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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF MUCOPOLYSACCHARIDES

EXAMINATION OF FUNDAMENTAL CONDITIONS FOR FRACTIONATION OF HEPARIN ON HYDROPHOBIC GELS

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SUMMARY

For hydrophobic interaction chromatography of mucopolysaccharides, some fundamental chromatographic conditions were examined mainly on a combination of Phenyl-Sepharose CL-4B gel and heparin. Every one of the conditions, such as column dimensions, amount of heparin applied, flow-rate, electrolyte and acidity of elution medium, and temperature, influenced the distribution of heparin among the fractions separated on the gel. The solutions of 4.0 *M*–1.0 *M* ammonium sulphate in water or in 0.01 *M* hydrochloric acid were excellent as elution media. Serious temperature effects were generally observed on the interaction between mucopolysaccharides and different types of hydrophobic gel.

Commercially available hydrophobic gels of two types were examined: (1) hydrophobic gels without any ionizable function —Phenyl- and Octyl-Sepharose CL-4B gels and Benzyl- and Octyl-Agarose gels, and (2) hydrophobic gels with some ionizable groups, such as isoureide and primary amino groups— Alkyl-Agarose and ω-Aminoalkyl-Agarose gels.

INTRODUCTION

We have reported previously on the separation of heparin into several fractions different in both chemical composition and anticoagulant activity by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B gel[1].

The present report describes an examination of fundamental conditions for hydrophobic interaction chromatography of mucopolysaccharides. Because heparin has been known to be the most heterogeneous of the molecular species among the mucopolysaccharides, the examination was mainly carried out on heparin.

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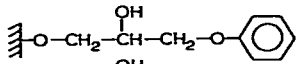
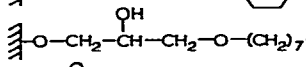
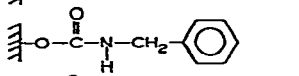
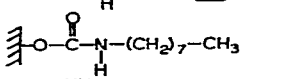
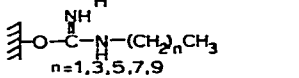
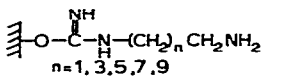
EXPERIMENTAL

Materials

The commercial hog-mucosal heparin (anticoagulant activity, 161 USP units/mg; Sigma, St. Louis, MO, U.S.A.), which contained dermatan sulphate in the amount of 3.4% (GalN/total HexN), was used throughout the examination. A heparin preparation freed from dermatan sulphate ("purified heparin") was prepared from the commercial heparin by the method of Cifonelli *et al.*[2,3]. Chondroitin 6-sulphate (sodium salt) was obtained from Seikagaku Kogyo, Japan. Various types of commercial hydrophobic gel were purchased from the suppliers listed in Table I, and their specifications are also summarized in the table. Sepharose 4B and Sepharose CL-4B gels were obtained from Pharmacia.

TABLE I

COMMERCIAL HYDROPHOBIC GELS AND THEIR SPECIFICATIONS

Hydrophobic gel	*Structure of hydrophobic ligand**		*Supplier*
Phenyl-Sepharose CL-4B	Sepharose		Pharmacia (Uppsala, Sweden)
Octyl-Sepharose CL-4B	Sepharose		
Benzyl-Agarose	Agarose		Pierce (Rockford, IL, U.S.A.)
Octyl-Agarose	Agarose		
Alkyl-Agarose**	Agarose		Miles Labs. (Elkhart, IN, U.S.A.)
ω-Aminoalkyl-Agarose**	Agarose		

* These schematic structures were based on the descriptions in refs. 12, 13 and 14.

** Products of Miles, which are those prepared by the reaction of alkylamines or ω-aminoalkylamines with agarose gels activated with cyanogen bromide, should contain imidocarbonate ($-O\diagdown\diagup O-C=N-$) and carbamate ($-O-\overset{O}{\overset{\|}{C}}-NH-$) linkages besides the indicated isoureide linkage in their structures[9].

