Epiphyseal Growth Zones in Acute Lathyrism.* Determinations of Water, Hydroxyproline and Mucopolysaccharides. (31549)

E. BERNTSEN (Introduced by G. Asboe-Hansen)
Connective Tissue Laboratory, Department of Dermatology, University of Copenhagen, Rigshospital, Denmark

The effects of lathyrogenic agents on the metabolism of various connective tissue structures have recently been reviewed(1,2). With regard to epiphyseal growth zones in particular, earlier biochemical studies on epiphyseal cartilage(3-7) tend to indicate a decrease in mucopolysaccharide synthesis and content together with an unchanged content of collagen. As part of a further investigation of growth zone changes in acute lathyrism(8), water determinations and analyses of hydroxyproline, hexosamine and uronic acid on epiphyseal cartilage and metaphyseal spongious bone have been carried out.

Material and methods. A 6% sterile solution of aminoacetonitrile (AAN), Fluka AG, Switzerland, was made up and adjusted to pH 7.3-7.4 with NaOH. Experimental animals received a dosage of 15 mg per 100 g body weight per day, while control animals received a corresponding volume of physiological saline. Injections were given subcutaneously into the back of the neck.

Thirty-one albino rabbits from 16 to 23 days of age were divided into 2 groups. Group A comprised 8 experimental and 7 control animals, and received one daily injection of AAN or saline through 2 days. Group B consisting of 8 experimental and 8 control animals was injected with AAN or saline through 4 days. All animals were sacrificed on the day after the last injection by an intraperitoneal overdose of Nembutal®.

By use of a dissection microscope, epiphyseal cartilage and corresponding metaphyseal spongiosa were dissected out from proximal humerus and tibia, distal radius, ulna and femur of all 4 extremities. Separating epiphysis from metaphysis through an incision along the epiphyseal line, cartilage was gently scraped off the epiphysis and metaphyseal surface. Thus, all layers of the cartilage plate were removed. After longitudinal splitting of the shaft and removal of marrow, spongious metaphyseal bone was cuvetted out down to the level of the cortex.

Wet weights were determined immediately after dissection. Samples were dried to constant weight over P_2O_5 in an Edwards tissue drier, at a pressure of less than 0.5 mm Hg. Following determination of dry weight, cartilage was crushed to a fairly uniform powder in a small glass grinder. Bone was defatted for one hour in ethanol and for one hour in ether on a mechanical shaker, dried again to constant weight and powdered in a Wiley Mill (micro model) using a 60-mesh sieve. Decalcification was not performed.

For analytical purpose, cartilage powder was homogenized for 5 minutes in 0.5 N NaOH (1 mg cartilage per ml NaOH) in a "VirTis 45" homogenizer running at "medium" speed, the glass being immersed in an ice bath.

Water was determined as the difference between wet and dry weights.

Biochemical analyses. All samples were run in duplicate and read on a Beckman spectrophotometer. For the scaled-down procedures, the DU-model with microcuvettes and diaphragm No. 7 was employed. In each analytical procedure, sample absorption curves matched those of standards, except bone hexosamine curves, which will be described in the *Results* section. Statistical evaluation of results was done according to Student's "t"-test.

Hexosamine. Cartilage. One ml homogenate samples were hydrolyzed in 4 N HCl in sealed glass ampoules at 118°C for 2 hours. After filtration and dilution, aliquots were dried in a vacuum oven at 60°C over NaOH and $CaCl_2$. Determinations were carried out according to a modified Elson and Morgan procedure(9-12), and using galactosamine as a reference standard. Optical densities were read at 540 mμ.

* Aided by grants from Danish Rheumatism Assn. and Danish State Research Foundation.

TABLE I. Wet Weights and Water Contents of Epiphyseal Cartilage and Metaphyseal Spongiosa of Lathyritic and Control Rabbits.

