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Degraded and undegraded carrageenans and antipeptic activity

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The antipeptic activities of a series of undegraded κ- and λ-carrageenans and degraded carrageenans have been examined by two methods. The activities differ quantitatively. Compared with the κ-carrageenans, the λ-carrageenans, as a group, have greater activity and this is associated with low 3,6-anhydro-D-galactose and high ester sulphate content. Within the series of undegraded carrageenans, both κ and λ, differences in antipeptic activity are significantly and positively related to differences in sulphate content. In addition to the effect of sulphate, 3,6-anhydrogalactose content appears to be inversely related to activity but in the λ- series only; differences in viscosity are without effect. Initial degradation without loss of sulphate resulted in loss of activity but further degradation, also without loss of sulphate did not reduce activity further. Antipeptic activity appears to depend on a combination of structural features of which the differences between κ-λ carrageenans, molecular size and polyanionic properties are aspects.

THE addition of sulphated polysaccharides to gastric juice or to solutions of pepsin results in a decrease in demonstrable proteolytic activity. This has been stated to be due to complex formation between the sulphated polysaccharide and the enzyme in the instance of chondroitin sulphate (Levey & Sheinfeld, 1954). For degraded carrageenan (Anderson, 1961), protection of the substrate rather than direct anti-enzyme action was shown to be principally responsible for the decrease in peptic activity.

Because of the interaction between sulphated polysaccharides and protein (substrate or enzyme, or both) at appropriate pH, which depends on the polyionic nature of the substances concerned, it has been inferred that ester sulphate is the part of the carrageenan molecule responsible for the magnitude of antipeptic activity. Indeed, it has been shown that for certain sulphated polysaccharides, sulphate content can be related to activity (Hawkins & Leonard, 1962; Ravin, Baldinus & Mazur, 1962). However, comparison of published work suggests that sulphate content is not the only part of the molecule concerned in antipeptic activity. The relationships are even less clear where different methods of measuring peptic activity have been used.

In the present work, the relationship between antipeptic activity and sulphate content, and other aspects of carrageenan structure, is examined using two methods of measuring antipeptic activity.

Experimental

MATERIALS AND METHODS

Carrageenans. Undegraded and degraded carrageenans were used, and were obtained from the principal seaweed sources, *Chondrus*, *Gigartina* and *Eucheuma* species. Of the undegraded carrageenans, unfractionated samples and samples fractionated into κ- and λ-components were used. With the exception of a degraded carrageenan from *Eucheuma spinosum*—C16—(Anderson, 1961), and Rees' carrageenan (Rees, 1963) kindly

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supplied by Dr. D. A. Rees, Chemistry Department, University of Edinburgh), all other carageenans (Black, Blakemore & others 1965) and the data in Table 1 were provided by the Arthur D. Little Research Institute, through the courtesy of Dr. E. T. Dewar.

Gastric juice. Gastric juice was obtained from duodenal ulcer patients undergoing the augmented histamine test and was used fresh. (It was provided through the courtesy of Dr. A. I. M. Glen, Western Infirmary, Glasgow.) Insoluble material was removed by centrifugation. Occasional juices devoid of acidity or discoloured were not used.

PEPTIC ACTIVITY

Method A was a modification of the method of Anson (1938). Two concentrations of carrageenan were used: 20 or 80 mg carrageenan was dissolved in 2 ml hydrochloric acid solution (pH 1·6) and this was added to 2 ml pepsin solution (20 mg/100 ml; pH 1·6; Armour crystalline porcine pepsin) giving concentrations of 0·5 and 2% of carrageenan respectively in the 4 ml of acid-pepsin at pH 1·6. This solution was incubated at 37° for 10 min and 2 ml haemoglobin solution (1·2%; pH 1·6; Armour bovine haemoglobin for proteolytic enzyme assay) was added, followed by incubation at 37° for 30 min. After incubation, 20 ml of 10% trichloroacetic acid was added, the flasks allowed to stand in the water bath for 1 hr and the contents filtered (Whatman No. 3). 5 ml filtrate was added to 10 ml N sodium hydroxide followed by 3 ml of a 1 : 2 aqueous dilution of Folin-Ciocalteu reagent. The extinction developing in 10 min was read at 660 mμ. Appropriate blanks and controls were included.

Method B was a modification of the method of Hunt (1948). Three concentrations of carrageenan were used: 8, 12 or 20 mg carrageenan was dissolved in 2 ml hydrochloric acid solution (pH 2·1) and 2 ml of centrifuged supernatant of the gastric juice added. (8 mg carrageenan in this 4 ml volume gave 0·2%.) 1 ml of this solution was used in the test for peptic activity. Appropriate controls and blanks were included. The remainder of the method was as described by Hunt. Extinction values were converted to Hunt units.

