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# The anticoagulant activity of carrageenan

W. ANDERSON AND J. G. C. DUNCAN

(With the technical assistance of Miss J. E. Harthill)

λ-Carrageenans and κ-carrageenans from samples of *Chondrus crispus*, carrageenan from *Polyides rotundus* and degraded carrageenan from *Eucheuma spinosum* have anticoagulant activity on intravenous injection in the rabbit. Anticoagulant activity appears to be caused by a general reaction with plasma protein. The undegraded carrageenans are acutely toxic on intravenous injection and form insoluble complexes with fibrinogen in neutral solution. Degraded carrageenan is very much less toxic and, like heparin, forms soluble complexes with fibrinogen. The λ-carrageenans from *C. crispus* have higher sulphate content, consistently prolong the clotting time more, and are more toxic than the corresponding κ-carrageenans. The differences in sulphate content between the various λ-carrageenans, and between the carrageenans from the other seaweeds tested, do not correspond directly with differences in anticoagulant action and toxicity.

THE carrageenans, which occur naturally in the red seaweeds, constitute a closely related group of sulphated galactans. While displaying the biological properties of sulphated polysaccharides in general, quantitative differences exist between the members of the group and between the readily separated κ- and λ-carrageenans which are present in certain of the members. Houck, Morris & Lazaro (1957) examined unfractionated whole extracts of a number of seaweeds for anticoagulant activity which they found only in *Gigartina acicularis*. Hawkins & Leonard (1962, 1963) fractionated one *Chondrus crispus* carrageenan into its κ- and λ-components and found greater anticoagulant activity in the λ-carrageenan which contained more ester sulphate than the κ-carrageenan. Rees (1963) has suggested that λ-carrageenan may be the biological precursor of κ-carrageenan. Whole extracts of seaweeds might therefore be expected to vary in the relative content of κ- and λ-carrageenans and hence in anticoagulant activity. This may explain largely negative findings such as those of Houck & others (1957).

We report a study of the anticoagulant activity of a group of carrageenans differing in ester sulphate content, molecular weight and source.

## Materials and methods

*Animals.* Male New Zealand white rabbits (2–4 kg) were used. Food, but not water, was withheld for 18 hr before testing. Each animal acted as its own control and none was used more than once.

*Collection of blood.* Blood was allowed to drip freely into Pyrex glass tubes from a small incision made on the marginal vein of the shaved, warmed and solvent-cleaned ear. The first ml of blood was discarded.

All clotting tests were made at 37°. Control results were obtained from blood withdrawn immediately before injection of carrageenan; test bloods were withdrawn 2 hr after the injection of carrageenan, or 0·5 hr in the heparin experiments. Preliminary experiments showed that the greatest anticoagulant effect of the carrageenans occurred 2 hr after injection while that for heparin occurred at 0·5 hr.

*Saline.* 0·85% w/v sodium chloride in water for injection, B.P.

From the Department of Pharmacy, University of Strathclyde, Glasgow, C.1.

*Carrageenans.* Degraded carrageenan (Ebimar, Evans Medical Ltd.). The other carrageenans, the $\kappa$- and $\lambda$-fractions (Black, Blakemore Colquhoun & Dewar, 1965), and the data in Table 1 were provided by the Arthur D. Little Research Institute through the courtesy of Dr. E. T. Dewar. The carrageenans were injected intravenously in 8 ml saline solution.

*Heparin.* Sodium heparin, 150·1 units/mg (Evans), kindly supplied by Dr. C. H. Smith.

*Thrombin solution.* Thrombin Topical (Maw) 20 u/ml in saline.

*Citrated plasma.* Blood (9 ml) was allowed to drip into trisodium citrate solution (1 ml; 3·13% w/v), mixed, and centrifuged at 1400 rpm for 10 min.

*Fibrinogen.* Prepared according to Biggs & Macfarlane (1962). A saline solution containing 1 mg/ml was used in the clotting tests.

*Whole blood clotting time.* The test of Lee & White (1913) in Pyrex glass tubes (10 × 75 mm) was used.

