Virchows Arch. path. Anat. 336, 580—591 (1963)

From the Orthopedic and Surgical Departments, the Academic Hospital, Uppsala, Sweden

The Effects of Thiouracil on Growing Cartilage in the Rat

By

A. Hulth and G. Nylander

With 6 Figures in the Text

(Received April 22, 1963)

The effects of reduced or lost thyroid function on skeletal growth have been the subject of many experimental investigations. These were reviewed by Silberberg and Silberberg (1943, 1954). The most prominent effect seems to be delayed growth of the long bones, which become generally thickened. This is brought about by retarded proliferation and ossification of the epiphyseal cartilage. The resorption of cartilage and primary bone is appreciably inhibited. The degeneration and breakdown of cartilage cells is slowed down and primary bone trabeculae remain unabsorbed for a longer time than normal.

In an earlier report (Hulth and Nylander 1962) we were able to demonstrate that propyl thiouracil fed to growing rats effectively retarded the growing epiphyseal cartilage. Signs of degeneration were observed, such as decreased height of the epiphyseal zone, decreased basophily of the cartilage cells and fibrillation of the matrix substance. The cartilage cells were preserved for a long time and were frequently found within the metaphyseal bone trabeculae, which were broader than those of untreated rats.

The incorporation of radio-sulphate in the chondrocytes, analysed by means of strip autoradiography, was substantially decreased in thiouracil-treated rats. The uptake of radioactivity in the individual cell was diminished as compared with that of untreated controls. Similarly, the emptying of the radio-sulphate from the cell into the extracellular matrix was retarded. The suggestion was put forward that the synthesis of chondroitin sulphuric acid was significantly inhibited in the chondrocytes of thiouracil-treated rats. It could not be concluded, however, whether this was an effect of the thyrostatic activity of thiouracil or if the substance directly influenced the metabolism of the epiphyseal certilage.

The effects of thiouracil were studied during an experimental period of 4 weeks. At the end of this period we were not able to state whether the therapeutic effects had reached a maximum or not.

The aim of the present investigation was to analyse this problem by extending the experimental period over 8 weeks. A further aim was to study the sulphate metabolism more in detail, with special reference to the effects of thiouracil on the chondrocytes. Finally we thought it worthwhile to investigate the alkaline phosphatase activity and presence of PAS-positive substance in the epiphyseal cartilage of thiouracil-treated rats.

Material and Methods

A total of 120 young rats was included. The initial weight of the animals varied between 34 and 40 g and averaged 37 g. The rats received the same standardized and adequate diet throughout the experimental period. The material was divided into two main groups:

1. Untreated controls.

2. Thiouracil-treated animals. These were fed with 0.05 g of propyl thiouracil per day. The substance was mixed with a portion of grated cheese, which was quantitatively consumed before the standard diet was given. All animals were weighed once a week.

Both groups were divided into two equal sub-groups, consisting of rats with an experimental time of 4 and 8 weeks respectively.

Radioactive sulphate (^{35}S—SJS 2, Radiochemical Centre, Amersham) was given intraperitoneally. The dose was in every case equivalent to 0.5 mC per 100 g of body weight. In each sub-group, the radio-sulphate was administered to 9 rats, which received the dose on one of the following occasions: 5 days, 2 days and 2 hours before death.

Oxytetracycline (Terramycin®, Pfizer) was given by subcutaneous injection to 10 animals of each sub-group. Those with 4 weeks' experimental time were injected on two different occasions (12 and 2 days before death) and those with 8 weeks' experimental time on three occasions (36, 12, and 2 days before death).

The animals were killed by ether. The parietal bone, both kneejoints with attaching femoral and tibial epiphyseal cartilages, and a cross-section of the tibial shaft were obtained from each animal.

The specimens destined for histologic and autoradiographic examination were fixed in 10 per cent neutral formalin, decalcified in formic acid and sodium citrate, embedded in paraffin wax, sectioned in 5 μ sections and stained with haematoxylin-eosin. The autoradiographic technique was in accordance with that of Engfeldt and Westerborn 1960, and Hulth and Westerborn 1962.

The parietal bones and the tibial shaft sections from the oxytetracycline-injected rats were embedded in methylmetachrylate. The sawing of the blocks, the grinding down of the sections to 100 μ and the UV-microscopic technique were described by Hulth and Olerud 1962.

Specimens obtained for histochemical investigations were fixed in cold acetone-ethanol and used for the demonstration of alkaline phosphatase activity according to the calcium-cobalt method of Gomori, and for PAS-positive substance according to the periodic acid Schiff technique of Hotchkiss (Pearse 1960).

