SHORT TITLE

# THE ROLE OF ELASTASE IN THE PATHOGENESIS OF ACUTE PANCREATITIS

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# THE ROLE OF PROELASTASE-ELASTASE ENZYME SYSTEM IN THE PATHOGENESIS OF EXPERIMENTAL PANCREATITIS

by

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# CHAPTER I

## INTRODUCTION

"On every scientist's desk there is a drawer labelled UNKNOWN in which he files what are at the moment unsolved questions, lest through guesswork or impatient speculation he come upon incorrect answers that will do him more harm than good. Man's worst fault is opening the drawer too soon. His task is not to discover final answers but to win the best partial answers that he can, from which others may move confidently against the unknown, to win better ones."

Homer Smith. From Fish to Philosopher; Boston; Little, Brown, 1953.

Acute pancreatitis is a disease of variable intensity, from mild oedema to a generalized fulminant necrosis; usually it is defined as an acute inflammatory response of pancreatic tissue to various types of injury.

The first accurate and comprehensive description of acute haemorrhagic pancreatitis was given in 1889 by Fitz (1), a Boston pathologist, and since that time this form of the disease has been widely discussed in the medical literature. As a result of observations during his experiments in haemorrhagic pancreatitis in animals, Archibald (2), in 1913, called attention to pancreatic oedema, and Zoepffel (3), in 1924, distinguished between cases of acute pancreatic oedema seen at operation and those characterized by haemorrhage and necrosis.

Most of the available information pertains to the fulminant form of the disease, and extensive experimental work has been directed largely toward the production and study of acute haemorrhagic pancreatitis. Recent literature

reflects a better understanding of the underlying physiological mechanisms and takes a broader view of the problem of pancreatitis, in an attempt to find a common denominator of its pathogenesis, whereas earlier work was devoted mainly to exploration of the validity and inconsistencies of the 'common-channel' theory propounded by Opie in 1901 (5-7).

Acute pancreatitis has been induced in various animals, including dogs, rats, cats, rabbits, goats and monkeys, by a variety of methods, e.g., ligation of the ducts in the presence of copious secretion, impairment of blood supply, mechanical or chemical trauma, metabolic derangement, and local anaphylaxis. All forms of experimental pancreatitis bear some resemblance in gross and microscopic pathology to certain stages of the disease in man. This resemblance may indicate that the pancreatic reaction to injury is somewhat stereotyped, depending more upon the rate and degree of insult than upon its nature. However, the findings in experimental pancreatitis cannot be related exactly to the disease in man, because of the anatomical and physiological differences between man and the experimental animal; any experimental model must be regarded as a compromise measure, since it is not known whether the aetiological factor(s) play the same role as in spontaneous pancreatitis in man.

Despite the accumulation of a vast amount of experimental data since the brilliant experiment princeps of Claude Bernard in 1856 (8), the controversy concerning the pathogenesis of the disease continues and it is not yet known which aetiological factors are responsible for the initiation of pancreatitis and which mechanisms contribute to its progression to extensive pancreatic necrosis and death.

The most plausible hypothesis is that of localized autodigestion of the pancreas, with disintegration of acinar cells, haemorrhage from necrotic

vessels, and fat necrosis, all of which have been ascribed to the effects of lipase and proteolytic enzymes which have been activated within the organ or in the ductal system and have gained access to the interstitial spaces. However, the details of such a mechanism are but poorly understood. The fact that active enzymes do not dissolve cells of the digestive tract has long excited the curiosity of physiologists; the outer layers of living cells, which appear to consist of muco- and lipoprotein, are resistant to digestion by common proteolytic enzymes, and while these layers remain intact the enzymes are not able to enter the sensitive cytoplasm. Surgeons have long been aware that chronic pure pancreatic fistulae do not cause necrosis. Also, an increase in active trypsin in homogenates of pancreatic tissue obtained from cases of all types of experimental pancreatitis has never been demonstrated, despite most diligent efforts. Recently, the entire concept of 'tryptic pancreatitis' has been challenged by Beck and his group (9), who found no active trypsin in homogenates of pancreatic tissue from dogs with acute bile pancreatitis.