Antipeptic activity. This term was calculated from $(1 - I/S) \times 100$ where I and S represent peptic activity with and without inhibitor respectively, corrected for blanks as necessary. Antipeptic activity is therefore the amount of inhibition, %.

Results and discussion

A representative group of carrageenans from species of the red seaweed families *Chondrus*, *Gigartina*, *Eucheuma* and *Polyides* has been examined for antipeptic activity by two methods which differ both in enzyme and substrate. Method A uses a purified enzyme, whilst method B uses fresh human gastric juice from duodenal ulcer patients. Two methods were used to increase the likelihood of finding a pattern of antipeptic activity amongst the carrageenans and, hence, not only a basis for a standard method of assessing their antipeptic activity, but also information

about their mode of action. These two methods have been used in, or have formed the basis of, most reports on antipeptic activity.

The carrageenans were selected from a number of such substances which became available during an extensive survey (Black & others, 1965) of carrageenan as a possible therapeutic agent for the treatment of peptic ulcer. κ- and λ-Components were studied because they differ in structure and properties (Rees, 1966). Undegraded and degraded carrageenans were examined because, although degradation usually reduces antipeptic activity, it is possible in therapy and in pharmacological investigation to use greater amounts of the less viscous, more rapidly soluble degraded product, thus obtaining greater activity and more information than would otherwise be possible with the highly viscous, slowly soluble, native

TABLE 1. SOURCES AND PROPERTIES OF THE CARRAGEENANS USED

	Code	SO_3Na (%)		η_{inh} (dl/g)		3,6-anhydro-D-galactose $C_6H_8O_4$ (%)	
		κ	λ	κ	λ	κ	λ
Undegraded							
Chondrus crispus	CMI[1]	27·6	32·2	8·6	9·4	24·8	9·8
" "	CSE[2]	29·8	32·3	14·3	13·8	25·2	9·1
" "	CBC[3]	29·6	34·6	11·6	14·4	22·9	4·7
" "	CNS[4]	28·4	34·9	20·8	21·7	25·3	4·1
" "	CY[5]	28·2	37·3	13·7	16·2	22·2	3·6
" "	CRF[6]	—	38·8	—	15·1	—	4·2
" "	CCB[7]	—	47·6	—	13·1	—	4·6
	CNB[8]	43·6 (unfractionated)				18·8	
Rees' carrageenan	R-λ[9]	—	41·1	—	23·2	—	1·5
Gigartina stallata	GS[10]	30·1	28·3	15·0	13·6	22·6	15·9
G. radula	GR[11]	31·0	35·6	15·7	15·7	23·2	9·6
G. pistillata	GP[12]	35·0	43·6	10·5	20·6	12·8	4·2
Eucheuma spinosum	ES[13]	37·7		7·2		19·0	
Polyides rotundus	PR[14]	36·3		5·1		2·3	
Degraded							
(*E. spinosum*)	C16[15]	36·1		0·3		21·0	
C. crispus	CY-λ-D1[16]	30·5		6·4			
" "	"-D2	29·7		3·0			
" "	"-D3	33·5		1·2			
" "	"-D4	38·2		3·8			
" "	"-D5	39·2		2·3			
" "	"-D6	37·3		1·35			
" "	"-D7	38·2		1·04			
" "	"-D8	37·2		0·66			
" "	"-D9	39·2		0·45			
" "	"-D10	39·2		2·80			
" "	"-D11	39·4		1·72			
G. pistillata	GP-λ-D2	37·7		2·6			
" "	"-D3	42·5		2·23			
" "	"-D4	40·6		1·58			
" "	"-D5	40·9		1·04			
" "	"-D6	40·9		0·72			
" "	"-D7	40·1		0·65			
E. spinosum	ED3	37·2		1·37			
" "	" 4	35·8		0·94			
" "	" 5	37·5		0·68			
" "	" 6	37·7		0·54			

Inherent viscosity (logarithmic viscosity number), $\eta_{inh} = c^{-1}\ln(\eta_{soln}/\eta_{solv})$dl/g where c = g solute in 100 ml solution, was measured at 25° in an Ostwald viscometer (M2 BSU/M) using 0·1M sodium chloride as solvent; for undegraded carrageenans, c = 0·02; for degraded carrageenans, c = 0·2.