Results

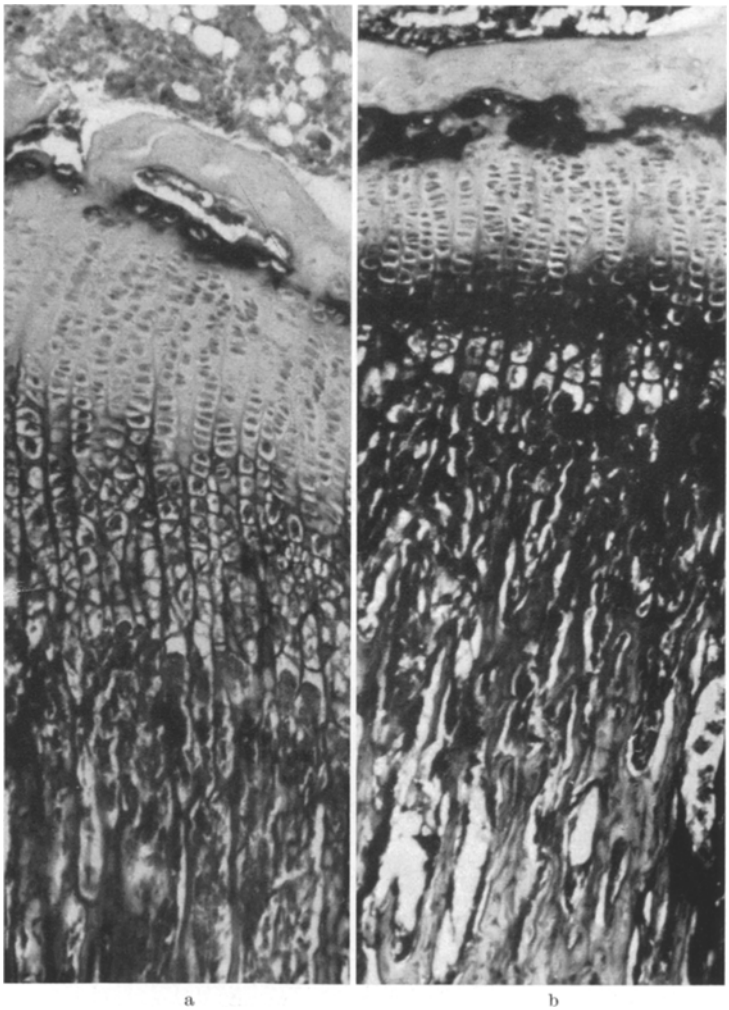
From the Table it can be seen that the thiouracil-treated rats increased considerably less in weight than the untreated controls. Further, they were appreciably inactive, the fur was matted and they shed a lot of hair. The thyroid gland was invariably enlarged and had the histological appearance of pronounced epithelial hyperplasia.

Table

Experimental group	Mean initial weight g	Mean weight — g and mean increase in weight — per cent of initial weight	
		4 weeks	8 weeks
Untreated controls	37.4	133.8 (258)	179.4 (380)
Thiouracil-treated animals	36.7	69.6 (90)	102.0 (178)

Conventional histology. In thiouracil-treated rats, the histological appearance of the epiphyseal growth zone was almost normal at the end of the first 4-week period, and remained so for the rest of the experimental time. As in the untreated rats, its height underwent continuous reduction with time, reflecting the physiological aging, but remained throughout somewhat lower in the thiouracil-treated

than in the untreated rats. In this material the degenerative alterations found in the epiphyseal cartilage at early stages of thiouracil treatment were absent or only suggested. The cartilage basophily was almost normal, the matrix only rarely fibrillated and the columnar grouping of the chondrocytes less sparse.



a b

Fig. 1a and b. Microphotographs demonstrating alkaline phosphatase activity in the epiphyseal cartilage of a) an untreated, and b) a thiouracil-treated rat. The enzymatic activity indicated by the black precipitate is definitely more pronounced in the latter specimen and occupies broader zones of the cartilage. Magnification: × 80

The articular cartilage of the knee-joint was similarly of normal appearance in the thiouracil-treated rats during the observation period, with one possible exception. Thus, at the end of the 8th week, the articular cartilage of the untreated rats covered an almost intact bone structure, whereas in the thiouracil-treated rats the bone was still subject to resorption indicating growth.

Histochemistry. *Alkaline phosphatase activity.* After 4 weeks this was demonstrated in a thin zone of the resting cartilage, as well as in the layer of hyper-

trophic cells. The enzymatic activity appeared both in the chondrocytes and in the cartilage matrix. In the epiphyseal growth zone of untreated rats, the intensity of the reaction was most pronounced in the hypertrophic cell layer. In the latter the enzymatic activity of the matrix continued into the primary bone trabeculae of the metaphysis, which were outlined by the black precipitate. Pronounced enzymatic activity was furthermore found in the osteoblasts of this region (Fig. 1 a).