Evidence for a key role of trypsin in acute pancreatitis was obtained by Polya in 1905 (10) and Rich and Duff in 1936 (11). The prominent feature of the pancreatic damage produced by the latter authors was necrosis of the vessel walls and associated tissue haemorrhage. Recently, this type of vascular injury, including the destruction of elastic fibres, has been attributed to elastase (12). It has been said that the role of the enzymes of the exocrine pancreatic secretion in the pathogenesis of pancreatitis will remain obscure until the physiological and biochemical mechanisms involved in their synthesis, activation, secretion and particularly inhibition are more fully understood (13).

In considering the action of intracellular enzymes and the part

they play in the destructive process in acute pancreatitis, it is noteworthy that, after death, the pancreas undergoes rapid autolysis and, with the possible exception of the mucosa of the gastro-intestinal tract and the adrenal cortex, is the most rapidly autolysed body tissue. Autolysis in necrotic tissue involves displacement of the normal equilibrium between synthetic and catabolic activities (14).

It has been shown that the pancreatic cell contains acid hydrolases which probably are lysosomal enzymes (15, 16). The lysosome concept was originally formulated by de Duve et al. in 1955 (17) to explain the 'latency' in sucrose suspensions of certain hydrolases, mainly nucleases, phosphatases, and cathepsins. These workers suggested that lysosomal enzymes may mediate physiological or pathological cell destruction. In amphibian metamorphosis, marked increases in activity of several lysosomal enzymes (cathepsins, beta-glucuronidase, and acid phosphatase) have been demonstrated in the regressing tail (18). It has long been suspected that cathepsins and other intracellular acid hydrolases participate in aseptic autolysis and necrosis of tissue, but no satisfactory explanation has been given of the processes responsible for their activation in dying or dead cells.

de Duve and Beaufay (19) reported that the earliest change observed in ischaemia in a rat liver lobe was the release of hydrolases. Holtzer and Van Lancker (20) investigated the fate of hydrolytic enzymes during controlled autolysis of mouse pancreas and found that rupture of zymogen granules, leading to the release of amylase and ribonuclease, is one of the first measurable events, and showed also that granules containing acid phosphatase are relatively more resistant to autolysis than are liver lysosomes. Beaufay and de Duve (21) observed that the addition of trypsin, chymotrypsin and

pancreatin resulted in the release of five acid hydrolases, including cathepsin, from a rat liver preparation. On the other hand, Greenbaum and Hirschkowitz (15) demonstrated activation in vitro of trypsinogen by cathepsin B, and presented evidence that endogenous trypsinogen in extracts of dog pancreas is activated under the same conditions. The role of the intracellular enzymes in the initial stages of acute pancreatitis and their interrelationship with the enzymes of the exocrine secretion are not well understood. Significant elevations of serum beta-glucuronidase have been observed in pancreatitis (22). The tissue damage that occurs in the Shwartzman reaction (one of the methods of inducing experimental pancreatitis) has been attributed recently to the release or activation of intracellular hydrolytic enzymes, effected by endotoxin, from granules of infiltrating polymorphonuclear cells at the site of injection (23). On the other hand, potentiation of the local haemorrhagic Shwartzman reaction by trypsin has been reported by Antopol and Chryssanthou (24); and heparin, which inhibits the Shwartzman phenomenon (25), inhibits the action of trypsin also (26). During most forms of tissue degeneration, whether induced by ischaemia, anoxia, or trauma, autolytic processes (effected by intracellular enzymes) are supplemented by the 'heterolytic' contribution of invading cells (27). The significance of such a heterolytic mechanism in acute pancreatitis is unknown.

Other agents which have recently been cited in the pathogenesis of acute pancreatitis are bradykinin, elastase, and collagenase. The ability of bradykinin to cause vasodilatation, increased vascular permeability and leukocyte accumulation suggests the possibility that this enzyme may be concerned in pancreatitis; the pancreas contains large quantities of trypsinogen and kallikreinogen, both of which when activated are capable of

releasing bradykinin from its precursor, bradykininogen (28). Schneider et al. (12) injected pancreatic elastase into the pancreatic ductal system of dogs and thereby induced pancreatitis; the vascular injury was comparable to that of the spontaneously occurring disease. The presence of collagenase in pancreatic tissue has been suspected for some time, but only recently has its activity been clearly distinguished from that of trypsin and chymotrypsin (29). It has been postulated that, in acute pancreatitis, collagenase initiates the process of parenchymatous disruption, after which trypsinogen is converted to trypsin with additional digestion of tissue(30).

