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Effect of Heparitin Sulfate Fractions on Coagulation and Hemostasis[1] (38135)

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(Introduced by Louis B. Jaques)

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We have shown recently that crude heparitin sulfate from beef lung tissue is composed of 4 distinct mucopolysaccharides (1). One of these heparitin sulfates (heparitin sulfate D) is very similar to heparin in chemical composition, metachromasy and electrophoretic migration in agarose gel. When both compounds are subjected to enzymatic degradation by a purified heparinase from *F. heparinum* the same products are formed, mainly a trisulfated disaccharide (70% yield) (2). Nevertheless, this compound can be differentiated from heparin using DEAE cellulose (1). Since it has been demonstrated that the only difference between heparitin sulfate D and heparin is the lower mol wt of the former (3), and since it has been suggested that the anticoagulant activity of heparin is related to its chemical and physical properties, such as molecular weight (4–6), sulfation, carboxylation and shape (7), it was then of interest to compare the activity of this compound and the other heparitins as anticoagulants and antihemostatics with the anticoagulant and antihemostatic activity of heparin.

The present paper reports the effect of heparitin sulfate fractions on coagulation and hemostasis compared to heparin. A preliminary account of this work has been presented (8).

Methods. Antihemostatic and anticoagulant activities. Bleeding time determinations were performed on rat tails essentially as previously described (9) and consisted of making a wound with a razor blade on the terminal part of the tail and immersing it in a small test tube containing 1 ml of the solution to be tested, held at 37° in a water bath. The blood flow from the vessels was observed with a dissecting microscope. Unless otherwise stated, the sequence of experiments was as follows: (a) the scarified tail was immersed into a saline solution (0.14 *M* sodium chloride) and the normal bleeding time observed; (b) the tail was removed from the water bath, then irritated by rubbing gauze forcefully along the wound; (c) the tail was immersed again in the test saline solution and the test bleeding time observed.

This experiment was repeated at least 10 times at 10 min intervals, giving bleeding times in the normal range (½–2 min). When heparin or other test substances were used, the tail, after the irritation, was left in contact with the heparin or test solution for 2 min and then washed with 100 ml of saline. The bleeding time was then observed in 1 ml of physiological saline (test saline).

The anticoagulant activity of the compounds was measured by U.S.P. assay. Chemical analytical methods were those used previously (1, 3).

Biochemical preparations. Heparitin sulfates were obtained from the Upjohn Co. (Kalamazoo, Mich.) through the courtesy of Dr. J. T. Correl. The 4 heparitin sulfate fractions, A, B, C, and D were prepared as previously described (1, 3). Chemically modified heparins were prepared as recently described (10). Chondroitin sulfates A, B, and C were purchased from Miles Laboratories (Elkhart, Indiana). Mollusc heparin (Mactin A from Mactrus pussula) was provided by Dr. L. B.

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TABLE I. Effect of Heparin Concentration on Hemostasis.

Heparin dose (mg/ml)	Bleeding time in heparin solution (min)	Bleeding time in test saline (min)
–	0.5	0
0.01	0.9	0
0.05	1.1	0
0.10	> 2.0	7
0.20	> 2.0	10
0.25	> 2.0	16
0.50	> 2.0	27
1.00	> 2.0	> 30

Jaques (University of Saskatchewan, Canada), and was originally donated by Dr. M. J. Fahrenbach, Lederle Laboratories Ltd., Pearl River, New York. Heparin was obtained from the Upjohn Co. and also from Abbott Laboratories Ltd. (Montreal, Quebec), Lederle Laboratories Ltd. (Pearl River, New York), and Hoffman-La Roche Ltd. (Montreal, Quebec), with anticoagulant activity of 150 U.S.P. units/mg.

Results. Antihemostatic activity of heparin and other mucopolysaccharides. Bleeding times of more than 30 min were obtained when the scarified rat tail was placed in a heparin solution of 1.0 mg/ml for 2 min. This antihemostatic effect remained after extensive washing of the rat tail with saline. The duration of the bleeding time in test saline was proportional to the concentration of the heparin solution (Table I). For each scarification the values were extremely constant. Repeat experiments have shown that the variation of the bleeding time for one specific dose was in the order of 15%. The repeat experiments used heparins of four different sources.

The prolongation of bleeding time was termed the heparin residual effect. Among the naturally occurring mucopolysaccharides only heparin had this antihemostatic activity. *N*-desulfation of heparin destroyed its antihemostatic activity. Substitution of the free amino-groups of this *N*-desulfated heparin with acetyl groups or dinitrophenol did not restore its antihemostatic property (Table II).

Antihemostatic and anticoagulant activities of heparitin sulfate fractions. Heparitin sulfate is a dextrorotatory mucopolysaccharide which is chemically and physically similar to heparin. We have recently fractionated this mucopolysaccharide obtained from beef lung

TABLE II. Effect of Mucopolysaccharides and Chemically Modified Heparins on Hemostasis.

Substance[a]	Dose (mg/ml)	Bleeding time	
		In solution (min)	In test saline (min)
Hyaluronic acid	2.0	0.7	0
Chondroitin sulfate A	2.0	1.0	0
Chondroitin sulfate B	2.0	1.8	0
Chondroitin sulfate C	2.0	1.0	0
N-desulfated heparin	1.0	1.0	0
N-desulfated, *N*-acetylated heparin	1.0	0.6	0
DNP-heparin	1.0	0.8	0
Heparin (beef)	0.25	> 2.0	> 15
Mactin (mollusc heparin)	0.25	> 2.0	> 15
Control (saline)	–	0.6	0

[a] The mucopolysaccharide solutions were prepared in saline.

TABLE III. Chemical Composition of Heparitin Sulfates.

