Carbohydrate Research, 159 (1987) 127–136
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

EVIDENCE FOR STRUCTURAL CHANGES IN DERMATAN SULFATE AND HYALURONIC ACID WITH AGING*

MARIA O. LONGAS†,
Department of Dermatology, The Mount Sinai School of Medicine, New York, New York 10029 (U.S.A.)

CHARLOTTE S. RUSSELL. AND XUE-YING HE
Department of Chemistry, The City University of New York, New York, 10036 (U.S.A.)

(Received September 9th, 1985; accepted for publication in revised form, June 11th, 1986)

ABSTRACT

Three dermatan sulfates (DS_{18}, DS_{28}, and DS_{35}) were isolated from women's skin of ages 19 ±2.5, 35 ±3.5, 47 ±1.7, 60 ±0.8, and 75 ±5 years. They sequentially precipitated with 18, 28, and 35% ethanol. Their sulfate content was: 23.5, 25.3, and 29.0% (w/w) for DS_{18} at ages 19–35, 47, and 60 years, respectively; 29.0, 24.0, and 18.8% for DS_{28}; and 18.0, 20.0, and 20.6% for DS_{35} at ages 19–47, 60, and 75 years, respectively. Both DS_{18} and DS_{28} decreased, respectively, from 0.030% (of wet-skin weight) to traces at age 75, and from 0.020 to 0.010% at 60 years. At age 75, DS_{28} apparently increased by 30%. The DS_{35} values (traces–0.006%) had no age-related trend. Hyaluronic acid (HA) precipitated with 45% ethanol, was 0.030% of skin-weight at ages 19–47, and decreased to 0.015 and 0.007% at 60 and 75 years, respectively. Its electrophoretic mobility was slower at age 47. In the oldest group, i.r. spectra of HA and DS_{35} displayed no bands at 1650–1600, 1380, and 1320 cm^{-1}, and a new band at 1560 cm^{-1}. Moreover, ninhydrin-positive material of HA and DS_{35} increased by 75 and 95%, respectively, and the reducing GlcNAc content of HA decreased. These data showed three chemically different dermatan sulfates (two of which were preponderant) and *N*-deacetylation of HA and DS_{35} of the oldest group. After age 47, total DS and HA considerably decreased, DS_{18} and DS_{35} were oversulfated, and DS_{28} became undersulfated with aging.

INTRODUCTION

Aging is a complex and natural process influenced by the genetic make-up of the individual and the environment. Its mechanism is presently an enigma and is difficult to discern because of the variety of factors involved[1].

*This work was supported by Grant AG-03877 from the National Institutes of Health.
†To whom correspondence should be addressed.

0008-6215/87/$ 03.50

Studies on the molecular aging parameters of human skin have concentrated on collagen[2]. Though the glycosaminoglycans (GAG)* are known to intermingle with this protein *in vivo* and have been suggested as regulators of its growth[3], their role on aging has been scarcely investigated. Existing data on the effect of senescence on human skin GAG are conflicting. Some workers found insignificant changes[4], whereas others[5–7] reported small decreases in both HA and DS.

Hyaluronic acid and DS, the major skin GAG, are extracellular constituents[8] of varying molecular weights which have high affinity for water[9]. Dermatan sulfate occurs covalently bound to protein in what has been termed proteoglycan[9]. Hyaluronic acid is a highly viscous, straight-chain GAG. *In vitro* studies have revealed that HA has a variety of regulatory functions, in addition to being a mechanical transducer[10]. Though it has been reported[11] that the major DS proteoglycans of porcine skin do not form aggregates with HA *in vitro*, in intact human skin, at least some DS proteoglycans and HA apparently form aggregates similar to those identified in cartilage[12]. This is important because human skin-proteoglycan aggregates may have specialized functions analogous to those suggested for the cartilage proteoglycans[3,9].

Proteoglycans and HA seemingly regulate the biological functions of the extracellular matrix and influence those of the cells[3]. Their postulated roles include space-filling and the control of molecular diffusion[3], in addition to the functions generated from their association with major intercellular components, such as collagen[3], fibronectin[13], and elastin[14], most of which are presently unknown. For example, by direct interaction with collagen, DS proteoglycans seem to control the orientation and growth of collagen fibers in various tissues including the skin[3,15].

