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Fibrinolytic activity evoked in the plasma of the normal and adrenalectomized rat by cellulose sulphate

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Summary

1. Cellulose sulphate, a kinin-releasing agent, produced fibrinolytic activity in plasma when administered intravenously to the rat but not when added to fresh rat plasma *in vitro*. The *in vivo* effect was maximal within 1 min and disappeared within 10–20 minutes. It was retained in plasma taken 1 min after the injection and kept at room temperature for 30 minutes.

2. A decrease of anti-fibrinolytic potency measured against urokinase-activated bovine plasmin, was shown to occur in plasma of rats given cellulose sulphate.

3. Activated rat plasma lysed heat-denatured fibrin: it probably contains free plasmin as well as plasminogen activator.

4. Adrenalectomized rats did not exhibit fibrinolytic activity nor statistically significant benzoyl-arginine ethyl ester-esterase activation in plasma after cellulose sulphate treatment.

5. Adrenalectomized rats had significantly increased levels of plasma kininogen, but were normally sensitive to the kininogen-depleting action of cellulose sulphate.

6. The increased plasma kininogen of adrenalectomized rats seems to be a consequence of the impairment of the plasminogen activating mechanism.

Introduction

It has been shown that cellulose sulphate (Rothschild, 1967, 1968; Eisen & Loveday, 1971), activates plasma arginine–ester esterase and depletes plasma kininogen in rat, guinea-pig and man, releasing kinins. In addition, Rothschild (1968) observed that following injection of cellulose sulphate, fibrinolytic activity appears in rat's plasma.

The role of the adrenal gland in the activation of fibrinolysis has been comparatively little studied. Adrenals contain plasminogen activators (Albrechtsen, 1959) and adrenaline, and situations leading to adrenaline release have been shown to stimulate fibrinolytic activity (Biggs, Macfarlane & Pilling, 1947; Sherry, Lindemeyer, Fletcher & Alkjaersig, 1959).

In the present work, the appearance of fibrinolytic activity after injection of cellulose sulphate has been examined in normal and adrenalectomized rats and

correlated with plasma kininogen levels as well as with the utilization of plasma kininogen and activation of arginine–ester esterase.

Methods

Cellulose sulphate was prepared from Whatman ashless cellulose powder according to Astrup, Galsmar & Volkert (1944).

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 hours. The bovine thrombin utilized was prepared according to the method of Eagle as described by Hawk, Oser & Summerson (1947). Fibrin plates contained 0·24% citrate and in the experiments on the effect of plasma on urokinase-induced fibrinolysis (Fig. 2) the plates also contained 0·01% calcium and 0·002% magnesium. Heat-denaturation of plasminogen in fibrin plates was accomplished by a 60 min heating period at 80°–85° C; its completeness was ascertained by noting the lack of response of the plate to urokinase.

Total plasma protein was estimated by the biuret method (Reinhold, 1953).

Kinin precursor (kininogen) was determined in plasma according to the method of Diniz & Carvalho (1963).

Esterolytic activity on benzoyl-arginine ethyl ester (BAEE), was determined by the colorimetric method of Brown (1960), under the conditions previously described (Rothschild, 1968). Enzymic activity was determined in whole blood added, immediately upon withdrawal, to 5 vol. of buffer.

Male Wistar rats (200–350 g) were used; neutralized solutions of cellulose sulphate were injected through the venous sinus of the penis. Samples of blood, rendered uncoagulable by 0·2% potassium oxalate, were withdrawn by cardiac puncture, under pentobarbitone (30–40 mg/kg) anaesthesia. Adrenalectomized animals were maintained on saline as drinking fluid and used 6–8 days after the operation. No difference between the total plasma protein content of rats subjected to adrenalectomy (4·4 ± 0·1 g %) or sham-operation (4·4 ± 0·1 g %), was noted.

Drugs

Bradykinin triacetate (Sandoz Ltd., Basle, Switzerland); N-benzoyl-L-arginine ethyl ester HCl and bovine fibrinogen (fraction I), containing at least 60% clottable protein (Sigma Chem Co., St. Louis, USA); urokinase, 48 CTA units (Abbott Labs., Chicago, USA).

Results

The intravenous injection of cellulose sulphate (1 mg/kg) into rats caused the appearance of fibrinolytic activity in plasma. This activity was maximal in samples withdrawn one minute after injection, decreased after 10 min, and was not detectable in plasma taken 20 min after treatment. Figure 1 illustrates this result, and shows that *in vitro*, plasma from blood taken 1 min after the injection of cellulose sulphate, retained its fibrinolytic activity for at least 30 min at room temperature.

The activity contained in the plasma of rats treated with cellulose sulphate was decreased, but not abolished, when tested on fibrin plates previously heated to

80°–85° C for 1 h; this treatment denatures plasminogen, a normal contaminant of commercially available fibrinogen. This result, shown in Table 1, suggests that plasma of cellulose sulphate-treated rats contains free plasmin as well as plasminogen activator.

