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Peptic inhibition by macroanions

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Heparin, dextran sulphate, dextran phosphate, chondroitin sulphate and degraded λ-carrageenan inhibit peptic activity by substrate occlusion or depletion. This holds for various substrates and enzyme preparations, but the amount of inhibition observed varies with method and inhibitor used. The most active inhibitors of the series are disulphated on at least alternate sugar residues and in addition to disulphation high molecular weight confers, in certain conditions, greatest activity. High and low molecular weight macroanions have different inhibition characteristics and it is concluded that activity may depend upon the structure of the substrate-inhibitor complexes formed. Inhibition is not observed when substrate-inhibitor interaction does not occur, as when pepsin or *N*-acetyl-L-phenylalanine-L-diiodotyrosine (APDT) are used as substrate.

INHIBITION of the proteolytic activity of acid gastric secretion by high and low molecular weight carrageenans is caused by occlusion or depletion of available substrate from the digestion system as a result of substrate-inhibitor interaction (Anderson, 1961; Anderson & Baillie, 1967). The effect of pH, order of addition of reagents, ratios and nature of interacting species, on the inhibition suggested that, to elucidate conditions existing *in vivo* with therapeutic or physiological sulphated polysaccharides, systems containing different substrates and macroanions should be investigated.

We used alternative substrates, various macroanions (heparin, dextran sulphate, dextran phosphate, chondroitin sulphate, degraded λ-carrageenan), and purified separated human gastric enzymes.

Experimental

MATERIALS AND METHODS

Heparin was highly purified sodium heparin (150·1 units/mg, batch H35460). *Chondroitin sulphate* was prepared from bovine trachea and contained S, 5·26; N, 3·6; sulphated ash, 24·0; and Na, 7·4% (batch 9). *Dextran sulphate* was PDS 242 11/256 (Glaxo). It contained S, 19·5%; specific rotation +90·7; average molecular weight was given as 6,000–8,000. *Dextran phosphate* was H 15 F1 (Glaxo). It contained P, 9·8; Na, 11%, 85% occurred as the monoester, average molecular weight was given as 9,000. *Carrageenans.* Undegraded λ-carrageenan (CY-λ) contained SO_3Na (37·3%), and was prepared from *Chondrus crispus*. Degraded carrageenans were: (a) degraded λ-carrageenan (GP-λ-D2), containing SO_3Na, 37·7% and prepared from the λ-carrageenan of *Gigartina pistillata* by hypochlorite degradation (Black, Blakemore & others, 1965); and (b) degraded *Eucheuma* carrageenan containing SO_3Na, 36·1%, and prepared from the κ-like carrageenan of *E. spinosum* by mild mineral acid degradation. Degraded λ-carrageenan and degraded *Eucheuma* carrageenan both have similar molecular weights (about 25,000, determined by light scattering). Undegraded and degraded λ-carrageenans and degraded *Eucheuma* carrageenans have been described

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by Black & others, 1965; Anderson & Harthill, 1967; Anderson & Baillie, 1967). *Pepsin.* Twice crystallized pig pepsin (Light-Koch) was used in the autodigestion experiments. *Haemoglobin* was haemoglobin substrate powder for proteolytic enzyme assay (Armour Laboratories Ltd.). *Synthetic pepsin substrate.* *N*-Acetyl-L-phenylalanine-L-diiodotyrosine (APDT) was obtained from Yeda Research and Development Co. Ltd., Rehovoth, Israel. *Plasma protein substrate* was dried human plasma protein. *Human gastric proteolytic enzymes.* The proteolytic activities of human gastric secretion having pH optima at pH 2·2 and 3·2 and referred to as human pepsin and gastricsin respectively, were separated from bulked gastric secretions obtained during augmented histamine tests according to Tang, Wolf & others (1959).

PROETOLYTIC ACTIVITY AND INHIBITION

The inhibition of peptic activity by heparin, chondroitin sulphate, dextran phosphate and sulphate and degraded λ-carrageenan was determined at pH 2·2 as described by Anderson & Baillie (1967) in which the macroanion is added to the enzyme before digestion (method *a*), or to the substrate before addition of the enzyme (method *b*).

