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Skeletal Changes in Growing Vitamin B Complex Depleted Rats and the Course of Repair.

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In continuation of our investigations on the response of the skeletal tissues to vitamin deficiencies, we have now analyzed the growth and development of the long bones in vitamin B complex depleted young rats. The course of repair after refeeding has also been studied.

Material and Methods. Fifty-five rats of the Wisconsin strain (24 males and 31 females), 19 to 22 days old, were divided into 3 groups, littermates and animals of the same sex being equally distributed among these groups.

Series I. 12 rats were kept on water and a stock diet composed as follows:

	g %
Yellow corn meal	51.55
Powdered whole milk	9.15
Powdered skim milk	9.15
Linseed meal	3.86
Wheat germ	1.44
Crude casein	9.63
Alfalfa leaf meal	9.63
Dried brewer's yeast	0.96
Sodium chloride	0.39
Calcium carbonate	0.39
Cod liver oil	0.96
Crisco	2.89
	100.00

These rats were killed after 18, 24, 30, or 40 days.

Series II. 21 animals received water and the following vitamin B-free diet:

	g %
Casein (Vit.-free)	18
Starch (Anheuser-Busch (Vit.-free)	62
Merck salt mixture No. 1	4
Cod liver oil (Mead Johnson)	2
Crisco	14
	100

The duration of the experiments was the same as in Series I, unless the poor condition of the animals necessitated an earlier sacrifice.

Series III. 22 rats received water and the deficient diet, but they were subsequently refed the stock diet for 5, 10, 21 or 30 days. As a rule, refeeding was begun after 3 weeks. The rats were weighed once a week. At the end of the experiment, the animals together with their controls were killed with chloroform. The upper end of the tibia and the lower end of the femur were removed as a whole, fixed in 10% formalin, decalcified in a formic acid-citrate mixture,[1] and embedded in paraffin. Slides, stained with hematoxylin and eosin, were prepared for histological studies. The width of the epiphyseal disc and of the cortex of the tibia was measured with an objectmicrometer, and average values were established. The number of trabeculae in the metaphysis of the tibia present in a standard area measuring 350 microns in diameter was counted.

Gross Observations. At the beginning of the observations, the mean weight of the males was 42 ± 1 g, that of the females 40 ± 6 g. During 18 days, the deficient animals, both male and female, gained 12 to 14 g, whereas the normal control males had gained 50 g and the females 68 g. Subsequently, the weights of the deficient animals dropped sharply. After 24 days, the loss was 7 ± ½ g in males and 4 ± 3 g in females, and after 30 days, 11 ± 1 g in males and 9 ± 2 g in females. The deficient state was tolerated for 34 days by 1 male that lost 18 g. Females were more resistant, 4 animals surviving for more than 30 days, and 1 for 40 days. These females lost 16 ± 4 g. After refeeding the stock diet for 5 days, the weight of the males had increased 46 ± 7 g, that of the females 40 ± 3 g, whereas the normal controls had gained 15 g in the corresponding period of time. After 10 days, the weights of the refed animals still remained 25 to 30% below those of the controls. After 21 days,

[1] Morse, A. J., *J. Dent. Res.*, 1945, **24**, 143.

the weights of the refed rats began to equal those of the normal ones, and after 30 days, the weights of 2 of the refed animals surpassed those of the controls.

Histologic Findings. In Males. After 18 days, the changes noted in the deficient animals were slight. The epiphyseal disc measured 320 microns (normal 355 microns). The cartilage cells were smaller and the cartilage cell rows shorter than usual. The latter were separated from each other by thin layers of chondromucoid ground substance. The single row was composed of 9 or 10 columnar and 3 or 4 hypertrophic cartilage cells (normal 12 columnar and 4 or 5 hypertrophic cells). There was mitotic proliferation of the columnar cells. The metaphysis was properly vascularized, and replacement of cartilage by bone was in progress. Whereas ordinarily the metaphyseal standard area contained 20 long osseous spicules, in the deficient animals only 16 short and thin trabeculae covered by small osteoblasts were counted. The articular cartilage cells were small. The cortex was 225 microns thick (normal 255 microns). The endosteum contained a continuous layer of oblong osteoblasts. The vascular canals were enlarged; the periosteum was dense; the bone marrow was hemopoietic.