		Cartilage		Bone	
		Wet wt	Water	Wet wt	Water
Group A	Exp (8)	364 ± 22*	85.5 ± .3†	323 ± 18‡	67.5 ± .9‡
	Controls (7)	279 ± 7	81.1 ± .4	346 ± 17	65.4 ± .4
Group B	Exp (8)	425 ± 26*	87.4 ± .5†	300 ± 10†	65.7 ± .4‡
	Controls (8)	313 ± 18	82.9 ± .2	511 ± 23	64.7 ± .4

Figures represent mean values ± standard error of mean.
Wet weight is given in mg. Water expressed as per cent of wet weight.
Group A: Experimental animals injected subcutaneously with aminoacetonitrile (AAN) 15 mg per 100 g body weight daily through 2 days.
Group B: Experimental animals injected with AAN as in Group A, but through 4 days.
Figures in brackets indicate No. of animals.
Experimental values differ significantly from control values at the 1% (*) or 0.1% (†) levels of probability. (‡) indicates no significant change.

Bone. Approximately 5 mg of bone powder was hydrolyzed in 2 N HCl applying the same procedure as for cartilage with the exception that volumes added to dried samples were scaled down to 50%. Spectrophotometric reading was done at 530 mμ with glucosamine as reference standard.

Hydroxyproline. One ml of cartilage homogenate or about 5 mg of bone powder were hydrolyzed in 6 N HCl in sealed glass ampoules at 138°C for 3 hours. Samples were filtered and diluted, and aliquots dried in a vacuum oven at 60°C over NaOH and $CaCl_2$. The modified(13) Neuman and Logan procedure(14) was followed, reading extinctions at 560 mμ.

Uronic acid. A modified Bollet procedure (15) was employed. Cartilage homogenates were left at 4°C for 24-48 hours before further procedure. Ten ml of the supernatant was dialyzed after precipitation of protein. Four hundred μl of 1% protamine sulfate was found necessary to precipitate all mucopolysaccharide, precipitates being eventually dissolved in 20 ml of 2 M potassium acetate buffer pH 5. Uronic acid was determined by the carbazole(16) as well as by the orcinol method(17). Carbazole sample extinctions were read at 530 mμ, orcinol sample extinctions at 670 mμ.

About 25 mg of bone powder were transferred to a centrifuge glass, suspended in 6 ml 0.5 N NaOH in a Vortex mixer and left at 4°C for 24-48 hours. Five ml samples were dialyzed after protein precipitation, adding 100 μl of 1% protamine sulfate for precipitation of mucopolysaccharide and 1.5 ml of 2 M potassium acetate buffer pH 5 for dissolving the precipitate. A scaled-down procedure(18) was employed, determining carbazole as well as orcinol values.

Results. Experimental animals of Group B presented symptoms typical of lathyrism, *viz.*, a ruffled fur, drooping of the ears and a waddling gait. In contrast, experimental animals of Group A were indiscernible from controls. In both groups, however, weight increase of experimental animals was arrested. Compared to control cartilage, lathyritic cartilage of both groups appeared swollen and edematous, having a soft and jelly-like consistency.

Wet weights and water contents are listed in Table I. In lathyrithic cartilage, both groups showed a significant increase in water content as well as in the amount of wet tissue recovered. In lathyritic metaphyses, insignificant increases in water content were noted. In Group A, the total amount of wet tissue was slightly lowered, but only in Group B was a significantly lower amount recovered from experimental animals.

Biochemical determinations on cartilage appear in Table II. The concentration of hydroxyproline in lathyritic samples increased significantly in both groups. In Group A, experimental hexosamine and uronic acid values were not significantly changed, whereas in Group B a significant decrease was seen both in hexosamine and uronic acid. While carbazole to orcinol ratios of neither group changed, a decrease in experimental mucopolysaccharide to hydroxyproline ratios was demonstrated in Groups A and B, both when

TABLE II. Hydroxyproline, Hexosamine and Uronic Acid Contents of Epiphyseal Cartilage of Lathyritic and Control Rabbits.

