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SEPARATION OF HEPARIN ON SEPHAROSE CL-4B IN THE PRESENCE OF HIGH CONCENTRATIONS OF AMMONIUM SULPHATE

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SUMMARY

Heparin was fractionated by chromatography on Sepharose CL-4B/3.8–2.0 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at 4°C based on a principle different from that of gel filtration —possibly due to multiple interaction mechanisms including those based on hydrophobic bonds. The results of the fractionation were very similar to those of chromatography on Phenyl-Sepharose CL-4B/3.8–2.0 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at room temperature. That is, the separation was related to the molecular size distribution, N-acetyl content and anticoagulant activities. As the result of studies on a set of Sepharose 4B gels with different degrees of cross-linking, it has been shown that the introduction of the cross-linked structure, $-O-CH_2-CH(OH)-CH_2-O-$, into the gel matrix enhances the interaction between the Sepharose CL-4B gel and heparin, indicating that heparin retention by the highly cross-linked Sepharose 4B gel surpasses that by Phenyl-Sepharose CL-4B.

INTRODUCTION

It has been reported that mucopolysaccharides such as heparin can be fractionated on a commercially available amphiphilic gel in a highly concentrated ammonium sulphate solution[1]. This fractionation has been considered to depend primarily on the interaction between the hydrophobic ligand of the gel and the hydrophobic residues of the polysaccharide, and it has been shown that the primary component of the polysaccharide structure participating in the interaction is the N-acetyl group[1,2].

It was reported previously[3] that the gel filtration behaviour of heparin on Sephadex G-100 and G-200 changes with the ionic strength of the eluent. At low ionic strengths the molecules of heparin are altered to such an extent that they are excluded by these gels, mainly due to the ion-exclusion effect between the gel network, which behaves as a very weak polyanion, and the negatively charged heparin. On the

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other hand, based upon the results of gel filtration of native and N-desulphated heparin on Sephadex G-200 in 0.2 *M* sodium chloride, it was suggested that a change in the shape of the heparin molecule significantly affects its gel filtration behaviour[4].

Recently, we have observed that Sepharose CL-4B, which possesses few hydrophobic ligands, retains heparin in a highly concentrated ammonium sulphate solution, especially at lower temperatures[5]. This phenomenon is considered to depend on a principle different from that of gel filtration[3,4]. The present report describes the separation of heparin with Sepharose CL-4B in the presence of high concentrations of ammonium sulphate, which is probably dependent on multiple interaction mechanisms including those involving hydrophobic interaction.

EXPERIMENTAL

Materials

A commercial hog intestinal heparin (anticoagulant activity, 169 USP units per mg) was obtained from Sigma (St. Louis, MO, U.S.A.). A heparin preparation free from dermatan sulphate (anticoagulant activity, 176 USP units per mg) was prepared from the commercial heparin by the method of Rodén *et al.*[6]. Another hog intestinal heparin (anticoagulant activity, 164 USP units per mg) obtained from Cohelfred Lab. (Chicago, IL, U.S.A.) was used for the thrombin-inactivation assay as a standard. Sepharose 4B and Sepharose CL-4B gels were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Analytical methods

Total sulphate content was analyzed by a turbidimetric method[7]. The N-sulphate content was determined by a previously reported method[8]. The N-acetyl content was determined by gas–liquid chromatography (GLC) after acid hydrolysis of the samples[9]. Uronic acid was determined by the carbazole method of Bitter and Muir[10]. Analytical gel chromatography on Ultrogel AcA-44 agarose–acrylamide gel (LKB, Bromma, Sweden) was performed using 0.3 *M* sodium chloride as solvent according to the procedure described previously[1].

Preparation of Sepharose 4B gels with different degrees of cross-linking

The cross-linking of Sepharose 4B gel was performed by the procedure described by Porath *et al.*[11]. To a suspension of the swelled gel (1 l) in 1 *M* sodium hydroxide (1 l) were added a variable amount of epichlorohydrin and a constant amount of sodium borohydride (5 g), and the mixture was heated at 60°C for 1 h. After filtration, the gels collected were washed with hot water, then suspended in water (500 ml). To this suspension, 2 *M* sodium hydroxide (100 ml) and sodium borohydride (2.5 g) were added, then the mixture was heated in an autoclave at 120°C for 1 h. The cross-linked gels were collected by filtration, washed with water and stored at 4°C. A set of alkaline epichlorohydrin reagents differing in the concentration of epichlorohydrin (2, 5, 10 and 20%, v/v) was used for preparing gels with different degrees of cross-linking (preparations 1–4 in Table II).