Analytical methods

The uronic acid content of heparin was determined by the carbazole method of Bitter and Muir[4], and that of chondroitin 6-sulphate was determined by a modification of the method described above[5].

Analysis of heparin distribution among the fractions separated on hydrophobic gels under different chromatographic conditions

A small 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 distilled water, then equilibrated with 3.8 *M*

ammonium sulphate in 0.01 *M* hydrochloric acid. The indicated amount of sample 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* → 1.0 *M* ammonium sulphate solutions in 0.01 *M* hydrochloric acid. Unless otherwise indicated, the elution was performed at room temperature (21–25°C) with a flow-rate of 15 ml/h, and each of the fractions pooled (30 ml) was analysed for uronic acid content.

Separation of heparin on Phenyl-Sepharose CL-4B by reversed linear gradient elution

The purified heparin (5.1 mg) dissolved in 1 ml of 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid, was loaded on a 6 × 0.6 cm I.D. Phenyl-Sepharose CL-4B column prepared in the same solution. The column was washed with 60 ml of the same solution at 4°C or room temperature, then was eluted with a reversed linear gradient comprising 3.8 *M*–2.0 *M* ammonium sulphate in 0.01 *M* hydrochloric acid (120 ml) at 4°C or room temperature. The flow-rate was 15 ml/h, and 2.0-ml fractions were collected. A sample of 0.5 ml was taken for the carbazole reaction and ionic strength measurement.

RESULTS AND DISCUSSION

Examination of chromatographic conditions for separation of heparin on Phenyl-Sepharose CL-4B

For hydrophobic interaction chromatography of mucopolysaccharides, several chromatographic parameters were examined for heparin using Phenyl-Sepharose CL-4B as hydrophobic chromatographic medium. Because heparin is known to be comprised of diverse molecular species, differing in the content of N- and O-sulphates and of N-acetylglucosamine, and in the composition of uronic acid and also in molecular size[6], the polysaccharide was selected as a representative of mucopolysaccharides for the present study.

Amount of heparin to be applied on hydrophobic gel. In our experience, the dimensions of the column and the amount of sample applied both affect the separation of some mucopolysaccharides on hydrophobic gels. After several trials, a small column (6 × 0.6 cm I.D.) packed with 1.7 ml of Phenyl-Sepharose CL-4B and a stepwise elution with 30 ml each of 3.8 *M*–1.0 *M* ammonium sulphate solutions in 0.01 *M* hydrochloric acid were selected as a standard procedure for the following experiments. As shown in Table II, the recoveries obtained from columns that had different amounts of heparin applied were almost quantitative. The 3.8 *M* fraction is a flow-through fraction composed of heparin species without affinity for Phenyl-Sepharose CL-4B, and the 3.4 *M*–1.0 *M* fractions are those composed of heparin species with more or less affinity for the gel. Therefore, the amount of heparin in the 3.4 *M*–1.0 *M* fractions is considered to be indicative of the ability of the column to retain heparin. As indicated in the table, the amount in the 3.8 *M* fraction increased with increasing amount of heparin applied, resulting in a decrease of the ratio of the amount of heparin adsorbed to the amount applied. Thus, these data indicate that the application of excess heparin should be avoided for proper separation. In the previous work[1], the amount of heparin applied on Phenyl-Sepharose CL-4B was 2.96 mg per millilitre of gel. These data also show that no irreversible adsorption of heparin on the gel occurred, irrespective of the amount of sample applied.

TABLE II

RELATION BETWEEN AMOUNTS OF HEPARIN APPLIED ON PHENYL-SEPHAROSE CL-4B COLUMN AND HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED

The indicated amount of heparin was applied on the column and chromatographed at 21–25°C.

