Changes in Endochondral Ossification of the Tibia Accompanying Acute Pantothenic Acid Deficiency in Young Rats.*† (17565)

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Retardation of growth of the tibia and grave impairment of chondrogenesis and endochondral ossification have been reported for chronic riboflavin deficiency in the rat.(2) These findings have prompted an investigation of the skeletal changes occurring in another B vitamin deficiency in the rat, namely pantothenic acid. Previous investigators have noted cartilage and bone marrow "hypoplasia" in pantothenic acid deficient rats.(3,4) Inhibition of growth and endochondral ossification has been observed also in pantothenic acid deficient mice.(5) In the present study the histological changes in the tibia accompanying pantothenic acid deficiency in young rats are described. The changes occurring in the oral mucosa and the mandibular joint of these rats have been reported.(6,7)

Experimental. Normal litters of 6 young together with the lactating mother (rats of the Long-Evans strain) were placed on the pantothenic acid-deficient or control diets at birth. Mother and young were weighed every fifth day; the young were weaned on the 21st day and continued on the same diet. This procedure is in contrast to the usual method of placing rats on experimental diets on the day of weaning (usually day 21) but has been found to produce the most acute pantothenic acid deficiency.(8) Suckling rats whose mothers were placed on the pantothenic acid deficient diet on the day of their birth, were severely retarded in growth and survived only a short time, *i.e.* the average survival for 226 rats was 35.4 ± 1.3 days (range 5-109 days) and only 13% lived past 60 days of age. In the majority of the young death occurred between their 25th and 40th day of age. The deficiency was so acute that many of the animals died before 30 days of age severely retarded in growth but without developing any other readily observable symptoms of the deficiency. In the animals that survived past 45 days of age, the characteristic symptoms of the deficiency (porphyrin deposition, dermatitis and greying) developed in some of the animals.

The pantothenic acid deficient diet was composed of 24% alcohol-extracted casein, 64% sucrose, 4% McCollum salts No. 185(9) and 8% hydrogenated cottonseed oil (Crisco). To each kilogram of diet was added 2 mg thiamine, 2 mg pyridoxine, 5 mg riboflavin, 5

* Aided by grants from the Board of Research and the Department of Agriculture of the University of California, the Rockefeller Foundation, New York City, the American Foundation for Dental Science, and the California State Dental Association. Crystalline B vitamins and synthetic alpha-tocopherol were generously furnished by Merck and Company, Rahway, N.J.

† A preliminary report of this study was given at the 24th General Meeting of the International Association for Dental Research, March, 1946.(1)

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1. Wainwright, W. W., and Nelson, M. M., *J. Dental Research*, 1946, v25, 185.

2. Nelson, M. M., Sulon, E., Becks, H., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 631.

3. Ashburn, L. L., *U. S. Publ. Health Rep.*, 1940, v55, 1337.

4. Daft, F. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H., *U. S. Publ. Health Rep.*, 1945, v60, 1201.

5. Levy, B. M., and Silberberg, M., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 380.

6. Wainwright, W. W., and Nelson, M. M., *Am. J. Orthodontics and Oral Surgery*, 1945, v31, 406.

7. Becks, H., Collins, D. A., Nelson, M. M., and Evans, H. M., *Am. J. Orthodontics and Oral Surgery* (in preparation).

8. Nelson, M. M., and Evans, H. M. (in preparation).

9. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, v33, 63.

TABLE I.
Effect of Acute Pantothenic Acid Deficiency in Male Rats on Tibial Length and Epiphyseal Cartilage Width.

	Pantothenic acid control			Pantothenic acid deficient		
Age, days	Body wt at autopsy, g	Tibial length, mm	Epiphyseal cartilage width, mμ	Body wt at autopsy, g	Tibial length, mm	Epiphyseal cartilage width, mμ
26	58	25.8	410	20	16.9	243
34	56	25.8	410	28	19.2	213
45	79	28.9	376	35	23.5	152
61	140	33.5	355	40	24.1	152
97	260	37.2	116	46	25.4	87*
Avg	119	30.2	333	34	21.8	179

* This excludes 50 micra of calcified scaling-off bone.

mg p-aminobenzoic acid, 10 mg nicotinic acid, 200 mg inositol and 500 mg choline chloride. During lactation the mother of each litter, both deficient and control, received weekly 1 cc of a fat-soluble vitamin mixture containing 6 mg alpha-tocopherol, 115 chick units vitamin D, 800 U.S.P. units vitamin A and 650 mg corn oil (Mazola). After weaning each individual rat received 0.5 cc of this fat-soluble vitamin mixture weekly by stomach tube. Control animals received the identical purified diet supplemented with 28 mg calcium pantothenate per kilogram of diet; in a few cases 500 μg calcium pantothenate was given weekly from the day of weaning. In addition, young together with their mothers were maintained from birth on a diet of natural foods§ as normal controls.

