BBA 3952

METABOLISM *IN VIVO* OF CONNECTIVE-TISSUE MUCOPOLYSACCHARIDES

II. CHONDROITIN SULFATE B AND HYALURONIC ACID OF SKIN

E. A. DAVIDSON AND WILLIAM SMALL

Department of Biochemistry and Center for the Study of Aging, Duke University Medical Center, Durham, N. C. (U.S.A.)

(Received September 24th, 1962)

SUMMARY

The gross composition of skin mucopolysaccharides and their metabolic activity has been investigated. The chondroitin sulfate and hyaluronic acid fractions appear to exist in more than one physical state and may change their properties following synthesis *de novo*. The biological half-lives of these polysaccharides are in agreement with those previously reported but the figures are accurate for either young animals or for only a portion of the total material. Testosterone causes a marked increase in hyaluronic acid content of skin. The effect of other hormones on the composition and metabolic activity of the skin polysaccharides was reported.

INTRODUCTION

The initial studies on the metabolism *in vivo* of skin heteropolysaccharides were carried out utilizing [^{14}C]acetate[1]; subsequently both glucose and sulfate were employed as tracers to follow the behavior of the polymers[2,3]. The results indicated that this tissue was in an active metabolic state; the half-life time of the hyaluronic acid component was 3–5 days and that of the chondroitin sulfate B was 10–14 days. Preliminary investigations in our laboratory showed these figures to be accurate for growing animals, but fully grown, mature animals exhibited turnover patterns which were somewhat different[4].

The effect of increasing age on the composition of connective tissue has been the subject of considerable recent investigation. Studies carried out on skin demonstrate an alteration in the composition of the polysaccharide fraction with an increase of the chondroitin sulfate B relative to the hyaluronic acid and a decrease in water content occurring with the passage of time[5]. Comparable results have been reported by LOEWI AND MEYER[6]. Dramatic changes in composition or polysaccharide pattern are not evident for this tissue in contrast to those reported for nucleus pulposus and cartilage mucopolysaccharides[7–11]. This study describes the age-dependent incorporation of glucose into the hexosamine moieties of chondroitin sulfate B and hyaluronic acid of rabbit skin. The effects of several hormones on the relative composition and

turnover of these heteropolymers were also studied. Preliminary reports have been presented[4, 12].

MATERIALS AND METHODS

The general experimental design, the age distribution of the animals, the details of hormone treatment, analytical and counting techniques have been previously described[13]. The number of animals utilized in each study as well as the method of isolation of the components under investigation is indicated in the pertinent figure legend.

Skin polysaccharides were isolated either by extraction or digestion as described below.

Digestion procedure

Animals were shaved, sacrificed by a blow on the head and the skin removed. The skin was milled under liquid nitrogen in a Wiley Mill, acetone powdered and dried. The powdered skin was subjected to proteolytic digestion with papain (48 h at pH 6 at 55°) and then trypsin (48 h at pH 8 at 37°), and the digest deproteinized with trichloroacetic acid. The trichloroacetic acid-soluble fraction was dialyzed, neutralized and the crude polysaccharides precipitated with calcium and alcohol as described by MEYER *et al.*[14], or with cetyl trimethylammonium bromide–celite after the procedure of SCHILLER *et al.*[15]. The calcium–alcohol precipitate was harvested by centrifugation and hydrolyzed with 4 N HCl for 16 h at 100° in a sealed tube to liberate the component hexosamines. The cetyl trimethylammonium bromide celite precipitate was fractionally eluted with salt and dialyzed aliquots were hydrolyzed as above. The amounts of hexosamine recovered and the specific activity of radioactive fractions indicated no difference between these two procedures. The resulting hydrolysates were fractionated on ion-exchange resin to resolve the glucosamine and galactosamine components[16]. The amino sugars were located by a modification of the ELSON–MORGAN procedure[17], positive fractions combined and hexosamine and radioactivity content quantitated. The identity of the individual hexosamines was confirmed by ninhydrin degradation according to the procedure of STOFFYN AND JEANLOZ[18].

Salt–alkali extraction procedure

Skin was removed after sacrifice and minced as fine as possible by hand. The skin was extracted with 5 vol. of 0.15 M KCl by homogenizing for 3 min in a Waring Blendor. The resulting suspension was centrifuged at 3000 × *g* for 15 min and the extraction repeated. The combined supernatants were dialyzed to remove excess salt and worked up as described below.

The KCl-insoluble residue was extracted for 24 h with 2 % KOH at 4° and the insoluble fraction remaining was reextracted for an additional 24 h under the same conditions. The combined extracts were neutralized and a crude polysaccharide fraction precipitated with calcium and alcohol at pH 5. Subsequent deproteinization of the salt or alkali extract and isolation of hexosamine markers was essentially identical to the procedure described above for the proteolytic digestion. Since the differential extraction procedure did not give identical results for the KCl or KOH fractions, nor were the results the same as those obtained by proteolytic digestion, both methods were regularly employed.

