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METABOLISM *IN VIVO* OF CONNECTIVE-TISSUE MUCOPOLYSACCHARIDES

I. CHONDROITIN SULFATE C AND KERATOSULFATE OF NUCLEUS PULPOSUS

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SUMMARY

The compositon of rabbit nucleus pulposus has been investigated as a function of age. The ratio of keratosulfate to chondroitin sulfate increases uniformly as a function of time. Several hormones, notably growth hormones, estrogens and androgens are able to alter the composition of mature tissues towards that represented by a younger age.

The metabolic activity of the polysaccharides has been examined and their half-lives estimated. The keratosulfate of the mature animal appears to be extremely inert following synthesis and probably has a half-life in excess of 120 days.

INTRODUCTION

Time-dependent changes in the composition of several discrete areas of connective tissue have recently been described by several investigators. One of the most striking of these is the alteration in the chondroitin sulfate C, keratosulfate and collagen content of human nucleus pulposus[1-3]. The keratosulfate and collagen components increase whereas the chondroitin sulfate C decreases with the passage of time. Rabbit nucleus pulposus exhibits time-dependent alterations similar to those found in human material and was chosen for further study.

The metabolism *in vivo* of the connective-tissue polysaccharides of skin has been studied by SCHILLER and co-workers who reported that the chondroitin sulfate B and hyaluronic acid present exhibited half lives of 5 and 10 days respectively. They also demonstrated that D-glucose served as a direct metabolic precursor of the monosaccharide moieties of both the chondroitin sulfate and the hyaluronic acid of skin[4,5]. Although comparable investigations have not been conducted with other tissues, sufficient knowledge is available regarding the pathways of hexosamine and uronic acid formation to permit the assumption that D-glucose functions as a precursor for the carbohydrate components of the chondroitin sulfate C and keratosulfate.

In view of the composition changes which occur in nucleus pulposus and the relative avascularity of this tissue, it was considered of interest to conduct a similar

investigation *in vivo* of the heteropolysaccharide metabolism of this tissue. An attempt was made to define those agents which may serve to regulate the rate of synthesis of the individual polymers concerned.

Preliminary reports have been presented[6,7].

EXPERIMENTAL

Rabbits of specified ages and weights were fed *ad libitum* on standard laboratory chow. During the course of experiments involving administration of a hormone, the body weights of the animals under study were also followed; no changes from the control animals were observed.

At specified time intervals, a set of animals was killed by a blow on the head and the nucleus pulposus removed as completely as possible from those intervertebral spaces which permitted satisfactory isolation*. The resulting gelatinous mass was extracted by stirring for 30 min with 10 ml of 0.15 M KCl, and insoluble material was removed by centrifugation. Additional hexosamine-containing material was recovered by extraction of the KCl-insoluble portion with 0.2 N KOH; this was not studied further. The KCl extract was treated with 0.05 vol. of 2 % cetyl trimethyl ammonium bromide resulting in the precipitation of a crude polysaccharide mixture containing both chondroitin sulfate C and keratosulfate. The physical composition of the nucleus pulposus is such that this simple procedure yields a relatively uncontaminated polysaccharide fraction; the insoluble component is mainly collagen. Measurements of recoverable hexosamine content indicated that more than 90 % of the total hexosamine present in the tissue was soluble in 0.15 M KCl. Analysis for methyl pentose or hexose showed less than 5 % of the hexosamine to be present as glycoprotein.

The crude precipitates were hydrolyzed with 4 N HCl at 100° for 16 h in a sealed vessel. After removal of the acid, the hydrolysates were fractionated on Dowex-50, H^+ to effect separation of the glucosamine and galactosamine components of the keratosulfate and chondroitin sulfate respectively[8]. The hexosamine fractions eluted from the column were quantitated by colorimetric analysis[9] and aliquots assayed for radioactivity. Radioactivity measurements were conducted with a Tri-Carb Liquid Scintillation spectrometer utilizing dioxane–anisole–dimethoxyethane (6:1:1) as solvent and 2,5-diphenyloxazole–1,4-bis-2(5-phenyloxazolyl)-benzene (40:1) as scintillators. The identity of the hexosamine-containing peaks was confirmed by paper chromatographic mobility on borate-treated paper in a butanol–pyridine–water (6:4:3) solvent system and by ninhydrin degradation to the corresponding pentoses which were chromatographically compared with standards[10]. The number of animals utilized for the determination of a single point are described in the pertinent figure legend.