The degree of cross-linking of the gel preparations obtained was estimated by nuclear magnetic resonance (NMR) spectrometry as follows. The cross-linked Sepharose 4B gel sample (10 ml, volume when wet) was washed with water (100 ml),

then with dry acetone (70 ml) on a glass filter. The gel washed was then dried in air, followed by drying *in vacuo* at 90°C for 2 h over phosphorus pentaoxide. To 0.5 ml of a solution of 0.1 *M* deuterium chloride in [$^2H_6$]dimethyl sulphoxide (1:10, v/v), the dried gel (*ca.* 20 mg, accurately weighed) was added, and the mixture was heated at 100°C until all had dissolved. The time necessary for solubilization varied from 10 to 75 min according to the degree of cross-linking of the gel sample. NMR spectra were then recorded at 80°C with a Varian EM-90 spectrometer. The ratio of the area of the signal due to protons of the cross-linked structure $-O-CH_2-CH(OH)-CH_2-O-$ and C_2-C_6 in anhydropolygalactose (δ 3–4 ppm) to the area of the signal due to anomeric proton (δ 4–5 ppm) was calculated from the spectrum obtained. The ratios obtained for the cross-linked Sepharose 4B preparations 1–4, starting Sepharose 4B and commercial Sepharose CL-4B are listed in Table II, and are a measure of the degree of cross-linking present in the gel samples.

Gel filtration of fibrinogen and heparin on cross-linked Sepharose 4B gels

A column (80 × 1.6 cm I.D.) packed with the cross-linked gel to be tested was equilibrated with 0.05 *M* Tris–hydrochloric acid (pH 7.5). A solution of fibrinogen (5 mg) and heparin (2 mg) in the same solvent (1 ml) was loaded on the column, and eluted with the same solvent at 21–25°C. The flow-rate was 21.3 ml/h, and 2.9-ml fractions were collected. An aliquot of 1 ml was taken for the assay of protein (280 nm) and the carbazole reaction (530 nm). The void volume, V_0 and the gel bed volume V_t were determined by the elution positions of Blue Dextran and 2.1 *M* sodium chloride, respectively. The K_{av} values obtained, according to $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, are shown below:

Cross-linked Sepharose 4B preparation	K_{av}	
	Heparin	*Fibrinogen*
2	0.60	0.36
3	0.56	0.38
4	0.58	0.36

Gel filtration of heparin on the cross-linked Sepharose 4B gels was also carried out using 0.15 *M* sodium chloride as solvent, according to the same procedure as described above. The K_{av} values obtained were almost identical with those shown above.

Analysis of heparin distribution among the fractions separated on Sepharose 4B gels with or without cross-linking

A column (6 × 0.6 cm I.D., 1.7 ml of gel), packed with the gel to be tested, was washed with ten volumes of water, then equilibrated with 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid. The amount of sample indicated in Table I dissolved in 1 ml of the same solution was loaded on the column, and eluted stepwise with 30 ml each of 3.8 *M*, 3.4 *M*, 3.0 *M*, 2.5 *M*, 2.0 *M* and 1.0 *M* ammonium sulphate solutions in 0.01 *M* hydrochloric acid. The elution was performed at the temperature indicated in Table I with a flow-rate of 15 ml/h, and each of the fractions pooled (30 ml) was analyzed for uronic acid content.

Preparative scale separation of heparin on Sepharose CL-4B gel at 4°C

A column (23 × 5.0 cm I.D., 450 ml of gel) packed with Sepharose CL-4B

TABLE I

HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON SEPHAROSE 4B AND SEPHAROSE CL-4B GELS

Heparin (5.1 mg) was applied on the column (6 × 0.6 cm I.D.) and chromatographed at the indicated temperature.