The identity of individual polysaccharide fractions was confirmed by analytical methods for uronic acid, hexosamine and sulfate as well as by susceptibility to the action of testicular hyaluronidase when assayed by a turbidity-reduction procedure[14].

RESULTS

The composition of the material solubilized by KCl extraction is presented in Table I. This fraction represents essentially hyaluronic acid since there is less than 5% galactosamine, the polymer is completely susceptible to the action of the testicular hyaluronidase and contains no sulfate*.

TABLE I

ANALYSES OF MATERIAL EXTRACTABLE BY KCl

Specific-activity measurements suggest that the hyaluronate first appears in this fraction but accurate figures were not obtained due in part to the rapidity of incorporation. Methods as summarized by MEYER *et al.*[14]. Quantitation shows this fraction to contain 17–25% of the total hyaluronate. This may decrease with increasing age but was not studied.

Analysis	*Result*
Hexosamine	32.6%
Uronic acid (carbazole)	30.7%
Nitrogen	3.20%
Sulfate	less than 1.0%
Hyaluronidase	better than 90% digested
$[\alpha]_D$	-62° (0.4, H_2O)

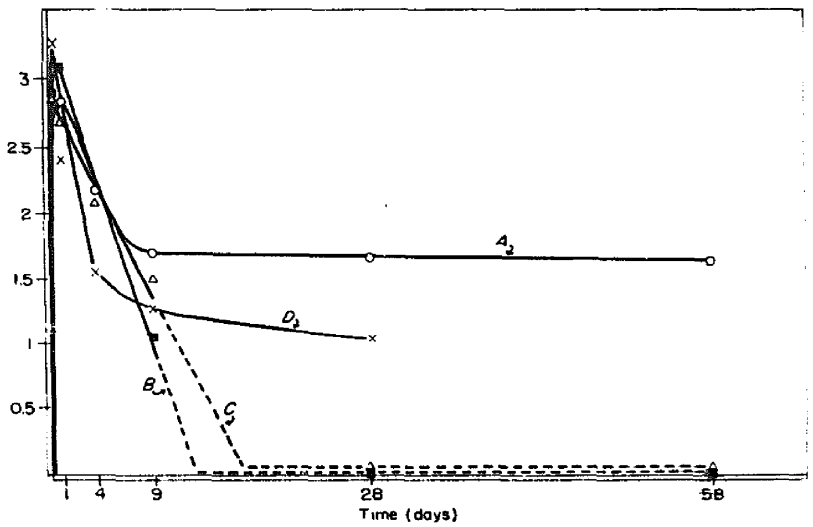

Fig. 1. Effect of hormone on metabolism of skin hyaluronic acid. Experimental details as described in text for proteolytic-digestion procedures except for thyroid- and growth hormone-treated animals, where the salt–alkali procedure was employed. Each point is average of at least three animals. Animals were 12–16 months of age. A, O, control; B, ■, estrogen (females) — $T_{\frac{1}{2}}$ 2 days; C, △, testosterone (males) — $T_{\frac{1}{2}}$ 2–3 days; D, ×, cortisone.

* This property of a portion of the hyaluronic acid of skin without any significant solubilization of the chondroitin sulfate B has also been observed by Dr. R. H. PEARCE (personal communication).

The time-dependent specific-activity changes of the hexosamine moieties of the skin polysaccharides are illustrated in Figs. 1 and 2 respectively. The results obtained for young animals confirmed those previously reported by DORFMAN and coworkers but the difference in the older animals is evident. The hyaluronic acid and chondroitin sulfate B fractions both appear to contain a relatively inert component not significantly catabolized even after extended time periods.