Heparin applied		*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed* (3.4 M–1.0 M)*	
mg	*mg/ml of gel*	*$(NH_4)_2SO_4$–0.01 M HCl*							*mg*	*mg/ml of gel*
		3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*			
0.85	0.5	40.8	31.6	19.7	2.7	1.9	3.4	100.0	0.50	0.30
1.7	1	55.3	25.3	12.7	3.0	2.2	1.6	104.7	0.80	0.47
5.1	3	56.4	27.7	12.4	2.3	0.7	0.6	98.7	2.22	1.31
10.2	6	69.9	19.9	8.4	0.6	1.1	0	103.3	3.06	1.80
17.0	10	80.4	11.6	5.6	1.2	1.2	0	95.8	3.33	1.96

* Total amount of heparin eluted with 3.4 *M*–1.0 *M* $(NH_4)_2SO_4$ in 0.01 *M* HCl.

Effect of acidification of elution media. The mucopolysaccharides are one of groups of natural anionic polyelectrolytes, and their structures are apparently lack hydrophobicity. Our previous studies on the hydrophobic interaction chromatography of heparin[1] and depolymerized chondroitin[7] indicated that the affinity of these polysaccharides for hydrophobic gels mainly depends on the number of N-acetyl groups in the polysaccharide molecules, necessarily relating to their molecular size. An electrolyte, such as ammonium sulphate, present in an elution medium in high concentration would increase the total hydrophobicity of large molecular polyelectrolytes, such as proteins and mucopolysaccharides. Acidification of a medium containing some anionic ionizable polyelectrolyte, such as heparin, was expected to increase its hydrophobicity owing to protonation of a number of carboxylic and sulphate groups in its molecule. The data listed in Table III substantiate this expectation, indicating an evident increase in the amount of heparin retained on the gel following acidification of the elution media. An increase in the amount of mucopolysaccharide retained would favour a proper separation.

Effect of different electrolytes in elution medium. The elution system of 3.8 *M*–1.0 *M* ammonium sulphate, which had been used in the chromatography of heparin[1] and depolymerized chondroitin[7], was compared with those of 3.8 *M*–1.0 *M* ammonium acetate, ammonium chloride, and sodium chloride. As shown in Table IV, no retention of heparin on Phenyl-Sepharose CL-4B occurred in the solutions containing electrolytes other than ammonium sulphate. It is not clear why a part of heparin or chondroitin (also dermatan sulphate and chondroitin 6-sulphate, see *Materials* and the data in Table VI) are retained by the gel exclusively in the solutions containing ammonium sulphate.

Effect of flow-rate. Separation of heparin was carried out at three different flow-rates. As shown in Table V, the 3.8 *M* fraction, a flow-through fraction, slightly increased in amount with increasing flow-rate. Recoveries among the three experiments were almost equal. In practice, a flow-rate of 9 ml/h per millilitre of gel is recommended.

TABLE III

EFFECT OF ACIDIFICATION OF ELUTION MEDIUM ON HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON PHENYL-SEPHAROSE CL-4B

Heparin (5.1 mg) was applied on the column and chromatographed at 21–25°C.

Elution medium	*pH at 3.8 M concentration*	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed (3.4 M–1.0 M, %)*
		Concentration of $(NH_4)_2SO_4$							
		3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
$(NH_4)_2SO_4$–H_2O	5.2	79.5	17.6	2.9	0	0	0	102.4	20.5
$(NH_4)_2SO_4$–0.01 *M* HCl	3.5	56.4	27.7	12.4	2.3	0.7	0.6	98.7	43.7

TABLE IV

EFFECT OF ELECTROLYTE IN ELUTION MEDIUM ON HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON PHENYL-SEPHAROSE CL-4B

Heparin (5.1 mg) was applied on the column and chromatographed at 21–25°C.

Elution medium	*pH at 3.8 M concentration*	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*
		Concentration of electrolyte						
		3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*	
$(NH_4)_2SO_4$	5.2	79.5	17.6	2.9	0	0	0	98.1
CH_3COONH_4	7.5	100	0	0	0	0	0	102.5
NH_4Cl	4.45	100	0	0	0	0	0	95.5
NaCl	6.3	100	0	0	0	0	0	104.5

TABLE V

EFFECT OF FLOW-RATE ON HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON PHENYL-SEPHAROSE CL-4B

Heparin (5.1 mg) was applied on the column and chromatographed at 17°C.