In the thiouracil-treated rats, the enzymatic activity in the resting cartilage zone was far more pronounced and the layer of positive reaction was appreciably broader than that of the untreated animals. The intensity of the enzymatic reaction was in this region as extreme as in the hypertrophic zone. In this, too, the enzymatic activity gave an impression of greater intensity than was the case in the untreated rats (Fig. 1 b).

Similarly, the joint cartilage of thiouracil-treated animals had increased phosphatase activity in the hypertrophic cells at the base of the cartilage (Fig. 2). A marked positive reaction was further observed in trabeculae of the metaphysis.

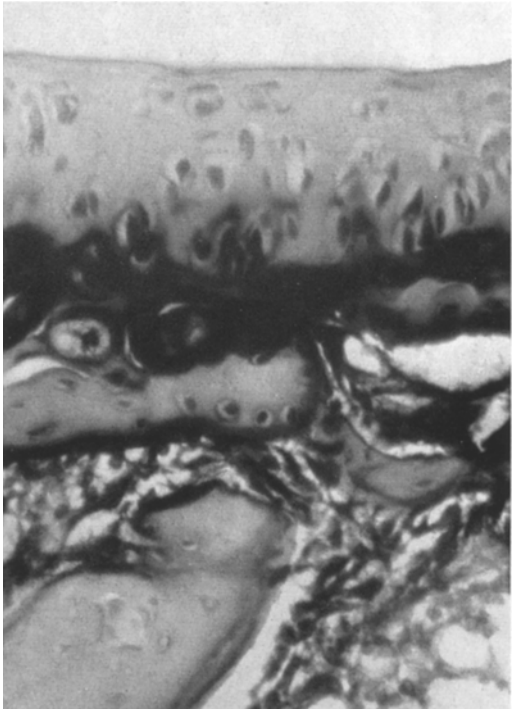


Fig. 2. Microphotograph showing alkaline phosphatase activity in the joint cartilage of an untreated rat. Note the considerable enzyme activity in the basal zone of the cartilage. Magnification: × 80

The *PAS reaction* in untreated rats after 4 weeks was only weakly positive in the matrix of the proliferating zone. The thin matrix septa between the large, vacuolized cells of the hypertrophic zone, as well as the primary bone trabeculae, took a somewhat deeper, red colour with PAS. Similarly, the matrix in a rather thin layer of the resting cartilage showed a rather strong, positive, PAS-reaction (Fig. 3 a).

In the thiouracil-treated animals, the PAS-reaction was as a rule stronger in the resting cartilage and its PAS-positive zone was definitely broader than that of untreated controls. In other respects, the epiphyseal growth zone of these animals corresponded to that of the controls (Fig. 3 b).

Autoradiography. After an *experimental period of 4 weeks* the density indicating radioactivity and appearing in the epiphyseal growth zone of thiouracil-treated rats was significantly less than that observed in the untreated controls. This feature was observed in all specimens and should be taken to indicate decreased uptake of radio-sulphate in this structure.

Five days after the administration of the dose, the radio-sulphate had completely disappeared from the chondrocytes and was found in the extracellular matrix. With its transformation into primary bone, the radio-sulphate was included in the primary bone trabeculae for a distance corresponding to that of epiphyseal growth during a 5-day period. In the thiouracil-treated rats this was approximately one-fifth less than that measured in the untreated controls.