Plasma of treated animals was less able to inhibit urokinase-induced lysis of bovine fibrin than that of normal rats. Figure 2 shows that statistically significant differences in the inhibitory power of plasma obtained from control and treated animals respectively, were noted when 20 to 30% of each plasma was present in the mixtures. It was found useful in these experiments to add calcium to the fibrinogen solution employed for the preparation of the fibrin plate. This cation was found to inhibit the fibrinolytic action generated by cellulose sulphate in rat plasma but not to interfere with the lytic action of urokinase. No correction for the lytic action due to the plasma from cellulose sulphate-treated animals was therefore necessary.

Table 2 shows that, in contrast to its effects in sham-operated animals, cellulose sulphate produced no detectable fibrinolytic activity in the plasma of adrenalectomized rats. The fibrinolytic system may play a role in the regulation of kinin

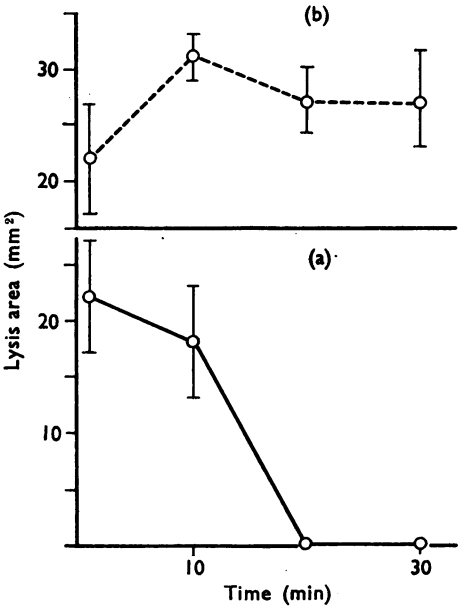

FIG. 1. Progressive loss *in vivo* and maintenance *in vitro*, of the fibrinolytic activity evoked in the plasma of rats injected intravenously with cellulose sulphate 1 mg/kg. (a) Blood was withdrawn from the animal 1, 10 and 20 min after the injection; (b) blood was withdrawn 1 min after the injection and aliquots taken at 10, 20 and 30 minutes.

TABLE 1. *Lytic effect of plasma from cellulose sulphate-treated rats on normal and heated bovine fibrin*

Substrate	Lysis area (mm²)	
Normal fibrin	58±10	$P<0.05$
Heated fibrin	23±4	

Plasma was obtained from blood withdrawn 1 min after the intravenous injection of 1 mg/kg of cellulose sulphate. Fibrin plates were heated to 80°–85° C for 60 minutes. Each result is the average of five paired experiments.

release in plasma (Eisen & Vogt, 1970). In order to investigate this relationship, the effect of cellulose sulphate on plasma kininogen and BAEE-esterase was examined after adrenalectomy. Table 2 shows that adrenalectomized rats showed a striking elevation of plasma kininogen, but in spite of this, they were as sensitive to kininogen-depletion by cellulose sulphate as sham-operated controls. BAEE-esterase activation by cellulose sulphate occurred only in sham-operated rats; it was not statistically significant in the adrenalectomized animals.

Whole plasma from either normal or adrenalectomized rats did not become fibrinolytically active on incubation with 1 to 1,000 μg/ml of cellulose sulphate for up to 20 min at 37° C.

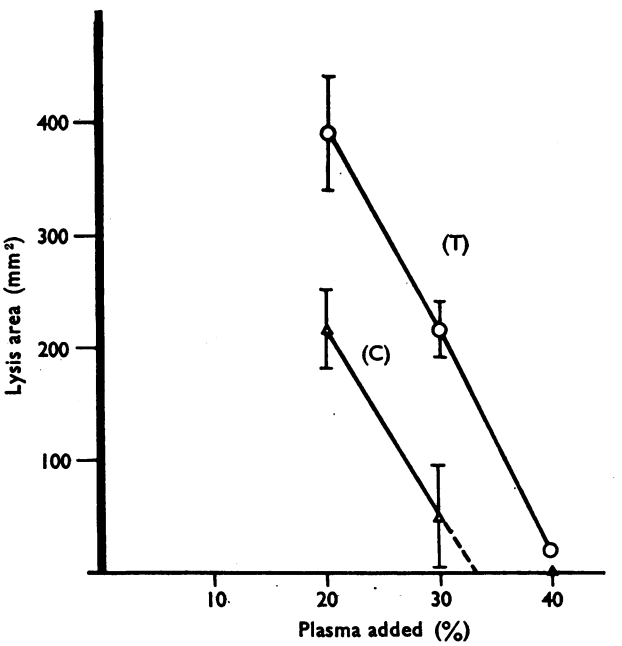

FIG. 2. Decrease of antifibrinolytic activity against urokinase-induced fibrinolysis in the plasma of rats injected intravenously with cellulose sulphate 1 mg/kg. 0·03 ml of plasma from control (C) and treated (T) rats, were separated from blood drawn 1 min after injection, mixed with urokinase and placed on the fibrin plate.