*Thrombin time.* Citrated plasma, (0·2 ml) and saline, (0·1 ml) were mixed and thrombin solution, (0·1 ml) was added. The time between addition of thrombin and solid clot formation was noted. Triplicate determinations were done on each plasma and results calculated as follows:

$$\frac{\text{average clotting time of control plasma}}{\text{average clotting time of test plasma}} \times 100$$

In the *in vitro* experiments (Table 3) saline (0·1 ml) in the control was replaced by carrageenan solution (0·1 ml) in the test and normal citrated rabbit plasma used.

*One-stage and two-stage prothrombin tests; prothrombin consumption test.* The methods of Biggs & Macfarlane (1962) were used and results were expressed as follows:

one-stage: $\dfrac{\text{average clotting time of control plasma}}{\text{average clotting time of test plasma}} \times 100$

(triplicate determinations on each plasma)

two-stage: $\dfrac{\text{area under test curve}}{\text{area under control curve}} \times 100$

prothrombin consumption index:

$$\frac{\text{minimum plasma clotting time}}{\text{minimum serum clotting time}} \times 100$$

*In vitro* prothrombin times (Table 3) were obtained using a one-stage test modified to include saline (0·1 ml) in the control and carrageenan solution (0·1 ml) in the test. 50% increase in time was taken as standard because certain carrageenans caused precipitation rather than clotting when used in excess of the amount required to prolong the clotting time by 50%.

*Fibrinogen-carrageenan precipitation.* Aqueous fibrinogen (1 ml; 0·4%) was mixed at room temperature with aqueous carrageenan (1 ml) of various concentrations. pH of the mixture was 6·8. Mixtures were allowed to stand 24 hr before reading against a fibrinogen solution as control.

Carrageenan was determined by the method of MacIntosh (1941).

*Platelet counts.* The method of Brecher & Cronkite (1950) was used.

## Results and discussion

The results are in Tables 2–7. All results from *in vivo* experiments are means from four rabbits.

*Anticoagulant action of the carrageenans.* In the whole blood clotting test all the λ-carrageenans were, in varying degree, active at 3 mg/kg (Table 2) but toxicity began to appear at 5 mg/kg. Table 2 reveals a trend to greater activity with higher sulphate content (Table 1) amongst the four λ-carrageenans, but the carrageenan with the highest sulphate content did not have the greatest activity, *Polyides* carrageenan having greatest effect. It is nevertheless clear that, for the *Chondrus* carrageenans,

TABLE 1. SOURCES AND PROPERTIES OF THE CARRAGEENANS USED

| Carrageenan | Code | Source | $SO_3Na$(%) | | 3,6-Anhydro galactose, (%) | | Inherent viscosity (dl/g) | |
|---|---|---|---|---|---|---|---|---|
| | | | κ | λ | κ | λ | κ | λ |
| *Chondrus crispus* | –CY | Yarmouth, Nova Scotia | 28·2 | 37·3 | 29·2 | 3·5 | 13·7 | 16·2 |
| ,, ,, | –CNS | Northumberland Strait, Nova Scotia | 28·4 | 34·9 | 25·3 | 4·1 | 20·8 | 21·7 |
| ,, ,, | –CSE | Sebasco Estates, Nova Scotia | 29·8 | 32·3 | 25·2 | 9·1 | 14·3 | 13·8 |
| ,, ,, | –CMI | Mud Island, Nova Scotia | 27·0 | 32·2 | 24·8 | 9·8 | 8·6 | 9·4 |
| *Polyides rotundus* | | Moose Head, Nova Scotia | 35·0 | | 2·3 | | 5·1 | |
| Degraded-λ from *C. crispus*-CNS | | | 30·7 | | — | | 1·3 | |
| Degraded carrageenan (*Eucheuma spinosum*) | | S.E. Asia (Evans Medical Ltd.) | 29·0 | | 21·0 | | 0·3 | |

Inherent viscosity, $\eta_{inh} = c^{-1}\ln(\eta_{soln.}/\eta_{solv.})$ dl/g where c = g solute in 100 ml solution, was measured at 25° in an Ostwald viscometer (M2 BS U/M) using 0·1 M sodium chloride as solvent. For undegraded carrageenans c = 0·02; for degraded carrageenans c = 0·2. Viscosity is taken as a comparative indication of molecular weights amongst these substances which all have similar structure.

