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Inhibitory Effect of Dioctyl Sodium Sulfosuccinate on Pepsin Activity

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Abstract □ The inhibitory effect of dioctyl sodium sulfosuccinate on hog pepsin activity was investigated over the pH 1.5–3.0 range. The inhibitory effect was studied using a natural substrate, hemoglobin, and a synthetic substrate, *N*-acetyl-L-phenylalanyl-L-diiodotyrosine. The mechanistic studies revealed that a substrate–inhibitor interaction was the major mechanism of inhibition with hemoglobin. However, some direct enzyme inhibition also was involved. With the synthetic substrate, the inhibition was due to a competition between the substrate and the inhibitor molecules for the enzyme. The possible therapeutic significance of the inhibitory effect of the medicinal surfactant is discussed.

Keyphrases □ Dioctyl sodium sulfosuccinate—mechanism of inhibition of pepsin activity □ Pepsin activity—mechanism of inhibition by dioctyl sodium sulfosuccinate □ Digestive enzymes—pepsin, mechanism of inhibition by dioctyl sodium sulfosuccinate □ Surfactants—dioctyl sodium sulfosuccinate, mechanism of inhibition of pepsin activity

Peptic ulcers can be defined as acute or chronic ulcerations of the digestive tract, occurring in an area accessible to gastric secretions. In spite of much recent effort, this disease continues to be an incompletely understood and a rather common disorder. Although little is known about the definite etiology of the disease, the interrelationship of a number of mucosal defensive and aggressive factors seems to determine an individual's susceptibility to ulcers (1, 2). Whatever the causative factors, it has now been unequivocally established that, through some unknown mechanism, the normal resistance of the gastric mucosa to acid and pepsin is compromised in all peptic ulcers (1).

Much experimental evidence is available indicating the importance of pepsin in the pathogenesis of the disease (3–6). In humans, the role of this enzyme in gastroduodenal ulceration was indirectly evidenced by the antiulcer activity shown by some macroanions, which inhibited peptic hydrolysis (7, 8). Oral administration of pepsin inhibitors such as degraded carrageenan, amylopectin sulfate, and a sulfated glycopeptide resulted in significant prevention and treatment of peptic ulcers in humans (9, 10). It was reported that the antipeptic effect of these sulfated macromolecules is related to the negative charge of the sulfate group and their high molecular weight (11–14).

Dioctyl sodium sulfosuccinate, an anionic surfactant that is widely used medicinally as a fecal softener, is a molecule with a relatively high molecular weight. Its inhibitory effect on tryptic activity was reported previously (15), and it strongly suppressed ulcer formation in restrained rats when given intraduodenally (16). These findings led to the present investigation, which dealt with the inhibitory effect of dioctyl sodium sulfosuccinate on the peptic activity of pepsin from hog stomach mucosa.

EXPERIMENTAL

Materials—Pepsin[1], hemoglobin[2], *N*-acetyl-L-phenylalanyl-L-diiodotyrosine[3], dioctyl sodium sulfosuccinate[4], and ninhydrin[5] were used as received. All other chemicals were either USP or reagent grade.

Methods for Antipeptic Activity—Two different methods for determining the antipeptic activity were used.

Method I—In this method, denatured hemoglobin was digested under standard conditions. The undigested hemoglobin was precipitated with trichloroacetic acid, and the amount of unprecipitated protein was estimated spectrophotometrically. The method used was a modification of Rajgopalan *et al.* (17).

The substrate (0.4–1.0 mg/ml), enzyme (10–60 μg/ml), and inhibitor (50–400 μg/ml) were prepared in hydrochloric acid (pH 1.8) or distilled water. All solutions were freshly prepared, adjusted to pH 1.8 immediately after preparation, and brought to 37° before mixing. One milliliter of the enzyme (pepsin) solution (I) was pipetted into each flask, already maintained at 37° in a metabolic water bath shaker[6]. Then 1 ml of the inhibitor (dioctyl sodium sulfosuccinate) solution (II) was added to each flask, followed by 2 ml of the substrate (hemoglobin) solution (III).

[1] From hog stomach mucosa, 3× crystallized and lyophilized, Nutritional Biochemicals Corp., Cleveland, Ohio.

[2] Denatured, standardized for protease assay, Nutritional Biochemicals Corp., Cleveland, Ohio.

[3] Sigma Chemical Co., St. Louis, Mo.

[4] Aerosol O. T. 100%, Sargent-Welch Scientific Co., Skokie, Ill.

[5] J. T. Baker Chemical Co., Philipsburg, N.J.

[6] Model G-77, New Brunswick Scientific Co., New Brunswick, N.J.

