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A Specific, Sensitive Method for the Determination of Hyaluronate[1]

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A sensitive and specific assay for hyaluronate was devised. Hyaluronate contained in biological mixtures was digested with a commercially available microbial hyaluronate lyase. The β-Δ4,5-eneglucopyranuronic acid residues contained at the nonreducing termini of the resulting oligosaccharides were oxidized with periodic acid to yield, among other products, formyl pyruvic acid. The latter compound reacted with thiobarbituric acid to yield a chromophore with an absorption maximum at 549 nm. Optimal conditions for quantitative assay of hyaluronate are described.

At present there is no simple method for the rapid and specific detection and quantitation of microamounts of hyaluronate. Classically, the concentrations of hyaluronate in biological solutions have been determined by turbidimetric methods (1), or by quantitative determination of its constituents after acid hydrolysis (2). However, these procedures are not specific for the determination of hyaluronate. Other methods depend upon isolation and chemical characterization and involve ion-exchange chromatography (3), cetylpyridinium chloride–cellulose microcolumns (4,5), and cellulose acetate electrophoresis (6). While generally satisfactory, these methods are time-consuming and are compromised if a large excess of other glycosaminoglycans are present in the sample. A highly sensitive and specific proteoglycan-binding assay for hyaluronate contained in mixtures of glycosaminoglycans has been reported but requires prior isolation of cartilage proteoglycans by density gradient centrifugation, column chromatography on agarose, and determination of the proteoglycan–hyaluronate complex elution profile by measurement of uronic acid concentration (7).

An alternative approach, utilized by a number of investigators for the determination of hyaluronate, has been the application of microbial hyaluronate lyases. In contrast to animal hyaluronidases (hyaluronate glycanohydrolase EC 3.2.1.35), the microbial hyaluronate lyases (hyaluronate lyase EC 4.2.2.1) are eliminases and hydrolyze hyaluronate to oligosaccharides containing terminal Δ4,5-unsaturated glucopyranuronic acid[3] units (8); the unsaturated compounds have a characteristic absorption maximum at 232 nm. This property served as the basis for the development of direct spectrophotometric means for the measurement of hyaluronate. However, biological fluids and tissue extracts usually contain high levels of ultraviolet-absorbing compounds such as protein and nucleic acids which interfere with the quantitative measurement of the Δ4,5-unsaturated uronic acid residues by

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[3] Δ4,5-Unsaturated glucopyranuronic acid oligosaccharides = (3-*O*-β-Δ4,5-ene-D-glucopyranuronic acid-2-acetamido-2-deoxy-D-glucose)$_n$.

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this methodology (9). The latter problem has in part been circumvented by colorimetric measurement of liberated *N*-acetylglucosamine end groups by a modification of the Morgan–Elson reaction (10). However, crude tissue extracts produce spurious color and the colorimetric determination is affected by the presence of even moderate protein concentrations (2). The enzymatic determination of hyaluronate in biological fluids is further limited by the observation that heparin, chondroitin 4-sulfate, and dermatan sulfate competitively inhibit some hyaluronate lyases (2). Ohya and Kaneko (11) have isolated a novel hyaluronate lyase from *Streptomyces hyalurolyticus* which is reported not to be inhibited by glycosaminoglycans. The enzyme is specific for hyaluronate, catalyzes the formation of oligosaccharides terminating in Δ4,5-unsaturated glucuronic acid units, and is stable at elevated temperatures.

Hascall *et al.* (12) observed that a periodate–thiobarbituric acid reagent reacted with Δ4,5-unsaturated uronic acid-terminating oligosaccharide chains (found on treatment of bovine nasal cartilage proteoglycan with microbial chondroitinases) to yield a chromophore with an absorption at 549 nm. This observation served as the basis for development of a convenient assay for the degradation of bovine nasal proteoglycan by chondroitinase ABC and AC activities. Koseki *et al.* (13) applied a similar periodate–thiobarbituric acid assay procedure, described by Warren (14), to the microdetermination of chondroitin 4- and 6-sulfate and dermatan sulfate after prior digestion with microbial chondroitinases.

The above observations served as the basis for development of a specific and sensitive enzymatic–colorimetric assay for hyaluronate described in this manuscript.