This study was initiated to determine age-mediated concentration changes of DS and HA, and to investigate both chemically and by infrared (i.r.) spectroscopy[16] the effect of senescence on the fine structure of these GAG.

EXPERIMENTAL

Materials. — Normal skin, pooled from mammoplasties on four women aged 17–23 years, was collected between 1 and 2 h after surgery. The subcutaneous fat was surgically removed, and skin was stored at −70° until used. Skin was also obtained from mastectomies on subjects aged 33–80 years and treated as indicated. Standard cartilage chondroitin 4- and 6-sulfate, porcine skin DS, bovine lung heparan sulfate and heparin, and human umbilical cord HA were a gift from Dr. Karl Meyer, Columbia University College of Physicians and Surgeons (New York, NY, U.S.A.). Hyaluronate lyase from *Streptomyces hyalurolyticus* (EC 4.2.2.1), chondroitinase ABC lyase (EC 4.2.2.2.2), and chondroitinase AC lyase (EC 4.2.2.5) were purchased from Miles Scientific/Division of Miles Laboratories, Inc.

*Abbreviations used: GAG, glycosaminoglycan(s); C-6-S, chondroitin 6-sulfate; HA, hyaluronic acid; DS, dermatan sulfate; DS_{18}, dermatan sulfate precipitated with 18% ethanol; DS_{28}, dermatan sulfate precipitated with 28% ethanol; DS_{35}, dermatan sulfate precipitated with 35% ethanol.

(Naperville, IL, U.S.A.). Cellulose acetate sheets (11 × 11 cm) were a product of Schleicher & Schuell, Inc. (Keene, NH, U.S.A.). L-Iduronic acid was isolated from rabbit skin, following the procedure previously described[17], and quantitatively determined by the orcinol method with L-IdoA as the standard[18]. Carbazole was used to estimate D-GlcA with D-GlcA as the standard[19].

Purification of GAG. — Skin was chemically delipidated at 4° overnight by use of 95% (v/v) ethanol (20 mL per g of tissue). The GAG were isolated mainly by sequential precipitation with 18, 28, 35, and 45% (v/v) ethanol from the skin homogenate obtained after exhaustive digestion with papain[20]. Further purification was accomplished with cetylpyridinium chloride as indicated earlier[21].

Electrophoresis on cellulose acetate. — Two-dimensional electrophoresis was carried out as described elsewhere[22]. This procedure was also employed with 0.47M formic acid–0.1M pyridine (pH 3.0) at 0.75 mA/cm for 90 min, the conditions of the first dimension only. Staining was done with 0.2% (w/v) Alcian blue in aqueous 5% (v/v) acetic acid. Both methods detected polysaccharides of molecular weight >7200 and were sensitive to 0.1 μg of GAG.

Degradation of GAG by chondroitinases ABC and AC. — Reaction mixture (55 μL) contained 0.1M sodium acetate, 0.15M Tris·HCl (pH 7.3), enzyme (0.025 unit), and GAG (50 μg). After 12 h at 37° (under toluene), the reaction was terminated by boiling for 2 min, followed by centrifugation at 1500*g* for 5 min. The supernatant was evaporated under reduced pressure, and the residue redissolved in water (50 μL) and used for electrophoresis. Control contained all reagents except the enzyme.

Digestion of GAG by Streptomyces *hyaluronidase.* — The reaction mixture (51 μL) contained 0.02M sodium acetate, 0.15M NaCl (pH 6.0), enzyme (1 unit), and GAG (50 μg). Incubation was at 60° for 2 h. Subsequent steps were as indicated above.

Sulfate determination. — Hydrolysis of DS (200 μg) was conducted in 24% (v/v) aqueous formic acid (1.0 mL) in sealed tubes at 100° for 24 h. Acid was removed under reduced pressure. The dry sample was dissolved in water (0.3 mL) and analyzed for sulfate in triplicate[23].