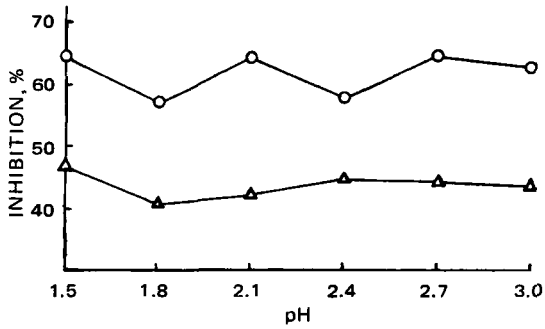

Figure 1—*Effect of pH and mixing orders on percentage inhibition of pepsin. Eight milliliters of digest contained 10 μg of enzyme, 2 mg of substrate (hemoglobin), and 200 μg of inhibitor. Key: ○, inhibitor added to enzyme first; and △, inhibitor added to substrate first. Each point represents the average of at least four determinations.*

All samples were incubated for 5 min with no shaking. At the end of the incubation period, 4 ml of 5% trichloroacetic acid was added to each sample in the same order and thoroughly mixed. The incubation mixtures were allowed to remain at 37° for 3 min and were then filtered[7]. The final volume of the incubation mixture was kept at 8 ml in all cases.

Blank determinations were carried out in exactly the same way as samples except that the substrate solution was added after trichloroacetic acid. The absorbance of all filtrates was determined spectrophotometrically[8] at 277 nm against distilled water. Controls were simultaneously run for every inhibition experiment. The controls differed from the samples in having the inhibitor solution replaced by an equivalent volume of hydrochloric acid (pH 1.8).

Differences between the absorbance of samples and their respective blanks represent the peptic activities and were used as measures of reaction velocities in all kinetic studies. The inhibition value, i, equals $1 - (v_i/v)$, where v_i and v represent reaction velocities with and without the inhibitor, respectively. Percentage inhibition values were calculated from the respective i values (15).

Method II—In this method, *N*-acetyl-L-phenylalanyl-L-diiodotyrosine (IV) was used as the substrate. Compound IV is a low molecular weight synthetic peptide which is easily hydrolyzed by pepsin. The extent of hydrolysis is determined by measuring the ninhydrin color given by the newly formed L-diiodotyrosine. The procedure used was that of Jackson *et al.* (18) with slight modifications.

A solution (20×10^{-5} *M*) of the substrate (IV) was prepared as described by Jackson *et al.* (18). Various concentrations of the enzyme (I) and the inhibitor (II) solutions were prepared as in Method I. The final volume of the reaction mixture was 9 ml in each case. Incubation of all samples was carried out at 37° for 10 min, and the reaction was stopped by adding 0.5 ml of 0.5 *N* sodium hydroxide into each test tube.

All controls and blanks were prepared as described for Method I. The absorbance of the reaction mixtures of both samples and blanks was read spectrophotometrically[8] at 570 nm against distilled water. All peptic activities, reaction velocities, and inhibition values were determined as indicated in Method I.

Various Orders of Mixing—Two different orders of mixing were used as described previously (15). The inhibitor was added to either the enzyme (Mixing Order A) or the substrate (Mixing Order B) before starting peptic hydrolysis. Mixing Order A was used in all studies except for the data of Fig. 1.

All experiments were performed at least in duplicate.

RESULTS AND DISCUSSION

The inhibitory effect of dioctyl sodium sulfosuccinate on pepsin activity was studied at various inhibitor concentrations using Method I. Data presented in Table I show a significant inhibitory effect at all concentrations. The effect of pH and mixing orders on the percentage inhibition was also studied using Method I (Fig. 1). Figure 1 shows

[7] Whatman No. 2 filter paper.

[8] Beckman ACTA CIII spectrophotometer, Beckman Instruments, Fullerton, Calif.

Table I—Effect of Various Concentrations of Dioctyl Sodium Sulfosuccinate on the Percentage Inhibition of Pepsin Activity at pH 1.8

Amounts of Components in 8 ml of Digest			Inhibition[b] of Control Activity, %
Enzyme, μg	Substrate[a], mg	Inhibitor, μg	
10	2	80	28.0
10	2	160	45.1
10	2	240	54.9
10	2	320	64.8
10	2	400	74.1

[a] Hemoglobin. [b] Average of at least two determinations.

a higher percentage inhibition with Mixing Order A (inhibitor added to the enzyme first) as compared to Mixing Order B (inhibitor added to the substrate first). Moreover, this increase in inhibition seemed to be almost constant over the pH 1.5–3.0 range. The marked difference in the inhibition pattern over the pH 1.5–3.0 range suggests that an enzyme–inhibitor interaction was taking place.