Concentrations of plasma protein substrate (S) and inhibitor (I) in the 6 ml digest were varied as follows: with (I) constant at 10 mg, (S) ranged from 50–250 mg; with (S) constant at 250 mg, (I) ranged from 5–20 mg for all inhibitors except chondroitin sulphate, for which the range was 10–40 mg.

The effects of undegraded λ-carrageenan and degraded *Eucheuma* carrageenan (examples of high and low molecular weight macroanions respectively) on human pepsin and gastricsin were studied as follows. Proteolytic activity was determined in 0·1M citrate-HCl buffers at pH 2·2 and 3·2 at 37°. To 0·8 ml enzyme solution (1·5 mg pepsin/100 ml; 1 mg gastricsin/100 ml), 2·2 ml of the appropriate carrageenan solution was added. The reaction was started by the addition of 1 ml haemoglobin solution in buffer at appropriate pH and the mixture was incubated 50 min. Trichloroacetic acid (2 ml; 10% w/v) was then added, the extinction of the filtrate read at 280 mμ and converted to equivalent mg/ml tyrosine from a standard reference curve. Appropriate blanks were included.

Variations of (S) and (I) in the 4 ml digest were as follows: (a) with (I) constant at 4 mg, (S) ranged from 10–35 mg; (b) with (S) constant at 25 mg (I) ranged from 0–6 mg (undegraded carrageenan) and 0–10 mg (degraded carrageenan).

There was no substrate inhibition at the substrate concentrations used and the uninhibited reaction progressed linearly with time during the digestion period.

Inhibition, $i = 1 - V_i/V$, where V_i and V represent digestion with and without inhibitor respectively.

The effect of undegraded λ- and degraded *Eucheuma* carrageenans on the peptic hydrolysis of APDT was studied using the methods of Chiang,

Sanchez-Chiang & others (1966) except that 30% isopropanol was substituted for 50% isopropanol. The carrageenan was dissolved in the diluted gastric juice before admixture with substrate.

Carrageenan and autodigestion of pepsin. 100 mg pig pepsin was dissolved in 6 ml of HCl (pH 2·2) with or without carrageenan (up to 100 mg degraded *Eucheuma*; up to 24 mg undegraded λ-carrageenan). Aliquots of 0·5 ml were removed at intervals for 24 hr and mixed with 5 ml trichloroacetic acid (10% w/v) the mixture being left in the water bath for 15 min. Filtration (Whatman No. 1) was followed by measurement of the extinction of the filtrate at 280 mμ. Appropriate blanks were included and the adjusted increase in extinction was used as a measure of autodigestion.

Results

The shapes of the double reciprocal plots (Figs 1A, 1B), plots (Figs 2A, 2B) of $1/V_i$ against I (Dixon 1953) and plots (Figs 3A, 3B) of I against $i/(1 - i)$ (Reiner, 1959) indicate a substrate-depletion mechanism of inhibition in all cases. In the absence of inhibitor, double reciprocal plots (compare Figs 1A, 1B) were linear over the substrate range studied. Figs 4A, 4B reveal the quantitative differences in activity between the macroanions under the conditions of study.

Effect on APDT hydrolysis. Undegraded λ-carrageenan, at up to 3 mg/ml and degraded *Eucheuma* carrageenan at up to 20 mg/ml failed to inhibit the peptic digestion of APDT.

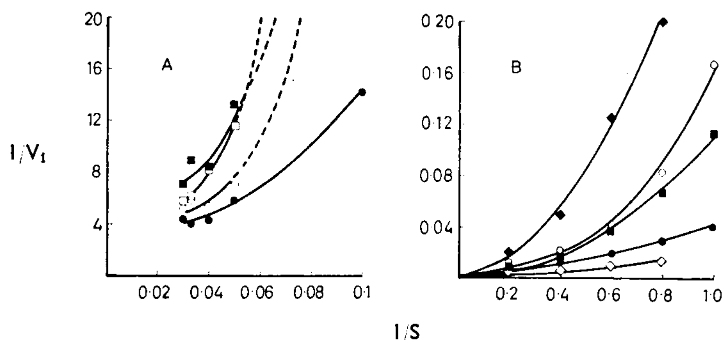

FIG. 1A. Plot of $1/V_i$ against 1/S. (I) constant at 4 mg/4 ml. Broken lines extend to $1/V_i$ values for $1/S = 0·1$ (undegraded carrageenan at pH 2·2, $1/V_i = 100$; degraded carrageenan at pH 2·2, $1/V_i = 125$; degraded carrageenan at pH 3·2, $1/V_i = 40$).