I.r. analysis. — Pellets were prepared with dry sodium GAG (0.5–1.9 mg) precipitated at pH 5.0 and KBr (150–200 mg). Spectra were recorded with a spectrophotometer Perkin–Elmer Model 247.

RESULTS AND DISCUSSION

Human skin contains three dermatan sulfates (DS_{18}, DS_{28}, and DS_{35}). They sequentially precipitated from the GAG pools isolated from women's skin of varying ages (19 ±2.5, 35 ±3.5, 47 ±1.7, 60 ±0.8, and 75 ±5 years) with 18, 28, and 35% (v/v) ethanol[20]. Their identities were ascertained by two-dimensional electrophoresis on cellulose acetate, where they migrated with standard porcine skin DS and displayed no other GAG (Fig. not shown). Their electrophoretic mobility was

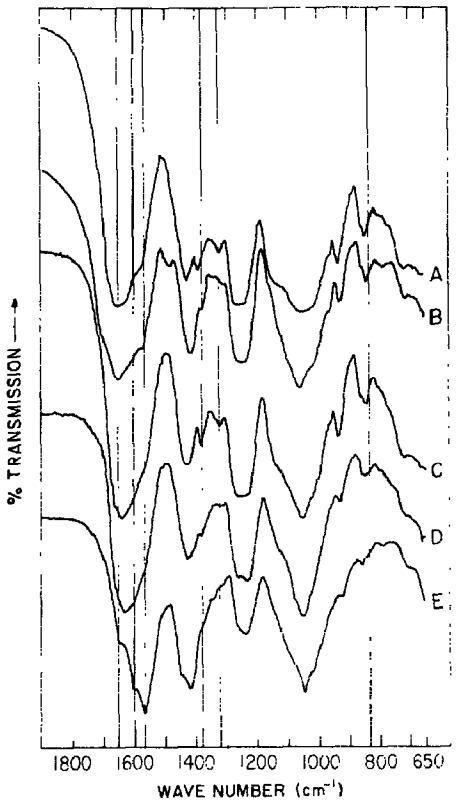

Fig. 1. Infrared spectra of standard and human skin dermatan sulfates: (A) Standard porcine skin DS. (B, C, and D) DS_{18}, DS_{28}, and DS_{35} of the 19 ±2.5-years old sets, respectively. The same profiles were obtained with dermatan sulfates isolated from the 35 ±3.5, 47 ±1.7, and 60 ±0.8-years old groups. (E) DS_{35} of the 75 ±5-year olds. Three identical spectra were obtained from different samples of every age. See the text for experimental conditions.

apparently unaffected by aging. Dermatan sulfates were degraded by chondroitinase ABC but seemingly undigested by chondroitinase AC and *Streptomyces* hyaluronidase. The identity of these GAG as DS was also established from their i.r. spectra (Fig. 1B–D).

The three dermatan sulfates reacted positively with carbazole and orcinol. Nevertheless, their yields were determined by weight (Table I). Carbazole and orcinol are reagents used to determine quantitatively GlcA and IdoA, the known DS uronic acids. Unfortunately, carbazole[19] gives more color for GlcA, and orcinol[18] for IdoA; therefore, DS yields estimated by these techniques give misleading results when the exact contents of IdoA and GlcA are unknown. The IdoA and GlcA compositions of human skin dermatan sulfates are presently under determination.

The dermatan sulfates reported herein were chemically distinct, as evidenced by the sulfate content, which was differently affected by aging. Thus, the sulfate

TABLE I

EFFECT OF AGE ON THE CONCENTRATION OF SKIN DERMATAN SULFATE

Age group (years)	*DS_{18} (%)*	*S.D.*[a]	*DS_{28} (%)*	*S.D.*[a]	*Total DS*[b] *(%)*	*Decrease (%)*
19 ±2.5	0.030	0.009	0.020	0.005	0.050	
35 ±3.5	0.029	0.004	0.018	0.006	0.047	6
47 ±1.7	0.026	0.005	0.012	0.002	0.038	24
60 ±0.8	0.020	0.001	0.010	0.001	0.030	40
75 ±5.0	Trace		0.026[c]	0.008	0.026	48

