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Antithrombin Activity of a Polyelectrolyte Synthesized from *cis*-1,4-Polyisoprene

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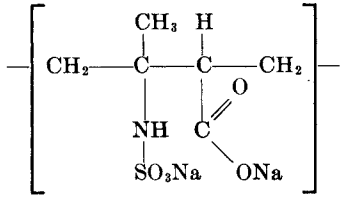
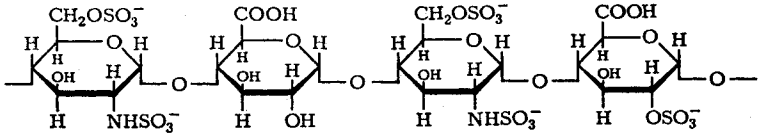
Summary

A polyelectrolyte synthesized from *cis*-1,4-polyisoprene, containing aminosulfonate and carboxylate groups was shown to have an anticoagulant activity of about 1/30 compared with heparin. Because the substance prevents the coagulation of plasma in the presence of thrombin it is assumed that it acts as an antithrombin.

Recently, the addition reaction of *N*-chlorosulfonyl isocyanate and *cis*-1,4-polyisoprene has been reported.[1] It was shown that the addition product upon treatment with alkali gave a water soluble polyelectrolyte (A) containing the following structural unit:



The aminosulfonate and carboxylate groups are also present in the tetrasaccharide unit of the anticoagulant heparin, for which the following structure has been suggested:[2]



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In seeking new thromboresistant polymeric materials we investigated the activity of A as an anticoagulant. The present results indicate that A acts as an antithrombin, with an activity of about 1/30 compared with heparin.

MATERIALS AND METHODS

The polyelectrolyte A was synthesized as described.[1] Citrated human and bovine plasma were stored at −26°C before use. Human plasma had a concentration of about $1.8.10^{-2}$ *M* disodium citrate and $2.0.10^{-2}$ *M* glucose. Prior to use the pH of the plasma was adjusted to 7.35 (unless otherwise stated) with the aid of 0.15 *M* HCl or 0.15 *M* Na_2CO_3.

Recalcification times of plasma were measured in Pyrex tubes with an outer diameter of 1.0 cm at 37°C. Part of the experiments were followed in an optical way as described by Matthews et al.[3] For this purpose a Vitatron colorimeter, Model UC 200 S, was used, equipped with a special thermostated vessel holder for the test tubes. Further, a 2 mm thick heat filter was placed between the lamp and vessel holder. Recalcification was carried out with 0.3 *M* $CaCl_2$ (pH 7.35) to a final concentration of $1.9.10^{-2}$ *M* to $3.5.10^{-2}$ *M* of added $CaCl_2$. Heparin, 152 E/mg, was purchased from Diosynth (Oss, Holland). Thrombin in 0.05 *M* phosphate buffer (pH 7.0), 250 NIH units/ml, was obtained from Sigma.

Stock solutions of 12 mg/ml for both heparin and A were used for all measurements. The pH of these solutions was adjusted to 7.35 prior to use.

RESULTS AND DISCUSSION

The anticoagulant activity of A compared with heparin in human plasma, is shown in Table I.

At concentrations up to about 0.4 mg A/ml plasma a real coagulation of the plasma took place after recalcification. In the case of 0.4 mg A/ml plasma the coagulation started after approximately 20 min and was completed in about 40 min. When the concentration was 0.8 mg A/ml, only some precipitate was formed at the interface of plasma and air after about 5 hr. When the experiment was continued longer than 8 hr a precipitate was also observed in the liquid. A similar behavior was found when heparin was present in

TABLE I

Anticoagulant Activity of A Compared With Heparin[a]

Concentration of heparin in plasma (mg/ml)	Concentration of A in plasma (mg/ml)	Beginning of coagulation or precipitation
no anticoagulant		clotting completed within 5 min
	0.4	21–28 min
	0.8	5–8 hr
	1.2	>8 hr
0.04		>6 hr
0.06		>8 hr

[a] Conditions: aliquots of 1.5 ml citrated human plasma, pH 7.35, were incubated with the anticoagulant during 5 min at 37°C. Recalcification was carried out to a final concentration of $3.5.10^{-2}$ *M* of added $CaCl_2$.

a concentration of 0.04 mg/ml plasma. When the concentration of A was 1.2 mg/ml no clotting or precipitation occurred even after 8 hr. From the experiments it follows that the activity of A in preventing clot formation is about 1/20 compared with heparin.

