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SOME PHARMACODYNAMIC PROPERTIES OF CELLULOSE SULPHATE, A KININOGEN-DEPLETING AGENT IN THE RAT

BY

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Recent observations (Rothschild & Gascon, 1966 have shown that water-soluble, sulphated polysaccharides (cellulose, starch or glycogen sulphates, carrageenin) promote the release of bradykinin when added to the plasma of the rat, guinea-pig or man. Preliminary results (Rothschild, 1967a) indicated that cellulose sulphate, at doses which cause extensive plasma kininogen depletion, was well tolerated by rats or guinea-pigs; these have led to a more detailed study of the pharmacodynamic properties of this compound. In the present investigation changes in arterial blood pressure, total protein and kininogen content, esterolytic and fibrinolytic activity, clotting time, platelet, leucocyte and haematocrit values in plasma or blood of rats treated with cellulose sulphate were investigated. The results obtained permit a better evaluation of the possible usefulness of this polysaccharide as a plasma kininogen-depleting agent in the rat.

METHODS

Cellulose sulphate was prepared from Whatman ashless cellulose powder according to Astrup, Galsmar & Volkert (1944); this procedure gave greater yields and a better reproducibility of the product than the technique described by Karrer, Koenig & Usteri (1943). Kinin precursor (kininogen) in plasma was determined according to Diniz & Carvalho (1963).

Esterolytic activity on benzoyl-arginine ethyl ester (BAEE) was determined by the colorimetric method of Brown (1960). Rapid spontaneous inactivation of the esterase occurred during the separation of plasma; enzymic activity was therefore determined in whole blood added, immediately upon withdrawal, to 5 vol. of the buffered substrate solution employed for incubation. This procedure, which probably did not entirely prevent the spontaneous inactivation of the enzyme, nevertheless permitted the demonstration of a high level of esterolytic activity in the blood of rats treated with cellulose sulphate.

Blood clotting times were determined by the Lee & White method, as described by Wintrobe (1961).

Total leucocyte counts in peripheral blood were performed using a Neubauer counting chamber. Platelets were estimated in peripheral blood diluted with 1.0% of ammonium oxalate, under the Zeiss phase contrast microscope, using a Fuchs-Rosenthal counting chamber.

Total plasma protein was determined according to Mokrasch & McGilvery (1956).

Fibrinolytic activity was determined by the bovine fibrin plate method of Astrup & Mullertz (1952). Incubations were performed at room temperature (21°–25° C) for a period of 17 hr. The

bovine thrombin utilized was prepared according to the method of Eagle as described by Hawk, Oser & Summerson (1947).

The isolated hindquarters of the rat were perfused under positive pressure with Tyrode solution entering the aorta cannulated just below the renal bifurcation. The perfused fluid was collected at a rate of approximately 0.5 ml./min from the inferior vena cava cannulated at the same level. Histamine was assayed on the isolated atropinized guinea-pig ileum, using a standard of histamine dihydrochloride.

Male Wistar rats (200–350 g) were used; neutralized solutions of cellulose sulphate were injected through the venous sinus of the penis or the polyethylene-cannulated carotid vein. Samples of blood were withdrawn by cardiac puncture, under light ether anaesthesia; each animal was bled only once to avoid the danger of haemorrhage caused by the anticoagulant activity of cellulose sulphate. Blood pressure changes were recorded from the carotid artery of 300–350 g male rats under pentobarbitone (30–40 mg/kg) anaesthesia.

Drugs

Synthetic bradykinin was obtained through the courtesy of Dr. A. Cerletti, Sandoz Ltd. (Basle, Switzerland). Compound 48/80 was obtained through the courtesy of Dr. R. A. Maxwell, Wellcome Laboratories (Tuckahoe, N.Y.). Histamine dihydrochloride (Mann); mepyramine (Neo-antergan, Rhodia); soya-bean trypsin inhibitor (Worthington); *N*-benzoyl-l-arginine ethyl-ester HCl (Sigma); bovine fibrinogen (fraction I, Sigma); polybrene (Hexadimethrine, Abbott).