MATERIALS

Glycosaminoglycans. The following glycosaminoglycans served as reference standards and were generous gifts of Dr. M. B. Mathews, University of Chicago: hyaluronate (human umbilical cord), chondroitin 4-sulfate (sturgeon cranial cartilage), chondroitin 6-sulfate (sturgeon notochord), dermatan sulfate (hog mucosa), keratan sulfate (bovine cornea), and heparin and heparan sulfate (beef lung). Hyaluronate was also prepared from human synovial fluid by electrodeposition (15). Chondroitin was prepared from chondroitin 4-sulfate by solvolytic desulfation with dimethyl sulfoxide (16).

Hyaluronate lyase. Hyaluronate lyase purified from *Streptomyces hyalurolyticus* (specific activity 3000 turbidity reducing units (TRU)/mg protein)[4] was obtained from Calbiochem.

Reagents. The following compounds were obtained from the indicated sources: sodium periodate and *n*-butyl alcohol, J. T. Baker Chemical Company; sodium arsenite and *p*-dimethylaminobenzaldehyde, Baker and Adamson; ammonium dihydrogen orthophosphate, BDH Chemicals Ltd.; and thiobarbituric acid, Eastman Chemical Company. All other reagents were of analytical grade or of the highest purity commercially available. Dialysis tubing with a molecular weight cutoff of 3500 was purchased from Arthur H. Thomas Company.

METHODS

Standard Assay Procedure

Incubation with hyaluronate lyase. Reactions were performed in 15 × 125-mm Pyrex tubes. Enzymatic digestions contained the following components in a total volume of 0.2 ml: sodium acetate buffer, pH 5.0, 4.0 μmol; 1.66 μg hyaluronate lyase (5 TRU); and 0 to 100 μg of sodium hyaluronate. Control tubes contained the same components but lacked either substrate or enzyme. The reaction mixtures were incubated at

[4] Enzyme activity is expressed in turbidity units (TRU) as defined by Tolksdorf *et al.* (17).

60°C for 5 h and enzyme action was terminated by the addition of 0.25 ml of 0.04 M $NaIO_4$ contained in 0.08 N H_2SO_4.

Measurement of eliminase products. The conditions used were similar to those described by Hascall *et al.* (12). After addition of the periodate–H_2SO_4 reagent the reactants were mixed and the tubes placed in a 37°C water bath for 1 h to allow oxidation to proceed. Excess periodate was destroyed by addition of 0.5 ml of 3% (w/v) $NaAsO_2$ contained in 0.5 N HCl. The reactants were mixed, allowed to stand at room temperature for 30 min, and 4 ml of 0.3% thiobarbituric acid contained in 0.012 N HCl were added to each tube. The contents were again mixed thoroughly and the tubes capped with marbles, heated at 100°C for 15 min, and cooled to room temperature. In contrast to the procedure of Hascall *et al.* (12) we routinely extracted the chromophore with 4 ml of 5% HCl in *n*-butyl alcohol to enhance the sensitivity of the assay procedure. Absorbance of the butanol layer was determined at 549 nm.

RESULTS AND DISCUSSION

Enzyme Incubation Conditions

Ohya and Kaneko (11) previously established that *Streptomyces* hyaluronate lyase is stable over a wide pH and temperature range; the presently described assay procedure was conducted at the established pH and temperature optima, pH 5.0 and 60°C, respectively. Routinely, the enzyme was dissolved in 0.04 M sodium acetate buffer, pH 5, at a level of 200 TRU/ml. No appreciable loss in activity (less than 5%) was observed on storage of the diluted enzyme at −19°C for 1 month.

In the standard assay procedure the sample is incubated with hyaluronate lyase for a period of 5 h. As shown in Fig. 1, this time of incubation resulted in complete product formation over the concentration range of hyaluronate employed. Maximal product formation was achieved with 5 TRU of hyaluronate lyase in each incubation. Lower amounts of hyaluronate lyase, i.e., less than 3 TRU per incubation, resulted in significantly lower initial rates and decreased product formation (Fig. 2). Variation of absorbance values on a day-to-day basis using the same standard hyaluronate solution was within ±5%.

