The column was washed with 1500 ml of hot water, the effluent was brought to near dryness by flash evaporation, and the residue was heated under reflux for 2.5 hr with 50 ml of concentrated hydrochloric acid. The hydrochloric acid was removed in a rotary evaporator, and the residual solution was dissolved in a small volume of water, decolorized with carbon, and adjusted to pH 3.1 with pyridine; then three volumes of ethanol was added.

After cooling at −15° overnight, the crystalline product was removed by filtration, washed with ethanol followed by ether, and dried at 70° overnight. The yield was 4.3 g (48%), mp 162–164°. On further heating, the melt solidified and remelted at 202–203° (2-phenylpyroglutamic acid). The analytical sample was crystallized from aqueous ethanol, mp 166–167° [lit. (5) mp 170°]; with further heating, the melting point was 205–206° [lit. (5) mp 207°].

Anal.—Calc. for $C_{11}H_{14}NO_4$: C, 59.19; H, 5.87; N, 6.27; O, 28.67. Found: C, 59.36; H, 5.95; N, 6.14; O, 28.89.

REFERENCES

(1) Y. C. Kim and G. H. Cocolas, *J. Med. Chem.*, **8**, 509(1965).

(2) A. Meister, *J. Biol. Chem.*, **210**, 17(1954).

(3) H. Sasaki and F. Takahashi, *Nippon Kagaku Zasshi*, **77**, 410(1956); through *Chem. Abstr.*, **52**, 9016b(1958).

(4) F. Sörm and V. Procházka, *Chem. Listy*, **46**, 409(1952).

(5) K. Pfister, III, W. J. Leanza, J. P. Conbere, H. J. Becker, A. R. Matzuk, and E. F. Rogers, *J. Amer. Chem. Soc.*, **77**, 697(1955).

(6) H. R. Henze and R. J. Speer, *ibid.*, **64**, 522(1942).

(7) M. Ikawa and E. E. Snell, *J. Biol. Chem.*, **235**, 1376(1960).

(8) R. E. Strange and C. B. Thorne, *Biochim. Biophys. Acta*, **24**, 199(1957).

(9) F. Zilliken, *Fed. Proc.*, **18**, 966(1959).

(10) W. A. Zygmunt, R. L. Evans, and H. E. Stavely, *Can. J. Microbiol.*, **8**, 869(1962).

(11) B. D. Davis and E. S. Mingioli, *J. Bacteriol.*, **60**, 17(1950).

(12) D. T. Warner and O. A. Moe, *J. Amer. Chem. Soc.*, **71**, 2586(1949).

(13) J. Done and L. Fowden, *Biochem. J.*, **51**, 451(1952).

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Inhibitory Effect of Dioctyl Sodium Sulfosuccinate on Trypsin Activity

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Abstract □ The inhibitory effect of dioctyl sodium sulfosuccinate on the proteolytic activity of trypsin was investigated over the pH 6–8 range. The antitryptic activity was determined using two different substrates: casein and *N*,α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride. The mechanistic studies revealed the substrate–inhibitor interaction to be the overall major mechanism of inhibition. This interaction was shown to involve substrate depletion, probably involving some primary sites of the natural substrate casein. Some inhibition was also shown to be due to an interaction between the enzyme and the inhibitor molecules. The interactions of the inhibitor with the enzyme and the substrate were irreversible. The possible therapeutic significance of the inhibitory effect of the surfactant is discussed.

Keyphrases □ Dioctyl sodium sulfosuccinate—trypsin inhibition, pH 6–8, two different substrates, therapeutic significance □ Trypsin—inhibition by dioctyl sodium sulfosuccinate, pH 6–8, two different substrates, therapeutic significance □ Enzyme inhibition—trypsin by dioctyl sodium sulfosuccinate, pH 6–8, two different substrates, therapeutic significance

The theories on the role of proteolytic enzymes in physiological and pathological states in humans and animals have been the topic of great controversy. The old belief that these enzymes catalyze protein synthesis no longer exists, yet it would be equally unjustified to restrict the role of body proteinases to a few processes such as cleavage of food proteins, mobilization of tissue proteins, and protein degradation usually associated with physiological wear and various traumas. In fact, it is now evident that these enzymes play a causative or adjunctive role in some disease processes such as inflammation, thromboembolic disorders, complement-dependent immune reactions, peptic and duodenal ulcers, and pancreatitis and even in some syndromes of malignant carcinomas (1).