At pH 1.8, using hemoglobin as the substrate, a plot of $1/v_i$ against 1/III (Fig. 2) and a plot of $1/v_i$ against II (Fig. 3) were made. Both plots are indicative of a substrate–inhibitor interaction as the major mechanism of inhibition (19). When using the data of Figs. 2 and 3, plots of III/v_i against III, v_i against v_i/III, and $1/i$ against 1/II also yielded curves typical of a substrate–inhibitor interaction (20). Further characterization of this mechanism was done by making a plot of II against $i/(1 - i)$ (Fig. 4), which shows substrate depletion to be the basic mechanism involved (21).

With the data of Fig. 2, a plot of i against III was also made, which indicates that an enzyme–inhibitor interaction seems to be involved in addition to a substrate–inhibitor interaction in the overall mechanism of inhibition (15). This finding explains the increased percentage inhibition observed with Mixing Order A (Fig. 1). Such an additional enzyme–inhibitor interaction could have been confirmed further through preincubation studies (15). However, such studies were not feasible due to the autodigestion of pepsin under the experimental conditions, as reported earlier (22) and found in the present investigation.

A double reciprocal plot (Fig. 5) was made using the synthetic substrate (Method II). Figure 5 is typical of a true competitive inhibition due to a competition between the substrate and the inhibitor molecules for the same site at the enzyme surface (23). Such a mechanism was further confirmed by making a plot of $1/v_i$ against II (Fig. 6). The value of the inhibitor constant, K_i, was also determined from this plot (19).

From the foregoing discussion, it is apparent that dioctyl sodium sulfosuccinate interacts with both the substrate and the enzyme when the substrate used is natural protein (hemoglobin). When the sub-

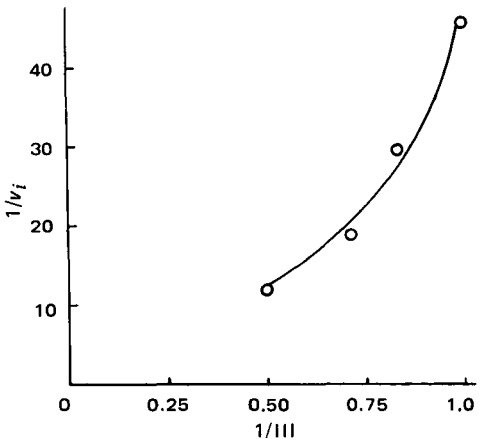

Figure 2—*Plot of $1/v_i$ (reciprocal reaction velocity) against 1/III (reciprocal substrate concentration) for antipeptic activity of dioctyl sodium sulfosuccinate at pH 1.8. Eight milliliters of digest contained 10 μg of enzyme, 200 μg of inhibitor, and 0.8–2.0 mg of substrate (hemoglobin).*

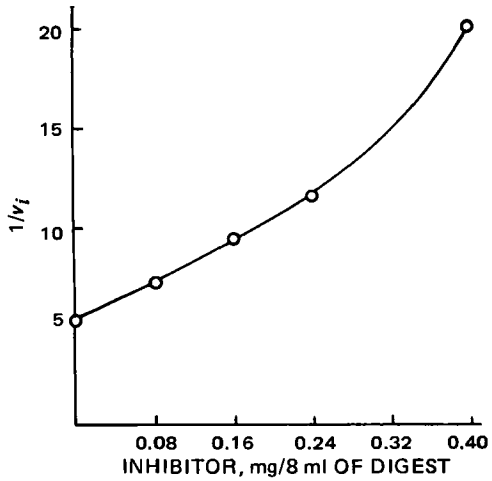

Figure 3—*Plot of* $1/v_i$ *(reciprocal reaction velocity) against II (inhibitor concentration) for antipeptic activity of dioctyl sodium sulfosuccinate at pH 1.8. Eight milliliters of digest contained 10 μg of enzyme and 2 mg of substrate (hemoglobin).*

strate is a synthetic dipeptide (IV), the inhibitor binds the enzyme molecule exclusively and shows no affinity for the substrate. This finding may indicate that the inhibitor probably alters the conformation of hemoglobin and that no involvement of the primary structure takes place. The lack of an interaction between the synthetic substrate and dioctyl sodium sulfosuccinate and its significant interacting capacity with hemoglobin are some of the typical inhibitory characteristics of the sulfated macroanion inhibitors of pepsin reported earlier (9).

