Biological implications of the structural, antithrombin affinity and anticoagulant activity relationships among vertebrate heparins and heparan sulphates

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We analysed the distribution, structural characteristics, antithrombin-III-binding properties and anticoagulant activities of heparins and heparan sulphates isolated from the tissues of a wide range of vertebrates. Heparin has a curiously limited distribution, since it was absent from lower aquatic vertebrate species, present in only certain organs such as intestine in many higher vertebrates, and completely absent from the rabbit among mammals examined. The heparins were structurally diverse, and they exhibited a broad range of anticoagulant activities, from approx. 50% to 150% of average commercial heparins. Although there was a rough correlation between the anticoagulant potency of the starting isolate and the proportional content of material exhibiting high-affinity binding to the proteinase inhibitor antithrombin III, activities of high-affinity fractions from heparins low in activity overlapped those of low-affinity fractions from highly active heparins. Heparan sulphates, which in contrast were isolated from nearly all vertebrate organs, contained high-affinity subfractions constituting up to 5% of the starting material and possessing anticoagulant potencies of 2–30 units/mg. In consideration of the heparin data, we infer that (1) its biological function is either species-specific or may be served by other molecular elements, and that (2) there exists considerable diversity in the antithrombin-III-binding sequence of heparin. The more-generally distributed glycosaminoglycan heparan sulphate possesses within its variable structure a small high-affinity subfraction with low anticoagulant potency, whether isolated from aorta or other tissues. Although heparan sulphate appears to have an essential function at the cellular level, we suggest that this is probably not that of providing heparin-like antithrombotic effects on vascular surfaces.

INTRODUCTION

Glycosaminoglycans (GAGs), a group of highly charged anionic polysaccharides, occur in most animal species. They constitute seven major polymers that are closely related in structure, but, as they have maintained their identity and vary in distribution, it is likely that each has distinctive biological functions. These functions, with perhaps the exception of cartilage proteoglycans, remain fairly elusive, though many of them are probably mediated by their strong negative charge. Recent investigative effort has focused on possible involvement of these substances in cell growth and differentiation. In this regard, the specific GAG heparan sulphate has been shown to exist as an intercalated cell-membrane proteoglycan (Kjellén *et al.*, 1981), at which location it may play a role in the mediation of cell–extracellular-matrix interactions (Lark *et al.*, 1985) or may function as a receptor for soluble factors such as transferrin (Fransson *et al.*, 1984). A crucial function in cell growth control may be inferred for heparan sulphate from the large amount of evidence showing a molecular perturbation in this polysaccharide consequent to spontaneous or induced neoplastic transformation of a number of cell lines (for review see Höök *et al.*, 1984).

Heparin merits separate consideration from the other

GAGs. It is more highly charged, appears to occur entirely in certain mast cells, and has the distinctive property of being an effective anticoagulant. Heparin has, however, many structural features in common with the heparan sulphates, which have a much more ubiquitous animal and tissue distribution. Linkage types, the presence of L-iduronic and D-glucuronic acid, *N*-acetylglucosamine and *N*-sulphated glucosamine are common to both. Biosynthetic pathways may also be similar. Despite these common features, there appears to be no complete structural overlap between these two groups of polymers. The more pertinent question is whether or not there is an overlap in biological function.

The present paper corroborates existing literature (Toledo & Dietrich, 1977; Gomes & Dietrich, 1982), which has shown that many animal species contain heparan sulphate but relatively few contain heparin. Even among the mammals it is possible to identify a species, the rabbit, that appears not to synthesize detectable amounts of heparin. Furthermore, in some species, such as oxen, heparin occurs in a large number of organs, whereas in others it is restricted to but a few. The hypothesis emerges, therefore, that the biological function(s) is usually served by heparan sulphate, and only on occasion by heparin. The anticoagulant properties of heparin (at that carried by only 25–30% of

Abbreviations used: GAG, glycosaminoglycan; AT III, antithrombin III; LA and HA fractions, low-affinity and high-affinity fractions respectively.
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the chains in a chemically pure polymer) may have obscured the true biological relationships between these two GAGs. Further evidence is herein presented supporting the existence within well-defined heparan sulphate preparations of small subfractions that bind to antithrombin III (AT III) and have some anticoagulant potency (Marcum & Rosenberg, 1984). We also extend the work on the AT III affinities and anticoagulant potencies of heparins, which to the present time have been limited to pig mucosal, ox lung and whale heparins, to preparations isolated from a number of other sources. Our results suggest that previous research has led to a simpler view than the complex mechanisms of AT III binding and anticoagulation effects may require.