Gel	*Temp. (°C)*	*Heparin eluted (% of total amount recovered) by $(NH_4)_2SO_4$–0.01 M HCl*						*Heparin adsorbed (%)**	*Recovery of heparin eluted (%)*
		3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
Sepharose 4B	21–25	96.5	1.3	1.2	0.5	0.5	0	3.5	96
	4	81.4	13.9	4.3	0.4	0	0	18.6	95
Sepharose CL-4B	22–25	83.0	14.1	2.7	0.2	0	0	17.0	97
	4	46.5	26.9	20.2	5.2	0.9	0.9	53.5	95

* Percentage of heparin adsorbed (3.4–1.0 *M*) to total heparin in 3.8–1.0 *M* ammonium sulphate.

(column I) was equilibrated with 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid (pH 3.3). A solution of heparin (1.37 g) in the same solvent (255 ml) was loaded on column I, and eluted with the same solvent (3 l) at 4°C. The flow-rate was 100 ml/h, and 20-ml fractions were collected, each of which was assayed for uronic acid content [3.8 *M* (1) in Fig. 1]. After the elution on column I, the column was washed with 1 l of water to recover all of the heparin retained, and the washings collected were freeze-dried. To the residue obtained, solid ammonium sulphate and 0.1 *M* hydrochloric acid were added to yield a solution of heparin (846 mg, determined by uronic acid analysis) in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid (370 ml). This was loaded on another column (31 × 3.0 cm I.D., 220 ml of gel) packed with Sepharose CL-4B (column II) prepared in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at 4°C. The elution on column II was performed stepwise at a flow-rate of 100 ml/h with 3.8 *M* (3 l), 3.4 *M* (1 l), 3.0 *M* (1 l), 2.5 *M* (500 ml) and 2.0 *M* (500 ml) ammonium sulphate in 0.01 *M* hydrochloric acid at 4°C. The effluent was collected as 20-ml fractions, each of which was assayed for uronic acid content. The fractionation on column II gave 3.8 *M* (2), 3.4 *M*, 3.0 *M*, 2.5 *M* and 2.0 *M* fractions as shown in Fig. 1.

The fractions [3.8 *M* (1), 1.35 l; 3.8 *M* (2), 850 ml; 3.4 *M*, 680 ml; 3.0 *M*, 430 ml; 2.5 *M*, 350 ml; 2.0 *M*, 400 ml] indicated by the length of the braces in the figure were isolated according to the procedure described previously[1].

Assays for anticoagulant activities

Clotting method. Anticoagulant activity was assayed by the whole-blood assay method of the United States Pharmacopeia, and the activity is expressed as units per mg.

Thrombin-inactivation assay. The assay was performed substantially according to the procedure of Björk and Nordenman[12] with some modification as described previously[1]. The thrombin-inactivation potency of the samples is expressed as a percentage of Cohelfred's heparin activity.

Affinity chromatography of heparin fractions on antithrombin III–Sepharose

The chromatography was performed essentially as described by Laurent *et al.*[13]. Approximately 2 mg of the heparin preparations were applied to a column (3 × 1.8 cm I.D., 7.6 ml) of antithrombin III–Sepharose CL-4B in 0.05 *M* Tris buffer (pH 7.4) + 0.05 *M* sodium chloride at 4°C, followed by elution with the same buffer (50 ml) and a linear gradient of sodium chloride increasing to 3.0 *M* (100 ml). The flow-rate was 25 ml/h and 3-ml fractions were collected, each of which was assayed for uronic acid and ionic strength. The areas of the absorbance peaks at 530 nm of the non-adsorbed (NA), low-affinity (LA) and high-affinity (HA) fractions were measured, and the proportions of the three types of heparin were calculated.

RESULTS AND DISCUSSION

Separation of heparin on Sepharose 4B and Sepharose CL-4B

A purified heparin was obtained from a commercial hog intestinal heparin by removing contaminated dermatan sulphate (*ca.* 2.5%, calculated by ratio of 2-amino-2-deoxy-D-galactose (GalN) to total 2-amino-2-deoxyhexose (HexN). Samples of

the purified heparin were loaded onto a Sepharose 4B column prepared in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid and onto a Sepharose CL-4B column prepared in the same solvent, and fractionations were carried out by stepwise elution with 3.8–1.0 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at room temperature and 4°C, respectively. The results of these fractionations, Table I, show that 53.5% of the heparin loaded onto the Sepharose CL-4B column was retained at 4°C. On the Sepharose 4B column, 18.6% of the loaded heparin was retained at 4°C. The results in Table I indicate: (1) that some species of heparin is retained on the anhydropolygalactose matrix of Sepharose 4B in the presence of 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid; (2) that Sepharose CL-4B cross-linked with epichlorohydrin retains heparin more strongly than Sepharose 4B devoid of cross-linking; (3) that heparin retention by both of these gels is marked at lower temperatures.

