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ANTI-INFLAMMATORY ACTION OF SULFATED POLYSACCHARIDES

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Abstract—A number of sulfated polymers: pentosan polysulfate, polyvinyl sulfate, polyethylene sulfonate, cellulose sulfate, dextran sulfate, sulfate amylopectin, in the same way as carragenin, degraded carragenin and dextran, produce edema in the rat's paw by local injection, to release kinins from fresh guinea pig and rat's plasma (*in vitro*) and to reduce the bradykininogen content of rat's blood when injected i.v. When given repeatedly by the i.p. route, cellulose sulfate and sulfated amylopectin reduced or abolished the edema produced in the rat's paw by a subsequent local injection of the same or other edematogenous polymers. This cross desensitization or anti-inflammatory action of the sulfated polymers was also observed if the animals received locally a fraction of the venom of *Agk. piscivorus*, which was found to split the ester bond of BAEE and release kinin when incubated with fresh plasma of different species. The possible significance of these findings in order to derive a new class of anti-inflammatory agents is discussed.

THE IMPORTANCE of the participation of the kinin system in inflammatory reactions (edema) induced in the rat's paw by local injection of sulfated polysaccharides (carragenin, cellulose sulfate, dextran sulfate, etc) has been stressed on the basis of the findings that cellulose sulfate and other sulfated polysaccharides produce a release of kinin, when incubated *in vitro* with the rat's and guinea pig's plasma.[1–4] Moreover, it was found that chronic treatment with cellulose sulfate or carragenin, leads to significant reductions in plasma levels of bradykininogen.[4]

This *in vivo* activation of the kinin system, with reduction of the available stocks of circulating BKg was the basis to understand the crossed desensitization which occurred in rats when the animals were chronically treated with one kind of sulfated polysaccharide and tested locally with another kind, to produce edema of the paw.[4] It is to be noted that other forms of local edema, as the thermic edema (45° for 30 min) are also reduced by a previous chronic treatment with cellulose sulfate or carragenin, given i.p.[4] This finding indicates that some fundamental pathway leading to the increased vascular (capillary) permeability, is common to the action of sulfated polysaccharides and the spontaneous swelling produced by immersing the rat's paw in a water bath at 45° for 30 min.[5] Furthermore, it was shown that hexadimethrine given before, not only reduced the edema produced by carragenin and cellulose sulfate, but also the one produced by heating of the paw to 45° for 30 min (thermic edema). Since hexadimethrine is known to interfere with the mechanism of kinin

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activation, these results once more indicate the importance of the kinin system in the production of swelling by sulfated polysaccharides and heating at 45°.

In the present paper, additional evidences are obtained by cross desensitization between carragenin, cellulose sulfate and the sulfated amylopectin (SN-263), currently assayed for its anti-peptic ulcer activity,[6] and also by the use of a fraction of the venom of *Agkistrodon piscivorus*, which is known to release bradykinin[7] and found to produce strong edema when injected in the rat's paw.

MATERIAL AND METHODS

Edema in the rat's paw. Adult male Wistar rats received injections in the sub-plantar region of the hindpaws, of 0·1 ml of the saline solution which did or did not contain the edematous agent. In each rat one paw received, as control, only saline and the other, the agent to be studied.

The volume of the paws, up to the tibio–tarsic junction was determined by plethysmography according to Winder *et al.*,[8] 0·5, 2 and 4 hr after the local injection, in animals receiving 30–40 mg/kg of pentobarbital i.p.

Data are given as percentages of increase of the average volume (V_t) of the paws of all animals submitted to a certain treatment over the control volume (V_0) of the paws injected with a certain volume of saline.

$$i\% = \frac{100\ V_t}{V_0} - 100$$

The details of all methods employed were described in a previous paper.[4]

Anti-inflammatory action. In the experiments in which the effect of a previous general treatment was tried to reduce or abolish the local effect of the edematous substances utilized, the animals received i.p., 2, 3 and 4 hr before, the indicated amounts of the sulfated polysaccharides (carragenin, dextran, cellulose sulfate and SN-263). In the experiments in which the anti-inflammatory action of sulfated polysaccharides was tested against the local treatment with the fraction of the venom of *Agk. piscivorus*, a more extensive schedule of chronic treatment was utilized, following the indications given in Garcia Leme *et al.* (1967).[4] Two hours after the last i.p. injection, the venom was injected locally in the hindpaw, in the amount corresponding to 5 μg of the fraction obtained as described below.

