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In vivo effects of cellulose sulphate on plasma kininogen, complement and inflammation

V. EISEN AND C. LOVEDAY

Rheumatology Research Department, The Middlesex Hospital Medical School, London, W1P 9PG

Summary

1. The *in vivo* action of cellulose sulphate was studied in an attempt to clarify the role of complement and kinin formation in inflammation.

2. Inflammatory oedema was produced in the rat paw by heat (45·5° C), and on the ear by xylene. The oedema was assessed by comparing the ratio of fresh wet weight to dry weight of corresponding injured and non-injured parts.

3. Following cellulose sulphate (6·5 mg/kg i.v.), plasma kininogen concentrations were promptly reduced by 90% or more. The reduction in complement titres was statistically significant and ranged from 17 to 65%. No toxic effects were observed. The oedema caused by heat or xylene was not reduced in these rats.

4. Cellulose sulphate (80 mg/kg i.p.) given over 3 days depleted plasma kininogen by about 90%, but reduced complement titres only slightly. These rats gained less weight and their condition was poor. Blood clotting was impaired and widespread haemorrhages were found. Heat and xylene produced significantly less oedema than in control rats. This diminished response is attributed to toxic side effects of cellulose sulphate, rather than depleted plasma kininogen and reduced plasma complement.

5. The results suggest that the inflammatory reactions to thermal and chemical injury can fully develop when plasma kininogen and complement are lowered.

Introduction

The sulphated polysaccharide cellulose sulphate induces formation of plasma kinin in rat and human plasma (Rothschild & Gascon, 1966) by activating clotting factor XII (Kellermeyer & Kellermeyer, 1969). Injected into rats, cellulose sulphate lowers the blood pressure and depletes plasma kininogen (Rothschild, 1968). This depletion has been used to study the role of kinin formation in inflammation. Leme, Schapoval & Rocha e Silva (1967) reported that, in rats, previous kininogen depletion with cellulose sulphate depressed the inflammatory response to injury by heat. Rats whose plasma kininogen had been depleted by intraperitoneal injections of cellulose sulphate or of other sulphated polysaccharides, responded to injections of these compounds into the hind paw with less local swelling than did rats with normal kininogen concentrations (Rocha e Silva, 1970). These findings were regarded as direct evidence for the participation of plasma kinins in acute inflammation. Edery & Lewis (1963) found that the kinin-forming potential in the lymph collected

from the dog's hind paw increased after the limb had been scalded at 80° C for 15 seconds. Experiments in rabbits suggested that thermal injury may lead to leakage of prekallikrein from plasma into lymph, where the enzyme is activated by tissue activators and then promptly neutralized by inhibitors (Lewis & Wawretschek, 1970). Other workers deny that kinin formation participates in the response to thermal damage. Although Urbanitz, Sailer & Habermann (1970) confirmed the activation of the plasma kinin-forming system in thermal injury (Rocha e Silva & Antonio, 1960; Starr & West, 1967), they concluded that this activation was not responsible for the development of oedema.

Like the related polysaccharide, carrageenin (Davies, 1963, 1965; Boros, Rapp & Crisler, 1964; Ward & Cochrane, 1965), cellulose sulphate acts not only on the kinin-forming system in plasma, but also lowers complement titres, both *in vitro* (Eisen & Loveday, 1970 and unpublished results) and *in vivo* (this report). It is therefore possible that the depression of inflammatory responses in rats pretreated with cellulose sulphate or carrageenin (Leme *et al.*, 1967; Rocha e Silva, 1970) was partly due to reduced complement activity, and not only to reduced kinin formation. Willoughby, Coote & Turk (1969) reported that the oedema produced in the skin by heat, was reduced in rats with lowered serum complement titres; they suggested that complement might have a role in non-immune inflammation. However, Spilberg & Osterland (1970) have cast doubt on such a role in the arthritis produced by intra-articular injections of monosodium urate crystals in rabbits, since this inflammation developed fully in animals whose complement had been inactivated by cobra venom.

In this work, the *in vivo* action of cellulose sulphate was studied in an attempt to clarify the role of complement and kinin formation in inflammation caused by thermal and chemical injury.

