HEPARIN-LIKE ANTICOAGULANT ACTION OF SULFONATED LIGNINS FROM COMMERCIAL WASTE SULFITE LIQUOR[1]

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Sulfonated lignins exist in large amounts in the waste sulfite liquor which is obtained as a by-product from the manufacture of paper pulp from wood (Braun, 1952). Preliminary experiments in this laboratory indicated that these sulfonated lignins possessed heparin-like anticoagulant activity. The existence of such sulfonated lignins in the waste sulfite liquor presents an abundant source of potentially active anticoagulant drugs. The present report is concerned with the fractionation of the sulfonated lignins as well as the investigation of the anticoagulant action and acute toxicity.

MATERIALS AND METHODS. *Preparation of the sodium lignin sulfonate fractions:* Commercial calcium base waste sulfite liquor was received directly from the digestors at the pulp mill. The fractions were prepared by a method similar to that described by Markham, Penniston and McCarthy (1949). Six liters of the crude liquor were stripped of dissolved sulfur dioxide by boiling and were then put through an ion exchange resin (Dowex #50) converting the calcium base lignins to the free acids. All ether soluble material was extracted using a continuous extraction apparatus. The ether was stripped from the solution with air and the lignins were converted to the barium salts by the addition of barium carbonate. Sufficient ethyl alcohol was added stepwise to precipitate up to a total of approximately 50 per cent of the total dissolved lignins as determined by ultraviolet optical density. Fractions of the precipitate were obtained as the precipitation was conducted with increasing amounts of alcohol. Fractions one and two were originally separated as a single fraction and then separated by additional alcohol fractionation from a water solution. All fractions were purified by redissolving the precipitate in water, centrifuging to remove excess barium sulfate and carbonate and reprecipitating from aqueous solution by alcohol. The precipitates were then dissolved separately in water and passed through the ion exchange column converting the barium salts to the sodium salts. The fractions were then reprecipitated from aqueous solution by the addition of 70 to 80 per cent by volume of acetone. The supernatants were discarded and the precipitates were vacuum dried. The fractions then consisted of the lignin sulfonates as the sodium salts. All procedures were conducted at room temperature. Throughout the procedure when the solution was allowed to stand overnight, toluene was added to saturation to prevent bacterial and fungus growth.

In vitro determination of anticoagulant action: A modified procedure for the determination of antithrombin activity was used. Oxalated human blood plasma was obtained from a single donor just prior to each test. Two-tenths ml. of plasma was added to 0.2 ml. of water, or of aqueous solution of the material to be tested for antithrombin activity in a concentration of 50 and 100 microgm. per ml. The mixture was incubated at 37°C. in a water bath for one hour. At the end of this time 4.0 units of thrombin in 0.6 ml. of water was added to each tube. The time required for the first sign of formation of fibrin threads to appear in the tube was taken as the clotting time for that tube. Duplicate determinations were made in each test.

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In vivo determination of anticoagulant activity: Mature mongrel dogs were used throughout the experiments. Clotting times were done according to the Lee and White method (1913). After the determination of control clotting time of blood obtained from the foreleg vein, the dogs were intravenously injected with the fraction of the sodium lignin sulfonate dissolved in water. The clotting time of the dogs blood was followed for the subsequent 36 hour period.

RESULTS. The properties of the various sodium lignin sulfonate fractions are summarized in table 1. A total of 48.9 per cent of the dissolved lignins in the original sulfite liquor is represented by the five fractions.

TABLE 1

Characterization of the sodium lignin sulfonate fractions. Duplicate determinations for sulfur departed not more than 0.05 per cent from the average. The % solids represent the per cent of total weight of the ultraviolet absorbing solids from the original waste sulfite liquor. Weight % of ethanol was calculated assuming just an ethanol-water mixture. The diffusion coefficients were determined by the solution to gel method of Felicetta et al. (1949) at 25°C. in 0.2 M potassium chloride solution. The molecular weights were calculated from the diffusion coefficients using the Einstein-Sutherland formula and assuming that the molecules are spheres.

