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STUDIES ON THE INHIBITORY EFFECT OF
DIOCTYL SODIUM SULFOSUCCINATE ON
CERTAIN BODY PROTEINASES AND ITS
POSSIBLE ROLE IN THERAPEUTICS

By

Gurcharan Singh Jodhka

B. Pharm. Panjab University, Chandigarh, India, 1972

Presented in partial fulfillment of the requirements for the degree of

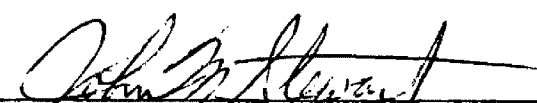
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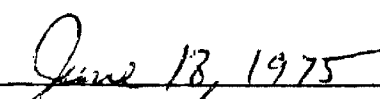
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Pharmacy

Studies on the Inhibitory Effect of Dioctyl Sodium Sulfosuccinate on Certain Body Proteinases and Its Possible Role in Therapeutics.

Director: M. Wafik Gouda

M. Wafik Gouda

Inhibitory effect of dioctyl sodium sulfosuccinate (DOSS), a medicinal surfactant, was studied on the proteinase activity of trypsin and pepsin *in vitro*, using both natural and synthetic substrates. The inhibitory effect of DOSS was studied under various reaction conditions such as inhibitor concentration, enzyme concentration, substrate concentration, pH and various mixing orders of the components. Mechanisms of inhibition were delineated using initial velocity studies and various graphical techniques.

Studies with trypsin both at pH 6.4 and pH 7.6, indicate that in the presence of a natural substrate, substrate-inhibitor interaction involving substrate depletion is the major mechanism of inhibition. Some direct enzyme inhibition may also occur. In the presence of a synthetic substrate, a similar inhibitory mechanism seems to exist at pH 8.2, but at pH 6.4 a true competitive enzyme inhibition forms the only mechanism. Results of the dialysis studies indicate that DOSS interacts both with the enzyme and the substrate in an irreversible fashion.

Using hemoglobin, as the natural substrate, mechanistic studies with pepsin revealed that the antipeptic activity of DOSS is due to a major mechanism of substrate-inhibitor interaction. With the synthetic substrate, however, the inhibition of pepsin was found to be due to competition between the substrate and the inhibitor molecules for the enzyme.

Possible therapeutic significance of the inhibitory studies was discussed. A possible use of dioctyl sodium sulfosuccinate in the medical treatment of pancreatitis and peptic ulcers was suggested. Certain implications of the study were also discussed from the points of view of biopharmaceutics and drug interactions.

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CHAPTER I

INTRODUCTION

Body proteinases are molecules of relatively small size and of compact, nearly spherical structure. They generally are among the simple enzymes which are devoid of allosteric regulatory mechanisms for controlling the enzymatic activity. Most of them do not need any activator ions. With the exception of pepsin, most proteinases can withstand rather extreme conditions of pH and temperature. Reversible denaturation is very common among proteolytic enzymes and one of the best investigated examples is the reversible denaturation of trypsin (6). Proteolytic enzymes are destructive agents which could rapidly destroy living cells including those which manufacture them. Therefore, protection against the unwanted effects of these enzymes has to be provided. This is accomplished in a number of ways: (1) Manufacture of the enzymes as inactive precursors, the so called zymogens. (2) The storage of the enzymes in zymogen granules whose membrane are resistant to proteolysis. (3) Presence of powerful and specific natural inhibitors of the active enzymes. Often more than one mechanism is available in the living organism.

Although there is no unique way of classifying these enzymes, various characteristics of these enzymes have formed the basis of different types of classifications (17, 46, 75). Trypsin is an endopeptidase with a serine residue included in the active center and belongs to

the enzymes of animal origin. Pepsin is also an endopeptidase of animal origin but this enzyme unlike trypsin, belongs to the acid proteinases.

Proteolytic enzymes have many similarities in their primary structure in that all of them can be basically compared to the structure of chymotrypsinogen (45). Another important structural feature among these enzymes is the position of disulfide bridges, which results in a similar gross disposition of the polypeptide chains of these enzymes.

Reactions catalyzed by proteolytic enzymes can be divided into five categories as follows: (1) Hydrolysis of peptide bonds. (2) Hydrolysis of amide of amino acids. (3) Peptide bond synthesis and transpeptidation reactions. (4) Hydrolysis of esters of amino acids. (5) Exchange of oxygen between water and the carboxyl group of amino acids. From all five of these types of reactions, hydrolysis of the peptide bond is the natural function of proteinases. Trypsin is maximally active at pH 7 and is rather specific for cleaving all peptide linkages in the substrate protein whose carbonyl groups are contributed by arginine and lysine. Pepsin initiates hydrolysis of native proteins by cleaving peptide linkages in which the amino function is contributed by aromatic and acidic amino acids.

The theories on the role of proteolytic enzymes in physiological and pathological states in man and animals have been the topic of great controversy during the recent years. 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 like 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 a number of disease processes such as inflammation, thromboembolic disorders, complement dependent immune reactions, peptic and duodenal ulcers, pancreatitis and even in some of the syndromes of malignant carcinomas (25). Trypsin and kallikrein which are present in the body tissues and fluids are capable of releasing pharmacologically active plasma kinins (94). These proteolytic enzymes play an indirect but significant role in some of the important conditions like hypertension, shock, certain pains, changed capillary permeability, edema, and leucocyte migration. Much evidence is available to support the central role of trypsin in the pathogenesis of pancreatitis (25, 38, 108). Direct as well as indirect evidences also indicate that through some yet unknown mechanism, the normal resistance of the gastric mucosa to acid and pepsin is compromised in all peptic ulcers (50). Therefore, the role of pepsin in the pathogenesis of peptic ulcers seems significant (67, 88, 97, 106). In the light of this knowledge, the proteinase inhibitors, both synthetic and natural, have gained new theoretical and practical importance (108). Recently attempts have been made to synthesize some active site-oriented inhibitors as well as to improve their inhibitory activity (11, 40, 75). Also, it is reported that proteolytic enzymes are inhibited by agents such as sodium dodecyl sulfate and some sulfated polysaccharides (2, 83).

Diocetyl sodium sulfosuccinate is an anionic surfactant that is widely used medicinally as a fecal softener (52). The effect of this surfactant on drug absorption through membranes of varying complexities has already been reported (41, 59, 71). This agent strongly suppresses the ulcer formation in restrained rats when given intraduodenally (69).

Absorption of dioctylsodium sulfosuccinate into the systemic circulation has been reported (28). These findings and the similarity of the chemical nature of this medicinal surfactant to some proteolytic inhibitors such as sodium dodecyl sulfate and sulfated polysaccharides and protein macroanions (96, 104) led to this investigation dealing with the inhibitory effect of dioctyl sodium sulfosuccinate on trypsin and pepsin *in vitro*. Such an *in vitro* investigation was deemed to be of interest because of the following reasons:

1. Dioctylsodium sulfosuccinate might play an important role in the medical treatment of pancreatitis and peptic ulcers through its inhibitory effects against trypsin and pepsin respectively *in vivo*.
2. The recent practice of polypharmacy, i.e., prescribing drug combinations has led to enormously increasing drug interactions (55, 64 103). The simultaneous use of certain digestive enzymes like trypsin and chymotrypsin along with dioctyl sodium sulfosuccinate may represent such an interaction. An *in vitro* inhibition study would help in predicting and/or explaining such drug-drug interactions.
3. The pharmacological action of many prodrugs is dependent on the action of proteinases of the gastro intestinal tract (98). Therefore, inhibition of these proteolytic enzymes by dioctyl sodium sulfosuccinate may be of significant therapeutic significance.
4. The studies of enzyme inhibitions also provide valuable information about the substrate specificity, the nature of the functional groups at the active site of the enzyme, mechanism of enzyme action and the participation of certain functional groups in maintaining the specific conformation of the enzyme molecule (66).

5. Inhibition studies provide important guidelines for the design of potent specific inhibitors which may prove useful therapeutic agents.

CHAPTER II

SURVEY OF LITERATURE

Role of Body Proteinases in Physiology and Disease

Proteolytic enzymes in protein digestion. Most historical and one of the important physiologic functions of proteinases has been associated with the digestion of food proteins in the gastrointestinal tract (73, 112). Protein digestion begins in the stomach where pepsin is liberated from the pepsinogen by the action of gastric hydrochloric acid. Pepsinogen is secreted by the chief or peptic cells of the stomach. Human gastric mucosa has been shown (95) to contain three chromatographically distinct pepsinogens which produce three different pepsins with slightly different properties (Pepsins I, II and III). Products of pepsin digestion are polypeptides of various sizes. A gelatinase that liquifies gelatin is also found in the stomach. Rennin is another enzyme of stomach responsible for milk clotting and is only found in young animals. Because pepsins have a pH optimum of 1.6 - 3.2, their action is terminated when the gastric contents are mixed with the alkaline pancreatic juice in the duodenum. The pH of the duodenal contents is about 6.5. In the small intestine, small polypeptides and dipeptides are formed by the action of the powerful protein splitting enzymes, trypsin and chymotrypsin which are major enzymes of the pancreatic juice secreted by the exocrine portions of the pancreas. Trypsin and chymotrypsin are produced as their inactive zymogens trypsinogen and several related chymotrypsinogens. Trypsinogen is converted to active trypsin

by enterokinase, an enzyme secreted by the duodenal mucosa. Enterokinase also seems to convert procarboxypeptidases of pancreatic juice to active carboxypeptidases. This enzyme contains about 41% polysaccharides that prevent it from being digested itself before it can exert its effect. Trypsin converts chymotrypsinogens into chymotrypsins. An elastase that hydrolyses fibrous proteins and a collagenase are also present in pancreatic juice. The pancreatic carboxypeptidases and the amino peptidases and dipeptidases of intestinal mucosa split polypeptide fragments into free amino acids. Amino acids may be liberated in the intestinal lumen or at the cell surfaces that line the luminal border of the mucosal cells (58, 77). Dipeptidases activities are low in the proximal part of the duodenum but increase rapidly in its distal part to reach maximum value in the jejunum and ileum (68). The rates of digestion of the dietary proteins vary rather widely, depending upon stomach emptying time, the nature of the protein, and the other food constituents ingested. Various enzymes involved in digestion are present in relatively large amounts, therefore hydrolysis *per se* cannot be considered as a rate limiting factor for absorption of amino acids (18). Thus conversion of many food proteins, collagen, nucleoproteins and certain endogenous proteins to the free amino acids is a prominent physiological function of the various proteolytic enzymes of gastric, pancreatic and intestinal secretions.

Proteolytic enzymes in blood coagulation. Proteolytic enzymes also play important roles in the process of blood coagulation. For over a century blood clotting has been thought to involve enzymatic action (84). While the classical theory of coagulation considered the clotting factors to be enzymes circulating in an inactive or zymogen

form (84) some earlier investigators believed coagulation was the result of a physical combination or stoichiometric reaction of the constituents (84, 92). Both theories turned out to be true, when a dozen separate plasma proteins or clotting factor activities were discovered to be involved in the process of coagulation. These factors are: I, fibrinogen; II, prothrombin; III, thromboplastin; IV, calcium; V, proaccelarin; VII, proconvertin; VIII, antihaemophilic factor (AHF); IX, plasma thromboplastin component (PTC) or Christmas factor; X, Stuart power factor; XI, plasma thromboplastin antecedent (PTA); and XII, Hageman factor (84, 113). Factor Ia is covalently bonded or urea insoluble fibrin; XIIIa is the activated form of the factor XIII (fibrinoligase), fibrin stabilizing factor and PL is Platelet phospholipid.

The coagulation of blood results both from enzymatic and stoichiometric reactions (56) and involves the activation of perhaps five zymogens to enzymes (XIIa, IXa, Xa, IIa, and XIIIa) and the formation by physical combination of three complexes (34). The fundamental reaction in the clotting of blood is conversion of the soluble plasma protein fibrinogen to fibrin. The fibrin molecules polymerise to form a loose mesh of insoluble interlacing strands and this polymer is converted by the formation of covalent cross linkages to a dense, tight aggregate. This latter reaction is catalyzed by factor XIII, the fibrin stabilizing factor or fibrinoligase, an important proteolytic enzyme. The clotting mechanism responsible for the formation of fibrin involves a complex series of reactions (Fig. 1). In this complex sequence the conversion of fibrinogen to fibrin is catalyzed by thrombin, another proteolytic enzyme. Thrombin is formed from its circulating precursor prothrombin, by the action of activated factor X. Factor X can be activated by reactions

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Intrinsic System

Extrinsic System

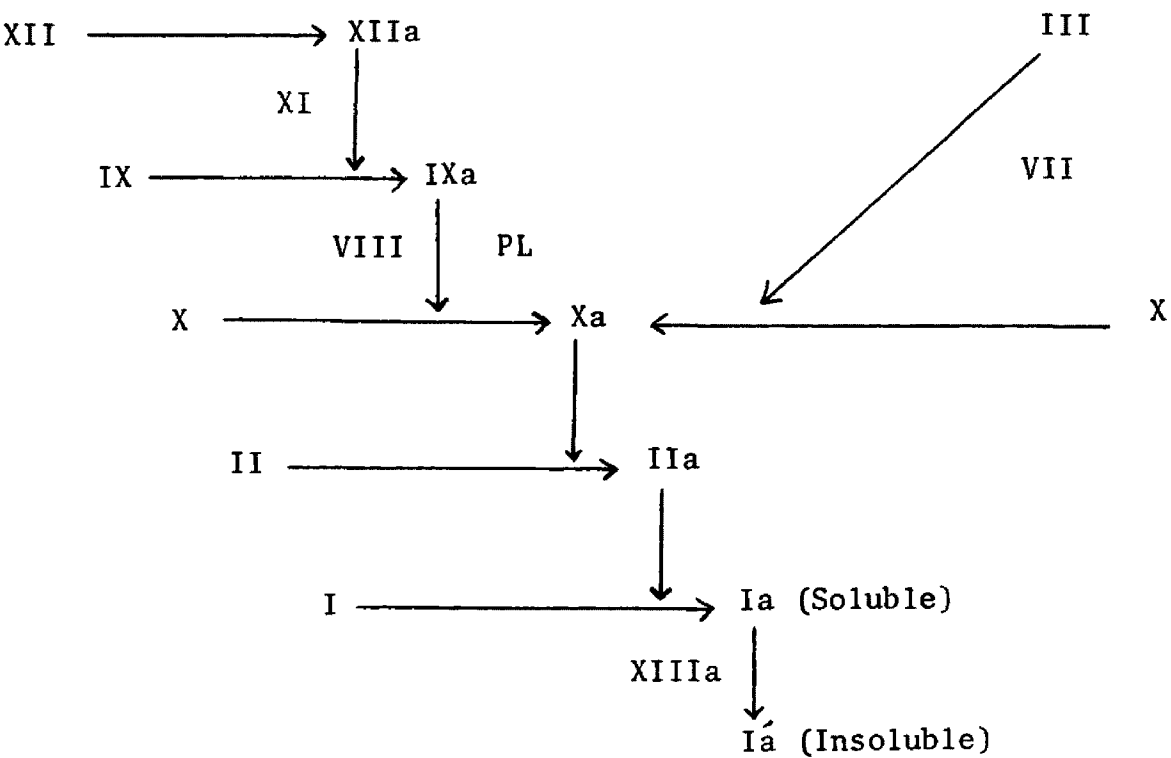


Figure 1.--Diagrammatic Summary of the Clotting mechanism.

that proceed along either of two pathways, an intrinsic and an extrinsic pathway as shown in Figure 1. The complicated and repetitive pattern of coagulation acts as a biologic amplifier in which a several fold gain of activity is achieved with each stage (70). Some product of coagulation accelerates the development of thrombin and fibrin, hence blood clotting can be termed an autocatalytic reaction. It is this chain reaction pattern and explosive characteristic of blood coagulation that contributes to the growth of a hemostatic plug or the propagation of a thrombus (78).

In addition to their role in blood clotting certain proteinases also seem to participate in the anticlotting mechanisms of blood. Fibrinolytic system of the body is such a mechanism that limits clotting. The active component of this system is a proteolytic enzyme, plasmin or fibrinolysin. This enzyme not only cleaves fibrin but also attacks factor V, factor VIII and fibrinogen, with the liberation of substances that inhibit thrombin.

Proteolytic enzymes in pancreatitis. Pancreatitis can be regarded as a chemical autolytic process the pathogenesis of which is well documented from postmortem, operative and experimental studies (15). The exact mechanism of the pathological process has yet to be established. However, evidence is available supporting the central role of certain body proteinases in the pathogenesis of the disease. It has been reported (25) that pancreatic duct obstruction coupled with intrapancreatic conversion of trypsinogen to trypsin is one of the most important mechanisms in the production of pancreatitis. This hypothesis was challenged by Beck and coworkers (14) who demonstrated that no free trypsin was found in hemorrhagic or necrotizing pancreatitis in dogs.

However, this can easily be accounted by the presence of pancreatic and serum inhibitors. The recent reports (74) on the usefulness of specific trypsin inhibitors such as trasylol (trypsin inhibitor from bovine organ) have further strengthened the case for the importance of trypsin in pancreatitis.

Furthermore, some workers (22, 23, 43, 80-82) have reported higher levels of plasma trypsin in cancer of pancreas and acute pancreatitis as compared to those in the normal subjects. Nevertheless, contradicting reports (9, 10, 25) also prevail claiming no differences between the plasma trypsin levels of patients with pancreatitis and those of normal individuals. These controversial claims may be explained due to the fact that different groups of workers used different synthetic substrates. Lack of a reproducible assay which is specific for trypsin in human plasma or serum may obviously be another reason.

Another mechanism through which trypsin may cause pancreatitis may be due to its ability to liberate certain vasodepressor kinins from serum globulin. Evidence has been obtained that trypsin is enhanced in its ability to produce pancreatitis by incubation with serum (1). The ability of bradykinin to cause hypotension, pain, vasodilatation, increased vascular permeability, smooth muscle stimulation, leucocyte migration, and leucocyte accumulation also suggests the possibility that bradykinin may play a part in a disease process such as acute haemorrhagic pancreatitis, characterised by hypotension, pain, haemoconcentration, dynamic fluid shifts, leucocytosis, edema and leucocyte infiltration of the gland (94). This hypothesis is further supported by the fact that pancreas contains large quantities of trypsinogen and kallikreinogen, both of which in active form, are capable of releasing

bradykinin from its inactive precursor, bradykininogen. Bradykininogen, as a normal pseudoglobulin of plasma, lymph and interstitial fluid is readily available to the pancreas.

Two important systems related to the proteolytic release of pharmacologically active kinins in the body have recently been described (76, 85). These are renin-angiotensin system and the kinin system. Renin is the proteinase involved in the renin system and the kinin system involves certain kininogenases, and kininases as the active enzymes. Plasmin and trypsin are also able to release bradykinin from their kininogen substrates. The particular proteolytic enzymes involved may be from a glandular source or of plasma.

Acute pancreatitis can also be related to the mechanism of activation of pancreatic proteinases (30). Studies on the enzyme mechanisms involved suggest that during activation of pancreatic proteinases, enterokinase converts trypsinogen into trypsin. Trypsin then reacts with trypsin inhibitor to form a trypsin-trypsin inhibitor complex. After interaction with bile, trypsin inhibitor decreases and free trypsin appears. Free trypsin then converts inactive zymogens of pancreas into active proteases. Trypsin inhibitor is thought normally to protect the pancreas against this premature activation within the gland. When trypsin inhibitor becomes depleted in a damaged pancreas (ischemia) it is postulated that the emerging proteinases digest the parenchyma and acute hemorrhagic pancreatitis results.

Trypsin also converts phospholipase A into the active enzyme, phospholipase A. This enzyme splits a fatty acid off lecithin forming lysolecithin. Lysolecithin damages cell membranes. The cause of acute pancreatitis may be activation of phospholipase A in the pancreatic

ducts, with the formation of lysolecithin from lecithin which is a normal constituent of bile. This may cause disruption of pancreatic tissues and necrosis of surrounding fat (38).

Proteolytic enzymes in peptic ulcers. Peptic ulcers can be defined as acute or chronic ulcerations of the digestive tract, occurring in an area accessible to gastric secretions. It is a common disorder of gastro intestinal tract that even today continues to be an incompletely understood disease. Although very little is known about the definite etiology of the disease, yet an interrelationship of a number of mucosal defensive factors and aggressive factors seems to determine an individual's susceptibility to ulcers (50, 93). Nevertheless it is important to note that none of these factors itself, has been shown to exert a strong enough effect to result in the large variations seen among various populations in the incidence of peptic ulcers (50). Whatever may be 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 (50). Some direct experimental evidence is available indicating the importance of pepsin in the pathogenesis of the disease (67, 88, 97, 106). In humans, the role of pepsin in gastroduodenal ulceration has been indirectly evidenced by the antiulcer activity shown by some macroanions which also inhibit peptic hydrolysis *in vitro* (96, 104). Pathophysiology of peptic ulcers may involve abnormalities of acid-pepsin secretion, presence of acid-pepsin at unusual sites or predisposition of the mucosa to ulceration (51). Such pathophysiological abnormalities may be a consequence of various physiological, anatomical, epidemiological, genetic or even environmental factors (38, 51).

Weiss and Serfontein (110) have recently indicated some of the qualitative and quantitative biochemical differences between the ulcerated tissues and the surrounding normal tissues. Compounds like hexosamines, hyaluronic acid, mucopolysaccharides and the glycoproteins seem to be present at higher concentrations and in more reactive forms at the ulcerated tissue than at the normal surrounding cells.