Source: [1]Mud Island, Nova Scotia; [2]Sebasco Estates, Maine, U.S.A.; [3]Co. Claire, Eire; [4]Northumberland Str., Nova Scotia; [5]Yarmouth, Nova Scotia; [6]Roscoff, Finistere, France; [7]Casco Bay, Maine, U.S.A.; [8]North Brittany, France; [9]Dr. D. A. Rees, University of Edinburgh (Rees, 1963); [10]Millport, Scotland; [11]South Africa; [12]Povoa de Varzim, Portugal; [13]S. E. Asia; [14]Moosehead, Nova Scotia; [15]Anderson (1961); [16]A. D. Little Research Institute (all this series + GP and ED series).

TABLE 2. ANTIPEPTIC ACTIVITY OF UNDERGRADED AND DEGRADED CARRAGEENANS

Carageenan	Method A				Method B			
	carrageenan concentration (%)				carrageenan concentration (%)			
	0·5		2		0·2		0·3	
	κ	λ	κ	λ	κ	λ	κ	λ
Undegraded								
CMI	10	33	40	62	10	23	17	29
CSE	26	37	32	57	28	12	27	38
CBC	26	67	69	80	13	52	40	100
CNS	16	62	76	79	20	81	100	100
CY	23	74	36	79	27	57	14	100
CRF	—	—	—			36	—	100
CCB	—	53	—	72	—	17	—	84
CNB	56		81		12		94	
R-λ	—	67	—	70	—	100	—	100
GS	20	29	69	61	7	8	100	100
GR	17	25	68	69	6	24	34	94
GP	39	78	28	86	18	100	57	100
ES	35		71		21		90	
PR	60		62		23		37	
Degraded								
C16	41				11[1]*			
CY-λ-D1					27			
»-D2					25	[2]		
»-D3					55			
»-D4					53			
»-D5	39				7			
»-D6	28				4			
»-D7	46				9			
»-D8	39				7	[3]		
»-D9	41				8			
»-D10	37				14			
»-D11	43				8			
GP-λ-D2	51				12[2]			
»-D3	43				15			
»-D4	45				14			
»-D5	46				11	[3]		
»-D6	49				13			
»-D7	45				8			
ED3	34				21			
» 4	32				21	[4]		
» 5	40				15			
» 6	36				13			

All values are the mean of 2 results. Most undegraded carrageenans gave 100% inhibition at 0·5% in method B.
Concentrations are explained in the text.
* Method of degradation.
[1] Dilute HCl; [2] periodate; [3] hypochlorite; [4] H^+ ion-exchange).

material. Degradation was carried out (Black & others, 1965) with the retention of sulphate as a principal objective, because it is generally believed that the basis of the activity of these substances lies principally in the sulphate content. Also, a degraded κ-carrageenan from *E. spinosum* which combines low viscosity and high sulphate content has been shown to have promising therapeutic effect (Bonfils & Lambling, 1960; Demole, 1963; Evans, Nowell & Thomas, 1965). Degradation with sulphate retention also permits the effect of variation of molecular weight with constant polyanionic properties to be observed. The results are in Tables 1 and 2.

UNDEGRADED CARRAGEENANS

The λ-carrageenans (with the exception of λ-carrageenan from *Gigartina stellata*, GS-λ) have higher sulphate and lower 3,6-anhydrogalactose

content than the corresponding κ-carrageenans, whilst the logarithmic viscosity number, η_{inh}, is, with the exception of carrageenan from *G. pistillata*, broadly similar for corresponding κ-and λ-components (Table 1). *Eucheuma*, a naturally occurring κ-like carrageenan, and *Polyides* carrageenan, which differs in structural details from the κ- and λ-carrageenans from other species of seaweed (Black & others, 1965) and therefore appears unique, have relatively low viscosities.

Analysis of activities. The antipeptic activities of the pairs of κ- and λ-carrageenans from eight samples of seaweed (5 C. *crispus*, 3 *Gigartina* species) were analysed separately. The 64 pairs of results were considered as a doubly replicated $2^3 \times 8$ factorial experiment, yielding Table 3.

