THE ANTIPEPTIC ACTIVITY OF SULPHATED POLYSACCHARIDES

By W. Anderson

From the Evans Medical Research Laboratories, Liverpool 24

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Sulphated polysaccharides reduce the proteolytic activity of pepsin principally by reacting with the substrate rather than with the enzyme; and the complex so formed is protected by the polysaccharide from digestion.

The claim by Babkin and Komarov (1932) that the "mucoitin- and chondroitin-sulphuric acid" components of gastric mucus are capable of diminishing peptic activity without altering the level of free acidity, and the subsequent interest in pepsin as a possible ulcerogenic factor received support from results (Matzner and Windwer, 1937; Schiffrin, 1940; Schiffrin and Warren, 1942) indicating that hydrochloric acid alone was not ulcerogenic but that pepsin, in the presence of hydrochloric acid at pH 1·1–1·5 (Schiffrin and Warren, 1942) was so. In the guinea-pig treated with large doses of histamine, Watt (1959) showed that the primary aetiologic factor in the initiation of the acute ulcerative process is the acid gastric juice. He claimed that the acidity was not as important as the excessive production and prolonged action of the acid gastric juice.

Antacid substances will inactivate pepsin by raising the pH and Shoch and Fogelson (1942) claimed that with the substances tested this was related to their influence on the acidity of the digest. Sodium lauryl sulphate, on the other hand, inhibited pepsin at high dilution without change in pH and increased the survival time of dogs given histamine.

The anionic properties of sodium lauryl sulphate are similar to those of sulphated polysaccharides and both will engage in salt formation with protein at or below the isoelectric point of the protein. Levey and Sheinfeld (1954) studied the inhibition of proteolytic action of pepsin by some sulphated polysaccharides, finding that *in vivo* and *in vitro* inhibition of peptic digestion occurred and they proceeded to show that oral chondroitin sulphate reduced the incidence of gastric ulcers in the Shay rat. These authors believed their results to indicate that the sulphated polysaccharide combined with the enzyme to form an inactive complex or one with reduced activity.

Tests by us with different sulphated polysaccharides did not support this interpretation and the antipeptic properties of some have been examined with the object of elucidating the way in which the peptic activity is reduced. The sulphated polysaccharide used in the present study has previously been reported to inhibit peptic activity and to prevent experimentally produced ulceration in the guinea-pig (Anderson and Watt, 1959a,b). Its antipeptic activity has been confirmed by Bonfils, Dubrasquet and Lambling (1959, 1960).

Materials and Methods

Degraded carrageenan was used as a model. Similar experiments have also been done with heparin, fucoidan, chondroitin sulphate, laminaran sulphate and dextran sulphate.

*Degraded carrageenan.** The physical properties of undegraded carrageenan (Stoloff, 1959) (carrageenan of commerce) were not ideal for this study for several reasons. Carrageenan was degraded by careful treatment with dilute hydrochloric acid and subsequent precipitation after neutralisation with sodium hydroxide (British Patent 840,623). The product was an off-white powder containing 28–30 per cent of unchanged bound sulphate. Typical data are: S, 9·4 per cent; Ca, 0·2 per cent; Na, 7 per cent; K, 1·3 per cent; $[\alpha]_D + 34°$. The specific viscosities at 25° (M3 U-tube viscometer) of a 0·05 per cent aqueous solution of the parent carrageenan and the degraded carrageenan are 1·52 and 0·06 respectively. Degraded carrageenan is therefore considerably less viscous than carrageenan, and is readily and completely soluble in water and acid.

Pepsin. Crystallised pepsin, Armour Laboratories Ltd. *Toluidine blue.* Michrome brand (Edward Gurr Ltd.). *Gastric mucin.* Armour Laboratories Ltd. *Artificial gastric juice.* Solution of pepsin in hydrochloric acid. *Human gastric juice.* Samples from any one peptic ulcer patient pooled and centrifuged. The clear supernatant was used.