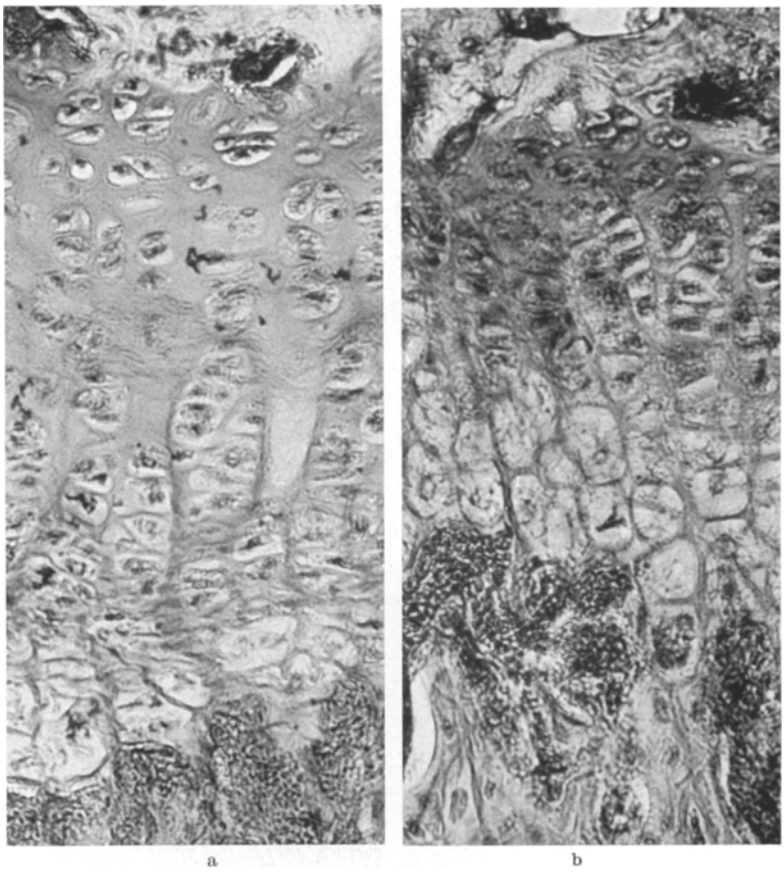


Fig. 3a and b. Microphotographs visualize the presence of PAS-positive material in the epiphyseal cartilages of a) untreated and b) a thiouracil-treated rat. In the latter the reaction is more pronounced throughout the cartilage, and especially in its resting zone.

When the radio-sulphate was given 2 days before the rats were killed, the radioactivity was principally found in the cartilage matrix. To some extent, however, the radio-sulphate was retained in the chondrocytes, contributing to the more diffuse density of the plates. The length of the bone trabeculae containing radioactive substance was considerably greater in the untreated than in the thiouracil-treated rats, and the density of the epiphyseal growth zone was much more pronounced in the former animals.

When the period between the injection of the dose and the sacrifice of the animals was shortened to 2 hours, the radio-sulphate was exclusively found within the chondrocytes. The radio-active labelling was very much greater in