MATERIALS AND METHODS

Materials

Chondroitinase ABC was obtained from Miles Scientific Co. (Naperville, IL, U.S.A.), testicular hyaluronidase from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and liver heparin from ICN Biochemicals (Cleveland, OH, U.S.A.). We acknowledge Dr. Thomas H. Taaffee and the Upjohn Co. (Kalamazoo, MI, U.S.A.) for providing the ox lung heparin and Dr. Zensaku Yosizawa (Tohoku University, Sendai, Japan) for providing the whale heparin. Heparitinase and heparinase were prepared as previously described (Linker & Hovingh, 1972).

Isolation of GAGs

GAGs were isolated as described previously (Linker & Hovingh, 1973). Briefly, acetone-dried tissues were treated with papain and Pronase, fractionated with cetylpyridinium chloride and NaCl, treated with trichloroacetic acid and Benedict reagent (to remove dermatan sulphate), and chromatographed on an anion-exchange column [AG-1 X2 (Cl⁻ form)] with stepwise NaCl gradients. Contaminating chondroitin sulphates and hyaluronic acid, if detected in the preparations, were removed by selective enzymic degradation. The final preparative step was ethanol fractionation. For minimal detection limits of these isolation methods, which depended upon the weight of the starting material, see the Results section.

Analytical procedures

The analytical methods applicable to hexosamines and uronic acids were essentially as described previously (Linker & Hovingh, 1973). For sulphate analysis see Terho & Hartiala (1971), and for *N*-acetyl analysis see Radhakrishnamurthy *et al.* (1968). Cellulose acetate electrophoresis was adapted from Seno *et al.* (1970). Column elution patterns were monitored by uronic acid analysis (Blumenkrantz & Asboe-Hansen, 1973). Identification of specific GAGs was obtained by the use of chondroitinase ABC, testicular hyaluronidase, heparinase and heparitinase (Linker & Hovingh, 1972) and by HNO₂ deamination under the low-pH conditions recommended by Shively & Conrad (1976).

Sources of animals

The animal species were obtained from the following sources. (1) Cephalaspidomorphi: lamprey (*Petromyzon marinus*) adults, John H. Howell, Hammond Bay

Fisheries, Millersburg, MI, U.S.A.; the ammocoetes, Bill Gaylord, Ludington, MI, U.S.A.; both through the courtesy of the U.S. Fish and Wildlife Service. (2) Chondrichthyes: shark (*Squalus acanthias*), Frank M. Mercker, Arrowac Fisheries, Fernsdales, WA, U.S.A. (3) Osteichthyes: northern pike (*Esox lucius*), caught by P. H. in the Northwest Territory, Canada; whitefish (*Coregonus clupeaformis*), Edwin Morin, Hay River, Northwest Territory, Canada; arctic char (*Salvelinus alpinus*), Roger Connally, Office of Economic Development and the Ekaloktotiak Eskimo Co-op, Cambridge Bay, Northwest Territory, Canada; carp (*Cyprinus carpio*) and white bass (*Morone chrysops*), Utah Division of Wildlife Resources, Salt Lake City, UT, U.S.A.; rock fish (*Sebastes marinus*), Roger Foulour, Caraquet Fisheries, Caraquet, New Brunswick, Canada. (4) Amphibia: bullfrogs (*Rana catesbeiana*), Jacques Weil Co., Rayne, LA, U.S.A.; tiger salamanders (*Ambystoma tigrinum*), collected by P. H. in the Wasatch National Forest, UT, U.S.A.; Mexican axolotls (*Ambystoma mexicanum*), George M. Malacinski, Department of Biology, Indiana University, Bloomington, IN, U.S.A. (5) Reptilia: sea turtle (*Chelonia mydas*), Harold H. Hirth, Department of Biology, University of Utah, Salt Lake City, UT, U.S.A.; rattlesnake (*Crotalus atrox*), Jim Glenn, Veterans Administration Hospital, Salt Lake City, UT, U.S.A. (6) Aves: starling (*Sturnus vulgaris*) and cowbird (*Molothrus ater*), James Mattsson, U.S. Fish and Wildlife Service, Lansing, MI, U.S.A. (7) Mammalia: wolf (*Canis lupus*), Jack Van Camp, Fish and Wildlife Service, Fort Smith, Northwest Territory, Canada; coyote (*Canis latrans*), Robert Reynolds, Animal Damage Control, U.S. Fish and Wildlife Service, Salt Lake City, UT, U.S.A.; harbour seal (*Phoca vitulina*), Thomas Poulter, Stanford Research Institute, Menlo Park, CA, U.S.A.; mule deer (*Odocoileus hemionus*), Utah Division of Wildlife Resources, Salt Lake City, UT, U.S.A.; pronghorn antelope (*Antilocapra americana*), Wesley Skidmore, Brigham Young University, Provo, UT, U.S.A.; mink, Peggy and George Mulligan, Salt Lake City, UT, U.S.A.; pig, sheep, ox, rabbit and goat, local abattoir and commercial suppliers; dog and human tissues, Veterans Administration Hospital, Salt Lake City, UT, U.S.A.