FRACTION	WEIGHT	% SOLIDS	WEIGHT % ETHANOL	% SULFUR	DIFFUSION COEFFICIENT	MOLECULAR WEIGHT
	gm.	*gm.*			*mm.²/day*	
1	33.6	4.9		5.72	4.3	310,000
2	38.6	10.2	39.4	5.91	5.5	150,000
3	41.2	9.3	42.6	6.24	7.1	68,000
4	68.7	11.6	48.2	6.72	8.8	36,000
5	73.6	12.9	55.3	6.69	10.8	20,000

TABLE 2

The intravenous LD_{50} in mice of the sodium lignin sulfonate fractions

FRACTION	LD_{50} (MGM./KGM.)	19/20 CONFIDENCE LIMITS
1	97	92–102
2	128	105–156
3	191	181–212
4	730	541–986
5	1000	938–1068
Heparin Na	2010	1218–3319

The intravenous LD_{50} in mice of the various fractions is presented in table 2. The LD_{50} increased as the molecular weight of the lignins represented by the fractions decreased. With the exception of fractions four and five the 19/20 confidence limits of each fraction, according to the method of Litchfield (1949), indicate that the LD_{50}'s are significantly different. Death did not usually occur until twelve to twenty-four hours following the injection. Respiratory failure was apparently the ultimate cause of death in the mice, however, an occasional animal showed with larger doses repeated bleeding arising from the site of injection or grossly bloody stools and bloody urine.

Table 3 represents the *in vitro* anticoagulant action of the fractions. Fractions one and two are more active than heparin at a concentration of 100 microgm. per

ml. than they are at a concentration of 50 microgm. per ml. Fractions three, four, and five are less active than heparin but have about the same relative activity to heparin irrespective of whether they are tested at the 100 microgm. per ml. or the 50 microgm. per ml. concentration.

TABLE 3

In vitro activity of sodium lignin suljonate fractions and of sodium heparin. Activity determined as antithrombin activity. Fraction/Heparin activity ratio calculated as: clotting time with fraction divided by clotting time with heparin

ANTICOAGULANT	CLOTTING TIME IN SECONDS AT ANTICOAGULANT CONCENTRATIONS (MICROGM./ML.)			FRACTION/HEPARIN, ACTIVITY RATIO, AT CONCENTRATIONS (MICROGM./ML.)	
	0	50	100	50	100
Heparin	9.3	26.2	79.5	2.3	10
Fraction I	9.3	60.9	1000		
Heparin	9.2	30.0	83.2	1.5	4.6
Fraction II	9.2	43.9	381.7		
Heparin	8.1	24.1	57.7	0.8	0.9
Fraction III	8.1	18.6	48.5		
Heparin	9.5	39.7	94.1	0.5	0.5
Fraction IV	9.5	18.0	45.2		
Heparin	8.5	31.3	57.0	0.3	0.2
Fraction V	8.5	10.5	12.8		

TABLE 4

In vivo activity of sodium lignin sulfonate fractions. Dose of each fraction was 15 mgm. per kgm. administered intravenously. Per cent increase in clotting time calculated from each animal's own control. Each figure represents the mean and standard deviation of effect on five dogs

% Increase in Clotting Time

FRACTION	TIME AFTER INJECTION (HOURS)						
	1	2	4	8	16	24	32
1	>1000	>1000	>1000	>1000	66 ± 20	0	
2	>1000	>1000	>1000	405 ± 235	290 ± 150	129 ± 83	51 ± 29
3	640 ± 334	490 ± 225	300 ± 120	130 ± 43	70 ± 38	49 ± 21	34 ± 20
4	783 ± 510	600 ± 274	240 ± 116	90 ± 40	47 ± 22	26 ± 10	14 ± 14
5	50 ± 10	30 ± 14	22 ± 13	0			

In the intact dog all of the fractions showed definite anticoagulant action. Table 4 indicates the duration of anticoagulant action following a single intravenous injection. Anticoagulant action following the injections was maximal within ten minutes. The order of intensity of activity of the fractions was the same as that observed in the *in vitro* experiments.