Substance	Molar proportions				Optical Rotation $[\alpha]D_{20}$	Average Molecular Weight
	Hexosamine	Acetyl groups	*N*-Sulfate groups	Total sulfate		
Heparitin sulfate A	1.0	0.97	0.04	0.45	+ 92.2	170,000
Heparitin sulfate B	1.0	0.58	0.35	0.95	+ 132.9	25,000
Heparitin sulfate C[a]	1.0	0.07	0.94	2.02	+ 18.7	—
Heparitin sulfate D	1.0	0	1.08	2.62	+ 53.6	3,800
Heparin	1.0	0	1.05	2.61	+ 53.0	12,000

[a] Contaminated with nucleic acids and chondroitin sulfate B (1).

tissue into 4 main fractions. The chemical composition of these fractions as well as some other properties are given in Table III. Heparitin sulfate D and heparin have the same chemical composition and optical rotation but the mol wt of heparitin sulfate D is about 3 times lower than that of heparin.

These compounds were then tested for antihemostatic and anticoagulant activity. These results are shown in Table IV. Among the heparitins only heparitin sulfate D had antihemostatic activity similar to heparin. Nevertheless this compound had a low anticoagulant activity. All the other heparitin sulfates were completely inactive as anticoagulants and as antihemostatics in the concentrations used.

The antihemostatic and anticoagulant activity of heparitin sulfate D compared to heparin is shown in Fig. 1. Whereas heparin and heparitin sulfate D have the same antihemostatic activity, heparitin sulfate D has only about 5% of the anticoagulant activity of heparin.

Discussion. Heparitin sulfates A and B which are larger molecules than heparitin sulfate D and heparin, are devoid of anticoagulant and antihemostatic activities. Since these compounds differ from heparin and heparitin sulfate D in exhibiting a lower degree of sulfation and the presence of *N*-acetyl groups, it is conceivable that the lack of action observed is due to these 2 basic differences. This has been previously suggested (7). This possibility is substantiated by the lack of antihemo-

TABLE IV. Effect of Heparitin Sulfate Fractions on Coagulation and Hemostasis.[a]

Substance	Bleeding time		Coagulation	
	Dose (mg/ml)	Test saline (min)	Dose (μg/ml)	Time (min)
Control (saline)	—	0	0	5.0 (± 1.0)
Heparitin sulfate A	0.25	0	1.25	5.0 (± 1.0)
	2.00	0	12.5	6.0 (± 1.5)
Heparitin sulfate B	0.25	0	1.25	5.0 (± 1.0)
	2.00	5 (± 0.4)	12.5	9.0 (± 1.2)
Heparitin sulfate C	0.25	0	—	—
	2.00	0	—	—
Heparitin sulfate D	0.25	> 15	1.25	5.5 (± 0.4)
	2.00	> 15	12.5	15.0 (± 3.8)
Heparin	0.25	> 15	1.25	25.0 (± 3.6)
	2.00	> 15	12.5	> 60

[a] Each bleeding and coagulation time is the mean of 6 determinations. Numbers in brackets are standard deviations.

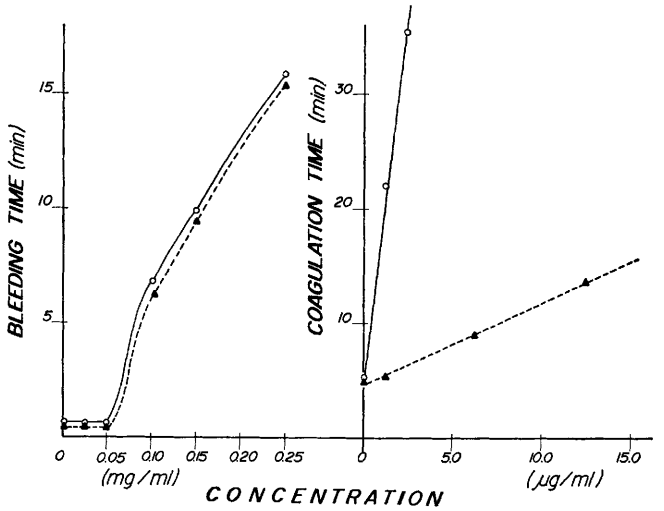

FIG. 1. Antihemostatic and anticoagulant activity of heparin and heparitin sulfate D. O—O heparin; Δ——Δ heparitan sulfate D. Each point is the mean of 8 determinations. The standard deviation was in the range of 10 to 25 percent.

static action of *N*-desulfated and *N*-acetylated heparin.

The findings reported in this paper confirm and extend the previous findings (4, 6) that there is a minimum molecular size requirement for the anticoagulant action of heparin, since heparitin sulfate D which differs from heparin only in molecular size is practically devoid of anticoagulant activity.

The similarity of action of heparin and heparitin sulfate D in hemostasis contrasted with the lack of action of heparitin sulfate D as an anticoagulant indicates that hemostasis and coagulation are not identical phenomena. These results substantiate earlier reports (9, 11).

The mechanism of action of heparin in coagulation and hemostasis at the molecular level as well as the physiological role of heparin are not yet known. Further comparison of the action of heparitin sulfate D and heparin (both naturally occurring mucopolysaccharides) in several processes where heparin has been involved (12) may be useful in the elucidation of the physiological role of this important compound.

Summary. The antihemostatic and anticoagulant activities of heparin and heparitin sulfate fractions were compared. Heparitin sulfate D has the same antihemostatic activity as heparin but negligible anticoagulant activity, demonstrating differences in the molecular mechanisms of these two biological processes. The effects of heparitin sulfate fractions and modified heparins on hemostasis and coagulation are also compared in order to determine the minimum structural and chemical requirements for activity in these two phenomena.

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