Separation of heparin on Sepharose 4B gels with different degrees of cross-linking

Then, the relationship between cross-linking and heparin retention on Sepharose CL-4B was examined. To prepare a set of cross-linked Sepharose 4B gels with different degrees of cross-linking, Sepharose 4B gel was cross-linked with an alkaline epichlorohydrin reagent according to the method of Porath *et al.*[11]. For these gel preparations, and a commercial cross-linked gel, Sepharose CL-4B, the relative degrees of cross-linking were estimated by the NMR spectrometry. The values listed in Table II are expressed in terms of the intensities of the NMR signal (δ 3–4 ppm) due to the protons of the cross-linked structure $-O-CH_2-CH(OH)-CH_2-O-$ and of the C_2–C_6 in the anhydropolygalactose and of the NMR signal (δ 4–5 ppm) due to the anomeric proton of the anhydropolygalactose. It is seen that the degree of cross-linking increased with increasing concentration of epichlorohydrin in the cross-linking reaction, and a comparison of the degree of cross-linking is possible between these gel preparations and Sepharose CL-4B. Porath *et al.*[11] reported that

TABLE II

COMPARISON OF DEGREE OF CROSS-LINKING AMONG CROSS-LINKED SEPHAROSE 4B PREPARATIONS

Gel	*Relative degree of cross-linking**
Sepharose 4B	2.69 (1.00)**
Sepharose CL-4B	2.85 (1.06)
Cross-linked Sepharose 4B preparation***	
1	2.72 (1.01)
2	3.24 (1.20)
3	3.40 (1.27)
4	3.52 (1.31)

* Expressed as the ratio of the area of the NMR signal due to protons (δ 3–4 ppm) of $-O-CH_2-CH(OH)-CH_2-O-$ and of C_2–C_6 in anhydropolygalactose to the area of the NMR signal due to anomeric proton (δ 4–5 ppm) of anhydropolygalactose.

** Ratios relative to the value on Sepharose 4B gel, 2.69, are given in parentheses.

*** Preparations 1–4 were obtained by cross-linking Sepharose 4B in alkaline solutions containing 2, 5, 10 and 20% (v/v) epichlorohydrin, respectively.

cross-linking of agarose with epichlorohydrin, which forms short cross-linkages between hydroxyl groups in the anhydropolygalactose matrix of the gel, affects primarily the structural strength of the gel but has no effect on the pore size. To confirm this, using the cross-linked Sepharose 4B samples (preparations 2–4), fibrinogen (mol.wt. 400,000) and heparin were subjected to analytical gel filtration. As described in the Experimental section, the K_{av} values obtained with these gel preparations were 0.56–0.60 for heparin and 0.36–0.38 for fibrinogen, thus indicating no significant difference in pore size between these gels.

Table III summarizes the results of heparin separation on these cross-linked Sepharose 4B gels. Apparently, there exists a correlation between the degree of cross-linking of the gel and heparin retention. Preparation 4 retained as much as 84% of the loaded heparin in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at 4°C. Also, Sepharose CL-4B, intermediate in degree of cross-linking between preparations 1 and 2, exhibited heparin retention intermediate between these two preparations. When these results are compared with that of heparin retention on Phenyl-Sepharose CL-4B, it can be concluded that the structure $-O-CH_2-CH(OH)-CH_2-O-$ contributes to the interaction between the gel and heparin, probably through an effect —hydrophobic interaction— analogous to that of the phenoxyglyceryl substitution in Phenyl-Sepharose CL-4B. However, it should be noted that retention of heparin on these gels is marked at lower temperatures. Since the hydrophobic interaction is generally strengthened with increasing temperature[14], the temperature dependence of the heparin retention in Tables I and III indicates that a considerable part of the retention depends on mechanism(s) other than that based on an hydrophobic one. This may also be the case for the retention of heparin on Phenyl-Sepharose CL-4B.