The effect of fraction three on the coagulation time was studied further in three additional groups of five dogs each. The results are presented in figure 1. There is a direct relationship between the dosage of the fraction and the intensity of effect on coagulation time and duration of action. Additional experiments showed that protamine sulfate is capable of reverting to normal the prolonged clotting time induced by the sodium lignin sulfonate. This evidence together with the *in vitro* anticoagulant results infer that the anticoagulant action of the lignin sulfonates is heparin-like in nature since the ability of protamine to block heparin anticoagulant action is a well recognized phenomenon.

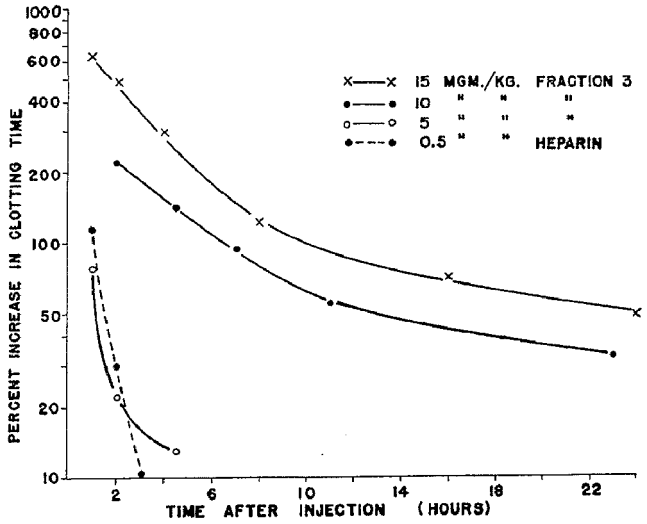

FIG. 1. Effect of various doses of fraction three of the lignin sulfonates on the coagulation time of intact dogs. The curves represent the mean effect on five dogs at each dosage level.

All animals completely recovered from the injections and there was no evidence of gross systemic malfunction. Some of the dogs were followed for three months following the injections. However, all of the fractions induced in some of the dogs a brief period of vomiting and/or defecation, some instability when standing, and a general sedative effect. These acute toxic effects became evident in five to ten minutes following the injection and lasted for twenty to thirty minutes following which the dogs again grossly appeared to be normal. This brief acute toxicity was seen most frequently in the animals receiving fractions one, two, and three and less frequent with fractions four and five. A series of four anesthetized animals showed no acute toxic symptoms; however, the first injection produced a marked but brief depressor response. Subsequent injections of the fraction in the same animal resulted in no depressor response.

DISCUSSION. Astrup and Piper (1946) investigated synthetic polysaccharide sulfate esters and found that although they were active anticoagulants, they caused precipitation of plasma fibrinogen. In the present experiments all blood samples obtained from intact animals which had been administered the lignin sulfonate fractions eventually did form a firm clot. This would indicate that if a reaction between the sulfonated lignins and fibrinogen took place in the intact animals it resulted in a loose combination and did not result in the breakdown or precipitation of fibrinogen. The ability of protamine to antagonize the anticoagulant action of the lignin sulfonates further indicates that if a reaction with plasma protein occurred as a result of the affinity of the sulfate ions with fibrinogen, the combination represented a readily reversible one.

The present experiments demonstrate a positive direct relation between lethal toxicity (and acute toxicity) and the molecular weight. This is similar in nature to the results described by Halse (1950) on synthetic sulfate polysaccharides. Also the present experiments demonstrate a positive direct relationship between molecular weight and anticoagulant activity.

Because of the well recognized absence of untoward acute toxicity to heparin, the acute toxicity observed following the use of the sulfonated lignin fractions in the present studies makes them unsatisfactory for immediate future use as substitutes for heparin in clinical anticoagulant therapy. However, because of the abundance of the waste sulfite liquor obtained in the commercial production of paper pulp and the ease by which highly potent anticoagulant material may be obtained it may serve the purpose of laboratory use in animal experimentation at the present time.

CONCLUSION

Commercial sulfite liquor which is a waste product from the manufacture of paper pulp from wood was investigated for the presence of active anticoagulant agents. The sulfite liquor was fractionated and a series of sulfonated lignins was obtained. All of these fractions showed anticoagulant activity, some acute toxicity but no obvious delayed toxicity.

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