Measurement of Peptic Activity

Method A. Modified Anson (1938) *method.* Two flasks each containing 1 ml. of pepsin solution at pH 1·6 and 1 ml. solution of hydrochloric acid also at pH 1·6 were incubated at 35° for 10 min. and 1 ml. haemoglobin (Armour, for proteolytic enzyme assay) solution at pH 1·6 and 35° was then added and incubated for 30 min. In a further two flasks treated similarly the 1 ml. of acid solution was replaced by 1 ml. of solution of the sulphated polysaccharide at pH 1·6. After incubation, 10 ml. of 10 per cent w/v trichloracetic acid was added, the flasks allowed to stand in the water bath for 15 min. and the contents then filtered (Whatman No. 1). Five ml. of filtrate was added to 10 ml. N NaOH followed by 3 ml of a 1 + 2 dilution with water of Folin and Ciocalteu's reagent. The optical density of the blue colour developing in 10 min. was measured in an EEL absorptiometer using 1 cm. cells and 607 filter. Blanks were also included to measure the contribution to the colour of the pepsin, haemoglobin, and pepsin + inhibitor. When human gastric juice was used in this method, 2 ml. of centrifuged supernatant of the juice was used to dissolve the degraded carrageenan and to this was added the 1 ml. of substrate solution.

Method B. (Hunt, 1948). When the peptic activity of artificial gastric juice was measured by this method there was no further dilution of the juice before incubation. This was to allow a greater range of activity especially in studies involving inhibition. When degraded carrageenan was added it was dissolved in the centrifuged juice.

* Ebimar (Evans Medical Ltd.).

Uniform agitation is required throughout the digestion period to effect and maintain dispersion of the coarse precipitate formed between inhibitor and substrate.

Antipeptic Activity

This term is calculated from $S-I/S \times 100$ where S is optical density obtained in the determination of peptic activity from the treated digest without inhibitor, corrected for blanks as necessary; I is the corrected optical density for the treated digest with inhibitor. Antipeptic activity is thus the amount of inhibition, per cent.

Estimation of Degraded Carrageenan using Toluidine Blue

Degraded carrageenan reacts with toluidine blue like heparin does and the method of MacIntosh (1941) for heparin proved suitable for its

TABLE I

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: HAEMOGLOBIN (Hb). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH ENZYME BEFORE ADDITION TO SUBSTRATE

Substrate (Hb) and inhibitor (DC) in reaction mixture			Antipeptic activity		
	Amount in mg./6 ml.		Pepsin added in amounts of		
Ratio	Hb	DC	50µg.	75 µg.	10 µg.
1	50	50	86	83	84
	10	10	34	20	7
5	50	10	39	40	37
10	50	5	21	23	21
	100	10	27	26	23
20	50	2·5	10	11	12
	200	10	20	22	21
30	50	1·7	5	8	9
	300	10	21	28	21

estimation. Optical densities were measured on a Hilger Spekker absorptiometer using filter OY2.

In the determination of degraded carrageenan remaining after reaction with protein, solutions in 0·2 per cent NaCl were made to contain 0·01 per cent w/v of degraded carrageenan and protein in amounts ranging from 0·005 per cent to 0·05 per cent w/v. The use of 0·2 per cent NaCl gave the same results as did 0·01 N HCl. The degraded carrageenan content was then estimated by calculating the toluidine blue removed from solution; this was assumed to be equivalent to the "free degraded carrageenan" which remained. The proteins studied were haemoglobin, human plasma protein, pepsin, and gastric mucoprotein.

RESULTS

Comparison of Sulphated Polysaccharides

The sulphated polysaccharides were estimated to have the following order of antipeptic activity, heparin, degraded carrageenan, fucoidan, chondroitin sulphate. Sodium sulphate and various non-sulphated polysaccharides (hyaluronic acid, agar, starch, tragacanth) were without

activity. The order of antipeptic activity found in the sulphated polysaccharides parallels the ester sulphate content of these substances.