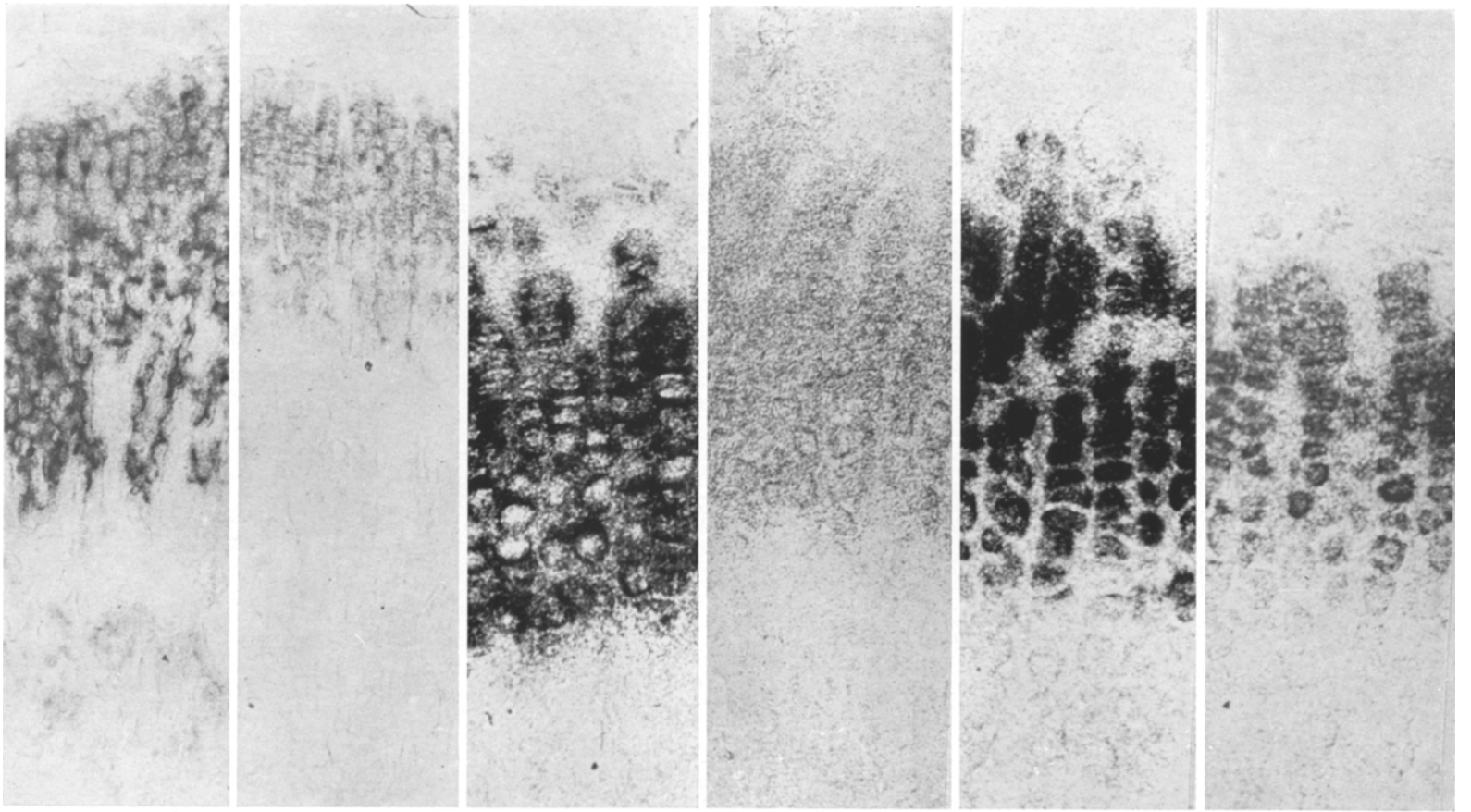
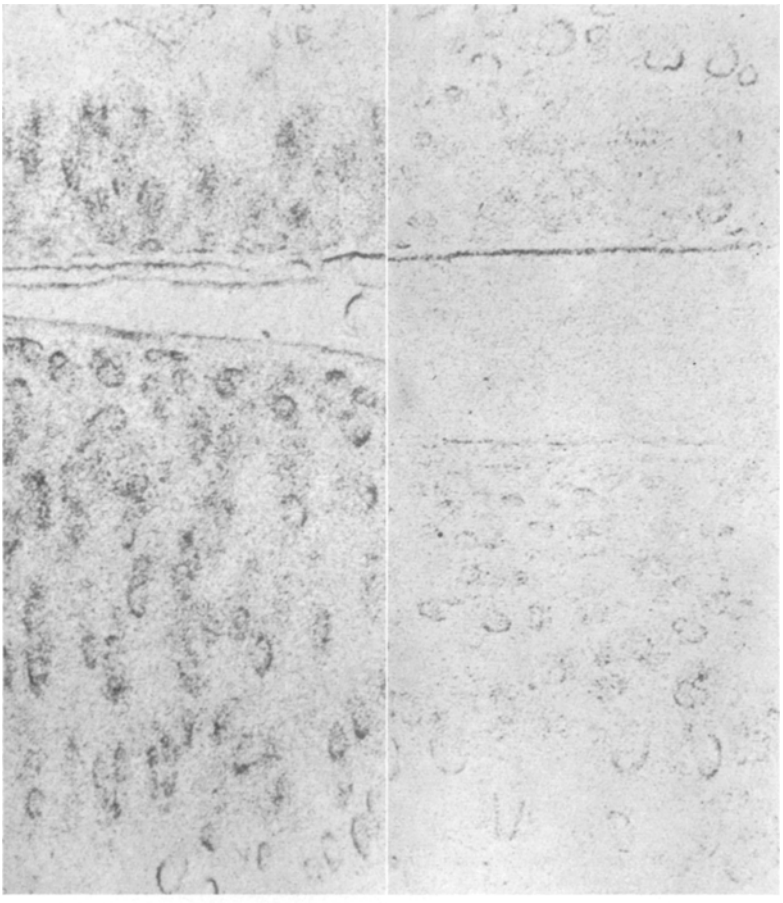


Fig. 4a—f. Autoradiographs. Experimental period of 8 weeks. Plates obtained from epiphyseal cartilages representing untreated (a, c, e) and thiouracil-treated rats (b, d, f). The radio-sulphate labelling was continued for 5 days (a, b), 2 days (c, d), and 2 hours (e, f) respectively. The increase in length is seen as radio-sulphate incorporation in the primary bone trabeculae. This was nil during the 5 days' period in the thiouracil-treated series presented in b. Magnification: a, b: × 50, c—f: × 130

the untreated rats. This impression might be due to the somewhat sparser distribution of cells within the cartilage of the thiouracil-treated rats, but the individual cells of these animals had without any doubt less density than those of the untreated rats.



