THE ANTICOAGULANT ACTIVITY AND TOXICITY OF LAMINARIN SULPHATE K

BY S. S. ADAMS AND HAZEL M. THORPE

From the Biology Division, Research Department, Boots Pure Drug Co. Ltd., Nottingham

Received January 11, 1957

Four samples of sulphated laminarin, with differing degrees of sulphation, and two samples of fucoidin were examined for anticoagulant activity. Laminarin sulphate K, with 1·83 sulphate groups per glucose unit, showed anticoagulant activity about a third as potent as heparin. It did not cause agglutination of rabbit platelets either *in vivo* or *in vitro*, but it proved fatal to rabbits when given intravenously twice daily for periods of seven to twelve days, at a dose approximately three times the clinical dose. It was extremely toxic to guinea pigs in single small intravenous doses and produced the "anaphylactoid" phenomenon associated with some other toxic synthetic anticoagulants. Laminarin sulphate K was too toxic to justify clinical trials.

LAMINARIN is a polysaccharide obtained from *Laminaria cloustoni* frond[1,2]. It is insoluble in cold water, but can be sulphated to produce water-soluble compounds with varying numbers of sulphate groups per glucose unit[3]. Fucoidin is a polysaccharide sulphate from the brown seaweeds and is believed to occur in the intercellular mucilage[4,5].

Four laminarin sulphates, with varying numbers of sulphate groups per glucose unit, and two samples of fucoidin were obtained from the Institute of Seaweed Research, Inveresk, Midlothian, Scotland.

The *in vitro* anticoagulant activity of these compounds was estimated by the sulphated whole-blood method of Adams and Smith[6], and the results are recorded in Table I in terms of the International Heparin Unit. Only laminarin sulphate K, which had the highest sulphur content, was sufficiently active to warrant further examination.

METHODS

Anticoagulant activity in rabbits. Clotting times were measured by the capillary tube technique on samples taken from the ear vein at 0, 15, 30 and 60 minutes, after the intravenous injection of heparin (100 units per mg.) at 1 mg./kg., and laminarin sulphate K at 3 mg./kg. Crossover tests were made with seven rabbits.

Acute intravenous toxicity. The acute intravenous toxicity was estimated in mice and guinea pigs and compared with heparin. Animals were kept for seven days after the injections.

Chronic intravenous toxicity. Rabbits were injected twice daily, on 5 days of the week, with laminarin sulphate K at 12·5 and 15 mg./kg. and heparin at 5 mg./kg. This was approximately three times the estimated clinical dose of the anticoagulants. Animals which died were examined after death.

Platelet counts. The effect of laminarin sulphate K on the platelet count of rabbit blood was measured both *in vivo* and *in vitro*. The

counting fluid devised by Lempert[7] was used, but no brilliant cresyl-blue was included as quite small amounts of heparin and laminarin sulphate K caused precipitation of the dye. In the *in vitro* experiments, which were similar to those of Astrup[8], rabbit blood was collected by heart puncture into one tenth of its volume of a 3·5 per cent sodium citrate solution.

TABLE I

SEAWEED ANTICOAGULANTS

Compound	Per cent sulphur	Number of sulphate groups per glucose unit	*In vitro* activity units per mg. (International Heparin Units)
Laminarin sulphate K	16·8	1·83	35
Laminarin sulphate L	14·5	1·37	9·4
Laminarin sulphate M	8·84	0·62	1·4
Laminarin sulphate N	5·97	0·37	<1·3
Fucoidin F13	9·33	*—	8·9
Fucoidin A	*—	*—	9·0

* Information not available.

Saline, heparin solutions or laminarin sulphate K solutions were added to the citrated blood, and platelet counts were made on each of the samples after 15 minutes. For the *in vivo* experiments, platelet counts were made before the intravenous injection of the anticoagulants and 15 minutes later.

RESULTS

Anticoagulant activity in rabbits. The results (Table II) showed that laminarin sulphate K was about a third as active as heparin (100 units per mg.). The clotting times suggested that the action of laminarin

TABLE II

MEAN CLOTTING TIMES OF SEVEN RABBITS AFTER INTRAVENOUS INJECTION OF HEPARIN AND LAMINARIN SULPHATE K

	Mean clotting time in minutes			
Time after injection, in minutes	0	15	30	60
Heparin 1 mg./kg.	1½	14	6	3½
Laminarin sulphate K 3 mg./kg.	2	11	9	5

sulphate K was slightly more prolonged than heparin, a finding which was confirmed at higher dose levels. The anticoagulant activity of laminarin sulphate K in rabbits could be neutralised by the intravenous injection of protamine sulphate.