		Hydroxy-proline	Hexos-amine	Uronic acid		Ratios		
				Carbazole	Orcinol	Carb/ Orc	Hex/ Hypro	UA/ Hypro
Group A	Exp (8)	15.5 ± .4†	54.3 ± 1.0§	82.4 ± 2.4§	51.4 ± 1.4§	1.6 ± .02§	3.5 ± .1‡	5.3 ± .2*
	Controls (7)	13.5 ± .4	56.1 ± 2.4	80.4 ± 2.0	49.6 ± 3.6	1.7 ± .10	4.1 ± .1	6.0 ± .2
Group B	Exp (8)	18.8 ± .9†	57.5 ± 2.1*	73.2 ± 1.1‡	48.1 ± .6‡	1.5 ± .03§	3.1 ± .2‡	4.0 ± .2‡
	Controls (8)	15.1 ± .4	65.4 ± 2.6	92.4 ± 2.0	56.8 ± .8	1.6 ± .04	4.3 ± .1	6.2 ± .1

Figures represent mean values ± standard error of mean, expressed as μg per mg dried tissue.
Animals of Groups A and B identical with those of Table I.
Ratios Carb/Orc, Hex/Hypro and UA/Hypro indicate proportions carbazole to orcinol, hexosamine to hydroxyproline and carbazole to hydroxyproline values, respectively.
Experimental values differ significantly from control values at the 5% (*), 1% (†) or 0.1% (‡) levels of probability. (§) indicates no significant change.

hexosamine and carbazole values were numerators.

In metaphyseal spongiosa (Table III), any alteration of experimental values opposed the corresponding cartilage values. Experimental hydroxyproline values decreased significantly in both groups. In Group A, no significant alterations in mucopolysaccharide content were observed. In Group B, a significant elevation of experimental hexosamine and uronic acid values was demonstrated. In contrast to cartilage, bone hexosamine was found to be in excess of uronic acid, and total hexosamine was therefore divided into 2 subgroups: 1) hexosamine corresponding to uronic acid (carbazole value × 179:194) and 2) excess hexosamine. Experimental values of both subgroups were significantly elevated. Compared to the 540 mμ absorption peak of cartilage hexosamine, control as well as experimental bone hexosamine absorption maxima shifted to 530 mμ, and ascending and descending parts of absorption curves ran close to the ascending part of glucosamine and descending part of galactosamine absorption curves. Carbazole to orcinol ratios were of the same order of magnitude throughout, ratios of individual sets not differing significantly from each other. A significant increase in experimental mucopolysaccharide to hydroxyproline ratio was found only in Group B.

Discussion. In lathyritic cartilage, the finding of increased water content and decreased relative amount of dry matter confirms earlier results(7,19). It appears from Table I that the average amount of total dry matter is the same(6) in control and experimental animals of both groups, and the increase in the amount of wet tissue may thus be explained by the increased water content. An explanation of the water increase based upon osmotic forces has been published(19). Increased vascular permeability seems also a possibility, and vascular damage is likely in animals given 4 AAN-injections, as cartilage in these cases is haemorrhagic(8). Since relative water content of metaphyseal bone was not significantly altered from control to experimental animals of both groups, it follows that the amount of total dry matter was significantly lower only in lathyritic animals of Group B. This was to be expected from the gross finding of reduction in metaphyseal height in this group(8).

The increased hydroxyproline concentration of lathyritic cartilage is not in accordance with earlier observations(3,4,7), in which an unchanged concentration was found. As estimated by mucopolysaccharide to hydroxyproline ratios, concomitant alterations in mucopolysaccharide content offer no explanation of the increase in cartilage and the decrease in bone hydroxyproline concentration. Osteoblastic function may be impaired in lathyrism, as deviations from normal cytomorphology have been demonstrated in metaphyseal osteoblasts in AAN-treated dogs(20).