TABLE 2. RABBIT WHOLE BLOOD CLOTTING TIMES 2 HR AFTER INTRAVENOUS CARRAGEENAN; LOWEST INTRAVENOUS DOSES OF CARRAGEENANS KILLING WITHIN 24 HR

| Carrageenan | Clotting time, min. mg/kg i.v. | | | | Lowest dose killing within 24 hr mg/kg i.v. | |
|---|---|---|---|---|---|---|
| | 5 | | 3 | | | |
| | κ | λ | κ | λ | κ | λ |
| *C. crispus* –CY | 11 | 14 | 6 | 10 | 3 | 1 |
| ,, –CNS | 8 | toxic | 9 | 13 | 5 | 1 |
| ,, –CSE | 7 | 8 | 6·5 | 7·5 | 10 | 5 |
| ,, –CMI | 6 | 10 | 5 | 9 | 15 | 5 |
| Degraded λ–CNS | | 8 | | 9 | | 10 |
| *P. rotundus* | | — | | 20 | | 5 |
| Degraded carrageenan | 200 mg/kg 17 | | 100 mg/kg 10 | | >1000 | |
| Heparin | (200 u/kg) 20 | | (75 u/kg) 8 | | — | |

Results are averages from four rabbits.

TABLE 3. WEIGHTS OF CARRAGEENANS REQUIRED TO PROLONG THE PROTHROMBIN AND THROMBIN TIMES *in vitro* BY STATED AMOUNTS

| Carrageenan | Weight required to prolong the prothrombin time by 50% μg | | Weight required to prolong the thrombin time by 100% μg | |
|---|---|---|---|---|
| | κ | λ | κ | λ |
| *C. crispus* –CY | 35 | 5 | 12 | <1 |
| ,, –CNS | 35 | 3 | 7 | 1 |
| ,, –CSE | 35 | 12 | 8 | 2 |
| ,, –CMI | 45 | 15 | 30 | 2 |
| *P. rotundus* | 5 | | 1 | |
| Degraded carrageenan | 200 | | 50 | |

Results are averages from plasmas of four rabbits.

each λ-carrageenan has higher sulphate content and greater effect than its κ-counterpart. Although this confirms the belief that the λ-carrageenans are generally more active anticoagulants than the corresponding κ-carrageenans, the results for all the carrageenans, and also the high dose required for degraded carrageenan, suggest that anticoagulant activity does not depend only on ester sulphate content. This conclusion is supported by the results (Table 3) of *in vitro* experiments in which different stages in the clotting mechanism are examined.

Degraded carrageenan is a degraded κ-carrageenan; it has a high 3,6-anhydrogalactose content and the parent carrageenan extracted from *Eucheuma spinosum* can be degraded by mild mineral acid treatment without serious sulphate hydrolysis. This degradation results in a much smaller molecule, permits a higher dose unaccompanied by the toxicity of the undegraded κ- and λ-carrageenans, and anticoagulant activity can be clearly demonstrated. But with undegraded κ-carrageenan, even at the highest safe dose, anticoagulant activity was either low or absent. Amongst the undegraded κ-carrageenans, the most active (κ-CY) was the most toxic and, conversely, the least toxic (κ-CMI) showed the least activity; it also had the lowest viscosity. It is unlikely that the difference in sulphate content (1%) between these two, accounts for the difference in anticoagulant activity, a conclusion which is supported by the data (Tables 1, 2 and 3) for the other two κ-carrageenans. Discussing a related group of polysaccharide sulphates, the laminarin sulphates, Adams, Heathcote & Walker (1962) stated that the laminarin "with the highest molecular weight and greatest degree of sulphation has high antilipaemic and anticoagulant activity, and toxicity".