[a]S.D., standard deviation of the mean. [b]Mean of the content (in %) of whole, surgically-defatted, wet skin from four people of every age. [c]Contains DS_{18} modified by aging to a DS_{28}-like GAG.

content of DS_{18} and DS_{35} increased from 23.5 and 18.0% (w/w) to 29.0 and 20.6%, respectively, whereas that of DS_{28} diminished from 29.0 to 18.8% (Table II). Two dermatan sulfates were identified in human[5] and rabbit[24] skins, in partial agreement with the present data.

The major human skin dermatan sulfates, DS_{18} and DS_{28}, represented 0.030 and 0.020% of the weight of whole, surgically-defatted, wet skin at age 19 years (Table I). Though DS_{18} was not significantly diminished between 19 and 60 years, it dropped to traces at age 75. The steady decrease of DS_{28} with senescence reduced its concentration to 0.010% at age 60. At age 75 year, DS_{28} apparently increased by 30%; the increment might originate from DS_{18} modified by senescence to a DS_{28}-like GAG. This assumption was supported by the yields of DS_{18} from the oldest group, which were only traces. The age-related DS decrease agree with previous

TABLE II

SULFATE CONTENT OF DERMATAN SULFATE FROM VARIOUS AGES[a]

Dermatan sulfate	*Age (years)*	*Sulfate*[b] *(%)*	*S.D.*[c]	*Increase (%)*	*Decrease (%)*
DS_{18}	19 ±2.5	23.5[d]	0.48		
	47 ±1.7	25.3	0.32	8	
	60 ±0.8	29.0	1.01	23	
DS_{28}	19 ±2.5	29.0[d]	0.16		
	47 ±1.7	29.0	1.40	0	
	60 ±0.8	24.0	0.50		17
	75 ±5.0	18.8	0.02		35
DS_{35}	19 ±2.5	18.0[d]	0.31		
	47 ±1.7	18.0	0.20	0	
	60 ±0.8	20.0	0.10	11	
	75 ±5.0	20.6	0.03	14	

[a]Experimental conditions are described in the test. [b]Mean of three separate determinations (done in duplicate) from every age. Expressed as a percentage of DS. [c]S.D., standard deviation of the mean. [d]The same results were obtained from the 35-years old group.

TABLE III

DERMATAN SULFATE$_{35}$ AND HYALURONIC ACID HEXOSAMINES AS A FUNCTION OF AGE[a]

Glycosaminoglycan	*Age group (years)*	*GlcN*	*Increase (%)*	*GalN*	*Increase (%)*
DS_{35}	19 ±2.5–60 ±0.8			5.4	
	75 ±5.0			103.0	95
HA	19 ±2.5–60 ±0.8	8.7			
	75 ±5.0	35.0	75		

[a]Data are expressed as the average of μg GalN/mg DS_{35} and μg GlcN/mg HA determined by the ninhydrin reaction in 0.1M pyridine[26]. Experiments, carried out in duplicate and performed in two different occasions, used specimens from three different people of every age.

reports[5,6], though the absolute values of the present data are much higher. The minor skin DS (DS_{35}) ranged from traces to 0.006% and displayed no age-mediated trend.

The i.r. spectra of DS_{35} isolated from the oldest group showed no band in the 1650–1600-cm^{-1} region and at 1380 and 1320 cm^{-1}, and a new band at 1560 cm^{-1} (Fig. 1E). The band(s) in the 1650–1600 cm^{-1} region was characteristic of C=O stretching with minor contributions from C–N–H in-plane bending of acetamido groups[16] (1644 cm^{-1}). It also arose from antisymmetric stretching modes of carboxyl groups in GAG sodium salts[16] (1620 cm^{-1}). The band at 1560 cm^{-1} originated from C–N stretching coupled to C–N–H in-plane bending of acetamido residues in monosaccharides and GAG. It was weak or shielded in the spectra of GAG sodium salts[16], but could also arise from primary and secondary amines[25]. The bands at 1380 and 1320 cm^{-1} stemmed, respectively, from C–H stretching and C–C–H vibrational modes of methyl groups in monosubstituted amides including those of GAG[16,25]. Therefore, loss of i.r. bands at the latter two wavenumbers together with the band(s) in the 1650–1600 cm^{-1} region indicated *N*-deacetylation. The appearance of the band at 1560 cm^{-1} suggested replacement of acetyl groups with hydrogen atoms in DS_{35} of the oldest group (Fig. 1E).