Trypsin and kallikrein, which are present in the body tissues and fluids, are capable of releasing pharmacologically active plasma kinins (2). These proteolytic enzymes play an indirect but significant role in such important conditions as hypertension, shock, certain pains, changed capillary permeability, edema, and leucocyte migration. In the light of this knowledge, the proteinase inhibitors, both synthetic and natural, have gained new theoretical and practical importance (3). Recent attempts have been made to synthesize some active-site-oriented inhibitors as well as to improve their inhibitory activity (4–6). Also, it is reported that proteolytic enzymes are inhibited by agents such as sodium lauryl sulfate (7) and some sulfated polysaccharides (8).

Dioctyl sodium sulfosuccinate is an anionic surfactant, widely used medicinally as a fecal softener. The effect of this surfactant on drug absorption through membranes of varying complexities has been re-

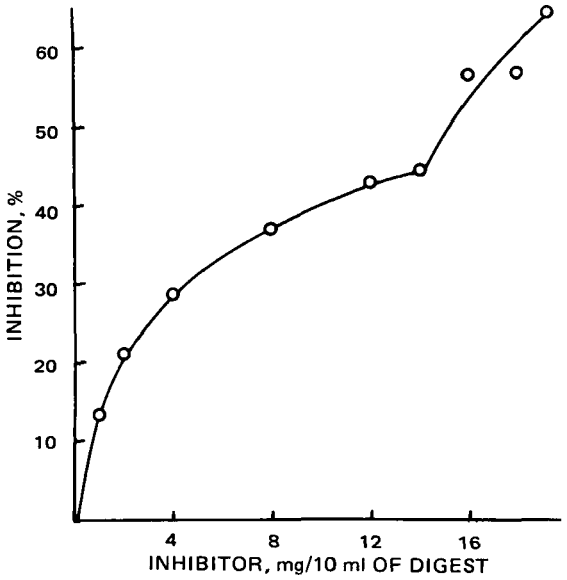

Figure 1—*Effect of various inhibitor concentrations on the percentage inhibition at pH 7.6. Ten milliliters of digest contained 10 μg of enzyme and 50 mg of substrate.*

ported (9–11), as has the absorption of dioctyl sodium sulfosuccinate into the systemic circulation (12). These findings and the similarity of the chemical nature of this surfactant to some proteolytic inhibitors such as sodium lauryl sulfate and sulfated polysaccharides led to this investigation of the inhibitory effect of dioctyl sodium sulfosuccinate on tryptic activity.

EXPERIMENTAL

Materials—Trypsin[1], casein[2], *N*,α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride[3], dioctyl sodium sulfosuccinate[4], and tromethamine[5] were obtained commercially. All other chemicals used were either USP or reagent grade.

Methods for Antitryptic Activity—*Method I*—Sørensen phosphate buffers (0.1 *M*) of various pH values were used. A fresh 1% casein (I) solution was prepared by suspending 1.0 g of casein in 100 ml of buffer and heating the suspension in a boiling water bath for 15 min. Enzyme (II) solutions of various concentrations, ranging between 10 and 50 μg/ml, were prepared by first dissolving 10 mg of trypsin in 10 ml of 0.001 *M* HCl and then diluting with the appropriate buffers. Various inhibitor (III) solutions were prepared by dissolving required amounts of the inhibitor in the buffers at the appropriate pH.

The assay was carried out using a modification of Kunitz's procedure (13). All solutions were freshly prepared and mixed at room temperature before incubation. The final volume of the digestion mixture was kept at 10 ml in all cases. In this method, two different mixing procedures were followed.

In one mixing procedure, inhibitor was added to the enzyme before digestion. One milliliter of enzyme solution was pipetted into flasks followed by 1 ml of the inhibitor solution. Five milliliters of the substrate solution was then added to each flask at 30-sec intervals. All flasks were incubated in a water bath metabolic shaker[6] maintained at 37° for 1 hr. Samples were shaken at 200 rpm throughout the digestion period. At the end of the incubation period, 3 ml of 10% trichloroacetic acid was added to each flask, in the same order, and the flasks were allowed to stand at room temperature for 10 min. Blanks were prepared in the same way as samples except that the casein solution was added after the addition of trichloroacetic acid.

