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Chronic Riboflavin Deficiency in the Rat. I. Ossification in the Proximal Tibial Epiphysis.*

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The importance of riboflavin in skeletal development has been recently emphasized by Warkany.[1] Warkany found that prenatal riboflavin deficiency in the rat resulted in the production of young with multiple skeletal abnormalities. Among the abnormalities were developmental disturbances of the mandible, shortening and distortion of the extremities, various forms of syndactyly of the hands and feet, and fusion of the ribs and sternal centers of ossification. Histologically, a more or less marked delay in ossification occurred with persistence of cartilage in many areas. Levy and Silberberg[2] recently reported that in the mouse deficiency of riboflavin from weaning resulted in retardation of endochondral ossification and increased "degeneration of cartilage."

In the present study the changes in endochondral ossification in the tibia that occurred in rats born from riboflavin-deficient mothers and maintaind for three to nine months on purified diets deficient in riboflavin are reported. The riboflavin deficiency produced under these experimental conditons was chronic in nature.

Experimental. Normal female rats of the Long-Evans strain, 2 to 3 months of age, were bred with normal males and placed on riboflavin-deficient or control diets the day of breeding. At birth the riboflavin-deficient young were carefully examined for skeletal abormalities. All litters† were weighed every 5 days, weaned on the twenty-first day and continued on the same diet as their parents.

Previous studies[3,4,5] have disclosed that riboflavin deficiency is accentuated by a high fat diet. For this reason 2 riboflavin-deficient diets were used; one was high in carbohydrate and the other, high in fat. The latter was given from 90 days of age to the end of the experiment. The high-carbohydrate diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco), and 4% salts No. 4.[6] The high-fat diet contained 48% instead of 8% hydrogenated cottonseed oil with a corresponding reduction in the proportion of sucrose. Both diets contained the following crystalline B vitamins per kilogram of diet: 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 50 mg calcium pantothenate, 400 mg inositol, and one gram choline chloride. A fat-soluble vitamin mixture was given weekly and contained 325 mg corn oil (Mazola), 400 U.S.P. units vitamin A, 58 Chick Units vitamin D, and 3 mg alpha-tocopherol. During the lactation period the lactating mothers received a double

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† Six young were kept per litter.

1 Warkany, J., *Vitamins and Hormones*, 1945, **3**, 73, Academic Press, Inc., New York, Publishers.

2 Levy, B. M., and Silberberg, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 355.

3 Mannering, G. J., Lipton, M. A., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 100.

4 Mannering, G. J., Orsini, D., and Elvehjem, C. A., *J. Nutrition*, 1944, **28**, 141.

5 Czackes, J. W., and Guggenheim, K., *J. Biol. Chem.*, 1946, **162**, 267.

6 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

TABLE I.
Riboflavin Deficiency in Male Rats from Birth.

Group	Animal No.	At autopsy		Length of tibia* (mm)		Width of proximal epiphyseal cartilage of tibia† (μ)	
		Age (day)	Wt (g)				
I	B8492	96	317	39.2		244	
Riboflavin control	BH8494	110	352	41.2		165	
group	BH8487	144	436	38.2	Avg 40.5	163	Avg 143
	B7985	194	537	41.8		130	
	GH7980	239	520	39.6		144	
	BH7986	278	630	42.9		110	
II	B6491	96	41	24.1		124	
Riboflavin deficient	GH2467	110	94	32.7		114	
group	B2498	144	122	35.0	Avg 34.3	114	Avg 111
	BH2499	194	169	37.9		123	
	B2495	237	127	36.9		100	
	B2493	277	188	39.3		89	

* The length of the tibia was measured with calipers before decalcification.
† As measured in the central portion of histologic sections with an ocular micrometer.