Key to FIGS 1A–4A (purified enzyme/haemoglobin system), ○ = undegraded carrageenan at pH 2·2 (pepsin); ● = undegraded carrageenan at pH 3·2 (gastricsin); □ = degraded carrageenan at pH 2·2 (pepsin); ■ = degraded carrageenan at pH 3·2 (gastricsin).

FIG. 1B. Plot of $1/V_i$ against 1/S. (I) constant at 10 mg/6 ml.

Key to FIGS 1B–4B (gastric juice/plasma protein system), ◆ = dextran sulphate; ○ = heparin; ■ = degraded λ-carrageenan; ◇ = dextran phosphate; ● = chondroitin sulphate.

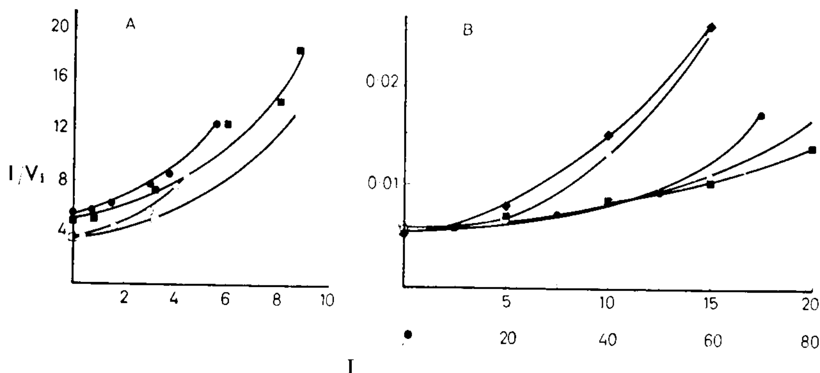

FIG. 2A. Plot of $1/V_i$ against I (mg/4 ml digest). (S) constant at 25 mg/4 ml.
FIG. 2B. Plot of $1/V_i$ against I (mg/6 ml digest). (S) constant at 250 mg/6 ml.

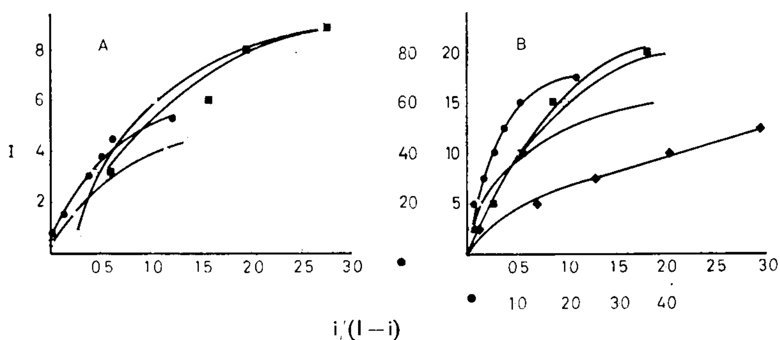

FIG. 3A. Plot of I (mg/4 ml digest) against $i/(1-i)$.
FIG. 3B. Plot of I (mg/6 ml digest) against $i/(1-i)$.

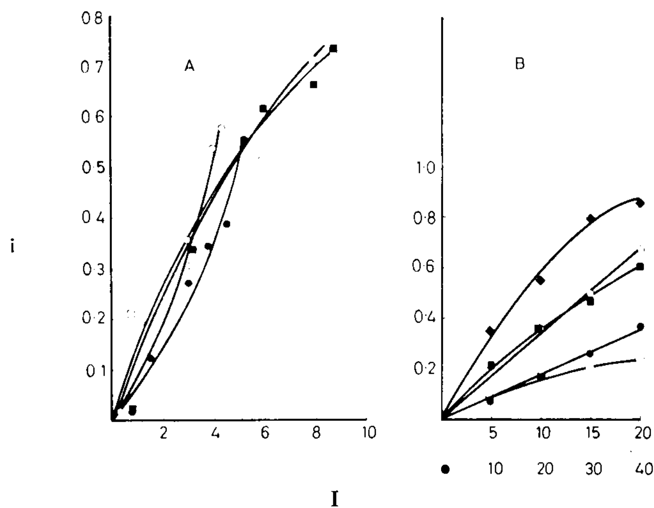

FIG. 4A. Plot of i against I (mg/4 ml digest).
FIG. 4B. Plot of i against I (mg/6 ml digest).