In Table II data on some coagulation experiments with bovine plasma in the presence of A are listed, for different concentrations of added $CaCl_2$ and different pH values. At low Ca^{2+} concentrations the coagulation times of plasma in the presence of A were less than those at high Ca^{2+} concentrations. The anticoagulant effect of A cannot be explained, however, by assuming a complex between A and Ca^{2+}. For instance, in the case of 0.2 mg A/ml plasma and $3.5.10^{-2}$ *M* $CaCl_2$ only 2% of the Ca^{2+} can be complexed, when it is assumed that the whole polymer consists of those units shown in the preceding structure (based on a ratio 1 unit: 1 Ca^{2+}). Furthermore, a conductometric titration of A and Ca^{2+} did not indicate any complex formation. The anticoagulant activity is also not caused by a "soap-effect" of the polyelectrolyte. Comparable amounts of sodium dodecylsulfate even enhanced the clotting of plasma. No significant differences in recalcification times were found for the experiments in which pH values of 7.37 and 7.98 were used.

The coagulation of bovine plasma is prevented at much lower concentrations of both the anticoagulants A and heparin compared to

TABLE II

Anticoagulant Activity of A in Bovine Plasma at Different Concentrations of Added $CaCl_2$ and Different pH Values[a]

Concentration of heparin in plasma (mg/ml)	Concentration of A in plasma (mg/ml)	Concentration of $CaCl_2$ (10^{-2} M)	pH	Beginning of coagulation
no anticoagulant		3.5	7.76	5.5 min
	0.008	3.5	7.76	7 min
	0.04	3.5	7.76	21–26 min
	0.08	3.5	7.76	70 min
	0.2	3.5	7.76	>8 hr
no anticoagulant		1.9	7.37	4 min
	0.04	1.9	7.37	6–9 min
	0.04	1.9	7.98	8 min
	0.06	1.9	7.98	12 min
	0.08	1.9	7.98	18–20 min
0.002		1.9	7.98	<12.5 min
0.003		1.9	7.98	11.5 min

[a] Aliquots of 1.5 ml plasma were incubated with A or heparin during 5 min at 37°C before recalcification.

human plasma. Because more precipitate is formed in bovine plasma than in human plasma during freezing, larger amounts of denaturated clotting factors may be present in the precipitate of bovine plasma.

A comparable polymer was synthesized, starting from 3,4-polyisoprene and *N*-chlorosulfonyl isocyanate. From the IR spectrum of the addition product it was expected that the alkaline hydrolysis product should contain less $NH\text{-}SO_3Na$-groups. In agreement with this assumption, the anticoagulant activity of this compound was found to be 4 to 5 times lower compared to A. A similar polymer derived from poly(2,3-dimethylbutadiene), which probably contains even fewer $NH\text{-}SO_3Na$-groups, was found to be even less active.

In Table III data from some experiments are given, in which thrombin, together with a small amount of $CaCl_2$, was added to bovine plasma that had been incubated with A or heparin. In the presence of 1.67 NIH units thrombin/ml plasma and $1.9.10^{-2}$ M Ca^{2+} the coagulation time of plasma *without* an anticoagulant was about 30 sec. In the presence of A or heparin the coagulation of the plasma is strongly delayed. The fact that A prevents the

TABLE III
Anticoagulant Activity of A in the Presence of Thrombin[a]

Concentration of heparin in plasma (mg/ml)	Concentration of A in plasma (mg/ml)	Beginning of coagulation
no anticoagulant		<30 sec
	0.08	9–11 min
	0.16	>2.5 hr
0.002		5 min
0.004		19 min
0.008		>2.5 hr

[a] Aliquots of 1.5 ml bovine plasma were incubated with A or heparin during 5 min at 37°C. Then 1.67 NIH units thrombin/ml plasma were added together with a solution of $CaCl_2$ to a final concentration of $1.9.10^{-2}$ M of added $CaCl_2$.

clotting of plasma in the presence of thrombin indicates that it acts as an antithrombin. Whether A also interferes in earlier stages of the blood coagulation process is not yet known. From the experiments, listed in Table III, in which the course of the clotting was also considered, it followed that the antithrombin activity of A is 1/20 to 1/40 compared with heparin. This order of magnitude is the same as the anticoagulant activity that can be derived from the Tables I and II.

Attempts will be made to synthesize an insoluble polymer derived from A. This can be done either by starting from a partly crosslinked *cis*-1,4-polyisoprene or by crosslinking the final product A.

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