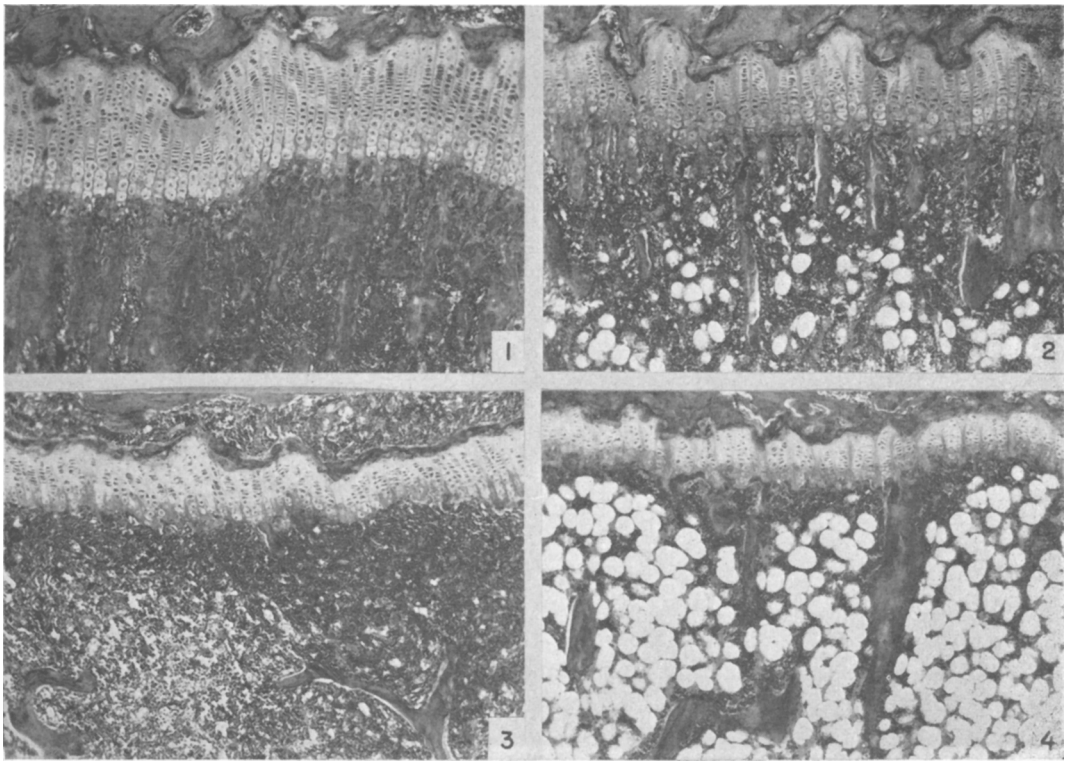
amount of the fat-soluble vitamin allotment. Control rats received the identical purified diet supplemented with 10 mg riboflavin per kilogram of diet.

For the histologic study of the tibia 6 riboflavin-deficient animals that were most severely affected as judged by external appearance and retardation of body weight were selected. These animals together with the riboflavin controls were autopsied at ages varying from 3 to 9 months, *i.e.* 96, 110, 144, 194, 237 and 278 days (Table I). The tibias were fixed in 10% neutral formol, roentgenographed and measured, decalcified in 5% nitric acid, embedded in nitrocellulose, sectioned at 8-10 micra, and stained with hematoxylin and eosin.

Results. During the first 90 days of the experiment on the high-carbohydrate diet, only a mild riboflavin deficiency was produced, the rats showing few deficiency symptoms other than retardation of growth. In agreement with the findings of previous investigators, the deficiency was accentuated by the high-fat diet. Dermatitis, alopecia, cataracts, spasticity, and marked decreases in growth occurred in many animals. However, the prolonged survival of many animals (see autopsy ages, Table I) showed that the deficiency was chronic rather than acute even with the high-fat diet. It may be mentioned that no skeletal abnormalities were observed in the young born from riboflavin-deficient mothers given either the high-carbohydrate or high-fat diets the day of breeding. Neither were abnormalities found in the second litters produced by these same mothers after they had received the deficient diets for 50-60 days at the time of the second breeding.

In comparing the tibial length and width of the epiphyseal discs marked differences were found between the riboflavin-deficient and the control group (Table I). The average length in the riboflavin-deficient group was 34.4 mm against 40.5 mm in the riboflavin-control group. Epiphyseal disc measurements averaged 111 micra for the deficient as compared with 143 micra for the control animals. Thus the decrease in epiphyseal disc width was aligned with shorter tibial length. The greatest changes in tibial length and cartilage disc width occurred in the youngest deficient animals. Severe decreases in body weight were found simultaneously (Table I).

