CHROM. 3491

Gel filtration behavior of heparin and N-desulfated heparin

The molecular weight of heparin has been variously reported in the range from about 6,000 to 16,000, depending on the source, purification procedure, and the analytical methods used[1-4]. In the course of gel filtration studies on heparin–protein complexes, we have observed that heparin is excluded from Sephadex G-75, which indicates a much larger molecular size. Exclusion of other strongly acidic macromolecules from dextran gel was reported by HAY *et al.*[5] and by POSNER[6]. Such anomalous behavior was attributed to the ionic interaction of polyanions with the carboxyl groups in the gel matrix. This type of interaction could account for the exclusion of heparin, a strongly acidic mucopolysaccharide, from Sephadex G-75. However, because of the sensitivity of molecular sieve coefficient to molecular asymmetry, the effect of molecular shape on the gel filtration behavior of heparin must be taken into consideration. Recent physical studies indicate that heparin is a highly asymmetric molecule.

The present investigation was undertaken to determine the gel filtration behavior of native and desulfated heparin, and to relate it to known hydrodynamic properties.

Materials and methods

Sodium heparinate (grade I, Sigma Chem. Co., St. Louis, Mo.) of 159 U.S.P. units/mg sp. ac. was employed in this study. Cytochrome c Type III from horse heart was obtained from Sigma Chem. Co. Sephadex G-200, 140–400 Mesh and blue dextran 2000 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.Y.

Partial and complete N-desulfation of heparin was accomplished by heating a dilute solution of heparin in 0.08 *N* HCl at 100° for 20 and 120 min time intervals, respectively. Following treatment, the solutions were cooled rapidly, neutralized by addition of 0.8 *N* NaOH, and used immediately or stored frozen for future use. Sephadex gel was prepared by the rapid swelling procedure recommended by the manufacturer. The gel was packed into a 2.5 cm × 50 cm column and equilibrated with 0.02 *M*, pH 7.4 phosphate buffer, made 0.2 *M* in NaCl. Three mg samples were applied to the top of the column in 2 ml of buffer-saline solution and eluted at room temperature with the same solvent at a flow rate of 24 ml/h. Column effluents were collected automatically in 3 ml fractions using a Beckman Model 132 fraction collector on a drop-count basis. The concentration of heparin and N-desulfated heparin in the effluent fractions was determined by means of the carbazole method[7]. Blue dextran 2000 and cytochrome c were estimated by light-extinction measurements at 640 mμ and 550 mμ, respectively.

Results and discussion

Sephadex chromatography of native and modified heparin resulted in typical gel filtration profiles with symmetrical peaks. The relative elution volume, V_e/V_0, where V_e is the elution volume of the substance and V_0 is the void volume, was 2.14 for the native heparin. Cytochrome c, the reference substance of 12,270 molecular weight, gave a value of 2.75. The correlation between the molecular weights of globular

proteins and their behavior on Sephadex columns is well established[8]. Assuming a conformance to the V_e–log mol. wt. relationship, the V_e/V_0 of 2.14 would correspond to a molecular weight of about 40,000. This is several times the estimates based on either sedimentation rate, light-scattering, or diffusion measurements. The N-desulfated heparin samples resulted in significantly higher relative elution volumes. Values of 2.43 and 2.67 were obtained for the 20 min and 120 min acid hydrolyzed heparin, respectively. These values indicate a progressive decrease in the apparent molecular weight with an increasing degree of desulfation. The apparent molecular weight of completely desulfated heparin was estimated at between 17,000 and 18,000 relative to the cytochrome c V_e/V_0 value. This figure is nevertheless markedly higher than the 11,000 to 12,000 estimate considered as the most probable molecular weight of heparin[9]. The observed deviations from the so-called standard gel filtration behavior of native and desulfated heparin cannot be attributed to the ion exclusion effect. The equilibration of the Sephadex column with 0.2 *M* NaCl solution is sufficient to eliminate the electrostatic interaction between the polyanion and fixed carboxyl groups in the gel matrix[10]. Consequently, the deviations are most likely due to molecular asymmetry of native and modified heparin.

It has been proposed that heparin is a linear polysaccharide composed of approx. 20 structural repeating units, namely, dimers of glucosamine and glucuronic acid linked through α-D-(1→4) glycosidic linkages[9,11]. In addition to a carboxyl group, the dimer carries one N-sulfate and one O-sulfate group[9]. Based on the optical rotatory dispersion properties of metachromic heparin, STONE[12] concluded that the heparin molecule has a helical conformation. Although LASKER AND STIVALA[4] found that heparin in solution exhibits substantial chain flexibility, high frictional ratio coefficients reported for heparin indicate an extended rod configuration. In fact, such a shape would be dictated by the high negative charge density and the resultant electrostatic interaction among anionic groups.