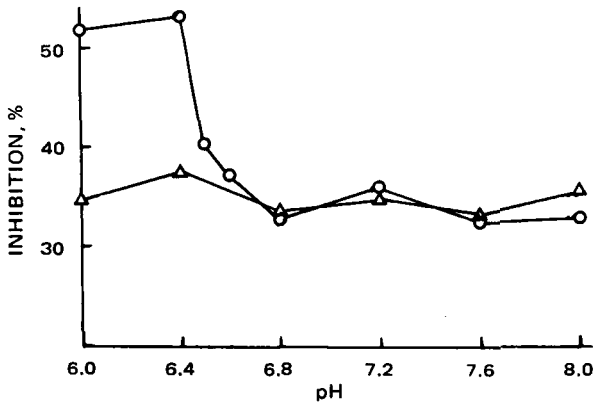

Figure 2—*Effect of pH and mixing orders on percentage inhibition. Ten milliliters of digest contained 20 μg of enzyme, 50 mg of substrate, and 10 mg of inhibitor. Key: O, inhibitor added to enzyme first; and Δ, inhibitor added to substrate first.*

Both samples and blanks were then centrifuged[7] at 8000 rpm for 20 min, and the supernates were filtered[8]. The absorbance of each filtrate was read[9] at 280 nm against the respective blank. Controls were run simultaneously for every inhibition experiment. The controls differed from the samples in having inhibitor solution replaced by equivalent amounts of buffers.

The absorbance values were used as measures of reaction velocities in the kinetic studies. The inhibition (i) equals $[1 - (v_i/v)]$, where v_i and v represent digestion velocities with and without the inhibitor, respectively. Percentage inhibition values were calculated from i values.

The other mixing procedure for digestion was the same as already described, except that the inhibitor solution was added to the substrate first and mixed, followed by addition of enzyme.

Method II—*N*,α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride solution (10^{-3} *M*) was prepared as described by Erlanger *et al.* (14). Various concentrations of enzyme and inhibitor solu-

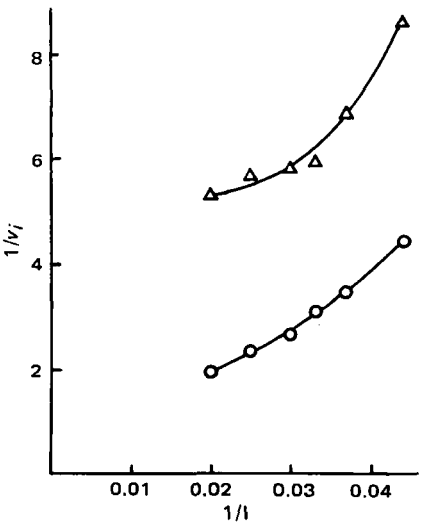

Figure 3—*Plots of $1/v_i$ against $1/I$ for dioctyl sodium sulfosuccinate at two pH values. Ten milliliters of digest contained 20 μg of enzyme, 10 mg of inhibitor, and 23–50 mg of substrate. Key: Δ, pH 6.4; and O, pH 7.6.*

[1] Twice crystallized, salt-free, and lyophilized beef pancreas, Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] Hammersten type, Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Schwarz/Mann, Dickinson and Co., Van Nuys, Calif.
[4] Aerosol O.T. 100%, Sargent-Welch Scientific Co., Skokie, Ill.
[5] Tris(hydroxymethyl)aminomethane, Sigma Chemical Co., St. Louis, Mo.
[6] Model G-77, New Brunswick Scientific Co., New Brunswick, N.J.
[7] Sorvall SS-4 superspeed centrifuge, Ivan Sorvall Inc., Norwalk, Conn.
[8] Whatman No. 2 filter paper.
[9] Spectronic-600 spectrophotometer, Analytical Instruments, Bausch & Lomb, Rochester, N.Y.