Fractionation of the venom of Agk. piscivorus. Gel filtrations on Sephadex G-200 and G-75 of the crude dry venom (Miami Serpentarium, Fla.), yielded the fraction utilized in the present experiments.[18] The fraction was devoid of any hemolytic activity and did not show histamine releasing activity when tested upon the isolated mast cells from the rat's peritoneal fluid. It was a powerful releaser of bradykinin when incubated with the rat's, the guinea pig's and dog's plasma and displayed a parallel activity in splitting the synthetic substrate benzoyl-L-arginine ethylester (BAEE). This esterasic activity ran parallel to the bradykinin releasing activity.

Bradykinin-forming activity. The experiments to show release of kinin activity by SN-263 were done as described previously, utilizing the oxalated guinea-pig plasma, to which 8-HQ (1·0 mg/ml) was added in order to preserve the formed kinin activity. Bradykininogen estimations were done according to the method routinely utilized in this laboratory.[9,10]

All assays were done on the isolated guinea pig ileum, in the presence of 0·1 μg

of atropine/ml and diphenhydramin (benadryl, 0·1 mg/ml). Synthetic bradykinin (BRS 640 Sandoz) was used as a standard.

Estimation of BKg in circulating blood. Male Wistar rats received i.v. 30 mg/kg of the indicated sulfated polymers (SN-263, polyvinyl sulfate and polyethylene sulfate); 1 ml heparinized blood samples were taken immediately and after 5 and 20 min from a carotid artery directly into siliconized needles and syringes. The processing of the sample for BKg estimation followed the micromethod described in this laboratory.[9,10] The amounts of BKg were calculated in μg of bradykinin (synthetic) per ml of plasma wherefrom the percentages of decrease were calculated and plotted in the graphs.

RESULTS

Comparative edematogenous actions of synthetic and natural sulfated polymers. A number of sulfated synthetic polymers were tried to produce local edema of the rat's paw, in comparison with natural sulfated polysaccharides. The results obtained are given in Table 1.

TABLE 1. COMPARATIVE EDEMATOUS ACTIONS OF SYNTHETIC AND NATURAL SULFATED POLYMERS

Agent	Dose inject. (μg/paw)	Volume increase of the rat paw (% of control-times in h_2 after administration of drug)		
		0·5	2	4
Pentosan polysulfate	500	10	18	28
Polyvinyl sulfate	500	70	80	49
Polyethylene sulfonate	500	48	49	30
Degraded carragenin	500	9·5*	19*	30*
Carragenin	500	16*	23*	32*
Cellulose sulfate (CS)	500	90	97	87
Cellulose sulfate (CS)	100	44	48	62
Cellulose sulfate (CS)	10	25*	19*	26*
Cellulose sulfate (CS)	1	12*	14*	16*
Dextran sulfate	500	110	107	125
Dextran	500	87	80	86
Sulfated amylopectin (SN-263)	500	50	46	44
Sulfated amylopectin (SN-263)	100	5	24	42

Each figure represents the average of five animals submitted to the experiment the same day; when the results of two different days were pooled, the average is indicated by *.

It is interesting that completely synthetic polymers as polyvinyl sulfate or polyethylene sulfate were able to produce edema in the rat's paw, indicating that the presence of a polysaccharide chain is not essential for the edematous activity. The weak effect of pentosan sulfate can be contrasted with the strong effect of polyvinyl sulfate. Carragenin and degraded carragenin were among the least active of the materials utilized, a result which is difficult to reconcile with that obtained by others, including our laboratory.[4] Dextran sulfate was the most potent agent utilized at the level of 500 μg/per paw; with cellulose sulfate a definite increase in the volume of the paw was obtained at the 1 μg level, confirming the results obtained previously. The fact that SN-263 (sulfated amylopectin) produced a strong edematous effect and that

it was active at the level of 100 μg, induced us to try its bradykinin releasing activity and its capacity to cross desensitize to other sulfated polysaccharides or to the edematous fraction of the venom of *Agk. piscivorus.*