JENSEN, SNELLMAN AND SYLVEN[1] have studied the change in physical properties of heparin during the inactivation caused by recrystallization of heparin from warm, dilute acetic acid. They reported an increase in the sedimentation rate and a higher degree of polydispersity, but no decrease in molecular weight. Since the frictional ratio coefficient decreased from 2.5–1.81, it was suggested that a change in the shape or hydration of heparin molecule had occurred. Subsequent studies[9,14] have shown that mild acid hydrolysis of heparin results in desulfation, which is limited primarily to the N-sulfate groups. The loss of sulfate groups is accompanied by a concomitant release of free amino groups, which causes a marked shift in net charge density. This results in the reduction of repulsive electrostatic interaction which in turn permits greater chain flexibility and a more compact structure. Moreover, the presence of oppositely charged groups makes possible the formation of structure stabilizing ionic bonds. In view of this, a conformational change should indeed be expected to occur upon desulfation of heparin.

The present findings are consistent with the postulated conformational difference between the native and desulfated heparin. The observed deviations from standard gel filtration behavior are in accord with existing hydrodynamic data. The V_e/V_0 values obtained for partially and completely N-desulfated heparin suggest relationship between the axial ratio and degree of desulfation. In this connection, it would be of interest to determine the correlation between the molecular sieve coefficient and other

physical parameters of heparin subjected to various degrees of N-desulfation. Such a study could include the investigation of structure-biological function relationship. Molecular sieve chromatography is particularly applicable to the investigation of heparin complexes with proteins and other substances.

Acknowledgement

The authors are indebted to Dr. GRANT H. BARLOW of Abbott Laboratories, North Chicago, Ill. for his valuable suggestions in the course of this study and during preparation of this manuscript. This investigation was supported by internal grant from IIT Research Institute.

Life Sciences Division, IIT Research Institute,
10 West 35th Street, Chicago, Ill. 60616 (U.S.A.)

GEORGE B. SUMYK
CHARLES F. YOCUM

1 R. JENSEN, O. SNELLMAN AND B. SYLVEN, *J. Biol. Chem.*, 174 (1948) 265.
2 F. PATAT AND H.-G. ELIAS, *Naturwiss.*, 46 (1959) 322.
3 G. H. BARLOW, N. D. SANDERSON AND P. D. MCNEILL, *Arch. Biochem. Biophys.*, 84 (1961) 518.
4 S. E. LASKER AND S. S. STIVALA, *Arch. Biochem. Biophys.*, 115 (1966) 360.
5 J. B. HAY, A. J. WICKEN AND J. BADDILEY, *Biochim. Biophys. Acta*, 71 (1963) 190.
6 A. M. POSNER, *Nature*, 198 (1963) 1161.
7 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
8 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
9 J. R. HELBERT AND M. A. MARINI, *Biochemistry*, 5 (1963) 1101.
10 J. C. JANSON, *J. Chromatog.*, 28 (1967) 12.
11 I. DANISHEFSKY, H. B. EIBER AND A. H. WILLIAMS, *J. Biol. Chem.*, 238 (1963) 2895.
12 A. L. STONE, *Biopolymers*, 2 (1964) 315.
13 A. B. FOSTER, E. F. MARTLEW AND M. STACEY, *Abstr. Am. Chem. Soc.*, 126 (1954) 6D.

Received March 5th, 1968

J. Chromatog., 35 (1968) 101–103

CHROM. 3490

Lattice entrapment of glycolytic enzymes

Entrapment of enzymes within a hydrophilic gel has been accomplished by several workers[1–4] using polyacrylamide. The technique is appropriate to the development of continuous-flow substrate conversion systems and to kinetic studies of enzyme catalyzed reaction sequences in which compartmental isolation is sought. In our present investigation, the use of polyacrylamide entrapment has allowed the isolation of four enzymes of the glycolytic sequence so that the end product of the sequence of reactions could be obtained as a column eluate.

The enzymes, obtained commercially, were entrapped in separate layers of a polyacrylamide gel column. Hexokinase, phosphoglucoisomerase (Sigma), phosphofructokinase (Calbiochem) and aldolase (Sigma) were entrapped separately and packed into the column in the same order as the reactions occur in nature. Enzymes were each within 8 cm long sections of the 2.5 cm diameter column and were separated by 2 cm