Proteinases may play an important role in the inflammatory process by releasing vasoactive peptides. From the fifth component of complement (C₅) they also release a potent chemotactic factor (C_{5a}) that can attract macrophages and polymorphonuclear leukocytes to the site of inflammation. Macrophage proteinase may therefore be an important mediator of parts of the chronic inflammatory process (72).

Miscellaneous. In addition to their presence in the oxyntic glands and the pyloric glands of the stomach, the pepsin zymogens are also reported to be present in the proximal duodenum, in seminal fluid (of seminal vesicles origin), in amniotic fluid (probably of fetal origin) and in blood and urine (96). Pepsin determination either as uropepsin or plasma pepsin (42, 79) has been a standard clinical practice. Uropepsin values are high in duodenal ulcers and low in pernicious anemia. Uropepsin is decreased in cirrhosis, Addison's disease, panhypopituitarism, and myxedema (25). Blood pepsin seems to reflect the total secretory potential of the stomach (102) yet in superficial gastritis with achlorhydria blood pepsin may rise due to regurgitation from glands blocked with inflammatory cells. The blood levels of pepsin may decrease if gastric atrophy predominates.

The use of chymotrypsin in ophthalmology for extracting cataract and in certain inflammatory diseases has been reported (25). Although

the role of trypsin in chemotherapy is controversial, the use of this proteolytic enzyme in pulmonary alveolar proteinosis, in promoting healing of opisthomyiasis and certain inflammatory processes has been indicated (25). Small amounts of trypsin have also been reported to accelerate blood clotting (35).

Elastase is another pancreatic proteolytic enzyme which can convert prothrombin to thrombin, thereby shortening the clotting time but it has no action on fibrinogen. Elastase has not been determined in serum but it has been claimed by certain workers that this enzyme plays a central role in atherosclerosis (12, 25). However, any role for this enzyme in human physiology and disease needs more investigations. Certain kallikreins in the human plasma and urine have been reported which differ from the pancreatic kallikrein in their immunologic properties. Besides their role in the pathogenesis of pancreatitis and certain other inflammatory diseases, this enzyme has also been implicated in the production of certain aspects of malignant carcinoid syndromes (25).

Plasmin, leucine amino peptidase, carboxypeptidases A and B, cathepsins and certain other proteinases of human body have been reviewed by Colman in relation to their role in physiology, pathology and therapeutics (25).

Proteinase Inhibitors

Enzyme inhibition is an extensively studied field of enzymology. An important aspect of this work is concerned with the identification of active site residues and structure and function of the active center of enzyme. Enzyme-inhibitor interactions are also important from the point of view of kinetics of the reaction. The practical importance of these studies lies with their utility in the control of certain diseases.

Various inhibitors of proteolytic enzymes can be classified into several categories according to their mode of action: (1) Inhibitors irreversibly destroying the enzyme. (2) Noncompetitive reversible inhibitors. (3) Competitive reversible inhibitors. (4) Active site directed irreversible inhibitors. (5) Specific protein inhibitors.

These proteolytic inhibitors, in particular the specific protein inhibitors of both synthetic and natural origin have proved very useful in certain diseases. Pancreatitis and peptic ulcers are two major disorders among such diseases in which trypsin and pepsin respectively have been shown to play a central role. Therefore, important inhibitors of these two proteolytic enzymes are discussed as follows.

Trypsin inhibitors. A reversible competitive trypsin inhibitor from the extract of Ascaris lumbricoides has been isolated by Pudles et al (89). One microgram of the inhibitor has been found to give 100% inhibition of 0.5 μ g of trypsin and the dissociation constant of the complex is 3×10^{-9} m. This inhibitor can also inhibit kallikrein. Along with trypsin inhibitor, in the ascaris extract, a pepsin inhibitor has also been reported. The presence of these inhibitors in Ascarides is thought to be a defense mechanism against the proteinases of the host organism.

Kunitz isolated a trypsin inhibitor from bovine pancreas (63). Pancreatic inhibitor is characterized by a high stability to acids, to heat and to pepsin. The stability decreases with increasing alkalinity. The isoelectric point is at pH 10 or 10.5. The inhibitor seems to form salts at acid pH values and is resistant to proteolytic degradation. This inhibitor has also been referred to as bovine trypsin kallikrein inhibitor, polyvalent inhibitor, Kunitz inhibitor and basic pancreatic

trypsin inhibitor. It is commercially available in highly purified form under the names Iniprol and Trasylol. Another trypsin inhibitor has been found in human, bovine and pig colostrum in an activity ratio of 1:10:67 (108). One milliliter of pig colostrum gives 100% inactivation of 2 mg of trypsin. One milliliter of cows' milk gives 50% inhibition of the proteolytic activity of 500 μ g of trypsin. The maximum inhibitor concentration in human milk is reached between the third and fifth day post partum and corresponds to the inhibition of 50-70 μ g of trypsin per milliliter of milk. The inhibitor is stable to acid and heat. It has an isoelectric point at pH 4.2. A trypsin inhibiting component has been detected in the placenta (108) and in the amniotic fluid (108), as well as in the heart, lungs, and brain of various species (21, 108).

Human blood serum has been reported to contain two trypsin inhibitors (16, 20, 24, 29, 32, 108). These inhibitors have been located to be present in the α_1 and α_2 globulin fractions. The inhibitor from the α_1 fraction is an inhomogenous inhibitor which is unstable to heat and acids. It is not dializable and is precipitated by trichloroacetic acid. Its isoelectric point is pH 4.0. The second trypsin inhibitor which belongs to α_2 globulin fraction, is characterized by a strong and instantaneous inhibiting capacity for plasmin. This inhibitor, therefore, is also known as α_2 -macroglobulin plasmin inhibitor. The inhibition is stoichiometric and reversible. This inhibitor is relatively stable to heat and can also inhibit chymotrypsin. One milligram of inhibitor inhibits 17-40 μ g of trypsin.

Haverback (47-48) found that human pancreatic juice obtained by drainage contained an inhibitor for trypsin and chymotrypsin which is soluble in trichloroacetic acid and is stable to trypsin and chymotrypsin.

In duodenal secretion the trypsin inhibitor of pancreatic juice is destroyed in the trypsin-inhibitor complex by excess trypsin, or chymotrypsin. The inhibitor level in the pancreatic juice can fall to minimal level in experimental pancreatitis.

In chronic pancreatitis the ratio of the inhibitor to the trypsin in the duodenal secretion is reduced from 4.1 to 1.3 by a high protein diet (19). This inhibitor is different from the Kunitz's pancreatic inhibitor in structure and properties.

A specific trypsin inhibitor from the extracts of ovarian tumors has been reported (19). The inhibition of trypsin is more pronounced for the extracts of benign tumors than those of malignant ones. The nature of the inhibitor is yet poorly characterized.

Trypsin inhibitors from various bovine organs like parotid glands, liver and lungs have also been described. Astrup found that human urine contains a trypsin-inhibiting component that can be separated from urokinase by fractionation on calcium phosphate gel (7 - 8). One microgram of the purified inhibitor inhibits 0.83 μ g of trypsin. The inhibitor shows practically no loss of activity at pH 2-3.5 after 30 minutes at 100° C. It is an acidic polypeptide, whose isoelectric point is pH 2.1.

An organ specific and sex specific trypsin inhibitor has recently been discovered. This inhibitor has been found in the vesicular glands and semen of all mammals including man (108). The inhibitor is a basic polypeptide which is stable to heat and acids. This inhibitor forms 1:1 complex with trypsin and the complex formed is reversible.

A trypsin inhibitor from the submandibular and sublingular glands has been isolated. The inhibitor is a strongly basic polypeptide with

isoelectric points of pH 12 and 12.5 (108). One microgram of inhibitor inhibits 3 μ g of trypsin and has maximum effect between pH 7-8.

A trypsin inhibiting principle from Clostridium-botulinum and a trypsin inhibitor from Aspergillus-soyae that preferentially inhibit the microorganism's own alkaline proteinase, have been described (54, 108). These inhibitors need further examination in detail.

A number of useful trypsin inhibitors have also been obtained from various plant sources. Soybean inhibitor is the most extensively studied of all the natural proteinase inhibitors. Trypsin is known to combine with this inhibitor in a stoichiometric, reversible, pH dependant reaction (60). It has been found to inhibit trypsin in a noncompetitive fashion in the presence of a natural substrate. Kinetic and thermodynamic and other biochemical studies of the interaction between this inhibitor and trypsin have been so extensive that this inhibitor has become a model substance for inhibitory studies of trypsin.

Sohonie isolated the trypsin inhibitor from mung beans (100). It has a molecular weight of 18,000, is relatively stable to heat and it inhibits trypsin stoichiometrically. Crystalline trypsin inhibitors have also been isolated from field beans and broad beans (37, 100-101). Two other trypsin inhibitors of plant source have been isolated from lima beans and garden beans respectively. The inhibitor from the lima beans inhibits trypsin in a molar ratio of 1:1 and has an inhibition constant of $10^{-8}m$.

Vogel et al (108) recently found another trypsin inhibitor from beet. The inhibitor is precipitated by trichloroacetic acid; it is stable to acid. However, it is not stable to heat and cannot be dialyzed. One gram of beet root tissue has been shown to give 50% inhibition of about 25 μ g of trypsin.

Trypsin inhibitors have also been isolated from several grains, e.g., whole wheat flour, cord seed, rye and wheat germ, and barley (108). The barley inhibitor is stable at 100° C for 15 minutes at pH 4.9 and to one hour's exposure to pH 2.1 at 35° C. It has equal effect on the tryptic digestion of casein, haemoglobin and certain synthetic substrates.

Various synthetic inhibitors of trypsin have also been described. Some of the competitive inhibitors of this kind are compounds such as phenyl-trans-4 amino methylcyclo hexane carboxylate, p-aminobenzamide, benzamide, benzyl ϵ -aminocaproate hydrochloride, thionine, 4-amino-methylbenzoic acid benzyl ester, proflavin, phenylguanidine, and benzylamine (75). N- α -tosyl-L-lysine chloromethyl ketone, ethyl-p-guanidino benzoate, bromoacetone, p-amidino-phenacyl bromide, and p-guanidino-phenacyl bromide are among the more important active site directed irreversible inhibitors of trypsin (75). Recently a number of diamidino- α , ω -diphenoxyalkanes have been synthesized by Geratz et al (39 - 40) which have proved to be active site directed reversible inhibitors of trypsin and certain other proteinases.

Pepsin inhibitors. Pepsin is inhibited by a host of natural inhibitors which also inhibit trypsin and certain other proteinases (108). Most of these inhibitors have already been described as trypsin inhibitors. However various synthetic inhibitors which specifically inhibit pepsin have also been synthesized (75). Among these, compounds such as N-acetyl-D-phenylalanyl-L-diiodotyrosine, L-phenylalanyl-L-phenylalanine methyl ester, and benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine inhibit pepsin competitively (75). Although these inhibitors represent some of the strong inhibitory agents of pepsin, their utility in the disease of peptic ulcers has not been indicated.

Among the therapeutically useful pepsin inhibitors are the macroanion inhibitors and certain glycopeptides as well as sulfated polysaccharides. Some of the more important characteristics of these inhibitors that seem to be related to their inhibitory activity and hence their antiulcer action, may be described as follows: (1) These inhibitors possess a net negative charge and the interaction of the negatively charged inhibitor with the positively charged protein substrate results in the inhibition of peptic hydrolysis. (2) Of all the physical and chemical parameters molecular size and degree of sulfation seem to be most significant in determining the antipeptic activity of the inhibitor. The molecule has to be a certain minimum size to show pepsin inhibitory activity. The inhibitory activity appears to be directly correlated with the sulfate content of the molecule. Antiulcerogenic activity also seems to be related to high sulfur content. (3) The effect of pH, order of addition of the reactants, ratio, and nature of the interacting species of different macroanions and substrates on the inhibition of peptic activity have been found to be important.

Barnes et al (94) have shown the antipeptic effect of chondroitin sulfate, heparin, carrageen and dextran sulfate on peptic activity *in vitro*. They also studied the effect of these inhibitors on the mortality and incidence of ulcers in pylorus ligated rats. Equivalent amounts of heparin, carrageen and dextran sulfate produced equivalent inhibition of peptic activity *in vitro*, but dextran sulfate was more effective both in decreasing the mortality and the incidence of ulcers.

A number of lignosulfonates have been shown to exert antipeptic activity as well as antiulcerogenic effects (105, 107). Inhibition of peptic activity by carbonoxolone and glycyrrhetic acid has been reported by Henman (49).

A concentration and pH dependant inhibition of pepsin by heparin, ribonucleic acid, chondriotin sulfate and a sulfated glycoprotein fraction from human gastric juice has been shown by some workers (3, 53). Significant inhibitory effect on pepsin and a reduction of the occurrence of peptic ulcers have been reported with SN-263 (sulfated amylopectin or depensen) and carrageenan (31).

Fogelson (36) in an uncontrolled study, treated 12 patients with gastric ulcer with a crude preparation of gastric mucin, which can inhibit pepsin. Calcium chondriotin sulfate and paritol C have also been found to possess some antipeptic activity.

Cook et al (26) have reported the antipeptic and antiulcerogenic activity of a number of synthetic sulfated polysaccharides. They also correlated a number of physical and chemical parameters of these inhibitors to their inhibitory effects and the antiulcerogenic activity.

The inhibition of the peptic activity of human gastric secretion by undegraded and degraded carrageenans of similar sulfate content has been examined at various pH values by Anderson and Baillie (2). These workers also showed the protective action of carrageenans against experimental ulcers in guinea pigs (4). Alkalies are among the most potent antagonists of pepsin. Any base raising the pH of the gastric juice to 7 or above will inhibit most of the pepsin within a few seconds. Sodium dodecylsulfate has also been shown to significantly reduce the pepsin activity within 2 hours (83).

By far the most active and specific inhibitor of pepsin is pepstatin, which is a pentapeptide with two γ -NH linkages, two β -OH groups and five branched aliphatic side chains. It has been reported to be nontoxic and is produced by Banyu Pharmaceutical Co. in Tokyo for the treatment of peptic ulcers (72).

Antipeptic activity of sulfate glycopeptide has been recently reported by Prino et al (88). The glycopeptide was shown to inhibit peptic activity of human gastric juice. It also proved effective in duodenal ulcers in man and animals when tested *in vivo*.

Thus, most of the macroanion pepsin inhibitors have proved to be of significant value in peptic ulcers of animals and man. However additional well controlled clinical studies are needed to completely delineate the efficacy of these inhibitors in the treatment of peptic ulcers.

CHAPTER III

OBJECTIVES OF RESEARCH

In spite of the fact that dioctyl sodium sulfosuccinate (DOSS) is widely used in medicine as fecal softener, no report is available of its systemic use. Probably because until recently DOSS has been claimed to show no absorption in the blood. Dujovne and Shoeman have now shown that this compound is significantly absorbed into systemic circulation (28). Lish (69) has shown that DOSS when administered intraduodenally strongly suppresses the ulcer formation in restrained rats. These findings and the similarity of the chemical nature of this surfactant to some proteolytic inhibitors such as sodium dodecyl sulfate and sulfated macroanions suggested possible inhibitory effects of DOSS against trypsin and pepsin. No study dealing with the inhibitory effect of DOSS against these enzymes has been previously reported. Since, it has been established that trypsin and pepsin play a central role in the pathogenesis of pancreatitis and peptic ulcers respectively, an *in vitro* investigation of the inhibitory effect of DOSS against these proteolytic enzymes was deemed necessary in order to delineate the possible use of DOSS in pancreatitis and peptic ulcers.

The objectives of this study are the following:

1. Determination of the nature and extent of inhibitory effect of DOSS on trypsin and pepsin activities *in vitro* using both natural and synthetic substrates.

2. To study the effect of various reaction conditions such as inhibitor concentration, enzyme concentration, substrate concentration, pH, and various mixing orders of the components.
3. To delineate the inhibitory mechanisms involved in the inhibitory action of DOSS against trypsin and pepsin through kinetic experiments.
4. Projection of the results of the *in vitro* inhibitory studies to the more realistic *in vivo* situations of disease in pancreatitis and peptic ulcers.

CHAPTER IV

STUDIES WITH TRYPSIN

I. EXPERIMENTAL

Materials

The following materials were used in these studies:

Trypsin (Beef pancreas, twice crystallized, salt free, lyophilized).

Nutritional Biochemicals Corporation.

Casein (Hammersten type).

Nutritional Biochemicals, Inc.

N α -benzoyl-DL-arginine-p-nitroanilide hydrochloride (DL-BAPNA).

Schwartz/Mann, Dickinson and Company.

Dioctyl sodium sulfosuccinate (DOSS).

"Aerosol O.T. 100%", Sargent-Welch Scientific Co.

Trizma base.

Tris (hydroxy-methyl) amino methane, Sigma Chemical Co.

Dimethyl Sulfoxide.

J. T. Baker Chemical Co.

All other chemicals used were either U.S.P. or reagent grade.

Procedures

Methods for antitryptic activity. Two different methods for determining antitryptic activity were used.

Method I. Sørensen phosphate buffers (0.1M) of various pH values were used. A fresh 1% casein (S) 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 minutes. Enzyme (E) solutions of various concentrations ranging between 10-50 μg per 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 (I) solutions were prepared by dissolving required amounts of DOSS in the buffers, at appropriate pH.

The assay was carried out by a modification of Kunitz's method (61). In this method casein is digested under standard conditions. The undigested casein is precipitated with trichloroacetic acid, and the amount of unprecipitated protein split products, which is a measure of the proteinase activity, is estimated spectrophotometrically in u. v. range. All the 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:

A. Inhibitor added to enzyme before digestion. One milliliter of enzyme solution was pipetted into 50 ml Erlenmeyer flasks followed by 1 ml of the inhibitor solution. Five ml of the substrate solution was then added to each flask at 30 second intervals. All the flasks were incubated in a water bath metabolic shaker¹ maintained at 37° C for one hour. Samples were shaken at 200 r.p.m. throughout the digestion period. At the end of incubation period, 3 ml of 10% trichloroacetic acid solution was added to each flask, in the same order, and allowed to stand at room temperature for 10 minutes. Blanks were prepared exactly in the same way as samples except that the casein solution was added after the addition of trichloroacetic acid. Both samples and blanks were then

¹Model G-77, New Brunswick Scientific Co., Inc., New Brunswick, N. J.

centrifuged at 8000 r.p.m. in a Sorvall SS-4 superspeed centrifuge² for 20 minutes and the supernatants filtered through Whatman #2 filter papers. The absorbance of each filtrate was read at 280 nm against the respective blank using a Spectronic-600 spectrophotometer³. Also, 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 all the kinetic studies. The inhibition (i) equals $(1 - \frac{v_i}{v})$, where v_i and v represent digestion velocities with and without the inhibitor respectively. Percentage inhibition values were calculated from values of (i).

B. Inhibitor added to substrate before digestion. The procedure for digestion was as in (A) above except that the inhibitor solution was added to the substrate first and mixed, followed by addition of enzyme.

Method II. In this method $N\alpha$ -benzoyl-DL-arginine-p-nitroanilide hydrochloride (DL-BAPNA), was used as the synthetic substrate. DL-BAPNA is a chromogenic substrate of trypsin and on tryptic hydrolysis releases p-nitroaniline which is yellow and can be estimated colorimetrically. The procedure used was that of Erlanger et al (33) with a few modifications.

Forty-three and five tenths (43.5) milligram DL-BAPNA was dissolved in one ml dimethyl sulfoxide and the solution was brought to 100 ml with 0.05 M tris buffer at pH 8.2 and containing 0.02 M $CaCl_2$. Care was taken to dissolve all of the DL-BAPNA in dimethyl sulfoxide.

²Ivan Sorvall, Inc., Norwalk, Conn.

³Analytical Instruments, Bausch & Lomb, Rochester, N. Y.

Presence of any crystals caused precipitation on standing. The temperature of the stock solution was never allowed to fall below 25° C.

Various concentrations of enzyme and inhibitor solutions were prepared as described in Method I except that the buffers used were 0.05 M tris (hydroxy methyl) aminomethane buffers of various pH values.

According to the assay procedure, all the solutions were allowed to equilibrate at 37° C for 5 minutes before mixing. One milliliter of enzyme solution was pipetted into each of the 50 ml Erlenmeyer flasks, which were already brought to 37° C. This was followed by addition of 1 ml of the inhibitor solution. At zero time, 5 ml of the substrate solution was added into each of the sample flasks. Appropriate blanks and controls were prepared in the same manner as described in Method I. Incubation of the samples, blanks and their respective controls was done at 37° C for 300 seconds in a water bath metabolic shaker except for data of Table 13, and Figure 12, in which case incubation was done for 600 seconds. All the flasks were shaken at 100 r.p.m. throughout the incubation period. The reaction was stopped by adding 1 ml of 30% acetic acid to each flask. The final volume of the reaction mixture was kept at 8 ml in each case. All incubation mixtures were centrifuged and then filtered as described in Method I. The absorbance of the filtrates of both samples and blanks was recorded at 410 nm against distilled water using a Beckman ACTA CIII Spectrophotometer. Difference between the absorbance of samples and blanks represent the proteolytic activity. Inhibition values (i) and the percentage inhibitions were calculated as described in Method I. Different orders of mixing were also carried out according to the two procedures used in Method I.

Pre-incubation studies. In the pre-incubation studies the inhibitor was allowed to react additionally for 15 minutes either with the enzyme or with the substrate before digestion was started. The controls were also pre-incubated for the same period of time. Digestion was carried out according to Methods IA and IB.

Dialysis studies. In order to know whether the interaction of inhibitor towards substrate and enzyme is reversible or irreversible, dialysis studies were carried out at pH 6.4 and pH 7.6.