AT III affinity chromatography

Affinity chromatography of heparins and heparan sulphates utilized ox AT III, which had been prepared by the method of Thaler & Schmer (1975); the AT III, complexed with pig mucosal heparin to block the binding site(s) on this serine proteinase inhibitor, was coupled to isocyanate-activated cellulose by Dr. G. Schmer (University of Washington, Seattle, WA, U.S.A.) and kindly provided to us. Before its use for the fractionation of our GAG isolates, the heparin bound to the affinity matrix was eluted with a high-salt buffer. The matrix was determined by us to have a capacity of approx. 2.5 mg of heparin per ml of hydrated gel.

The affinity fractionation was conducted on columns of different volumes depending on the weight of the starting material. Except where stated otherwise, the ratio of approx. 5 mg of heparin or heparan sulphate per ml of hydrated gel was used. Each sample, dissolved in less than 1 ml of start buffer (0.05 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.5), was applied to the column, and the low-affinity (LA) fractions were eluted with 30 ml of start buffer. Then

Table 1. Presence of heparin in selected animal tissues

Source	Heparin content (mg/100 g dry wt. of tissue)				
	Stomach	Intestine	Liver	Lung	Kidney
Lamprey		< 1	< 1	< 0.1*	
Shark	< 0.1	< 1	< 0.1		
Pike	< 0.1	< 0.1	< 0.1	< 1*†	
Whitefish			< 0.1	< 0.1*†	
Arctic char	< 0.1	< 0.1	< 0.1	< 0.1*†	
Carp		< 1		< 1*†	
White bass	< 1	< 10	< 1	< 1*, < 10†	
Rock fish		< 1	< 0.1	< 1*†	
Bullfrog	< 1	46	< 0.1	57	
Tiger salamander‡	< 1	< 1	< 1	< 10	
Mexican salamander§	< 10	< 10	< 1	< 10	
Sea turtle	< 1	< 1	< 0.1	< 1	
Rattlesnake	< 0.1	< 1	8	< 1	
Starling	< 1	< 1	< 1	< 1	
Cowbird		< 1	< 1	< 1	
Man		< 0.1		2	
Wolf	22	29	5	13	< 0.1
Coyote	136	50	28	34	< 1
Dog	18	49	27	39	< 1
Mink		89	< 0.1	< 0.1	< 1
Harbour seal	1	2	20	< 1	< 1
Rabbit	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Pig	< 1	40	< 0.1	< 1	< 1
Mule deer	< 0.1	52	< 0.1	< 1	< 1
Antelope		18	< 0.1	< 0.1	
Goat	< 1	50	< 0.1	< 1	< 1
Sheep	< 0.1	32	< 0.1	< 1	< 1
Ox		45	10	48	14

* Gills.

† Air (swim) bladder.

‡ Aquatic young of the year. Less than 1 mg/100 g dry wt. in the gills. No detectable heparin in throat or abdominal skin.

§ Less than 1 mg/100 g dry wt. in the gills. Heparin (47 mg/100 g dry wt.) was isolated from the throat skin, and no heparin was detected in the abdominal skin. Heparin was similarly found in the adult terrestrial tiger-salamander throat skin.

the high-affinity (HA) fractions were eluted with linear 0.05–2.5 M-NaCl gradients (in 0.05 M-Tris/HCl buffer, pH 7.5) at a flow rate of 0.5 ml/min, 3–4 ml fractions being collected at 4 °C.

Anticoagulation assays

Anticoagulant potency of heparin and heparan sulphate preparations was determined by the activated partial thromboplastin time (Proctor & Rapaport, 1961), with as the standard a pig mucosal heparin (Sigma Chemical Co.) assumed to have the anticoagulant activity specified by the manufacturer (168 units/mg by the U.S.P. J-A standard). Selected preparations were also tested by the anti-(Factor Xa) assay with the chromogenic substrate S-2222 (Coatest Heparin; KabiVitrum, Stockholm, Sweden); this assay utilizes normal human plasma as one of the reagents.