Release of kinin activity by sulfated polymers. Confirming previous results by Rothschild and Gascon[1] and Garcia Leme *et al.*[3,4] we have found that cellulose sulfate is able to release active kinins when incubated with the oxalated plasma from guinea pig and rat. Other sulfated polymers such as SN-263, polyethylene sulfate and polyvinyl sulfate were also able to activate the kinin system in the above mentioned plasmas. Surprisingly, pentosan sulfate was practically devoid of the capacity to release kinin from guinea-pig and rat plasma. Table 2 and Fig. 1 summarize the experiments done on fresh guinea-pig plasma incubated with the mentioned sulfated polymers. Table 3 gives the results obtained with fresh rat's plasma incubated with varying amounts of the same polymers.

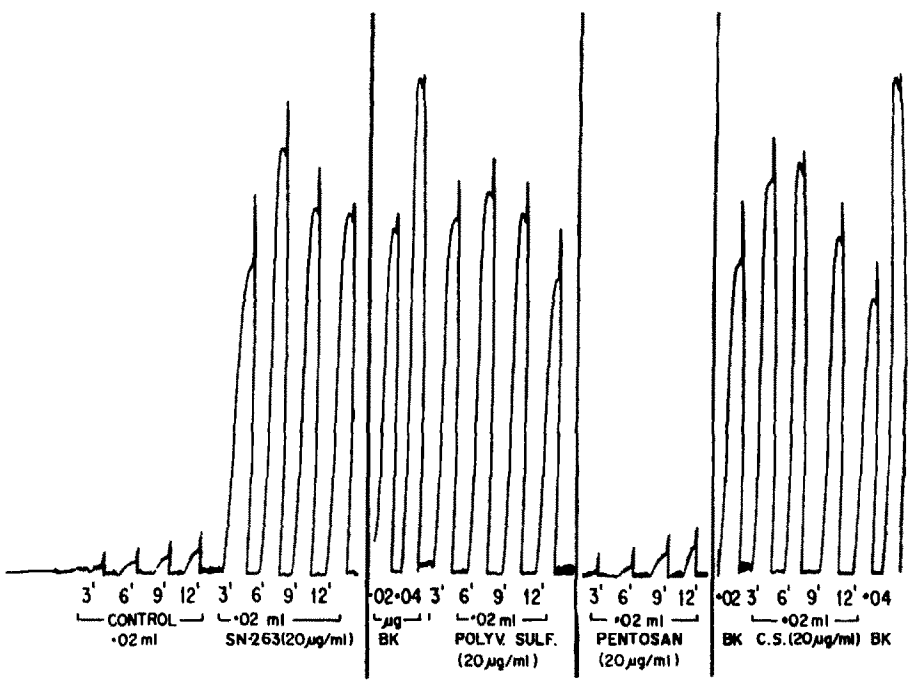

FIG. 1. Guinea pig ileum. Assay of activity released by incubation of fresh guinea pig plasma with the indicated sulfated polymers: SN-263 = sulfated amylopectine; Polyv. sulf. = polyvinyl sulfate; pentosan sulfate; C.S. = cellulose sulfate. The figures indicate time of incubation in minutes; BK = synthetic bradykinin. Control = fresh plasma + buffer (Tris pH = 7·8) + saline.

For a control, the sulfated polymers were incubated for increasing lengths of time with pure synthetic bradykinin. Even 60 μg of the different polymers with 1 μg of the polypeptide (Sandoz 640 BRS) incubated for 12 min failed to change the response of the gut to 0·1 ml of the solution in comparison to the response to 1 μg of BK alone. We can therefore conclude that the sulfated polymers in spite of their strong acidic characters are unable to combine with the polypeptide.