Regenerated cellulose dialysis tubings⁴ were cut into pieces of 6-9 inches and thoroughly washed with distilled water. The tubings were then immersed in 500 ml of 10 mM sodium edetate solution and heated at 70° - 80° C for 2 hours. After this, the tubings were allowed to cool, washed again thoroughly with double distilled water and stored in distilled water at 2° - 4° C in a refrigerator. Prior to use, the tubings were soaked in appropriate buffer for 18-24 hours.

The inhibitor was added to the enzyme or substrate and the mixture incubated at 37° C for 60 minutes in a metabolic shaker at 200 r.p.m. A portion of the mixture was then assayed for tryptic activity using Method I and the rest of the mixture was put in a pre-conditioned dialysis tubing sac. The sac was immersed in 1000 or 2000 ml of 0.1 M phosphate buffer of appropriate pH and dialysis carried out in a cold room at 4° C for 96 hours. The external buffer was continuously stirred with a stirring magnet and was replaced every 24 hours during the dialysis period, at the end of which the volume of the contents was measured and assayed for its tryptic activity again. No significant change in the

⁴No. 20, 5/8 inches in flat width for trypsin dialysis and No. 36, 9/8 inches in diameter for casein dialysis. Average pore sizes 24°A, Van-Waters and Rogers, Inc., San Francisco, Calif.

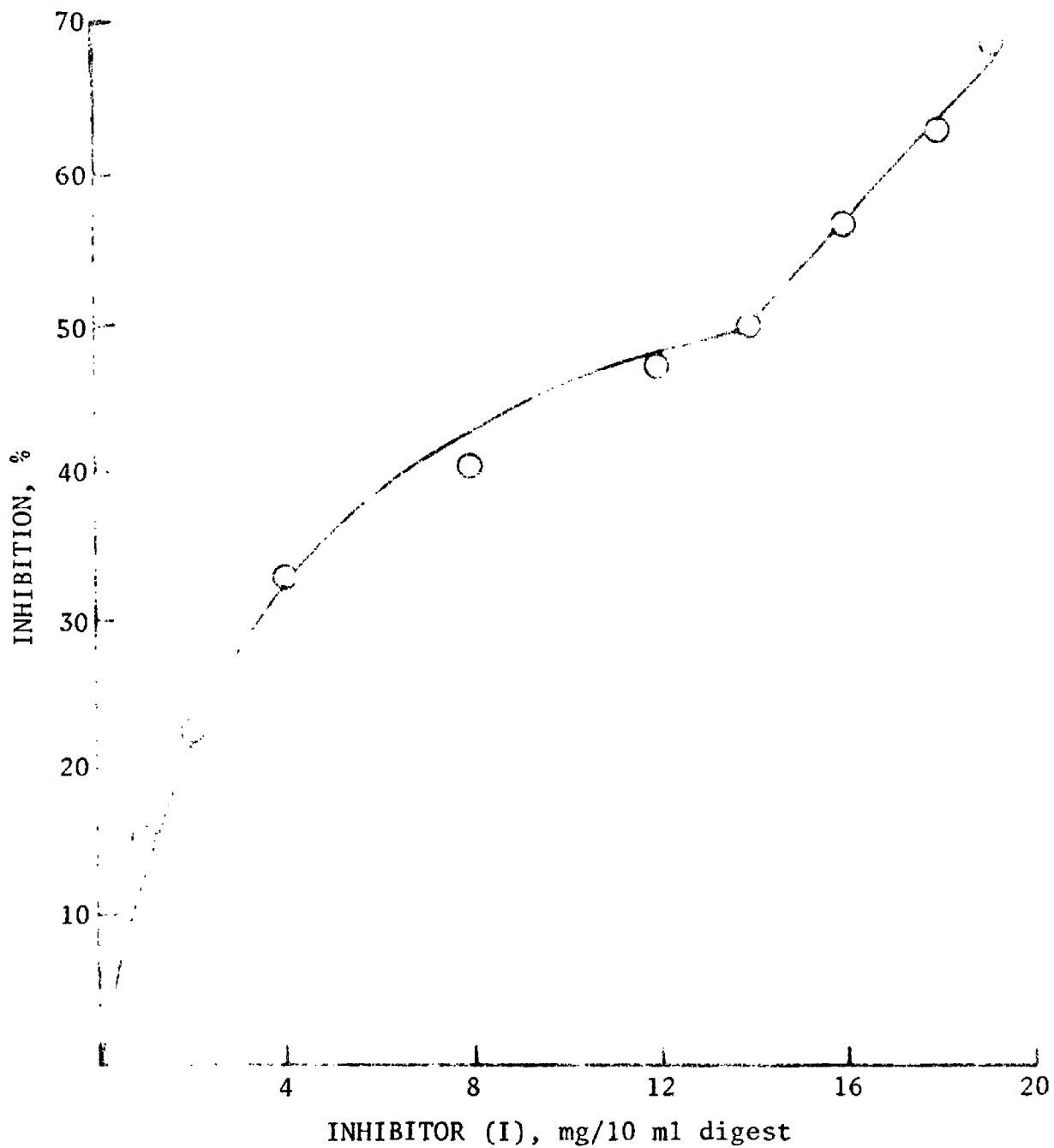


Figure 2.--Effect of various inhibitor concentrations on the percentage inhibition of trypsin activity at pH 7.6. Ten ml of digestion mixture contained 10 μ g enzyme (E) and 50 mg casein (S).

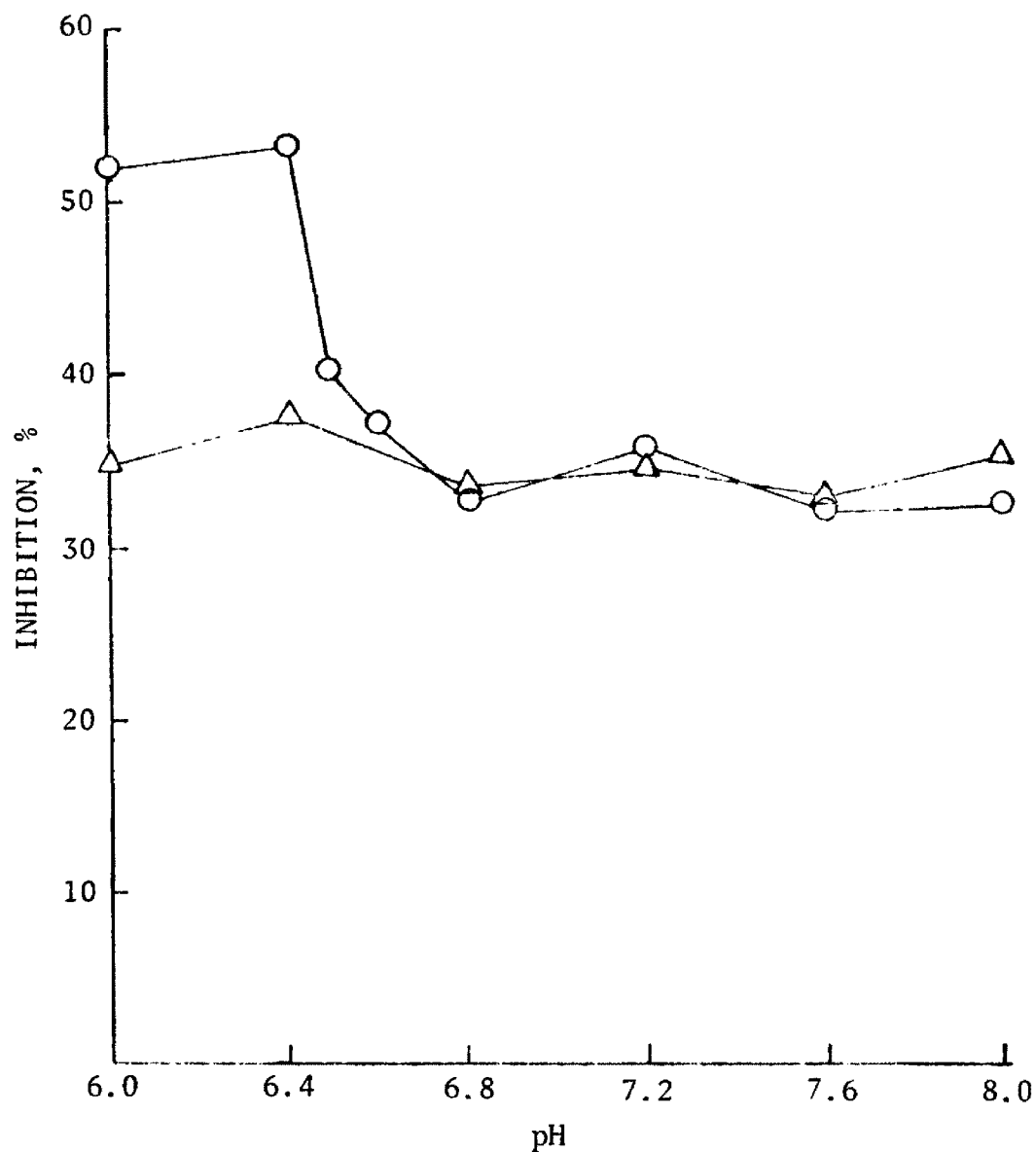


Figure 3.--Effect of pH and mixing orders on percentage inhibition of trypsin activity. Ten ml digest contained, E=20 μ g; S=50 mg; I=10 mg. Key: O, inhibitor added to enzyme first; and Δ , inhibitor added to substrate first. Substrate used was casein.

TABLE 1

Effect of Various DOSS Concentrations on the Percentage Inhibition of Trypsin Activity at pH 7.6

Amount of the Components in 10 ml of Digestion Mixture			Absorbance ^b at 280 nm against Respective Blanks	Inhibition, % of Control Activity
Enzyme (µg)	Substrate ^a (mg)	DOSS (mg)		
10	50	Control	0.511	--
10	50	1	0.423	18.0
10	50	2	0.394	23.0
10	50	4	0.340	34.0
10	50	8	0.303	41.0
10	50	12	0.266	48.0
10	50	14	0.253	51.0
10	50	16	0.218	58.0
10	50	18	0.187	64.0
10	50	20	0.158	69.0

^aCasein

^bAverage of at least three determinations.

volume of the sac contents was noticed after dialysis. A control dialysis experiment was run simultaneously in the same way except that the inhibitor solution was replaced by equivalent amount of buffer solution. Percentage inhibition values before and after dialysis were calculated as mentioned in Method I.

All the above experiments were carried out at least in duplicate.

II. RESULTS AND DISCUSSION

In all the trypsin studies, the inhibitor was added to the enzyme first (Mixing Order A) except for the data of Table 2 and Figure 3 in which case both Mixing Orders A and B were used.

The inhibitory effect of DOSS on tryptic activity was studied at various inhibitor concentrations using Method I (Table 1). A biphasic plot (Fig. 2) resulted on plotting the percentage inhibition against the inhibitor concentration. It is possible that at lower concentrations DOSS inhibits only by interacting with either the substrate or the enzyme but at higher concentrations of DOSS additional denaturation of the protein (trypsin or casein) also takes place. The biphasic plot in Figure 2 can also be explained as due to the saturation of either the enzyme or the substrate with the inhibitor. This explanation seems more likely in the light of the mechanistic studies to be discussed later.

The effect of pH and mixing orders on the percentage inhibition was also studied using Method I. The results are shown in Table 2. A plot of these results was made as shown in Figure 3. Figure 3 shows that when the inhibitor was added to the substrate first (Mixing Order B), the percentage inhibition remained reasonably constant throughout the pH range studied. When the inhibitor was added to the enzyme first

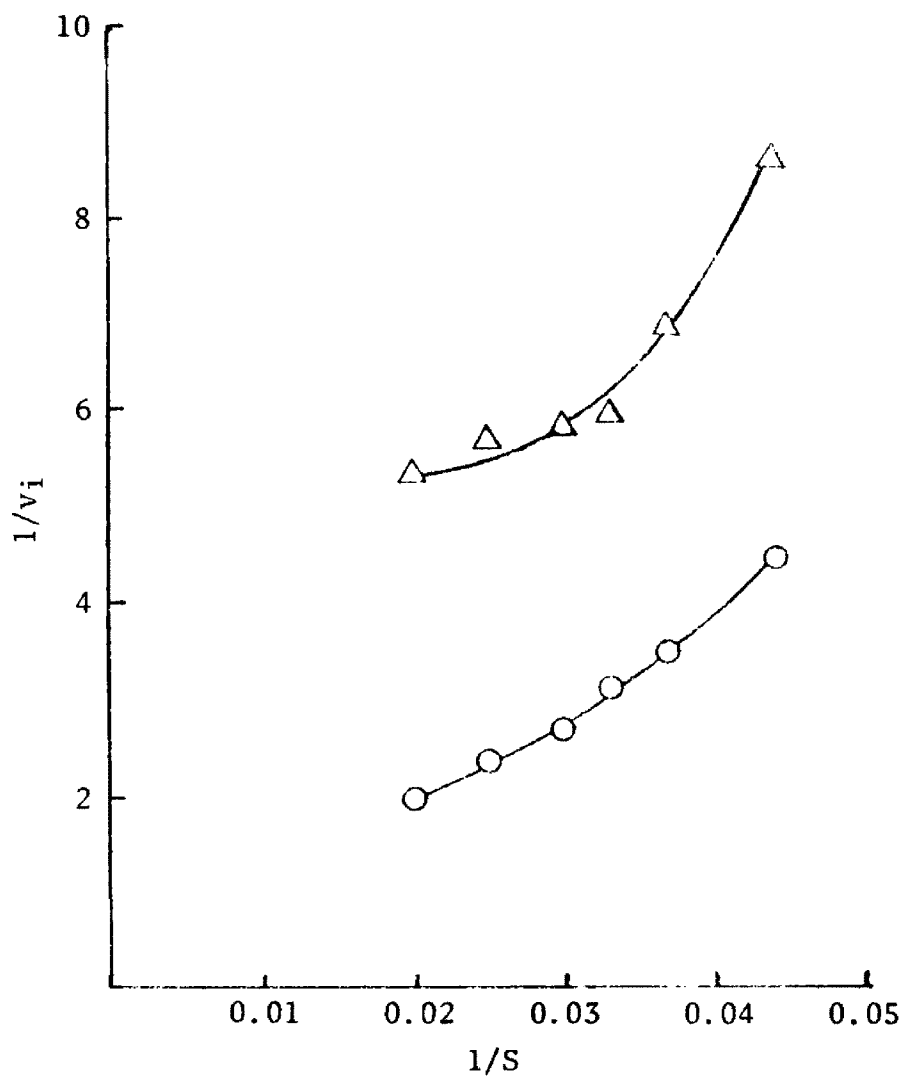


Figure 4.--Plots of $1/v_i$ against $1/S$ for antitryptic activity of DOSS at two pH values. Ten ml digest contained, E=20 μ g; I=10 mg; S=23-50 mg. Key: Δ pH 6.4; and \circ , pH 7.6. Substrate used was casein.

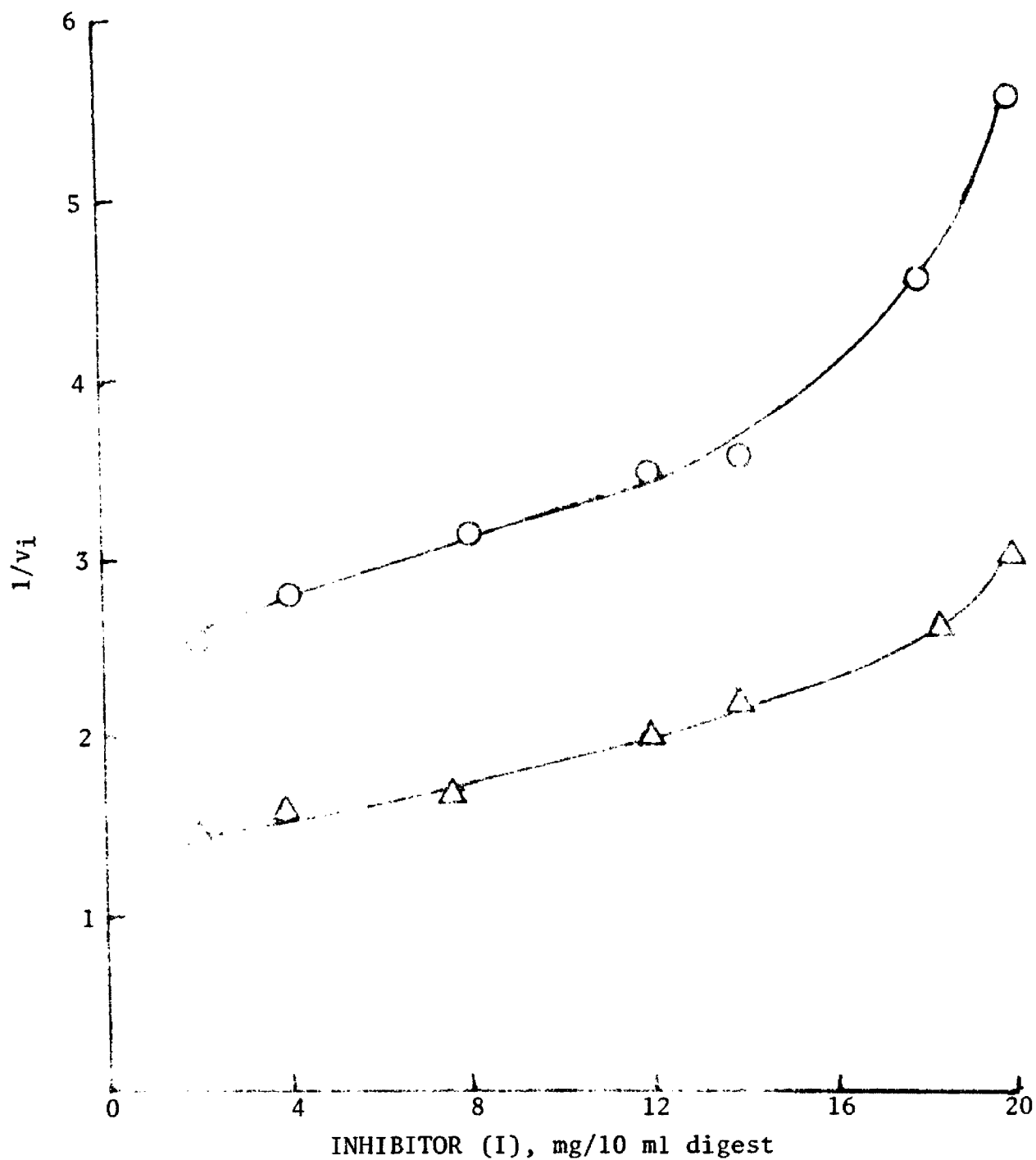


Figure 5.--Plots of $1/v_i$ against I for antitryptic activity of DOSS at pH 7.6. Key: O, ten ml digest contained E=10 μ g, S=50 mg; and Δ , ten ml digest contained E=20 μ g; S=50 mg. Substrate used was casein.

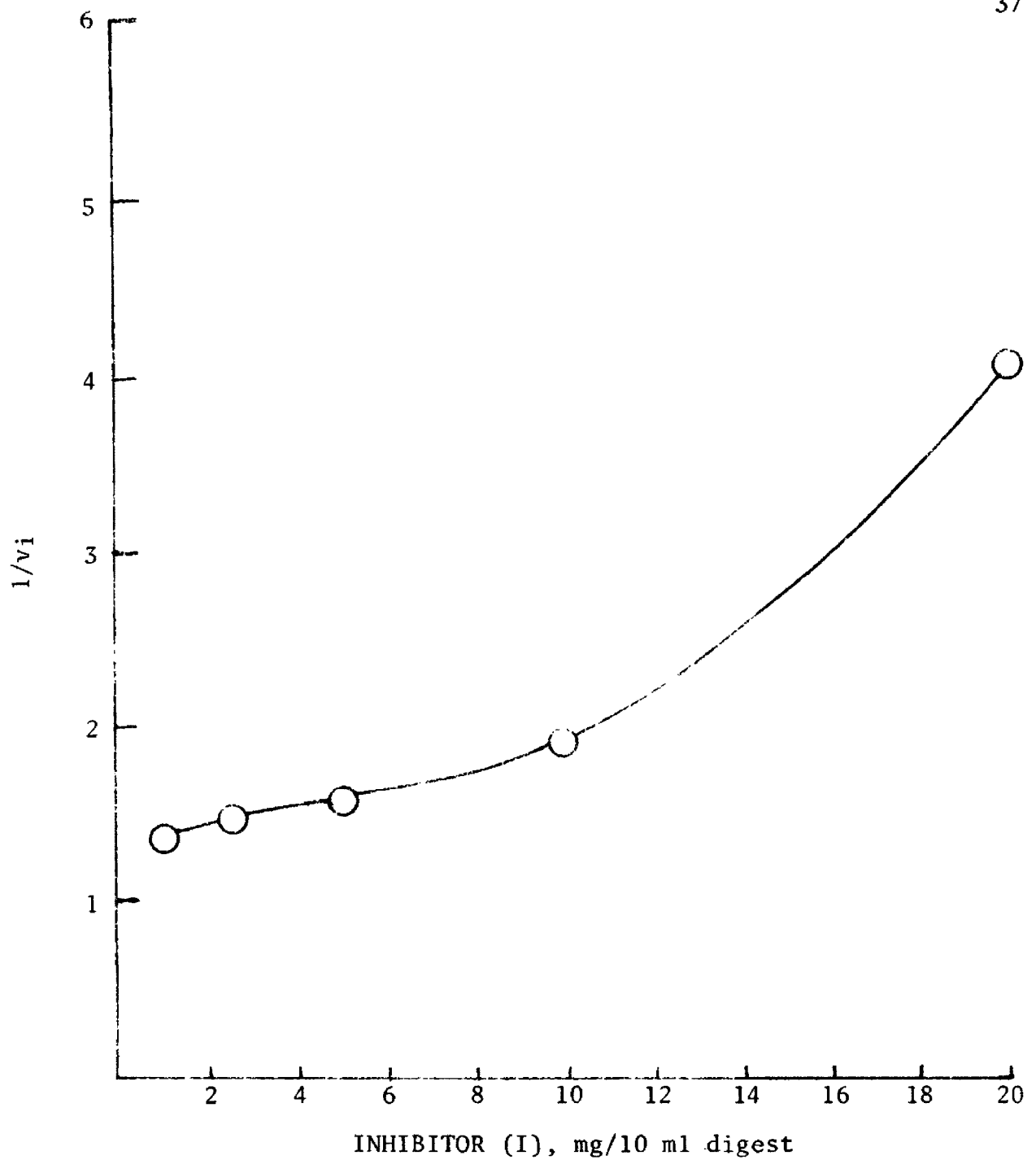


Figure 6.--Plot of $1/v_i$ against I for antitryptic activity of DOSS at pH 7.6. Ten ml digest contained E=30 μ g; S=40 mg. Substrate used was casein.