Flow-rate (ml/h per ml of gel)	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed (3.4 M–1.0 M, %)*
	$(NH_4)_2SO_4$–0.01 M HCl							
	3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
18	52.4	28.9	15.4	1.2	1.5	0.6	99.3	47.6
9	49.6	31.4	17.1	0.6	1.4	0	100.1	50.5
3	46.9	31.9	16.5	3.0	1.2	0.5	104.0	53.1

Effect of temperature. Temperature may influence, in a positive way, the adsorption of proteins on hydrophobic matrices owing to an entropy effect. However, the temperature may also provoke other effects, such as increased solvation and decreased surface tension, which may reduce the ability of molecules such as proteins to interact with hydrophobic matrices[8]. In the case of mucopolysaccharides, as shown in Table VI, the amount of heparin retained on the gel markedly increases as the temperature is lowered. When 10 mg of heparin per millilitre of gel were applied on the column at 4°C, the amount of heparin retained was 4.55 mg/ml of gel (*cf.* the heparin retained at 21–25°C (1.96 mg/ml of gel) in Table II). Although data are not shown, an excellent reproducibility was obtained on the separation of heparin at 4°C, when 3 mg of heparin per millilitre of gel were applied.

Separation of bovine serum albumin on Phenyl-Sepharose CL-4B was performed at 4°C or room temperature (21–25°C) by successive elution with 1.0 *M*–0.1 *M* ammonium sulphate (pH 5.2–5.5), 0.01 *M* phosphate buffer (pH 6.8), and 10–40% ethanol in 0.01 *M* phosphate buffer. The results obtained showed no sign of the effect of temperature that was observed with heparin (data not shown). To confirm whether the latter effect is common to other mucopolysaccharides, separation of chondroitin 6-sulphate on Phenyl-Sepharose CL-4B was carried out at different temperatures, and

a remarkable increase in the affinity at a lower temperature was again detected (Table VI). The temperature effect, which is possibly specific to the mucopolysaccharides, seems to be due to a temperature-dependent fluctuation of individual hydrophobic interaction between the hydrophobic elements common to these polysaccharide structures (such as the N-acetyl group) and hydrophobic ligands on the gel matrices.

The purified heparin, which was freed from contaminant dermatan sulphate, was chromatographed on Phenyl-Sepharose CL-4B with a reversed linear gradient comprising 3.8 *M*–2.0 *M* ammonium sulphate in 0.01 *M* hydrochloric acid at 4°C or room temperature (21–25°C). As shown in Fig. 1, the heparin distribution between the non-adsorbed (3.8 *M*) and adsorbed (3.4 *M*–2.0 *M*) fractions obtained at 4°C was largely different from that at room temperature. We had reported that most of the contaminant dermatan sulphate in commercial heparin was concentrated in the fraction eluted with 2.0 *M* ammonium sulphate by stepwise elution on Phenyl-Sepharose CL-4B column at room temperature[1]. Both elution diagrams of Fig. 1 indicate that any material positive to the carbazole reaction was not found in the fractions less than