The histologic aspect of the epiphyseal cartilage of the tibia in normal rats and changes occurring with increasing age have been recently presented in detail.[7] In this study measurements of tibial length and epiphyseal disc width of the riboflavin control animals were slightly but consistently below normal at every age, although histologic differences were slight. A microscopic study of the process of endochondral ossification in the tibia revealed decided differences between the



Proximal epiphyseal region of the tibia of male rats, H. and E. stain, × 75.
FIG. 1. Riboflavin control, 96 days of age. Spec. No. 8908, Plate 9426.
FIG. 2. Riboflavin control, 144 days of age. Spec. No. 8912, Plate 9427.
FIG. 3. Riboflavin-deficient rat, 96 days of age. Spec. No. 8896, Plate 9431.
FIG. 4. Riboflavin-deficient rat, 144 days of age. Spec. No. 8900, Plate 9429.

riboflavin-deficient and the control group.

Riboflavin Control Group. At 96 and 110 days the histologic aspect of the proximal epiphysis shows the normally thick surface cartilage, dense lamellar coat of bone, numerous coarse trabeculae, and a continuous layer of bone resting against the cartilaginous plate. The epiphyseal cartilage and subjacent diaphyseal area of the riboflavin control animal at 96 days of age are illustrated in Fig. 1. The trabeculae are numerous, close and coarse but do not extend far into the diaphysis. Few anastomoses are seen and erosion is very active. At 144 days (Fig. 2) the epiphyseal cartilage of the control animal shows a reduction in width, an increase of matrix and a more pronounced conical pattern of chondrocytes than usual for this age. The bone trabeculae are shorter and coarser and in many areas the marrow tissue is found in direct contact with the zone of enlarged cells. Many osteoclasts are seen on the surface of the bone trabeculae. In the more advanced age groups at 194, 237, and 278 days of age, the cartilaginous plate became progressively narrower but richer in matrix and the columnar arrangement of cells appeared still more irregular and frequently lost even the Dawsonian pyramidal aspect of normal old age. The bone trabeculae disappeared for the most part except for a few lying horizontally some distance below the disc. As contrasted with animals reared on a diet of natural foods[7] ossification was subnormal and trabeculation somewhat disturbed.

Riboflavin Deficient Group. At 96 days the epiphyseal surface cartilage was well-formed

[7] Becks, H., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1945, **92**, 109.

but the adjacent lamina of bone was so delicate as to be almost non-existent. The trabeculae were sparse in number and fine in structure. In the epiphysis, there was a thin, nearly continuous, horizontal layer of lamellar bone resting directly upon the cartilage (Fig. 3). The cartilaginous epiphyseal disc consists of 5 to 9 flattened cells and only one to 2 vesicular cells. The matrix is abundant in the basophilic region with bands occasionally traversing the entire width of the plate. Calcification of the matrix from the diaphyseal side is in progress and is enclosing the majority of vesicular cells. Osteogenesis has practically ceased and trabeculation consists only of short projections covered with true bone. This stage foreshadows the sealing-off of the epiphyseal cartilage from the marrow cavity by bone. Erosion is rare. The marrow shows an unusual wealth of osteoclasts but is normal in fat content.

At 110 days the epiphysis was somewhat larger; otherwise the appearance was the same as that of the 96-day-old tibia. The cartilage disc was slightly reduced in width. There was no evidence of fat infiltration in the marrow.

At 144 days the changes were more severe. The epiphysis showed advanced ossification. The bone lamina beneath the articulating cartilage, the trabeculae, and the layer of bone above the epiphyseal disc were increased in thickness. The cartilaginous disc (Fig. 4) is very narrow and the chondrocytes in the basophilic zone are arranged conically. The vesicular cells are enclosed in calcified matrix having a well-advanced marginal deposit of lamellar bone sealing it from the marrow. Three or 4 heavy isolated trabeculae extend into the diaphysis. Numerous osteoclasts are present in the marrow cavity and the number of large fat cells is increased markedly.

The findings in the tibias of the older age groups were similar. The margins of the epiphyseal cartilage were highly irregular and the matrix between the pyramidal groups of cells was increased. At 277 days the cartilage disc was the narrowest observed in this experiment and was reduced in a few places to 40 micra. The matrix lay in wide tracts in vertical or oblique planes across the disc, often penetrating the marrow on the diaphyseal side and forming short trabeculae which showed lamellar bone on their surface. Much of the disc was separated from the marrow by true bone. The 3 or 4 remaining lamellar trabeculae evinced no particular orientation with respect to the diaphysis. The marrow contained an unreduced amount of fat cells.