TABLE 3. ANALYSIS OF VARIANCE FOR THE ANTIPEPTIC ACTIVITY OF THE κ- AND λ-CARRAGEENANS FROM FIVE CHONDRUS AND THREE GIGARTINA SEAWEEDS

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F-ratio
M	42·8	1	42·8	
C	29,585·3	1	29,585·3	
F	24,255·0	1	24,255·0	302·7
S	21,539·0	7	3,077·0	
MC	780·1	1	780·1	
MF	420·51	1	420·51	5·25
MS	4,106·7	7	586·7	
CF	21·13	1	21·13	0·26
CS	12,294·2	7	1,756·3	
FS	11,083·0	7	1,583·3	
MCF	442·5	1	442·5	
MCS	3,095·9	7	442·3	
MFS	2,183·0	7	311·9	
CFS	4,128·4	7	589·8	
MCFS	2,906·5	7	415·2	
Total = treatments	(116,884)	(63)		
Error	5,128	64	80·125	
Total	122,012	127		

M corresponds to the method factor (2 levels); C to the concentration factor (2 levels); F to the fraction factor (2 levels); S to the seaweed factor (8 levels).
0·1% point for F (1,64) $\doteq$ 11·94 and for F (7,64) $\doteq$ 7·07; 1% point for F (1,64) $\doteq$ 7·07 and for F (7,64) $\doteq$ 2·94; 5% point for F (1,64) $\doteq$ 4·00 and for F (7,64) $\doteq$ 2·17.

Study of this Table and the percentage points of the F-distribution with the appropriate degrees of freedom shows that of the 15 treatment combinations, all but two, namely the effect of the method used and the interaction of concentration with fraction (i.e. κ or λ), will have significantly large F-ratio values. This may be attributed to the relatively small error sum of squares obtained by measuring the variation of the results within each of the 64 cells. Of these 64 cells, 18 contained an equal pair of results and this would reduce the error sum of squares considerably. Although there is no difference between the results of the two methods, it has to be remembered that different concentrations of carrageenan have been used in the two methods. The conclusions drawn are: that despite the size of the error sum of squares there is a highly significant difference in the effects of the two fractions, κ and λ; that there is no interaction between the concentration and the fraction used, i.e. the effect of the different fractions is the same regardless of concentration; and finally, that the interaction of method used and fraction studied is slightly significant. Since the antipeptic activity of undegraded carrageenan is probably

mediated by substrate occlusion (as for degraded carrageenan), this could indicate differences in structure of the complexes formed between the κ- and λ-carrageenans and the proteins used in the methods.

Again, considering only the eight pairs of undegraded carrageenans, a multiple regression analysis was performed by computer, treating 3,6-anhydrogalactose (*g*), sulphate (*s*) and viscosity (*v*) as independent variables, and the logarithm of antipeptic activity (*y*) as dependent variable (both methods, 3 concentrations) for both κ- and λ-carrageenans. The antipeptic activities of the higher concentration in method B were omitted from the calculation because the proportionately large number of duplicate activities of 100 would tend to blunt the perception of the analysis. The regressions of *y* on *s*, *y* on *s* and *v*, *y* on *s* and *g* and *y* on *g*, s and v were studied separately for both κ- and λ-carrageenans.

Sulphate content. The regressions of antipeptic activity on sulphate were very highly significant (level of significance lower than 0·1 of 1%): differences in sulphate content determined differences in antipeptic activity amongst both κ- and λ-carrageenans in both methods at all concentrations studied, high sulphate content being associated with high activity. Differences in viscosity made no significant contribution to this conclusion.

3,6-*Anhydrogalactose content.* Higher 3,6-anhydrogalactose content, on the other hand, had significant association with lower effect (after due allowance for the effect of sulphate) only in the λ-carrageenans in method B and at the higher concentration in method A. With κ-carrageenans, where 3,6-anhydrogalactose content is higher than in the more active λ-carrageenan (*t* test showed significance at 0·1 of 1%), differences in 3,6-anhydrogalactose content did not contribute to differences in activity independently of sulphate.

Further interpretations of differences in activity require more knowledge of carrageenan structure and the nature of the interaction with substrate protein on which inhibition depends.

Molecular size. An apparent absence of effect of molecular size (as indicated by viscosity) is almost certainly due to the similarity which exists in this series of undegraded carrageenans.

Unfractionated extract. The effect of the κ-component in an unfractionated extract cannot yet be discerned, but the antipeptic activity of CNB carrageenan (Table 1), for example, does not match the high sulphate content. That this might be due solely to the presence of κ- carrageenan in the unfractionated extract tends to be discounted by the antipeptic activity of CRF-λ and CCB-λ where high sulphate content, especially in CCB-λ, is not associated with the highest antipeptic activity. There would therefore be unexplained exceptions to a "sulphate rule." It is not likely that incomplete fractionation of the κ- and λ-components could account for the exceptions which probably have their origins in other structural differences. κ- and λ-Carrageenans [and the "third component" (Rees, 1966)], are stages in a biosynthetic process and cannot, therefore, under conditions of growth and harvest, be expected to be at all times identical.