RESULTS

Presence of heparin

In examining the distribution of heparin in vertebrates (Table 1), several general observations emerged. First, heparin may not be present in lower aquatic vertebrates

(ordinary teleost fish, sharks and lampreys). Of the ordinary teleost fish examined, the rock fish is of marine origin, the pike, whitefish and char are cold-water fishes, and the carp and white bass are warm-water species. Secondly, with the exception of the ox, the hoofed herbivores contained heparin only in intestine, of those tissues examined. Thirdly, the bullfrog and salamander adults both contained heparin in rich yields in some organs, whereas the two reptiles and the two birds examined contained either no heparin or only small amounts. Finally, a comparison of the material isolated from wolf, coyote and dog showed that two of the animals contained similar amounts of heparin in the same organ whereas the third either had much more or much less in that organ. We confirmed that the rabbit does not have any measurable heparin and that man has very little of this material in the examined organs (intestine and lung).

An analysis of the more interesting heparins is presented in Table 2. The data show that the sulphate content varied from 18 to 35%, iduronic acid content from 45 to 80%, and indole hexosamine from 16 to 28%. Acetyl content in each was low (less than 1.4%). Dog and ox lung heparins were chemically quite similar. Wolf, coyote and dog heparins were structurally the most

Table 2. Analysis of heparin from different sources

Source	Composition (% , w/w)					
	Uronic acid	Iduronic acid*	Sulphate	Acetyl	Hexosamine†	
					Total	N-Sulphate
Wolf intestine 0–30‡	34	60	18	0.2	18	23
Wolf intestine 30–50‡	33	60	24	0.8	19	25
Dog intestine	26	55	25	0.4	19	25
Coyote intestine	42	65	23	0.6	21	28
Wolf stomach 0–30‡	35	45	24	0.6	19	19
Wolf stomach 30–50‡	30	55	24	1.4	17	16
Dog lung	38	80	35	0.4	17	16
Coyote liver 0–30‡	46	70	32	0.5	19	27
Coyote liver 30–50‡	45	65	25	0.7	19	24
Oxen lung§	38	70	34	0.4	20	27
Oxen liver§	40	55	31	1.8	21	26
Whale§	34	60	25	2.5	18	20

* As percentage of total uronic acid.

† Total hexosamine as measured by Ehrlich's reagent, N-sulphate hexosamine as measured by indole reagent.

‡ Fractionation into 0–30% - and 30–50% -ethanol fractions

§ Reported previously by Linker & Hovingh (1979).

diverse. Preliminary analysis of the anticoagulant activity of the foregoing preparations demonstrated that a wide range of activities exists. For example the wolf intestinal material was quite low in activity, whereas the coyote liver heparin was highly active. In addition to similarities in structural composition, dog lung heparin anticoagulant activity was comparable with that of ox lung heparin. Representative examples of the unusual heparins for which sufficient material existed were evaluated further by AT III affinity chromatography.

Affinity chromatography

To exemplify results of typical chromatographic elution from AT III affinity columns, those obtained with pig mucosal and ox lung heparins and with ox lung heparan sulphates are depicted in Fig. 1. The HA-fraction elution maxima for the mucosal, lung and a low- M_r (5900 by analytical ultracentrifugation) lung heparin occurred between approx. 0.7 M- and 1.0 M-NaCl. The HA fractions accounted for between 32 and 43% of the starting material for these heparins. In agreement with results obtained by other investigators, we found no difference in M_r distribution by Sephadex G-150 filtration of the HA and LA heparin preparations, since the K_{av} for the HA and LA mucosal fractions was 0.36, that for the lung heparin fractions 0.33, and that for the low- M_r lung heparin fractions 0.52.