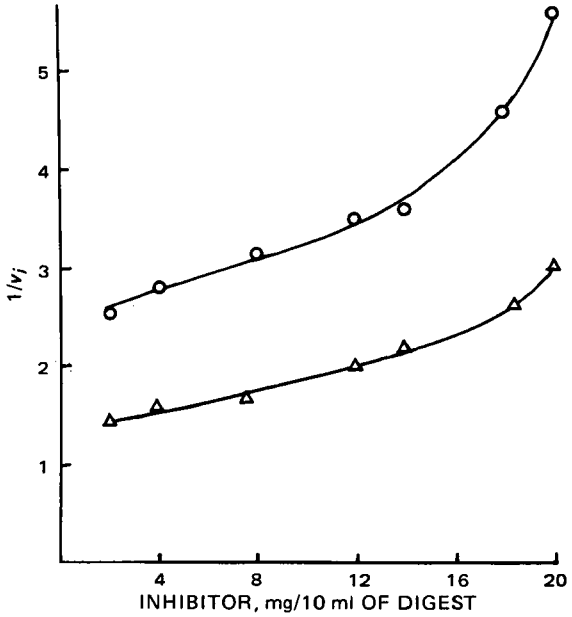

Figure 4—*Plots of $1/v_i$ against III for dioctyl sodium sulfosuccinate at pH 7.6. Key: O, 10 ml of digest contained 10 μg of enzyme and 50 mg of substrate; and Δ, 10 ml of digest contained 20 μg of enzyme and 50 mg of substrate.*

tions were prepared as described in Method I, except that the buffers used were 0.05 *M* tromethamine at various pH values. An amidase assay procedure was used with a few modifications (14). The final volume of the reaction mixture was 8 ml in each case. Incubation of the samples was done at 37° for 300 sec, and the reaction was stopped by adding 1 ml of 30% acetic acid to each flask.

All incubation mixtures were centrifuged and then filtered as described in Method I. Blanks and controls were prepared in the same manner as described in Method I. The absorbance of the filtrates of both samples and blanks was recorded[10] at 410 nm against distilled water. Differences between the absorbance of samples and blanks represent the proteolytic activity. Inhibition values (i) and the percentage inhibitions were calculated as already described. Different orders of mixing were carried out according to the two procedures used in Method I.

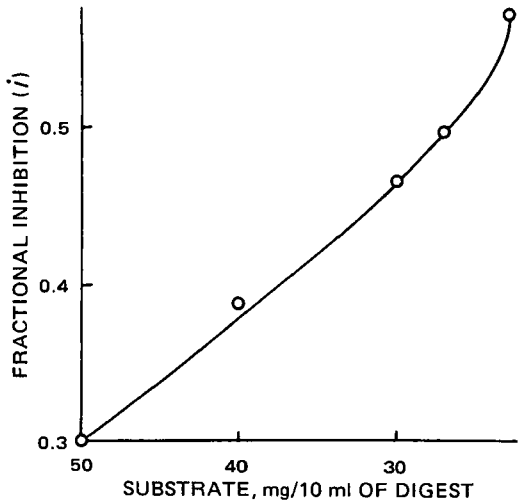

Figure 5—*Plot of* i *against I for dioctyl sodium sulfosuccinate at pH 7.6. Ten milliliters of digest contained 20 μg of enzyme and 10 mg of inhibitor.*

[10] Beckman Acta CIII spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

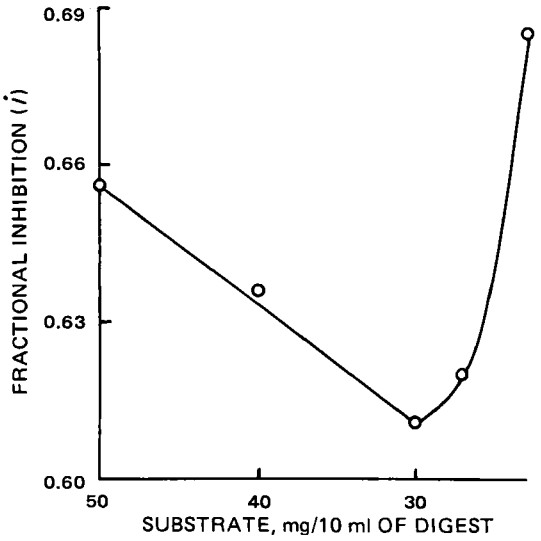

Figure 6—*Plot of* i *against I for dioctyl sodium sulfosuccinate at pH 6.4. Ten milliliters of digest contained 20 μg of enzyme and 10 mg of inhibitor.*

Preincubation Studies—In the preincubation studies the inhibitor was allowed to react for an additional 15 min either with the enzyme or with the substrate before digestion was started. The controls were also preincubated for the same period. Digestion was carried out according to both procedures described under *Method I*.