TABLE 2. *Influence of adrenalectomy and cellulose sulphate on kininogen, fibrinolytic and BAEE-esterase activities of rat plasma*

Assay	Fibrinolysis (mm^2)		Kininogen (μg/ml)		BAEE-esterase ((μmol/ml blood)/h)	
Treatment	Sham-operated	Adrenalectomized	Sham-operated	Adrenalectomized	Sham-operated	Adrenalectomized
Saline	0	0	6·1±0·7	10·2±0·7	22·6±1·8	24·7±2·0
Cellulose sulphate (1 mg/kg)	42±3*	0	2·8±0·2*	5·2±0·4*	34·0±2·8*	28·9±2·2

*Effect of cellulose sulphate, significant at $P<0·05$.
Each value represents the average of 5 experiments.

Discussion

Plasma of rats given cellulose sulphate intravenously develops the ability to lyse bovine fibrin. Although reduced to approximately one-half, this action is still detected on heat-denatured fibrin which, according to Lassen (1952), is no longer sensitive to plasminogen activators, but retains sensitivity to directly-acting proteolytic enzymes like plasmin. It has been suggested (Sherry *et al.*, 1959), that free plasmin is rarely encountered in plasma, fibrinolytic activity being the result of the activation by circulating activators, of plasminogen adsorbed into fibrin. The present experiments show, that given a strong enough stimulus, the rat can release not only plasminogen activator, but also free plasmin into the circulation.

The fibrinolytic activity evoked by cellulose sulphate is transitory and could not be detected in plasma collected 20 min after injection. Extra-plasma factors are probably involved in this inactivation since it did not occur *in vitro*. Previous work has shown that inhibition of the benzoylarginine ester esterase activity arising in the plasma of the cellulose sulphate-treated rat, followed the same time course as that of the fibrinolytic inactivation. This esterase activity is, in part, probably due to plasma kallikrein and was shown to disappear as rapidly *in vivo* as *in vitro* (Rothschild, 1968). This suggests that the mechanisms responsible for the spontaneous inactivation of the cellulose sulphate-activated kallikrein and the fibrinolytic enzymes are different.

Urokinase is a powerful activator of plasminogen, an effect which can be measured in bovine fibrin plates prepared from plasminogen-containing fibrinogen. The plasmin generated during this process is readily inactivated by plasma inhibitors. Plasma of cellulose sulphate-treated rats showed a decreased anti-fibrinolytic potency in comparison to control plasma. This difference may be a consequence of the consumption of part of the inhibitor reserve of plasma, a phenomenon which may occur in severe fibrinolytic states (Bang, 1971).

Adrenalectomized rats failed to exhibit the increased levels of fibrinolytic activity observed in the plasma of normal or sham-operated controls injected with cellulose sulphate. Previous results had shown that rapid and substantial formation of kinin occurs in plasma following the injection of cellulose sulphate (Cordeiro & Rothschild, 1971). Kinins release adrenaline directly, as well as by reflex mechanisms set off by the strong hypotensive action of kinin, and both mechanisms might be involved in this phenomenon (Haddy, Emerson, Scott & Daugherty, 1970).

The present results indicate that adrenaline may be of importance in mediating the occurrence of fibrinolytic activation by cellulose sulphate; additional work will be required to clarify the exact role of the hormone. Considerable evidence shows that adrenaline owes most of its biological actions to the activation of adenyl cyclase and the synthesis of cyclic AMP, a stimulant of many forms of cellular secretory activity; further work will be required to demonstrate whether lack of adrenal gland hormones prevents the secretion of substances required for the activation of plasminogen.

Adrenalectomized rats had heightened levels of kininogen in their plasma. This finding, also made by others (Werle, personal communication), remains unexplained. It may reflect decreased kininogen consumption rather than enhanced plasma protein synthesis, since no evidence of an increased total plasma protein content was found in adrenalectomized rats. It is worth noting that the breakdown of kininogen caused by cellulose sulphate administration seemed not to be

impaired in the adrenalectomized rat: the same relative decrease of total kininogen as that occurring in sham-operated animals, was found. Two major enzyme systems seem to be able to generate kinin in plasma: the kallikreins (kininogenases) and the fibrinolytic (plasmin) system (Eisen & Vogt, 1970). Adrenalectomized animals seem to be able to respond to cellulose sulphate injection by activating the former but not the latter. Since such animals exhibit increased levels of kininogen in plasma, this could mean that plasmin has a greater role in a possibly physiological, adrenal gland-controlled breakdown of kininogen.

The fibrinolytic activation of whole rat plasma by cellulose sulphate could not be reproduced *in vitro*. Nevertheless, this treatment gives rise to a plasminogen activator, which can, however, only be detected after separation from plasma by centrifugation. This activator was markedly increased in plasma obtained 1 min after the injection of cellulose sulphate (Rosa, Rothschild & Rothschild, 1971). Its nature and role in the *in vivo* activation of fibrinolysis due to cellulose sulphate are currently under investigation.

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