RESULTS

Figure 1 shows that 3 mg/kg of cellulose sulphate administered intravenously caused intense hypotension in the pentobarbitone-anaesthetized rat. The fall of blood pressure, observed after a latency of about 60 sec, was followed by a slight, transient rise appearing

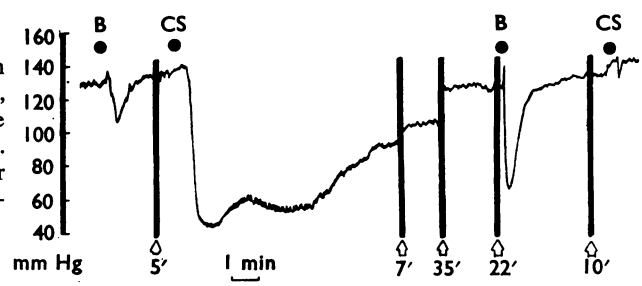

Fig. 1. Effect of cellulose sulphate on rat arterial blood pressure. At B, bradykinin 1 μg; CS, cellulose sulphate 3 mg/kg, intravenously. Note tachyphylactic effect after the first injection of cellulose sulphate.

about 150 sec after the injection, a renewed fall and gradual recovery which was complete within 60 min. This pattern was typical for the response of four other rats submitted to identical treatment. A second injection of the same dose of the polysaccharide failed to elicit another hypotensive response. This tachyphylactic effect was not due to loss of the animal's sensitivity to the hypotensive effect of bradykinin or of histamine and acetylcholine. Samples of plasma from such desensitized animals, however, failed to yield kinin material when incubated with powdered glass or cellulose sulphate.

The hypotensive action of cellulose sulphate did not seem to be due to the release of histamine. Figure 2 shows that the response of the rat to the polysaccharide was quite pronounced in spite of the animal's pre-treatment with a histamine-antagonist, mepyramine. Two other experiments confirmed this observation. The absence of a

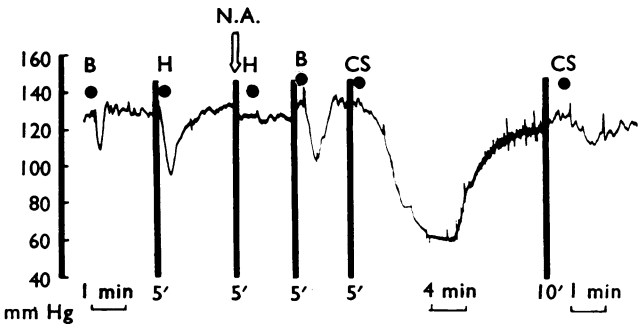

Fig. 2. Effect of an antihistamine on the hypotensive response of the rat to cellulose sulphate. At B, bradykinin 1 μg; H, histamine dihydrochloride 2 μg; N.A., neoantergan (Mepyramine) 200 μg/kg; CS, cellulose sulphate, 400 μg/kg.

histamine-releasing effect of cellulose sulphate could also be demonstrated by perfusion experiments. Figure 3 shows that cellulose sulphate was unable to release histamine from the perfused hindquarters of the rat under conditions in which a well known histamine liberator, compound 48/80, released copious amounts of the amine.

Previous results (Rothschild & Gascon, 1966) had indicated that the crystalline trypsin inhibitor from soya-beans (SBTI) inhibited the release *in vitro* of bradykinin elicited by cellulose sulphate from rat or human plasma. The same inhibition seems to prevail

