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Acid Mucopolysaccharides in the Skin of Lathyritic Rats.* (29816)

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Ponseti and Shepard(1) suggested that the tissue mucopolysaccharides (MPS) might be responsible for the dramatic changes produced in mesenchymal tissue by lathyrogenic agents. Several reports have since appeared concerning the concentration and metabolism of MPS in experimental lathyrism. Such reports suggest a defect of the synthesis of MPS in tissues such as epiphyseal plates and bone callus(2-5). A clear cut evidence of such a defect in other tissues is lacking.

Rat skin appears to be particularly suitable for the study of the effect of lathyrogenic agents on MPS for many reasons: Its MPS content has been extensively studied(6,7) and is known to consist of chondroitinsulphate B (ChsB), hyaluronic acid (HA) and heparin (Hep); MPS turnover is active in this tissue (8,9) and gross changes have been demonstrated in the skin of lathyritic rats(10,11). The present report is concerned with the isolation and characterization of MPS from the skin of rats treated with aminoacetonitrile (AAN), known to be the most powerful lathyrogenic agent in these animals(12).

Procedure and methods. In 5 separate studies, 50 Holtzman male rats weighing 50 to 60 g were kept on a diet of ground Purina Chow containing 0.075% AAN for 3 weeks; 50 male control animals of the same size received the same amount of food, but without AAN. The food intake of the control animals was restricted so that at the end of the 3 weeks the weight of the experimental and control animals was approximately the same. Roentgenograms were obtained at the end of the experiment to verify the degree of skeletal changes typical of lathyrism. The rats were sacrificed with ether and weighed. Immediately after death, skins were removed and stored at —20°C. At the moment of use the skins were cut into strips; hair, epidermis and subcutaneous fat were removed by dissection. The skins were then minced, dehydrated in 4 changes of acetone for 24 hours and defatted in boiling n-pentane in a Soxhlet apparatus for 2 days with 5 changes of solvent.

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TABLE I. Analyses of MPS Extracted from Skins of Normal and Lathyritic Rats. Mean values of 5 extractions each.

	Uronic acid, %		Hexosamine, %	Neutral sugars	N total, %
	Carbazole	Orcinol			
Control	19.1	22.7	17.7	0	8.5
Lathyritic	17.6	23.6	19.9	0	8

The extraction of MPS was carried out essentially as described by Schiller *et al*(7) with some modifications. Digestion of the tissue was carried out with crude Papain (5 mg/g dry weight) in 0.5 M acetate buffer pH 5.5 (12 ml/g dry weight) containing 0.005 M cysteine and 0.005 M diNa EDTA, at 65°C for 24 hours. During digestion more Papain (1 mg/g dry weight) was added every 6 hours.

The mixture was then cooled and trichloroacetic acid was added slowly with shaking to a final concentration of 10%. After standing in the cold for 2 hours the precipitate was centrifuged off. The supernatant solution was dialyzed against frequently changed distilled water at 4°C for 48 hours. To avoid any loss of MPS during dialysis, of Heparin in particular, the dialysis tubes were heated for 3 days at 85°C in a dry oven, washed with 1 M NaCl and rinsed with distilled water(13).

The solution was assayed for uronic acid by the carbazole method of Dische(14) and concentrated in a rotating evaporator to approximately 1 ml/mg of MPS. The MPS were completely precipitated by adding an excess of cetylpyridinium chloride (CPC) (3 mg/mg MPS) and NaCl to a concentration of 0.04 M, allowing the mixture to incubate for one hour at 30°C.

After centrifuging the precipitate was dissolved in 3 M NaCl to dissociate the complex MPS-CPC and the MPS precipitated with 2.5 vol ethanol. After standing for 24 hours in the cold, the Na salts of the MPS were collected by centrifugation. This procedure was repeated twice.

The MPS were dried with ethanol and ether, weighed, analyzed and then fractionated with increased concentrations of ethanol according to Meyer *et al*(6). Three fractions were obtained at 25, 36 and 50% ethanol. Analyses of the fractions were carried out by the methods described elsewhere(15). Uronic acid was assayed by the carbazole method (14) and by the orcinol method of Brown (16).

The total hexosamine content of the dry skin was determined in each experiment by hydrolyzing one or more samples in sealed tubes with 4 N HCl at 100°C for 10 hours. The hydrolysates were then dried, redissolved in water and purified with Dowex 50 H according to Boas(17). The eluates from the columns were dried and redissolved in 0.3 N HCl for determination of the total aminosugar content(18). Samples hydrolyzed as described before but without purification have been used for chromatographic separation of glucosamine and galactosamine according to Gardell(18).

Results. One hundred rats were used for the experiments. Five extractions of MPS were carried out. Total hexosamine was essentially the same, 1.39 and 1.61 mg/g dry tissue in normal and lathyritic skin respectively. The glucosamine : galactosamine ratio was approximately 7:3 in both cases. Also the yield of MPS was the same in normal and lathyritic rats and was comparable with that found by others in normal rat skin(7,19) 6.6 mg/g dry tissue for the normal and 6.8 mg/g dry tissue for the lathyritic animals. The composition of the total skin MPS (Table I) did not show significant differences in experimental and control animals; even the slight decrease in the carbazole/orcinol ratio does not seem to be significant. The degree of purification of the MPS is not very high, as can be seen by the relatively low content of hexosamine and uronic acid. Probably a certain amount of protein was still bound to the polysaccharides, as demonstrated by high nitrogen values. We tried unsuccessfully to eliminate this contamination by further pepsin-trypsin digestion. The fractionation of the MPS yielded 3 fractions of almost equal consistency and again the pattern found in the treated animals could not be distinguished from that of the

TABLE II. Analyses of the MPS Fractions.