Growth hormone which was obtained from the National Institutes of Health, assayed 1.53 units/mg; 0.5 mg/kg body weight was administered every other day. When turnover experiments were being performed the hormone was administered for 7–10 days prior to the injection of isotope. Thyroid-stimulating hormone was obtained through the courtesy of Armour and Company and assayed 1.2 units/mg. This preparation was not assayed for other pituitary trophic activity but was stated to be

* Since quantitative removal of the nucleus is not possible, results are on a relative concentration basis rather than related to tissue dry weight, nitrogen content or some other parameter.

minimally contaminated. The dose schedule for this preparation was the same as that employed for growth hormone. Estrogen-treated female animals and androgen-treated males were injected with 10 mg of hormone (Parke-Davis Co.) per kg body weight on alternate days. Cortisone was administered in doses of 25 mg/kg in early experiments and was noted to have severe systemic effects. In subsequent studies therefore, the dosage was reduced to 5 mg/kg/day without a significant change in observed isotope patterns or tissue composition. Control animals of comparable ages received mock injections and were otherwise handled in an exactly analogous fashion. No attempt was made to correlate the results of the pituitary hormone or cortisone studies with the sex of the animal.

Glucose uniformly labeled with ^{14}C was purchased from a commercial source and assayed approx. 100 $\mu C/\mu$mole. Animals that were at least 1 year of age received a single 250-μC dose of glucose in an intramuscular injection. Animals 4 weeks to 4 months of age received 75 μC of uniformly labeled glucose in a similar manner. The day of injection was designated as day "0". At varying subsequent time intervals, the animals were killed as described above.

The only heteropolysaccharides present in the nucleus pulposus are chondroitin sulfate C and keratosulfate. These contain galactosamine and glucosamine respectively and thus the metabolic activity of the individual polymers can be assessed by measurements conducted on the isolated hexosamine fractions. The turnover of the sulfate groups in this tissue will be the subject of a future report.

RESULTS

The relation between animal age and the relative composition of the nucleus pulposus polysaccharide fraction is shown in Fig. 1. These results are quite similar to those reported by HALLEN in a study on human discs[1]. The effect of various hormones on this composition is illustrated in Fig. 2. Testosterone, estrogens and growth hormone have similar net effects, *i.e.*, a marked alteration of the composition of mature tissue

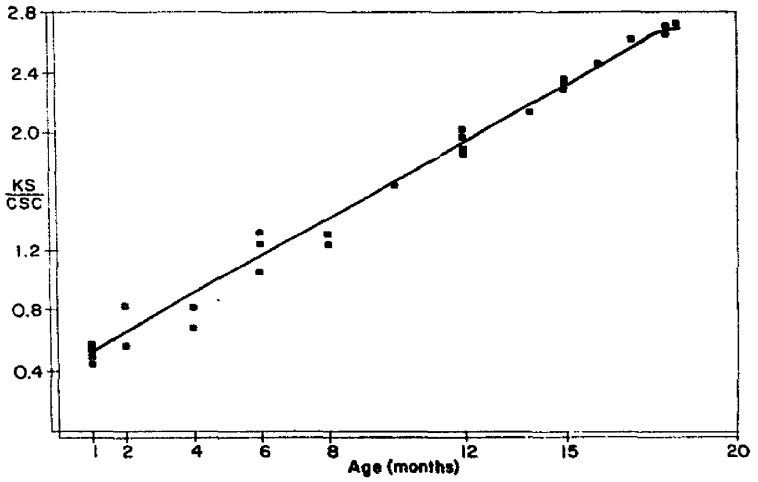

Fig. 1. Effect of age on the keratosulfate–chondroitin sulfate C ratio of rabbit nucleus pulposus. Polysaccharide content is derived from hexosamine values as described in text. Each point represents average figures for at least three pairs of animals.

toward that of an animal of much younger age; this composition-alteration effect is quite reproducible.

After the composition of the tissue had been altered by pre-treatment with hormone, the reversion of the tissue following cessation of hormone administration was evaluated. The results are illustrated in Fig. 3. The pattern appears to be very similar to that found in "normal" aging. Thus, if this alteration continues in a linear fashion, this tissue will take approx. 8 months to return to the pre-treatment polysaccharide composition.