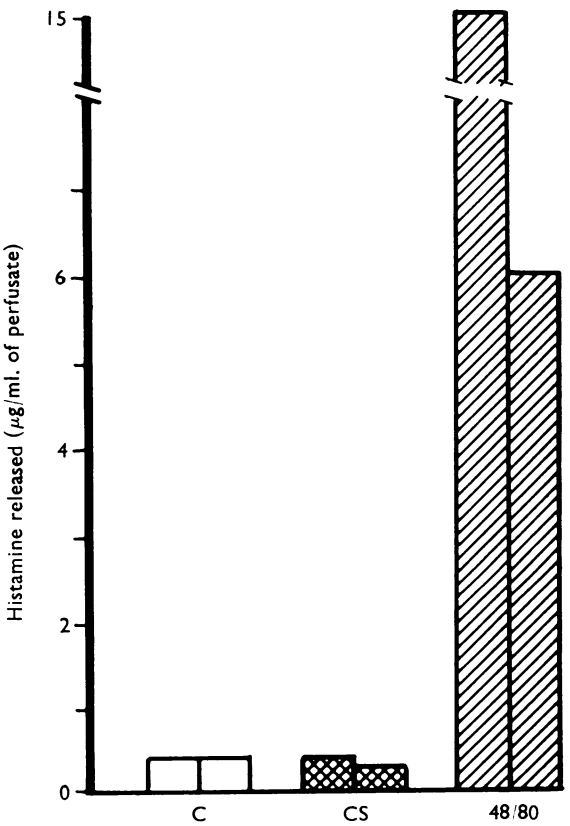

Fig. 3. Determination of the histamine-releasing capacity of cellulose sulphate in the perfused hindquarters of the rat. Each column refers to the histamine content of fluid collected during successive 10 min perfusion periods using: C, Tyrode solution alone; CS, with cellulose sulphate 1 mg; 48/80, with compound 48/80 50 μg. Assays of histamine were performed on the atropinized guinea-pig ileum. Results are expressed in terms of the free base.

in vivo because, as shown in Fig. 4, the hypotensive response of the rat to cellulose sulphate could be prevented by pre-treating the animal with SBTI 12 mg/kg. This measure failed to inhibit the response of the animal to bradykinin, an indication that the effect of SBTI was not due to an unspecific decrease of its sensitivity to hypotensive agents.

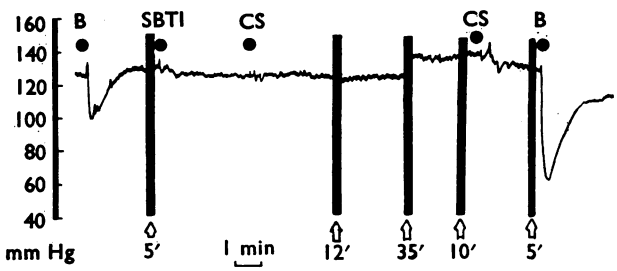

Fig. 4. Effect of soya-bean trypsin inhibitor (SBTI) on the hypotensive response of the rat to cellulose sulphate. At B, bradykinin 1 μg; SBTI, 12 mg/kg; CS, cellulose sulphate 3 mg/kg.

The effects of cellulose sulphate on plasma kininogen, blood esterase, leucocyte and platelet levels, plasma protein and haematocrit values of the rat are shown in Fig. 5. The intravenous injection of the polysaccharide (3 mg/kg) depleted the rat of approximately 70% of its plasma kininogen within 5 min. The animal remained in this condition for at least 1 hr; the return to normal concentrations of kininogen was observed within 12 hr after treatment. A sharp increase of the esterolytic activity on benzoylarginine ethyl ester (BAEE) of blood followed the injection of the sulphopolysaccharide. This effect, observed already within 1 min of treatment, waned rapidly and was no longer noticed in blood drawn 12 min after treatment. A similar, gradual loss of the esterolytic activity on BAEE had been noted in human plasma treated with cellulose sulphate *in vitro* (Rothschild & Gascon, 1966). Figure 6 shows that the inactivation curves of the cellulose sulphate-induced enzyme followed approximately parallel courses *in vivo* and *in vitro*.

Blood platelets seemed to be unaffected by cellulose sulphate, except for a slight tendency to clumping and a small decrease in total number observed in samples drawn 2.5 min after treatment. In contrast, the total leucocyte content of peripheral blood increased after the administration of the polysaccharide. This effect was statistically significant ($P<0.05$), in blood samples collected 5 min after injection, increased ($P<0.01$) within 1 hr and had disappeared after 12 hr. No significant change was observed in the total plasma protein content or the haematocrit level of rats treated with cellulose sulphate.