For the histologic study, 20 pantothenic acid deficient young (13 male and 7 female rats) that were most severely affected as judged by retardation of body weight and external appearance were sacrificed at ages ranging from 3 to 16 weeks. These rats were usually moribund at the time of autopsy. Rats receiving the purified diet supplemented with calcium pantothenate and normal rats reared on a diet of natural foods were autopsied at the same time. The tibias were fixed in 10% neutral formol, roentgenographed and measured, decalcified in 5% nitric acid, embedded in nitrocellulose, sectioned at 8 micra, and stained with hematoxylin and eosin.

Results. The histologic aspect of the epiphyseal cartilage of the tibia in normal rats reared on a diet of natural foods and the changes occurring with increasing age have been published in detail.(10) In this study tibial lengths and epiphyseal cartilage widths of the pantothenic acid-supplemented animals maintained on the purified diet were slightly but consistently below normal at every age. Histologically, increased lipid deposition in the marrow and slight involvement of hematopoiesis were observed. Rats receiving calcium pantothenate only from weaning did not approach normality as closely as rats receiving the vitamin throughout their entire life. Similar findings with other purified diets have recently been reported in a study of chronic riboflavin deficiency in the rat.(2) Tibial measurement showed considerable differences between the pantothenic acid-deficient and supplemented animals (Table I). The average tibial length for the deficient group was 21.8 mm as contrasted with 30.2 mm for the vitamin-supplemented group. The width of the epiphyseal cartilage also reflected corresponding decreases, averaging 179 and 333 micra for the deficient and supplemented

§ This diet is McCollum's Diet I, slightly modified, and contains 67.5% ground whole wheat, 15% technical casein, 10% whole milk powder, 5.25% hydrogenated vegetable oil (Crisco or Primex), 1.5% calcium carbonate, and 0.75% iodized sodium chloride, 3.5 g Sardilene (fish oil concentrate containing 3,000 U.S.P. units vitamin A and 400 Chick units vitamin D per g) are added to each kilogram of diet.

10. Becks, H., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1945, v92, 109.

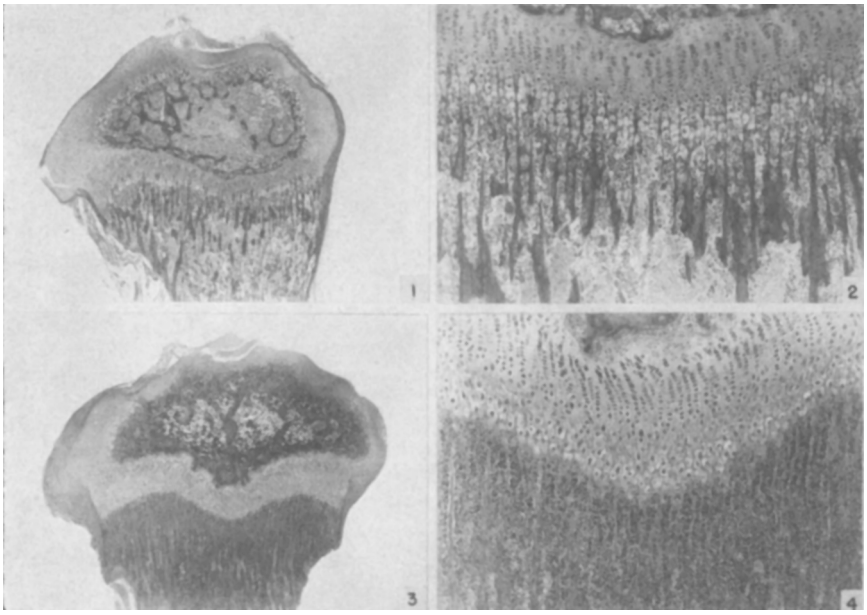

PLATE I.
Proximal epiphysis of the tibia of male rats. Hematoxylin and eosin stain.
FIG. 1. Pantothenic acid deficient, 26 days of age, plate 9680, × 30.
FIG. 2. Pantothenic acid deficient, 26 days of age, plate 9696, × 140.
FIG. 3. Control, 26 days of age, plate 9703, × 30.
FIG. 4. Control, 26 days of age, plate 9706, × 140.

groups, respectively. The histologic changes observed in the tibia pantothenic acid-deficient rats fall into four distinct classes which do not necessarily correlate with age. Due to variations in individual response to the deficiency, increasing severity of morphologic disturbance has been used as the sole criterion for classification.