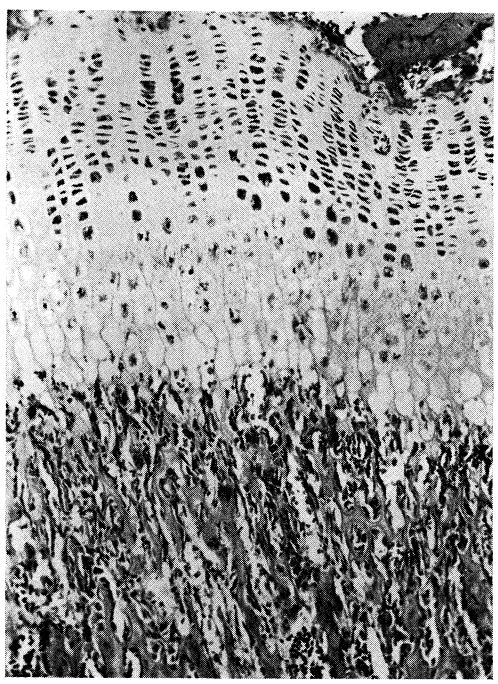

FIG. 1.
Section through the epiphyseal disc at the upper end of the tibia of a male rat, 44 days old, receiving the stock diet for 25 days. Magnification 104 ×.

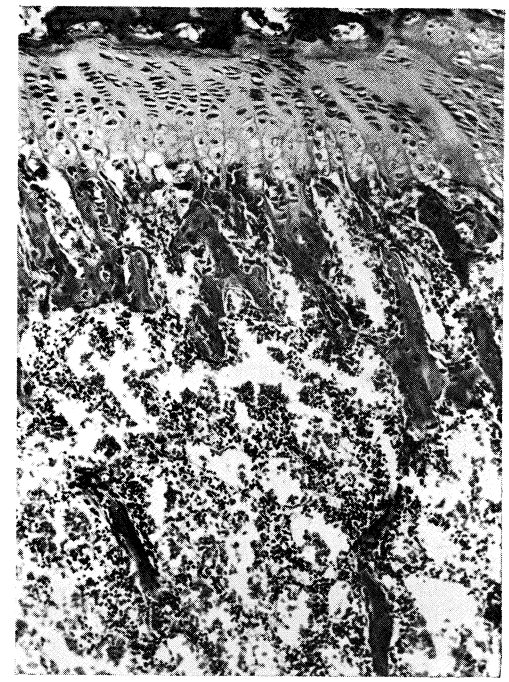

FIG. 2.
Section through the epiphyseal disc at the upper end of the tibia of a male rat, 43 days old, kept on the deficient diet for 21 days. Magnification 104 ×. Compare with Fig. 1. The epiphyseal disc is narrowed and bone formation is markedly decreased.

After 24 days, the epiphyseal discs of the deficient animals had narrowed down to 135 microns (Fig. 1, 2). The shortened cell rows were composed of 6 to 8 small columnar and 2 or 3 small sclerosed "hypertrophic" cartilage cells. Mitoses were not seen. The cartilaginous matrix was abundant and hyalinized. The vascularization of the metaphysis was diminished, and only 5 trabeculae were found in the standard area. The spicules were very short, and frequently only fragments of bone were seen. The cartilage cells of the pressure and hypertrophic zones of the joint were less numerous and smaller than ordinarily. The cortex measured 205 microns. The endosteum consisted of a discontinuous layer of small

osteoblasts or spindle cells with elongated cytoplasm. The vascular canals were filled with edematous connective tissue. The periosteum was loosened. In the bone marrow, foci of serous atrophy were noted. Subsequently, the changes were still more pronounced.

After 30 days, growth of cartilage and bone was at a standstill. The single cartilage cell row contained 5 or 6 columnar and 2 small hypertrophic cells. Much hyaline matrix appeared between the cartilage cell rows, and a layer of preosseous material was seen in the distal third of the atrophic epiphyseal disc. Metaphyseal trabeculae were absent. Instead, a thin transverse bony lamella delimited the epiphyseal cartilage from the marrow cavity. The cartilaginous covering of the joint was markedly atrophic. The cortex of the shaft measured 135 microns. At its inner surface, a discontinuous layer of small spindle cells had taken the place of osteoblasts. The Haversian canals were enlarged and contained engorged vessels and edematous connective tissue. The periosteum was poor in cells. The bone marrow showed an advanced state of serous atrophy.

After 34 days, the epiphyseal disc was only 65 microns wide, and the single cartilage cell row was composed of 4 inactive columnar cells and 1 cell of hypertrophic type. The conditions were otherwise comparable to those seen after 30 days.

In Females. The changes were of the same kind as in the males, but their progress was slower. Thus after 18 days on the deficient diet, the epiphyseal disc was 330 microns wide (normal 340 microns), and the single cartilage cell row contained 10 or 11 columnar and 4 hypertrophic cells. The decrease in the size of the cartilage cells, however, was conspicuous. There were 18 trabeculae in the metaphyseal standard area (normal 20). The cortex was of usual diameter (245 microns). The endosteum and periosteum were unchanged.