Table 1 illustrates the relationship between clotting time, plasma kininogen content and leucocyte count in the blood of rats treated with different doses of cellulose sulphate. It can be seen that a significant effect on blood clotting time only appeared after the injection of at least 3 mg/kg of the polysaccharide. Cellulose sulphate (300 μg/kg) caused a fall of approximately 25% in circulatory kininogen; this effect was markedly increased in animals injected with 1.0 or 3.0 mg/kg; further increases of dosage led to even greater kininogen depletion, but even the highest dose used failed to deplete fully the animal of plasma kinin precursor. The leucocytosis induced by cellulose sulphate

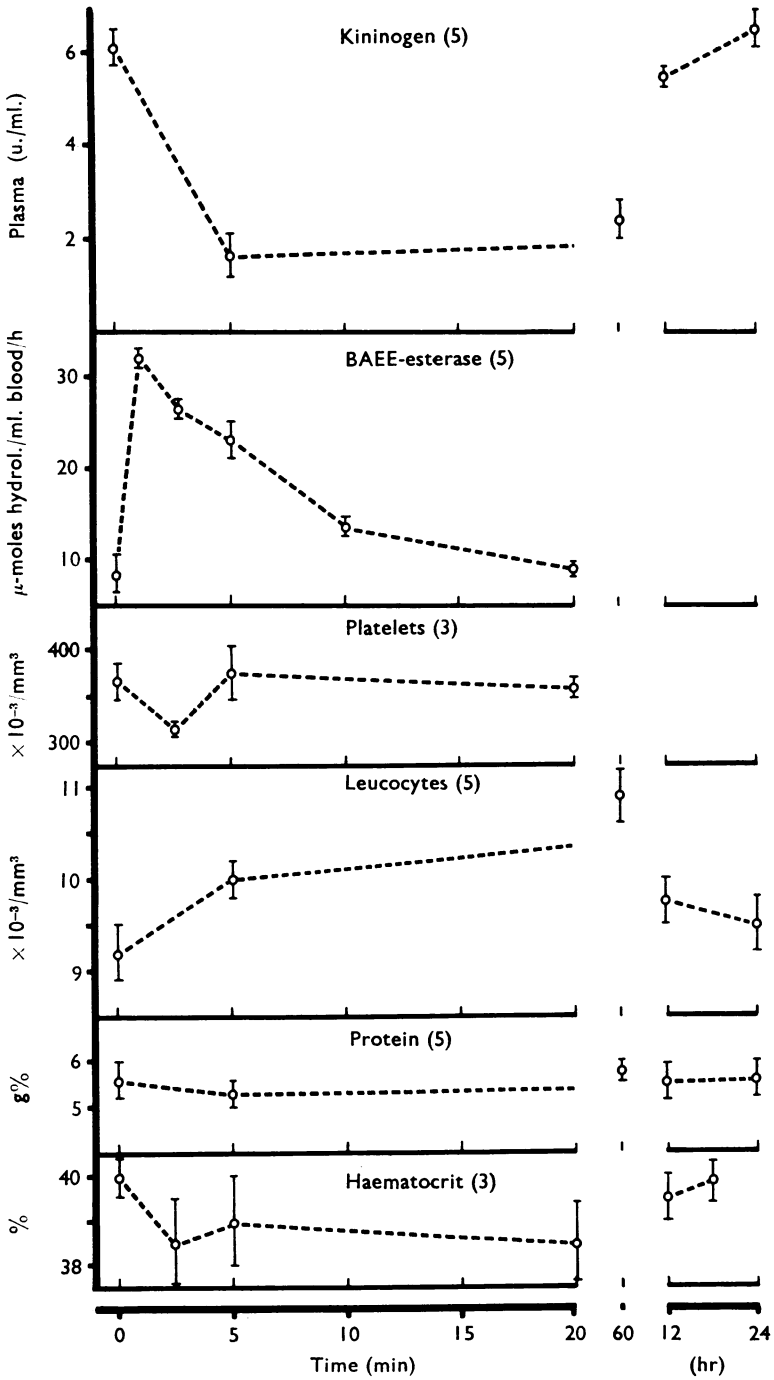

Fig. 5. Effect of the intravenous administration of cellulose sulphate on blood components of the rat. Figures in parenthesis refer to the number of experiments performed. One unit of kininogen is equivalent to 0.44 μg of synthetic bradykinin ; BAEE, benzoylarginine ethyl ester.