Analysis of anticoagulant activity of HA and LA preparations

In consideration of the structural diversity and the unexpected range in anticoagulant activities of the heparin isolates, especially those from wolf and coyote, they were fractionated on matrix-immobilized AT III, as above, in order to determine if heparins with low activity contained any highly active subfractions and, contrariwise, if heparins with high activity contained appreciable quantities of subfractions with low anticoagulant

activities. After following the procedure generally used in these fractionations, which involves overloading AT III columns with excessive amounts of heparin (or, alternatively, using high molar ratios of heparin to the protein in solution, followed by chromatographic separation of the products) (see, e.g., Jordan *et al.*, 1979; Atha *et al.*, 1984), we noticed that considerable variations in results occurred when the ratio of the amount of heparin to column volume was varied. Specifically, at low ratios of GAG to matrix, there appears to be either 'non-specific' binding or, alternatively, binding of GAG molecules with low intrinsic affinity for the proteinase inhibitor and hence poor resolution of HA subfractions. This effect could be demonstrated with pig mucosal heparin when starting amounts ranging from 5 to 100 mg were fractionated on a column of fixed size (7 ml). The respective proportions of HA and LA subfractions changed substantially over the examined range, with the apparent content of HA material exceeding the usual 25–35% range when the heparin/matrix volume ratio declined below 4 mg/ml (Table 3). At this low ratio and below, the anticoagulant activities by both assays declined somewhat for both HA and LA fractions, whereas at very high ratios (> 8 mg/ml) the activities for each fraction increased. With the particular gel matrix used for these studies, the best resolution of the subfractions occurred with 4–8 mg of heparin/ml of matrix. This factor became of importance during the affinity fractionation of the parent heparin with high activity from coyote liver (see below). It seems therefore that there is an artifact element complicating the affinity fractionation of heparin on matrix-immobilized AT III.

In light of this, Table 3 shows the proportional fraction of the starting material present in LA and HA preparations, and their corresponding anticoagulant activities, for several heparins, including data for the heparins of most interest, namely the wolf stomach and coyote liver isolates. For unfractionated coyote liver

Fig. 1. AT III affinity chromatography of some heparins and heparan sulphates

These chromatograms represent the typical elution patterns found for the various isolates. The proportional fractions of the starting material present in the LA and HA subfractions are given in parentheses. Elution of the latter fractions occurred between approx. 0.7 M- and 1.0 M-NaCl. For details of the chromatographic conditions see the Materials and methods section.

heparin, which has quite high anticoagulant potency (225 units/mg), the proportional content of HA material was 40% with a heparin/matrix ratio of 4.5 mg/ml, whereas a ratio of 0.8 mg/ml gave an apparent HA fraction of 67%. Similarly, for the parent wolf stomach heparin, having a much lower activity (100 units/mg), the HA fraction accounted for only 18% of the starting material at the higher ratio of 6.0 mg/ml, but a value of 52% for a 1.2 mg/ml ratio. Data in Table 3 allow a comparison of these values with those obtained from pig mucosal heparin under similar conditions of affinity chromatography. The activities of the HA fractions differed considerably: 270 units/mg for the HA fractions from coyote liver and 139 units/mg for the wolf stomach material, with the use of values obtained at high heparin/matrix ratios. HA fractions obtained from the wolf intestinal heparins were also low in anticoagulant activity. The anticoagulant potencies of even the LA fractions obtained from coyote liver heparin at both heparin/matrix ratios were quite high. These findings contrast with results showing a 2–3-fold enrichment in activity of HA compared with LA subfractions obtained

from the most commonly studied heparins, namely those from ox lung and pig mucosa (Table 3). The comparisons among the coyote liver, wolf stomach and intestine and two commercial heparins permit the general observation that the unfractionated heparin derived from the coyote source and possessing unusually high anticoagulant activity contained mostly highly active material that was not further resolved to any substantial extent by AT III affinity chromatography. Moreover, both the HA and LA subfractions obtained from the wolf sources that had low activity were each relatively low in activity. Note that there were no large differences in the estimates for the anticoagulant activities of the preparations by the two assays used.

Heparan sulphates

In contrast with heparin, heparan sulphate was detected in all species shown in Table 1 (Table 4). As heparan sulphates have been implicated in anticoagulant effects on endothelial surfaces (Marcum & Rosenberg, 1984; Stern *et al.*, 1985), we analysed aortic vascular tissues for their GAG content. All samples contained heparan sulphate, chondroitin sulphate and dermatan sulphate. Heparin, however, was not detected. The heparan sulphate content varied among species, as exemplified by the high value in dog aorta (97 mg/100 g dry wt.) compared with the low values in wolf and coyote aorta (5–6 mg/100 g dry wt.). In the latter tissues, chondroitin sulphate and dermatan sulphate contents were half those of the dog aorta. Thus aortic tissues display a wide range in GAG composition among species, indicating no clear-cut relationship in terms of tissue specificity. The other organs also evidenced a range in heparan sulphate content (Table 4). Chondroitin sulphate and dermatan sulphate show very similar distributions (results not shown).