STAGE I: This stage was found in rats at 21, 26 and 33 days of age and is best represented by the tibia of the 26-day-old rat (Figures 1 and 2). Although the epiphysis is small and possesses thick articular cartilage, much early calcification is seen along the inner margin. The epiphyseal trabeculae are thin and scant. The epiphyseal cartilage is one-half the width of the corresponding vitamin-supplemented control, the decrease occurring almost entirely in the basophilic layer. The columns are slightly less regular than normal and are separated by increased amounts of matrix. Capillary tufts are directed against the cartilage cells of the vesicular zone and erosion appears active. The diaphyseal trabeculae are short, coarsened and are seldom covered with osteoblasts. However, osteoclasts possessing 8-10 nuclei are profuse. Along the endosteum the proliferation of osteoblasts is retarded and osteoblasts undergoing lysis may be seen. The diaphyseal medulla is granulocytopenic and erythrocytopenic and contains no fat cells. The marrow is highly edematous and characterized by many large connective tissue cells. In addition, cells somewhat similar to macrophages and connective tissue cells but larger and containing much clear substance without macrophagic inclusions are seen. The tibia of the 26-day-old rat supplemented with calcium pantothenate is shown in Fig. 3 and 4 and represents a typical picture for the age range of 21-36 days. The epiphysis is larger than that just described and many fine trabeculae are directed centrally. The metaphysis is wide and composed of long, straight columns of basophilic cells separated by little matrix. Erosion is proceeding rapidly and the trabeculae are slender and long with little anasto-

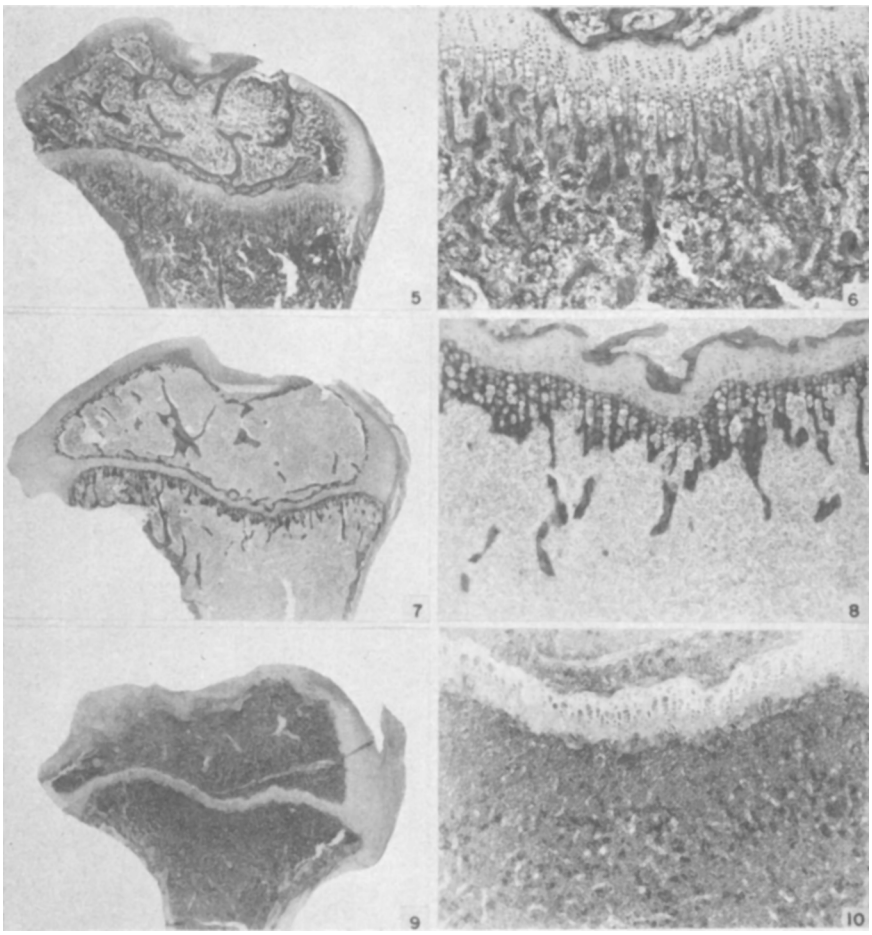

PLATE II.