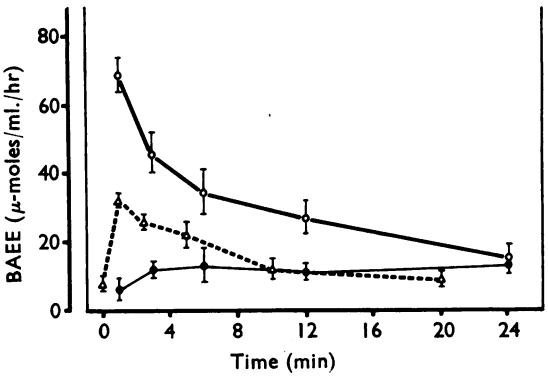

Fig. 6. Appearance and progressive loss of the esterolytic activity on benzoylarginine ethyl ester (BAEE) in the blood of rats injected intravenously with cellulose sulphate 3 mg/kg (△----△) and of rat plasma treated *in vitro* with cellulose sulphate 25 μg/ml. (○——○), (●——●), Esterolytic activity *in vitro* of untreated rat plasma. Samples at zero time were withdrawn before the injection of cellulose sulphate.

TABLE 1

EFFECT OF CELLULOSE SULPHATE ON RAT PLASMA KININOGEN CONTENT, BLOOD CLOTTING TIME AND CIRCULATORY LEUCOCYTE COUNTS

Each result is the average of three experiments; control values refer to fifteen anmals. * The effect observed was statistically significant ($P<0.05$) when compared with controls by Student's t test on paired samples.

	Cellulose sulphate (mg/kg, intravenously)								
	0	0·3		1·0		3·0		10·0	30·0
		Interval between injection and collection of blood sample (min)							
		5	20	5	20	5	20	5	5
Clotting time (sec)	142	120	120	120	120	7,200	7,200	7,200	21,600
Range	(90–240)								
Kininogen (u./ml.)	5·3	4·0*	4·0*	1·8*	1·8*	2·3*	2·1*	1·1*	1·0*
Range	(4·8–6·5)								
Leucocytes (per mm³)	6,960	8,050*	7,140	8,560*	11,770*	7,550*	7,990*	9,200*	7,830*
Range	(6,350–7,610)								

was statistically significant in all animals except those examined 20 min after the injection of 300 μg/kg of the compound.

Table 2 shows that intravenous injections of cellulose sulphate 300 μg/kg repeated once or twice within a period of 30 min led the rat to a condition of plasma kininogen deple-

TABLE 2

EFFECT OF REPEATED INJECTIONS OF CELLULOSE SULPHATE ON PLASMA KININOGEN CONTENT OF THE RAT

* Means of three animals ± S.E.M.; 10 min elapsed between successive injections.

	Cellulose sulphate		
No. of injections	Dose (mg/kg)	Kininogen level* (u./ml. plasma)	Fall (%)
0	—	5·3±0·5	
1	0·3	4·0±0·3	25
2	0·3	2·4±0·4	55
3	0·3	1·5±0·1	68
1	1·0	1·8±0·2	66

TABLE 3

EFFECT OF REPEATED INJECTIONS OF CELLULOSE SULPHATE 300 μg/kg ON RAT BLOOD PRESSURE

* Measured by calculating the area between the original base line and the recorded tracing during 10 min after cellulose sulphate injection.