λ-Carrageenans have a low, and κ-carrageenans a high, 3,6-anhydrogalactose content, and this is one of the distinguishing features between the κ- and λ-carrageenans (Table 1). That the presence of 3,6-anhydrogalactose is not responsible for lower activity of the κ-carrageenans is shown by the anticoagulant activity of degraded carrageenan at adequate dosage.

*Intravenous toxicity of carrageenans.* Large doses of sulphated polysaccharides can be given orally to man and animals, little or no absorption occurs, and direct systemic toxicity by this route has never been observed.

TABLE 4. CARRAGEENAN-FIBRINOGEN REACTION IN WATER (pH 6·8)

| Carrageenan | μg carrageenan mixed with 4 mg fibrinogen in 2 ml mixture | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 160 | 80 | 40 | 20 | 10 | 5 | 2·5 | 1·25 | 0·6 |
| *C. crispus* –λ–CY .. .. | — | — | — | ± | ± | ++ | +++ | +++ | +++ |
| ,, λ–CNS .. | — | — | — | 0 | 0 | ++ | +++ | +++ | +++ |
| ,, λ–CSE .. .. | — | — | — | — | 0 | + | ++ | ++ | ++ |
| ,, λ–CMI .. | — | — | — | — | — | 0 | 0 | + | + |
| ,, κ–CY .. .. | — | — | 0 | + | + | ± | + | + | + |
| ,, κ–CNS .. .. | 0 | 0 | 0 | + | + | + | + | + | + |
| ,, κ–CSE .. .. | — | — | — | — | — | 0 | + | + | ± |
| ,, κ–CMI .. .. | — | — | — | — | — | 0 | 0 | 0 | + |
| *P. rotundus* .. .. .. | — | — | — | — | 0 | + | ++ | ++ | |
| Degraded carrageenan .. | — | — | — | — | — | — | — | — | — |
| Heparin .. .. .. | — | — | — | — | — | — | — | — | — |

— = clear solution after mixing (see text); 0 = mixture has same opalescence as control (i.e. no evidence of complex formation); ± = increased opalescence (finely dispersed precipitate); ++ = flocculated precipitate; +++ = copious flocculated precipitate.

Nevertheless, these substances are toxic when administered intravenously. Astrup (1953) and Walton (1954) found toxicity by this route in certain high molecular weight dextran sulphates and concluded that the toxic reactions were initiated by formation, in the circulation, of insoluble fibrinogen complexes which entrapped platelets and blood cells. The carrageenans can be grouped according to the nature of their reaction with fibrinogen at pH 6·8, which is above its isoelectric point. In distinction from the less active, less toxic, κ-carrageenans which formed small particle dispersions with fibrinogen, the λ-carrageenans formed coarser precipitates which were flocculent or coagulated. Both types of precipitate were soluble in excess of the respective carrageenan. Table 4 shows that insoluble complex formation was much more pronounced with the CY-carrageenans, and slightly more pronounced with the CNS-carrageenans, than with the other carrageenans. They were also the most active and toxic of the *Chondrus* group. The λ-carrageenans did not all form similar types of precipitate: thus λ-CY and λ-CNS formed fibrous floating coagula immediately, but λ-CSE and λ-CMI required up to 0·5 hr to form particulate precipitates. Assuming Walton's (1953) conclusions regarding the trapping of platelets by dextran sulphate-fibronogen precipitates to be relevant to the present case, the differences in character of the formed precipitates could be related to the different activities and toxicities of the λ-carrageenans. With the κ-carrageenans, on the other hand, there was little or no evidence to suggest a relationship between sulphate content, precipitation, and anticoagulant activity.

Maximum precipitation occurred for most carrageenans when 2·5 μg reacted with fibrinogen (4 mg; in 2 ml); greater concentrations of carrageenan resulted in less precipitation and eventually in soluble complex formation, indicated by the clearing of the opalescence contributed by the fibrinogen, and by results of estimation of free carrageenan in solution.

Soluble complex formation, seen at all concentrations with degraded carrageenan and heparin is associated in these two substances with relative absence of toxicity. Toxicity is associated with sulphated polysaccharides which can form insoluble complexes with fibrinogen at neutral pH.