Methods

Synthetic bradykinin was obtained from Sandoz Ltd., trypsin from the Boehringer Corp., sheep blood in Alsever's solution and horse haemolytic serum for sheep red cells from Wellcome Reagents Ltd. Cellulose sulphate was prepared by the method of Astrup, Galsmar & Volkert (1944); it was dissolved so that the dose to be injected was contained in 0·2–0·3 ml of saline.

Total complement

This was measured as 50% haemolytic units (C'H50) using the method of Osler, Strauss & Mayer (1952). The normal range obtained with this method in forty-five rats was M=48·4 units/ml, S.D.= ±18·0, S.E.M.= ±2·7.

Kinin concentrations

The isolated rat uterus suspended from an iso- or auxotonic lever was used for assays (Eisen, 1963).

Plasma kininogen

The concentration was expressed as the bradykinin in nmol/ml released from 10 times diluted plasma or serum by trypsin (250 μg/ml) in presence of sodium edetate (2 mg/ml). Tests with synthetic trypsin substrates showed that this concen-

tration of trypsin was not neutralized by the inhibitors in plasma for at least 10 minutes. The kinin release was completed in 3 min at 37° C. The trypsin was then inhibited by soya bean trypsin inhibitor 1 mg/ml.

Arterial blood pressure

The intracarotid pressure of rats anaesthetized with pentobarbitone sodium (45 mg/kg) was recorded with a Devices pressure transducer type 3-327-L222 and a M4 recorder.

Cellulose sulphate was administered to Wistar rats (150–200 g) in two ways: 1. Acute treatment. A dose of 6·5 mg/kg was injected into the tail vein over 15 min, starting 45 min before injury by heat or by application of xylene. 2. Three-day course. Two doses of 10 mg/kg and three doses of 20 mg/kg were injected intraperitoneally at 12 h intervals; the final injection was given 1 h before injury. (This was the same dosage as used by Leme *et al.* (1967) for kininogen depletion.)

Thermal injury

Rats were anaesthetized with pentobarbitone (45 mg/kg i.p.), and one hind paw was immersed up to the tibio-metatarsal joint in a water bath at 45·5° C for 5 minutes.

Chemical injury

Xylene was applied with a cotton wool bud to one ear of anaesthetized rats and left for 20 minutes.

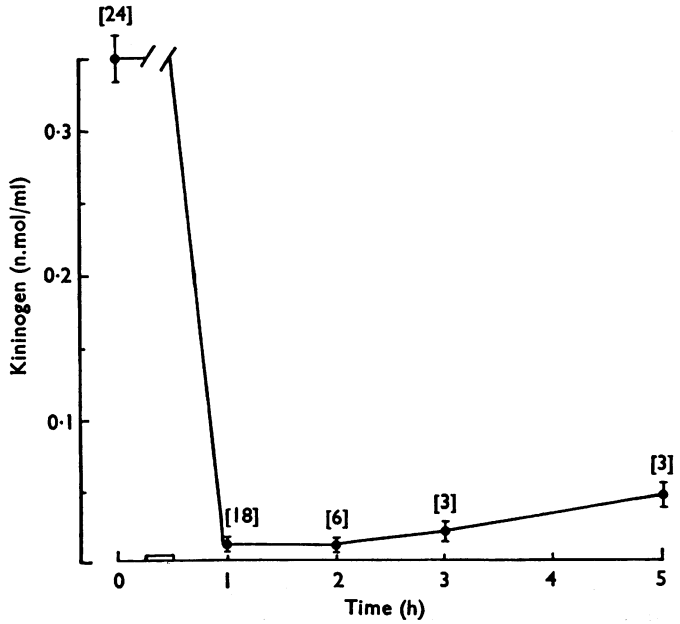

FIG. 1. Reduction of plasma kininogen by cellulose sulphate (6·5 mg/kg infused i.v.) at 15–30 min (□□). Means and S.E.M. are shown. Figures in brackets give numbers of animals.