		Number of injections							
		1		2		3		4	
Exp.	Initial blood pressure (mm Hg)	Maximal fall (mm)	Hypotension area* (mm^2)	Maximal fall (mm)	Hypotension area (mm^2)	Maximal fall (mm)	Hypotension area (mm^2)	Maximal fall (mm)	Hypotension area (mm^2)
I	150	40	340	78	925	38	225	10	40
II	155	5	25	88	955	44	295	5	25
III	168	13	65	30	315	70	525	25	85
Mean ± S.E.		19·3±10·6	143±99	65·3±17·9	732±210	50·6±9·8	345±91	13·3±6·0	50±18

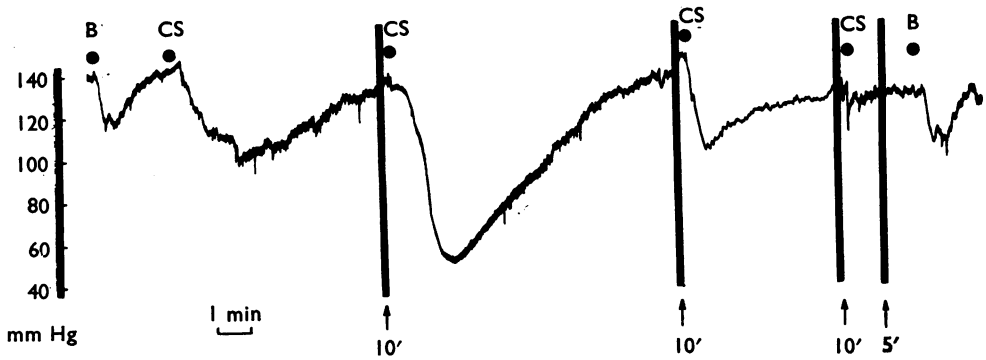

Fig. 7. Effect of a series of injections of small doses of cellulose sulphate on the arterial blood pressure of the rat. At B, bradykinin 0.5 μg ; CS, cellulose sulphate 300 μg/kg. Note cumulative effect after the second and tachyphylactic effect after the third injection of cellulose sulphate.

tion equivalent to that following a single injection of a 1 mg/kg dose of the compound. Figure 7, which depicts a typical experiment, and Table 3, which shows the result of three further experiments, present the changes in arterial blood pressure observed in rats receiving four successive injections each of cellulose sulphate 300 μg/kg at 10 min intervals. A cumulative effect was observed, the first injection evoking only a slight response but leading to a markedly larger hypotension with a second dose of polysaccharide ; the third injection produced a response which was smaller than the second in two experiments and larger in a third ; clear-cut tachyphylaxis was obtained after the fourth injection in all cases.

Figure 8 shows that the injection of cellulose sulphate 1 mg/kg into the rat evoked fibrinolytic activity, demonstrable on bovine fibrin, in its plasma. Treatment with higher

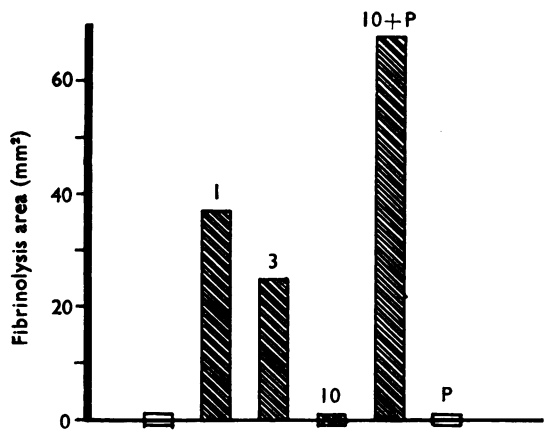

Fig. 8. Effect of the intravenous administration of cellulose sulphate on the fibrinolytic activity of rat plasma on bovine fibrin. Activity is expressed in mm² representing the product of the perpendicular diameters of the area of dissolved fibrin. Light bars refer to the activity of plasma collected before the injection of cellulose sulphate. Hatched bars refer to the activity of plasma obtained 5 min after the injection of cellulose sulphate 1 mg/kg (1), 3 mg/kg (3) and 10 mg/kg (10). P, refers to samples mixed with polybrene 50 μg/ml. of plasma before application to the fibrin plate. All results represent averages of three experiments.

doses of the polysaccharide diminished this effect, which disappeared from the plasma of animals treated with 10 mg/kg of the compound. This result seemed to be the result of an inhibitory effect of excess sulphopolysaccharide on the test system employed. When the plasma of an animal which had been treated with cellulose sulphate 10 mg/kg was

mixed, before assay, with polybrene, a cationic compound capable of reacting with negatively charged macromolecules (Kimura, Young, Stein & Richards, 1959), clear-cut fibrinolytic activity could be demonstrated. Polybrene alone failed to induce such activity in normal rat plasma.