TABLE 2

Effect of pH and Mixing Orders on Percentage Inhibition of Trypsin Activity by DOSS in the Presence of a Natural Substrate, Casein

Amounts of Components in 10 ml of Digestion Mixture			pH	Inhibition ^a , % of Control Activity with	
Enzyme (μg)	Substrate (mg)	DOSS (mg)		A	B
20	50	10	6.0	51.67	34.66
20	50	10	6.4	53.24	37.54
20	50	10	6.5	40.15	---
20	50	10	6.6	37.04	---
20	50	10	6.8	32.55	33.31
20	50	10	7.2	35.64	34.38
20	50	10	7.6	31.97	32.73
20	50	10	8.0	32.25	35.15

^aAverage of at least four determinations.

^AInhibitor added to enzyme first.

^BInhibitor added to substrate first.

TABLE 3

Effect of Different Shaking Speeds on the
Percentage Inhibition of Trypsin
Activity by DOSS at pH 7.6

Amounts of Components in 10 ml of Digestion Mixture			Shaking Speed (r.p.m.)	Inhibition ^b , % of Control Activity
Enzyme (μ g)	Substrate ^a (mg)	DOSS (mg)		
20	50	10	0	32.56
20	50	10	50	32.05
20	50	10	100	32.37
20	50	10	200	31.96

^aCasein

^bAverage of at least five determinations.

TABLE 4

Effect of DOSS on the Reaction Velocities of Casein Digestion by Trypsin at pH 7.6 at Varying Concentrations of Substrate and at Fixed Levels of Enzyme and Inhibitor

Serial #	Amounts of Components in 10 ml of Digestion Mixture			Absorbance ^a at 280 nm against Respective Blanks	
	Enzyme, E (μg)	Substrate, S (mg)	Inhibitor, I (mg)	Controls (v)	With Inhibitor (v _i)
1	20	23	10	0.522	0.224
2	20	27	10	0.575	0.290
3	20	30	10	0.598	0.320
4	20	33	10	0.620	0.378
5	20	40	10	0.693	0.424
6	20	50	10	0.728	0.512

^aAverage of two determinations.

TABLE 5

Effect of DOSS on the Reaction Velocities of Casein Digestion by Trypsin at pH 6.4 at Varying Concentrations of Substrate and at Fixed Levels of Enzyme and Inhibitor

Serial #	Amounts of Components in 10 ml of Digestion Mixture			Absorbance ^a at 280 nm against Respective Blanks	
	Enzyme, E (µg)	Substrate, S (mg)	Inhibitor, I (mg)	Controls (v)	With Inhibitor (v _i)
1	20	23	10	0.368	0.116
2	20	27	10	0.392	0.145
3	20	30	10	0.432	0.168
4	20	33	10	--	0.172
5	20	40	10	0.481	0.175
6	20	50	10	0.546	0.188

^aAverage of two determinations.

TABLE 6

Effect of DOSS on the Reaction Velocities of Casein Digestion by Trypsin at pH 7.6 at Varying Concentrations of the Inhibitor and at Different Fixed Levels of Enzyme and Substrate

Serial #	Amounts of Components in 10 ml of Digestion Mixture		Absorbance ^a at 280 nm against Respective Blanks (v _i)	
	Substrate, S (mg)	Inhibitor, I (mg)	A	B
1	50	-	0.505	0.775
2	50	2	0.398	0.700
3	50	4	0.360	0.630
4	50	8	0.318	0.600
5	50	12	0.288	0.500
6	50	14	0.280	0.460
7	50	18	0.219	0.390
8	50	20	0.180	0.338

^aAverage of two determinations.

^AAmount of enzyme used was 10 µg.

^BAmount of enzyme used was 20 µg.

TABLE 7

Effect of DOSS on the Reaction Velocities of Casein Digestion by Trypsin at pH 7.6 at Varying Concentrations of the Inhibitor and at Different Fixed Levels of Enzyme and Substrate

Serial #	Amounts of Components in 10 ml of Digestion Mixture		Absorbance ^a at 280 nm against Respective Blanks (v _i)	
	Substrate, S (mg)	Inhibitor, I (mg)	A	B
1	40	--	0.845	0.913
2	40	1.25	0.750	0.800
3	40	2.50	0.680	0.732
4	40	5.00	0.635	0.662
5	40	10.00	0.520	0.535
6	40	20.00	0.245	0.290

^aAverage of at least two determinations.

^AAmount of enzyme used was 30 µg.

^BAmount of enzyme used was 40 µg.

(Mixing Order A), the resulting percentage inhibition was found to be significantly higher ($p < 0.001$ as determined by the student's *t* test for unpaired data) at pH below 6.5. This marked difference in inhibition pattern below pH 6.5 will be discussed later.

Data in Table 3 shows the effect of different shaking speeds on the antitryptic activity of DOSS at pH 7.6. In this study casein was used as the substrate. The results (Table 3) show that the percentage inhibition values remain unchanged at various shaking speeds which indicates that no mechanical and/or physical forces of shear and viscosity are involved in the process of inhibition. Therefore, some stoichiometric chemical interactions between the various components of the reaction must be operative which results in the inhibitory effect of DOSS.

In order to know the mechanism of inhibition, the effect of DOSS on the trypsin activity at both pH 7.6 and pH 6.4 were determined using casein as the substrate (Tables 4-5). In these studies, the concentrations of the inhibitor and the enzyme were kept constant, whereas the substrate levels were varied. Effect of DOSS on the reaction velocities of casein digestion by trypsin at pH 7.6 was also studied varying the concentration of the inhibitor but keeping the enzyme and the substrate at different fixed levels (Tables 6-7). Various graphical methods were used in order to delineate the particular mechanism of inhibition involved.

Using the data from Tables 4 and 5, double reciprocal plots both at pH 6.4 and 7.6 (Fig. 4) and plots of $1/v_i$ against I at pH 7.6 with different inhibitor concentrations (Fig. 5-6), indicate substrate-inhibitor interaction as the major mechanism of inhibition (27, 109). Using the data of Tables 4 and 5 plots of S/v_i against S (Wolf plots),

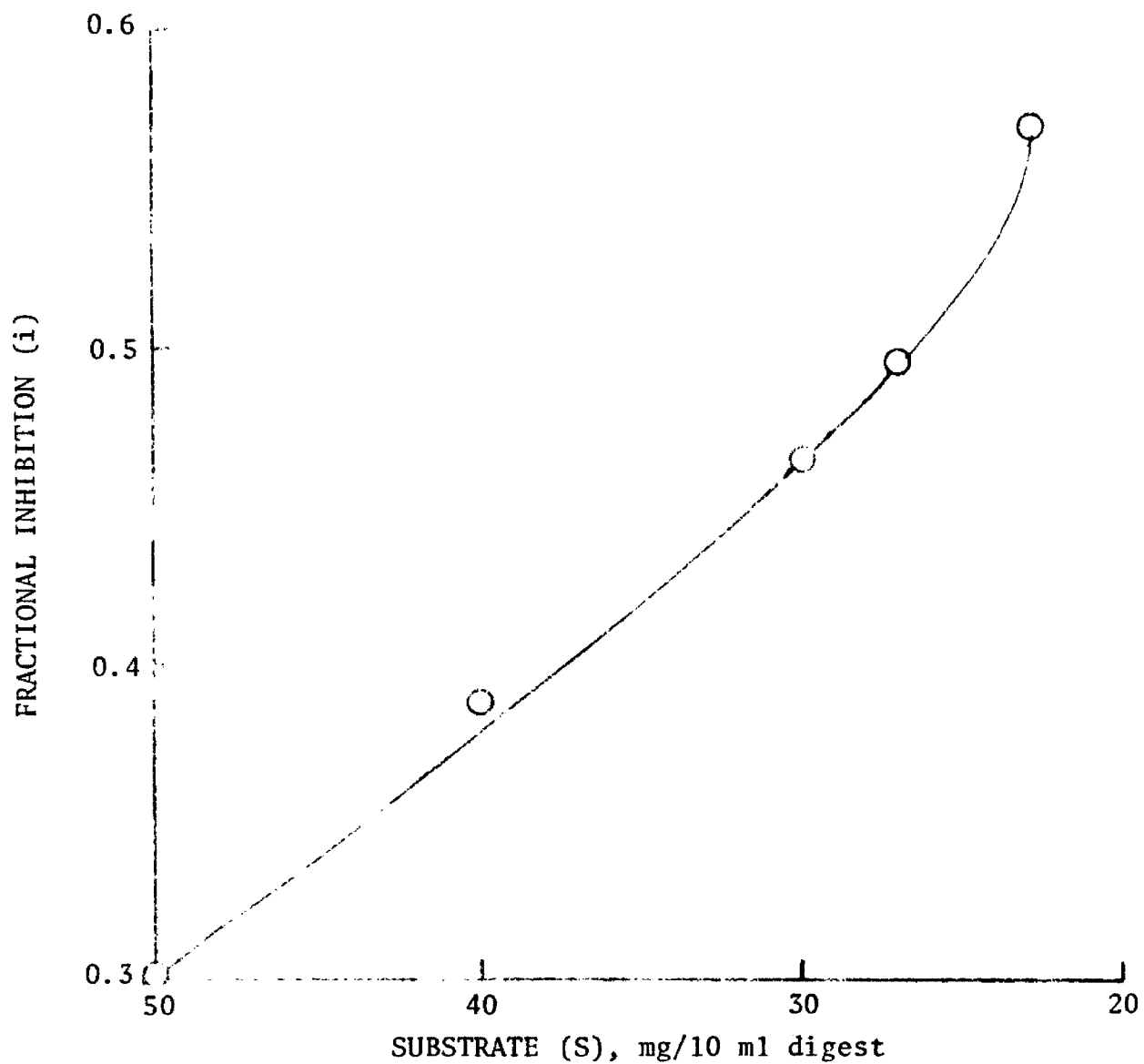


Figure 7.--Plot of (i) against S for antitryptic activity of DOSS at pH 7.6. Ten ml digest contained E=20 μ g; I=10 mg. Substrate used was casein.

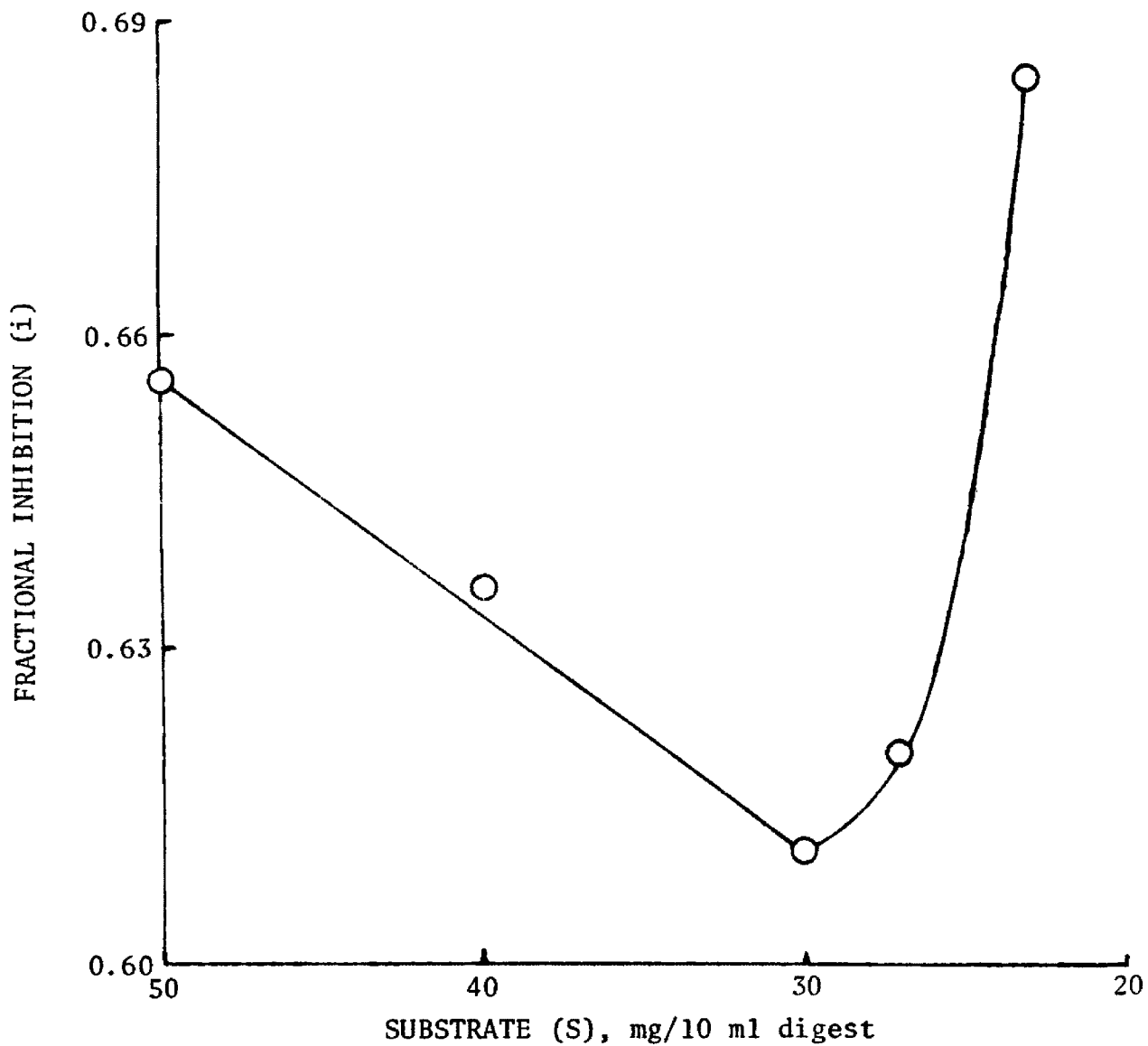


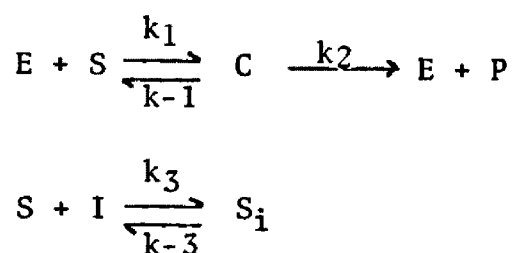
Figure 8.--Plot of (i) against S for antitryptic activity of DOSS at pH 6.4. Ten ml digest contained E=20 μ g; I=10 mg. Substrate used was casein.

v_i against v_i/S (Hofstee plots) and plots of $1/v$ against $1/I$; plots of i against I from Tables 6 and 7 also yielded curves typical of substrate-inhibitor interaction (27, 109). Plots of fractional inhibition (i) against S at pH 7.6 (Fig. 7) and at pH 6.4 (Fig. 8) were made. These plots suggest that in addition to substrate-inhibitor interaction, enzyme-inhibitor interaction also contributes to the overall inhibition. This contribution seems to be least at pH 7.6 (Fig. 7) and most at pH 6.4 (Fig. 8). 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 DOSS is a molecule carrying a net negative charge and the trypsin possesses an isoelectric point in the vicinity of pH 7.0 (62). Therefore, increased amount of inhibition below pH 6.5 is expected. This explains the increased inhibition below pH 6.5 observed in Figure 3. Also, the biphasic curve of percentage inhibition against the inhibitor concentration (Fig. 2), can be explained on the basis of a dual mechanism of inhibition involving both the substrate and the enzyme.

Considering the fact that casein has an isoelectric point at pH 4.2, negatively charged DOSS cannot be expected to interact with the natural substrate, casein at both pH 6.4 and 7.6. However a DOSS-casein interaction is possible in which conformation of the natural protein, casein is changed involving no electrostatic binding.

A general inhibitory mechanism involving a substrate can be due to (1) substrate depletion, (2) inhibition of the enzyme by a substrate-inhibitor complex, (3) combination of both mechanisms. Mechanistic schemes, conservation equations and the various forms of the Michaelis equation describing these inhibitory systems have been described by Reiner (91) in detail. For the case where the effective concentration

of the substrate decreases as a result of interaction with the inhibitor, the following scheme may be considered:



Where E, S, I, C, P and S_i are, respectively, the concentrations of free enzymes, free substrate, free inhibitor, enzyme-substrate complex, product and substrate-inhibitor complex and the k's are rate constants. If E_t , S_t and I_t respectively stand for the total amounts of enzyme, substrate and the inhibitor, the conservation equations for the above mechanism can be written as follows:

$$E_t = E + C$$

$$S_t = S + S_i \quad (\text{Ignoring } S \text{ bound in } C).$$

$$I_t = I + S_i$$

Considering these conservation equations and making certain mathematical manipulations, a very useful form of the Michaelis-Menton equation can be obtained as described by Reiner (91) and given below:

$$I_t = \left[1 + \frac{S_t}{K_m} \right] \left\{ \frac{\left[\frac{i}{1-i} \right] (K_3 + S_t)}{1 + \left[1 + \frac{S_t}{K_m} \right] \left[\frac{i}{1-i} \right]} \right\}$$

In this equation I_t and S_t have their usual meaning as described earlier whereas i , K_3 and K_m are, respectively, fractional inhibition, dissociation constant for the substrate-inhibitor complex and the Michaelis constant. According to this equation, a plot of I_t against $(i/1-i)$ must be hyperbolic in shape with linear asymptotes and different slopes in the various regions. When the variable $(i/1-i)$ is small, the second term in the braces is approximately equal to $(S_t \cdot i/1-i)$. Thus the initial

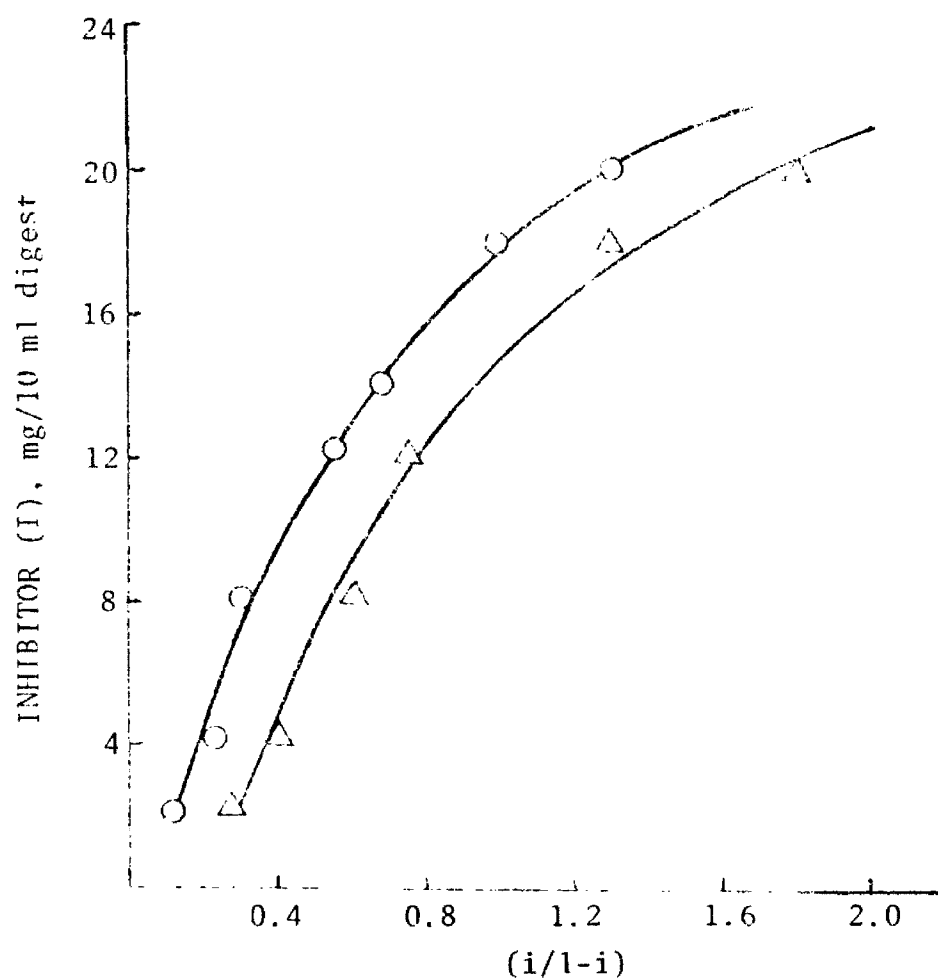


Figure 9.--Plots of I against $(i/l-i)$ for antitryptic activity of DOSS at pH 7.6. Key: O, 10 ml digest contained E=20 μ g; S=50 mg; and Δ , 10 ml digest contained E=10 μ g; S=50 mg. Substrate used was casein.