We quantitatively analysed the cell surface-associated heparan sulphate synthesized *in vitro* by a mouse cutaneous fibrosarcoma cell line (Piepkorn *et al.*, 1985) in order to provide a comparison with the GAG content of tissues. It was found that 1.6×10^7 cells, which were analysed for heparan sulphate, had a dry weight of 5.1 mg. After the sequential preparative steps involved in the isolation, including treatment with testicular hyaluronidase, the material was analysed for uronic acid content and electrophoresed on cellulose acetate. These determinations showed that there was 14 mg of heparan sulphate/100 g dry wt. of cells. Using an average M_r of 15000, we calculate that there are approx. 2×10^5 molecules of this GAG species per cell. The weight value falls in the mid-range of the heparan sulphate content derived from dried tissues. Thus heparan sulphate present as cell-surface-associated material could quantitatively account for most of that isolated from whole organs.

Since the rabbit has not been found to contain heparin by isolation procedures that are limited in sensitivity, we next examined the GAG composition of lung and intestinal tissue from this species after intravenous injection of $\text{Na}_2^{35}\text{SO}_4$. Each of three animals received 2 mCi of the radioisotope as a single bolus. After the animals had been killed 24 h later, lung and intestinal tissue were determined to contain respectively 286000 and 1330000 c.p.m. of ^{35}S radioactivity/g of dried tissue. The results from these isotopic data, shown in Table 5, demonstrated a diversity in the GAG composition, but

Table 3. Anticoagulant activities of heparins and heparan sulphates

Abbreviations: APTT, activated partial thromboplastin time; Anti-Xa, anti-(Factor Xa) assay; N.D., not done.

Source	Heparin/ matrix volume ratio (mg/ml)	Preparation	Proportion of starting material (%, w/w)	Anticoagulant activity by APTT/Anti-Xa (units/mg)
I. Heparins				
Coyote liver 0–30*	(a) 4.5	Unfractionated	100	225
		Low-affinity	60	158/195
		High-affinity	40	270/285
	(b) 0.8	Low-affinity	33	140/158
		High-affinity	67	215/230
Wolf stomach 30–50*	(a) 6.0	Unfractionated	100	100
		Low-affinity	82	65/82
		High-affinity	18	139/150
	(b) 1.2	Low-affinity	48	55
		High-affinity	52	120
Wolf intestine 0–30*	3.7	Unfractionated	100	N.D.
		Low-affinity	70	75
		High-affinity	30	130
Wolf intestine 30–50*	4.7	Unfractionated	100	N.D.
		Low-affinity	68	52
		High-affinity	32	110
Dog lung	3.4	Unfractionated	100	N.D.
		Low-affinity	65	68
		High-affinity	35	189
Ox lung	5.3	Unfractionated	100	N.D.
		Low-affinity	57	70
		High-affinity	43	195
Pig mucosa	(a) 5.4	Unfractionated	100	168
		Low-affinity	61	68
		High-affinity	39	245
	(b) 1.2	Low-affinity	43	51
		High-affinity	57	193
Ox lung (low- M_r)	5.3	Unfractionated	100	N.D.
		Low-affinity	68	50/72
		High-affinity	32	171/190
II. Heparan sulphates				
Ox aorta		Unfractionated	100	N.D.
		Low-affinity	95	0.8
		High-affinity	5	2.0
Rabbit aorta		Unfractionated†		0.8
		Unfractionated†		1.2
Ox lung 0.9 m‡		Unfractionated	100	2.4
		Low-affinity	97	1.2
		High-affinity	3	10.4
Ox lung 1.2 m‡		Unfractionated	100	2.4
		Low-affinity	98	2.9
		High-affinity	2	10.0
Ox lung 1.4 m‡		Unfractionated	100	5.5
		Low-affinity	96	2.0
		High-affinity	4	32.3
Rabbit lung 1.0 m‡		Unfractionated	100	N.D.
		Low-affinity	95	1.1
		High-affinity	5	1.9
Rabbit lung 1.3 m‡		Unfractionated	100	N.D.
		Low-affinity	95	3.8
		High-affinity	5	7.7

* Fractionation into 0–30% - and 30–50% -ethanol fractions.

† Insufficient material for affinity chromatography.

‡ Denotes salt fraction.