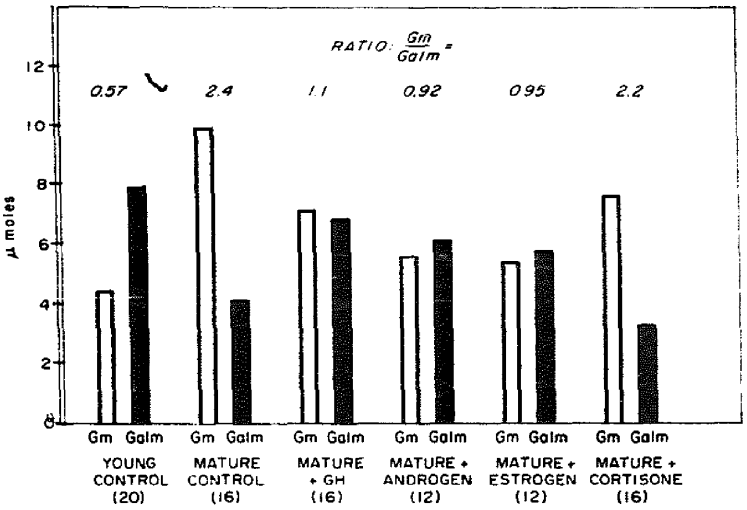

Fig. 2. Effect of hormones on the polysaccharide composition of rabbit nucleus pulposus. Mature animals were 15 months of age at the start of each experiment. Hormones were administered for 14 days prior to sacrifice. Figures in parentheses represent the number of animals used for each determination.

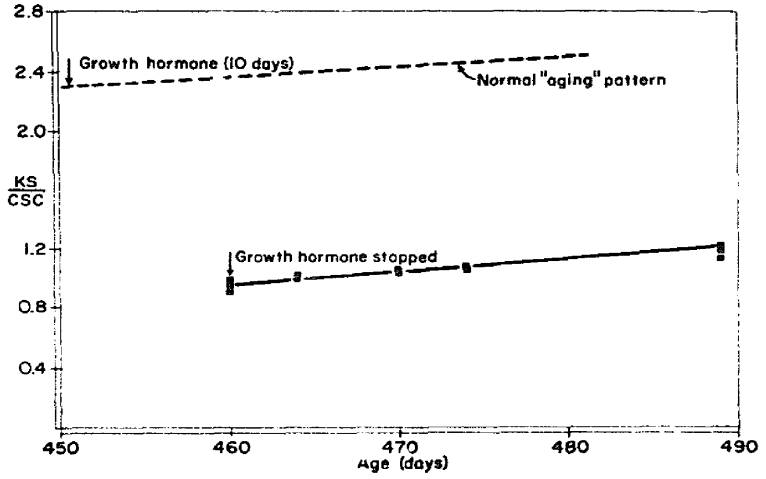

Fig. 3. Alteration in polysaccharide composition of rabbit nucleus pulposus following pretreatment with growth hormone or steroids and cessation of stimulus. Patterns are essentially the same regardless of hormone used. Each point represents average of 4 animals.

The rate of incorporation of D-glucose into the hexosamine moieties of the respective polysaccharides is illustrated in Fig. 4. There is a striking difference between young and old animals as well as an extremely slow rate of appearance of isotope in

