The calcium-wasting effect of this hormonal disturbance observed with the aid of Ca^{45}, and the mainly unchanged calcium accretion rate by the bone, indicate that the loss of bone minerals was due to increased bone resorption. This seems to be the essential factor in the pathogenesis of this type of osteoporosis, which is further supported by the absence of quantitative or qualitative changes in the bone glycosaminoglycans.

11. The essential features of the investigation are summarized in the general discussion. The results are discussed in relation to reported contradictory results of animal experiments. The importance of calcium homeostasis in the pathogenesis of osteoporosis is pointed out.

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