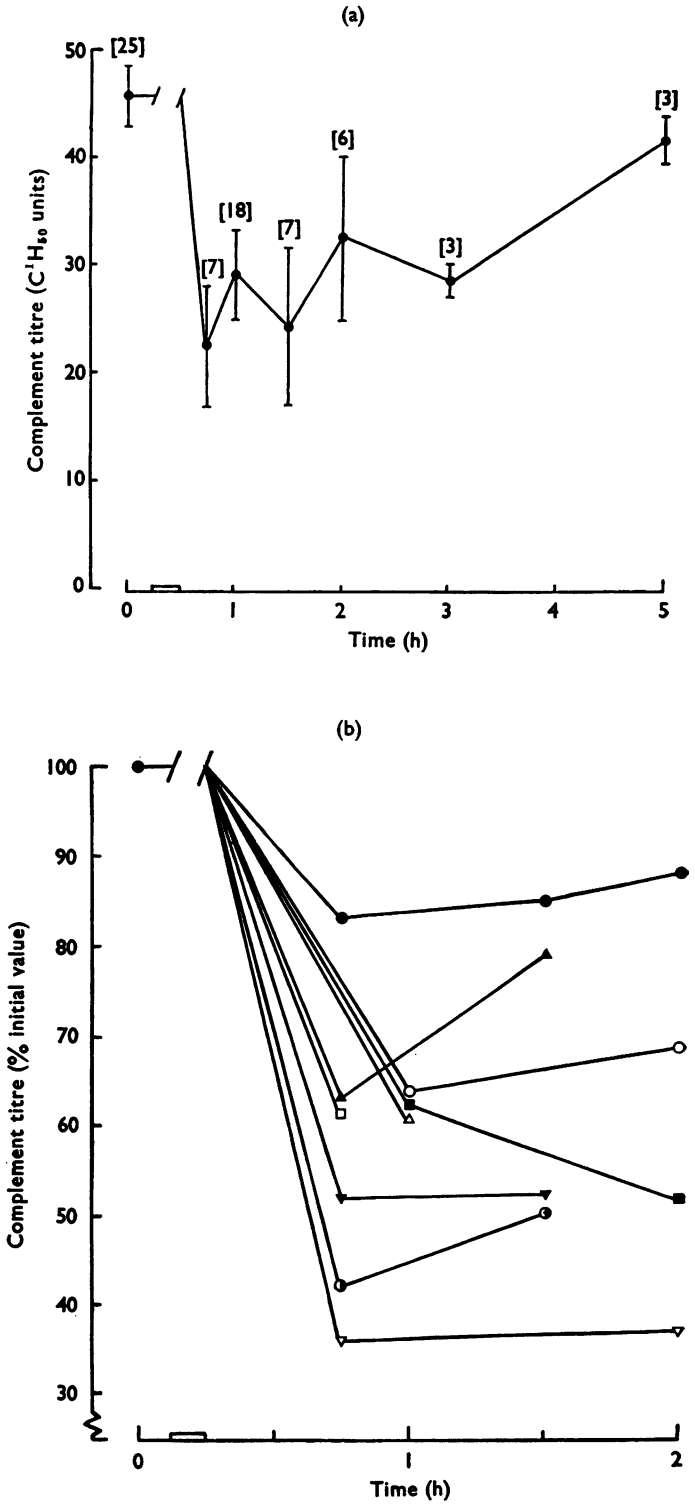

FIG. 2. Reduction of serum complement (C'H50) by cellulose sulphate infused as in Fig. 1. (a) Mean titres and S.E.M. Figures in brackets give numbers of rats. (b) Percentage fall in titres, followed individually in nine rats. Blood was collected from carotid cannulae.

Collection of blood

Cardiac puncture was carried out 15 min after the injury, unless stated otherwise.

Assessment of oedema

Paws and ears were cut off as uniformly as possible 20 min after injury, and promptly weighed. They were then dried in an oven at 120° C to constant weight for 3–4 days. Oedema was estimated by comparing the ratio of fresh wet weight to dry weight of injured and of normal paws and ears, respectively.

