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SC 2246

Specific analysis of terminal reducing glucosamine and glucuronic acid groups
Hyaluronate hydrolysis by hyaluronate lyases

Specific microanalytical methods for terminal reducing glucuronic acid and glucosamine are particularly desirable in the study of hyaluronate lyases (EC 4.2.99.1, formerly known as hyaluronidase). Such procedures would not only establish the nature of the linkage undergoing cleavage but also reveal the extent of participation of the ubiquitous enzymes, β-glucuronidase (EC 3.2.1.31) and acetyl-β-glucosaminidase. The latter enzymes are capable of further degrading the mucooligosaccharide products of hyaluronate hydrolysis.

Recently, YUKI AND FISHMAN[1] applied the alkaline hypoiodite oxidation of the glucuronyl aldehydic function[2] to the differential analysis of glucuronate, glucosiduronate and hyaluronate. Such a method would be capable of measuring terminal reducing glucuronic acid groups of mucooligosaccharides in relation to the total glucuronic acid present.

In a subsequent attempt to analyze mixtures of glucosamine and hyaluronate, hypoiodite oxidation of the glucosaminyl aldehyde was first explored. It was abandoned when success was not realized in reducing the level of contaminating cations (introduced to remove iodide ions) to a value which did not interfere with the subsequently applied color reaction for glucosamine (ELSON–MORGAN[3]).

Oxidation of reducing glucosamine proved to be satisfactory and convenient with cupric ions* (pH 9.7) and, fortunately, the excess cupric ions could be removed completely by converting them to the highly insoluble cuprous oxide by glucose

* The pH of the cupric ion oxidation of the glucosaminyl aldehyde group must be regulated between 9.3 and 9.7, since above the latter pH the glucosaminidic linkages in hyaluronate are unstable (see also refs. 4 and 5).

oxidation. This maneuver has been incorporated in the present procedure* which consists essentially of two analyses. One is the measurement of total hexosamine (A) by the ELSON–MORGAN[3] procedure and the other, of hexosamine remaining after oxidation (B). The difference (A—B) in the two values corresponds to the amount of reducing hexosamine in the sample.

Oxidation of reducing hexosamine. The sample (2.0 ml) and 1 ml of alkaline copper solution** are pipetted into a 6-ml capacity test-tube which is fitted with a glass stopper and heated for 15 min in a boiling-water bath. Under these conditions up to 200 μg of free glucosamine are oxidized. Following the addition of 0.1 ml of 0.25 % glucose, the mixtures are heated for another 5 min and are centrifuged after cooling***.

TABLE I

DETERMINATION OF VARIOUS MIXTURES OF GLUCOSAMINE AND HYALURONATE

Figures in parentheses are the experimental values.

Expt.	*Composition of mixtures*	
	Glucosamine (μg) +	*Hyaluronate* (μg)*
1	10.0 (11.4)	10.4 (9.0)
2	10.0 (10.7)	41.5 (40.8)
3	10.0 (10.6)	72.6 (72.0)
4	40.0 (39.9)	10.4 (10.5)
5	40.0 (38.0)	41.5 (43.5)
6	40.0 (40.6)	72.6 (72.0)
7	70.0 (68.4)	10.4 (12.0)
8	70.0 (70.2)	41.5 (41.3)
9	70.0 (70.8)	72.6 (71.8)

* Chugai Pharmaceutical Co., Ltd., Tokyo. Glucuronic acid, 37 %; glucosamine, 41.7 %.

Measurement of hexosamine remaining after oxidation. Duplicate samples (1 ml) are pipetted into test tubes. To each is added 0.4 ml of conc. HCl and the stoppered tubes are heated for 4 h in the case of hyaluronic acid in a boiling-water bath to complete the hydrolysis. After cooling, the mixture is neutralized to phenolphthalein with 24 % Na_2CO_3 (usually 1.5 ml), and then 1 ml of acetylacetone–1 M Na_2CO_3 solution[3] is added. The tubes are heated in a boiling-water bath for 15 min. After cooling, 3 ml of ethanol and 1 ml of Ehrlich reagent[3] are added. (Sometimes centrifu-

* The hexosamine color values are all developed from free hexosamine following complete hydrolysis of the glucosaminidic linkages. In this way, the error is avoided of variation in extinction coefficients which are characteristic of the type of linkage of hexosamine in the mucopolysaccharide[6,7].

** This is made up fresh every working day by mixing 0.2 ml of Solution A (15 % $CuSO_4 \cdot 5H_2O$) with 2.5 ml of Solution B (25 g Na_2CO_3, 20 g $NaHCO_3$, 25 g potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 3 g Na_2SO_4 are dissolved in 1 l of water) and diluting to 50 ml with distilled water. An amount greater than 2.5 ml of Solution B is required when the analytical specimen is already buffered at an acid pH. Thus, 10 ml of Solution B is needed for the alkaline copper solution in the analysis of an enzyme digest buffered at pH 5.6 (0.1 M acetate buffer). The amount of Solution B needed can be determined in advance so that the pH of the final mixture remains between 9.3 and 9.7.