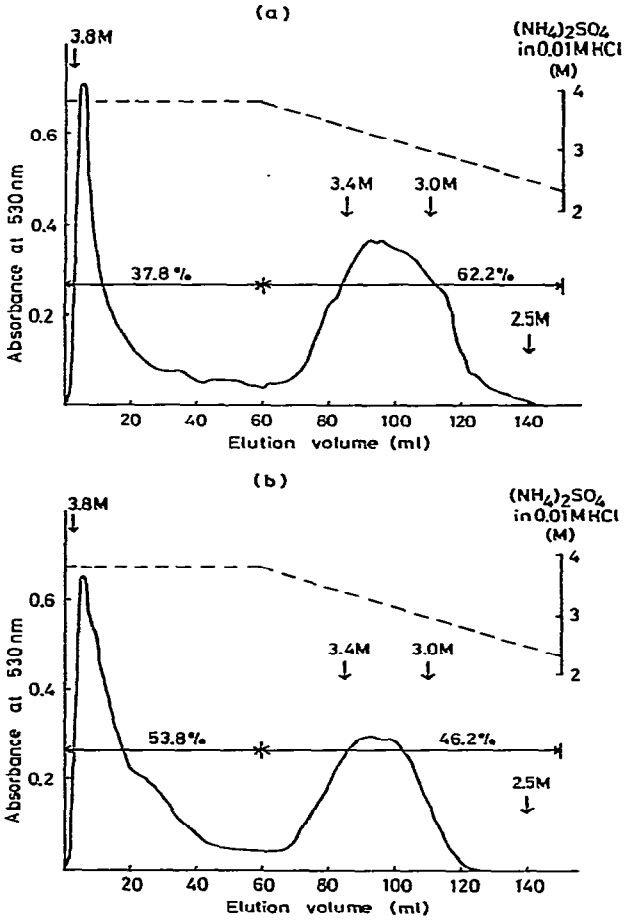


Fig. 1. Separation of heparin on Phenyl-Sepharose CL-4B by reversed linear gradient elution. (a) Elution diagram at 4°C. (b) Elution diagram at room temperature (21–25°C).

TABLE VI

EFFECT OF TEMPERATURE ON HEPARIN AND CHONDROITIN 6-SULPHATE DISTRIBUTIONS AMONG FRACTIONS SEPARATED ON PHENYL-SEPHAROSE CL-4B

Temp. (°C)	*Amount applied*		*Heparin or chondroitin 6-sulphate eluted (% of total amount recovered)*							*Recovery of heparin or chondroitin 6-sulphate eluted (%)*	*Heparin adsorbed (3.4 M–1.0 M)*	
	mg	*mg/ml of gel*	*$(NH_4)_2SO_4$–0.01 M HCl*								*mg*	*mg/ml of gel*
			3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.5 M*	*1.0 M*			
Heparin												
50	5.1	3	91.2	6.7	1.3	0.9	0		0	104.0	0.45 (8.9)*	0.26
30	5.1	3	70.5	18.4	8.3	1.3	1.6		0	103.5	1.51 (29.6)	0.89
21–25	5.1	3	56.4	27.7	12.4	2.3	0.7		0.6	98.7	2.23 (43.7)	1.31
4	5.1	3	28.7	28.4	30.0	11.1	1.0		0.8	102.0	3.65 (71.3)	2.15
4	17	10	54.5	12.3	19.7	11.1	2.4		0	103.6	7.74 (45.5)	4.55
Chondroitin 6-sulphate												
21–25	5.1	3	0	0	0.3	5.1	42.9	49.9	1.7	97.7		
4	5.1	3	0	0	0	0	27.3	58.5	14.2	103.0		

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

2.5 *M* in ammonium sulphate concentration, suggesting that there was no contamination with dermatan sulphate in the purified heparin used.

Examination of repeated use of gels. Affinity between hydrophobic gels and mucopolysaccharides seemed to be so feeble that it was sensitive to various factors as tested above, and the hydrophobic gels available commercially are fairly expensive at present. For these reasons, variations in the affinity between heparin and Phenyl-Sepharose CL-4B gel following repeated use of the gel were examined. The data in Table VII obtained at 30°C show that the affinity of heparin for the gel tends to decrease with repeated use. Regeneration of the used gel was performed by the washing procedure described in ref. 14. If any appropriate procedure for washing is devised, a perfect recovery of the intrinsic hydrophobicity of gels may be possible. In either case, it should be remembered that hydrophobic gels have a tendency to decrease in affinity following repeated use.

TABLE VII

CHANGE OF HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON REPEATEDLY USED PHENYL-SEPHAROSE CL-4B

Heparin (5.1 mg) was applied on the column and chromatographed at 30°C.