The relationship of fibrinogen precipitation *in vitro* to toxicity and

activity *in vivo* is obscure. Concentrations in the precipitation experiments were chosen to simulate *in vivo* concentrations as far as was possible; thus a carrageenan dose of 3 mg/kg, given intravenously to a rabbit having

TABLE 5. PERCENTAGE REDUCTION OF PLATELETS IN THE RABBIT 2 HR AFTER INTRAVENOUS CARRAGEENANS

| Carrageenan | Dose mg/kg i.v. | Percentage reduction in platelet count |
|---|---|---|
| *C. crispus* κ–CY | 5 | 24 |
| ,, κ–CNS | 5 | 39 |
| ,, κ–CSE | 5 | 25 |
| ,, κ–CMI | 5 | 0 |
| ,, λ–CY | 5 | 62 |
| ,, λ–CNS | 5 | 59 |
| ,, λ–CSE | 5 | 42 |
| ,, λ–CMI | 5 | 31 |
| *P. rotundus* | 3 | 42 |
| Degraded carrageenan | 200 | 32 |
| Heparin | 200 u/kg | 0 |

Results are averages from four rabbits.

a blood volume of 200 ml, would, if completely mixed with the blood, give 90 μg carrageenan in 2 ml blood. It was below this concentration that precipitation *in vitro* began, and maximum precipitation was only seen when the carrageenans were even more dilute (Table 3).

In similar experiments thrombin-carrageenan complexes also precipitated, but with thrombin, as distinct from fibrinogen, heparin and degraded carrageenan formed insoluble precipitates at appropriate concentrations. Carrageenan affected all the tests of clotting examined (Tables 2, 6, 7) and this suggests that their reaction with plasma proteins, especially those concerned with clotting, is a general one and not restricted to fibrinogen; it is possible that insoluble complex formation with other proteins, whether engaged in the clotting or not, could also contribute to toxicity by embolism formation. Complexes formed with clotting proteins need not, on the other hand, be insoluble to affect the clotting reaction.

Platelet counting showed (Table 5) that injection of λ-carrageenans resulted in lower platelet counts than the injection of κ-carrageenans, and amongst the λ-carrageenans the most active anticoagulants caused greatest reduction in count. Although such a trend was obscure amongst the κ-carrageenans, it is noteworthy that κ-CMI caused no reduction in platelet count and was the least active and the least toxic of the κ-carrageenans. Heparin and degraded carrageenan, both relatively non-toxic, caused little or no reduction in platelet count. When the platelet counts were very low, agglutinated platelets were never seen, but with normal or slightly reduced counts (as seen with the κ-carrageenans) agglutination was occasionally seen. It is appropriate to speculate that the structure of the κ-carrageenan-plasma protein complex is unable to trap platelets which are nevertheless caused to agglutinate by the κ-carrageenans. The structure of the λ-carrageenan-plasma protein complex is able to trap the agglutinated platelets, resulting in depleted numbers of unagglutinated platelets in the circulation. Such differences

in structure are more likely to be due to difference in configuration of the sulphated polysaccharides rather than to relatively small differences in sulphate content and molecular weight. This view is supported by the fact that degraded λ-carrageenan is much more toxic than degraded

TABLE 6. EFFECTS OF INTRAVENOUS CARRAGEENANS ON THROMBIN AND PROTHROMBIN TESTS IN THE RABBIT

| Carrageenan | Thrombin time | | | | One-stage prothrombin | | | | Two-stage prothrombin | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/kg i.v. | | | | mg/kg i.v. | | | | mg/kg i.v. | | | |
| | 5 | | 3 | | 5 | | 3 | | 5 | | 3 | |
| | κ | λ | κ | λ | κ | λ | κ | λ | κ | λ | κ | λ |
| *C. crispus* –CY | 83 | 68 | 91 | 76 | 78 | 58 | 95 | 69 | 55 | 38 | 61 | 50 |
| ,, –CNS | 85 | toxic | 85 | 87 | 84 | toxic | 81 | 49 | 63 | toxic | 56 | 36 |
| ,, –CSE | 87 | 55 | 82 | 74 | 79 | 62 | 78 | 75 | 66 | 57 | 59 | 59 |
| ,, –CMI | 102 | 67 | 97 | 94 | 92 | 63 | 87 | 70 | 71 | 68 | 82 | 56 |
| *P. rotundus* | | | 72 | | | | 58 | | | | 43 | |
| Degraded carrageenan 200 mg/kg | 60 | | | | 69 | | | | 17 | | | |
| Heparin | | | | | | | | | | | | |
| 200 u/kg | <1 | | | | 86 | | | | 9 | | | |
| 75 u/kg | 8 | | | | 100 | | | | 24 | | | |