*** If cuprous oxide does not precipitate well, it can be adsorbed on $BaSO_4$ with which it will coprecipitate when 2 % $BaCl_2$ (0.1 ml) is introduced.

gation is necessary to clear the solution of insoluble matter.) After 30 min, the absorbancy is measured in an Evelyn Photocolorimeter (540-mμ filter) and is converted to micrograms of non-reducing hexosamine (B). In the preparation of the calibration curve, glucosamine (0–80 μg) is added to the mixture after glucose oxidation.

The performance of the method has been tested by analyzing mixtures of glucosamine and hyaluronate (Table I). As a rule, the procedure succeeds in this test to within 2 μg.

Application of methods for terminal reducing glucosamine and glucuronic acid[1].

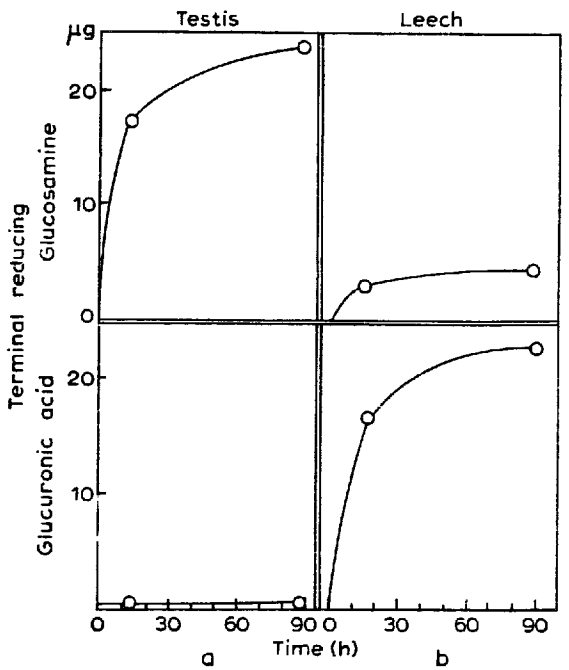

Fig. 1. Release of reducing glucosamine and glucuronic acid linkages during hyaluronate lyase-catalyzed hydrolysis of hyaluronate. Fig. 1a: Bovine testis hyaluronate lyase, 3 mg (Sigma 320 U.S.P. units/mg) was incubated with 2.5 mg of hyaluronate in 15 ml of 0.06 N acetate buffer (pH 5.6) at 38°. Fig. 1b: Leech enzyme[9] (68 units/1 ml) was incubated with 2.5 mg of hyaluronate in 15 ml of 0.06 N acetate buffer (pH 5.6) at 38°. In both experiments, aliquots were removed and heat inactivated (100°, 2 min) prior to analysis.

The hydrolysis of hyaluronate by two hyaluronate lyases has been studied as a function of time. As seen from Fig. 1a there was a liberation of reducing glucosamine but not of reducing hexuronic acid groups. This result is the expected one for testis hyaluronate lyase (free of β-glucuronidase) since the mucopolysaccharase splits glucosaminidic bonds[8]. In contrast, the leech preparation[9] (Fig. 1b) releases reducing glucuronic acid groups as one would predict[4]. However, the liberation of glucosamine groups was not expected but can now be attributed to contamination by acetyl-β-glucosaminidase[10]. The latter enzyme presumably attacks mucooligosaccharides by cleaving their terminal acetylglucosamine radicals.

In conclusion, these two methods should help in establishing linkage specificity of mucopolysaccharases and in furthering studies on reducing glucosamine and glucuronate end groups of mucooligosaccharides.

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SC 2263

Purification of C-reactive protein, an acute phase protein of human serum

An alternative method of isolation of C-reactive protein which would avoid the troublesome preliminary preparation of pneumococcal C-polysaccharide[1] employed in the classical isolation procedure of this protein devised by MCCARTY[2] would facilitate study of the biochemistry of CRP. In addition, the production of clinically useful CRP antiserum would be simplified.

We have utilized chromatographic technics employing DEAE-cellulose and have devised a simple procedure which gives good yields of CRP.

Within the typical protocol, given below, figures for total protein concentration were estimated from the absorbancy of samples at 280 mμ. CRP was assayed on dilutions of samples utilizing the capillary precipitin test[3].

A 1.75-l sample of pleural exudate having a CRP titer of 1:64, obtained from a patient with carcinoma of the tongue with pulmonary metastasis, was brought to 0.5 saturation with $(NH_4)_2SO_4$, filtered and the filtrate raised to 0.75 saturation with the same salt. The precipitate, recovered by filtration, was slurried in sufficient distilled water to permit transfer to dialysis tubing, dialyzed against running tap water for approx. 4 h and then dialyzed at 4° against several changes of Buffer A (0.05 M sodium citrate, pH 7.0, containing 0.1 M NaCl). This fraction, containing approx. 17.4 g of protein in 700 ml of solution, was passed at a rate of approx. 1.5 ml/min through a 2.2 × 20-cm column of DEAE-cellulose which had been previ-

Abbreviation: CRP, C-reactive protein.