*Number of times used**	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed (3.4 M–1.0 M, %)*
	$(NH_4)_2SO_4$–0.01 M HCl							
	3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
1	69.8	19.4	8.2	1.3	1.4	0	102.9	30.3
2	74.7	15.6	7.1	1.1	1.5	0	101.3	25.3
3	77.7	14.3	5.0	1.9	1.0	0	99.1	22.2

* After washing successively with distilled water (200 ml), 1 *M* sodium chloride (200 ml), and distilled water (200 ml), the used gels were re-equilibrated with 3.8 *M* ammonium sulfate in 0.01 *M* hydrochloric acid ready for further use.

Examination of chromatographic characteristics of different types of hydrophobic gel

Separation of heparin on commercially available hydrophobic gels. Specifications of the hydrophobic gels obtained are summarized in Table I. The products of Pharmacia and of Pierce, the hydrophobic ligands of which were bound to gel matrices through a glycidyl ether or carbamate linkage, contain none of ionizable group in their structures. On the other hand, the products of Miles, which were prepared by the reaction of alkyl and ω-aminoalkyl amines with the agarose gel activated by cyanogen bromide, usually contain isoureide linkages on the gel matrices to link the hydrophobic ligands, such as alkyl and ω-aminoalkyl groups[9]. Because the isoureide groups are ionizable at the pH values used, the products of Miles are able to function as anion-exchangers as well as hydrophobic chromatographic media.

To examine the affinity of mucopolysaccharides for the gel matrix without any hydrophobic ligand, fractionation of heparin on Sepharose 4B or Sepharose CL-4B was carried out under the conditions indicated in Table VIII. The data obtained show that a part of the heparin applied (3.2%) was retained on the Sepharose 4B column in the solution of 3.8 *M* ammonium sulphate in 0.01 *M* hydrochloric acid. Because the

TABLE VIII

HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON DIFFERENT TYPES OF HYDROPHOBIC GEL

Heparin (5.1 mg) was applied on the column and chromatographed at 21–25°C.

Gel tested	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed (3.4 M–1.0 M, %)*
	$(NH_4)_2SO_4$–0.01 M HCl							
	3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
Sepharose 4B	96.8	0.5	0.5	0.9	1.3	0	94.0	3.2
Sepharose CL-4B	83.3	12.1	3.8	0.8	0	0	101.6	16.7
Phenyl-Sepharose CL-4B	56.4	27.7	12.4	2.3	0.7	0.6	98.7	43.7
Octyl-Sepharose CL-4B	67.4	21.7	8.7	1.2	0.7	0.4	101.7	32.7
Benzyl-Agarose	37.0	31.9	26.1	4.2	0.9	0	94.0	63.1
Octyl-Agarose*	43.7	36.1	17.6	1.8	0.8	0	100.3	56.3
Octyl-Agarose**	28.5	23.9	26.7	15.5	4.2	1.2	99.5	71.5
ω-Amino-octyl-Agarose	31.3	24.5	23.2	16.8	4.2	0	99.1	68.7

* Product of Pierce.
** Product of Miles Labs.

purified heparin, free of dermatan sulphate, was almost quantitatively eluted from the column with the same solvent system, most of the material retained on Sepharose 4B gel was assumed to be dermatan sulphate which had been contained in the commercial heparin. On the other hand, Sepharose CL-4B gel retained distinctly a part of the heparin species, suggesting a possible contribution of the structure –O–CH_2–CH(OH)–CH_2–O– at the cross-linkages in the gel matrix to the observed hydrophobic interaction.

The affinity of heparin for Phenyl-Sepharose CL-4B was always a little higher than that for Octyl-Sepharose CL-4B, as mentioned previously[1]. Benzyl- and Octyl-Agarose (Pierce), the hydrophobic ligands of which were bound to agarose gel through a carbamate linkage (supposedly formed by the use of agarose activated with carbonyl di-imidazole), were superior in retaining heparin to Octyl- or Phenyl-Sepharose CL-4B (Pharmacia).

