Effect of Sulfate Content of Several Anionic Polymers on *in Vitro* Activity of Pepsin

By L. J. RAVIN, J. G. BALDINUS, and M. L. MAZUR

Carrageenan was degraded with acid to give materials with sulfate contents varying from 5–30%. Antipeptic activities of these materials correlated closely with the sulfate content. Other sulfated polymers such as polyvinyl sulfonate, sulfated rice starch, amylose sulfate, heparin, etc., gave a similar correlation. All these polymers precipitated protein in the antipeptic test. Compounds such as sodium lauryl sulfate and *m*-xylene sulfonate possessed significant antipeptic activity but did not precipitate protein, and no correlation between sulfate content and antipeptic activity was found. The precipitation of protein by carrageenan depends upon the pH, becoming negligible at a pH above 4.5.

THAT SULFATED polysaccharides possess antienzyme activity has been established by a number of workers. Levey and Sheinfeld (1) found that the proteolytic action of pepsin on casein is inhibited by chondroitin sulfate, heparin, and sodium polyanhydromannuronic acid sulfate. Fellig and Wiley (2) demonstrated that heparin and various synthetic anionic polymers are inhibitors of pancreatic ribonuclease. More recently, Anderson and Watt (3, 4) and Houck, Bhayana, and Lee (5) reported that the incidence of ulcers in stress rats and in cortisone- and histamine-treated dogs and rats can be reduced by the oral administration of carrageenan, a sulfated polymer. Anderson (6) states that the degree of peptic inhibition parallels the sulfate content of the polymer. This paper deals with the effect of the sulfate content of several synthetic and naturally occurring anionic polymers on the *in vitro* activity of pepsin with emphasis placed on carrageenan and its degraded forms.

Carrageenan is a heterogenous, water-soluble polysaccharide obtained from marine algae such as *Chondrus crispus*. The two main components are kappa- and lambda-carrageenan, which can be separated by selective precipitation with potassium ions. These components differ in molecular size, viscosity, optical rotation, sugar units, and sulfate content (7–16).

Carrageenan is sensitive to acid. The glycosidic bonds undergo hydrolysis under relatively mild conditions, whereas the sulfate groups require more drastic conditions for their removal. This principle has been utilized by Evans Medical Limited to make C-16, a form of carrageenan with lower viscosity than the starting material yet with essentially the same sulfate content (17). Because of its low viscosity, C-16 is easier to administer orally (6). In the present study C-16 has been subjected to further acid hydrolysis; we will refer to the resulting materials with varying sulfate content as degraded carrageenan. The remaining sulfated polymers used in this study were obtained through commercial sources or through the generosity of several individuals. Amylose sulfate was prepared by the method of Roubal, Placer, and Vokac (18).

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EXPERIMENTAL

Materials.—C-16, carrageenan, Evans Medical Ltd., Speke, Liverpool 24, England; lambda-carrageenan; kappa-carrageenan; Sea Kem, type 8, Marine Colloids, Inc., Rockland, Me.; pectin N.F.; sulfated rice starch; polyvinyl sulfonate; amylose sulfate; heparin sodium, 100 units/mg., Nutritional Biochemicals Corp., Cleveland, Ohio; sulfated polyvinyl alcohol, Chemicals Procurement Co., New York 36, N. Y.; polyvinyl alcohol; sodium alginate; carboxymethylcellulose; sodium lauryl sulfate, U.S.P.; sodium heptadecyl sulfate; sodium *m*-xylene sulfonate; sodium 1,2-ethylene disulfonate dihydrate; alginic acid sulfate, Naurylande SARL, Venetta, France; pepsin, 1–10,000, Nutritional Biochemicals Corp., Cleveland, Ohio; hemoglobin, lyophilized, salt free, Mann Research Laboratories, New York, N. Y.; albumen (bovine) fraction V, Nutritional Biochemicals Corp., Cleveland, Ohio; blood plasma, normal, human, irradiated, dried, Hyland Laboratories, Los Angeles, Calif.

Degradation of Carrageenan.—C-16 (100 Gm.) was suspended in a 10% (v/v) solution of concentrated hydrochloric acid in acetone. The suspension was shaken periodically to facilitate hydrolysis. The resulting material was collected on filter paper, washed with anhydrous acetone, dissolved in a minimum quantity of water, and the solution adjusted to pH 7.0 with 40% (w/v) sodium hydroxide. After treatment with charcoal, the solution was filtered and the clear filtrate was added to anhydrous acetone, with constant stirring, until precipitation was complete. The precipitate was washed with anhydrous acetone, dried overnight at 50° and analyzed for antipeptic activity and sulfate content. By varying the time of hydrolysis, carrageenans with different sulfate contents were obtained.

