Hageman Factor Activation and Kinin Formation in Human Plasma Induced by Cellulose Sulfate Solutions* (33780)

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Cellulose sulfate was shown to be an anticoagulant by Astrup *et al.* (1). It is a potent activator of the kinin generating system in the plasma of rat, guinea pig, and man as shown by Rothschild and Gascon (2) and Rothschild (3). Rothschild and Castania recently demonstrated that intravenous injection of cellulose sulfate into a dog causes transient hypotension and a concurrent fall in kininogen levels of the plasma (4). They proposed that this transient hypotension was mediated by bradykinin. The anticoagulant properties of cellulose sulfate *in vitro* is confirmed by our data. However, at low concentrations we found the coagulation mechanism is enhanced. This procoagulant effect is a direct result of the activation of Hageman factor (factor XII of the intrinsic clotting mechanism). Further, we have demonstrated that release of kinins in the plasma by cellulose sulfate *in vitro* is dependent on the activation of Hageman factor. Activated Hageman factor has previously been reported to initiate a series of enzymatic reactions in plasma which results in the generation of kinins (5-7).

Materials and Methods. Cellulose sulfate was prepared by a method described by Astrup *et al.* (1). The pyridine was distilled, and the fraction between 113–115° was taken for use in the synthesis of cellulose sulfate. The final yield of the procedure was 6.3 g of cellulose sulfate starting with 2.5 g cellulose. Solutions of cellulose sulfate were prepared in barbital–saline buffer.

Platelet deficient plasma was prepared in equipment coated with silicone so that the plasma did not come in contact with glass. The venous blood was drawn into 30-ml chilled syringes, mixed with 1/10 vol of 0.13 *M* sodium citrate, pH 5.0, in 40-ml Lusteroid tubes, and centrifuged 30 min at 700*g* at 1°. The plasma was then transferred to silicone-coated thick walled Servall centrifuge tubes (15 × 120 mm), and centrifuged at 30,000*g* at 0° for 30 min. The plasma was then transferred to plastic containers in approximately 1.0-ml aliquots, and was stored at —20° until used. Plasma deficient in Hageman factor was prepared in a similar manner from blood obtained from an individual with Hageman trait.

Barbital–saline buffer and de Jalon solution was prepared as previously described (8, 9). Soy bean trypsin inhibitor (SBTI) (Worthington) was prepared fresh in the desired concentrations in barbital–saline buffer. *o*-Phenanthroline (Fischer) was made up to the desired concentration in barbital–saline buffer, and stored at 4°. Anti-Hageman factor globulin and normal rabbit globulin were prepared by a method previously described (10). Vascular-permeability enhancing activity was assayed by determining the average diameter (mm) of the blue spot that developed following the intradermal injection of 0.1 ml of the test substance into each of 4 guinea pigs previously injected with Pontamine sky blue (11). Recalcified clotting times of platelet deficient plasma were measured in plastic tubes (Falcon Plastics, 10 × 75 mm) in duplicate by a method previously described (12).

Kinin formation in normal human plasma was studied by the following procedure. Two-tenths ml of platelet deficient plasma, 0.1 ml of test substance, and 0.05 ml of *o*-phenanthroline (0.01 *M*) were placed in plastic tubes (Falcon Plastics, 10 × 75 mm) and mixed. The tubes were allowed to stand at room temperature (22°) for 5 min and then

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TABLE I. The Effect of Cellulose Sulfate on the Recalcified Clotting Time of Normal Platelet-Deficient Plasma.

Test substance (μg/ml)	Clotting time (min)
Buffer	16 , 16
Cellulose sulfate[a]	
670	>60 , >60
67	>60 , >60
6.7	10.5, 10.5
3.3	6.5, 6.5
1.3	5.0, 5.0
0.13	13.0, 13.0

[a] These are final concentrations in a total volume of 0.3 ml containing 0.1 ml plasma and 2.5×10^{-3} meq of $CaCl_2$.

a 0.15-ml sample was tested in the isolated rat uterus preparation (13). The response was measured by using a linear tranducer (Brush Instrument Co., model 33-03-981) and a Grass polygraph (Grass Instruments, model 5D). The de Jalon solution was heated to 29° before entering the muscle bath. The muscle was washed 3 times between contractions, allowing 5 min after the last wash until the time of adding the next test sample. Only the contraction occurring within 1 min after addition of the sample was considered to be significant. The uterus was isolated from virgin albino female rats (Sprague-Dawley strain) treated with 1.0 ml subcutaneous injection of diethylstilbestrol (Lilly, 100 μg/ml in olive oil) 18–24 hr prior to sacrifice. The uterus was placed in de Jalon solution and stored less than 12 hr at 4° until used.

Results. The effect of cellulose sulfate on the coagulation of normal human plasma. The recalcified clotting time of normal human plasma is increased beyond 1 hr by cellulose sulfate at concentrations of 67 ng/ml or greater (Table I). When the cellulose sulfate is added to the clotting system at lower concentrations (Table I), the recalcified clotting time is reduced to less than 1/3 of the control value.