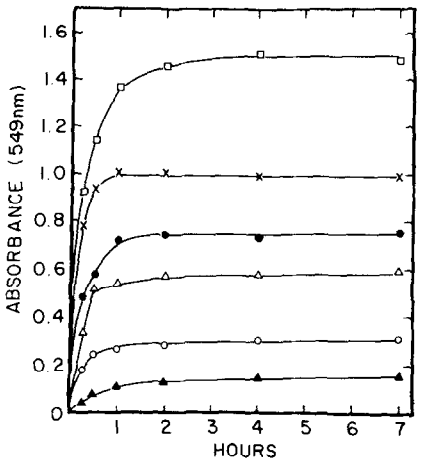

FIG. 1. The formation of periodate–thiobarbituric acid reactive compounds from hyaluronate as a function of time of incubation with *Streptomyces* hyaluronate lyase: effect of substrate concentration. The incubation conditions were those described for the standard assay procedure except the hyaluronate concentrations (expressed in μg/incubation) were as follows: (▲) 10, (○) 20, (△) 35, (●) 50, (X) 75, and (□) 100.

Enzyme Specificity

Ohya and Kaneko (11) reported that *Streptomyces* hyaluronate lyase specifically catalyzes the hydrolysis of hyaluronate and, in contrast to other microbial hyaluronate lyases, is not inhibited by other glycosaminoglycans. In view of the importance of these considerations for the specific determination of hyaluronate in biological samples, we reexamined the substrate specificity of *Streptomyces* hyaluronate lyase and the ability of other glycosaminoglycans to inhibit *Streptomyces* hyaluronate lyase. The following glycosaminoglycans were studied: chondroitin 4- and 6-sulfate, chon-

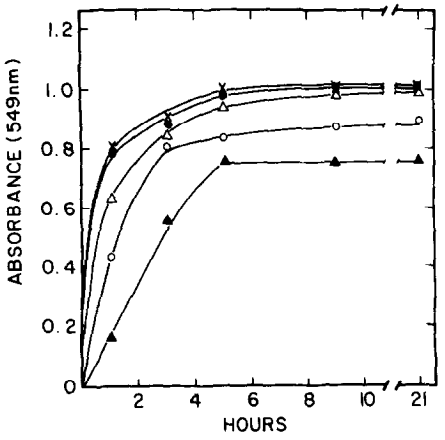

FIG. 2. The formation of periodate–thiobarbituric acid-reactive compounds from hyaluronate as a function of time of incubation with *Streptomyces* hyaluronate lyase; effect of enzyme concentration. The incubation conditions were those described for the standard assay except the hyaluronate lyase concentrations (expressed in TRU/incubation) were as follows: (▲) 1, (○) 2, (△) 3, (●) 4, and (X) 5.

droitin, dermatan sulfate, heparin, heparan sulfate, and keratan sulfate. The standard assay conditions were employed. For specificity studies 50 μg of a single glycosaminoglycan was added to a reaction mixture as a substrate and for inhibition studies each reaction mixture contained 50 μg of hyaluronate and 500 μg of another glycosaminoglycan. In agreement with the findings of Ohya and Kaneko no absorbance over the blank value was obtained with any glycosaminoglycan other than hyaluronate. Furthermore, the absorbance obtained from hyaluronate was not altered when the other glycosaminoglycans were present during the incubation.

Factors That Affect Periodate–Thiobarbituric Assay

As shown in Fig. 3, the sensitivity of the colorimetric procedure described by Hascall *et al.* (12) was enhanced approximately 2.5 times by extraction of the chromophore into acidified *n*-butyl alcohol, a method previously described by Aminoff for enhancement of the sensitivity of the same chromophore derived from sialic acid (18). Unextracted and *n*-butyl alcohol-extracted samples lost less than 8% of their color in 6 h and under the assay conditions described, the formation of *n*-butyl alcohol-extractable chromophore was linear with hyaluronate concentrations between 5 and 100 μg per reaction mixture.