The presence of free amines in DS_{35} of the 75-year old sets was confirmed by the results of the ninhydrin reaction which showed a 95% increase in ninhydrin-positive material (determined as 2-amino-2-deoxygalactose) between the ages of 60 and 75 years (Table III). It is worth noting that no *N*-deacetylation was detectable in DS_{18} and DS_{28} of the ages studied.

Hyaluronic acid was isolated by precipitation with 45% (v/v) ethanol from the GAG pools depleted of DS. Its identity was defined by two-dimensional electrophoresis on cellulose acetate, where it migrated with standard umbilical cord HA and displayed no other GAG (Fig. not shown). This GAG was degraded by chondroitinases ABC and AC, and *Streptomyces* hyaluronidase. Its electrophoretic mobility dropped from 1.0 to 0.9 after 47 years. Its i.r. spectra indicated that it was highly pure (Fig. 2).

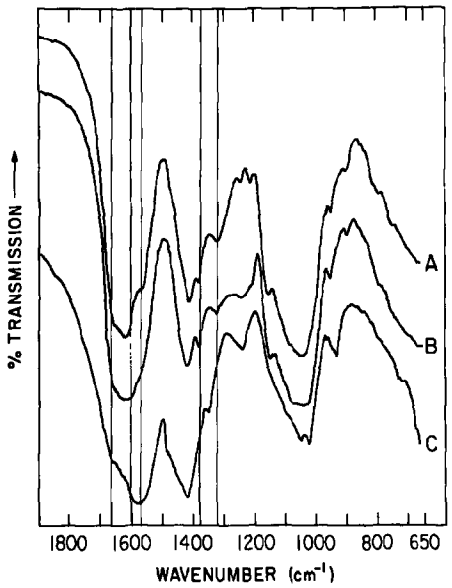

Fig. 2. Infrared spectra of standard and human skin hyaluronic acids: (A) Standard HA of human umbilical cord. (B) HA of the 19 ±2.5 years old group. The same profiles were obtained with HA isolated from 35 ±3.5, 47 ±1.7, and 60 ±0.8 years old sets. (C) HA of the 75 ±5-year olds. Three identical spectra were obtained from different samples of every age, as described in the text.

Hyaluronic acid represented 0.030% of the weight of whole, surgically-defatted, wet skin from 19 to 47 years and dropped to 0.015 and 0.007% at ages 60 and 75 years, respectively (Table IV). The age-mediated HA decrease agreed with previous reports[5,7], though the absolute values of the present data were much higher.

The i.r. spectra of HA isolated from the 75-year olds displayed loss of bands in the 1650–1600 cm^{-1} region and at 1380 and 1320 cm^{-1} with concomitant appearance of the band at 1560 cm^{-1}, which suggested *N*-deacetylation (Fig. 2C). The loss

TABLE IV

EFFECT OF AGE ON THE CONCENTRATION OF SKIN HYALURONIC ACID

Age group (years)	*Hyaluronic acid*[a] *(%)*	*S.D.*[b]	*Decrease (%)*
19 ±2.5	0.030	0.005	
35 ±3.5	0.030	0.005	
47 ±1.7	0.030	0.006	
60 ±0.8	0.015	0.003	50
75 ±5.0	0.007	0.001	77

[a]Mean of the content (in %) of whole, surgically-defatted, wet skin from four people of every age.
[b]S.D., standard deviation of the mean.