Determination of Antipeptic Activity.—Hunt's (19) method was modified as follows: each run required a control, a carrageenan standard arbitrarily designated as having a 100% antipeptic activity, the unknown, and the appropriate blanks. The control consisted of 1 ml. pepsin, 1 ml. human plasma, and 1 ml. acidified water; the standard had 1 ml. pepsin, 1 ml. plasma, and 1 ml. carrageenan standard; the unknown, 1 ml. pepsin, 1 ml. plasma, and 1 ml. test substance. Concentrations were: pepsin, 0.25 mg./ml.; human plasma, 5%; test substance and standard, 0.5%; pH of these solutions was 1.6 adjusted with hydrochloric acid only.

The mixtures were incubated at 37°; after exactly 1 hour, 10 ml. trichloracetic acid (10% aq.) was added; 15 minutes later, the mixtures were filtered. To 1 ml. filtrate were added: 10 ml. aq. sodium hydroxide (0.4 *N*) and 3 ml. dilute (1 part reagent and 2 parts water) Folin-Ciocalteu reagent. After 15–20 minutes, the absorbances (*A*) were read on a Klett-Summerson colorimeter (No. 66 filter). Results were calculated using the following equation

$$\frac{A \text{ control} - A \text{ test substance}}{A \text{ control} - A \text{ standard carrageenan}} \times 100 = \% \text{ antipeptic activity}$$

When hemoglobin was used to check the method, the antipeptic values were similar to those obtained with human plasma.

Determination of Interaction with Protein.—One-half per cent solutions of the sulfated polymers were adjusted to pH 1.6, 3.0, 4.0, 4.5, and 5.0, respectively, with hydrochloric acid. Then, 4 ml. of each solution was mixed with 1 ml. of 5% solutions of blood plasma; albumen, bovine, fraction V; and hemoglobin; similarly adjusted to the same pH values with hydrochloric acid. The resulting mixtures were kept at 37° for 1 hour, then they were filtered and the tyrosine in the filtrate was measured colorimetrically, as described above. This method does not differentiate between unreacted protein and a soluble protein-sulfated polymer complex.

Determination of Sulfate.—Samples were burned in a Schöniger flask which contained 0.01*N* sodium hydroxide as an absorbent solution. After combustion was complete, the flask contents were shaken and allowed to stand for 1 hour to insure complete absorption of the oxides of sulfur. After treatment with fuming nitric acid, the contents were evaporated to dryness and the residue dissolved in water and neutralized to a phenolphthalein end point. The resulting sulfate was titrated with barium chloride solution (0.02 *N*) using tetrahydroxyquinone as indicator (20–22).

RESULTS AND DISCUSSION

***Interaction between Protein and Carrageenan* (C-16) at Various pH's.**—It is thought that carrageenan exerts its antipeptic activity *in vitro* by precipitating the protein, and *in vivo* by forming a protective coating on the stomach walls (6). Carrageenan does not precipitate pepsin, and recently Piper and Fenton (23), on the basis of paper electrophoresis studies, concluded that there is no interaction at all between pepsin and carrageenan. Carrageenan precipitates protein by virtue of its many negative charges which can interact with positively charged proteins to form insoluble complex salts. The ability of carrageenan to precipitate several proteins has been determined at various pH's; these data are summarized in Table I. It is apparent that protein precipitation depends upon the pH and that the amount of precipitation decreases as the pH rises. At pH 4.5 and higher, little or no protein is precipitated. The reaction between carrageenan and protein is completely reversible; a protein precipitated with carrageenan can be brought back into solution simply by raising the pH. These facts suggest that antacids may be contraindicated when carrageenan is administered.

TABLE I.—EFFECT OF PH ON THE INTERACTION BETWEEN C-16 AND SEVERAL PROTEIN SUBSTRATES

	Interaction, %		
pH	Blood Plasma	Albumen (Bovine) Fraction V	Hemoglobin
1.6	100	100	100
3.0	99	83	79
3.5	...	75	82
4.0	88	8	39
4.5	47	...	...
5.0	0	0	0

Sulfate Content *vs.* Antipeptic Activity.—C-16 was hydrolyzed with acid to decrease the sulfate content and was then assayed for antipeptic activity. Table II lists the sulfate content and antipeptic activity of several degraded samples. These data are also plotted in Fig. 1. It is clear that the antipeptic activity correlates closely with sulfate content. At the lower sulfate contents, however, the antipeptic activity tends to level off.