Dialysis Studies—To find out whether the interaction of inhibitor toward substrate and enzyme is reversible or irreversible, dialysis studies were carried out at pH 6.4 and 7.6. Regenerated cellulose dialysis tubings[11] were cut into pieces of 15–23 cm (6–9 in.) and thoroughly washed with distilled water. The tubings were then immersed in 500 ml of 10 m*M* edetate sodium solution and heated at 70–80° for 2 hr. Then the tubings were allowed to cool, washed again thoroughly with double-distilled water, and stored in distilled water at 2–4°. Prior to use, the tubings were soaked in an appropriate buffer for 18–24 hr.

The inhibitor was added to the enzyme or substrate, and the reaction was allowed to proceed at 37° for 60 min in a metabolic shaker at 200 rpm. A portion of the mixture was then assayed for tryptic activity using Method I, and the rest of the mixture was put in a preconditioned dialysis tubing sac. The sac was immersed in 1000 or 2000 ml of 0.1 *M* phosphate buffer of appropriate pH, and dialysis was carried out at 4° for 96 hr. The external buffer was replaced every 24 hr during the dialysis period, at the end of which the contents were again assayed for their tryptic activity. A control dialysis experiment was run simultaneously in the same way, except that the inhibitor solution was replaced by an equivalent amount of buffer solution. Percentage inhibition values before and after dialysis were calculated as already described.

All experiments were performed at least in duplicate.

RESULTS AND DISCUSSION

The inhibitory effect of dioctyl sodium sulfosuccinate on tryptic activity was studied at various inhibitor concentrations using Method I. Percentage inhibition was plotted against inhibitor concentration (Fig. 1). In all studies, the inhibitor was added to the enzyme first, except for the data of Fig. 2 in which case both mixing procedures were used. The biphasic plot in Fig. 1 can be explained as due to the saturation of either the enzyme or the substrate with the inhibitor at the point of inflection.

The effect of pH and mixing orders on the percentage inhibition was also studied using Method I (Fig. 2). Figure 2 shows that when the inhibitor was added to the substrate first, the percentage inhi-

[11] No. 20, 1.6 cm (0.62 in.) in flat width, for trypsin dialysis and No. 36, 2.85 cm (1.1 in.) in diameter, for casein dialysis were used. Average pore size was 24 Å. Van-Waters and Rogers, Inc., San Francisco, Calif.

Table I—Irreversible Binding of the Inhibitor to Enzyme and Substrate[a]

pH	Component Bound to the Inhibitor before Dialysis	Amount of the Components in 10 ml of Digestion Mixture			Inhibition, %	
		Enzyme, μg	Substrate, mg	Inhibitor, mg	Before Dialysis	After Dialysis
6.4	Trypsin	10	50	1.0	100.0	100.0
	Trypsin	10	50	0.1	65.0	57.5
	Trypsin	20	50	0.1	94.4	95.0
	Casein	20	50	10.0	42.0	38.0
7.6	Trypsin	10	50	1.0	65.6	67.6
	Casein	20	50	10.0	36.7	34.3

[a] Casein.

bition remained reasonably constant throughout the pH range studied. When the inhibitor was added to the enzyme first, the resulting percentage inhibition was significantly higher ($p < 0.001$ as determined by the Student t test for unpaired data) at pH <6.5. This marked difference in inhibition pattern below pH 6.5 will be discussed later.

Using casein as the substrate, double reciprocal plots in the presence of the inhibitor at both pH 6.4 and 7.6 (Fig. 3) and plots of $1/v_i$ against III at pH 7.6 (Fig. 4) indicate the substrate–inhibitor interaction as the major mechanism of inhibition (15). When using the data of Figs. 3 and 4, plots of I/v_i against I, v_i against v_i/I, and $1/i$ against 1/III also yielded curves typical of substrate–inhibitor interaction (16).

Plots of fractional inhibition (i) against I at pH 7.6 (Fig. 5) and 6.4 (Fig. 6), with the data of Fig. 3, suggest that the enzyme–inhibitor interaction as well as the substrate–inhibitor interaction also contributes to the overall inhibition. This contribution seems to be least at pH 7.6 (Fig. 5) and most at pH 6.4 (Fig. 6). This apparent increased involvement of the enzyme with the inhibitor as a consequence of a change in pH can be well explained by the fact that dioctyl sodium sulfosuccinate is a molecule carrying a net negative charge and that trypsin possesses an isoelectric point in the vicinity of pH 7 (17). Therefore, increased amount of inhibition below pH 6.5 is expected. This explains the increased inhibition below pH 6.5 observed in Fig. 2. Also, the biphasic curve of percentage inhibition against the inhibitor concentration (Fig. 1) can be explained on the basis of a dual mechanism of inhibition involving both the substrate and the enzyme.