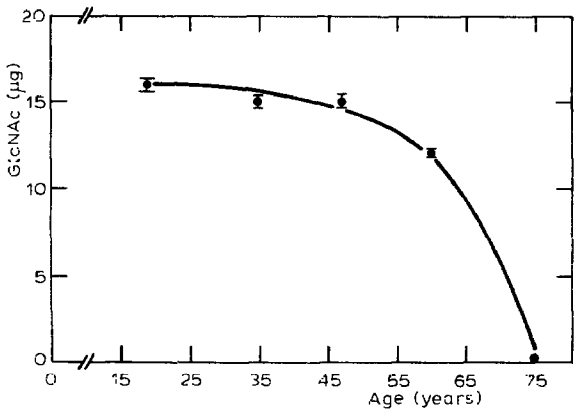

Fig. 3. Effect of age on the concentration of reducing 2-acetamido-2-deoxyglucose generated upon digestion of hyaluronic acid by *Streptomyces* hyaluronidase. HA (100 μg) was degraded by 2 urbidity units of enzyme under the conditions described in the text. Each point represents the mean ±s.d. of triplicate results from two separate experiments, which used HA from three different subjects of every age. The assay was sensitive[27] to 0.1 μg of standard D-GlcNAc/0.875 mL.

of acetyl groups was confirmed by the yields of reducing GlcNAc[27], obtained after HA digestion by *Streptomyces* hyaluronidase, which diminished significantly at 60 years and became undetectable by age 75 (Fig. 3). There was also a 75% increase in ninhydrin-positive material (quantified as GlcN) between 60 and 75 years (Table III). These results, together with those shown in Fig. 2C and the yield of reducing GlcNAc (Fig. 3), indicated *N*-deacetylation and replacement of acetyl groups by hydrogen atoms in HA.

Total DS/HA ratios (Table V) were in accord with the data of Meyer *et al.*[20] who identified DS > HA in adult porcine skin. The DS_{28}/HA ratios of the 19- and 35-year old groups correspond to the values reported by Mier and Wood[28] for adult human skin[28], but the other ratios did not. The discrepancies might originate in the method used to purify GAG, and from the way the results are expressed. In the present study, GAG were determined by weight, whereas Mier and Wood did it by uronic acid determination.

The DS and HA yields displayed a rather wide range but were within accept-

TABLE V

AGE-RELATED VARIATIONS IN THE RATIOS OF DERMATAN SULFATE TO HYALURONIC ACID

Age group	*DS_{18}/HA*	*DS_{28}/HA*	*Total DS/HA*
19 ±2.5	1.00	0.67	1.67
35 ±3.5	0.97	0.60	1.63
47 ±1.7	0.87	0.40	1.27
60 ±0.8	0.80	0.33	1.13
75 ±5.0	[a]	3.71	3.71

[a]Not determined.

able deviations from the means. Of the 23 skins analyzed, two contained DS and HA in proportions much higher than those reported in Tables I and IV, and one had considerably lower values. These individuals did not fall in with the majority of the population. Skin used was either from mammoplasties on perfectly healthy women, or from mastectomies on subjects who had no history of illness other than the breast malignancy. Furthermore, to minimize error, the following parameters were carefully controlled: skin was examined by a pathologist to ascertain that it was normal; skin was frozen 2 h after surgical removal, to avoid enzymic degradation of GAG; and all the intermediates during purification were tested with the carbazole[19] and orcinol[18] reaction to ensure thorough extraction and to prevent loss of significant GAG amounts. Therefore, the variations observed should stem from innate differences perhaps influenced by diet, or environment, or both as previously suggested[28].

The age-mediated changes reported herein should be the consequence of true physiologic aging, since sun-protected healthy skin was examined[1]. However, the effect of unknown environmental factors cannot be ignored. This study used women's skin only to further control the parameters, because the concentration of skin HA has been reported to be higher in men than it is in women[29]. Thus, if the aging mechanism of human skin is sex-dependent, the present data are not applicable to all people.

The data show three chemically different dermatan sulfates (two of which were preponderant) and *N*-deacetylation of HA and DS_{35} of the oldest group. After age 47, total DS and HA considerably decreased, DS_{18} and DS_{35} were oversulfated, and DS_{28} became undersulfated with aging.