The effect of cellulose sulfate on the coagulation of plasma deficient in Hageman factor. Cellulose sulfate at concentrations of 6.7 μg/ml or greater caused prolongation of the recalcified clotting time (Table II). At concentrations of 3.3 and 1.3 μg/ml, there was a slight decrease in the clotting time compared to the control.

Induction of permeability-enchancing activity in normal plasma. Cellulose sulfate induces the development of permeability-enhancing activity in normal human plasma

TABLE II. The Effect of Cellulose Sulfate on the Recalcified Clotting Time of Plasma from a Patient with Hageman Trait.

Test substance (μg/ml)	Clotting time (min) Plasma deficient in Hageman factor[a]		
	Citrated	Platelet deficient A	Platelet deficient B
Buffer	37, 40	>133, >133	40 , 45
Cellulose sulfate[b]			
670	>60, >60		
67	>60, >60		
6.7	>60, >60	133, 133	52 , 48
3.3	36, 36	98, 95	29 , 29
1.3	22, 22	80, 65	24.5, 24.5
0.13	22, 22		

[a] Plasma from three different patients with Hageman trait were tested. "Citrated" plasma was plasma collected in silicone coated equipment, anticoagulated with 1/10 vol of citrate and the plasma separated by centrifugation (1000*g*, 4°, 20 min). The "Platelet deficient" A and B plasmas were prepared from blood anticoagulated with citrate collected in silicone coated containers as described previously (8).

[b] The concentrations given are final.

TABLE III. The Induction of Permeability Enhancing Activity by Cellulose Sulfate in Normal Plasma and Plasma Deficient in Hageman Factor.

Miture tested[a]	Permeability activity (mm)[b]
Normal plasma + buffer	3.1
Normal plasma + cellulose sulfate (0.001 mg/ml)	7.1
Plasma deficient in Hageman factor + buffer	2.8
Plasma deficient in Hageman factor + cellulose sulfate (0.001 mg/ml)	3.1
Soy bean trypsin inhibitor (0.25 mg/ml)	1.4
Normal plasma + soy bean trypsin inhibitor (0.25 mg/ml)	1.8
Normal plasma + cellulose sulfate (0.001 mg/ml) + soy bean trypsin inhibitor (0.25 mg/ml)	2.0
Cellulose sulfate (0.001 mg/ml) + buffer	2.0

[a] The concentrations given are for the final volume.

[b] These values represent the average diameter of the blue spot that develops following the intradermal injection of 0.1 ml of test material into each of four guinea pigs previously injected with Pontamine sky blue.

(Table III). The development of this activity was blocked by the addition of SBTI to the plasma prior to the addition of cellulose sulfate (Table III). Neither cellulose sulfate (0.001 mg/ml) nor SBTI alone induced any increase in vascular permeability (Table III).

Failure to induce an enhancement of vascular permeability in plasma deficient in Hageman factor. When cellulose sulfate was combined with plasma deficient in Hageman factor and then injected intradermally into guinea pigs "blued" with Pontamine sky blue, there was no increase in vascular permeability compared to the controls (Table III).

Kinin generation in normal human plasma and plasma deficient in Hageman factor. When cellulose sulfate (670 and 67 μg/ml) was incubated with normal human plasma, kinins were released as evidenced by the contraction of smooth muscle when an aliquot of this plasma was added to the rat uterus preparation (Table IV). This activity was destroyed by the addition of carboxypeptidase B after the generation of the kinin activity had occurred (Table IV). The incubation of cellulose sulfate (670 and 67

TABLE IV. The Formation of Kinin Activity in Normal Human Plasma and Plasma Deficient in Hageman Factor Incubated with Cellulose Sulfate.

Mixture tested	Kinin releasing activity[a]
Normal plasma + buffer	0
Normal plasma + buffer + phenanthroline	0
Normal plasma + cellulose sulfate (670 μg/ml) + phenanthroline	+
Normal plasma + cellulose sulfate (67 μg/ml) + phenanthroline	+
Plasma deficient in Hageman factor + cellulose sulfate (670 μg/ml) + phenanthroline	0
Plasma deficient in Hageman factor + cellulose sulfate (67 μg/ml) + phenanthroline	0
Normal plasma + cellulose sulfate (67 ng/ml) + carboxypeptidase B (0.007 μg/ml)	0
Normal plasma + cellulose sulfate (67 μg/ml)	+

[a] + indicates that a maximal contraction was produced, and 0 indicates a lack of contraction during the period of observation.

TABLE V. Inhibition by Anti-Hageman Factor of the Induction of Permeability Enhancing Activity by Cellulose Sulfate in Normal Human Plasma.