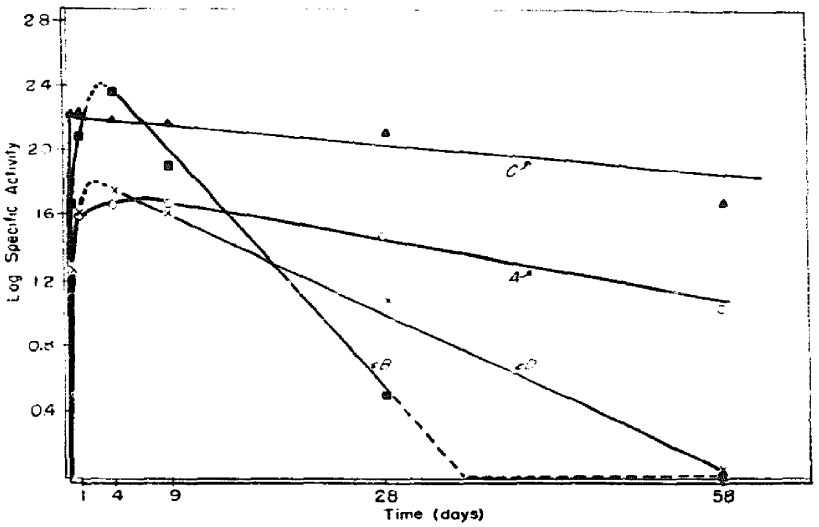

Fig. 2. Effect of hormone on metabolism of chondroitin sulfate B. Experimental details as described in text for proteolytic-digestion procedures except for thyroid- and growth hormone-treated animals, where the salt–alkali procedure was employed. Each point is average of at least three animals. A, O, control—$T_{\frac{1}{2}}$ 25 days; B, ■, estrogen (females)—$T_{\frac{1}{2}}$ 6 days; C, ▲, testosterone (males)—$T_{\frac{1}{2}}$ 48 days; D, ×, cortisone—$T_{\frac{1}{2}}$ 10 days.

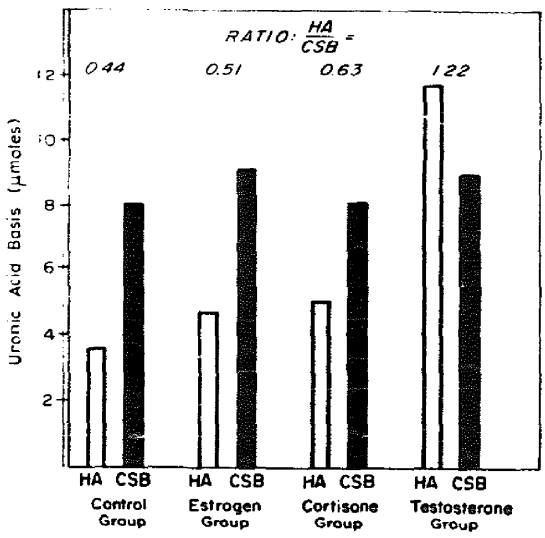

Fig. 3. Effect of hormones on relative mucopolysaccharide content of skin. Proteolytic-digestion procedure employed except that hyaluronate expressed as hyaluronidase-sensitive glucosamine-containing fraction.

The effect of various hormones on the composition of the skin polysaccharides is illustrated in Fig. 3. The hyaluronic acid component undergoes a striking increase following testosterone treatment. Although the chondroitin sulfate B also is increased, the magnitude of the change is not nearly as great.

Cortisone appears to be inhibitory for polymer chain synthesis possibly by regulation of the mode of glucose utilization.

DISCUSSION

The non-linearity of the semi-logarithmic plot of specific activity of the isolated hexosamine component *versus* time strongly suggests the existence of more than one pool of the same chemical entity. It is possible that there exists a continuous family of both condroitin sulfate B and hyaluronic acid molecules, which, following their *de novo* synthesis from monosaccharide precursors, become less readily extractable or subject to the action of degradative enzymes. This may be analogous to the reduced amount of "soluble collagen" that is present in skin with increasing age[19]. The KCl-soluble fraction may contain those molecules most recently synthesized whereas the proteolytic-digestion procedure misses all molecules of the same chemical structure. From these data, it would appear that the half-life concept may not be rigorously applied to these molecules.

The action of skin extracts in promoting the degradation of chondroitin sulfate B has been reported but the mechanism of such breakdown is unknown[20]. Reports have also appeared indicating the existence of hyaluronidase-like activities from mammalian sources other than testes[21]. These are apparently non-enzymic and other mechanisms for the breakdown and removal from tissue locus of these polymers should be considered. These include degradation of a protein complex, cleavage of the polysaccharide from the non-reducing end and simple diffusion. Skin extracts do not contain mucopolysaccharide–protein complexes with properties similar to those demonstrable in cartilage[22] but rather the presence of other sugar components including galactose, rhamnose and mannose in structures of unknown complexity[24,25]. These complexes may represent intermediate stages in the formation of fibrous elements but their relationship, if any, to the synthesis of hyaluronic acid and chondroitin sulfate B is unknown.

The effect of testosterone on the hyaluronic acid content of male skin is noteworthy. A similar effect may be demonstrable with female animals but was not studied. This increase in hyaluronate content is qualitatively similar to that observed on rooster comb[23] and represents a stimulation of the synthesis of this polymer since the chondroitin sulfate B fraction does not show a corresponding increase. The hyaluronic acid content of other tissues such as heart valves was not examined and it is not known whether the observed effect is limited to skin. There was also an increase of polysaccharide content in skin after estrogen administrations; however, both polysaccharides were approximately equally affected and the net increase was not striking.