While the current studies were in progress, a micromethod for the determination of chondroitin 4- and 6-sulfate and dermatan sulfate, after prior digestion with chondroitinase ABC and AC, was reported by Koseki *et al.* (13). The methodology applied a modification of the periodate–thiobarbituric acid method of Warren (14) to the assay of Δ4,5-unsaturated uronic acid-containing disaccharides derived from these glycosaminoglycans. The authors reported their assay procedure was approximately threefold more sensitive than that described by Hascall *et al.* (12). When the periodate–thiobarbituric acid conditions of the Japanese investigators were applied to the determination of hyaluronate (using the incubation conditions described in the pres-

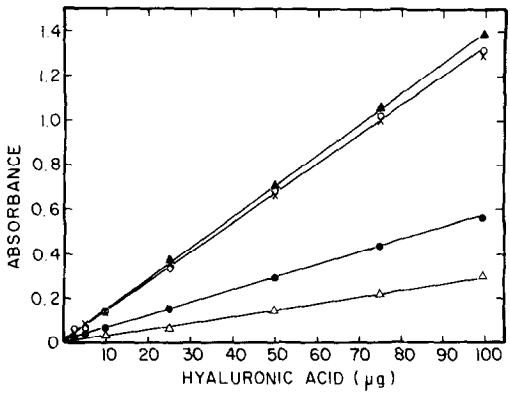

FIG. 3. Determination of the absorbance (color yield) generated by Δ4,5-unsaturated glucopyranuronic acid-containing oligosaccharides in *Streptomyces* hyaluronate lyase digests of hyaluronate by the following colorimetric and spectrophotometric procedures: (△) absorption at 232 nm (8); (●) 549 nm, Hascall *et al.* (12); (X) Koseki *et al.* (13); (○) 549 nm, determined by the methodology described in this manuscript; and (▲) 585 nm, Morgan–Elson reagent as modified by Reissig *et al.* (10).

ent manuscript), less than a 1% difference in color yield was observed from that obtained by the presently described methodology (Fig. 3). In our experience, the procedure described originally by Hascall *et al.* (12) coupled with extraction of the chromophore with acidified *n*-butyl alcohol is technically easier, is less time-consuming, and requires fewer reagents than the procedure of Koseki *et al.* (13).

The absorbance values obtained at several hyaluronate concentrations with the modified Morgan–Elson procedure of Reissig *et al.* (10) and direct spectrophotometric estimation at 232 nm are also presented in Fig. 3. Nearly equivalent absorbance values were obtained with the Morgan–Elson and the periodate–thiobarbituric acid procedures at equivalent hyaluronate concentrations; the absorbance values obtained by direct measurement of the unsaturated products at 232 nm were approximately fivefold lower than those obtained by the periodate–thiobarbituric acid or Morgan–Elson procedures.

Assay of Hyaluronate in Biological Fluids and Extracts

Accurate measurement in crude biological preparations of products arising from the enzymatic degradation of hyaluronate by direct spectrophotometric measurement or by the Morgan–Elson procedure is restricted for the reasons cited in the introduction. The previous successful application of the periodate–thiobarbituric acid reagents to the determination of free sialic acid in crude biological mixtures sugggested that the unsaturated oligosaccharides derived from hyaluronate could also be measured in such mixtures. While the presently described methodology has not been extended to a large number of tissues, we are reporting its application to the determination of hyaluronate in lung extracts, as an example of a tissue which contains interfering substances, and in synovial tissue, where the presence of hyaluronate is well documented and can be determined by classical methods. Extracts of the soft tissue of lung are rich in compounds that have the potential to contribute to elevated blank values. These compounds include sialic acid-containing glycoproteins (14,18), deoxyribose-containing nucleic acids (19), and lipids (20).

Neuraminidases present in lung extracts were found to release large amounts of free sialic acid from sialic acid-containing lung glycoproteins. Interference attributable to periodate oxidation of free sialic acid was avoided by dialyzing the extract to remove free sialic acid followed by heating at 100°C for 3 min to inactivate neuraminidase. As shown in Table 1, this heating step produced tolerable blank values with the lung extracts. The results presented in Table 1 also show that hyaluronate was not detected in lung extracts (less than 5 μg/100 μl). Analysis of the pooled lung extract for uronic acid (after papain digestion by the procedure of Schiller *et al.* (3)) showed it contained 0.24 μmol uronic acid/100 μl extract. However, results described in Table 1 show that hyaluronate was not detected by the coupled enzyme–colorimetric procedure. These results suggest that in our lung preparation, hyaluronate contributes to less than 6% of total uronic acid. This is surprising in view of the reported prevalence of hyaluronate in lung tissue (22) and at present, no explanation can be offered for this apparent contradiction. When exogenous hyaluronate was added to lung extracts prior to heating, more than 95% was recovered by the coupled enzyme–colorimetric procedure (Table 1).