a b

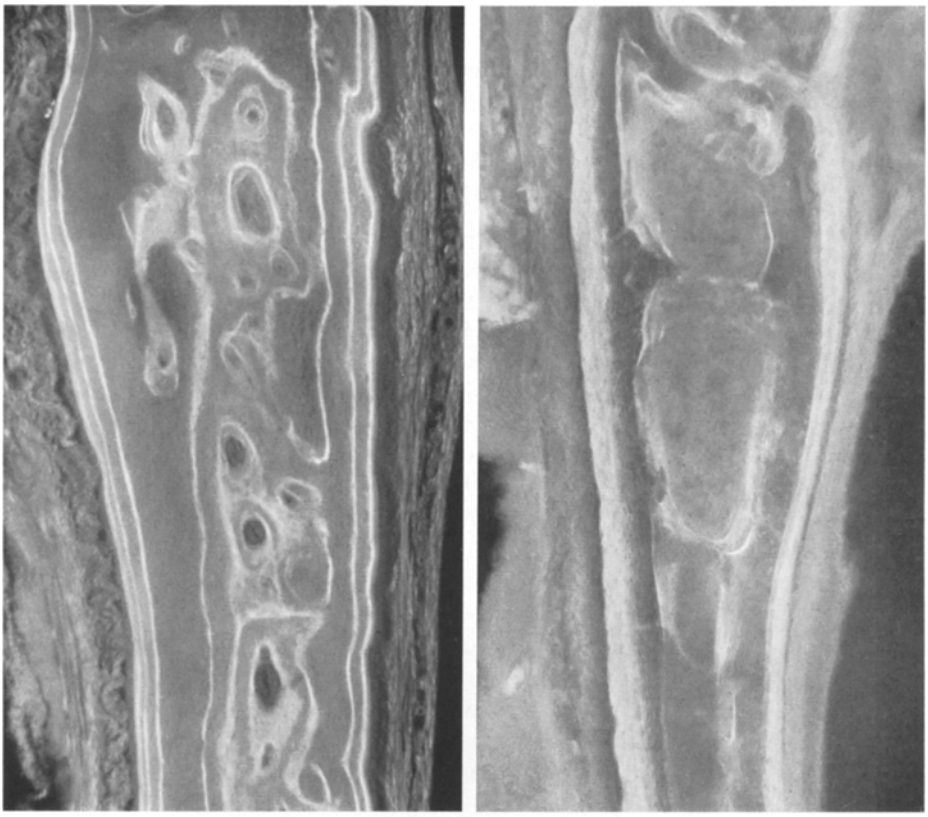
Fig. 5a and b. Autoradiographs representing joint cartilages of untreated (a) and thiouracil-treated (b) animals. Radiosulphate was given 2 days before death. The difference in radioactive labelling is clearly seen. Magnification: ×130

After an *experimental period of 8 weeks* (Fig. 4a—f) the reduced radioactive labelling in the epiphyseal growth zone of the thiouracil-treated rats was still more apparent.

The specimens representing 5 days (Fig. 4a—b) of radio-active labelling demonstrated in the untreated rats that the epiphyseal growth was appreciably less than after 4 weeks and in accordance with the lower height of the epiphyseal growth zone. The differences obtained between specimens from untreated and thiouracil-treated rats were striking. In the latter, weak, almost diffuse density was found in all the cartilage, and the epiphyseal growth, as indicated by radio-

active labelling of primary bone trabeculae, was substantially reduced as compared with untreated controls.

From the plates obtained after 2 days of radioactive labelling (Fig. 4c—d), the physiologically reduced epiphyseal growth in the controls was evident. In



a b

Fig. 6a and b. Tetracycline-fluorescence microphotograph of the parietal bone of an untreated (a) and a thiouracil-treated rat (b). Experimental time was 8 weeks. The agent was given at 3 different occasions before the animal was killed (see text). In the specimen representing the untreated rat three clear cut lines are distinguished (a). In that representing the thiouracil-treated rat the lines are not discerned from one another but appear as one. Magnification: ×50

the thiouracil-treated animals, the pathological reduction in growth and the weak and diffuse labelling of the epiphyseal cartilage was likewise obvious.

The preparations representing labelling with radio-sulphate for 2 hours (Fig. 4e—f), were analogous with those obtained after 4 weeks, the intracellularly localized density being much reduced in the thiouracil-treated series.

The *articular cartilage* (Fig. 5a—b) revealed in principle the same characteristics in these respects. Compared with untreated controls, the thiouracil-treated rats had considerably less radio-sulphate incorporated into the matrix and chondrocytes. As regards the accumulation of radioactivity within the cells and its transfer to the cartilage matrix as a function of time, the specimens of articular cartilage were identical with those of the epiphyseal growth zone. In these

respects, no certain differences could be established between untreated and thiouracil-treated rats.

From the preparations demonstrating *tetracycline-induced fluorescence* in the parietal bones and the cross-section of the tibia, decreased appositional growth was established in the thiouracil-treated series.

Controls which had received three injections of the agent on different occasions demonstrated at the end of the 8th week three separate and distinct lines of fluorescence both endo- and ectocranially. Corresponding specimens from thiouracil-treated rats were different. The lines of fluorescence could not be distinguished from one another, but coincided in one rather diffuse line as a manifestation of the disturbed and retarded appositional growth (Fig. 6a—b).