The adsorption of proteins by a hydrogel such as Sephadex (a cross-linked dextran product, Pharmacia Fine Chemicals) at higher ionic strengths is explained as due to hydrophobic interactions based on bonds at multiple points, although each interaction occurs very weakly between the hydrophobic area of the protein molecule and $-CH_2-$ and $=CH-$ groups in the hydrogel when both approach each other through structural changes in the water molecules —elimination of co-ordinated water— existing between the protein molecule and hydrogel[15]. Recently, Holmes *et al.*[16] reported that *Escherichia coli* tRNA was retained on Sepharose 4B gels in the presence of high concentrations of ammonium sulphate at pH 4.5 and the tRNA species were eluted individually by a reverse gradient of ammonium sulphate. More recently, Spencer and Binns[17–19] described the existence of two mechanisms in the fractionation of tRNA on Sepharose 4B. The first mechanism, believed to be interfacial precipitation, progressively releases tRNA species as the salt concentration is reduced. A second mechanism, adsorptive retardation, in which molecules lag behind the solvent; this process, widely believed to be unimportant in the chromatography of macromolecules with multiple binding sites, is mainly responsible for the fractionation of the tRNA species.

The heparin retentions on Sepharose 4B and cross-linked Sepharose 4B gels as shown in Tables I and III can be considered as due to plural mechanisms analogous to those in the cases of the above protein and tRNA. That is, in the presence of high concentrations of ammonium sulphate, the water molecules surrounding heparin molecules will be greatly changed in structure and may be considered to be in a pre-salting-out state. In this situation, some species of heparin molecules will be

TABLE III

HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON CROSS-LINKED SEPHAROSE 4B GELS WITH DIFFERENT DEGREES OF CROSS-LINKING

Heparin (5.1 mg) was applied on the column (6 × 0.6 cm I.D.) and chromatographed at the indicated temperature.

Cross-linked Sepharose 4B preparation	*Temp. (°C)*	*Heparin eluted (% of total amount recovered) by $(NH_4)_2SO_4$–0.01 M HCl*						*Heparin adsorbed (%)*	*Recovery of heparin eluted (%)*
		3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
1 (1.01)*	22–25	91.7	6.9	0.9	0.5	0	0	8.3	92
	4	53.6	26.8	14.6	4.0	0.5	0.5	46.4	90
2 (1.20)	22–25	84.8	13.4	1.8	0	0	0	15.2	94
	4	42.1	27.4	23.2	6.5	0.8	0	57.9	94
3 (1.27)	22–25	52.5	27.7	16.8	1.6	1.0	0.4	47.5	102
	4	21.8	23.9	31.8	19.9	1.5	1.2	78.2	93
4 (1.31)	22–25	41.0	36.3	20.3	1.5	1.0	0	59.0	104
	4	15.9	18.3	30.6	30.9	2.9	1.4	84.1	100
Phenyl-Sepharose CL-4B**	21–25	56.4	27.7	12.4	2.3	0.7	0.6	43.7	99
	4	28.7	28.4	30.0	11.1	1.0	0.8	71.3	102

* Relative degree of cross-linking (see Table II).
** Data reproduced from ref. 5.

retained by the gel through "interfacial precipitation" on the gel matrix and will be released progressively from the gel as the salt concentration is reduced. Although the solubility of heparin at different temperatures could not be determined due to its extremely high solubility in concentrated ammonium sulphate solutions, the results in Tables I and III —temperature dependence of the heparin retention— strongly suggest that the fractionation of heparin on the gels depends largely on such a mechanism as described by Spencer and Binns[17–19]. On the other hand, if the marked elimination of co-ordinated water from heparin molecules under the conditions used is taken into consideration, it is also very probable that the heparin retention is partly due to hydrogen bonds, as in the effect of urea on the adsorption of hyaluronic acid —a non-sulphated mucopolysaccharide— on Phenyl-Sepharose CL-4B[20].

Another important result in these tables —a relationship between the degree of cross-linking and heparin retention— indicates a marked contribution of the cross-linked structure, $-O-CH_2-CH(OH)-CH_2-O-$, to heparin retention, and suggests that a fair part of the retention may depend on hydrophobic interaction. That is, although heparin (similar to other mucopolysaccharides) is by far less hydrophobic than protein or tRNA, the heparin molecules devoid of co-ordinated water in solutions at high concentration of ammonium sulphate may be considered to participate in binding with the gel, through a hydrophobic interaction mainly based on bonds between the cross-linked structure in the gel and CH_3-CO-, $-CH_2-$ and $=CH-$ groups in the heparin molecules.