These similarities in the inhibitory characteristics between dioctyl sodium sulfosuccinate and macroanion inhibitors may be a consequence of their structural similarities. However, the interaction between dioctyl sodium sulfosuccinate and pepsin in the presence of both a natural and a synthetic substrate is an additional inhibitory feature of this surfactant and is not exhibited by macroanion inhibitors (9). Such an enzyme-inhibiting property may be due to a higher water solubility, surface-active properties, or the lower molecular weight of dioctyl sodium sulfosuccinate. It is also possible that dioctyl

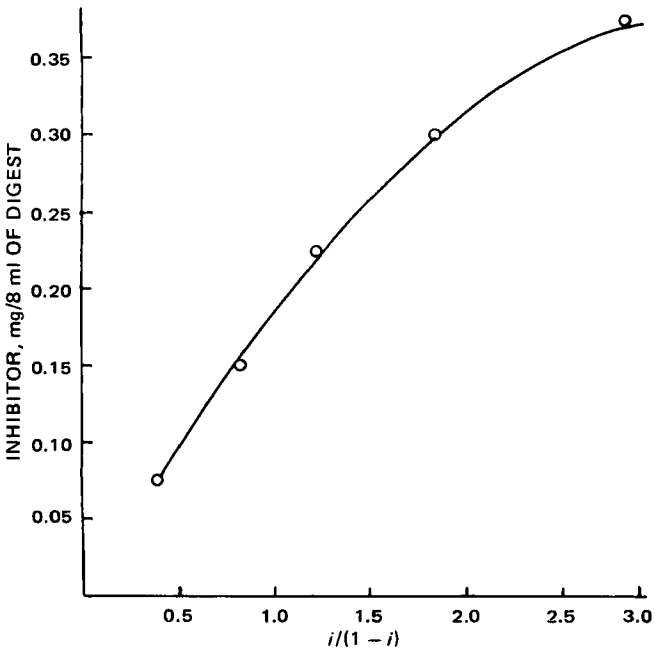

Figure 4—*Plot of II (inhibitor concentration) against* $i/(1-i)$ *for antipeptic activity of dioctyl sodium sulfosuccinate at pH 1.8. Eight milliliters of digest contained 10 μg of enzyme and 2 mg of substrate (hemoglobin).*

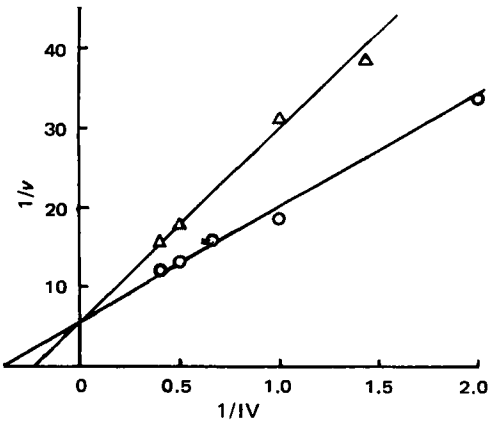

Figure 5—*Plot of 1/v (reciprocal reaction velocity) against 1/IV (reciprocal substrate concentration) for antipeptic activity of dioctyl sodium sulfosuccinate at pH 2.0. Nine milliliters of incubation mixture contained 60 μg of enzyme and* 10×10^{-8}–50×10^{-8} *mole of substrate (IV). Key: ○, no inhibitor added; and △, inhibitor = 50 μg. Both lines were drawn by the method of least squares: △,* $y = 22.94x + 6.55$ ($r = 0.990$); *and ○,* $y = 13.358x + 6.2712$ ($r = 0.9972$).

sodium sulfosuccinate has a molecular size and shape that favor the hydrophobic and steric considerations so important in the orientation and proximity effects necessary for its binding to the enzyme molecule.

The mechanistic studies also reveal that dioctyl sodium sulfosuccinate seems to possess a lower affinity for pepsin than for hemoglobin in the presence of the two. Such behavior was reported for some macroanion inhibitors (24).

Weiss and Serfontein (25) indicated some of the qualitative and quantitative biochemical differences between ulcerated tissues and surrounding normal tissues. Compounds like hexosamines, hyaluronic acid, mucopolysaccharides, and the glycoproteins are present at higher concentrations and in more reactive forms at the ulcerated tissues. The antipeptic activity of most macroanion sulfated polysaccharides as well as certain other antiulcer agents has been shown to be due to their ability to form insoluble complexes with these tissue degradation products or other proteins present at the ulcerated surface (26). An ulcerated site covered with such a complex is believed to be shielded from further exposure to the damaging effects of the acidic gastric juice and pepsin, thus facilitating healing of the ulcer. In view of the mechanistic evidence presented, dioctyl sodium sulfosuccinate may provide possible antiulcer action through such an indirect mechanism.