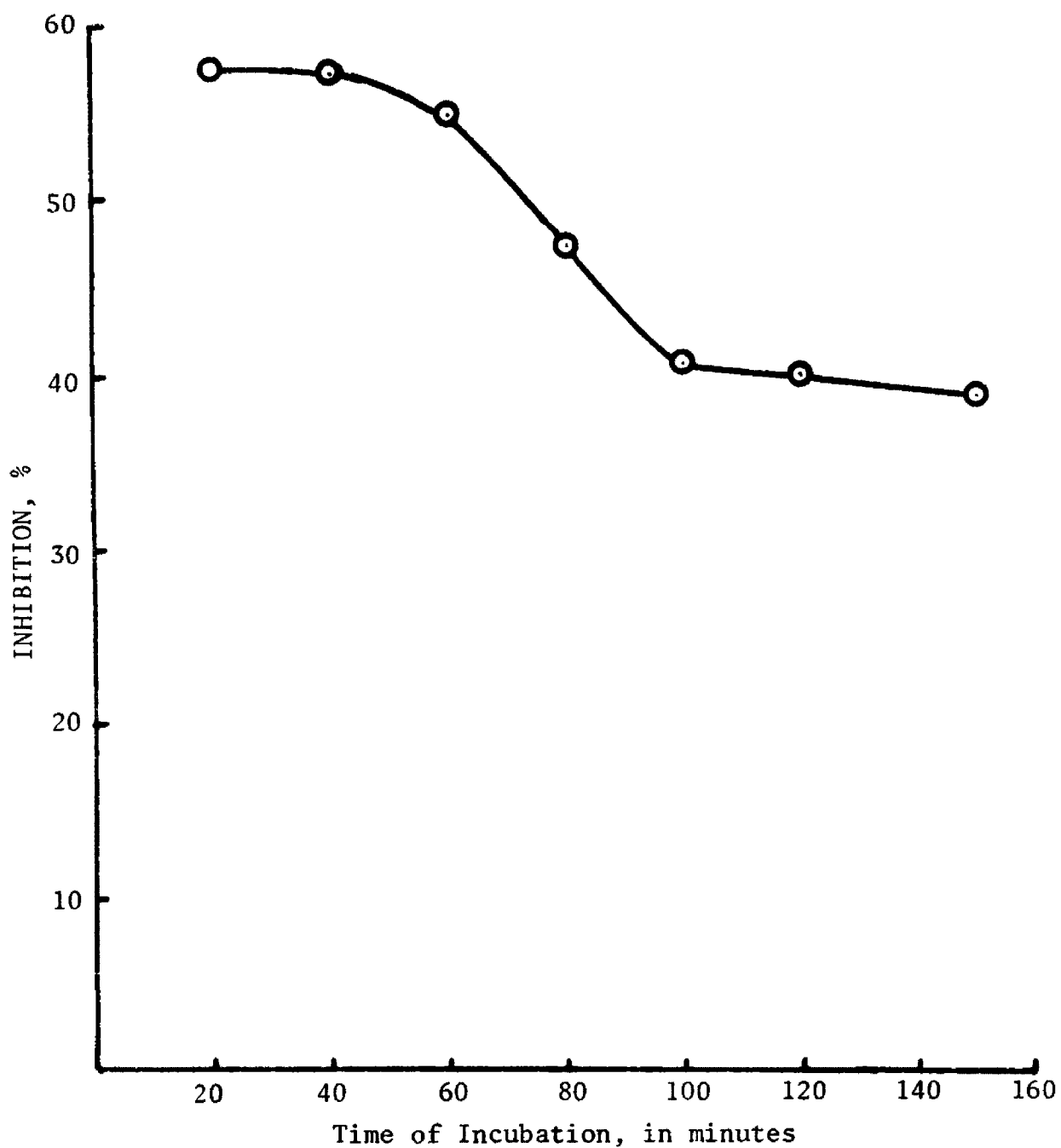


Figure 10.--Effect of incubation time on the percentage inhibition of trypsin activity at pH 7.6. Ten ml of digestion mixture contained E=10 μ g; S=50 mg; and I=10 mg. Substrate used was casein.

TABLE 8

Effect of Pre-Incubation on Antitryptic Activity
of DOSS at pH 7.6

Amounts of Components in 10 ml of Digestion Mixture			Substrate/Inhibitor	Inhibition ^b , % of Control Activity		
Enzyme (μ g)	Substrate ^a (mg)	Inhibitor (mg)		A	B	C
30	40	20	2	71.0	66.5	88.6
30	40	10	4	38.5	39.2	66.4
30	40	5	8	25.0	25.0	45.5
30	40	2.5	16	19.5	21.8	36.7
30	40	1.25	32	11.2	10.0	23.4

^aCasein.

^bAn average of two determinations.

A No pre-incubation.

B The inhibitor pre-incubated with casein for 15 minutes.

C The inhibitor pre-incubated with trypsin for 15 minutes.

TABLE 9

Irreversible Binding of DOSS to Enzyme (Trypsin)
and Substrate (Casein)

pH	Component Bound to the Inhibitor before Dialysis	Amount of the Components in 10 ml of Digestion Mixture			Percentage Inhibition ^a of Control Activity	
		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

^aAverage of at least two determinations.

TABLE 10

Effect of Incubation Time on Percentage Inhibition
of Trypsin Activity by DOSS at pH 7.6

Amounts of Components in 10 ml of Digestion Mixture			Incubation Time (Hrs)	Inhibition ^b , % of Control Activity
Enzyme (μ g)	Substrate ^a (mg)	Inhibitor (mg)		
10	50	10	0.333	57.5
10	50	10	0.666	57.3
10	50	10	1.000	55.0
10	50	10	1.333	47.6
10	50	10	1.666	41.0
10	50	10	2.000	40.4
10	50	10	2.500	39.1

^aCasein.

^bAverage of two determinations.

portion of the curve is linear, with slope $y_1 = (K_3 + S_t)(1 + S_t/K_m)$. As $(i/1-i)$ becomes infinite (as will be the case when i approaches 1), the entire second term in the braces approaches a constant. The first term is still linear. Hence the asymptote should be again linear, with slope $y_2 = K_3 (1 + S_t/K_m)$, which is smaller than the initial slope.

The shapes of such plots (Fig. 9) for dioctyl sodium sulfosuccinate at pH 7.6, using casein as the substrate and resulting from Table 6, are typical of substrate depletion. Similar plots showing substrate depletion as the basic mechanism were also obtained using different substrate and enzyme concentrations (Table 7).

Pre-incubation studies (Table 8) show that pre-incubation of the substrate with the inhibitor bears no significant effect on the inhibition. Pre-incubation 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 substrate-inhibitor interaction, enzyme-inhibitor interaction is also a part of the overall mechanism of inhibition at pH 7.6. This is in good agreement with what has already been concluded from the mechanistic studies.

The results of the dialysis studies (Table 9) show that percentage inhibition values after dialysis are similar to the ones before dialysis, indicating no net gain in enzyme activity due to dialysis. Therefore, it seems that DOSS binds both casein and trypsin irreversibly both at pH 6.4 and pH 7.6.

Effect of incubation time on the antitryptic activity of DOSS was studied using Method I at pH 7.6 (Table 10). A plot of percentage inhibition against the incubation time was made (Fig. 10). Figure 10 indicates an initial slow fall in the percentage inhibition, followed by

TABLE 11

Effect of pH and Mixing Orders on the Antitryptic Activity of DOSS in the Presence of a Synthetic Substrate, DL-BAPNA

Amounts of Components in 8 ml of Reaction Mixture			pH	Percentage inhibition ^a of Control Activity with	
Enzyme (μg)	Substrate (mg)	Inhibitor (μg)		A	B
20	2.175	250	6.4	29.20	9.43
20	2.175	250	8.2	27.00	12.70

^aAverage of at least three determinations.

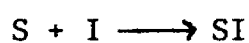
^AInhibitor added to enzyme first.

^BInhibitor added to substrate first.

a rapid decrease and a successive slow fall in the percentage inhibition. Such a continuous decrease in the percentage inhibition with time can be explained by considering the following simultaneous reaction taking place in the system:



It seems possible that in the beginning, reaction (B) proceeds at a comparatively faster rate (Probably due to higher affinity of the inhibitor for S as compared to that of E for S), followed by saturation of I with S to give the SI complex which is then followed by a relatively slower rate of reaction (A) in the forward direction [due to depletion of S and accumulation of products in (A)]. Such an explanation of Figure 10 is consistent with the mechanistic evidence and the pre-incubation studies discussed previously. The shape of curve in Figure 10 can also be explained by considering the following mechanism of inhibition:



According to this mechanism a reversible enzyme-inhibitor complex is formed, followed by substrate-inhibitor interaction which then saturates and thus results into a successive decrease in percentage inhibition with time in Figure 10. The occurrence of such a mechanism in the present studies is very unlikely since the results of dialysis studies indicate that the formation of EI complex is irreversible.

The effect of mixing orders on the antitryptic activity of DOSS was studied both at pH 6.4 and pH 8.2 using DL-BAPNA as the synthetic substrate (Table 11). The results suggest the involvement of an enzyme-

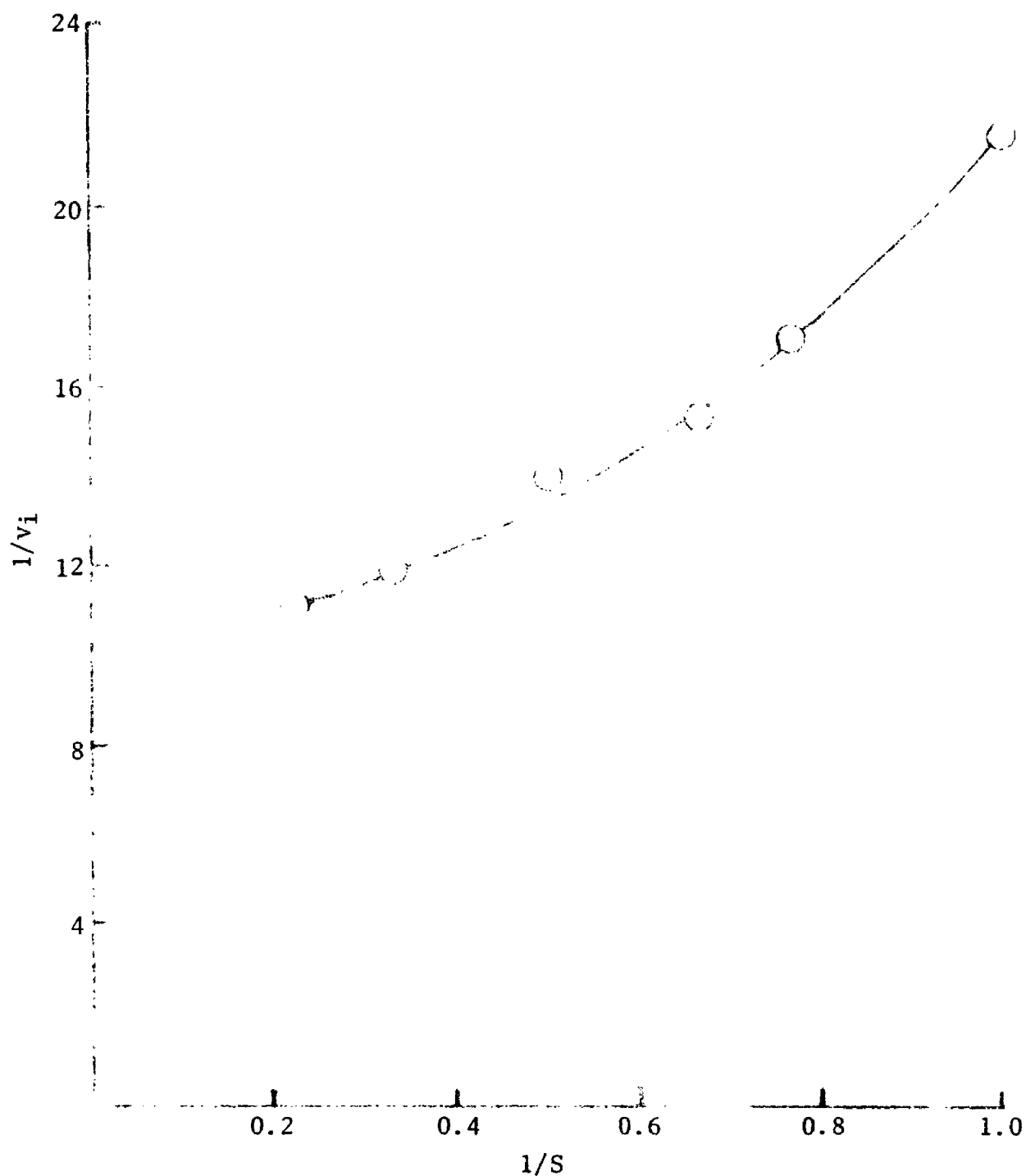


Figure 11.--Plot of $1/v_i$ against $1/S$ for antitryptic activity of DOSS at pH 8.2. Eight ml of incubation mixture contained $E=40$ μ g; $I=0.25$ mg; $S=1 \times 10^{-6}$ - 5×10^{-6} moles. Substrate used was DL-BAPNA.

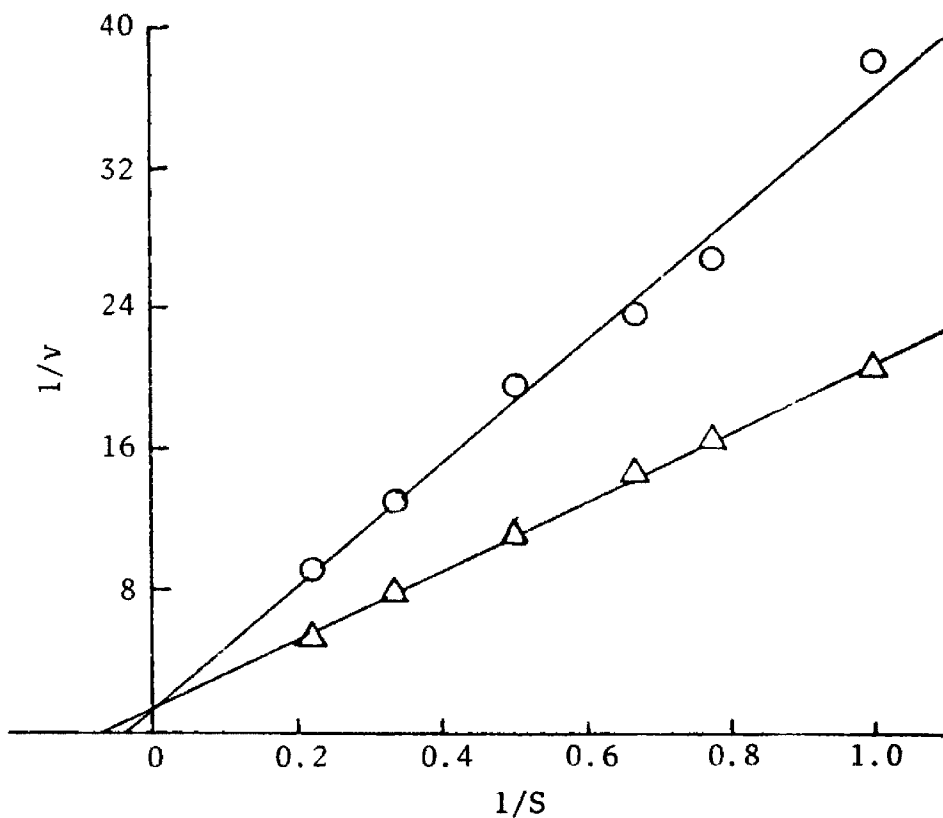


Figure 12.--Plot of $1/v$ against $1/S$ for inhibition of trypsin activity by DOSS at pH 6.4. Eight ml of incubation mixture contained $E=40 \mu\text{g}$; $S=1 \times 10^{-6} - 5 \times 10^{-6}$ moles. Key: Δ , no inhibitor added; and O , $I=0.25 \text{ mg}$. Substrate used was DL-BAPNA. Both lines were drawn by the method of least squares. Δ , $y=19.97 x + 1.14$ ($r=0.990$); O , $y=35.94 x + 0.95$ ($r=0.990$).

TABLE 12

Effect of DOSS on the Reaction Velocities of DL-BAPNA Hydrolysis by Trypsin at pH 8.2 at Varying Concentrations of the Substrate and at Fixed Levels of Enzyme and Inhibitor

Serial #	Amounts of Components in 8 ml of Reaction Mixture			Difference in Absorbance ^a of Samples and Their Respective Blanks at 410 nm Against Distilled Water	
	Enzyme, E (μg)	Substrate, S (μm)	Inhibitor, I (μg)	Controls (v)	With Inhibitor (v _i)
1	40	1.0	250	0.098	0.046
2	40	1.3	250	0.120	0.058
3	40	1.5	250	--	0.065
4	40	2.0	250	0.174	0.071
5	40	3.0	250	0.191	0.084
6	40	4.5	250	--	0.090
7	40	5.0	250	0.263	--

^aAverage of two determinations.

TABLE 13

Effect of DOSS on the Reaction Velocities of DL-BAPNA Hydrolysis by Trypsin at pH 6.4 at Varying Concentrations of the Substrate and at Fixed Levels of Enzyme and Inhibitor

Serial #	Amounts of Components in 8 ml of Reaction Mixture			Difference in Absorbance ^a of Samples and Their Respective Blanks at 410 nm Against Distilled Water	
	Enzyme, E (μg)	Substrate, S (μm)	Inhibitor, I (μg)	Controls (v)	With Inhibitor (v _i)
1	40	1.0	250	0.048	0.026
2	40	1.3	250	0.060	0.037
3	40	1.5	250	0.068	0.042
4	40	2.0	250	0.089	0.051
5	40	3.0	250	0.126	0.077
6	40	4.5	250	0.169	0.108

^aAverage of two determinations.

inhibitor interaction at both pH values. However, no conclusion can be drawn as far as the involvement of a substrate-inhibitor interaction is concerned. Therefore, data for double reciprocal plots (Tables 12-13) were obtained using a synthetic substrate (Method II). A double reciprocal plot (Fig. 11) at pH 8.2 indicates that the substrate-inhibitor interaction is the overall mechanism of inhibition, however, direct enzyme inhibition may also be involved. Such a mechanism was further confirmed to exist by making Hofstee and Woolf plots using the data of Table 12. This mechanism of inhibition seems to be similar to the one observed when casein was used as the substrate (Fig. 4). This similarity in mechanisms, when two different substrates were used, may indicate that the interaction between the inhibitor and casein molecules involves some primary sites of the natural proteins. However, a double reciprocal plot at pH 6.4 with DL-BAPNA as substrate (Fig. 12) was found to be typical of a true competitive inhibition involving the enzyme-inhibitor interaction but no substrate-inhibitor interaction. Such a mechanism was further confirmed by making corresponding Woolf and Hofstee plots. Lack of substrate-inhibitor interaction in the presence of synthetic substrate at pH 6.4 may be attributed to the higher affinity of trypsin for DOSS which is expected at pH 6.4, considering the facts that trypsin has an isoelectric point of 7.0 and that DOSS assumes a net negative charge.

In summary, inhibition of tryptic activity by DOSS in the presence of a natural substrate, involves substrate-inhibitor interaction involving substrate depletion as the major mechanism at both pH 6.4 and pH 7.6, however some direct enzyme inhibition may also occur. Direct inhibitory effect of DOSS against trypsin seems to be enhanced at pH

values below 6.5. In the presence of a synthetic substrate, at pH 8.2 the inhibitory mechanism seems to be similar to the one outlined for the natural substrate, but at pH 6.4 a true competitive enzyme inhibition forms the only mechanism.

Pancreatitis is a chemical autolytic process with a well documented pathogenesis (15). Although various mechanisms underlying the pathological process have been reported (1, 25, 30, 38, 94) no single exact mechanism has been established. Nevertheless, evidence is available that trypsin plays a central role in the pathogenesis of the disease irrespective of the mechanism involved (22, 23, 43, 74, 80-82). It is then apparent that means of controlling the undesirable hypertryptic activity at the site of disease could serve as an approach to alleviate and control the disease. In fact the use of various trypsin inhibitors such as soybean inhibitor, ovomucoid inhibitor and trasylol, a bovine inhibitor (74, 108) has been reported in the treatment of pancreatitis. Although trasylol has found significant place as an antipancreatitis agent in human medicine, other inhibitors have proven effective and useful only *in vitro* and/or in animals. Therefore in view of the various possible mechanisms of the disease process and even more complicated situation *in vivo*, one must exercise great care in projecting the *in vitro* data to the clinical situation. In summary, an effective and useful antipancreatitis agent *in vivo* must exhibit the following characteristics: (1) The inhibitor must not be species specific and should possess a reasonably broad inhibition spectrum. (2) Molecule should have a low molecular weight to permit faster diffusion into the focus of inflammation and should possess a membrane permeability equivalent to that of the enzyme molecule. (3) It should irreversibly bind the enzyme and

should show significant inhibition in the presence of both natural and synthetic substrates. (4) The inhibition constant (K_i) must be smaller than the affinity constant between the enzyme and the substrate. This is absolutely necessary because concentration of the enzyme to be inhibited *in vivo* are roughly 10^{-7} - 10^{-9} moles/liter. (5) The inhibitor must have high compatibility, nontoxicity, nonallergic and noninflammatory properties. (6) It should not interfere with the trypsin inhibiting power of plasma. (7) It should have few accessory sites of binding, and low rates of degradation and excretion thus ensuring higher bioavailabilities at the site of disease. (8) It should be appreciably absorbed into systemic circulation.