After 24 days, the epiphyseal disc was 165 microns high. The cartilage cell rows were short and composed of 7 to 9 columnar and 4 hypertrophic cells. Both cell types were considerably smaller than usual. Hyalinization of the cartilaginous matrix was less accentuated than in the male. In the metaphysis, 8 short trabeculae covered by small osteoblasts were counted. The cortex was 225 microns thick. The changes in the endosteum and periosteum were now comparable to those seen in the male. In the bone marrow, a few foci of serous atrophy were noticeable.

After 30 days, conditions in the female had approached those in the male. After 40 days, the epiphyseal disc had narrowed down to 70 microns, the single cartilage cell row containing 3 atrophic columnar cells and 1 hyalinized "hypertrophic" cell. Trabeculae were lacking. The articular cartilage cells were markedly atrophic. The cortex of the shaft was 145 microns thick. The bone marrow had undergone complete serous atrophy.

Effects of Refeeding. After 5 days, the growth of the epiphyseal and articular cartilages as well as endochondral and periosteal

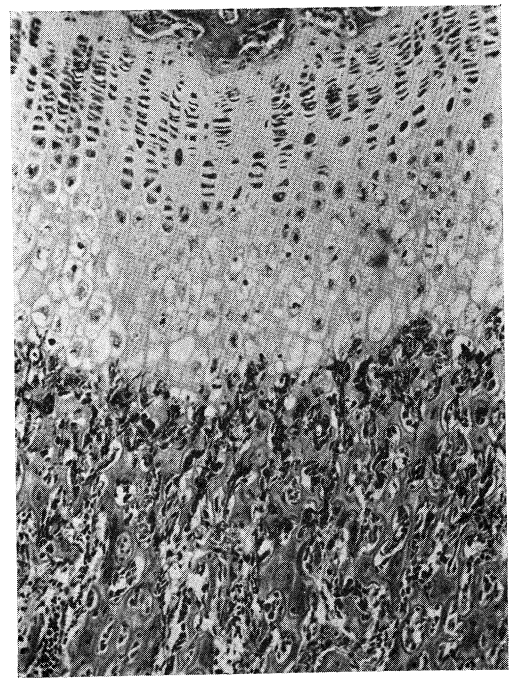

FIG. 3.

Section through the epiphyseal disc at the upper end of the tibia of a male rat, 45 days old, kept on the deficient diet for 21 days, and refed the stock diet for 5 days. Magnification 104 ×. Compare with Fig. 2. Growth of the epiphyseal cartilage and endochondral ossification are resumed.

ossification was resumed with remarkable intensity. Mitotic proliferation of the columnar cartilage cells was accentuated, and their conversion into hypertrophic cells was intensified. In the male, the epiphyseal disc was 355 microns high (Fig. 3). There was little intercartilaginous ground substance present. The single cartilage cell row was composed of 12 or 13 columnar and 4 or 5 hypertrophic cells. The standard area of the metaphysis contained 18 thick and long spicules covered by large mitotically proliferating osteoblasts. The cortex measured 235 microns. The endosteum was again composed of large osteoblasts, and the bone marrow was completely regenerated. After 10 days, the epiphyseal disc was 400 microns high. The single cartilage cell row contained 13 or 14 columnar and 5 large hypertrophic cells. There were 27 thick interlacing trabeculae in the standard area. The cortex was 265 microns thick. Endosteum and periosteum were not remarkable. The intensification of ossification was even more pronounced in the female than in the male. Gradually, the growth processes declined and assumed a normal rate in both sexes. After 21 or 30 days of refeeding, the histological appearance of the skeletal tissues did not differ from that of the normal controls of corresponding sex and age.

Comment. In vitamin B complex deficiency, skeletal development continues for some time, although at a reduced rate. The vitamin reserves of the organism are apparently sufficient to permit these processes to go on for a time. The subsequent cessation of growth and the serous atrophy of the bone marrow are findings typical of inanition. They suggest that the food ingested was not utilized, probably because the vitamin reserves had become exhausted. The severity of these changes manifests itself when they are compared with those obtained previously in underfed growing guinea pigs[2] and rats.[3] In animals kept on a quantitatively restricted but adequately balanced diet, skeletal growth proceeded at a reduced rate, but it lasted longer than usual. Thus in rats allowed to gain only 5 g every 50 days throughout their life, the long bones were only 13 to 18 % shorter than in normally fed animals.[3] In other words not unless the utilizable food energy is at a minimum and probably incompatible with life, can the inherent growth tendency of the skeleton be completely suppressed. The lack of thiamine in the diet may be responsible for loss of appetite and consequently decreased intake of food. However, the advanced skeletal changes seen in vitamin B complex-depleted rats were out of proportion to the diminished intake of food and must thus be attributed to the lack of the vitamin B complex. Again, the male responded to the vitamin deficiency more readily than the female. At this early age, the growth rate of the male is higher than that of the female. Any factor injurious to growth must, under otherwise identical conditions, necessarily affect the former more severely than the latter. If refeeding was begun before the animals were moribund, skeletal growth was quickly resumed, surpassed temporarily the normal rate and finally returned to normal. These findings are in agreement with observations in the skeleton of undernourished and refed animals.[2-5]