Earlier studies(3,5,6) have demonstrated a

TABLE III. Hydroxyproline, Hexosamine and Uronic Acid Contents of Metaphyseal Spongiosa of Lathyritic and Control Rabbits.

		Hydroxy-proline	Hexosamine			Uronic acid		Ratios		
			Total	UA-Hex	Excess	Carbazole	Orcinol	Carb/Orc	Hex/Hypro	UA/Hypro
Group A	Exp (8)	12.6 ± .21	4.75 ± .10§	1.21 ± .06§	3.55 ± .07§	1.31 ± .07§	.79 ± .04§	1.7 ± .05§	.38 ± .01§	.11 ± .01§
	Controls (7)	13.5 ± .2	4.92 ± .09	1.17 ± .09	3.75 ± .14	1.26 ± .10	.70 ± .06	1.8 ± .05	.36 ± .01	.09 ± .01
Group B	Exp (8)	11.5 ± .4*	4.97 ± .11†	1.46 ± .06†	3.51 ± .15*	1.58 ± .07*	1.00 ± .05*	1.6 ± .03§	.43 ± .01‡	.14 ± .01*
	Controls (8)	12.5 ± .2	4.11 ± .19	1.19 ± .07	2.92 ± .21	1.29 ± .07	.82 ± .04	1.6 ± .03	.33 ± .02	.11 ± .01

Figures represent mean values ± standard error of mean, expressed as μg per mg dried defatted tissue.
Animals of Groups A and B identical with those of Table I.
UA-Hex = hexosamine corresponding to uronic acid = hexosamine calculated on a 1:1 molar basis with uronic acid = carbazole value × 179:194. Ratio Carb/Orc as in Table II. Ratios Hex/Hypro and UA/Hypro indicate proportions of total hexosamine to hydroxyproline and carbazole to hydroxyproline values, respectively.
Experimental values differ significantly from control values at the 5% (*), 1% (†) or 0.1% (‡) levels of probability. (§) indicates no significant change.

decrease in the concentration and enzymatic synthesis of hexosamine in epiphyseal cartilage of lathyritic rabbits, while in lathyritic rat epiphyseal cartilage hexosamine and uronic acid were found unchanged and decreased, respectively(7). In the present experiment, hexosamine as well as uronic acid were significantly lowered in lathyritic cartilage, but not until 4 AAN-injections had been given.

Also in metaphyseal bone, 4 AAN-injections were necessary to bring about changes in mucopolysaccharide content. Presupposing excess hexosamine to be due to plasma glycoproteins, the significant increase in excess hexosamine in Group B favors the idea of vascular damage. This would lead to exudation of plasma and thus increase neutral hexosamine.

The observed fragility of lathyritic bone (8) may make it susceptible to microfractures, which in turn would induce repair processes. The acid mucopolysaccharide increase seen in healing fractures was impeded in lathyritic animals(21,22).

Judging from carbazole to orcinol ratios (23), chondroitin sulfate A and/or C should be the dominating mucopolysaccharide of both cartilage and bone, but the presence of hyaluronic acid in these tissues was previously demonstrated by electrophoresis(8). As cartilage hexosamine absorption curves corresponded closely to that of galactosamine, it is likely that the observed hexosamine decrease is related to galactosamine-containing chondroitin sulfate. The different shapes of cartilage and bone hexosamine absorption curves are suggestive of qualitative differences in the hexosamine pattern of these tissues. Whether the increase in acid mucopolysaccharide in lathyritic bone samples of Group B is related to chondroitin sulfate or to hyaluronic acid cannot be established.