DISCUSSION

Cellulose sulphate lowered the arterial blood pressure of rats. Unlike other polysaccharides, such as dextran and ovomucoid, which release histamine in this species (Rocha e Silva & Rothschild, 1955), or of dextran and dextran sulphate, which do so in rabbit blood (Haining, 1956), cellulose sulphate did not seem to owe its hypotensive effect to histamine release. Rats treated with an anti-histamine drug (mepyramine) were quite sensitive to the sulphopolysaccharide, which also lacked histamine-releasing activity when perfused through the isolated hindquarters of the rat. Leme, Schapoval & Rocha e Silva (1967), by showing that the oedema of the rat's paw produced by locally injected sulphopolysaccharide was little inhibited by anti-histamine or anti-5-hydroxytryptamine agents but was quite pronounced in histamine-depleted animals, have also provided evidence against the participation of histamine in the vascular response of the rat to cellulose sulphate.

Although no attempts to demonstrate free kinins in the circulation of treated animals have been made, bradykinin seems to be a likely mediator of the rat's response to this polysaccharide. The extensive decrease in circulatory kininogen which followed the intravenous administration of the compound was completed within the time interval required for the maximal fall of blood pressure ; the formation of substantial amounts of bradykinin in rat plasma treated with cellulose sulphate has been demonstrated *in vitro* (Rothschild & Gascon, 1966). The desensitization which followed the administration of cellulose sulphate must have resulted from the depletion of available plasma kininogen, because it did not extend to the hypotensive effect of bradykinin but was accompanied by the exhaustion of the *in vitro* kinin forming capacity of the treated animal's plasma.

The inhibition of kinin release *in vitro* by cellulose sulphate in rat plasma containing crystalline soya-bean trypsin inhibitor has been demonstrated by Rothschild & Gascon (1966) ; a similar inhibition could explain the absence of a hypotensive response to the sulphopolysaccharide of rats pretreated with SBTI.

Depletion of plasma kininogen by cellulose sulphate was a comparatively short-lasting effect, restoration of normal plasma concentrations being complete within 12 hr. This period is similar to that reported by Gautvik & Rugstad (1967) for the regeneration time of plasma kininogen in rats treated with ellagic acid, but seems to be somewhat shorter than the time span required for the repletion of plasma kininogen in the guinea-pig treated with cellulose sulphate (Rothschild, 1967a).

Hamberg & Rocha e Silva (1957) suggested that enzymes capable of releasing bradykinin are also able to split arginine esters ; this relationship also seems to exist in the blood of rats treated with cellulose sulphate.

The observation that the inactivation of the BAEE-esterase of rat blood followed a very similar time course *in vivo* and in plasma *in vitro* suggests that a humoral process is involved in both instances.

Glycogen and certain other polysaccharides have been shown to cause leucopenia and thrombocytopenia in the rabbit (Rocha e Silva, 1950). Such effects could also be obtained when cellulose sulphate 25 mg/kg was given to this species (Rothschild, 1968); however, neither leucopenia nor loss of circulatory platelets were observed in rats treated with cellulose sulphate at concentrations causing maximal kininogen breakdown. On the contrary, such animals exhibited a significant leucocytosis: this change could be caused by released bradykinin, since, as shown by Cardoso, Amorim, Grellet, Coutinho & Manço (1966), infusions of the polypeptide seem to lead to a transiently increased leucocyte content of peripheral blood.

The anticoagulant action of cellulose sulphate and other synthetic anionic polysaccharides has been described by Bergström (1935), Karrer *et al.* (1943) and Astrup *et al.* (1944). Nevertheless, there seems to be no correlation between the anticoagulant and the kinin releasing actions of cellulose sulphate, for this compound caused extensive kininogen depletion at doses which entirely failed to affect the clotting time of the blood of the rat. A dissociation between these effects has also been demonstrated by results showing that heparin, while 4–6 times more active an anticoagulant than cellulose sulphate (Karrer *et al.*, 1943), was less efficient than the former in causing the release of bradykinin from rat plasma *in vitro* (Rothschild, 1967a).