A double reciprocal plot (Fig. 7) also was made using a synthetic substrate (Method II), and the mechanism of inhibition seems to be similar to the one observed when casein was used as the substrate. This similarity in mechanisms when two different substrates were used indicates that the interaction between the inhibitor and casein molecules involves some primary sites of the natural protein.

A general inhibitory mechanism involving a substrate can be further distinguished into three basic mechanisms by plotting III against $i/(1 - i)$ (18). These mechanisms are: (a) substrate depletion, (b) inhibition of the enzyme by a substrate–inhibitor complex, and (c) combination of both mechanisms. The shapes of such plots (Fig. 8) for dioctyl sodium sulfosuccinate at pH 7.6, using casein as a substrate, are typical of substrate depletion. Similar plots showing substrate depletion as the basic mechanism were also obtained using different substrate and enzyme concentrations. The results of the dialysis studies (Table I) indicate that dioctyl sodium sulfosuccinate binds both casein and trypsin irreversibly at both pH 6.4 and 7.6.

Preincubation studies (Table II) show that preincubation of the substrate with the inhibitor has no significant effect on the inhibition. Preincubation of the enzyme with the inhibitor prior to digestion seems to increase the percentage inhibition at almost all substrate–inhibitor ratios. These results suggest that, besides the substrate–inhibitor interaction, the enzyme–inhibitor interaction is a part of the overall mechanism of inhibition at pH 7.6. This finding is in good agreement with what has already been concluded from the mechanistic studies.

The effect of various mixing orders on percentage inhibition was also studied using the synthetic substrate. Results were similar to those obtained with casein.

The effect of various shaking rates was investigated using casein as the substrate. The degree of inhibition was unaltered at various shaking speeds ranging between 0 and 200 rpm.

Pancreatitis can be regarded as a chemical autolytic process, the pathogenesis of which is well documented from postmortem, operative, and experimental studies (19). The exact mechanism of the pathological process has yet to be established. However, evidence supports the central role of trypsin in the pathogenesis of the disease (1, 3, 20). It is then apparent that means of controlling the undesirable hypertryptic activity at the site of disease could serve as an approach to alleviate the disease. The present inhibitory studies with dioctyl sodium sulfosuccinate and the recent finding that this compound is absorbed into the systemic circulation (12) suggest a possible use of this medicinal surfactant in pancreatitis. The interaction of dioctyl sodium sulfosuccinate with casein

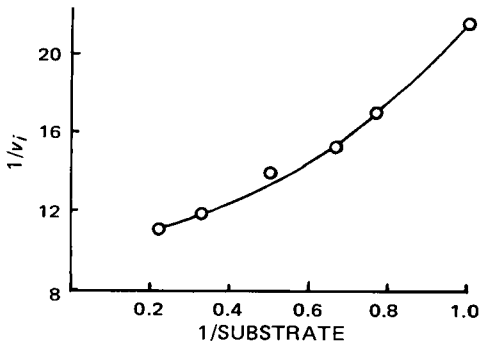

Figure 7—*Plot of* $1/v_i$ *against 1/substrate for dioctyl sodium sulfosuccinate at pH 8.2. Eight milliliters of the incubation mixture contained 40 μg of enzyme, 0.25 mg of inhibitor, and* $1–5 \times 10^{-6}$ *mole of substrate. Substrate used was* N,α-benzoyl-DL-arginine-p-nitroanilide hydrochloride.

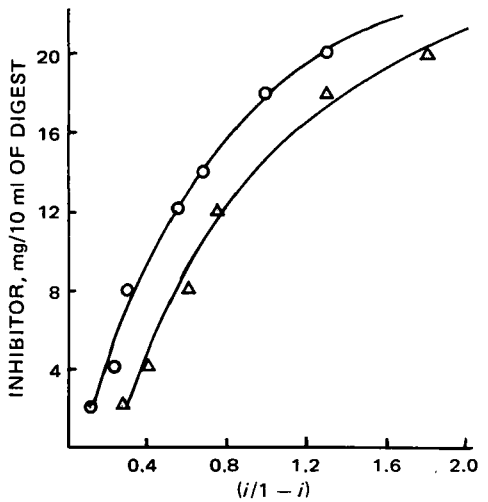

Figure 8—*Plots of III against* (i/1 − i) *for dioctyl sodium sulfosuccinate at pH 7.6. Key:* O, *10 ml of digest contained 20 μg of enzyme and 50 mg of substrate; and* Δ, *10 ml of digest contained 10 μg of enzyme and 50 mg of substrate.*