Autodigestion. Failure to inhibit peptic autodigestion was observed with both undegraded λ-carrageenan and degraded *Eucheuma* carrageenan.

Discussion

Anderson & Baillie (1967) showed the mechanism of inhibition of peptic activity by high and low molecular weight carrageenans to be depletion or protection of substrate, following interaction with inhibitor, when a plasma protein substrate is used. The present results (Figs 1–3 show that inhibition of haemoglobin proteolysis is also of this type and that other macroanions with different anionic groups and polysaccharide structure are similar in antipeptic action to carrageenan, indicating the general nature of this type of activity.

The results with purified pepsin and gastricsin were similar to those where whole gastric juice was used, showing that the non-enzymic components of gastric juice have no marked effect. The results in Figs 1–4 and the absence of evidence for direct anti-gastriscin activity indicate an inhibition based on a substrate-inhibitor interaction at pH 3·2 for this enzyme. Also, protein interaction with carrageenan at this pH is the same as that at pH 2, suggesting that differences in antipeptic activity of carrageenan with changing pH reflect differences in the structure of the substrate-inhibitor product. The inhibition of the proteolytic activity of human acid gastric juice, pepsin and gastricsin by sulphated polysaccharides is accomplished by substrate depletion and may be a common mechanism for the gastric protease spectrum.

Differences between methods. The high and low molecular weight carrageenans have similar antipeptic activity when observed in the pepsin-haemoglobin or gastricsin-haemoglobin systems (Fig. 4) whereas in the gastric juice-plasma protein system the undegraded carrageenan (high molecular weight) was previously observed to be more active. However at low substrate concentrations (Fig. 1) in the present haemoglobin system, undegraded carrageenan can appear less active than degraded carrageenan, especially at the higher pH. Decrease in the activity of undegraded carrageenan with increase in pH also occurred in a plasma protein system (Anderson & Baillie, 1967). The activity of undegraded carrageenan is therefore more readily affected by type of substrate protein and pH than degraded carrageenan. This influence of method on activity has been observed for a series including both types of carrageenans (Anderson & Harthill, 1967). In the plasma protein system the difference between the undegraded and degraded carrageenans was attributed to the structure of the substrate protein-polysaccharide complex being different; the present observations on the two carrageenans can be adequately explained similarly.

The mechanism of peptic inhibition by macroanions is common to all systems so far studied and the practical implications of the observed effects are twofold: first, the protective activity of low molecular weight macroanions *in vivo* would be expected to be affected less than that of

the higher molecular weight group by variation of conditions of free acidity and protein present; second, care is required in comparison of quantitative results of antipeptic activity when different methods of examination are used.

Inhibition by different macroanions. Dextran sulphate and phosphate, heparin, degraded λ-carrageenan and chondroitin sulphate gave increasing inhibition of peptic activity with increase in concentration (Fig. 4b). Each substance effects similar inhibition whether added to the enzyme or to the substrate first. In both these respects the activity of this group therefore conforms to low molecular weight macroanion inhibition typified (Anderson & Baillie, 1967) by degraded carrageenan. Although differences in activity emerge between members of the low molecular weight group a pattern is not readily discernible; dextran sulphate might be expected to be more active than dextran phosphate of similar molecular weight, on the basis that the sulphate ester groups are more electronegative than the phosphate ester group, resulting in a substrate complex which is less accessible to proteolysis. Chondroitin sulphate, even though it has the highest molecular weight of all members of the group, is the least active. Matthews (1964) has found that chondroitin sulphate B has a much greater binding affinity than chondroitin sulphate A or C, except when carboxyl group ionization is strongly suppressed, when binding affinities become similar. Hence, it is doubtful if the isomeric chondroitin sulphates would exhibit enhanced antipeptic activity or substrate interaction in experiments of the present type. The low activity of chondroitin sulphate reflecting low substrate binding, is adequately explained on the basis of its low sulphate ester content, which provides only one sulphate ester group per repeating disaccharide unit, less than the other macroanions in the group. Degraded λ-carrageenan and heparin, of similar activity, have one 2,6-disulphated sugar per disaccharide unit with occasional sulphation in the other sugar, whereas degraded *Eucheuma* carrageenan, being a κ-type with high 3,6-anhydro-D-galactose content, features 2,6-disulphated galactose in place of the 3,6-anhydride only occasionally, the more usual repeating unit being 3-linked β-D-galactose 4-sulphate with 4-linked 3,6-anhydro-α-galactose 2-sulphate. The influence of 2,6-disulphation is seen in the requirement of 15 mg of degraded λ-carrageenan and heparin for $i = 0{\cdot}5$ whereas with degraded *Eucheuma* carrageenan 20 mg was required (Anderson & Baillie, 1967). More active (9 mg for $i = 0{\cdot}5$) than these is dextran sulphate, completely sulphated (S, 19·5%) and of molecular weight about 8,000, but, on the other hand undegraded λ-carrageenan (S, 11·5%), which is less highly sulphated than dextran sulphate, requires just under 2 mg (Anderson & Baillie, 1967) thus indicating the profound effect of high molecular weight (800,000) in this particular method, in the presence of 2,6-disulphate esterification.