REFERENCES

1 B. A. Gilchrest (Ed.), *Skin and Aging Process*, CRC Press, Boca Raton, Florida, 1984, pp. 5–36.
2 T. Miyahara, A. Murai, T. Tanaka, S. Shiozawa, and M. Kameyama, *J. Gerontol.*, 37 (1982) 651–655.
3 H. Muir, *Biochem. Soc. Trans.*, 11 (1983) 613–622.
4 M. Breen, H. G. Weinstein, R. L. Johnson, A. Veis, and R. T. Marshall, *Biochim. Biophys. Acta*, 201 (1970) 54–60.
5 R. Fleishmajer, J. S. Perlish, and R. I. Bashey, *Biochim. Biophys. Acta*, 279 (1972) 265–275.
6 J. M. J. Van Lis, T. Kruiswijk, W. H. Mager, and G. L. Kalsbeek, *Br. J. Dermatol.*, 88 (1973) 355–361.
7 J. H. Poulsen and M. K. Cramers, *Scand. J. Clin. Lab. Invest.*, 42 (1982) 545–549.
8 L. Rodén, in W. J. Lennarz (Ed.), *The Biochemistry of Glycoproteins and Proteoglycans*, Plenum Press, New York, 1980, 267–373.
9 V. C. Hascall and C. K. Hascall, in E. D. Hay (Ed.), *Cell Biology of Extracellular Matrix*, Plenum Press, New York, 1981, pp. 39–63.
10 B. Chakrabarti and J. W. Park, *CRC Crit. Rev. Biochem.*, 8 (1980) 225–313.
11 S. P. Damle, L. C. Coster, and J. D. Gregory, *J. Biol. Chem.*, 257 (1982) 5523–5527.
12 H. Maeda, H. Ishikawa, and S. Ohta, *Br. J. Dermatol.*, 105 (1981) 239–245.
13 A. Garcia-Pardo, E. Pearlstein, and B. Frangione, *J. Biol. Chem.*, 258 (1983) 2670–2674.
14 M. B. Contri, C. Fornieri, and I. P. Ronchetti, *Connect. Tissue Res.*, 13 (1985) 237–249.
15 M. O. Longas and R. Fleischmajer, *Connect. Tissue Res.*, 13 (1985) 117–125.
16 J. J. Cael, D. H. Isaac, J. Blackwell, J. L. Koenig, E. D. T. Atkins, and J. K. Sheeban, *Carbohydr. Res.*, 50 (1976) 169–179.

17 M. O. LONGAS AND K. MEYER, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 6225–6228.
18 A. H. BROWN, *Arch. Biochem.*, 11 (1946) 269–278.
19 T. BITTER AND J. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
20 K. MEYER, E. DAVIDSON, A. LINKER, AND P. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506–518.
21 J. ŠVEJCAR AND W. VAN B. ROBERTSON, *Anal. Biochem.*, 18 (1967) 333–350.
22 R. HATA AND Y. NAGAI, *Anal. Biochem.*, 45 (1972) 462–468.
23 K. S. DODGSON AND R. G. PRICE, *Biochem. J.*, 84 (1962) 106–110.
24 P. E. GREGOIRE, C. DICTUS-VERMEULEN, AND J. P. AMERYCKX, *Biochim. Biophys. Acta*, 279 (1972) 102–117.
25 R. M. SILVERSTEIN AND G. C. BASSLER (Eds.), Wiley, New York, 1967, pp. 64–85.
26 S. MOORE, *J. Biol. Chem.*, 243 (1968) 6281–6283.
27 J. L. REISSIG, J. L. STROMINGER, AND L. F. LELOIR, *J. Biol. Chem.*, 217 (1955) 959–966.
28 P. D. MIER AND M. WOOD, *Br. J. Dermatol.*, 181 (1969) 528–533.
29 R. H. PEARCE, in E. A. BALAZS AND R. W. JEANLOZ (Eds.), *The Amino Sugars*, Vol. IIA, Academic Press, New York, 1965, pp. 149–193.