Degraded Carageenan

Factors affecting the inhibition of pepsin. The duration of exposure and pH. Increasing the time of contact between pepsin and sulphated polysaccharide to 1 hr. did not modify the inhibition of peptic activity.

TABLE II

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: HAEMOGLOBIN (Hb). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH SUBSTRATE BEFORE ADDITION OF ENZYME

Substrate (Hb) and inhibitor (DC) in reaction action			Antipeptic activity		
Ratio	Amount in mg./6 ml.		Pepsin added in amounts of		
	Hb	DC	50 μg.	75 μg.	100 μg.
1	50	50	91	87	84
	10	10	39	45	26
5	50	10	41	44	46
10	50	5	26	25	25
	100	10	14	19	21
20	50	2·5	15	12	14
	200	10	0	2	1
30	50	1·7	9	8	11
	300	10	3	8	16

TABLE III

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: PLASMA PROTEIN (PP). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH ENZYME BEFORE ADDITION TO SUBSTRATE

Substrate (PP) and inhibitor (DC) in reaction mixture			Antipeptic activity		
Ratio	Amount in mg./6 ml.		Pepsin added in amounts of		
	PP	DC	100 μg.	200 μg.	300 μg.
1	50	50	82	85	86
	10	10	43	64	64
5	50	10	30	59	64
10	50	5	25	32	34
	100	10	26	29	29
20	50	2·5	15	21	19
	200	10	18	28	8
30	50	1·7	7	12	15
	300	10	9	14	11

Similarly, varying the pH of the medium between the limits pH 1·1-5·0 did not affect the inhibition.

The concentration of sulphated polysaccharide. Increasing the concentration of degraded carrageenan with either haemoglobin or plasma protein as substrate and constant enzyme and substrate levels caused an increasing inhibition of enzyme (Tables I, II, III and IV). The effect is independent of the enzyme concentrations employed; whether the inhibitor is mixed with substrate first or with enzyme first makes little or no difference. Peptic activity was measured using Method B.

The ratio of substrate (protein) to inhibitor (degraded carageenan). At different substrate concentrations similar variations in enzyme concentration did not alter the inhibition. Tables I to IV.

Experiments with gastric juice from ulcer patients. Similar experiments were made with human gastric juice, and the results are given in Tables V to VII.

Variation of the substrate (protein): inhibitor (degraded carageenan) ratio. Method A was used and it is seen in Table V that the antipeptic activity increases as the protein: degraded carrageenan ratio decreases.

Variation in enzyme concentration. Method A was used and Table VI shows that dilution of enzyme had little or no effect on antipeptic activity for a constant protein: degraded carrageenan ratio.

TABLE IV

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: PLASMA PROTEIN (PP). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH SUBSTRATE BEFORE ADDITION OF ENZYME

Substrate (PP) and inhibitor (DC) in reaction mixture			Antipeptic activity		
Ratio	Amount in mg./6 ml.		Pepsin added in amounts of		
	PP	DC	100 µg.	200 µg.	300 µg.
1	50	50	88	89	88
1	10	10	83	73	76
5	50	10	64	67	65
10	50	5	27	25	26
10	100	10	35	31	34
20	50	2·5	13	14	13
20	200	10	27	21	17
30	50	1·7	7	10	11
30	300	10	24	16	10

TABLE V

VARIATION IN ANTIPEPTIC ACTIVITY OF DEGRADED CARRAGEENAN IN HUMAN GASTRIC JUICE WITH VARYING SUBSTRATE (PLASMA PROTEIN, PP): INHIBITOR (DEGRADED CARRAGEENAN, DC) RATIOS

Reaction mixture	Antipeptic activity	Ratio substrate: inhibitor
2 ml. gastric juice + 15 mg. DC + 120 mg. PP ..	36	8
2 ml. gastric juice + 15 mg. DC + 50 mg. PP ..	70	3·3
2 ml. gastric juice + 30 mg. DC + 50 mg. PP ..	85	1·7
2 ml. gastric juice + 30 mg. DC + 20 mg. PP ..	84	0·7

Variation of the substrate concentration. Method B was used with a bulked sample of gastric juice from patients with duodenal ulcer. The results are given in Table VII.