Mucopolysaccharides are considered to play an active part in the initiation of the calcification process, or, controversially, to inhibit the process(24). The latter conception is, to some extent, favored by the present experiment. Histologically(8), v. Kossa-stained specimens showed calcium to concentrate pericellularly approaching the primary calcification area, and was thus found to line up at the proper anatomical site where formation

of primary spicules begin. The regularity of this pattern decreased in lathyritic specimens. Secondly, biochemical analyses show a precipitous drop in mucopolysaccharide concentration from epiphyseal cartilage to metaphyseal bone, coincident with the onset of ossification. This reflects the cation-binding capacity of acid mucopolysaccharides, and may signify a release of calcium at the cartilage-metaphysis junction and a transfer to non-mucopolysaccharide components as soon as the ossification zone is entered. In lathyrism, the decrease of acid mucopolysaccharide and the abnormal cellular pattern of epiphyseal cartilage would then cause a decreased calcium-binding capacity and a distribution of calcium unfavorable to the onset of ossification. Alterations in mucopolysaccharide composition and physical state of collagen have previously been correlated with the deranged endochondral ossification in lathyritic growth zones(25).

Summary. Biochemical analyses of growth zones of young rabbits suffering from acute lathyrism showed that an increase in water and hydroxyproline in epiphyseal cartilage and a decrease of hydroxyproline in metaphyseal spongiosa constitute the initial alterations. In the more pronounced lathyritic state, hexosamine and uronic acid decreased in cartilage and increased in bone. Acid mucopolysaccharides may play a regulating part in the process of endochondral ossification.

1. Bickley, H., J. Am. Geriat. Soc., 1964, v12, 717.
2. Tanzer, M. L., Int. Rev. Connect. Tiss. Res., 1965, v3, 91.
3. Castellani, A. A., Castellani-Bisi, C., Proc. Soc. Exp. Biol. and Med., 1958, v98, 318.
4. Follis, R. H., Tousimis, A. J., ibid., 1958, v98, 843.
5. Castellani, A. A., Castellani-Bisi, C., Frigerio, E. R., Arch. Biochem. Biophys., 1959, v80, 57.
6. Pedrini, V., Pedrini-Mille, A., Proc. Soc. Exp. Biol. and Med., 1959, v101, 358.
7. Karnovsky, M. J., Karnovsky, M. L., J. Exp. Med., 1961, v113, 381.
8. Berntsen, E., Acta Path. Microbiol. Scand., in press.
9. Elson, L. A., Morgan, W. T. J., Biochem. J., 1933, v27, 1824.
10. Boas, N. F., J. Biol. Chem., 1953, v204, 553.
11. Kirk, J. E., Dyrbye, M. O., J. Geront., 1956, v11, 273.
12. Dyrbye, M. O., ibid., 1959, v14, 32.
13. Martin, C. J., Axelrod, A. E., Proc. Soc. Exp. Biol. and Med., 1953, v83, 461.
14. Neuman, R. E., Logan, M. A., J. Biol. Chem., 1950, v184, 299.
15. Bollet, A. J., Anderson, D. V., Simpson, W. F., J. Clin. Invest., 1958, v37, 858.
16. Dische, Z., J. Biol. Chem., 1947, v167, 189.
17. Brown, A. H., Arch. Biochem., 1946, v11, 269.
18. Marckmann, A., Proc. Soc. Exp. Biol. and Med., 1965, v119, 794.
19. Borle, A. B., Karnovsky, M. J., Nichols, G., Am. J. Physiol., 1959, v197, 1224.
20. Engfeldt, B., Tegner, B., Bergquist, E., Acta Path. Microbiol. Scand., 1960, v49, 39.
21. Bolognani, L., Proc. Soc. Exp. Biol. and Med., 1961, v108, 70.
22. Pedrini-Mille, A., Pedrini, V., ibid., 1965, v119, 322.
23. Hoffmann, P., Linker, A., Meyer, K., Fed. Proc., 1958, v17, 1078.
24. Weidmann, S. M., Int. Rev. Connect. Tiss. Res., 1963, v1, 339.
25. Hartmann, F., Verh. d. Dtsch. Ges. f. Path., 1963, v47 Tag., 161.

Received July 6, 1966. P.S.E.B.M., 1966, v123.