Results

Figure 1 shows that following cellulose sulphate (6·5 mg/kg i.v.) plasma kininogen concentrations were promptly reduced from a mean value of 0·35 nmol/ml to about 0·015 nmol/ml. Recovery to normal concentrations required 24–36 hours. The reduction in complement was smaller and more transient (Fig. 2a). The effect on complement was also rather variable (Fig. 2b), since in the 60 min following the start of the cellulose sulphate infusion, falls in complement titres of nine rats followed individually, ranged from 17 to 65%.

After this dose of cellulose sulphate, rats were in good condition, alert and lively. Only a few rats seemed less active for 5–10 min after the infusion had started, possibly due to the hypotension which lasted 10–15 min; they soon recovered. No evidence of damage to organs and tissues was found.

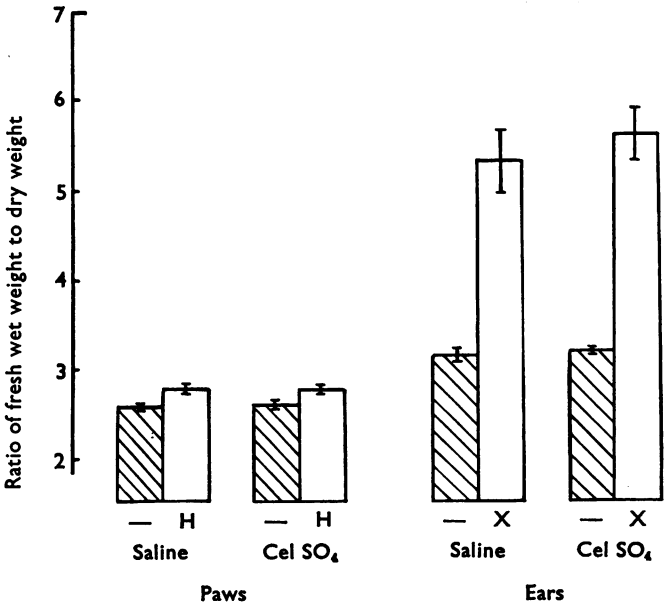

FIG. 3. Effect of cellulose sulphate (6·5 mg/kg i.v.) on inflammation. Oedema was produced in the rat paw by heating (H) (45·5° C/min) and on the ear by xylene (X) (xylene/20 min) at time 0. Cellulose sulphate was infused from −45 to −30 minutes. The clear columns show the oedema expressed as the ratio of fresh wet weight to dry weight of the injured parts. The shaded columns give the ratio of fresh wet weight to dry weight of uninjured parts. The vertical bars show S.E.M. The mild injury by heat produced significant oedema in the paws of depleted ($P<0·001$) and of undepleted ($P<0·001$) rats. The oedema produced by xylene was highly significant ($P\ll0·001$). There was no significant difference between depleted and undepleted rats, either in the swelling of paws ($P>0·1$) or of ears ($P>0·5$).

When intravenous treatment was repeated after 24 h, plasma kininogen and complement fell to the same level as after the first treatment. However, the 3-day course of intraperitoneal injections (total 80 mg/kg) depleted only plasma kininogen, whilst little or no reduction in complement was found.

Inflammation after acute treatment

Rats treated with cellulose sulphate (6·5 mg/kg i.v.) 45–30 min before mild thermal or moderate chemical injury, developed inflammatory oedema of the same size as did control rats (Fig. 3). Cellulose sulphate lowered plasma kininogen in all test rats to less than 15 pmol/ml. The C'H50 titres (M=35·36±3·69 (S.E.M.) units/ml) of the test rats were significantly lower ($P<0·01$) than in the control group (M=52·87±3·95 (S.E.M.) units/ml). The correlation between C'H50 titres and the swelling produced by heat or xylene was not significant and in one case even negative (Fig. 4).