Although trasylol is an effective antipancreatitis agent, it has a very high molecular weight and inhibits reversibly with a very low rate of inhibition. Soybean inhibitor shows lower inhibitory effect with natural substrates than with synthetic ones (108). It does not inhibit the release of kinins by trypsin *in vivo*.

DOSS is a molecule with relatively lower molecular weight and is currently used in medicine as a fecal softener to relieve constipation associated with hard, dry stool. Until recently this compound has been generally promoted as nonabsorbable, nontoxic and a systemically inert pharmacological agent. However recent investigations of Dujovne and Shoeman (28) have shown that DOSS is considerably absorbed into the systemic circulation in rats and humans after its oral administration. Along with this finding, the present inhibitory studies with DOSS, suggest a possible use of this medicinal surfactant in pancreatitis. The results of the present studies indicate that besides its direct inhibitory effect against trypsin, the interaction of DOSS with casein may

suggest the possibility of a similar interaction between the inhibitor molecule and the glycoproteins at the site of inflammation. Such an interaction *in vivo* may prove to be useful in protecting the site from necrosis and erosion (5). Moreover irreversible binding of DOSS with trypsin and casein, its smaller molecular weight as compared to protein inhibitors, and its excretion in bile (28) may prove to be useful characteristics of DOSS as an antipancreatitis agent.

The inhibitory studies with DOSS also suggest that possible drug interactions may arise in the course of therapy with DOSS in situations and conditions where concomitant use of proteolytic enzymes like trypsin and chymotrypsin is desirable for the simultaneous treatment of disorders like indigestion, inflammation, injury, burns, pulmonary alveolar proteinosis, surgery; episiotomies and blood clotting. Drug interactions are also possible with drug esters like penamecillin and chloramphenicol palmitate and certain N-acylated drugs when given orally along with DOSS. Such drugs are usually acted by proteinases in the gastrointestinal tract resulting in changed chemical and/or pharmacological activity. These drug interactions may be difficult to recognize, particularly when DOSS is used as an excipient, either for the formulation of these drugs mentioned above or any other drug being used simultaneously.

Present studies also suggest that the use of DOSS as one of the adjuvants in insulin dosages may improve its oral absorption as certain proteinase inhibitors such as trasylol have been shown to increase insulin absorption when administered together (44, 65). Such an enhancement of absorption can be further augmented by the recent studies of Gouda (41, 59, 71) in which DOSS has been shown to enhance the absorption of drugs through membranes of various complexities.

CHAPTER V

STUDIES WITH PEPSIN

I. EXPERIMENTAL

Materials

The following chemicals were used in these studies:

Pepsin (from hog stomach mucosa, 3 x crystallized, lyophilized).

Nutritional Biochemicals Corporation.

Hemoglobin (denatured, standardized for protease assay).

Nutritional Biochemicals Corporation.

N-Acetyl-L-phenylalanyl -L-diiodotyrosine (APD).

Sigma Chemical Company.

Diocetyl sodium sulfosuccinate (DOSS).

"Aerosol O. T. 100%", Sargent-Welch Scientific Company.

Ninhydrin.

J. T. Baker Chemical Company.

Isopropyl alcohol (2-propanol).

J. T. Baker Chemical Company.

Methyl Cellosolve (ethylene glycol monomethyl ether).

Fisher Scientific Company.

All other chemicals used were either U.S.P. or reagent grade.

Procedures

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

Method I. In this method, denatured hemoglobin is digested under standard conditions. The undigested hemoglobin is precipitated with trichloroacetic acid, and the amount of unprecipitated protein is estimated spectrophotometrically in u. v. range. The method used was essentially that of Rajgopalan, Moore and Stein (90). However a few modifications in the procedure were made. The substrate, enzyme and inhibitor solutions of various concentrations were prepared either in HCl (pH 1.8) or distilled water. All solutions were finally adjusted to pH 1.8, were freshly prepared and were brought to 37° C before mixing.

One milliliter of the enzyme solution was pipetted into each of the flasks already maintained at 37° in a metabolite water bath shaker. Subsequent to this 1 ml of the inhibitor solution was added to each flask followed by 2 ml of the substrate solution. All the samples were incubated for 5 minutes at 37° C 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 final volume of the incubation mixture was kept at 8 ml in all cases. The incubation mixtures were allowed to remain at 37° C for 3 minutes and were then filtered through Whatman #2 filter paper. Blank determinations were carried out exactly in the same way as samples except that the substrate solution was added after the addition of trichloroacetic acid. The absorbance of all the filtrates was read at 277 nm against distilled water using a Beckman ACTA CIII Spectrophotometer. Controls were simultaneously run for every inhibition experiment. The controls differed from the samples in having inhibitor solution replaced by equivalent amounts of HCl (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. Inhibition values (i) and the percentage inhibitions were calculated as described previously under Method I for antitryptic activity.

Method II. In this method, N-Acetyl-L-phenylalanyl-L-diiodo-tyrosine (APD) is used as the substrate. APD is a low molecular weight synthetic peptide that is easily hydrolysed by pepsin. The extent of hydrolysis is determined by measuring the ninhydrin color given by the newly formed L-diiodotyrosine. The assay procedure used was that of Jackson et al (57) with slight modifications. Various reagents used in this method were prepared as follows:

Enzyme and inhibitor solutions. Various concentrations of the enzyme and the inhibitor solutions were prepared as in Method I except that all the solutions were finally adjusted to pH 2.00.

Substrate solutions. The substrate was extremely difficult to dissolve directly at pH 2.00. Therefore, a stock solution ($20 \times 10^{-5}M$) of the substrate was prepared by first dissolving 12.444 mg of APD in about 40 ml of 0.01 N NaOH solution and then adjusting the pH of the solution to 2.00 with HCl. Final volume of the substrate solution was made to 100 ml with HCl at pH 2.00. Various concentrations used could be obtained by further diluting the stock solution with HCl. These solutions were used within 30-60 minutes after preparation since material precipitates on prolonged standing.

Acetate buffer (pH 5.3-5.4). Sixty-seven milliliter of glacial acetic acid and 360 g of sodium acetate ($CH_3COONa \cdot 3H_2O$) were dissolved in 270 ml of distilled water and made up to 1000 ml with H_2O .

Cyanide-acetate buffer solution. Forty-nine milligram of this solution was taken into a volumetric flask and made up to 1000 ml with more acetate buffer.

Ninhydrin solution. Three grams of ninhydrin was dissolved in 100 ml of methyl cellosolve (ethylene glycol monomethyl ether).

All the substrate, enzyme and inhibitor solutions were freshly prepared and were brought to 37° C before mixing. The final volume of the reaction mixture was kept at 9 ml in each case.

A series of test tubes were set up and preequilibrated at 37° C in a metabolic water bath. One milliliter of the enzyme solution was added to each tube followed by the addition of 1 ml of the inhibitor solution. Three milliliter of the substrate solution was then added to each of the tubes and the samples incubated at 37° C for 10 minutes with no shaking. The reaction was stopped by adding 0.5 ml of N/2 NaOH into each of the test tubes. All the tubes were well shaken. Five-tenths milliliter of cyanide-acetate buffer solution was then added to each tube followed by 0.5 ml additions of ninhydrin solutions. Without any shaking all the tubes were placed in a boiling water bath for 15 minutes, after which 2.5 ml of isopropylalcohol was added to each tube and allowed to cool at room temperature. The extent of hydrolysis was determined by measuring the ninhydrin color given by the newly formed L-diiidotyrosine. This was done by recording the absorbance of the reaction mixtures of both samples and blanks at 570 nm against distilled water using a Beckman ACTA CIII Spectrophotometer.

All blanks and controls were prepared simultaneously as described for Method I. The peptic activities, reaction velocities and the inhibition values were determined as indicated in Method I.

Various orders of mixing. Two different orders of mixing were used, namely Mixing Order A and Mixing Order B. These have already been described in Chapter IV. In all the pepsin studies involving Method I,

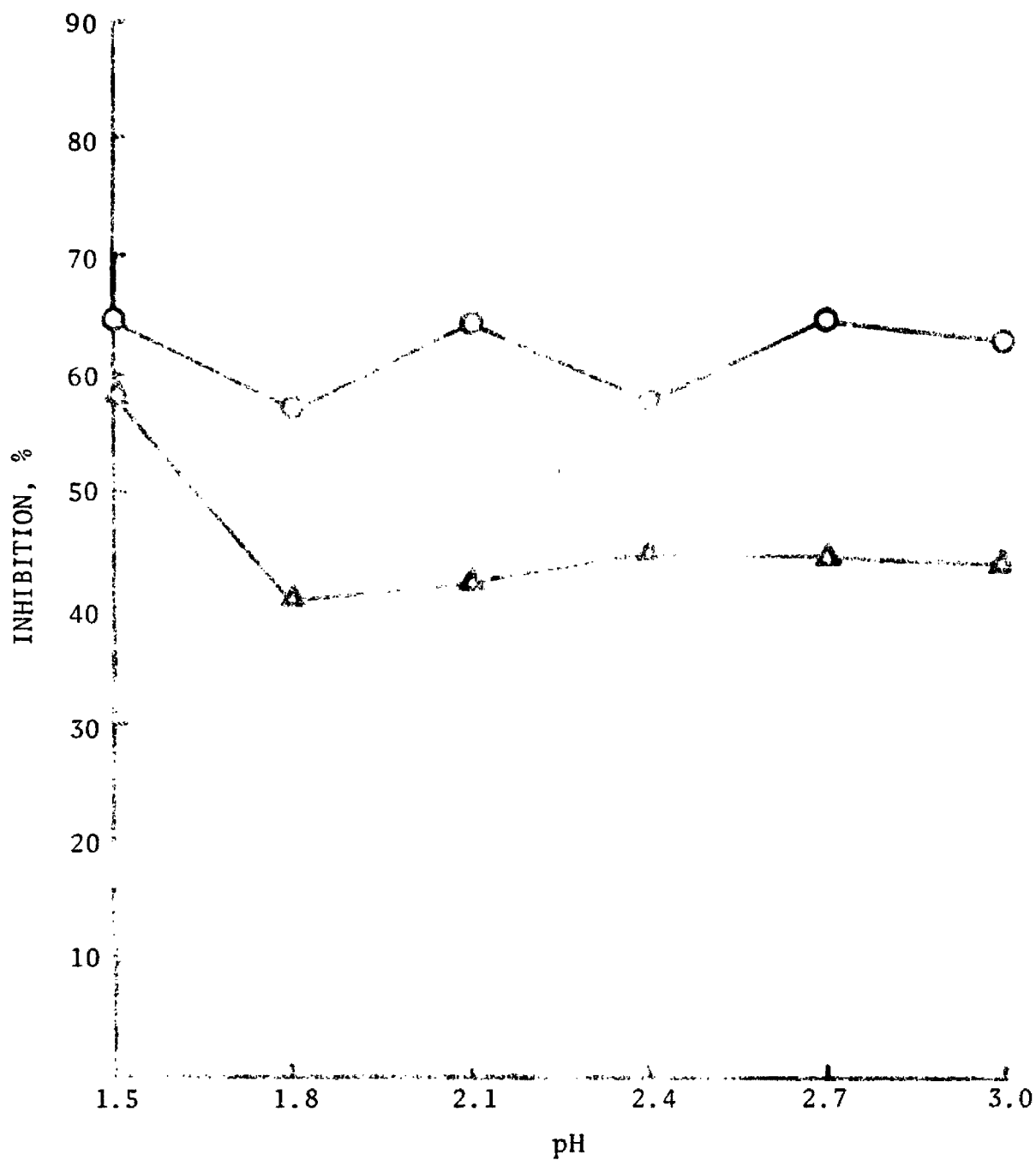


Figure 13.--Effect of pH and mixing orders on percentage inhibition of pepsin. Eight ml of digest contained E=10 μ g; S=2 mg; I=200 μ g. Key: O, inhibitor added to enzyme first; and Δ , inhibitor added to substrate first. Substrate used was hemoglobin.

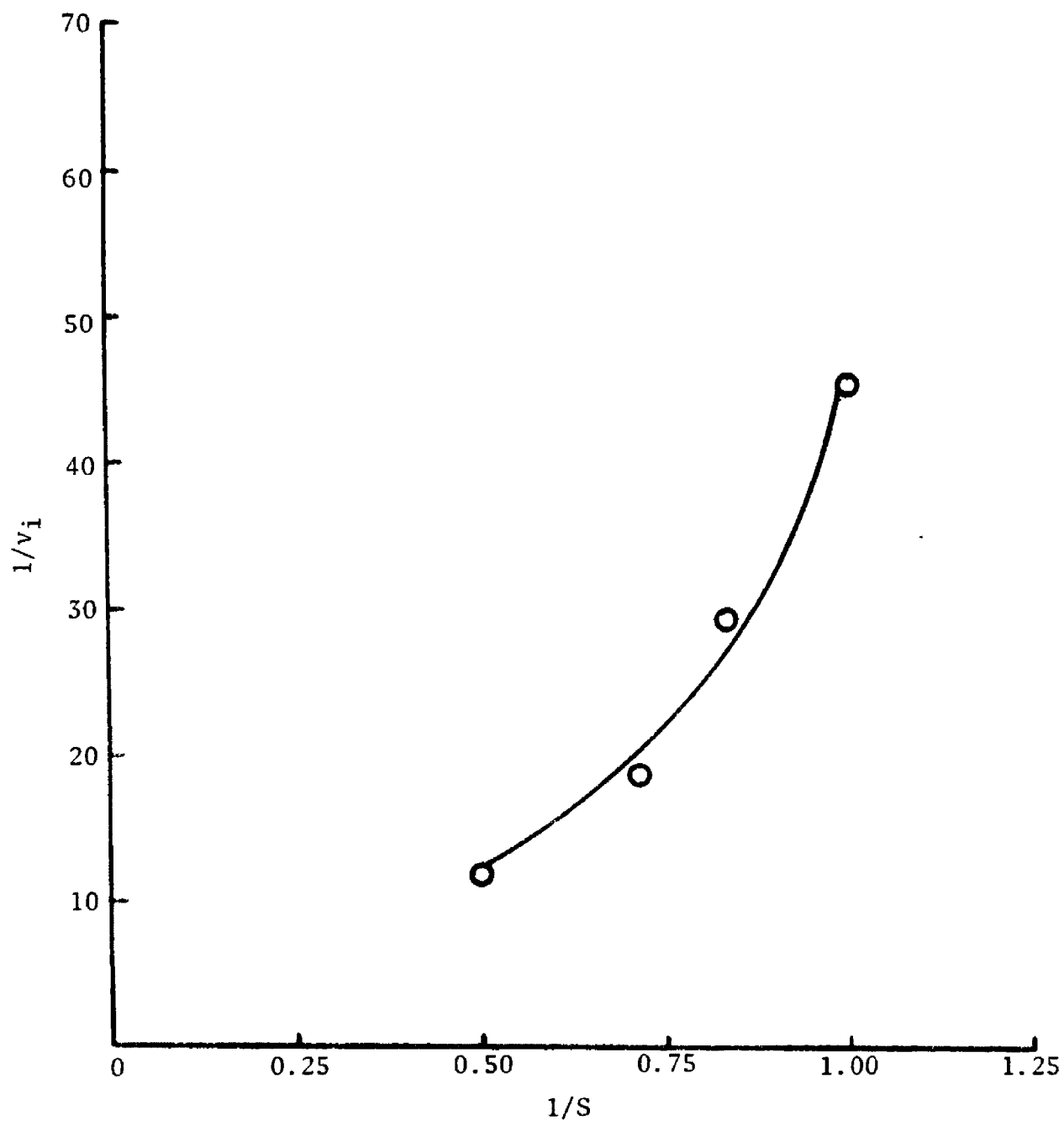


Figure 14.--Plot of $1/v_i$ against $1/S$ for antipeptic activity of DOSS at pH 1.8. Eight ml digest contained $E=10 \mu\text{g}$; $I=200 \mu\text{g}$; $S=0.8-2.0 \text{ mg}$. Substrate used was hemoglobin.

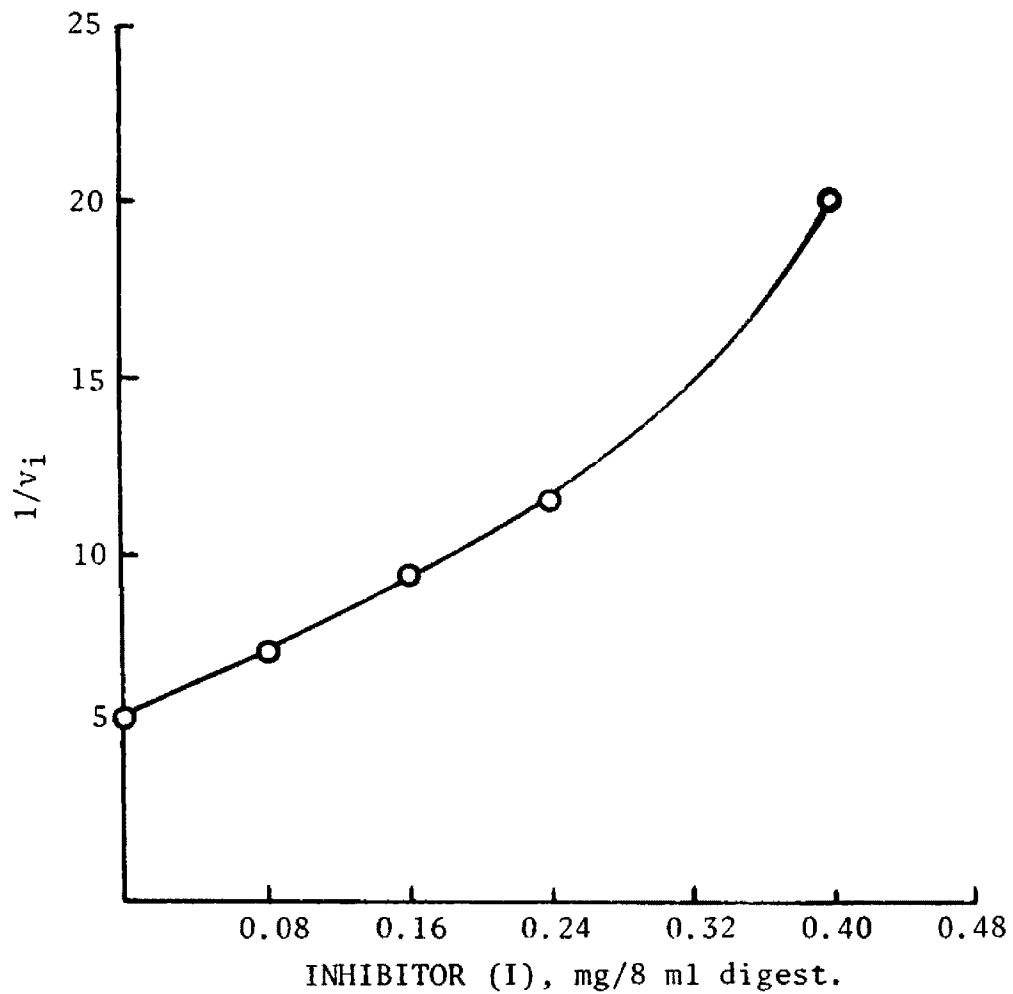


Figure 15.--Plot of $1/v_i$ against I for antipeptic activity of DOSS at pH 1.8. Eight ml digest contained $E=10 \mu\text{g}$; $S=2 \text{ mg}$. Substrate used was hemoglobin.

TABLE 14

Effect of Various Concentrations of DOSS on the
Percentage Inhibition of Pepsin
Activity at pH 1.8

Amounts of the Components in 8 ml of Digest			Percentage Inhibition ^b of Control Activity
Enzyme (μ g)	Substrate ^a (mg)	Inhibitor (μ g)	
4	1.2	50	--
4	1.2	100	--
4	1.2	200	72.0
4	1.2	300	100.0
4	1.2	500	100.0
4	1.2	1000	100.0

^aHemoglobin

^bAverage of at least three determinations.

TABLE 15

Effect of pH and Mixing Orders on Percentage Inhibition of Pepsin Activity by DOSS in the Presence of a Natural Substrate, Hemoglobin

Amounts of Components in 8 ml of Digest			pH	Percentage Inhibition Control Activity	
Enzyme (μ g)	Substrate (mg)	Inhibitor (μ g)		A	B
10	2.0	200	1.5	64.40	58.10
10	2.0	200	1.8	57.20	41.00
10	2.0	200	2.1	64.90	42.50
10	2.0	200	2.4	58.20	45.20
10	2.0	200	2.7	65.20	44.90
10	2.0	200	3.0	63.40	44.10

^aAverage of four determinations.

^AInhibitor added to enzyme first.

^BInhibitor added to substrate first.

TABLE 16

Effect of DOSS on the Reaction Velocities of Hemoglobin Digestion by Pepsin at pH 1.8 at Varying Concentrations of Substrate and at Fixed Levels of Enzyme and Inhibitor

Serial #	Amounts of Components in 8 ml of the Digest			Difference in Absorbance ^a of Samples and Their Respective Blanks at 277 nm Against Distilled Water	
	Enzyme, E (μg)	Substrate, S (mg)	Inhibitor, I (mg)	Controls (v)	With Inhibitor (v _i)
1	10	1.0	0.2	0.080	0.022
2	10	1.2	0.2	0.102	0.034
3	10	1.4	0.2	0.124	0.054
4	10	2.0	0.2	0.184	0.085

^aAverage of two determinations.