Proximal epiphysis of the tibia of male rats. Hematoxylin and eosin stain.

FIG. 5. Pantothenic acid deficient, 61 days of age, plate 9978, × 30.
FIG. 6. Pantothenic acid deficient, 61 days of age, plate 9924, × 140.
FIG. 7. Pantothenic acid deficient, 45 days of age, plate 9683, × 30.
FIG. 8. Pantothenic acid deficient, 45 days of age, plate 9697, × 140.
FIG. 9. Pantothenic acid deficient, 97 days of age, plate 9981, × 30.
FIG. 10. Pantothenic acid deficient, 97 days of age, plate 9704, × 140.

mosing. Occasionally osteoclasts are encountered subjacent to the epiphyseal cartilage. Osteoblasts are present in abundance at the capillary tips and along the trabeculae and the endosteum. The diaphyseal marrow is slightly granulocytopenic and fat cells may be noted in the marrow cavity.

STAGE II: This stage is observed over an age range of 33 to 63 days and is represented by the 61-day-old tibia (Fig. 5 and 6). The cartilage is less than half the width of the corresponding vitamin-supplemented control. Erosion is decreased. The trabeculae are blunt and thick and osteoclasts are present. The osteoblasts at the remaining capillary tufts are so small and fibroblast-like as to merit comment and osteoblastic proliferation along the endosteum has ceased. Small pointed fibroblasts frequently act as replacements. In the marrow varying degrees of granulocytopenia or erythrocytopenia are encountered. In the vitamin-supplemented controls for this early age range (not illustrated), the larger epiphyses, wide epiphyseal cartilage, numerous

long trabeculae and the profusion of osteoblasts contrast with the marked retardation of endochondral ossification in the deficient rats.

STAGE III: In this stage the deficiency has now resulted in the virtual cessation of growth. Calcification has sealed off the inner margin of the epiphysis and the diaphyseal side of the epiphyseal cartilage. Chosen from an age range of 45 through 72 days, the 45-day-old animal exemplifies this condition (Fig. 7 and 8). The fragile appearance of the epiphysis with its marked absence of trabeculae as the result of continued resorption is striking. The osteoblasts remaining at the juxtadiaphyseal region are small; fibroblasts line some of the few trabeculae and no endosteal proliferation is evident. The absence of erythrocytes is pronounced in the 45 and 48-day-old tibias. Otherwise the granulocytopenia previously noted in the marrow persists.

STAGE IV: This stage represents the final arrest of growth. All tibias for rats between 78 and 109 days reflected similar histologic pictures and the 97-day-old tibia may serve as an illustration for this period (Fig. 9 and 10). The epiphysis has not increased in size and persists in its frailty and absence of trabeculae. Bone growth is impaired by sealing-off bone of approximately 50 micra in the seven animals investigated. The epiphyseal cartilage is narrow and shows much matrix in the basophilic zone. Only a few very small osteoblasts and osteoclasts remain. No fat storage is seen in the marrow cavities. Hematopoiesis in some rats was improved with regard to erythrocytes but not to granulocytes. Occasional heavy basophilic staining of the marrow is due to the high incidence of blast forms, premyelocytes and metamyelocytes and the paucity of older forms. The 78-109 day old rats supplemented with calcium pantothenate showed a larger epiphysis with many heavy ramifying trabeculae. The epiphyseal cartilage was progressively reduced with age(10) but lacked the sealing-off bone observed in the deficient group. Through this age range the vitamin-supplemented rats showed diminished osteogenic activity whereas in the deficient group complete cessation of osteogenesis had occurred as early as 78 days.

Discussion. The data reported in this study demonstrate that pantothenic acid is a vital factor for the stimulation and maintenance of normal chondrogenesis, osteogenesis and hematopoiesis in the rat. Histologic study of the tibia has revealed severe disturbance to morphologic structure depending more upon individual response to the deficiency than upon the age of the animal. In all stages of the deficiency growth of the tibia was retarded; chondrogenesis and endochondral ossification were drastically reduced. Hematopoiesis was usually severely affected, the bone marrow being granulocytopenic and/or erythrocytopenic and lacking megakarocytes. In Stages III and IV the onset of calcified sealing-off bone along the inner margin of the epiphysis and the diaphyseal margin of the epiphyseal cartilage showed that growth of the tibia had stopped. In normal rats of the Long-Evans strain the beginning of sealing-off bone in the tibia has been shown to occur at approximately 170 days of age(10) but complete sealing-off never occurs.