Discussion. The foregoing experiment was planned to study the effects of prenatal riboflavin deficiency on skeletal growth in the young. In contrast to Warkany, no skeletal abnormalities were observed at birth and the animals were sacrificed at ages varying from 3 to 9 months. The pathological changes observed were not necessarily progressive with age since the animals had varying degrees of the deficiency at the time of autopsy. Some of the older animals had lived considerably longer before showing outward signs of the deficiency and were less affected than animals autopsied at an earlier age. Also histological changes in the tibia due to age may have masked some of the changes due to riboflavin deficiency in the older animals. However, even at 278 days, the tibia of the deficient animal, although showing the characteristic changes of advanced age, differed markedly from that of the riboflavin control.

The principal histologic changes observed in the riboflavin-deficient group were retarded development of the epiphysis, progressive decrease in the width of the epiphyseal cartilage, increased hyalinization of its matrix, calcification and separation of the epiphyseal cartilage from the marrow cavity by a thin layer of bone. Osteogenesis ceased with an almost complete disappearance of the diaphyseal trabeculae. Unusual fat infiltration with reduction of hematopoietic tissue occurred in most of the older animals and was similar to the myeloid hypoplasia recently reported by Endicott *et al.*‡,[8] for acute riboflavin de-

‡ Endicott *et alia*[8] also found that both riboflavin and "folic acid" were concerned in this hypoplasia. Further studies will be necessary to separate the effects of these two vitamins on endochondral ossification.

[8] Endicott, K. M., Kornberg, A., and Ott, M., *Blood*, 1947, **2**, 164.

ficiency in the rat. In general, the changes observed in this study of the rat were similar but much more severe than those reported for the mouse by Levy and Silberberg.[2]

Reasons for the failure to duplicate the Warkany findings (*i.e.* multiple skeletal abnormalities) are not obvious. The experimental conditions and the purified high-carbohydrate diet were similar but not identical with those used by Warkany. The principal dietary difference was the use of an alcohol-extracted casein, probably containing slightly more riboflavin[9] than that used by Warkany, at a 24% instead of 18% level in the diet. The use of a high-fat diet to accentuate the deficiency should have overcome this difference in the protein components of the diets. At the present time 40 litters comprising 350 riboflavin-deficient young have been carefully examined at birth. While clearing with microscopic examination might have revealed some skeletal defects, abnormalities such as shortening of the mandible with protrusion of the tongue and syndactylism of the extremities which occurred with high frequency in Warkany's abnormal young could have been easily recognized by external inspection. Further studies on this problem are in progress.

Summary. The tibias of 6 male rats of the Long-Evans strain born from riboflavin deficient mothers and maintained on riboflavin deficient diets for 3 to 9 months were studied histologically and compared with an equal number of controls reared upon the same diet but with riboflavin added.

In the deficient rats the growth of the tibia was retarded and endochondral ossification was gravely impaired. Marked reduction in chondrogenesis and in capillary erosion and ossification were characteristic for all degrees of the deficiency. The formation of a thin calcified plate on the diaphyseal side of the epiphyseal cartilage is a finding in consonance with the early arrest of growth. Hematopoietic tissue was replaced by fat in all animals after 144 days on the deficient diet.

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A New Method for the Production of Non-Specific Capsular Swelling of the Pneumococcus.

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Neufeld,[1] and Neufeld and Etinger-Tulczynska[2] first described the phenomenon of capsular swelling of the pneumococcus when specific antiserum was added to the organism. This capsular swelling, known as the "quellung" reaction, is dependent upon the reaction of capsular polysaccharide with its specific antibody. It has been shown that an excess of specific polysaccharide,[3] heat,[4] papain digestion,[5] and repeated washing with water[6] can produce a reversal of the specific "quellung" reaction.

Non-specific capsular swelling of pneumo-

1 Neufeld, F., *Z. f. Hyg. u. Infectionskrankh.*, 1902, **40**, 54.

2 Neufeld, F., and Etinger-Tulczanska, R., *Ibid.*, 1931, **112**, 492.

3 Nungester, W. J., and Kempf, A. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 705.

4 Etinger-Tulczynska, R., *Z. f. Hyg. u. Infectionskrankh.*, 1933, **114**, 769.

5 Kalmanson, G. M., and Bronfenbrenner, J., *Science*, 1942, **96**, 21.

6 Kempf, A. H., and Nungester, W. J., *J. Inf. Dis.*, 1942, **71**, 50.