TABLE 2. RELEASE OF KININS FROM FRESH GUINEA-PIG PLASMA BY SULFATED POLYMERS

Sulfated polymers	Amount added to samples* (μg/ml)	Kinin release† after incubation (min) 3′	6′	9′	12′
Cellulose sulfate + 8-HQ	60	1·5	1·5	1·5	1·4
	40	0·5	0·7	0·6	0·6
	20	1·8	1·5	1·2	1·0
SN-263 + 8-HQ	60	1·5	1·5	1·5	1·4
	40	0·8	0·7	0·7	0·6
	20	1·5	1·4	1·0	0·9
Polyethylene sulfate + 8-HQ	60	2·0	2·0	1·8	1·4
	40	2·0	1·8	1·9	1·9
	20	1·8	1·6	1·3	1·0
Polyvinyl sulfate + 8-HQ	60	1·0	1·0	0·8	0·8
	40	2·0	2·2	1·9	1·9
	20	1·0	0·8	0·8	0·5
Controls: Plasma + buffer + 8-HQ		traces (0·01)——0·1 μg/ml			
Bradykinin + polymers + 8-HQ		no change in activity of pure BK			

* Each sample contained 0·5 ml of fresh plasma + 0·2 ml of "Tris" buffer (pH 7·8) + 0·01 ml 8-HQ (mg/ml); volume completed to 1·0 ml with saline to which the proper amount of the releaser was added.

† Results are given in μg of synthetic bradykinin (640 BRS, Sandoz), per ml of plasma.

TABLE 3. RELEASE OF KININS FROM FRESH RAT'S PLASMA BY SULFATED POLYMERS

Sulfated polymers	Amount added* (in μg/ml)	Kinin release† after incubation (min) 3′	6′	9′	12′
Cellulose sulfate + 8-HQ	60	0·7	0·7	0·7	0·5
	40	1·0	2·8	2·6	2·6
	20	0·5	0·5	0·5	0·5
SN-263 + 8-HQ	60	1·6	1·8	1·8	2·0
	40	1·6	1·8	1·8	2·0
	20	2·0	2·0	2·0	2·0
Polyethylene sulfate + 8-HQ	60	1·6	1·5	1·5	1·5
	40	1·8	1·9	2·0	1·8
	20	1·4	2·0	1·5	1·4
Polyvinyl sulfate + 8-HQ	60	1·1	1·2	1·2	1·2
	40	1·4	1·6	1·8	2·0
	20	0·9	1·0	1·0	1·0
Controls: Plasma + buffer + 8-HQ		traces			

* Composition of samples was the same as indicated in Table 2, using rat plasma instead of guinea pig plasma.

† The results are given in μg of synthetic bradykinin (640 BRS, Sandoz), per ml of plasma.

TABLE 4. BRADYKININOGEN CONTENT OF THE BLOOD OF RATS TREATED WITH SULFATED POLYMERS

Sulfated polymers (30 mg/kg i.v.)	Number of animals	Bkg (μg/ml) in circulating blood*		
		Control	5′	20′
SN-263	4	1·85	1·46	1·00
Polyvinyl sulfate	4	2·50	1·75	1·20
Polyethylene sulfonate	3	2·80	2·10	1·50

* The figures indicate the averages of bradykininogen content after incubation with the polymers, expressed as μg of synthetic bradykinin (640-BRS, Sandoz) per ml of plasma.

Experiments *in vivo* showed a significant reduction of BKg in the circulating blood after 5 or 20 min of the i.v. injection of the indicated polymers (Table 4).

Crossed desensitization by SN-263, and cellulose sulfate. Oral treatment. In an attempt to investigate any possible anti-inflammatory action of SN-263 by the oral route, rats were given 40–100 mg of the drug at 2, 3 and 4 hr before the local treatment with cellulose sulfate (100 μg). Though, in two of six experiments, the animals treated orally with 40 and 50 mg of SN-263 presented a diminished swelling after local injection of 100 μg of cellulose sulfate, the groups receiving 80–100 mg of SN-263 rather showed enhancement of the swelling produced by local injection of C.S. in one of the hind paws. The results are summarized in Table 5.

TABLE 5. EFFECT OF ORAL TREATMENT BY SN-263, UPON THE RAT'S PAW EDEMA INDUCED BY LOCAL INJECTION OF CELLULOSE SULPHATE

Exp. No.	SN-263 mg, orally	Maximal swelling obtained in animals* receiving 100 μg C.S., following oral treatment (hr)			Controls
		2	3	4	
I	100	72	39	64	52
II	80	71	73	76	51
III	80	55	37	55	33
IV	50	47	55	47	86
V	50	44	94	74	36
VI	40	51	51	53	66
Averages:		57	58	61	54

* Maximal swelling of the rat hind paw, by local injections of 100 μg C.S. after oral treatment (in hrs) of SN-263.