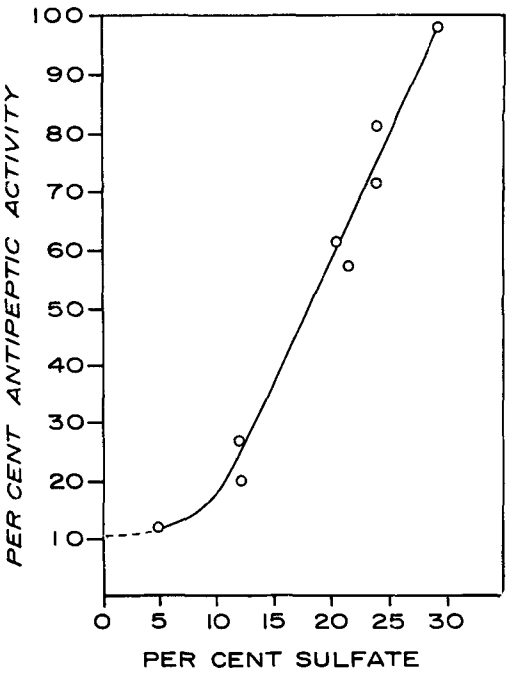

Fig. 1.—Relationship between *in vitro* antipeptic activity and sulfate content for degraded carrageenan.

TABLE II.—RELATIONSHIP BETWEEN SULFATE CONCENTRATION AND ANTIPEPTIC ACTIVITY FOR DEGRADED CARRAGEENAN

Hydrolysis Time, hr.	Sulfate, %	Antipeptic Activity, %
0.0	29.0	98.5
0.5	23.7	81.0
1.0	23.7	71.0
3.0	21.4	57.0
6.0	20.3	61.0
24.0	12.0	27.0
48.0	12.2	20.0
96.0	4.9	12.0

Sulfate Content of Various Natural and Synthetic Polymers *vs.* Antipeptic Activity.—The fact that carrageenan's antipeptic activity parallels its sulfate content prompted us to test other sulfate and sulfonated polymers with varying degrees of sulfation. Table III lists the polymers tested, their antipeptic activity, and their sulfate content. In all cases, the antipeptic activity is that compared with a standard sample of C-16. Figure 2 is a plot of these data. Again, the antipeptic activity correlates with the sulfate content; *i.e.*, the greater the sulfate content, the greater the antipeptic activity.

Since all the polymers precipitated protein in the antipeptic test we can assume that the mechanism of pepsin inhibition is similar to that of carrageenan. In summary, then, for those polymers that precipitate protein, the antipeptic activity is completely dependent upon the sulfate content. Other chemical or physical properties of these polymers may come into play, but these effects are not apparent by the methods used in this study.

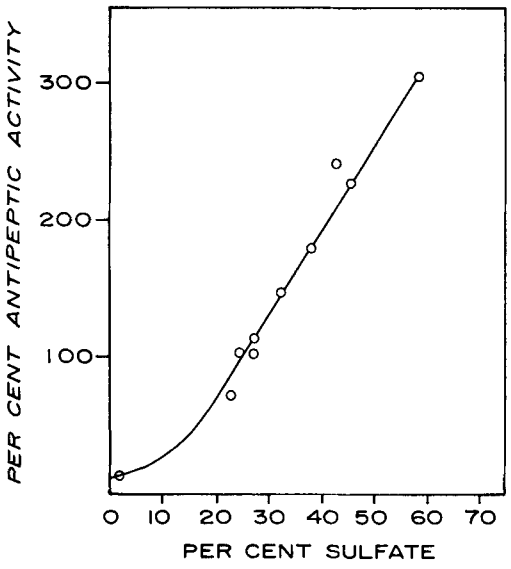

Fig. 2.—Relationship between *in vitro* antipeptic activity and sulfate content for several anionic polymers.

Comparison of Antipeptic Activity of Lambda- and Kappa-Carrageenan Mixtures.—The effect of varying ratios of lambda- to kappa-carrageenan on the *in vitro* activity of pepsin is shown in Fig. 3 which shows that the higher the concentration of lambda fraction the greater the antipeptic activity. Kappa- and lambda-carrageenan differ greatly in viscosity, yet their antipeptic activity correlates with their sulfate content (Table III). *In vivo*, it is possible that the viscosity of carrageenan may have a bearing on its antipeptic activity; *in vitro*, however, it is not the viscosity but the sulfate content that determines its antipeptic effectiveness.