The same features were observed in the cross-sections of the tibia.

Discussion

Under the given experimental conditions, the effects of propyl thiouracil on the rat's epiphyseal growth zone were most prominent during the first weeks of therapy. The very definite, degenerative alterations observed during the first weeks and reported in an earlier study (HULTH and NYLANDER) were appreciably less when the agent had been administered for longer periods. In fact, almost normal histology was found after 4 and 8 weeks of treatment, with the important exception that growth was considerably retarded throughout the experimental period. Although less marked, the same tendency was valid for the changes in the sulphate metabolism of the cartilage, which were recorded by autoradiographic identification of incorporated radio-sulphate.

These features argue strongly in favour of the assumption that the noxious effect of thiouracil is not a local one but is mediated by the thyrostatic action of the agent. Whether lack of thyroxine only or — as in the case of surgical thyroidectomy — secondary deficiency of growth hormone (ASLING, SIMPSON and EVANS 1951) is the main factor is not established (WERNER 1955).

The noxious effect of substances known to cause degenerative changes in the epiphyseal growth zone, on the other hand, has been shown to increase with duration of therapy. This was demonstrated for aminonitriles (SELYE 1957) and for papain (HULTH 1958 and WESTERBORN 1961).

It would thus seem that with prolonged thiouracil therapy the epiphyseal cartilage undergoes an adaptation to the decreased thyroid hormone activity.

In the epiphyseal cartilage of thiouracil-treated rats, the incorporation of radio-sulphate was constantly reduced. Every specimen obtained from this series had less density than those from corresponding controls. Though quantitative evaluation of strip autoradiographs is not strictly accurate, the interpretation that a quantitative difference existed in these respects seemed justifiable. The same tendency was constantly observed in the joint cartilages as well. The diminished uptake of radio-sulphate in the chondrocytes should be interpreted as an expression of decreased sulphate and, accordingly, of chondroitin sulphate turnover in the growth cartilage of thiouracil-treated rats. This finding may be supported by that of DZIEWIATKOWSKI (1951, 1957), who demonstrated decreased

deposition of radioactive sulphur in the growth cartilage of hypo-thyreotic rats by quantitative methods.

In the epiphyseal growth zone, as well as in the articular cartilage, the presence of alkaline phosphatase activity was demonstrated. In agreement with FOLLIS and BERTHRONG (1949) and SCHAJOWICS and CABRINI (1954) we found the enzymatic activity of the growth cartilage most pronounced in the hypertrophic cell layer and along the metaphyseal bone trabeculae. In contrast to these authors, however, we were able to visualize intense enzymatic activity in the resting cartilage zone too. As far as we know, this has not been pointed out before, and the presence of alkaline phosphatase activity in this zone has been denied by FOLLIS et al. The controversial findings could perhaps be explained by the different kinds of epiphyseal cartilage which were investigated. The resting zone of the growth cartilage in the upper tibia is close to the ossification centre of the epiphysis, while a corresponding layer is lacking in the rib. The growth cartilage of the rib was investigated by FOLLIS et al.

The essential point, however, was that the enzymatic activity was substantially increased in the epiphyseal and joint cartilages of the thiouracil-treated series. The intensity of the reaction was not only stronger, but the zone revealing such enzymatic activity broader. This was especially the case in the resting zone of the epiphyseal cartilage. Conclusions as to the significance of increased enzymatic activity in the epiphyseal and joint cartilages of thiouracil-treated animals should not be drawn without taking into account the retarded growth and sulphate-chondroitin sulphate metabolism in the cartilage. It might thus be interpreted as an accumulation of the enzyme due to decreased metabolism requiring its contribution.

The increased amounts of PAS-positive material in the epiphyseal cartilage of thiouracil-treated animals was a constant feature. Considering the controversial and difficult interpretations of this reaction, we are unable to comment upon this finding and thus only report this feature and the interesting fact that the strongest reaction was found in the same zone, the resting cartilage, that revealed the strongest alkaline-phosphatase reaction.

Thiouracil therapy resulted in diminished increase in weight and retarded enchondral growth. During the last 5 days of the 8-week period the thiouracil rats did not demonstrate any growth contrary to the controls, in which the growth amounted to a distance corresponding to the height of the epiphyseal plate. Repeated labelling with tetracycline revealed that the appositional growth, too, was retarded in the thiouracil-treated series. The current concept is that hypothyroidism does not affect appositional bone growth. Thiouracil-treated rats had parietal bones of the same thickness as that of untreated controls. We were further unable to demonstrate decreased bone resorption in the parietal bone. Similarly the newly formed bone trabeculae of the tibial metaphysis were of the same thickness and appearance in both groups and the resorption of these was not appreciably reduced in the thiouracil-treated rats.