Antipeptic activity. The effect on antipeptic activity of the method used to determine it is seen in the individual cases of R-λ and GP-λ (Table 2). The high activity at lower concentration in method B for these carrageenans was not reproduced in method A, suggesting that these carrageenans may have some particular affinity for the enzyme or substrate in method B. With these carrageenans, and also with CY-λ and CNS-λ to some extent, it was observed that when they were added to gastric juice, insoluble globule-like structures were formed and it was usually when this occurred that extinction of peptic activity occurred. 100% antipeptic activity was not observed in the absence of this occurrence. Differences were also observed in the size of the floccules formed in the reaction between the carrageenans and the substrate, large floccules tending to produce greater inhibition than small ones. Attention has been drawn to a similar occurrence in the reaction between fibrinogen and carrageenans (Anderson & Duncan, 1965). Molecular size probably influences antipeptic activity by determining such differences in the nature of the reaction with substrate protein. R-λ and GP-λ, together with CNS-λ and CY-λ, have the highest viscosities of the series (Table 1). This suggests that there may be an optimum association of sulphate content and molecular properties for activity.

The antipeptic activity of *Polyides* carrageenan with its unique structure (Black & others, 1965) shows that activity is not monopolized by the κ-λ carrageenans. The activity of *Eucheuma* carrageenan, which occurs naturally without a λ-component, indicates that, with adequate sulphate the κ- configuration and a high 3,6-anhydrogalactose content do not preclude marked antipeptic activity.

DEGRADED CARRAGEENANS

Undegraded carrageenans generally dissolve slowly and to a limited extent in water to give solutions of very high viscosity. This raises practical problems in the study of the actions and uses of carrageenans. The anti-ulcer action of carrageenan follows inhibition of peptic activity (Anderson, 1961), mucosal coating (Anderson & Watt, 1959) and anti-secretory activity (Anderson, Marcus & Watt, 1962), and for these actions rapid dissolution after administration is desirable. Reduction of molecular weight with retention of activity would therefore be desirable to increase solubility. Of the λ-carrageenans, GP-λ and CY-λ were chosen, on the grounds of activity and availability, for degradative study in which sulphate content was left intact as far as possible. *Eucheuma* carrageenan was also studied because of its relatively high sulphate content and naturally low viscosity (Table 1), and also because of its susceptibility to degradation under mild acid conditions (British Patent, 840,623) explained by Black & others (1965) as being possible on account of the presence of the acid-labile 3,6-anhydrogalactose in the molecule.

Results for η_{inh} (Table 1) show that much degradation could be effected in the series without serious loss of sulphate. However, with the exception of CY-λ-D3 and CY-λ-D4 (and to some extent CY-λ-D1 and CY-λ-D2) antipeptic activity was reduced as a result of degradation,

especially when measured by method B; it was also reduced, but to a lesser extent, in method A. Whether the retention of activity by CY-λ-D3 and -D4 is due to periodate degradation as opposed to hypochlorite degradation used for the others, is not known, but the antipeptic activity of GP-λ-D2, also periodate degraded, was about half that of CY-λ -D3 or -D4. CY-λ-D3 and -D4 have retained more sulphate than CY-λ-D1 or -D2. However, if this were the only factor CY-λ-D5 to -D9 might have been expected to be more active.

Degraded carrageenans were also examined at the higher concentrations in both methods, but the activities were either similar to, or less than, those at the lower concentrations. It is suspected that, at the higher concentrations, the degraded carrageenans may be forming trichloroacetic acid-soluble complexes with the substrate protein, with consequent interference with the estimation of the split products of the proteolysis. These results have therefore been omitted.

Otherwise adequate sulphate content turns out to be insufficient for high activity when certain λ-carrageenans are degraded by hypochlorite, and differences shown by the two methods of assessing activity are rather marked, suggesting that structures other than the sulphate are concerned in interaction with the different proteins in the two methods.

The analysis of the antipeptic activities of the undegraded carrageenans showed that such differences as existed in viscosity did not contribute to activity differences. However the reduction in viscosity effected by degradation, in the presence of substantially unaltered sulphate, suggests that marked changes in molecular size do affect antipeptic activity. The evidence therefore suggests that antipeptic activity is a function of the whole molecule, sulphate content and molecular size being principal features.

In use it may well be advantageous to employ degraded carrageenan the lower viscosity of which enables greater dosage to be used and faster dissolution to be achieved with more rapid distribution over the mucosa

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