Reaction of Degraded Carrageenan with Proteins

A reaction between degraded carrageenan and proteins can readily be demonstrated by mixing acid solutions of both in suitable proportions when a precipitate may form which contains both substances. When the conditions of concentration or solubility do not allow the formation of a precipitate a decrease in the concentration of the free degraded carrageenan in solution can usually be detected. To demonstrate the relative affinity of degraded carrageenan for substrates and pepsin, under conditions where enzymic digestion is not employed, mixture of degraded

carrageenan and each protein were prepared and the degraded carrageenan remaining in solution was estimated by the toluidine blue method. The relative affinities of a series of proteins for degraded carrageenan is shown in Fig. 1, from which it appears that the greater the protein: degraded carrageenan ratio the less the degraded carrageenan which is then available to react with the toluidine blue, giving a lower curve. When pepsin is the protein, a ratio of 5 causes the "removal" of less degraded carrageenan than when either haemoglobin or plasma protein

TABLE VI

ABSENCE OF EFFECT ON ANTIPEPTIC ACTIVITY, IN HUMAN GASTRIC JUICE, OF VARIATION IN ENZYME CONCENTRATION FOR CONSTANT VALUES OF SUBSTRATE (PLASMA PROTEIN, PP) AND INHIBITOR (DEGRADED CARRAGEENAN, DC)

Reaction mixture 3 ml.	Antipeptic activity	Ratio substrate: inhibition
2 ml. gastric juice + 20 mg. DC + 75 mg. PP ..	56	3·8
2 ml. gastric juice diluted 1 : 5 + 20 mg. DC + 75 mg. PP	62	3·8
2 ml. gastric juice diluted 1 : 10 + 20 mg. DC + 75 mg. PP	62	3·8

TABLE VII

VARIATION IN ANTIPEPTIC ACTIVITY IN HUMAN GASTRIC JUICE WITH VARIATION IN SUBSTRATE (PLASMA PROTEIN) CONCENTRATION; I.E., ALTERATION IN SUBSTRATE: INHIBITOR RATIO

Degraded carrageenan mg./ml. juice	Antipeptic activity mg. plasma protein substrate		
	140	280	420
35	93	75	84
30	89	69	70
25	84	69	63
20	77	57	57
10	51	43	18

is used in a ratio of 2. The reaction of degraded carrageenan with pepsin is small, less than with mucoprotein and much less than with the plasma protein or haemoglobin.

DISCUSSION

Undegraded carrageenan of commerce yields viscous solutions at low concentrations, and hence might not be expected to disperse readily or react rapidly in the stomach, in the contents, or on the mucosa. For this reason carrageenan was degraded to give a product having the original ester sulphate content. It was only slightly less active in antipeptic property than purified heparin and has the significant advantage of being without anticoagulant effect.

Levey and Sheinfeld (1954) consider that the antipeptic properties of sulphated polysaccharides are the result of the action of these substances upon the protein enzyme. But if this were correct, it might reasonably be expected that inhibition of peptic activity could be effected with a

suitable sulphated polysaccharide in much lower concentration than is actually required, and the addition of excess quantities of substrate should not lower the amount of inhibition. On the contrary, it is clear from Tables I to IV that increasing the concentration of the substrate diminishes the inhibition and decreasing the enzyme concentration does not affect the amount of inhibition, which might be expected to have been increased had the reaction mainly been inactivating salt formation