Inflammation after 3-day treatment

In rats treated with cellulose sulphate for 3 days (total dose 80 mg/kg i.p.) as described by Leme *et al.* (1967), thermal and chemical injury produced significantly less oedema than in control rats injected with saline (Fig. 5). The plasma kininogen of these rats (M=26·8±2·7 (S.E.M.) pmol/ml) was not lower than after the acute application of cellulose sulphate (6·5 mg/kg); the C'H50 titres (M=47·0±6·8 (S.E.M.) units/ml) differed little from titres in control rats of the same batch (M=53·1±6·1 (S.E.M.) units/ml; $P>0·5$). There was no correlation between C'H50 titres and swelling of paws heated after cellulose sulphate or saline ($r=0·362$ and $r=0·112$, respectively). This was also true of swelling induced by xylene ($r=0·031$

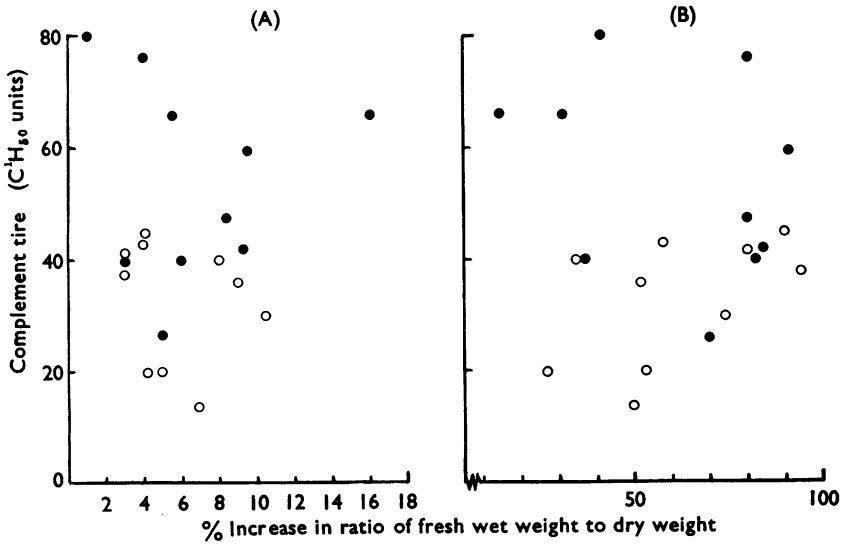

FIG. 4. Oedema in relation to complement titres in control rats (●) and after cellulose sulphate (6.5 mg/kg) (○). (A), Paws; (B), ears. Abscissa: percentage increase in ratio of fresh wet weight to dry weight produced by injury. Ordinate: C'H50 titres. There was no significant positive correlation between C'H50 titres and oedema. (A), Saline $r=-0·43$; cellulose sulphate $r=-0·214$. (B), Saline $r=-0·509$; cellulose sulphate $r=0·511$.

and $r=0{\cdot}280$, respectively). However, after cellulose sulphate (80 mg/kg i.p.), rats were lethargic and in a poor state of health; they had gained less weight (M $=3{\cdot}6\pm1{\cdot}7$ (S.E.M.) g) than control rats injected with saline (M $=11{\cdot}3\pm1{\cdot}1$ (S.E.M.) g). The snouts, paws and eyes of test rats were distinctly paler than in control rats. The clotting time was 20–60 min, instead of the normal 1–2 minutes. Haemorrhagic lesions in skeletal muscles, lungs, kidneys, liver and intestine were found macro- and microscopically.

Discussion

This study shows that cellulose sulphate, a polymerized cellobiose-sulphate ester, acts on complement *in vivo* in the same way as the polymerized galactose sulphate, carrageenin (Davies, 1963, 1965; Borsos *et al.*, 1964; Ward & Cochrane, 1965). The effect of these two polysaccharides on plasma kininogen is also similar (Leme *et al.*, 1967; Rothschild, 1968; Van Arman & Nuss, 1969).

Cellulose sulphate appeared to deplete kininogen more completely (Fig. 1) than was reported by Rothschild (1968). This discrepancy was probably due to the different techniques of estimating kininogen. In this work, rat plasma was not denatured before kininogen was measured. In such rat plasma, trypsin and plasma kininogenases form similar amounts of kinin (Collins, Eisen & Glanville, 1970). In plasma denatured by boiling in acid (Rothschild, 1968), trypsin digests additional kininogen, whereas the effect of plasma kininogenases is not enhanced.