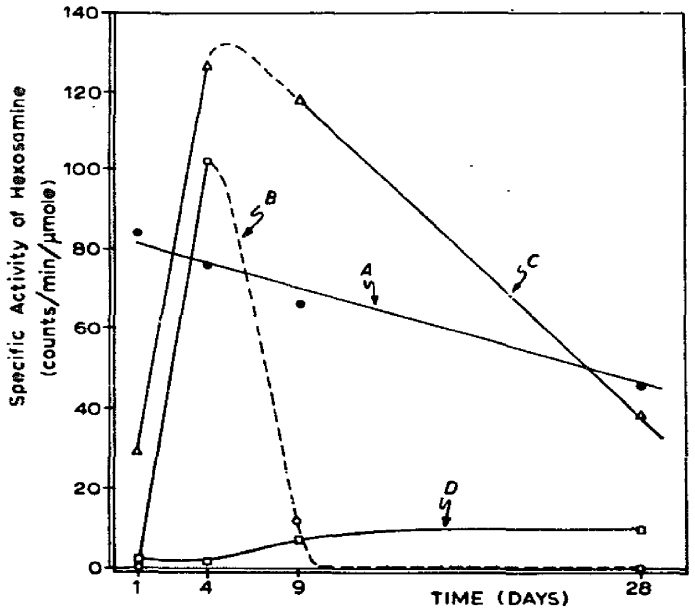

Fig. 4. Metabolic activity of nucleus-pulposus polysaccharides. Young animals were 4 weeks old at day zero: mature animals were 15 months of age. A, young-animal chondroitin sulfate C; B, mature-animal chondroitin sulfate C; C, young-animal keratosulfate; D, mature-animal keratosulfate.

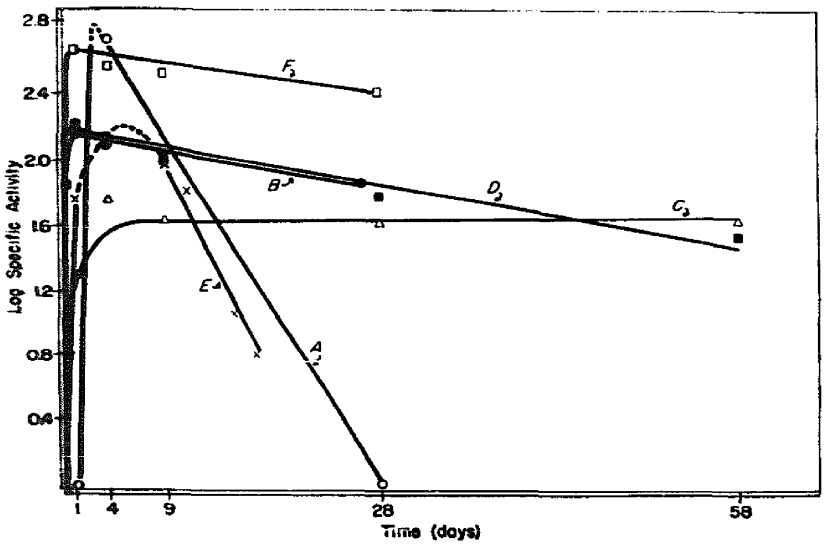

Fig. 5. Effect of hormone on metabolism of nucleus-pulposus chondroitin sulfate C. Half lives are estimated from slope of decay curve. Experimental details as described in text. Each point is average of at least 4 animals. A, ○, control—$T_{\frac{1}{2}}$ 3–5 days; B, ●, growth hormone—$T_{\frac{1}{2}}$ 28–30 days; C, △, testosterone (males)—$T_{\frac{1}{2}}$ not measurable; D, ■, estorgen (females)—$T_{\frac{1}{2}}$ 25 days; E, ×, cortisone—$T_{\frac{1}{2}}$ 3–4 days; F, □, young (2 weeks) control—$T_{\frac{1}{2}}$ 28–30 days.

keratosulfate of a fully grown animal. This latter phenomenon is consistent with the general metabolic behavior of keratosulfate which appears to increase in amount uniformly throughout life. The reason for the delay in appearance of radioactivity in the keratosulfate fraction is not understood at present. Since the radioactivity of the body glucose pool declines to essentially 0 in 36 h, the isotope appearing in keratosulfate of both the young and old animal must reflect events no longer in equilibrium with this pool.

The effects of various hormones on the metabolic activity of the polysaccharides are illustrated in Figs. 5 and 6. The similar alterations in composition brought about by estrogens and androgens appear to be the result of different mechanisms. Neither of these hormones elicited response patterns akin to that exhibited by growth hormone.