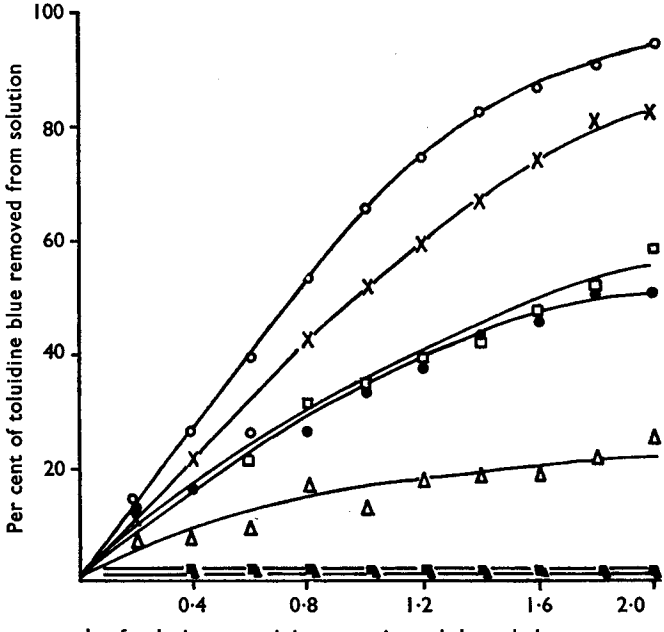

FIG. 1. The relative affinities of some proteins for degraded carrageenan.

		protein : degraded carrageenan
○——○	degraded carrageenan only	0
X——X	„ „ + pepsin	5
□——□	„ „ + plasma protein ..	2
●——●	„ „ + mucoprotein ..	5
△——△	„ „ + haemoglobin ..	2
■——■	„ „ + plasma protein ..	5
▲——▲	„ „ + haemoglobin ..	5

between enzyme and inhibitor. The ratio of protein substrate to sulphated polysaccharide appears to determine the inhibition. Furthermore, for similar ratios the total concentrations of each are shown to affect the inhibition, and this is probably so because of the nature of the complex salt formed. Important among these considerations would be its dissociation and solubility, as well as purely physical factors such as the ease or difficulty of mixing and diffusion of enzyme and soluble substrate in a medium in which floccules of varying size and amount are being formed.

These considerations are important because of the conditions frequently prevailing in the stomach which favour reaction of a sulphated polysaccharide with any protein present. Plasma protein and haemoglobin were thought to be realistic substrates because, apart from food protein, these two are likely to be encountered in the stomach or duodenum of the peptic ulcer patient. The reaction between sulphated polysaccharides involves salt formation (Jaques, 1943) and is instantaneous, and it might therefore be expected that upon presentation to an ulcerated surface, an immediate reaction would occur with deposition of the polysaccharide-protein complex and subsequent protection of the site from peptic erosion. On the basis of the results in Tables I to IV it is likely that the greater the dose of sulphated polysaccharide the greater the protection from peptic erosion, assuming the amount of protein remains constant. It therefore follows that if maximum deposition of the sulphated polysaccharide is to be obtained upon the sites to be protected the material should be administered at times when the food protein in the stomach is as low as possible.

From Fig. 1 it seems that the affinity for pepsin protein is lower than for plasma protein or haemoglobin or for gastric mucoprotein. This supports the conclusions of the digestion studies which indicate that the amount of pepsin present is not as important as the amount of substrate protein in determining the inhibition to be observed.

Increased pepsin concentrations occasionally gave slightly lower inhibition but this was never a consistent finding and the impression fitted with the results of the non-digestion study of the reaction between proteins and degraded carrageenan, which showed that some pepsin did react but that it was not a strong reaction. A precipitate of the pepsin-carrageenan complex was not obtained at the concentrations used in the digestion studies, although this in itself is no indication of absence of complex formation. The occasional lower inhibition at higher pepsin levels may reflect merely the greater chance of effective union of some enzyme with some substrate during the reaction between sulphated polysaccharide and substrate. Nevertheless, it appears that the inhibition of peptic activity is mediated by protection of the substrate, and it is probably desirable that this mode of action operates, because if pepsin is a causal factor in peptic ulcer the "substrate" becomes gastric or duodenal tissue. If now these tissue substrates are protected by an antipeptic substance complexed with mucoprotein the concentration of pepsin in the gastric juice and the loss of "free" sulphated polysaccharide due to gastric emptying become of small importance.

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