Intraperitoneal route. A definite anti-inflammatory action was obtained by treating the animals with 100 mg of SN 263, i.p., and injecting locally 2, 3 and 4 hr thereafter, 100 μg of cellulose sulfate, into one of the hind paws. The results obtained with the four groups of rats are summarized in the graph of Fig. 2. The most striking result was obtained when C.S. was injected 3–4 hr after the i.p. treatment.

Edema produced in the rat's paw by local injection of the venom of Agkistrodon

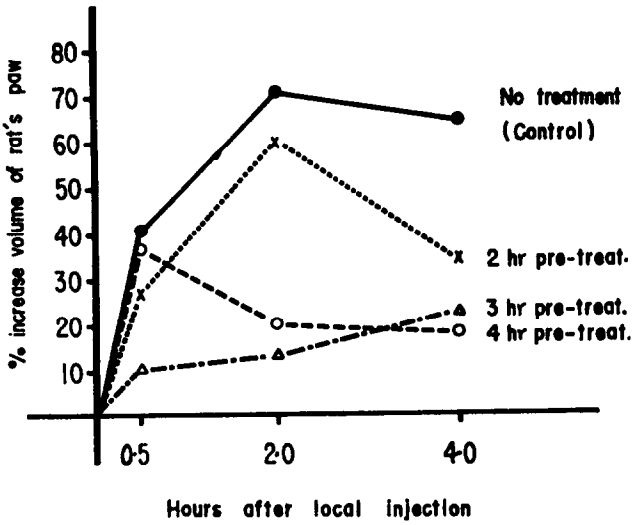

FIG. 2. Swelling of the rat hindpaw by local injection of 100 μg of cellulose sulfate (C.S.) after i.p treatment with 100 mg/kg of SN-263; ●—●, controls, treated with saline instead of SN-263; ×—×, 2 hr; △—△, 3 hr and ○—○, 4 hr after the i.p. treatment with SN-263.

piscivorus *and the esterase fraction F.C.* As shown in Figs. 3 and 4, the local injection of the venom of *Agkistrodon piscivorus* and the fraction isolated therefrom by two passages through Sephadex columns (Fraction C), induces strong reaction, that was greatest with 50 μg of the total venom and with 5 μg of Fraction C. Fraction C, utilized in the experiments presented in Fig. 5, displayed a strong esterase activity when assayed upon the arginine ester BAEE (benzoyl-L-arginine ethyl ester) and

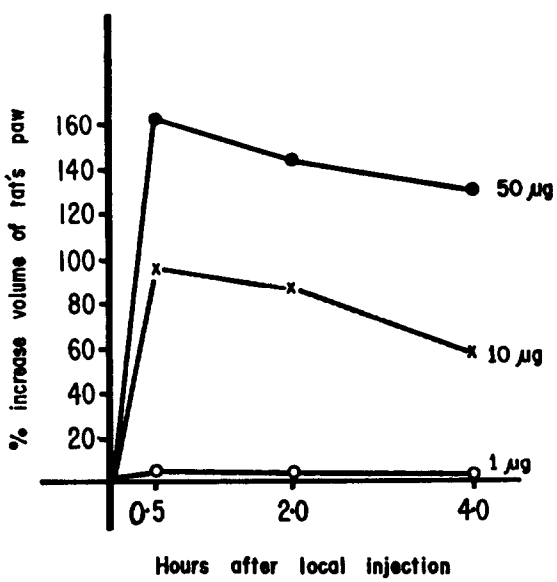

FIG. 3. Swelling of the rat hindpaw by local injection of the venom of *Agk. piscivorus* (total venom).