Interaction with protein. Differences in the extent of interaction of the macroanions with protein can be measured by determining the ratio mg protein : mg macroanion at which free macroanion appears in solution

(Anderson & Baillie, 1967). The ratios observed, heparin 5·5, dextran sulphate 5, dextran phosphate 4·5, chondroitin sulphate 4·5, degraded λ-carrageenan 3, give no indication of the order of antipeptic activity, suggesting that the structure of the inhibitor-substrate complex is important in determining the antipeptic activity, rather than the reacting quantities.

Activity in systems where substrate interaction is low or absent. Absence of antipeptic activity is observed in systems where either pepsin or APDT is used as substrate. Interaction between degraded or undegraded carrageenan and pepsin or APDT could not be demonstrated. This is to be expected in the case of pepsin with an isoelectric point at or below pH 1 precluding the assumption of active cationic character for the enzyme in the digestion system and therefore eliminating the possibility of interaction with the anionic groups of the sulphated polysaccharides. Massive concentrations of degraded carrageenan can be shown to cause slight inhibition in these systems but this is interpreted as a simple ionic effect causing incomplete association between enzyme and substrate and can be demonstrated even for microions. The absence of inhibition in these experiments is further evidence that interaction with the enzyme does not occur and supports the interpretation of other data that inhibition of peptic activity by sulphated polysaccharides is brought about solely by protection of the substrate from digestion.

The study of macroanionic inhibition in the strongly acid conditions of the peptic digest, where the enzyme itself behaves as an anion, provides a special case of the type of inhibition described by Spensley & Rogers (1954) for enzyme inhibition by macroanions in systems where the enzyme behaved as a macroion and is consequently engaged in direct interaction with the inhibitor. Also, the reduced (or even absence of) inhibition observed when undegraded carrageenan is added to substrate first constitutes an exception to the principle (Levey & Sheinfeld, 1954; Dellert & Stahmann, 1955; Kornguth & Stahmann, 1960), that this suggests interaction with the enzyme; the reduction in this instance can be explained by the substrate - inhibitor interaction.

This mechanism of inhibition for sulphated polysaccharides probably reflects a physiological function, for example in conditions of excessive pepsin secretion or mucosal susceptibility, and it is of interest to consider the recent demonstration of chondroitin sulphate A- or C-like substance in the chief cells of the gastric mucosa and the fundic gastric juice of the dog (Gerald, DeGraeff & others, 1967). The present work shows that degree of antipeptic activity of sulphated polysaccharides can vary according to conditions in the digest, and the order of activity reported for *in vitro* systems may not necessarily hold for *in vivo* or intracellular systems, for example, those involving chondroitin sulphate. Nevertheless, the mechanism of activity, namely protection of protein from peptic hydrolysis without destroying or inactivating a physiological enzyme has been shown to hold in all the systems studied. This type of inhibition of peptic activity differs fundamentally from direct pepsin inhibition

observed *in vitro* for the natural pepsin inhibitor (Herriott, 1941), poly-amino-acids (Katchalski, Berger & Neumann, 1954; Dellert & Stahmann, 1955), α-keto analogues of amino-acids (Geratz, 1965) and substrate-like irreversible inhibitors (Ong & Perlmann, 1967).

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