Tube	Reaction mixture[a]			Permeability-enhancing activity (mm)[c]
	A[b]	B[b]		
1	Normal plasma	Buffer		2.5
2	Normal plasma + anti-Hageman factor	Buffer		3.1
3	Normal plasma	Cellulose sulfate + buffer		6.8
4	Normal plasma + anti-Hageman factor	Cellulose sulfate + buffer		2.3
5	Normal plasma + normal rabbit globulin	Buffer		2.4
6	Normal plasma + normal rabbit globulin	Cellulose sulfate + buffer		6.8
	C[b]	D[b]	E[b]	
7	Normal plasma + cellulose sulfate		Buffer	5.3
8	Normal plasma + cellulose sulfate	Anti-Hageman factor	Buffer	5.4
9	Cellulose sulfate + buffer			2.4
10	Normal plasma + cellulose sulfate	Normal rabbit globulin	Buffer	5.3
11	Normal plasma + cellulose sulfate + buffer			6.8
12	Normal plasma + normal rabbit globulin	Cellulose sulfate + buffer		5.4

[a] The final volume of the reaction mixture was 1.0 ml; the final concentration of cellulose sulfate was 0.001 mg/ml; the final dilution of the plasma was 1:100.

[b] (A) Mixture incubated 16 min at 0°. (B) Reactants listed here were added to mixture A and incubated an additional 10 min at 37°. Following the incubation (A + B), a 0.1 ml sample was injected into each guinea pig. (C) Mixture incubated 10 min at 37°. (D) Reactants listed here were added to C mixture and incubated an additional 16 min at 0°. (E) The final volumes were equalized following the incubation in D. Then 0.1-ml sample was injected into each guinea pig.

[c] These values represent the average diameter of the blue spot that develops following the intradermal injection of 0.1 ml of test material into each of four guinea pigs previously injected with Pontamine sky blue.

μg/ml) with plasma deficient in Hageman factor did not result in the generation of kinin activity (Table IV).

Abolition of permeability-enhancing activity of cellulose sulfate by Hageman factor specific antibody. After normal human plasma was incubated with anti-Hageman factor globulin, the addition of cellulose sulfate did not result in the development of permeability-enhancing activity (Table V). Normal rabbit globulin incubated with normal human plasma did not suppress the development of the permeability-enhancing activity. When cellulose sulfate was incubated with normal plasma (37°, 10 min) and then further incubated with anti-Hageman factor antibodies (0°, 16 min), there was no suppression of permeability-enhancing activity (Table V).

Discussion. Activated Hageman factor can initiate two series of enzymatic reactions in human plasma. One series of reactions, the intrinsic clotting pathway, is dependent on the sequential interaction of clotting factors XI, IX, VIII, X and prothrombin (II), resulting in the formation of thrombin (14). Hageman factor also initiates the sequential interaction of another group of proteins, namely kallikrein, PF/dil, and kininogen that results in the release of kinin (5–7, 14). Both kallikrein and PF/dil will induce increased vascular permeability in the skin of the "blued" guinea pig and both are inhibited by SBTI. The initiation of this second series of reactions *in vivo* following the activation of Hageman factor by ellagic acid results in the consumptive depletion of a portion of the plasma kininogen (18). The kinin formed *in vivo* following the administration of ellagic acid produces only a transient hypotension because it is quickly destroyed by a hydrolytic enzyme called kininase (19).

The activation of Hageman factor in nor-

mal human plasma accelerates clotting, induces kinin release, and causes the formation of a vascular-permeability enhancing activity independent of kinin (14). We demonstrated that cellulose sulfate can produce all three of the above activities in normal human plasma but not in plasma deficient in Hageman factor. Since Hageman factor is the only recognized factor missing from this plasma, the mode of action of the cellulose sulfate must have been through the activation of Hageman factor. Inhibition of Hageman factor by the anti-Hageman factor globulin and prevention of the formation of the vascular-permeability enhancing activity induced by cellulose sulfate is also consistent with this interpretation. Inhibition by SBTI of the induction of vascular permeability enhancing activity by cellulose sulfate suggests that PF/dil or kallikrein participates in the development of this activity.

Until the discovery by Ratnoff and Crum in 1964 that ellagic acid and related compounds activated Hageman factor (12) all previous known activators of Hageman factor had been insoluble particles including glass, kaolin, and silica dioxide (14–16). Hubbard made the observation that the particulate activators of Hageman factor had a negative surface charge (17). Although both ellagic acid and cellulose sulfate can have a negative charge, comparison of their structures or chemical properties does not further aid in determining the molecular alteration of Hageman factor that results in activation.

Summary. Cellulose sulfate was previously reported as an anticoagulant and as a substance that will induce kinin formation in normal human plasma *in vitro*. The present data confirm these findings but in addition indicate that at lesser concentrations it is a procoagulant as well. Further, cellulose sulfate will induce permeability-enhancing activity in normal human plasma. Cellulose sulfate does *not* induce the formation of kinins, or permeability-enhancing activity, nor does it enhance clotting when added to human plasma deficient in Hageman factor. Further the induction of permeability-enhancing activity in normal human plasma is blocked by the addition of rabbit anti-Hageman serum prior to the addition of cellulose sulfate. These data therefore indicate that cellulose sulfate, like ellagic acid, activates Hageman factor in normal human plasma *in vitro*. On the basis of this datum, it is suggested that the mechanism of kinin formation and kininogen depletion in dogs given cellulose sulfate (4) is the same as that proposed for ellagic acid, namely the activation of Hageman factor and sequential interaction of Hageman factor, PF/dil, kallikrein, and kininogen resulting in a consumptive depletion of kininogen (18).

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