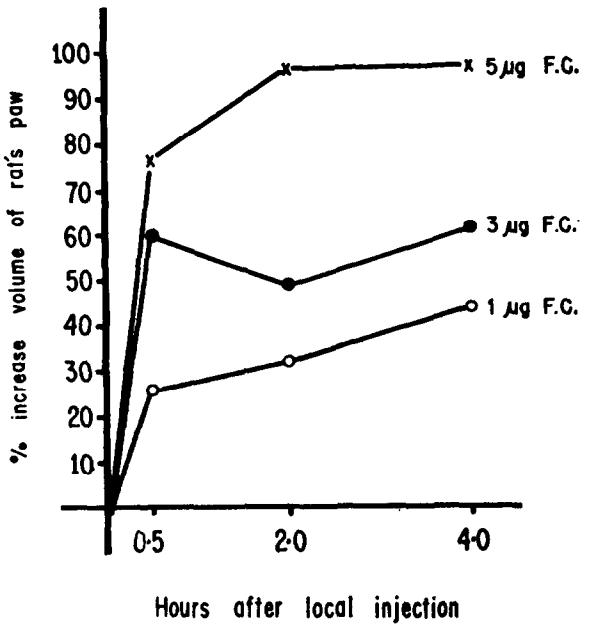

FIG. 4. Swelling of the rat hindpaw by local injection of Fraction C of the venom of *Agk. piscivorus*.

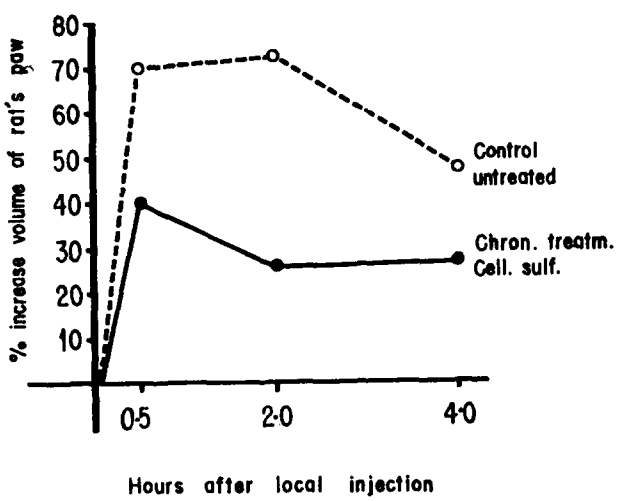

FIG. 5. Swelling of the rat hindpaw by local injection of 5 µg of Fraction C of *Agk. piscivorus* before and after chronic treatment with cellulose sulfate.

almost no activity upon the synthetic substrate ATEE (acetyl-tyrosine ethyl ester). The capacity of the fraction in releasing bradykinin when incubated with the guinea pig's or the dog's plasma, ran parallel with its capacity in splitting BAEE.[18]

The chronic treatment of rats with cellulose sulfate and the sulfated amylopectin (SN-263) resulted in a sharp decrease of the edemas produced by 5 µg of Fraction C as shown in Figs. 5 and 6.

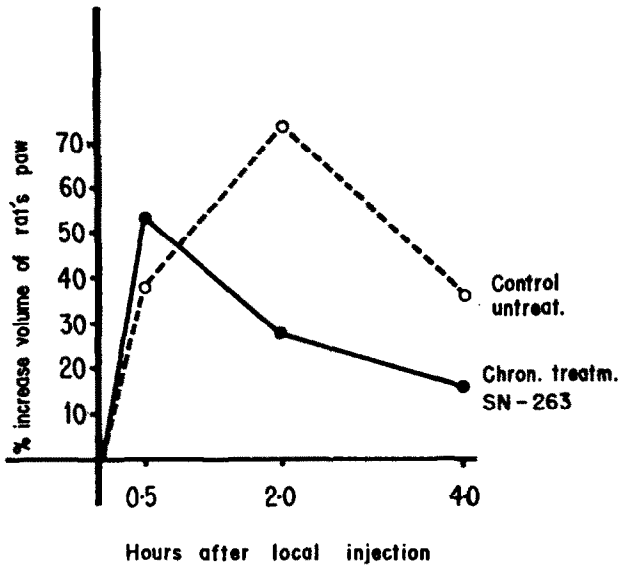

FIG. 6. Swelling of the rat hindpaw by local injection of 5 μg of Fraction C of the venom of *Agk. piscivorus* before and after chronic treatment with SN-263.