Table 4. Heparan sulphate contents in vertebrate organs

Organ	No. of species	Heparan sulphate content (mg/100 g dry wt.)		Species without detectable heparan sulphate
		Minimum	Maximum	
Lung	20	7	150	Cowbird (< 0.3) Starling (< 0.4)
Stomach	17	2	60	
Liver	25	2	200	Goat (< 1.7)
Intestine	26	3	75	
Kidney	11	2	140	
Gills	9	6	27	
Air bladder	6	8	40	
Ventricle	7	8	44	
Spleen	10	9	47	
Aorta	22	3	325	

Table 5. Analysis of GAGs in rabbit organs by incorporation of [³⁵S]sulphate

Organ	Glycosaminoglycan	Composition of sulphated GAGs (%)	
		By isolation	By radiolabel
Lung	Heparan sulphate	27	41
	Chondroitin sulphate	45	33
	Dermatan sulphate	28	4
	Other (not heparin)	0	22
	Total:	100	100
Intestine	Heparan sulphate	85	15
	Chondroitin sulphate	0	7
	Dermatan sulphate	15	4
	Other (not heparin)	0	74
	Total:	100	100

they also confirmed that neither tissue contains heparin, within the minimum detection limits of the procedures (which we estimate to be 3000 c.p.m. for detection of heparin). The intestinal tissue contained a large amount of sulphated glycoprotein that was eluted from the ion-exchange column between 0.5 M- and 3.0 M-NaCl. As no heparin could be detected, we present in Table 6 a chemical analysis of the heparan sulphates isolated from this species (see also the next subsection).

Anticoagulant properties of heparan sulphates

Analysis by AT III affinity chromatography of the heparan sulphates (with the exception of that from the rabbit aorta, for which insufficient material existed) demonstrated that the HA fractions constituted 5% or less of the starting material, the proportional fraction being no greater for the ox aorta heparan sulphate than for that from the other sources (Table 3 and Fig. 1). Anticoagulant activities for the HA material ranged from 1.9 to 32.3 units/mg, compared with 0.8–3.8 units/mg for the LA fractions. Note that the activities obtained from the aortic tissues were less than those obtained from the non-aortic sources. The ox lung fractions are very well characterized (Linker & Hovingh, 1973). It is apparent that, although these heparan sulphates contain small subfractions with high-affinity binding potential to matrix-immobilized AT III, the anticoagulant potencies

of these HA preparations are rather modest. Cellulose acetate electrophoresis, standardized by authentic GAGs, revealed that each HA and LA heparin and heparan sulphate migrated in a fashion identical with that of the starting material. Thus there was no heparin contamination in the HA fractions to account for any of the augmented anticoagulant activity of these fractions compared with that of the starting material.

DISCUSSION

Structural diversity among heparins from different animals sources (Ototani *et al.*, 1984, and the Results section), in conjunction with its apparent absence from many vertebrates, has contributed to the difficulties in defining the biological function of this GAG. Our results have suggested, additionally, that the tissue distributions of heparin species varying widely in their anticoagulant potency (e.g. coyote liver heparin compared with the wolf intestinal material) appear random. Furthermore, we have been unable to demonstrate this highly sulphated GAG in aortic tissue. These considerations suggest that the anticoagulant activity of heparin is an incidental property of its primary structure. The function of heparin in mast-cell granules (Lagunoff, 1974) should be considered in light of evidence that certain mucosal

Table 6. Analysis of rabbit lung and aorta heparan sulphates

Tissue source	Optical rotation (°)	Composition (% w/w)						
		Uronic acid	Iduronic acid*	Sulphate	Acetyl	Hexosamine†		10 ⁻³ × M _r
						Total	N-Sulphate	
Lung 1.0 M‡	+67	38	14	8	4.7	23	8	8–20
Lung 1.2 M‡	+73	46	23	12	4.3	23	13	20–32
Lung 1.4 M‡	+39	43	35	13	4.3	22	12	20–32
Aortic arch		35	20	3	5.3	22	7	8–26
Thoracic aorta§	+36	28	30	3	3.1	17	11	23–26
Abdominal aorta	+47	42	20	7	5.3	25	10	10–26

* As percentage of total uronic acid.

† Total hexosamine as measured by Ehrlich's reagent, N-sulphate hexosamine as measured by indole reagent.

‡ 1.0 M-, 1.2 M- and 1.4 M-salt fractions.

§ Contained about 30% nucleic acid.

mast-cell populations lack this GAG (Enerbäck *et al.*, 1985).