Effect of laminarin sulphate K on platelets in vitro *and* in vivo. Laminarin sulphate K at 1 mg. and 4 mg./ml. of rabbit blood had no effect on the platelet count *in vitro*. There was also no evidence that laminarin sulphate K, in doses up to 500 mg./kg. intravenously in rabbits, caused any pronounced drop in the platelet count 15 minutes after

injection. In the course of all the platelet counts there was no indication that laminarin sulphate K caused precipitation or aggultination.

Acute Toxicity

From Table III it will be seen that the intravenous average lethal dose in mice of laminarin sulphate K was about 1000 mg./kg., and that the substance was about twice as toxic as heparin. The way in which the animals reacted to the two compounds was quite different. The toxic effect of heparin appeared immediately after injection, whereas the animals dosed with laminarin sulphate K showed no effects for one to two hours.

TABLE III

ACUTE INTRAVENOUS TOXICITY OF LAMINARIN SULPHATE K AND HEPARIN IN MICE AND GUINEA PIGS

Animals	Substance	I.V. dose mg./kg.	Deaths
Mice	Laminarin sulphate K	1000 1500	6/10 9/10
	Heparin (100 units/mg.)	1500 2500	2/10 5/5
Guinea pigs	Laminarin sulphate K	20 40	8/11 3/5
	Heparin (100 units/mg.)	250	0/5

The intravenous toxicity of laminarin sulphate K was about fifty times greater in guinea pigs than in mice (Table III). While laminarin sulphate K was only twice as toxic as heparin to mice, it was more than twelve times as toxic in guinea pigs. Thus 20 mg./kg. of laminarin sulphate K produced toxic effects in all guinea pigs, while 250 mg./kg. of heparin produced no reactions of any kind.

All the guinea pigs which received laminarin sulphate K showed the same signs to a greater or lesser degree. There were signs of pulmonary oedema ("bubbly breathing") shortly after injection, a bout of jactitating movements and finally limpness, with the animal lying on its belly or side. Death occurred in some animals within an hour of injection, but in others a day or two later. These symptoms were similar to those reported by Walton and Ricketts in guinea pigs given certain synthetic anticoagulants[10], and termed by them "anaphylactoid" phenomena.

Chronic Toxicity in Rabbits

Three animals were given 5 mg./kg. of heparin intravenously twice daily for 5 days weekly for 3 weeks, and were killed at the end of this time by intravenous sodium pentobarbitone. Throughout this period the animals were in good health.

Nine rabbits were given 12·5 or 15 mg./kg. of laminarin sulphate K intravenously twice daily on five days of the week. Seven died after seven to twelve days of dosing. The other two were killed by intravenous sodium pentobarbitone when moribund, in order to obtain fresh autopsy material. Three died overnight, and autolytic changes had occurred by

the time they were examined. The other six were examined very soon after death and the outstanding macroscopical and pathological findings were recorded. The most consistent feature was a pronounced loss of tone of the ileum and sometimes colon, and the presence of liquid contents in these parts of the gut. In three animals a small ulcerated area was found in the stomach. Diarrhoea was a prominent symptom in six of the nine test rabbits, but was absent in the three heparin controls.

Microscopic changes in the mucous membrane of the small intestine were present in all animals, but were slight in the controls. In three animals there was an associated change in the submucosa suggesting that the necrosis was inflammatory. Degenerative changes in the convoluted tubules of the kidney were present in four animals, and in one rabbit this was associated with distension of the glomerular capillaries by fibrin clots. Fibrin clots were also seen in the pulmonary arteries of two animals, and in the portal vein of one.

Discussion

Our results suggest that laminarin sulphate K is an anticoagulant with about one third the activity of heparin.

Hawkins and O'Neill[9] tested four sulphated derivatives of laminarin, one of which contained 1·7 sulphate groups per glucose unit and was one third as active as heparin. It was probably similar in composition to laminarin sulphate K, with 1·83 sulphate groups. They found that a single intravenous injection of this substance in rats and dogs did not affect the platelet counts.