Octyl-Agarose and ω-Amino-octyl-Agarose (Miles), the *n*-octyl or ω-amino-octyl group of which was bound to agarose gel through a positively ionizable linkage, mainly isoureide, were comparable in retaining heparin to the gels carrying hydrophobic ligands through a carbamate linkage. Comparing the distributions of heparin among the 3.4 *M*–1.0 *M* fractions separated on Octyl-Agarose and ω-Aminooctyl-Agarose (Miles) with those on Benzyl-Agarose and Octyl-Agarose (Pierce), the 2.5 *M* fraction obtained from the former gels contained more heparin than that from the latter gels. The data in Table VIII indicate that an increase in the amount of heparin eluted with 2.5 *M* ammonium sulphate in 0.01 *M* hydrochloric acid seems to be common to the gels carrying positively ionizable hydrophobic ligands (*cf.* the data in Table IX). In either case, the data in Table VIII indicate that the affinity for heparin of hydrophobic gels carrying ionizable groups mainly depends on the hydrophobic interaction between them under the conditions used.

Separation of heparin on Alkyl-Agarose gels of different alkyl chain lengths. The effect of the alkyl chain length on the heparin distribution among the fractions separated on hydrophobic gels substituted with different alkyl groups was examined using a series of Alkyl-Agarose gels (Miles). Table IX shows that Agarose itself retains some heparin (6.2%). As described above (Sepharose 4B in Table VIII), it was found that agarose retained scarcely any heparin species, but did retain contaminant dermatan sulphate in the commercial heparin used (data not shown).

TABLE IX

HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON ALKYL-AGAROSE GELS OF DI FERENT ALKYL CHAIN LENGTHS

Heparin (3 mg) was applied on a column (1.3 × 0.75 cm I.D., 1.0 ml of gel) packed with the gel to be tested, and w eluted stepwise with the elution media indicated at 21–25°C.

*Alkyl-Agarose**	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed (3.4 M–1.0 . %)*
	$(NH_4)_2SO_4$–0.01 M HCl							
	3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
Agarose*	93.7	3.7	0.8	0.1	1.3	0.3	97.0	6.2
Ethyl-Agarose	37.5	14.9	20.3	20.3	6.2	0.9	100.1	62.6
n-Butyl-Agarose	27.1	15.7	24.5	24.3	6.9	1.5	103.5	72.9
n-Hexyl-Agarose	28.5	16.8	24.2	23.2	6.6	0.6	98.6	71.4
n-Octyl-Agarose	27.5	23.8	22.8	20.1	4.4	1.4	99.7	72.5
n-Decyl-Agarose	48.1	21.8	18.1	11.0	1.0	0	105.1	51.9

* Products of Miles Labs.

The retention on Ethyl-Agarose was greater than or comparable with that on the gels of Pharmacia and of Pierce. According to Shaltiel and Halperin[10], the density of charge (microequivalents per millilitre of settled gel) on the gels carrying alkyl groups through a positively ionizable linkage (for example, the products of Miles) increases with decreasing alkyl chain length. Although the degree of participation of the ionic interaction to the affinity between heparin and Ethyl-Agarose is not clear, the contribution of the hydrophobic interaction between them seems to be considerable, considering that the heparin retained on the gel was quantitatively eluted with solutions of decreasing concentration of ammonium sulphate. The data for Ethyl-Agarose also indicate that the alkyl group to be substituted on agarose gel does not need to be a higher one for the retention of heparin. Heparin distributions among the fractions separated on *n*-Butyl-, *n*-Hexyl- and *n*-Octyl-Agarose gels were almost equivalent to each other. If any effect due to ionization at the linkage region of these gels can be ignored under the conditions used, the data of Table IX show that there is little variation in hydrophobic interaction with heparin among the C_4, C_6 and C_8 alkyl groups. As can be seen in the table, there is a decrease in the retention of heparin on *n*-Decyl-Agarose. A marked increase in the amount in the 3.8 *M* ammonium sulphate fraction clearly indicates that the *n*-decyl group is not suitable as a hydrophobic ligand to interact with heparin, contrary to the case of proteins[11].