Preparative-scale separation of heparin on Sepharose CL-4B at 4°C

As shown in Table I, Sepharose CL-4B gel retained 53.5% of the loaded heparin in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at 4°C. On the other hand, Phenyl-Sepharose CL-4B retained 43.7% of the loaded heparin at 21–25°C in the same solvent (Table III). Heparin separation on a preparative scale on Sepharose CL-4B was carried out at 4°C, and the fractionated heparins were isolated for exam-

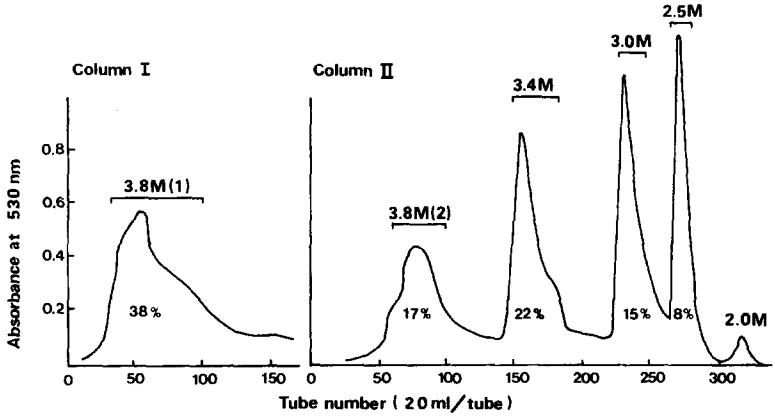

Fig. 1. Hog intestinal heparin (1.37 g) chromatographed successively on column I (23 × 5.0 cm I.D., 450 ml of gel) and column II (31 × 3.0 cm I.D., 220 ml of gel) of Sepharose CL-4B by stepwise elution with ammonium sulphate (3.8–2.0 *M*) in 0.01 *M* hydrochloric acid. Fractions (20 ml per tube) were analyzed for uronic acid content. Each of the pooled fractions indicated by the length of the braces was isolated. The percentages of material fractionated were calculated from the areas of each fraction in the chromatograms.

ination of their chemical and biological properties. Since desalting or other operations must be carried out with as small volumes as is possible, fractionation was conducted according to the two-step elution employing column I (large volume) and column II (small volume). As the result, the chromatograms shown in Fig. 1 were obtained. On a Sepharose CL-4B column (23 × 5 cm I.D., gel volume 450 ml) prepared in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid, column I, were applied 1.37 g of heparin. The fraction eluted with 3 l of the same solvent is the 3.8 *M* (1) fraction. All of the heparins retained on column I were recovered by elution with 1 l of water, and charged onto the second Sepharose CL-4B column (31 × 3 cm I.D., gel volume 220 ml) prepared in 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid, column II. The fraction eluted with 3 l of the same solvent is the 3.8 *M* (2) fraction. Subsequently, by stepwise elution with decreasing ammonium sulphate concentration, 3.4–2.0 *M*, other fractions were obtained. The yield of each fraction expressed in terms of the amount of heparin (based on uronic acid analysis) calculated from each peak area in Fig. 1 is shown in Table IV, and the loaded heparin was found to be recovered almost quantitatively. These heparin fractions were isolated as sodium salts and subjected to chemical analysis and analytical gel chromatography.

The 3.8 *M* (1) and 3.8 *M* (2) fractions comprise 38% and 17% of the total amount of heparin, respectively, and they consist of heparin species having the lowest affinity for Sepharose CL-4B. As shown in Table IV, there is no meaningful difference in analytical values between them, except for the K_{av} value, thus indicating that the 3.8 *M* (1) fraction contained the smallest heparin species. In contrast, the 2.5 *M* fraction comprising 8% of the total heparin fractionated —consisting of heparin species having the highest affinity for the gel— was found to have the lowest sulphate content and the highest N-acetyl content. As for the molecular size distribution, the 2.5 *M* fraction gave the lowest K_{av} value, indicating that it contained the largest

TABLE IV

YIELDS AND ANALYTICAL DATA OF HEPARIN FRACTIONS SEPARATED ON SEPHAROSE CL-4B AT 4°C

The hog intestinal heparin (1.37 g) was loaded on the column. Each of the pooled fractions indicated by the length of the braces in Fig. 1 was isolated and subjected to quantification and analyses.