	Ethanol conc., %	Yield, %	Uronic acid, %		Carbazole/orcinol ratio	SO_4^-, %	Aminosugars by paper chromatography
			Carbazole	Orcinol			
Control	25	31.5	5.1	25.4	.2	3.6	Galactosamine
Lathyritic	25	31.3	5.5	22.9	.24	3.6	"
Control	36	37.8	36.5	25.4	1.43	7.6	Glucosamine
Lathyritic	36	37.1	36.2	22.9	1.41	5.6	"
Control	50	30.9	38.2	24	1.59	4.9	"
Lathyritic	50	31.7	35.7	26.4	1.35	4.6	"

controls. From the analysis of the fractions (Table II), it appears that fraction precipitated at 25% ethanol is likely to be ChS B, while fractions precipitated at 36 and 50% ethanol are a mixture of more than one MPS, probably HA and Hep. To calculate the amount of HA in these 2 fractions, they were subjected to digestion with testicular hyaluronidase(20) and the free acetyl-hexosamine, determined by the method of Reissig *et al*(21) was taken as an index of the content of HA. In the 36% ethanol fraction the HA content was 85.3% and 79.7% for the control and lathyritic groups respectively while in the 50% fractions HA accounted for 89.7% in the control and 99% in the lathyritic group. No difference, therefore, exists in the total HA content of skin in experimental and control animals.

Discussion. From our data it appears that the MPS content of the skin of lathyritic rats is practically indistinguishable from that of the controls. This result is in good agreement with the data of Grant *et al*(22) who did not find abnormalities in the hexosamine and uronic acid content of the skin of rats given a sweet pea diet, and with the histochemical findings of Gillespie and Burns(10).

It is of interest that in this very tissue striking abnormalities of the intramolecular cross-linking of the collagen chains have been demonstrated in lathyrism(23). Therefore, the finding of a normal pattern of MPS in the skin, suggests that these substances are not essential, at least in this tissue, for development of the typical lesions in collagen due to lathyrogenic agents.

On the other hand, differences in MPS content between control and treated animals have been found in other tissues, such as bone callus and epiphyseal plates(2-5). Since in these tissues the MPS pattern is different from that of the skin, it seems that the AAN intoxication affects the metabolism of only some of the various MPS of connective tissue.

Summary. The MPS composition of normal and lathyritic rat skins has been studied. Determination of the total hexosamine content, chromatographic separation of glucosamine and galactosamine, extraction and purification of the MPS, identification through analysis of their components did not demonstrate any difference in the MPS composition between normal and lathyritic skin.

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Effect of Parathyroid Extract on Succinic Dehydrogenase Activity in Young Rat Bone and Skin.* (29817)

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While studying the mechanism of parathormone action on bone matrix, it was observed that at very early time intervals enzymatic changes could be detected in bone. Goldhaber, using mouse calvaria in tissue culture found that bone resorption was oxygen dependent (1) and that malonate inhibited bone resorption presumably by inhibition of succinic dehydrogenase(2). He also observed large amounts of succinic dehydrogenase in osteoclasts associated with active bone resorption after addition of parathyroid extract (PTE) to culture medium(3). This increase in succinic dehydrogenase has also been reported by other investigators(4-6) employing histochemical techniques. However, Laskin and Engel(7) observed a decrease in succinic dehydrogenase in bone in weanling rabbits following injection of parathyroid extract. This effect of PTE was measured only at 28 hours after the last injection. It would appear that this difference in effect of PTE could be accounted for by the length of time after the hormone injection. The investigations of Jeffay and Bayne(8) which showed that PTE resulted in an early breakdown of bone (the radioactivity of serum calcium rose to a maximum in 6 hours after administration of PTE) and Nichols(9) who found a PTE effect in 2 hours *in vitro* led us to the following experiment which we felt might reconcile the above observations. We assayed young rat bone at 2, 4, 6, 12, 24, and 72 hours after administering 1 unit PTE/g body weight for succinic dehydrogenase activity using the modified triphenyltetrazolium chloride method of Kun and Abood(10). The effect of parathormone on the level of succinic dehydrogenase in skin was also investigated.

Methods. Experimental animals. Male rats of the Holtzman strain 35 to 60 days old, weighing between 70 and 200 g and maintained on a natural stock diet‡ were used. Parathyroid extract (Lilly) was administered intraperitoneally, 1 unit/g body weight to the experimental group while the controls were injected a solution of 0.9% saline, 0.2% phenol, 1.6% glycerol. The animals were sacrificed at 2, 4, 6, 12, 24, and 72 hours after the injection and heart blood was collected for calcium determinations(11), as a check on the potency of the parathyroid extract.

Tissue preparation. The bone was removed and carefully cleaned of connective tissue, periosteum and bone marrow. The epiphyses and cartilage caps of long bones were discarded and the metaphyseal region separated from the diaphyses. The bones of each animal were divided into metaphyses, diaphyses and calvaria, pooled, and frozen in dry ice-ether mixture. They were then crushed in a frozen stainless steel mortar and pestle, and the entire bone sample, after weighing, was quantitatively transferred to the incubation

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