κ-carrageenan even though both have similar sulphate content and only a small difference in viscosity. The different effect on platelets could also be causally concerned in the different anticoagulant activities of the κ- and λ-carrageenan, although this suggestion is, with present knowledge, not amenable to searching experimental examination.

The general pattern of the fibrinogen reaction of the carrageenans therefore resembles that of certain dextran sulphates, and the picture of respiratory and circulatory collapse with generalised congestion of abdominal organs, in rabbits given a fatal dose, conforms to the description of dextran sulphate overdosage described by Walton (1954) and by others who have observed the toxic effects of parenterally-administered macromolecules.

*Localisation of anticoagulant action.* The reaction of the carrageenans with plasma protein can explain their toxicity; fibrinogen depletion, caused by its reaction with carrageenan, could affect the final clotting stage. However, the rapid removal of some fibrinogen from the circulation, cannot wholly account for the anticoagulant action which was not fully developed in the rabbit for any of the carrageenans until 2 hr after injection, whereas that of heparin was present at 0·5 hr.

Hawkins & Leonard (1962) deduced that the anticoagulant action of carrageenan was antithrombic in nature on the basis that thrombin time increased with increasing plasma concentration of carrageenan; in agreement with the same authors (1963), Table 3 shows that more carrageenan was required to prolong prothrombin than thrombin time *in vitro* but no quantitative relationship can be obtained from this comparison. Antithrombin action could obviously affect all clotting tests but this does not appear to be the sole mode of action. Thrombin time (Table 6)

was only slightly affected by the carrageenans as compared with heparin which gave a marked effect even at the low dose. This is in contrast to the

TABLE 7. PROTHROMBIN CONSUMPTION INDEX BEFORE AND AFTER INTRAVENOUS INJECTION OF CARRAGEENANS

| Carrageenan | mg/kg i.v. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 | | | | 3 | | | |
| | κ | | λ | | κ | | λ | |
| | before | after | before | after | before | after | before | after |
| *C. crispus* –CY.. .. | 6 | 31 | 11 | 42 | 11 | 30 | 5 | 49 |
| ,, –CNS .. | 7 | 46 | toxic | | 9 | 72 | 6 | 52 |
| ,, –CSE .. | 5 | 42 | 24 | 115 | 13 | 22 | 4 | 29 |
| ,, –CMI .. | 3 | 19 | 7 | 66 | 9 | 29 | 14 | 56 |
| *P. rotundus* .. .. | | | | | before 13 | | after 146 | |
| Degraded carrageenan | 200 mg/kg | | | | | | | |
| | before 11 | | | | after 105 | | | |
| Heparin .. .. | 200 u/kg | | | | 75 u/kg | | | |
| | before 5 | | after 335 | | before 5 | | after 5 | |

similarity in effect in the whole blood clotting time, between *Chondrus* carrageenans and the low dose of heparin, and between degraded carrageenan and the high dose of heparin. This difference between carrageenan and heparin suggests that carrageenan may affect the earlier stages of clotting which precede prothrombin conversion. These earlier stages are associated with prothrombin activator (thromboplastin) formation which can be followed by the prothrombin consumption test. Reduced prothrombin consumption (Table 7) is compatible with the actions of carrageenan already shown, namely platelet count reduction, and effects on fibrinogen and probably on other clotting factors.

The results of the one-stage and two-stage tests (Table 6) support the suggestion that carrageenans interfere with clotting factors in a non-specific manner.

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