Heparin fraction	*Yield** (mg)*	*Total sulphate*** (%)*	*N-sulphate*** (%)*	*N-acetyl*** (%)*	*K_{av} on Ultrogel AcA-44*
Starting heparin		12.21 (2.27)	4.40 (0.82)	1.02 (0.14)	0.36
3.8 *M* fraction (1)	498	12.53 (2.37)	4.55 (0.86)	0.96 (0.13)	0.44
3.8 *M* fraction (2)	228	12.47 (2.35)	4.47 (0.84)	0.97 (0.14)	0.39
3.4 *M* fraction	285	12.24 (2.28)	4.39 (0.82)	0.97 (0.14)	0.37
3.0 *M* fraction	200	12.11 (2.24)	4.00 (0.74)	1.04 (0.14)	0.33
2.5 *M* fraction	109	11.78 (2.14)	4.13 (0.75)	1.04 (0.15)	0.27
2.0 *M* fraction	2.6				
Sum	1322.6				
Recovery	97.2%				

* Yields of heparin (based on uronic acid determination) were calculated from the areas of each peak in the chromatogram of Fig. 1.

** Total sulphate and N-sulphate contents as sulphur. Numbers of moles are given in parentheses.

TABLE V

BIOLOGICAL PROPERTIES OF HEPARIN FRACTIONS SEPARATED ON SEPHAROSE CL-4B AT 4°C

Heparin fraction	*Anticoagulant activity*		*Fraction separated on antithrombin III–Sepharose column (%)*		
	USP units/mg	*Thrombin-inactivation potency*	*Non-adsorbed*	*Low affinity*	*High affinity*
Starting heparin	176	146	1.5	49.2	49.3
3.8 *M* fraction (1)	67	12	19.0	64.4	16.6
3.8 *M* fraction (2)	210	97	1.6	55.6	42.9
3.4 *M* fraction	210	142	0.9	46.7	52.4
3.0 *M* fraction	234	219	1.0	27.8	71.2
2.5 *M* fraction	257	308	1.7	15.0	83.3

heparin species. The chemical and physical properties of the respective heparin fractions summarized in Table IV are very similar, although not identical with the heparin fractions previously separated on Phenyl-Sepharose CL-4B[1].

As seen in Table IV, the 3.8 *M* (2) fraction had a more similar N-acetyl content and K_{av} value to the 3.4 *M* fraction rather than to the 3.8 *M* (1) fraction. This is due to the two-step chromatographic procedure employed, the tailing region of the 3.8 *M* (1) peak comprising species similar in properties to the heparin species constituting the 3.4 *M* fraction which should form the next peak obtained by stepwise elution (note the tailing of the elution pattern from column I, in Fig. 1). On column II, the tailing region of this 3.8 *M* (1) fraction is eluted with 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid to form the 3.8 *M* (2) peak distinctly retarded compared with the 3.8 *M* (1) fraction (see the corresponding tube numbers for columns I and II, on the abscissae in Fig. 1). Accordingly, the 3.8 *M* (2) fraction should contain heparin species of similar properties to the 3.4 *M* fraction. Table V shows the anticoagulant properties of these heparin fractions and the results of affinity chromatography on an antithrombin III–Sepharose column. The heparin species low in blood-clotting activity and thrombin-inactivation potency were found to be concentrated in the 3.8 *M* (1) fraction. As a matter of course, this fraction was low in heparin species with high affinity for antithrombin III. As shown in Table IV, the 3.8 *M* (1) fraction and 3.8 *M* (2) fraction clearly differed in K_{av} values, although only slightly in chemical analytical data. However, the difference in biological activities between these fractions was much greater than the difference in chemical or physical properties in Table IV. The similarity in biological properties between the 3.8 *M* (2) fraction and the 3.4 *M* fraction shown in Table V surpassed greatly the similarity in chemical or physical properties between these fractions shown in Table IV. Of the subsequent fractions, those with higher affinity for Sepharose CL-4B gel were higher in anticoagulant activities, and the ratio of the heparin species with high affinity for antithrombin III was markedly higher. Thus, the biological activities of the heparin fractions summarized in Table V were very similar to those of the heparin fractions previously separated on a Phenyl-Sepharose CL-4B column[1], suggesting a similarity in fractionation mechanism between Sepharose CL-4B and Phenyl-Sepharose CL-4B gels.

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