TABLE III.—RELATIONSHIP BETWEEN SULFATE CONTENT AND ANTIPEPTIC ACTIVITY FOR SEVERAL NATURALLY OCCURRING AND SYNTHETIC POLYMERS

Polymer	Antipeptic activity, %	Sulfate, %
Polyvinyl sulfonate	310	59.0
Sulfated polyvinyl alcohol	247	42.5
Sulfated rice starch	230	45.3
Amylose sulfate	182	38.0
Lambda-carrageenan	150	32.2
Kappa-carrageenan	110	24.0
Heparin	103	27.0
Carrageenan, C-16	114	27.1
Sodium alginate	13	0
Carboxymethylcellulose	0	0
Polyvinyl alcohol	15	0
Pectin N.F.	12.5	2.07
Sea Kem, type 8	73	23.0
Alginic acid sulfate	40	15.8
Carrageenan, standard	100	29.0

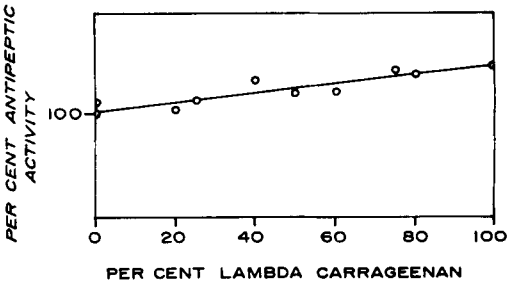

Fig. 3.—Effect of various ratios of lambda- to kappa-carrageenan on the *in vitro* activity of pepsin. Concentration is expressed as per cent lambda-carrageenan.

Compounds That Did Not Precipitate Protein.—During our screening program we found several nonpolymeric compounds with very high antipeptic activities that did not correlate with the sulfate content (Table IV). Since these compounds did not precipitate protein in the antipeptic test it can be assumed that they interact with protein or pepsin to form soluble complexes. In these interactions, factors such as the conformation of the compounds, the size of the micelles in solution, etc., probably predominate so that the role played by the sulfate content becomes insignificant.

TABLE IV.—ANTIPEPTIC ACTIVITY AND SULFATE CONTENT OF COMPOUNDS THAT DID NOT PRECIPITATE PROTEIN

	Sulfate, %	Antipeptic Activity, %
Sodium lauryl sulfate	31.0	408
Sodium heptadecyl sulfate	41.36	240
Sodium *m*-xylene sulfonate	12.84	93
Sodium 1,2-ethylene disulfonate dihydrate	71.50	29

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Synthesis of Some New Derivatives of N^4-*p*-Cyanobenzoyl Sulfanilamide

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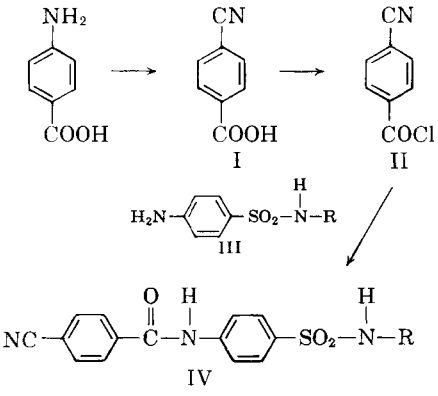
Six new derivatives of N^4-*p*-cyanobenzoyl sulfanilamide were prepared: N^4-*p*-cyanobenzoyl sulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyrimidinylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-(4-methyl-2-pyrimidinyl)sulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-thiazolylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyridylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyrazylsulfanilamide. Infrared spectra of the new compounds were determined. Common absorption bands of the compounds occurred at 2240–2210 cm.$^{-1}$, 1160–1130 cm.$^{-1}$, 1325–1310 cm.$^{-1}$, and 1580–1560 cm.$^{-1}$.

N^4-SUBSTITUTED sulfanilamides have shown important pharmacological actions. N-Sulfanilylbenzamide, which is similar to the derivatives prepared here, has been shown to be effective against the bacillary dysentery organism (1) and against pneumococcus in mice (2).

Some N - alkyl - *p* - cyanobenzamides have been prepared and shown to possess local anesthetic and antimicrobial properties (3). The amidines and imidazolines prepared from these compounds have also shown significant activity (4).

The synthesis of some new sulfonamides containing the *p*-cyanobenzoyl and sulfanilyl moieties was successfully completed.

The synthesis of the N^4-*p*-cyanobenzoyl sulfanilamide derivatives was initiated with the preparation of *p*-cyanobenzoic acid I, which was prepared according to the procedure of Miller and Gisvold (5). *p*-Cyanobenzoyl chloride II was prepared by refluxing the acid with thionyl chloride and recrystallizing from petroleum ether.



Equimolar quantities of *p*-cyanobenzoyl chloride and the finely powdered sulfanilamide derivative III were refluxed in *p*-dioxane for 2 hours. Mechanical stirring was required if the reactants were not completely soluble in dioxane. The solvent was removed and the N^4-*p*-cyanobenzoyl sulfanilamide derivative IV was treated with dilute-hydrochloric acid to remove any unreacted material. Recrystallization was from a mixture of solvents.

The solubility characteristics of the new derivatives (see Table I) were generally predictable.

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