TABLE 17

Effect of DOSS on the Reaction Velocities of Hemoglobin Digestion by Pepsin at pH 1.8 at Various Concentrations of the Inhibitor and at Fixed Levels of Enzyme and Substrate

Serial #	Amounts of Components in 8 ml of the Digest			Difference in Absorbance ^a of Samples and Their Respective Blanks at 277 nm Against Distilled Water
	Enzyme, E (μg)	Substrate, S (mg)	Inhibitor, I (mg)	
1	10	2.0	--	0.193
2	10	2.0	0.08	0.139
3	10	2.0	0.16	0.106
4	10	2.0	0.24	0.087
5	10	2.0	0.32	0.068
6	10	2.0	0.40	0.050

^aAverage of two determinations.

Mixing Order A was used except for the data of Figure 13 and Table 15 in which case both mixing orders were used. Only Mixing Order A was used in all the studies using Method II.

II. RESULTS AND DISCUSSION

The inhibitory effect of DOSS on pepsin activity was studied at various inhibitor concentrations using Method I. The data is presented in Table 14, which shows a significant inhibitory effect at all concentrations above 12.5 $\mu\text{g/ml}$ in the system. The effect of pH and mixing orders on the percentage inhibition was also studied using Method I (Table 15). The data was plotted as shown in Figure 13. Figure 13 shows 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). This increase in inhibition as a consequence of mixing order seems to be almost constant over the pH range 1.8-3.0. However at pH 1.5, no significant increase in the percentage inhibition is indicated. The marked difference in the inhibition pattern over the pH range 1.8-2.0 may suggest an enzyme-inhibitor interaction taking place. This will be discussed later. However lack of increased inhibitory effect of DOSS at pH 1.5 may be accounted for by the fact that pepsin possesses a pH optimum range of 1.6-3.2 for its maximum activity.

The effect of DOSS on the reaction velocities of hemoglobin digestion by pepsin at pH 1.8 was studied using various concentrations of substrate (Table 16) and inhibitor (Table 17). Using these data, a plot of $1/v_i$ against $1/S$ (Fig. 14) and a plot of $1/v_i$ against I (Fig. 15) were made. Both of these plots indicate substrate-inhibitor interaction as the major mechanism of inhibition (27, 109). Using the data of

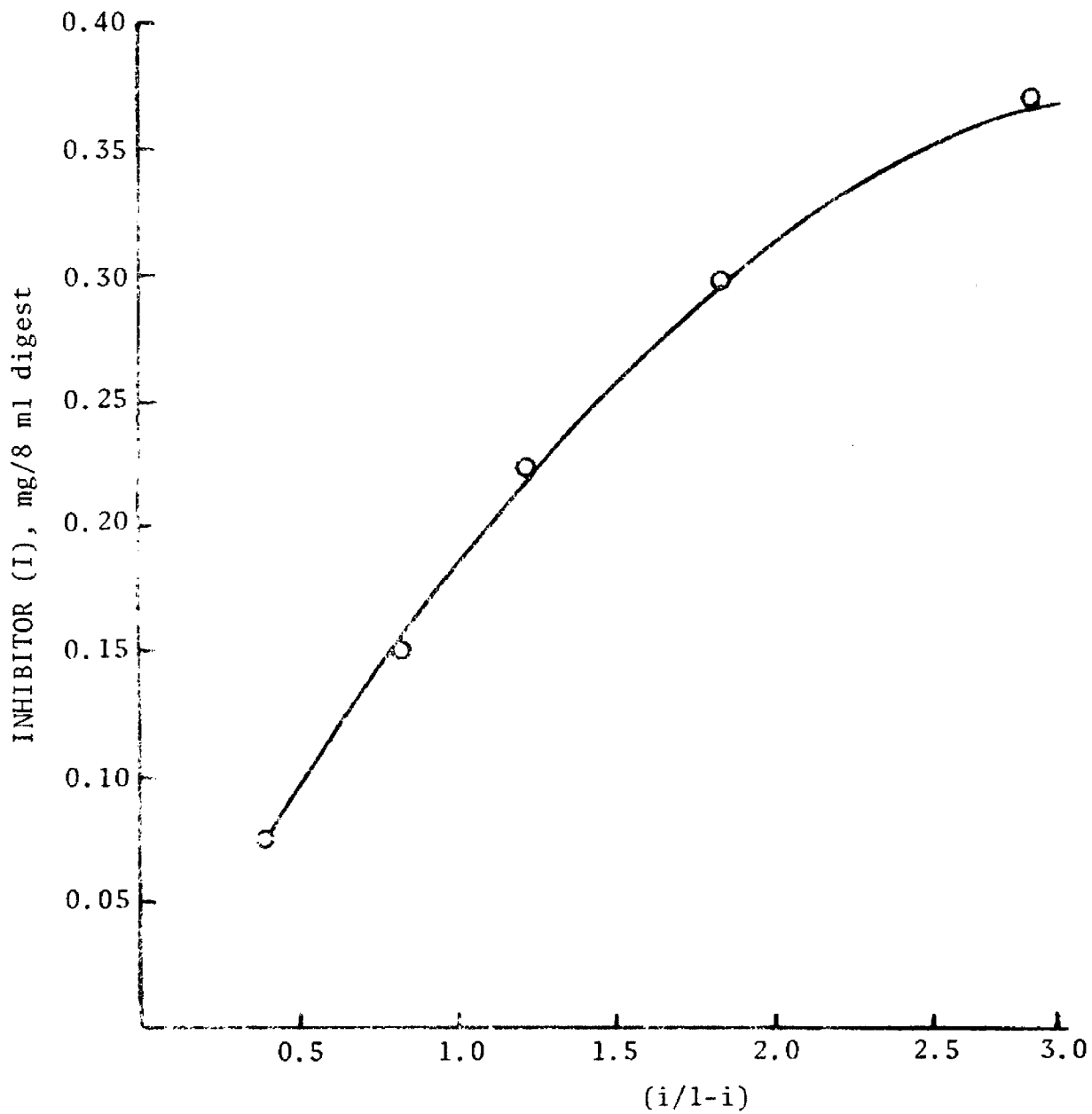


Figure 16.--Plot of I against $(i/l-i)$ for antipeptic activity of DOSS at pH 1.8. Eight ml digest contained E=10 μ g; S=2 mg. Substrate used was hemoglobin.

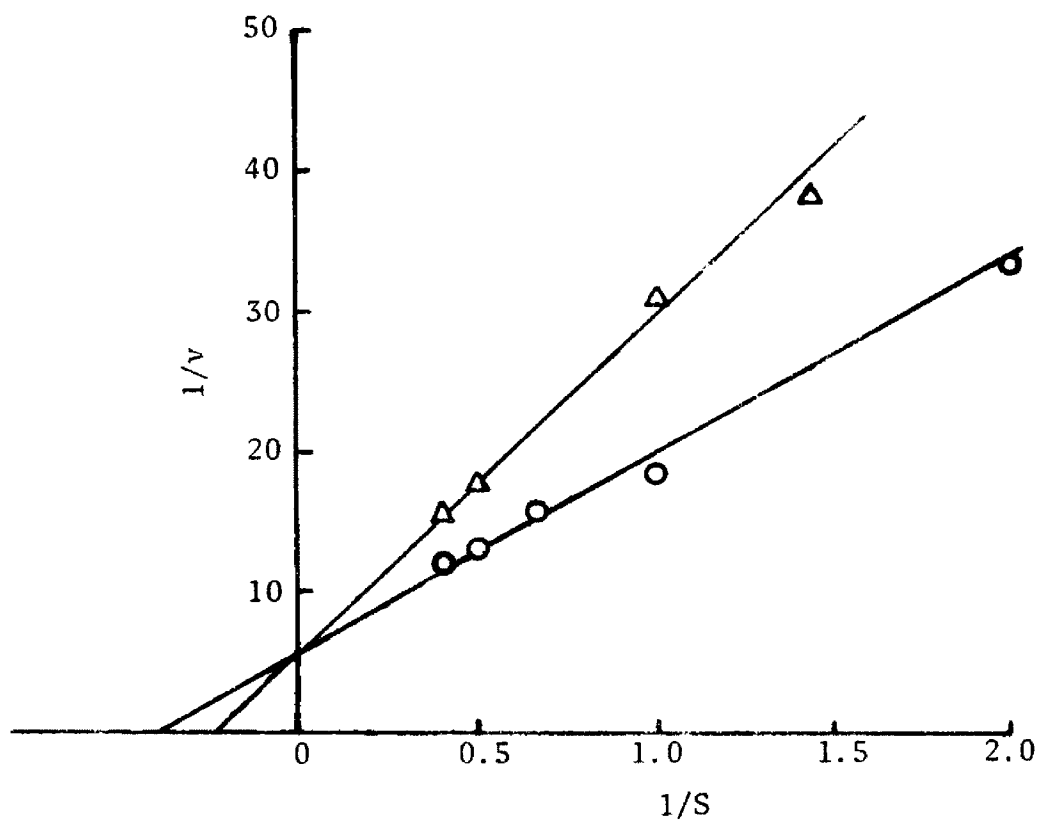


Figure 17.--Plot of $1/v$ against $1/S$ for antipeptidic activity of DOSS at pH 2.0. Nine ml of incubation mixture contained $E=60 \mu\text{g}$; $S=10 \times 10^{-8}$ - 50×10^{-8} moles. Key: O, no inhibitor added; and Δ , $I=50 \mu\text{g}$. Substrate used was APD. Both lines were drawn by the method of least squares. Δ , $y=22.9400 x + 6.5500$ ($r=0.9900$); O, $y=13.3580 x + 6.2712$ ($r=0.09972$).

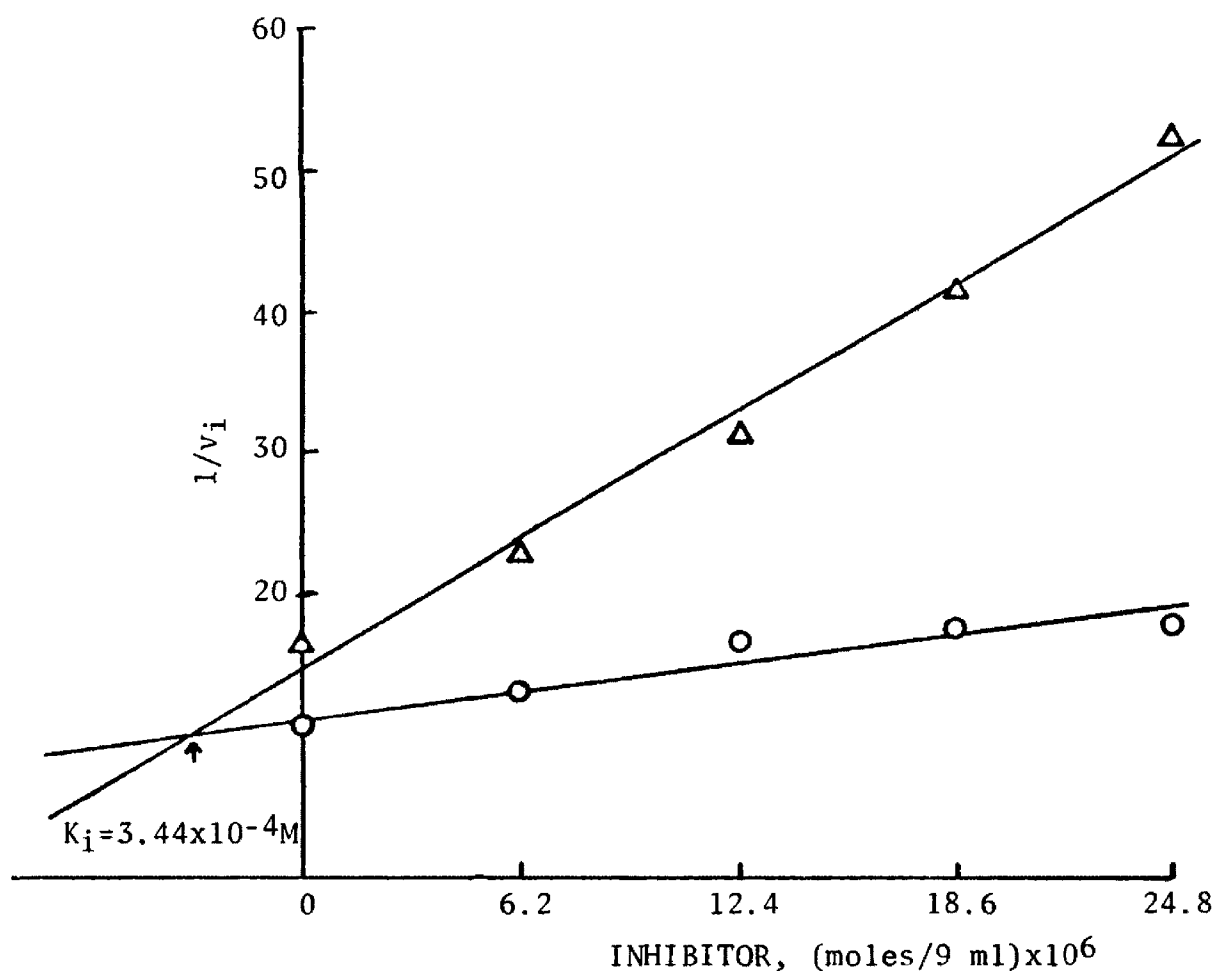


Figure 18.--Plot of $1/v_i$ against I for antipeptic activity of DOSS at pH 2.0. Nine ml of incubation mixture contained $E=60 \mu\text{g}$.
 Key: Δ , $S=2.2 \times 10^{-8}$ moles; O , $S=4.4 \times 10^{-8}$ moles. Substrate used was APD. Both lines were drawn by the method of least squares.
 Δ , $y=1.4729 x + 14.694$ ($r=0.9948$); O , $y=0.3166 x + 11.1520$ ($r=0.9415$).

TABLE 18

Effect of DOSS on the Reaction Velocities of APD Hydrolysis by Pepsin at pH 2.0 Using Various Concentrations of the Substrate and at Fixed Levels of Enzyme and Inhibitor

Serial #	Amounts of Components in 9 ml of Reaction Mixture			Difference in Absorbance ^a of Samples and Their Respective Blanks at 570 nm Against Distilled Water	
	Enzyme, E (μg)	Substrate, S (20x10 ⁻⁸)m	Inhibitor, I (μg)	Control (v)	With Inhibitor (v _i)
1	60	0.5	50	0.030	--
2	60	0.7	50	--	0.026
3	60	1.0	50	0.054	0.033
4	60	1.5	50	0.064	--
5	60	2.0	50	0.077	0.058
6	60	2.5	50	0.084	0.067

^aAverage of two determinations.

TABLE 19

Effect of DOSS on the Reaction Velocities of APD Hydrolysis by Pepsin at pH 2.00 at Various Concentrations of the Inhibitor and at Different Fixed Levels of the Substrate and Enzyme

Serial #	Amounts of Components in 9 ml of Reaction Mixture		Difference in Absorbance ^a of Samples and Their Respective Blanks at 570 nm Against Distilled Water	
	Enzyme, E (μg)	Inhibitor, I (μg)	A	B
1	60	--	0.061	0.097
2	60	25	0.044	0.077
3	60	50	0.032	0.059
4	60	75	0.024	0.057
5	60	100	0.019	0.056

^aAverage of two determinations.

^AAmount of substrate used was (20×10^{-8}) moles.

^BAmount of substrate used was (40×10^{-8}) moles.

Figure 14 and Figure 15, plots of S/v_i against S , v_i against v_i/S ; and $1/i$ against $1/I$ also yielded curves typical of substrate-inhibitor interaction (27, 109). Further characterization of this mechanism was done by making a plot of I against $i/(1-i)$ (Fig. 16), which shows substrate depletion to be the basic mechanism involved (91).

Using the data of Figure 14, a plot of i against S was also made. This plot indicated that in addition to substrate-inhibitor interaction, enzyme inhibitor interaction may also be involved in the overall mechanism of inhibition. This seems to explain the increased percentage inhibition with Mixing Order A found over the pH range 1.8-3.0 in Figure 13. Such an additional enzyme-inhibitor interaction could have been further confirmed to exist through preincubation studies but such studies were not feasible due to the autodigestion of pepsin under the conditions as reported earlier (86) and also found in the present investigation.

Effect of DOSS on the reaction velocities of APD hydrolysis by pepsin at pH 2.00 (Method II) was studied using various concentrations of the substrate (Table 18) and inhibitor (Table 19). A double reciprocal plot (Fig. 17) was made using the data of Table 18. Figure 17 is typical of a true competitive inhibition due to a competition between the substrate and the inhibitor molecules for the similar site at the enzyme surface (87). Such a mechanism was further confirmed by making a plot of $1/v$ against I (Fig. 18). The value of the inhibitor constant (k_i) was also determined from this plot (27).

From the foregoing discussion, it is apparent that DOSS interacts with both the substrate and the enzyme, when the substrate used is a natural protein, hemoglobin. On the other hand, it binds the enzyme molecule exclusively and shows no affinity for the substrate when the

substrate used is a synthetic dipeptide, N-Acetyl-L-phenylelanyl-L-diidotyrosine (APD). Lack of interaction between APD and DOSS and significant interacting capacity of DOSS for hemoglobin are some of the typical inhibitory characteristics of the sulfated macroanion inhibitors of pepsin reported earlier (88). These similarities in the inhibitory characteristics between DOSS and macroanion inhibitors may be a consequence of their structural similarities. However, the interaction between DOSS and pepsin in the presence of both a natural and a synthetic substrate seems to be an additional inhibitory feature of DOSS and is not exhibited by macroanion inhibitors (88). Such an enzyme inhibiting property may be attributed to higher water solubility, surface active properties, or a relatively lower molecular weight of DOSS. It is also possible that DOSS may have a molecular size and shape that highly favors the hydrophobic and steric considerations important in the orientation and proximity effects necessary for its binding to the catalytic site at the surface of the enzyme. The mechanistic studies also reveal that DOSS seems to possess a lower affinity for pepsin than for hemoglobin in the presence of the two. Such a behavior has also been reported to be shown by some macroanion inhibitors of pepsin (5). Lack of interaction between DOSS and APD but significant interaction between DOSS and hemoglobin, may also indicate the fact that DOSS probably alters the conformation of the natural protein, hemoglobin and no involvement of the primary structure takes place.

Medical treatment of peptic ulcers has been as controversial as the nature of the disease itself. The nature of treatments proposed have ranged from gastric freezing to a wide variety of possible diets to psychotherapy (50). Today, the most reliable clinical regimens of

treatment depend on an elaborate group of intertwined therapeutic approaches (50). These may include rest, diet, antacids, anticholinergics, sedatives, tranquilizers, and psychotherapy. Although these approaches, when chosen and applied rationally to the individual patient, have proven useful in relieving symptoms and in facilitating healing, none of these modes of treatment have proven effective in initiating and accelerating the healing of ulcers. In this regard pepsin inhibitors both direct and indirect have gained much significance in experimental and clinical ulcers (2-4, 13, 26, 31, 36, 49, 53, 72, 88, 105, 107). However these antiulcer agents are not capable of providing the quick symptomatic relief characteristic of antacids. Thus, an effective ulcer therapy has three aims: alleviation of the complaints, acceleration of the healing and prevention of recurrency. The important therapeutic properties of a successful antiulcer agent may be outlined as follows: (1) It should immediately neutralize the gastric hyperacidity and preferably should elevate the gastric pH to the range between 4 and 5. This will help in removal of pain and elevating the pH would ensure inactivation of existing pepsinogen and pepsin in the gastric juice. (2) A direct inhibitory effect on peptic activity of gastric juice. Such an action may be achieved through complexation, inactivation or adsorption of the active enzyme on the inhibitor molecule. (3) An indirect inhibitory effect of peptic activity which may be achieved by interaction of the inhibitor with one or more components which act as the natural substrates for pepsin. (4) The therapeutic agent must be capable of initiating the onset of healing. Such an effect is possible if the inhibitor can irreversibly complex with the cell components of the mucosal tissue and thereby form an insoluble crater at the ulcerated site. Such a

complexation will also be helpful in protecting from further necrosis and erosion. (5) It should decrease the gastric secretion of acid and pepsin that is due to vagal stimulation. (6) The therapeutic agent must have long gastric half life and should decrease the motility. (7) It should have reasonably greater buffering capacity and should also inhibit the release of kinins and any chemotactic factor involved in the process of inflammation.

Compounds like hexosamines, hyaluronic acid, mucopolysaccharides and certain glycoproteins seem to be present at higher concentration and in more reactive forms at the ulcerated tissue than at the normal surrounding cells (110). Many macroanion sulfated polysaccharides and even some other compounds such as ulcerine, carbanoxolone sodium and bicitropeptide (a bismuth proteinate) have been shown to possess their antipeptic activity and hence their antiulcerogenic action due to their ability to form insoluble complexes with these tissue degradation products (99, 110, 111). An ulcerated site covered with such a complex has been believed to be shielded from further exposure to the damaging effects of the acidic gastric juice and pepsin and thus resulting into healing of the ulcer.

In view of the mechanistic evidence, DOSS may provide a possible antiulcer action through such an indirect mechanism. It may also exert additional healing effect due to its direct inhibitory effect against pepsin. Pepstatin, a pentapeptide marketed by Banayu Pharmaceutical Co. is perhaps the most effective pepsin inhibitor in ulcers which acts through such a direct inhibitory action against pepsin (72). Furthermore, the findings by Lish (69) that DOSS exerts a potent inhibitory effect on the volume of secretions and on the amount of acid secreted

when administered intraduodenally to rats, may further enhance the anti-ulcerogenic effect of this compound.

A reduction in the digestive capacity of the gastric juice for food proteins may be a consequence of the use of DOSS as an antiulcer agent. Drug interactions with certain prodrugs and proteolytic enzymes are possible. These interactions have already been discussed in Chapter IV.