Table II—Effect of Preincubation on Antitryptic Activity of Dioctyl Sodium Sulfosuccinate at pH 7.6

Substrate[a], mg	Inhibitor, mg	Substrate/Inhibitor	Percentage Inhibition[b]		
			A	B	C
40	20	2	71.0	66.5	88.6
40	10	4	38.5	39.2	66.4
40	5	8	25.0	25.0	45.5
40	2.5	16	19.5	21.8	36.7
40	1.25	32	11.2	10.0	23.4

[a] Casein. [b] Amount of enzyme used was 30 μg. A, no preincubation; B, the inhibitor was preincubated with casein for 15 min; and C, the inhibitor was preincubated with trypsin for 15 min.

suggests the possibility of a similar interaction between the inhibitor molecule and the polysaccharide proteins at the site of inflammation. Such an interaction *in vivo* may prove to be useful in protecting the site from necrosis and erosion (21).

These inhibitory studies with dioctyl sodium sulfosuccinate also suggest that possible drug interactions may arise with this compound in situations and conditions where concomitant use of proteolytic enzymes such as trypsin and chymotrypsin is desirable. Drug interactions are also possible with drug esters such as penamecillin and chloramphenicol palmitate and certain *N*-acylated drugs when given orally along with dioctyl sodium sulfosuccinate. Such drugs are usually acted upon by proteinases in the GI tract resulting in changed chemical and/or pharmacological activity. These drug interactions may be difficult to recognize, particularly when dioctyl sodium sulfosuccinate is used as an excipient for the formulation of any drug being used simultaneously.

REFERENCES

(1) R. W. Colman, *Clin. Pharmacol. Ther.*, **6**, 598(1965).

(2) J. W. Ryan, J. G. Moffat, and A. G. Thompson, *Nature*, **204**, 1212(1964).

(3) R. Vogel, I. Trautschold, and E. Werle, "Natural Proteinase Inhibitors," Academic, New York, N.Y., 1968, pp. 112–119.

(4) B. R. Baker, "Design of Active Site Directed Irreversible Enzyme Inhibitors," Wiley, New York, N.Y., 1967, pp. 129–149.

(5) E. Mihalyi, "Application of Proteolytic Enzymes to Protein Structure Studies," C. R. C. Press, Cleveland, Ohio, 1972, p. 90.

(6) J. D. Geratz, A. C. Whitemore, M. C. F. Cheng, and C. Piantadosi, *J. Med. Chem.*, **16**, 970(1973).

(7) C. A. Nelson, *J. Biol. Chem.*, **246**, 3895(1971).

(8) W. Anderson and A. J. Baillie, *J. Pharm. Pharmacol.*, **19**, 720(1967).

(9) M. W. Gouda, *Can. J. Pharm. Sci.*, **9**, 37(1974).

(10) S. N. Malik, D. H. Canaham, and M. W. Gouda, *J. Pharm. Sci.*, **64**, 987(1975).

(11) N. Khalafallah, M. W. Gouda, and S. A. Khalil, *ibid.*, **64**, 991(1975).

(12) C. A. Dujovne and D. W. Shoeman, *Clin. Pharmacol. Ther.*, **13**, 602(1972).

(13) M. Kunitz, *J. Gen. Physiol.*, **30**, 291(1947).

(14) B. F. Erlanger, N. Kokowsky, and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271(1961).

(15) M. Dixon, *Biochem. J.*, **55**, 170(1953).

(16) J. L. Webb, "Enzyme and Metabolic Inhibitors," vol. 1, Academic Press, London, England, 1963, p. 85.

(17) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **16**, 295(1935).

(18) J. M. Reiner, "Behavior of Enzyme Systems," 2nd ed., Van Nostrand Reinhold, New York, N.Y., 1969, pp. 192–199.

(19) P. B. Beeson and W. McDermott, "Textbook of Medicine," 11th ed., vol. II, Saunders, Philadelphia, Pa., 1963, p. 944.

(20) W. F. Ganong, "Review of Medical Physiology," 5th ed., Lange Medical Publications, Los Altos, Calif., 1971, p. 367.

(21) W. Anderson, *J. Pharm. Pharmacol.*, **13**, 139(1961).

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