DISCUSSION

In previous publications of this laboratory,[1,3,4,11,12] it was shown that cellulose sulfate is able to activate the kinin system in rat and guinea-pig plasma and to induce cross desensitization when given i.p. before the local injection of any one of the other sulfated polysaccharides injected in the rat's paw. It was shown that cellulose sulfate and carragenin were able to increase the resistance of the rats to the so-called thermic edema, produced in the rat's paw by heating it at 45° for 30 min.[4,5,12] In the present report we have extended these findings by utilizing sulfated polymers such as polyvinyl and polyethylene sulfate, and a sulfated amylopectin (SN-263). The definite release of kinin activity by sulfated polymers, including those which are not polysaccharides, indicate that the main active part function in these polymers is the SO_4 grouping and not the polysaccharide chain. This observation might open up the possibilities of obtaining compounds which would be able to deplete the plasma of its easily activatable kinin-forming system.

It was shown before that a previous treatment of plasma with glass powder abolishes its capacity of releasing kinin by incubation with cellulose sulfate.[4] On the other hand it has been shown that the stock of kininogen which is depleted by glass treatment of fresh guinea-pig plasma represents 30–50 per cent of the whole stock of kininogen and that substances like cellulose sulfate or enzymes, such as chymotrypsin or trypsin, act mainly through activation of this part of kininogen in fresh plasma.[13] To explain cross desensitization and the anti-inflammatory effect of the sulfated polymers one might venture the idea that such compounds act mainly by depleting the circulating plasma of that part of the kininogen system that is easily activated by glass. The remaining stock of BKg, depends probably on more drastic activation processes such as heating at pH 2·0 or trypsin treatment upon denatured plasma.

A partial blockade of the edema produced in the rat's paw by local injection of an

esterase fraction of the venom of *Agk. piscivorus* indicates that the increased resistance induced by sulfated polymers might be of practical importance to reduce other kinds of inflammatory reactions, such as those produced by cellulose sulfate, carragenin and other sulfated polymers, and also the so-called thermic (45°, 30 min) edema. This crossed resistance might indicate, as stressed before, that in the endogenous activation of the kinin system, sulfated polymers might be involved. Another evidence in favour of this possibility is the fact that hexadimethrine is not only able to block the activation of the kinin system, but also to reduce the thermic edema as shown previously.[4]

Recently, Starr and West[14] reported confirmatory evidences on the participation of bradykinin in the thermic edema (45°, 30 min) of the rat's paw, using many anti-inflammatory drugs and dibenzyline to produce a partial inhibition of the edema, as well as by direct estimations of bradykinin released from the heated paw. The fact mentioned in this paper that hexadimethrine did not reduce the thermic edema, is at variance with our own results,[4,2] and might be derived from differences in the conditions of the experiments (for instance, the rats were previously treated with dextran, in Starr and West's experiments). Another finding by Ankier and Starr[15] namely that histamine is probably a primary agent in the edema production by dextran, and that kinins might play only a subsidiary role in this type of anaphylactoid reaction agrees with our previous results showing that treatment of the rats with promethazine + BOL delayed in a significant way the edema produced by dextran, but had no effect on the edema produced by carragenin or cellulose sulfate, and also that hexadimethrine had less effect on the edema produced by dextran than upon that produced by the sulfated polymers.[4] However, the contention by Ankier and Starr[15] that dextran sulfate did not enhance vascular permeability in the rat, is difficult to reconcile with our own findings that dextran sulfate is a very potent agent in producing edema in the rat's paw when injected locally. Here again, the degree of sulfation introduces differences in experimental conditions and may account for the differences in the results presented by Ankier and Starr.[15]

Some of the sulfated polysaccharides, such as carragenin and degraded carragenin and the amylopectin sulfate (SN-263), have been used as anti-peptic agents in the experimental treatment of gastric ulcers. The anti-ulcerogenic activity of carragenin has been studied in rats[16] and the similar, but apparently more pronounced effects of amylopectine sulfate, with the trademark Depepsen (G. D. Searle and Co, Chicago, Illinois), have been extensively investigated in rats and humans.[6,7] Though part of the effects of such sulfated polysaccharides might be due to their antipeptic activity, we have to consider now the contribution of the anti-inflammatory effects, possibly due to removal of part of the activatable kininogen system, as described in the present paper. The fact, however, that a polysaccharide component of the molecule is not essential for the anti-inflammatory action of sulfated polymers might expand the possibilities for the synthesis of sulfated macromolecules displaying such anti-inflammatory activity.

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