Growth hormone resulted in a generally increased synthetic activity; the decay curve indicated replacement by newly synthesized material.

The early portion of the specific activity–time curve (the first 10 days) yields a half life in agreement with that previously reported by DORFMAN *et al.*[2,8] but extension

of the experimental time period to 58 days indicated a component present in this fraction which did not rapidly turn over.

The composition of human skin is altered in several pathological states including those which yield a decrease in insoluble collagen and an increased content of polysaccharide[26]. The identity of these polysaccharide components has not been established and thus no correlation can be made between these states and normal aging changes in the same sense that such a relationship may be extended between nucleus pulposus and pathological herniated disc material[9]. The small changes that occur in skin composition as a function of age appear to be overshadowed by the ability of various hormones to alter the normal pattern. This is in contrast to that observed in nucleus pulposus where hormone administration results in the apparent reversion of the tissue to an earlier chronological age[13]. The only comparable effect would be the relatively large increase in hyaluronate content following testosterone administration.

ACKNOWLEDGEMENTS

This work was supported by grants A 2903 (C-2), A 4315 (C-1) and H-3582 and M-2109 from the National Institutes of Health, Bethesda, Md. (U.S.A.).

REFERENCES

1 S. SCHILLER, M. B. MATHEWS, L. GOLDFABER, J. LUDOWIEG AND A. DORMAN, *J. Biol. Chem.*, 212 (1955) 531.
2 S. SCHILLER, M. B. MATHEWS, H. JEFFERSON, J. LUDOWIEG AND A. DORFMAN, *J. Biol. Chem.*, 211 (1954) 717.
3 S. SCHILLER, M. B. MATHEWS, A. CIFONELLI AND A. DORFMAN, *J. Biol. Chem.*, 218 (1956) 139.
4 E. A. DAVIDSON, W. SMALL, P. PERCHEMLIDES AND W. BAXLEY, *Biochim. Biophys. Acta*, 46 (1961) 189.
5 K. MEYER in *Polysaccharides in Biology*, Vol. 4, Josiah Macy Foundation, New York, 1958.
6 G. LOEWI AND K. MEYER, *Biochim. Biophys. Acta*, 27 (1958) 453.
7 A. HALLEN, *Acta Chem. Scand.*, 12 (1958) 1869.
8 A. HALLEN, *Acta Chem. Scand.*, 16 (1962) 705.
9 E. A. DAVIDSON AND B. WOODHALL, *J. Biol. Chem.*, 234 (1959) 2951.
10 D. KAPLAN AND K. MEYER, *Nature*, 183 (1959) 1267.
11 R. KUHN AND H. J. LEPPELMAN, *Ann. Chem.*, 611 (1958) 254.
12 E. A. DAVIDSON, W. SMALL AND P. PERCHEMLIDES, *Federation Proc.*, 20 (1961) 162.
13 E. A. DAVIDSON AND W. SMALL, *Biochim. Biophys. Acta*, 69 (1963) 445.
14 K. MEYER, E. A. DAVIDSON, A. LINKER AND P. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506.
15 S. SCHILLER, G. A. GLOVER AND A. DORFMAN, *J. Biol. Chem.*, 236 (1961) 983.
16 S. GARDELL, *Acta Chem. Scand.*, 7 (1953) 207.
17 C. J. M. RONDLE AND W. T. J. MORGAN, *Biochem. J.*, 61 (1955) 586.
18 P. J. STOFFYN AND R. W. JEANLOZ, *Arch. Biochem. Biophys.*, 52 (1954) 373.
19 W. G. BANFIELD, *Anat. Record.*, 114 (1952) 157.
20 E. A. DAVIDSON AND J. G. RILEY, *Biochim. Biophys. Acta*, 42 (1960) 566.
21 H. GIBIAN, *Z. Physiol. Chem.*, 291 (1952) 6.
22 S. M. PARTRIDGE, H. F. DAVIS AND G. S. ADAIR, *Biochem. J.*, 79 (1961) 15.
23 J. A. SZIRMAI, *Proc. Soc. Exptl. Biol. Med.*, 93 (1956) 92.
24 G. E. W. WOLSTENHOLME AND M. O'CONNOR, *Chemistry and Biology of Mucopolysaccharides*, Little Brown Co., Boston, 1958.
25 I. MALAWISTA AND E. A. DAVIDSON, *Nature*, 192 (1961) 571.
26 J. G. SMITH, E. A. DAVIDSON, J. P. TINDALL AND W. M. SAMS, *Proc. Soc. Exptl. Biol. Med.* 108 (1961) 533.