CHAPTER VI

SUMMARY

Diocetyl sodium sulfosuccinate is an anionic surfactant widely used in medicine as a fecal softener. Inhibitory effect of this medicinal surfactant on the proteinase activity of trypsin and pepsin was studied *in vitro* using both natural and synthetic substrates. The influence of various reaction conditions such as pH, mixing order, ratio of the components, preincubation, incubation time, and shaking rates on the inhibitory effect of the medicinal surfactant were studied. Kinetic studies were carried out using initial velocities and the mechanisms of inhibition were delineated employing various graphical techniques.

Mechanistic studies indicate that the inhibition of tryptic activity by dioctyl sodium sulfosuccinate in the presence of a natural substrate, involves substrate-inhibitor interaction involving substrate depletion as the major mechanism at both pH 6.4 and pH 7.6. However some direct enzyme inhibition may also occur. In the presence of a synthetic substrate, at pH 8.2, the inhibitory mechanism seems to be similar to the one outlined for the natural substrate, but at pH 6.4 a true competitive enzyme inhibition forms the only mechanism. The interactions of the inhibitor with the enzyme and the substrate were found to be irreversible through dialysis studies.

Mechanism of inhibition for pepsin was found to be similar to that of trypsin with the natural substrate. With the synthetic substrate,

the inhibition of pepsin at pH 2.00 was also found to be due to a competition between the substrate and the inhibitor molecules for the enzyme.

Possible therapeutic significance of the inhibitory effect of this medicinal surfactant was discussed suggesting possible use of dioctyl sodium sulfosuccinate in the treatment of peptic ulcers and pancreatitis. Biopharmaceutical implications of the study were also discussed.

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APPENDIX

APPENDIX

BASIC KINETIC MECHANISMS OF ENZYMES

Enzymes may be defined as the biocatalysts in the living organism. The most characteristic property and function of enzymes is the catalysis of chemical reaction. These reactions are complicated processes which are made up of a series of steps involving a number of intermediates. Following are some of the important experimental approaches which have contributed to the present knowledge of the various characteristic behaviors of enzymes and the mechanisms of the reactions catalyzed: (1) Various protein studies related to the structure, size, shape, and physicochemical properties of the enzymes. (2) Studies on the organic reaction models for enzyme action¹. (3) Kinetic methods.

Enzyme kinetics deals with the measurement of reaction rates and the derivation of kinetic constants for enzyme systems. Special kinetic techniques like continuous flow methods, stopped flow methods, relaxation methods and temperature jump measurements can give very useful information about the enzymic catalysis in some cases¹. However, the types of more usual kinetic approaches² are as follows: (1) Initial velocity studies. (2) Inhibition studies. (3) Isotopic exchange studies. (4) Variation of kinetic parameters with pH. (5) Use of non-linear reciprocal plots. (6) Use of inorganic cations.

¹S. Bernhard, "The Structure and Function of Enzymes", W. A. Benjamin, Inc., Menlo Park, Calif., 1968.

²W. W. Cleland, In: The Enzymes. (Boyer, P. D., Vol. II, Third Ed.) 1970, Acad. Press, N. Y., pp. 1-61.

I. INITIAL VELOCITY STUDIES

A determination of initial velocity patterns usually involves variation of the concentration of one substrate at different fixed levels of the other components and in the absence of products. The normal method for determining initial velocity pattern is to use separate reaction mixture for each substrate concentration. But it is possible to determine initial velocities as a function of substrate concentration from a single progress curve of the reaction if the equilibrium constant is high and the products do not inhibit the reaction.

In most simple enzyme systems only one of the reactants is rate limiting. However, more complex systems may involve more than one reactants, substrates, and/or intermediates which may be rate limiting. Kinetic equations describing such complex systems are tedious mathematical expressions. Nevertheless, rate equations of all enzyme systems can be reduced to one single rate equation, universally known as Michaelis-Menton equation³. Following is the derivation and some important features of Michaelis theory:

Michaelis-Menton Equation

Although it is useful to consider the rate equations of a number of types of possible mechanisms⁴, a minimum requirement for devising mechanisms for consideration is an understanding of the kinetic behavior of a given mechanism. In order to illustrate the Michaelis theory, one may consider the following simple type of enzymic mechanism involving a single intermediate:

³H. L. Segal, In: The Enzymes, (Boyer, P. D., Lardy, H. and Myrback, K., 2nd Ed., Vol. 1) 1959, Acad. Press, N. Y., p. 1.

⁴R. A. Alberty, Advances in Enzymology, 17, 1 (1956).

The mechanism



Where E, S, ES and P are, respectively, the concentrations of free enzyme, free substrate, enzyme substrate complex and product and the k's are rate constants.

Further one may consider the following assumptions about the mechanism: (1) There is actually formation of an intermediate enzyme-substrate complex, (ES). (2) Constant 'k₃' is unidirectional as 'k₄' is very very small. (3) The concentration of substrate is very high as compared to that of enzyme, i.e., S >> E. (4) Products are released rapidly from the enzyme. (5) Products bear no effect on the interaction between enzyme and substrate. (6) Concentrations of the individual components represent the molar activities and the latter remain unaltered with respect to their charges in the system. (7) pH of the system does not change during reaction. (8) Solubilities of the reactants do not change during the reaction.

Velocity of the reaction "v" at any time must be:

$$v = k_3 (ES)$$

moreover

$$\frac{d(ES)}{dt} = k_1[(E)(S)] - k_2(ES) - k_3(ES)$$

$$\text{or } \frac{d(ES)}{dt} = k_1[(E)(S)] - (k_2 + k_3)(ES) \quad (1)$$

at steady state:

$$\frac{d(ES)}{dt} = 0$$

Therefore, equation 1 can be written as:

$$k_1 [(E)(S)] = (k_2 + k_3) (ES) \quad (2)$$

We have the conservation equation;

$$(Et) = (E) + (ES) \quad \text{Where } (Et) \text{ represents total enzyme concentration.}$$

$$\therefore (E) = (Et) - (ES)$$

Substituting this value of (E) into Equation (2), we get:

$$k_1 [(Et)(S) - (ES)(S)] = k_2 (ES) + k_3 (ES)$$

$$\text{or } k_1 (Et)(S) = k_2 (ES) + k_3 (ES) + k_1 (ES)(S)$$

$$\text{or } k_1 (Et)(S) = (ES) [k_2 + k_3 + k_1(S)]$$

$$\therefore (ES) = \frac{k_1 (Et)(S)}{k_2 + k_3 + k_1(S)}$$

Dividing both numerator and denominator of R.H.S. by k_1 :

$$(ES) = \frac{(Et)(S)}{\frac{k_2 + k_3}{k_1} + (S)}$$

$$\text{or } ES = \frac{(Et)(S)}{K_m + S} \quad \text{where } K_m = \frac{k_2 + k_3}{k_1}$$

Substituting this value of ES into the original equation for the reaction velocity:

$$v = \frac{k_3 (Et)(S)}{K_m + (S)} \quad (3)$$

If ' V_{\max} ' is the maximum velocity and

$$V_{\max} = k_3(Et)$$

Then equation (3) becomes:

$$\boxed{v = \frac{V_{\max} (S)}{K_m + (S)}} \quad (4)$$

This is called the Michaelis and Menton Equation. According to this equation, a plot of v vs (S) will result in a hyperbola with an asymptot parallel to abscissa. In this equation K_m represents the Michaelis-Menton constant. K_m is not usually identical with the dissociation constant of the enzyme-substrate complex, but rather it represents the apparent dynamic dissociation constant under steady state

conditions. The Michaelis-Menton equation can describe the enzyme behavior at all the concentrations of the substrate studied. K_m of a system may vary for different substrates, temperature, solvent, and pH. K_m has the same units as the substrate. On the other hand V_{max} has the units of velocity and can also vary for different substrates, pH and temperature. ' V_{max} ' is dependant upon the enzyme concentration in the system, whereas K_m is independant.

Some Implications of Michaelis-Menton Equation

If $(S) \gg K_m$, equation (4) would reduce to:

$$v = V_{max}$$

which means that 'v' is the maximum velocity obtainable for a given enzyme concentration. Under these conditions, the velocity remains unchanged for any increase in substrate concentration. This situation is called zero order kinetics. If (S) is 100 times K_m , the deviation is 1%.

When $(S) \ll K_m$, equation (4) becomes:

$$v = \frac{V_{max} (S)}{K_m} = \frac{V_{max}}{K_m} (S) = \text{Constant} (S)$$

This equation shows that velocity 'v' of the reaction is directly proportional to substrate concentration (S). Therefore, reaction may be designated as of 'first order' kinetics. If $(S) = 0.01 K_m$, the deviation from first order is 1%; if $(S) = 0.1 K_m$, the deviation from first order is 9%.

Reaction rate is considered to be of mixed order for any concentration of substrate between $0.1 K_m$ and $10 K_m$ and is described by equation (4).

When $(S) = K_m$; equation (4) becomes:

$$v = 1/2 V_{max}$$

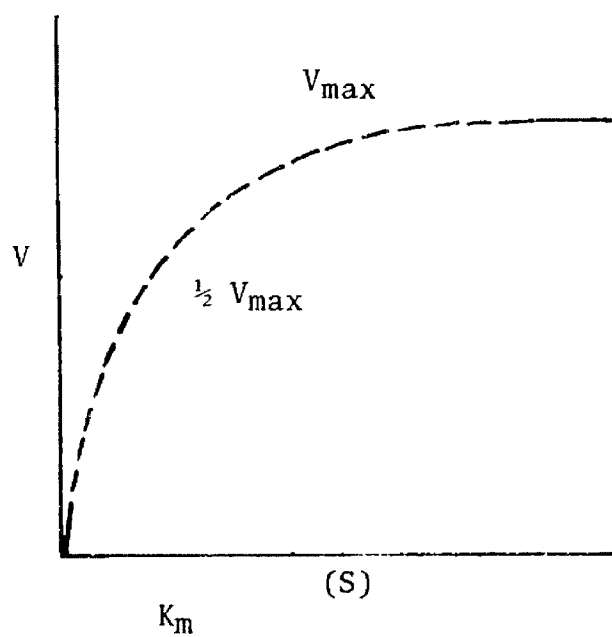


Figure 19.--Effect of substrate concentration on the rate of an enzyme-catalyzed reaction.

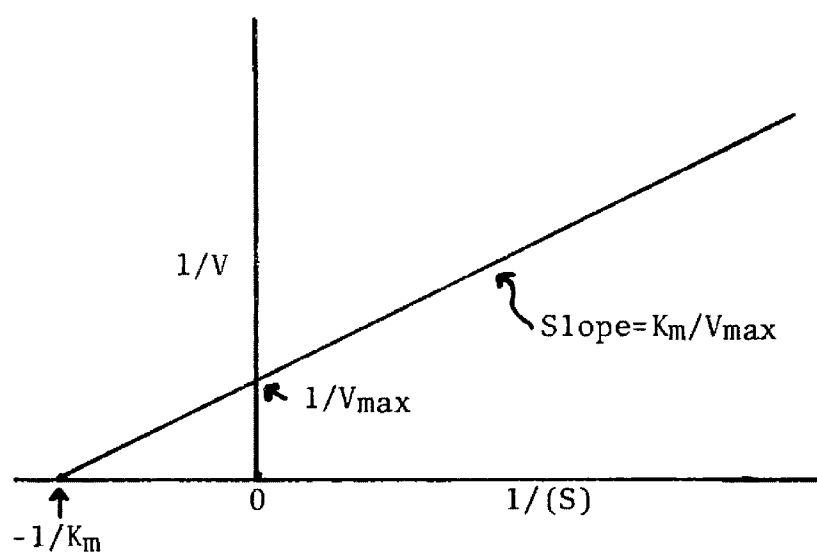


Figure 20 -- Lineweaver-Burk Plot

which means ' K_m ' equals the concentration of the substrate at which the reaction velocity equals half the maximum velocity obtainable.

Linear Transformations of Michaelis Equation

(1) Hyperbolic plot (Fig. 19) of v vs (S) is an inaccurate, inconvenient and cumbersome graphical method of determining the kinetic parameters, V_{max} and K_m . (2) High concentrations of substrate must be used in order to obtain V_{max} ; such high concentrations may not be attainable due to low solubility of substrate and furthermore substrate inhibition may occur.

In an effort to overcome these difficulties, various linear transformations of the Michaelis-Menton equation have been developed. These may be as follows:

Lineweaver Burk Plot⁵. Inverting equation (4) yields:

$$1/v = \frac{k_m + (S)}{V_{max} (S)}$$

$$\text{or } 1/v = \frac{K_m}{V_{max}} \frac{1}{(S)} + \frac{1}{V_{max}} \quad (5)$$

This is an equation of a straight line with slope K_m/V_{max} and intercept of ordinate, $1/V_{max}$. A plot of $1/v$ vs $1/S$ (Fig. 20) is helpful in determining V_{max} and K_m conveniently. It is also informative for enzyme inhibition studies. But this plot is fairly insensitive at high substrate concentrations. However, this problem can be overcome by plotting $1/v$ vs $\log \frac{1}{(S)}$.

Eadie Hofstee Plot⁶. Dividing both the numerator and denominator of R.H.S. of equation (4), by (S) yields:

⁵H. Lineweaver and D. Burk, J. Amer. Chem. Soc., 56, 658 (1934).

⁶G. S. Eadie, J. Biol. Chem., 146, 85 (1942).

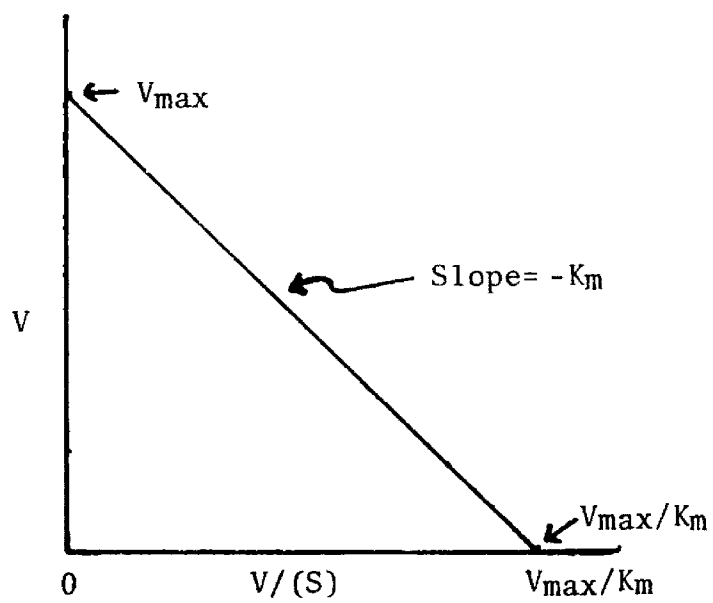


Figure 21 -- Eadie Hofstee Plot

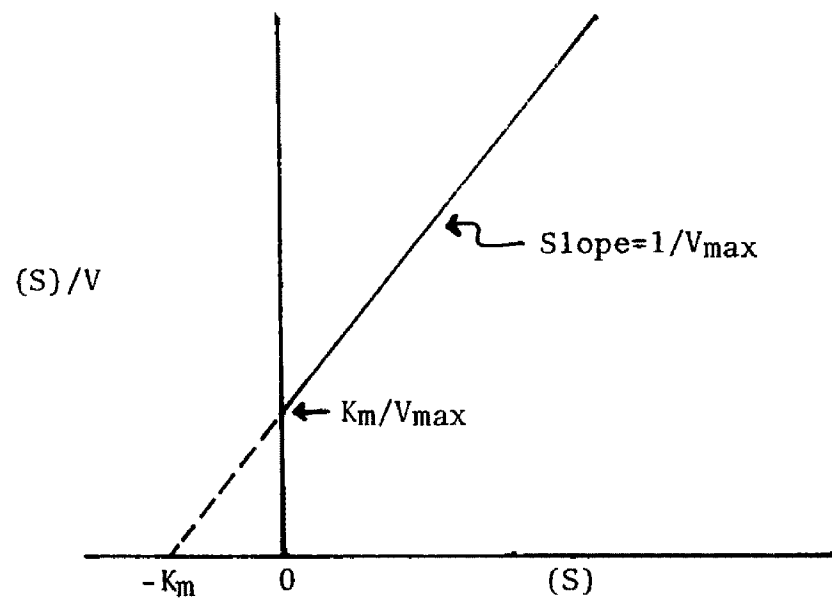


Figure 22 --Woolf Plot

$$v = \frac{V_{\max}}{1 + \frac{K_m}{(S)}}$$

$$\text{or } v + v \frac{K_m}{(S)} = V_{\max}$$

$$\text{or } v = V_{\max} - \frac{v}{(S)} \cdot K_m \quad (6)$$

According to this equation a plot of v vs $v/(S)$ results in a straight line with the slope equal to $-K_m$, the intercept on $v/(S)$ axis equal to V_{\max}/K_m and the intercept on v axis equal to V_{\max} (Fig. 21). This plot gives a more even distribution of the experimental points and the two constants appear separately. Therefore, any nonlinearity not shown by Lineweaver-Burk plot becomes apparent.

Wolf Plot⁷. Another linear transformation of equation (4) can be obtained by multiplying both sides of equation (5) by (S) :

$$\frac{(S)}{v} = \frac{K_m}{V_{\max}} + \frac{1}{V_{\max}} (S)$$

According to this equation a plot of $(S)/v$ vs (S) gives a straight line with the slope equal to $1/V_{\max}$, intercept on $(S)/v$ axis equal to K_m/V_{\max} and the intercept on the (S) axis equal to $-K_m$ (Fig. 22).

II. INHIBITION STUDIES

The significance of inhibition studies has already been discussed. It has now been realized that rarely a kinetic mechanism can be worked out without extensive resort to inhibition experiments⁸. A compound that slows down the rate of an enzyme catalyzed reaction is called

⁷H. Christensen and H. Palmer, "Enzyme Kinetics", W. B. Saunders Co., Philadelphia, 1967, p. 103.

⁸W. W. Cleland, In: The Enzymes, (Boyer, P. D., Vol. II, Third Ed.) 1970, Acad. Press, N. Y., pp. 1-61.

an inhibitor. Inhibitors usually form complexes with various enzyme forms and thus lower the amount of enzyme available for participation in the normal reaction sequence. However, inhibitors can also inhibit by complexing with a substrate, coenzyme or some other component on the system. It is possible that the substrate or products may be inhibitory and thus act as the inhibitors for some form of the enzyme. When a molecule which neither acts as a substrate nor as a product but forms complexes with one or more enzyme forms, is called a dead-end inhibitor. Inhibitory effect on the enzymic catalysis may also be exerted by an alternate substrate or an alternate product which may act as inhibitors. Denaturation of an enzyme by general protein denaturants is usually not considered as inhibition at all, but rather as protein destruction.

Most of the nonallosteric enzyme inhibitions involving enzyme-inhibitor interaction can be broadly classified into irreversible or reversible types. Irreversible inhibition usually involves the destruction or modification of one or more functional groups of the enzyme. An irreversible inhibitor may or may not contain structural elements of the substrate. If the catalytic course of an enzyme reaction involves the formation of metastable covalent enzyme-substrate intermediate, other molecules which have the ability to form similar but stable covalent enzymes compounds will function as irreversible inhibitors. Reversible inhibition on the other hand can be competitive, uncompetitive, noncompetitive, or even mixed types. In the competitive type, the presence of the inhibitor prevents the access of substrate to the catalytic site and the EI complex is dissociable. Therefore, competitive inhibition can be reversed by increasing the substrate concentration. In the uncompetitive type of inhibition, the presence of the inhibitor at the catalytic site

does not necessarily exclude the substrate, but the inhibitor prevents chemical catalytic action by the enzyme. EI complex is dissociable even in this case. The special case of uncompetitive inhibition in which the presence of the inhibitor has no effect on the binding of the substrate to the catalytic site, is known as noncompetitive inhibition.

Distinction between these mechanisms can easily be made by using Lineweaver-Burk plots, i.e., plots of $1/v$ vs $1/S$ at varying concentrations of inhibitor. If only the slope varies with inhibitor concentration, competitive inhibition is present. If only the intercept varies, one has uncompetitive inhibition. The case in which both slope and intercepts are function of inhibitor concentrations is termed noncompetitive inhibition. In truly competitive inhibition, double reciprocal plots are characterized by straight lines of differing slope intercepting at a common intercept on the $1/v$ axis. In the presence of a competitive inhibitor V_{max} is not altered but K_m is increased. In noncompetitive inhibition, the plots differ in slope but do not share a common intercept on the $1/v$ axis. V_{max} decreases but K_m may or may not remain unchanged in this case. In the most usual cases the slope or intercept variation with the inhibitor concentration is a linear function. In more complex cases, the slopes or intercepts give replots which are hyperbolas or parapolas.

Inhibitors that have the potential of interacting with the substrate, may affect enzyme action. Such an inhibitor can affect the rate of enzyme catalyzed reaction in three ways: (a) The inhibitor may combine with the substrate and decrease its effective concentration. (b) The inhibitor may combine with the substrate and the complex formed may act as an inhibitor for the enzyme. (c) A combination of the above two mechanisms.

These types of inhibitions are frequently difficult to distinguish from those where apoenzyme is attacked, if the usual kinetic analysis is applied uncritically. The most common graphical methods used to distinguish such inhibitory mechanisms have been described by Webb⁹ and Reiner¹⁰ in detail. Among these methods plot of fractional inhibition (i) against the inhibitor concentration (I), and plots of I vs $i/(1-i)$ seem to be the most important graphical techniques.

⁹J. L. Webb, "Enzyme and Metabolic Inhibitors", Vol. 1, Acad. Press, London, 1963, p. 85.

¹⁰J. M. Reiner, "Behavior of Enzyme Systems", 2nd Ed., Van Nostrand Reinhold Co., N.Y., 1969, pp. 192-199.