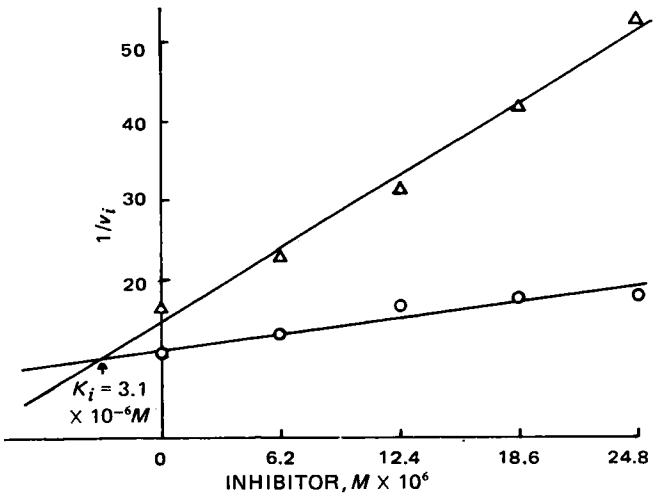

Figure 6—*Plot of* $1/v_i$ *(reciprocal reaction velocity) against II (inhibitor concentration) for antipeptic activity of dioctyl sodium sulfosuccinate at pH 2.0. Nine milliliters of incubation mixture contained: △,* 20×10^{-8} *mole of substrate (IV) and 60 μg of enzyme; and ○,* 40×10^{-8} *mole of substrate (IV) and 60 μg of enzyme. Both lines were drawn by the method of least squares: △,* $y = 1.4729x + 14.694$ ($r = 0.9948$); *and ○,* $y = 0.3166x + 11.1520$ ($r = 0.9415$).

It may also exert some additional healing effect due to its direct inhibitory effect against pepsin. Pepstatin, an antiulcer agent, has been shown to act through this direct inactivation of pepsin (26).

The results of the present studies strongly suggest a significant inhibition of gastric peptic activity by the surfactant at concentrations well below the dosages of dioctyl sodium sulfosuccinate presently used in laxative therapy. Such an inhibition of pepsin may explain the antiulcerogenic effect of dioctyl sodium sulfosuccinate already observed in rats (16). An additional antiulcer effect of this medicinal surfactant may be due to its potent ability to decrease the volume of secretions and the amount of acid secreted as indicated by Lish (16).

Although dioctyl sodium sulfosuccinate may provide an antiulcer action, a reduction in the digestive capacity of the gastric juice for dietary proteins and possibly certain drug interactions (15) must be considered when evaluating this agent for possible therapy of peptic ulcer patients.

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GLC Determination of Aprindine: Quantitation and Stability Measurement

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Abstract □ A GLC method of analysis of a new antiarrhythmic agent, aprindine, is described. The raw material of the new drug substance, supplied as the hydrochloride salt, is dissolved in deionized water, and the base is liberated by a 10% aqueous solution of sodium carbonate. Aprindine is extracted with chloroform and mixed with the internal standard, 5α-cholestane. GLC is performed on a glass column packed with 3.8% W-98 on Chromosorb W-HP. Quantitation is achieved by computer calculation of the peak area ratios. GLC–mass spectral analysis indicates that the observed peak is that of aprindine, with a molecular ion at *m/e* 322. The retention times of aprindine and the internal standard are 2.0 and 5.8 min, respectively. All synthetic precursors show a shorter retention time than aprindine. This GLC method is applied to the quantitative determination of aprindine as raw material and in capsule and ampul formulations. The method is also used to measure the stability of aprindine to acid, base, dry heat, refluxing, and UV light and to pH variations.

Keyphrases □ Aprindine—GLC analysis in pharmaceutical formulations, effect of dry heat, refluxing, UV light, and varying pH on stability □ GLC—analysis, aprindine in pharmaceutical formulations □ Stability—aprindine in pharmaceutical formulations, effect of dry heat, refluxing, UV light, and varying pH □ Antiarrhythmic agents—aprindine, GLC analysis in pharmaceutical formulations, effect of dry heat, refluxing, UV light, and varying pH on stability

Aprindine (I), *N,N*-diethyl-*N'*-(2-indanyl)-*N'*-phenyl-1,3-propanediamine, is a new potent antiarrhythmic agent (1) which can be administered both orally and intravenously. The drug is presently being used in humans (1, 2) and has a long-lasting action when taken orally (1, 3).