The residual color noted in the blanks from lung extracts has an absorption maximum at 532 nm and may arise from periodate oxidation of deoxyribose or unsaturated lipids. Oxidation of these compounds gives malonaldehyde which in turn reacts with thiobarbituric acid to give a colored complex with an absorption maximum at 532 nm. The concentration of this chromophore

TABLE 1

ASSAY OF HYALURONATE IN LUNG EXTRACT BY A *Streptomyces* HYALURONATE LYASE–PERIODATE–THIOBARBITURIC ACID PROCEDURE

Lung extract[a] (μl)	Hyaluronate added (μg)	Absorbancy (549 nm)[b]		Hyaluronate found (μg)
		Without hyaluronate lyase	With 5 TRU hyaluronate lyase	
Heating step omitted				
100		0.545	0.520	0
Heated at 100°C for 3 min				
100		0.072	0.077	0
100	5	0.078	0.142	6.5
100	25	0.069	0.344	27.0
100	50	0.065	0.636	55.5
100	100	0.069	1.056	95.8

[a] Soft tissue was scraped from six freshly excised, perfused guinea pig lungs. The tissue (3.7 g) was ground in a motor-driven Teflon-glass homogenizer with 10 ml 0.1 M sodium phosphate buffer, pH 6.0. The homogenate (20 mg protein/ml) was dialyzed at 4°C for 4 h against the buffer used for homogenization and 100-μl aliquots (with or without added hyaluronate) heated at 100°C for 3 min.

[b] Samples were treated with hyaluronate lyase and assayed by the periodate–thiobarbituric acid procedure as described under Methods.

obtained from lung extracts was not significantly reduced by prior extraction with 2 vol of chloroform–methanol (2:1). The residual blank value may therefore arise from the presence of deoxyribonucleic acid.

Synovial fluid is a rich source of hyaluronate (15). Analysis of a limited number of synovial fluid samples for hyaluronate by the coupled enzyme–colorimetric procedure (Table 2) yielded values similar but not identical to those obtained from computations based on uronic acid content. These tissues gave negligible blank values without the heating and dialysis steps that were required for analysis of the lung extracts. Analysis of two samples of synovial fluid

TABLE 2

ASSAY OF HYALURONATE IN SYNOVIAL FLUID BY A *Streptomyces* HYALURONATE LYASE–PERIODATE–THIOBARBITURIC ACID PROCEDURE

Synovial fluid[a]	Diagnosis[b]	Absorbancy at 549 nm[c]		Hyaluronate found (mg/ml)	Uronic acid found[d] (μmol/ml)
		Without hyaluronate lyase	With 5 TRU hyaluronate lyase		
S.Y.	RA	0.025	0.116	0.37	1.6
M.W.	RA	0.016	0.535	2.02	7.8
L.C.	SLE	0.016	1.183	3.92	9.8

[a] Individual synovial fluid samples were received from the clinical laboratory of the Arthritis Division and were obtained as by-products of routine diagnostic procedures.

[b] RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

[c] Aliquots of the synovial fluids (25 μl) were treated with hyaluronate lyase and assayed by the periodate–thiobarbituric acid procedure as described under Methods.

[d] Uronic acid analyses were performed by the procedure of Bitter and Muir (21).

from rheumatoid arthritis patients indicated that hyaluronate accounted for approximately 66% of the uronic acid content whereas hyaluronate accounted for nearly all the uronic acid in a sample of synovial fluid from a patient with systemic lupus erythematosus.

CONCLUSIONS

We believe the coupled enzyme–colorimetric assay described above will prove particularly suitable for the determination of hyaluronate in partially purified mixtures of glycosaminoglycans, and with proper precautions, in crude biological extracts. The methodology is sensitive, specific, and is not inhibited by the presence of other glycosaminoglycans.

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