From the above mentioned results the impression was gained that the effect of thiouracil therapy on the enchondral as well as appositional growth is a retarding one.

Summary

In spite of almost totally inhibited growth, the epiphyseal cartilage of thiouracil-treated rats revealed practically normal micromorphology at the end of the 8-week experimental period. Deposition of administered radio-sulphate in cells and matrix of epiphyseal and joint cartilages was reduced indicating decreased chondroitin-sulphate turnover. Alkaline phosphatase activity was appreciably increased, in the resting cartilage layer of the epiphyseal growth zone; increased amounts of PAS-positive material appear in the epiphyseal cartilage.

Both enchondral and appositional growth were delayed by thiouracil. The bone resorption was approximately on a level with the decreased formation of new bone, which explained the relatively normal histological appearance.

Die Wirkung von Thiouracil auf den wachsenden Knorpel bei der Ratte

Zusammenfassung

Trotz des fast völlig aufgehobenen Wachstums zeigte der Epiphysenknorpel von thiouracilbehandelten Ratten am Ende der 8 Wochen betragenden Versuchszeit praktisch ein völlig normales mikroskopisches Aussehen. Die Ablagerung von Radiosulfat in Zellen und Grundsubstanz des Epiphysen- und Gelenksknorpels war allerdings verringert, was auf einen verringerten Chondroitin-Sulfat-Stoffwechsel hinweist; alkalische Phosphataseaktivität war am ruhenden Knorpel der epiphysären Wachstumszone wesentlich vermehrt; weiter trat hier eine erhöhte Menge von PAS-positivem Material auf.

Sowohl das enchondrale sowie das appositionelle Knochenwachstum werden durch Thiouracil verzögert. Die Knochenresorption entspricht der verringerten Knochenneubildung, was das verhältnismäßig normale histologische Aussehen des Knochens erklärt.

References

ASLING, C. W., M. E. SIMPSON and H. M. EVANS: Effects of pituitary factors and of thyroxin on skeletal morphogenesis in the rat. Quoted by S. Werner 1955.

DZIEWIATKOWSKI, D.: Effect of thyroxine and thiouracil on S^{35} deposition in articular cartilage. J. biol. Chem. **189**, 717 (1951).

— Synthesis of sulfomycopolysaccharides in thyreoid-ectomized rats. J. exp. Med. **105**, 69 (1957).

ENGFELDT, B., and O. WESTERBORN: An autoradiographic study of the epiphyseal cartilage in normal rabbits after administration of radiosulphate. Acta path. microbiol. scand. **49**, 73 (1960).

FOLLIS, R. H. and M. BERTHRONG: Histochemical studies on cartilage and bone. I. The normal pattern. Bull. Johns Hopk. Hosp. **85**, 281 (1949).

HULTH, A.: The growth inhibiting effect by papain on young rabbits. Acta orthop. scand. **27**, 167 (1958).

—, and G. NYLANDER: Effect of thyroxin and propyl thiouracil on the epiphyseal cartilage in growing rats. A histologic and autoradiographic study. Acta Soc. Med. upsalien. **67**, 273 (1962).

—, and S. OLERUD: Tetracycline labelling of growing bone. Acta Soc. Med. upsalien. **67**, 219 (1962).

—, and O. WESTERBORN: Effect of cortisone on the epiphyseal cartilage. Virchows Arch. path. Anat. **336**, 209 (1963).

Pearse, A. G. E.: Histochemistry, theoretical and applied, sec. Edit. London: J. & A. Churchill Ltd. 1960.

Schajowics, F., and R. L. Cabrini: Histochemical studies of bone in normal and pathological conditions. J. Bone Jt Surg. B **36**, 512 (1954).

Selye, H.: Lathyrisme. Rev. canad. Biol. **16**, 1 (1957).

Silberberg, M., and R. Silberberg: Influence of the endocrine glands on growth and aging of the skeleton. Arch. Path. **36**, 512 (1943).

— — Skeletal effects of radio-iodine induced thyroid deficience in mice as influenced by sex, age and strain. Amer. J. Anat. **95**, 263 (1954).

Werner, S. C.: The thyroid. New York: Paul B. Hoeber, Inc. 1955.

Westerborn, O.: The effect of papain on epiphyseal cartilage. Acta chir. scand., Suppl. **270**, 1 (1961).

Doz. Dr. A. Hulth,
Sahlgrenska Sjukhuset, Orthopädische Abteilung, Göteborg (Schweden)