The binding sequence, or sequences, for AT III binding are present in the examined heparan sulphates, in agreement with the report of Hubbard *et al.* (1984) and our earlier suggestion (Linker, 1979). The high-affinity fractions present in heparan sulphate preparations have anticoagulant potencies up to approx. 30 units/mg and constitute 5% or less of the starting material. This activity would be sufficient to serve heparin-like functions with respect to antithrombotic effects on endothelial surfaces. Further support for this putative function, however, might be expected to have been provided by the detection of heparan sulphate species containing a higher proportion of high-affinity material, or of material with somewhat greater anticoagulant activity, in the preparations isolated from aorta. Table 3 demonstrates this not to be the case. Nevertheless, endothelial-cell heparan sulphate, whose existence has been shown by a number of investigators (e.g. Buonassisi & Root, 1975; Oohira *et al.*, 1983), probably constitutes the heparin-like GAG on vascular surfaces that, although not well-characterized structurally, clearly appears to have potential anticoagulant effects (Marcum & Rosenberg, 1984; Stern *et al.*, 1985). Data reported by Marcum & Rosenberg (1984) concerning both the anticoagulant activities of high-affinity fractions and the proportional share of the starting material represented by these fractions for the heparin-like GAGs they isolated from vascular tissue are very comparable with the results that we report here for heparan sulphates isolated from both aortic and non-aortic tissues. We therefore suggest that alternative functions for this GAG be considered more likely than that of contributing to blood homeostatic functions, such as the possibility that they constitute cell-surface receptors for growth factors (Klagsbrun & Shing, 1985) or contribute to the growth regulation of subendothelial smooth-muscle cells (Fritze *et al.*, 1985). Using different approaches, Lollar *et al.* (1984) and Hatton & Moar (1985) have reached conclusions similar to ours.

Although an approximate correlation exists between the anticoagulant potency of a heparin or heparan sulphate and the proportion of the starting material demonstrating high affinity for AT III, these studies show that low-affinity preparations can have a broad range in

activity by assay *in vitro*, which in the case of the low-affinity preparation obtained from coyote liver (Table 3) can be substantial. Our results suggest either that selection of HA and LA fractions by AT III affinity chromatography is to a certain extent an artifact, excluding molecules that possess some AT-III-binding affinity, or that high-affinity binding to AT III is not the only determinant of high anticoagulant potency. In fact, it does appear that an additional site on heparin, adjacent to the AT-III-binding domain, is necessary for thrombin binding in the formation of a ternary complex (Laurent *et al.*, 1978; Ototani *et al.*, 1984). Furthermore, the data obtained for wolf and coyote heparin reported here and for whale heparin (Ototani *et al.*, 1984) indicate that in given heparin preparations it is not only the number of chains containing an AT-III-binding sequence that determines final activity, but that variations in the binding site itself and in additional sites may be relevant. Our results with heparan sulphates, in which about 5% of chains can bind to AT III with high affinity but yet have only about 30 units/mg or less anticoagulant activity, support this argument. The likely existence of two domains on the protein for interaction with heparin (Piepkorn *et al.*, 1978, 1980; Oosta *et al.*, 1981; Stone *et al.*, 1982) provides potential for further binding sequence diversity. The fact that sulphated pentosans (Scully *et al.*, 1983) and chitosans (Hirano *et al.*, 1985) do have anticoagulant activity but yet lack the specific AT-III-binding site(s) of heparin and the role of surface charge (Heuck *et al.*, 1985) have similar implications. The point is not to detract from the valuable studies on binding sites (Rosenberg & Lam, 1979; Lindahl *et al.*, 1984; Atha *et al.*, 1984), but rather to stress the complexities. Taken together, these considerations suggest that there exist multiple sequences capable of binding the proteinase inhibitor, which in their entirety cover a broad range of affinities, ranging up to that of the oligosaccharide sequence described by Rosenberg & Lam (1979) and Lindahl *et al.* (1979), and which interact with one or both of the domains on AT III as well as with a site on thrombin. The final activity of a given heparin sequence would thereby be proportional to the number of bonding interactions between the carbohydrate residues and subsites on the proteins.

The general abundance of heparan sulphates, compared

with the curiously limited distribution of heparin, indicates a more fundamental function for the former GAG. Also, the structural similarity could indicate that heparan sulphates may fill the role of heparin where that is absent. There is, however, no indication that heparan sulphates are different in species or organs not containing heparin. Furthermore, neither amounts nor structural characteristics of heparan sulphates bear an obvious relationship to a particular organ system. Our observations support the conclusion that heparan sulphates are involved in processes not peculiar to particular tissues but rather those that are basic to cellular function.

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