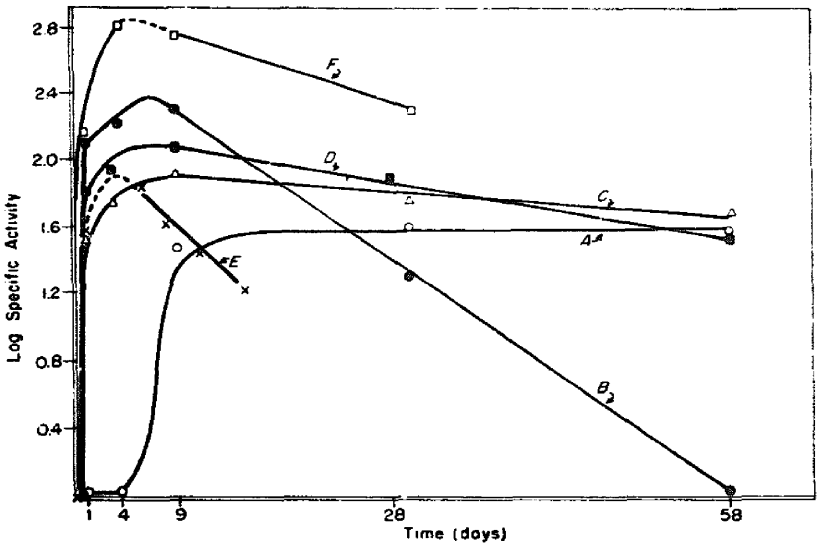

Fig. 6. Effect of hormone on metabolism of nucleus-pulposus keratosulfate. Half lives are estimated from slope of decay curve. Experimental details as described in text. Each point is average of at least 4 animals. A, ○, control—$T_{\frac{1}{2}} > 60$ days; B, ●, growth hormone—$T_{\frac{1}{2}} > 8$ days; C, △, testosterone (males)—$T_{\frac{1}{2}}$ 47 days; D, ■, estrogen (females)—$T_{\frac{1}{2}}$ 21 days; E, ×, cortisone—$T_{\frac{1}{2}}$ 5 days; F, □, young (2 weeks) control.

The pattern of metabolic activity imprinted upon the tissue by the administration of growth hormone appears to mirror very closely that found in a very young animal, except that the absolute specific activities of the various fractions are much lower, as would be expected from a larger pool. Results with TSH were not uniform but suggested a reduction in the rate of appearance of isotope in the polysaccharides.

The results of cortisone administration are consistent with previous reports that this hormone effectively stops the incorporation of ^{35}S-labeled inorganic sulfate into newly synthesized connective-tissue polysaccharides.

DISCUSSION

The gross homogeneity of the spinal-disc material suggests that the changes of specific activity that have been observed are indicative of the metabolic activity of the polysaccharides under consideration. This would not necessarily be true in tissues where a

clear distinction can be made between material that has been deposited for some period of time and that which has been newly synthesized[11,12]. Due to the relatively acellular structure of the nucleus pulposus, the term "polymer synthesis" cannot be restricted to the formation of the macromolecules examined but of necessity includes the time required for appearance at the locus studied.

The ability of growth hormone to affect both chondroitin sulfate C and keratosulfate metabolism in an essentially identical way, that is, to convert their turnover patterns to those found in younger animals suggests that this hormone influences some event not enzymically a part of polymer synthesis such as tissue permeability, cellular glucose entry or activation.

Qualitatively, estrogens and androgens produce similar alterations in polysaccharide composition. However, the mechanism of this effect appears to be different for each hormone and from that of growth hormone as well. Since the effects are not the same for both polysaccharides and in neither case do they lead to metabolic patterns which are the same as those seen in young animals, the interpretation may be made that these hormones are more directly related to polymer synthesis or degradation.

The effects of cortisone are in keeping with observations on the ability of this hormone to inhibit sulfate fixation and wound healing[13,14]. This may be a function of the inability to synthesize the sulfate acceptors, or may be due to changes in capillary flow which would alter the availability of nutrients (glucose) to the cells involved.

After withdrawal of each hormone, the tissue appears to behave as defined by its composition rather than by the chronological age of the animals. This is illustrated in the rate of reformation of keratosulfate following pretreatment with hormone and cessation of the stimulus. Since this is the only tissue wherein such composition effects have been demonstrated, it is not known whether similar alterations can be achieved in other tissues, whether these effects can be repeated several times on the same tissue, or whether the tissue ultimately becomes refractory.

The failure of the keratosulfate moiety to change significantly in specific radioactivity even after extended time periods suggests that this component in rabbit nucleus pulposus has a metabolic half life of at least 120 days. This slow turnover is in marked contrast to that previously reported for other skin polysaccharides, but may be quite general for keratosulfate. In any event, this polymer appears to resemble fibrous collagen, both in its net positive balance as a function of time and in its very low metabolic activity.

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