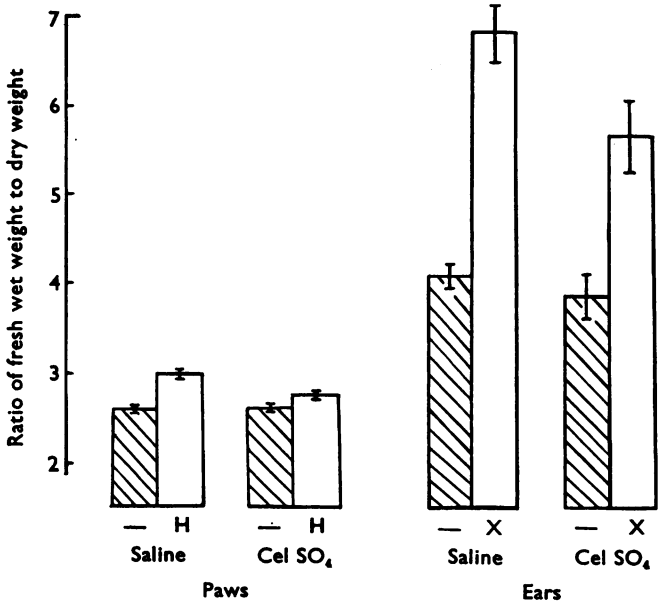

FIG. 5. Effect of cellulose sulphate (80 mg/kg i.p.) on inflammation. Oedema was produced in the rat paw by heating (H) (45·5°C/5 min) and on the ear by xylene (X) (xylene/20 min). Cellulose sulphate was injected over 3 days before injury. The clear columns show the oedema expressed as the ratio of fresh wet weight to dry weight of the injured parts. The shaded columns give the ratio of fresh wet weight to dry weight of uninjured parts. The vertical bars show S.E.M. Heat and xylene produced significant oedema of paws ($P<0{\cdot}001$) and ears ($P<0{\cdot}001$), respectively. Rats treated with cellulose sulphate developed significantly less oedema in the paws ($P<0{\cdot}001$) and in the ears ($P<0{\cdot}01$), than did control rats.

In view of the described effects on the kinin-forming and complement systems, it seems necessary to assess both systems whenever inflammation is studied in animals pretreated with carrageenin or cellulose sulphate, since both kinin formation and complement may influence the type of inflammation used in the experiment.

The experiment with rats given cellulose sulphate (80 mg/kg) over 3 days shows that toxic side effects of depleting agents may interfere with inflammation. The depletion of plasma kininogen by this dose was no more complete than after acute depletion by 6·5 mg/kg of cellulose sulphate; C'H50 titres were not significantly lower than in the control group. The pronounced depression of inflammation therefore could not be attributed to depletion of plasma kininogen or reduced plasma complement. However, these experiments do not exclude the possibility that cellulose sulphate (80 mg/kg) (but not 6·5 mg/kg) may have depleted kininogen not only in plasma but also in lymph and interstitial fluid, and that this may have resulted in a depression of the inflammatory response.

The very obvious toxicity of 80 mg of cellulose sulphate was probably at least partly responsible for the reduced swelling. In this dose, cellulose sulphate had a powerful anticoagulant effect (cf. Astrup *et al.*, 1944; Kellermeyer & Kellermeyer, 1969), which was probably responsible for the widespread haemorrhages. These would tend to lower blood pressure and elicit reflex sympathetic vasoconstrictor activity. Constriction of precapillary sphincters would reduce the blood flow through skin and other tissues, and less fluid and protein would leave the capillaries when their permeability was increased by injury. Toxic doses of cellulose sulphate may also depress inflammation by other mechanisms, not yet fully understood. Lowered inflammatory reactivity has been reported in several other toxic conditions such as diabetic acidosis and ketosis, and chronic renal failure (Perilie, Nolan & Finch, 1962; Seegmiller, Laster & Howell, 1963).

The finding that inflammatory oedema developed fully in rats depleted of plasma kininogen by cellulose sulphate (6·5 mg/kg i.v.), suggests that kinin formation in plasma is not essential for this response. Since complement was only partially reduced in this experiment, sufficient may have remained to mediate inflammation, and its role cannot fully be excluded.

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