The inhibition of chondrogenesis and endochondral ossification observed in this study is much more severe than that previously reported for pantothenic acid deficient rats (or mice). This is to be expected in view of the acute syndrome produced by instituting the deficiency much earlier in life. Sealing-off bone has not been observed previously in pantothenic acid deficient animals although its formation at a comparable early age has been reported for rats deficient in vitamin A(11) and for hypophysectomized rats.(12)‖ The marked impairment of hematopoiesis observed in this study is in agreement with the findings of Daft *et alia*(4) on pantothenic acid deficient rats. These investigators also reported a variable degree of edema in the bone marrow in contrast to the markedly edematous condition found in the youngest animals of this study. A similar markedly edematous bone marrow has been reported for rats de-

11. Wolbach, S. B., *J. Bone and Joint Surgery*, 1947, v29, 171.

12. Beeks, H., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1945, v92, 121.

‖ In this laboratory sealing-off bone at this early age has also been observed in acute protein deficiency in the rat (to be published).

ficient in pteroylglutamic acid(13) and "serous atrophy" of the bone marrow, possibly a comparable condition, for rats restricted in calories(14) and for mice depleted of the entire vitamin B complex.(15) The inhibition of chondrogenesis and endochondral ossification previously observed in chronic riboflavin deficiency in the rat(2) is similar to that reported here for acute pantothenic acid deficiency. In comparison, the acute pantothenic acid deficiency was characterized by marked resorptive activity which led to the complete absence of diaphyseal trabeculae and the formation of sealing-off bone at a much earlier age. In addition, fatty infiltration of the bone marrow, which was marked in riboflavin deficiency, was not observed in this acute pantothenic acid deficiency.

Despite the severity of the morphologic changes produced by the lack of pantothenic acid and the prevention of practically all changes by the administration of the synthetic vitamin, it is not to be inferred that all of these changes are due specifically to the lack of this vitamin. Biotin and pteroylglutamic acid were not present in the purified diets used in this study nor in the diets used by other investigators carrying out pathological studies of pantothenic acid-deficient animals. Daft *et alia*(4) have presented evidence indicating that the granulocytopenia occurring in pantothenic acid-deficient rats is due to an induced deficiency of pteroylglutamic acid. These authors also found evidence indicating that the anemia observed in such animals might be due either to pantothenic acid deficiency or to a deficiency of an unknown vitamin. The effects of pteroylglutamic acid deficiency and caloric restriction have been mentioned in connection with the edematous bone marrow. Caloric restriction, as well as pantothenic acid deficiency, also induces a pteroylglutamic acid deficiency(16) and it is probable that some of the effects previously attributed to caloric restriction may actually be due to deficiencies of pteroylglutamic acid or of still unknown vitamins. The role of caloric restriction, pteroylglutamic acid and of unknown factors in the changes in endochondral ossification accompanying acute pantothenic acid deficiency in young rats obviously needs further investigation.

Summary. The changes in endochondral ossification of the tibia accompanying acute pantothenic acid deficiency in young rats have been investigated. Twenty deficient animals, 13 male and 7 female rats, were autopsied together with their controls at ages ranging from 3 to 16 weeks. The histologic changes observed were decreased growth of the tibia with marked impairment in chondrogenesis, osteogenesis and hematopoiesis.

Four stages of morphologic changes were observed. Stage I, in which the ages of the rats ranged from 21 to 33 days, was characterized by unusual trabecular resorption, decreased osteoblast proliferation and edema of the bone marrow. In Stage II the ages ranged from 33 to 63 days and marked retardation of osteogenesis with some calcification in the epiphysis and blunt diaphyseal trabeculae was observed. The dominant feature of Stage III, from 45 to 72 days, was the beginning of epiphyseal cartilage calcification and the cessation of osteogenesis. Stage IV, from 78 to 109 days, was characterized by complete absence of trabeculae and the formation of a heavy layer of sealing-off bone below the epiphyseal cartilage.

The role of pantothenic acid and of additional factors such as pteroylglutamic acid, unknown vitamins and caloric restriction in these histologic changes is discussed.

13. Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Pathology*, 1945, v40, 364.

14. Saxton, J. A., Jr., and Silberberg, M., *Am. J. Anat.*, 1947, v81, 445.

15. Silberberg, M., Levy, B. M., and Younger, F., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 185.

16. Ershoff, B. H., and Adams, A. D., Jr., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 154.

Received November 4, 1949. P.S.E.B.M., 1950, v73.