INVESTIGATIONS ON THE CHEMISTRY OF HEPARIN. II. PRESENCE OF A URONIDIC LINKAGE WITH CARBON-6 OF GLUCOSAMINE

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The comparative resistance of the glycosidic linkages of heparin to acid hydrolysis is probably the major source of difficulty in the determination of its structure by graded hydrolysis experiments. Thus the conditions required to effect appreciable reaction also cause extensive degradation of the uronic acid. A previous paper from this laboratory described the conversion of heparin to a desulfated N-acetylated derivative containing nitrogen, sulfur and acetyl in a molar ratio of 1:0.5:1 (Danishefsky et al., 1960). It was also shown that the acid hydrolysis of this product proceeds at a significantly higher rate than heparin itself. A subsequent study was, therefore, carried out on the desulfated acetamido heparin. This report describes the isolation of a disaccharide from the hydrolyzate and presents evidence as to some of its structural characteristics.

EXPERIMENTAL

A solution of 4 g. of the desulfated acetamido heparin in 100 ml. of 1 N H_2SO_4 was heated at 100° C. for 6 hrs. and after cooling the mixture was neutralized to pH 4 with $Ba(OH)_2$. The clear filtrate obtained upon removal of the $BaSO_4$ was passed through a column of Dowex-50 (H-form). The column was washed with 2 liters of water followed by dilute HCl of increasing normality. Ten ml. fractions of the effluent were collected on a fraction collector and the amount of reducing material in each tube was determined by the Nelson copper reduction method (Nelson, 1944). A number of peaks

were obtained in this manner, the best defined material being eluted with 0.04 and 0.3 N HCl. The separate fractions were lyophylized and the residues recrystallized from aqueous ethanol. This yielded 190 and 165 mg. in the respective fractions. Each of the materials when submitted to paper chromatography with different solvents (butanol-acetic acid-water, 10:3:7 and butanol-pyridine-water, 6:4:3) and various stains (alkaline $AgNO_3$, ninhydrin, aniline hydrogen phthalate) gave a single spot. In butanol-acetic acid-water the $R_{glucose}$ for the 0.04 N HCl eluted fraction was .42 while that for the 0.3 N HCl eluted fraction was .79. With the pyridine-containing solvent the $R_{glucose}$ values were .04 and .71, respectively.

The fraction eluted with 0.3 N HCl proved to contain glucosamine on the basis of its specific rotation (+73°, 2 % in water at 23° C.) analysis for hexosamine by the Elson-Morgan reaction (Boas, 1953) and as evidenced by the fact that it is converted to arabinose when treated with ninhydrin (Stoffyn and Jeanloz, 1954). Results on the fraction eluted with 0.04 N HCl were consistant with that of a disaccharide of glucosamine and uronic acid. Specifically these were as follows: reducing power (glucose as standard), 41.64 %; anhydroglucosamine (Elson-Morgan reaction after acid hydrolysis), 38.75 %; uronic acid (carbazole reaction), 56.92 %; anhydroglucosamine without prior hydrolysis, 20.45 %. Acetylation of the disaccharide yielded an acetamido derivative which gave a Morgan-Elson result (Aminoff et al., 1952) for acetylhexosamine consistant with the Elson-Morgan hexosamine analysis.

CONCLUSIONS

That the glucosamine is on the reducing end of this disaccharide is indicated by the positive Elson-Morgan and Morgan-Elson reactions since disaccharides of hexosamine and uronic acid with the latter on the reducing end give only a limited color value (Linker et al., 1960). A disaccharide with hexosamine on the reducing end would also be expected by analogy with disaccharides obtained on hydrolysis of hyaluronic acid and chondroitin

sulfate which contain uronic acid and acetylhexosamine. The positive Morgan-Elson reaction with the acetamido derivative of the disaccharide eliminates the possibility that the uronic acid is linked to carbon-4 of the glucosamine (Jeanloz and Tremege, 1956). Furthermore, the fact that there is no enhancement of this reaction indicates that carbon-3 of the hexosamine is not involved in the uronidic linkage. This is further substantiated by the fact that when the Elson-Morgan reaction is carried on the free amino compound without prior hydrolysis, the ratio of the optical densities at 540 to 510 millimicrons is 1.15. This is inconsistant with a 3-O-substituted hexosamine where the ratio is less than 1 (Cifonelli and Dorfman, 1958). Assuming a pyranose ring for the glucosamine, these findings indicate that heparin contains a disaccharide unit in which the uronic acid is linked to carbon-6 of glucosamine. This is of special significance since no other mucopolysaccharide has been reported to contain such a linkage. The possibility exists that this structure may be responsible, at least in part, for the specific physiological properties of heparin.

It should be noted that a number of other fractions, in addition to the two described above, were obtained from the effluents from the column. One of these contained a small amount (approximately 30 mg.) of another disaccharide composed of hexosamine and uronic acid. Preliminary results indicate that this disaccharide contains the uronic acid on the reducing end. The other fractions contained higher oligosaccharides of the same components. The question whether carbon-6 of glucosamine is the only one which is involved in the uronidic linkages and whether branching occurs in the heparin polymer is presently being investigated.

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