The plasma of rats treated with cellulose sulphate lysed bovine fibrin. This substrate was, like most preparations obtained from commercial fibrinogen, contaminated with plasminogen. It is probable that the fibrinolytic effect observed results from the action of a plasminogen activator, a plasminoplastin (Iatridis & Ferguson, 1962), because such an agent has been demonstrated to arise in rat plasma treated with cellulose sulphate *in vitro* Rothschild, Rosa & Rothschild, 1968).

The decreased fibrinolytic activity of the plasma of rats treated with high doses of cellulose sulphate can be explained as the result of an inhibitory effect of excess sulphopolysaccharide. Polybrene, a cationic polymer capable of combining with polysaccharide sulphates (Kimura *et al.*, 1959), was able to restore appreciable fibrinolytic activity to such plasma. This observation parallels that of Robinson, Harris & Ricketts (1959), who have only been able to estimate the enhanced lipoprotein lipase activity in the plasma of rats treated with dextran sulphate after neutralizing excess sulphopolysaccharide with protamine, a functional congener of polybrene.

Although cellulose sulphate is a water-soluble compound, it seems to release kinin in plasma by a process similar to that evoked by particulate materials such as glass or kaolin (Eisen, 1964). This is indicated by results showing that *in vitro* kinin release by the sulphopolysaccharide is greatly retarded in Hageman factor-deficient human plasma, unhindered by ε-amino caproic acid and effectively inhibited by small increases of the ionic strength (Rothschild, Rosa & Rothschild, unpublished).

Cellulose sulphate has been used to deplete kininogen in rats for investigations on the role of kinins in passive cutaneous anaphylaxis (Rothschild, 1967b), and thermic oedema (Leme *et al.*, 1967), and in dogs used for studies on the origin of endotoxin shock (Rothschild & Castania, 1968). The present work indicates that in order to induce plasma kininogen depletion, the injection of several small doses of cellulose sulphate may be preferable to the injection of a single larger dose, because the latter causes intense and relatively long-lasting hypotension. It is worth noting that even the intravenous adminis-

tration of a single dose of cellulose sulphate 3 mg/kg, which led to the rapid breakdown of an amount of kininogen corresponding to up to 100 μg of bradykinin/kg, never caused irreversible shock or an otherwise severe outcome.

SUMMARY

1. Intravenously administered cellulose sulphate caused intense but surmountable hypotension in the rat, which was accompanied by a marked fall of plasma kininogen and seemed to be due to the release of bradykinin. The hypotension was absent in rats pretreated with the crystalline trypsin inhibitor from soya-beans, but occurred in animals pretreated with a histamine antagonist.

2. Cellulose sulphate failed to release histamine from the perfused hindquarters of the rat.

3. The depletion of plasma kininogen caused by cellulose sulphate was accompanied by a marked but short-lasting elevation of the esterolytic action of the treated animal's blood on benzoylarginine ethyl ester, by moderate leucocytosis, but not by changes in blood platelet, plasma protein, or haematocrit values. Control kininogen and leucocyte values were found in the blood of rats examined 12 hr after treatment.

4. The anticoagulant effect of cellulose sulphate was only noticeable in the blood of rats receiving doses of polysaccharide at least 3 times higher than those required to induce extensive plasma kininogen depletion.

5. Fibrinolytic activity could be demonstrated in the plasma of rats receiving cellulose sulphate. The apparent interference of excess of sulphopolysaccharide on components of the fibrinolysis assay system could be overcome by polybrene, a polymeric quaternary ammonium salt.

6. A series of three or four injections of small amounts of cellulose sulphate, while leading to the same degree of kininogen depletion as the administration of a single, larger dose, caused smaller hypotensive responses and seemed to be the better way of producing the plasma kininogen-depleted state in the rat.

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