Many of the synthetic anticoagulant polysaccharide polysulphuric acid esters are known to be toxic because they cause agglutination of the blood platelets and precipitate fibrinogen[10–17].

Astrup[8] showed that rabbit blood platelets could be agglutinated *in vitro* by several synthetic anticoagulants, including "Paritol", a compound which has been in clinical use. Piper[11] recorded very great reductions in the platelet counts of rabbits after the intravenous injection of several synthetic sulphated polysaccharides in doses as low as 1 mg. per kg. Using similar *in vitro* and *in vivo* techniques, we found that laminarin sulphate K had no effect in doses higher than those which these two workers had used for their compounds. With *in vivo* tests we used very high doses without causing abnormal effects. We concluded that laminarin sulphate K did not cause platelet agglutination.

The chronic toxicity tests in rabbits, however, indicated that laminarin sulphate K was too toxic for clinical use. The occurrence of diarrhoea in six of these animals was very interesting, since this was one of the reasons why "Treburon" (a polyhexuronic acid ester) was abandoned clinically[19].

Walton[18] found that the higher molecular weight members of a series of dextran sulphates precipitated human fibrinogen and caused platelet agglutination in human blood. Injected intravenously into guinea pigs these same compounds caused an "anaphylactoid" response[10]. This is Walton and Ricketts' term for the typical symptoms of jactitating movements and respiratory distress that follow an intravenous injection of a

toxic anticoagulant, and is not to be confused with anaphylactic shock. They found that two other toxic synthetic anticoagulants, a sulphuric ester of xylan, and sodium polyanethol sulphonate ("Liquoid"), also produced the "anaphylactoid" phenomena in guinea pigs. We found that laminarin sulphate K at the very low doses of 20 mg./kg. and 40 mg./kg. produced the same toxic reaction, which supported our conclusion that this substance possesses some of the toxic properties shown by other synthetic heparin-like anticoagulants, and is too toxic for clinical use.

Acknowledgements. We wish to thank Dr. F. N. Woodward of the Institute of Seaweed Research, Inveresk, for supplying us with the compounds, Mr. K. L. Smith for the *in vitro* anticoagulant results, and Dr. H. E. Harding for his examination of the post-mortem material. We are also indebted to Dr. M. R. Gurd for his helpful criticism and Mr. C. A. Burrows for technical assistance.

References

1. Connell, Hirst and Percival, *J. chem. Soc.*, 1950, 3494.
2. Black, Cornhill, Dewar and Woodward, *J. appl. Chem.*, 1951, **1**, 505.
3. Black and Dewar, *J. Sci. Fd. Agric.*, 1954, **5**, 176.
4. Black, *Rep. Progr. Chem.*, 1953, **50**, 322.
5. Montgomery and Smith, *Ann. Rev. Biochem.*, 1952, **21**, 79.
6. Adams and Smith, *J. Pharm. Pharmacol.*, 1950, **2**, 836.
7. Lempert, *Lancet*, 1935, **1**, 151.
8. Astrup, *Scand. J. clin. Lab. Invest.*, 1953, **5**, 137.
9. Hawkins and O'Neill, *Canad. J. Biochem. and Phys.*, 1955, **33**, 545.
10. Walton and Ricketts, *Nature, Lond.*, 1954, **173**, 31.
11. Piper, *Acta physiol. scand.*, 1945, **9**, 28.
12. Piper, *Acta pharm. tox., Kbh.*, 1946, **2**, 317.
13. Astrup and Piper, *Acta physiol. scand.*, 1946, **11**, 211.
14. Barsøe and Selsø, *Acta pharm. tox., Kbh.*, 1946, **2**, 367.
15. Astrup and Piper, *Acta physiol. scand.*, 1945, **9**, 351.
16. Walton, *Proc. R. Soc. Med.*, 1951, **44**, 563.
17. Walton, *Brit. J. Pharmacol.*, 1952, **7**, 370.
18. Walton, *ibid.*, 1953, **8**, 340.
19. Field, Ramsay, Attyah and Starr, *J. Lab. clin. Med.*, 1953, **41**, 208.