Effect of temperature on separation of heparin with different types of hydro-

TABLE X

EFFECT OF TEMPERATURE ON HEPARIN DISTRIBUTION AMONG FRACTIONS SEPARATED ON DIFFERENT TYPES OF HYDROPHOBIC GEL

Heparin (5.1 mg) was applied on the column, and the operation was carried out according to the standard procedure.

Hydrophobic gel	*Temp. (°C)*	*Heparin eluted (% of total amount recovered)*						*Recovery of heparin eluted (%)*	*Heparin adsorbed (3.4 M–1.0 M, %)*
		$(NH_4)_2SO_4$–0.01 M HCl							
		3.8 M	*3.4 M*	*3.0 M*	*2.5 M*	*2.0 M*	*1.0 M*		
Octyl-Agarose*	21–25	43.7	36.1	17.6	1.8	0.8	0	100.3	56.3
	4	17.2	25.7	41.3	12.6	2.2	1.0	104.4	82.8
Octyl-Agarose**	21–25	28.5	23.9	26.7	15.5	4.2	1.2	99.5	71.5
	4	5.8	10.1	20.7	29.3	22.8	11.2	98.4	94.1
ω-Amino-octyl-Agarose**	21–25	31.3	24.5	23.2	16.8	4.2	0	99.1	68.7
	4	5.2	8.0	19.3	26.8	30.7	9.9	104.8	94.7

* Product of Pierce.

** Products of Miles Labs.

phobic gel. The data in Table VI indicated a notable temperature effect on the retention of heparin with Phenyl-Sepharose CL-4B. Three kinds of gel with different hydrophobic ligands or different structures of the linkage region were examined for the effect of temperature. As shown in Table X, the amount of heparin retained by these gels markedly increased at 4°C. Octyl-Agarose and ω-Amino-octyl-Agarose (Miles) showed similar patterns of heparin distribution at 4°C and room temperature, but they were different from that of Octyl-Agarose (Pierce), especially for the 2.0 *M* and 1.0 *M* ammonium sulphate fractions. On the other hand, the variation in heparin distribution due to the difference in temperature was very close in the cases of Phenyl-Sepharose CL-4B and Octyl-Agarose (Pierce). The data in Tables VI and X suggest that a proper combination of hydrophobic gel and operating temperature would make a promising separation of heparin and other mucopolysaccharides possible.

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REFERENCES

1 A. Ogamo, H. Uchiyama and K. Nagasawa, *Biochim. Biophys. Acta*, 626 (1980) 477.
2 J. A. Cifonelli and J. King, *Biochim. Biophys. Acta*, 320 (1973) 331.
3 L. Rodén, J. Baker, J. A. Cifonelli and M. B. Mathews, *Methods Enzymol.*, 28 (1972) 73.
4 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330.
5 M. Kosakai and Z. Yoshizawa, *Anal. Biochem.*, 93 (1979) 295.
6 U. Lindahl, *Int. Rev. Sci., Org. Chem.*, 7 (1976) 283.
7 H. Uchiyama, A. Ogamo and K. Nagasawa, *Carbohydr. Res.*, in press.
8 J.-L. Ochoa, *Biochimie*, 60 (1978) 1.
9 R. Axén and S. Ernback, *Eur. J. Biochem.*, 18 (1971) 351.
10 S. Shaltiel and G. Halperin, in E. Hoffman, W. Pfeil and H. Aurich (Editors), *FEBS 12th Meeting, Dresden, Protein: Structure, Function and Industrial Applications*, Vol. 52, Pergamon Press, Oxford, 1978, pp. 441–451.
11 S. Shaltiel, in R. Epton (Editor), *Chromatography of Synthetic and Biological Polymers*, Vol. 2, Ellis Horwood, Chichester, 1978, pp. 13–41.
12 S. Hjertén, J. Rosengren and S. Påhlman, *J. Chromatogr.*, 101 (1974) 281.
13 Pierce Chemical Co., *Protein Digest*, Vol. 4, No. 1, 1980, p. 11.
14 *Miles Biochemicals 1979/80*, Miles Labs., Elkhart, IN, 1979, p. 138.