The present findings in vitamin B complex-depleted rats were similar to but more marked than those seen in mice deficient in pantothenic acid, or pyridoxine.[6] Lack of riboflavin, on the other hand, inhibited growth less but produced more degenerative changes in the epiphyseal cartilage than total B complex deficiency.[7] Serous atrophy of the bone marrow was not observed if only one of the aforementioned components of the vitamin B complex was lacking in the diet.

Investigations are in progress to determine

[2] Silberberg, M., and Silberberg, R., *Arch. Path.*, 1940, **30**, 675.

[3] Saxton, J. A., Jr., and Silberberg, M., *Am. J. Anat.*, 1947, **81**, 455.

[4] Jackson, C. M., *The Effects of Inanition and Malnutrition upon Growth and Structure*, P. Blakiston's Son & Co., Philadelphia, 1925.

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, **18**, 95; 1915, **23**, 439.

[6] Silberberg, R., and Levy, B. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, in press.

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

whether the changes seen in complete vitamin B complex deficiency represent a cumulative effect of all the constituents of the complex; or whether in the absence of one of the constituents the others, even if present in the diet, cannot be properly utilized. The latter condition might then be comparable to that shown to exist relative to the essential amino acids.[8]

Summary. In growing vitamin B complex-deficient rats, there is retardation and, finally, cessation of growth of cartilage and bone associated with serous atrophy of the bone marrow. The male is more sensitive to the deficiency than the female. After refeeding a stock diet, skeletal growth is resumed at an intensified rate resulting in early restoration of normal conditions.

[8] Cannon, P. R., Steffee, C. H., Frazier, L. J., Rowley, D. A., and Stepto, R. C., *Fed. Proc.*, 1947, **6**, 390.

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Toxicity of Equine Serum Treated by Alkali.

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Observations on the removal of antigenicity of proteins by alkali were early made by Wells,[1] TenBroeck[2] and have been confirmed and extended by a number of later workers. Recently, Davis and Eaton,[3] as well as Kazal, DeFalco and Arrow,[4] paid special attention to the toxicity of the resulting products but came to apparently conflicting conclusions. The former found alkali-treated bovine serum albumin to be non-toxic for white mice, rabbits, and for 2 dogs when given intravenously. On the other hand, Kazal *et al.* confirmed an earlier report by Lewis[5] that purified bovine plasma protein treated by sodium hydroxide gained acute toxicity for guinea pigs as soon as its antigenicity was lost. In view of the importance of these findings especially in relation to possible practical application, it seems worthwhile to make a similar study on horse serum treated by alkali and to observe its toxicity in rabbits, guinea pigs, and white mice. The results obtained are herewith presented.

After several preliminary trials, it was found that one-quarter normal solution of sodium hydroxide would, after a period of 30 days at 37°C, render normal horse serum so altered in antigenicity that rabbits immunized with the treated serum produced no precipitin against both normal and treated sera, gave occasional complement fixation reaction with either, and in half of these immunized animals, skin allergy to normal serum alone was demonstrated. Furthermore, of 10 guinea pigs sensitized to treated serum, only 3 out of 7 reacted mildly with normal, and 4 out of 6 similarly to alkali-treated serum without a single death. At the same time, 4 controls died when shocked with normal serum. Nine rabbits tolerated as much as one gram of treated serum protein per kilo body weight without any symptoms, whereas only 5 out of 12 immunized rabbits reacted with an elevation of body temperature to half a degree Centigrade following intravenous injections of 20 ml (800 mg protein) of the treated serum. Among 26 white mice weighing 20 g given intravenously 0.5 ml of the same (20 mg protein), 2 died in 6 hours, and one died in a week. The rest survived 4 weeks, the period of observation. However, among 9 guinea pigs weighing 200-250 g, only 2 out of 3 receiving less than 100 mg of treated serum protein intracardially survived, while all 6

[1] Wells, H. G., *J. Inf. Dis.*, 1909, **6**, 506.

[2] TenBroeck, C., *J. Biol. Chem.*, 1914, **17**, 369.

[3] Davis, H. A., and Eaton, A. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **59**, 246.

[4] Kazal, L. A., DeFalco, R. J., and Arrow, L. E., *J. Immunol.*, 1946, **54**, 245.

[5] Lewis, J. H., *Science*, 1943, **98**, 371 (quoted in [4]).