It is obvious from the preceding brief considerations that a better understanding of the pathogenesis of acute pancreatitis must await elucidation of several pertinent points which relate to the complex enzymatic mechanisms involved:

1. The role of intracellular hydrolases, especially cathepsins, in initiation of aseptic autolysis and activation of precursors of exportable pancreatic enzymes.
2. The part played by collagenase in the destruction of collagen in pancreatic septa and vessel walls.
3. The extent of the catalytic and autocatalytic role of trypsin and its degree of inactivation by pancreatic trypsin inhibitor.
4. The importance of the bradykinin system.
5. The role of the proelastase--elastase system in the production of vascular injury, and the progression of pathology in the early stages of acute pancreatitis; this particular point constitutes the subject of this thesis.

# CHAPTER II

## HISTOPATHOLOGY OF ACUTE PANCREATITIS

The essential histopathological features of acute pancreatitis, as it occurs spontaneously in man and as produced experimentally in animals, in order of increasing severity, are:

Oedema and inflammatory cellular infiltration of interstitial spaces;

Necrosis and disintegration of acinar cells;

Intrapancreatic and extrapancreatic fat necrosis; and

Haemorrhage from necrotic blood vessels.

All gradations occur in the experimental and the naturally occurring disease. Oedema, inflammatory cellular infiltration and parenchymal necrosis are common expressions of inflammatory processes in all organs. Fat necrosis and the peculiar variety of vascular necrosis with haemorrhage are distinctive and unique characteristics of pancreatic inflammation. The lesions may be confined to the pancreas or they may involve contiguous structures or may even be widespread throughout the abdominal cavity. Acute haemorrhagic pancreatitis is accompanied by an extravasation of bloody fluid which may be of sufficient magnitude to produce sanguineous ascites.

### A: OEDEMA

In the milder forms of pancreatitis, varying amounts of oedema fluid are lost into the interstitium of the gland and surrounding tissues. Oedema may occur in any area of the pancreas but usually is more severe in the head. The region affected becomes pale and indurated and its blood vessels more congested (31). Popper (32) accepts the concept that oedema is the earliest response of the pancreas to injury and that necrosis and haemorrhage represent advanced stages of oedematous pancreatitis: he frequently noted that a

pancreas which was only oedematous at the time of laparotomy was necrotic a few days later at autopsy. He and his associates (33) found that temporary occlusion of the pancreatic arteries transformed into pancreatic necrosis the pancreatic oedema which had been produced by duct ligation and exocrine stimulation. On the other hand, it has been suggested (34) that haemorrhagic pancreatitis differs from pancreatic oedema both pathologically and biochemically, although oedema is a prerequisite to the development of necrosis.

Safadi, Pfeffer and Hinton (35) made a closed duodenal-loop obstruction in the dog and produced haemorrhagic pancreatitis. Histological studies showed that the earliest lesion was an extravasation of red blood cells that disrupted and interfered with parenchymal nutrition at the acinar level. They concluded that a vascular lesion quickly initiates fulminating pancreatitis. Further experiments by the same group, in which sterile polyethylene microspheres were injected under controlled pressures into the superior pancreatico-duodenal artery of dogs, resulted in varying degrees of pancreatitis, oedematous through haemorrhagic (36). The severity of the lesion was inversely proportional to the size of the microspheres.

Morris (37) studied the development of pancreatic necrosis in the mouse by observing the chronological events that followed ductal ligation in an exteriorized pancreas with an intact blood supply in a chamber under a microscope. The most important irreversible change was oedema of the intercalated ducts, which was accompanied by dilatation of acini and decreased capillary flow with sludging. Tissue ischaemia and, finally, acinar rupture with interstitial haemorrhage occurred terminally. Thal (38) found that when bile was injected forcibly into the pancreatic duct of rabbits, blood flow almost ceased, because of rapid vascular injury; and Anderson (39) observed in dogs that portal venous-outflow occlusion converted enzyme-induced

pancreatic oedema into severe necrotizing pancreatitis.

B: FAT NECROSIS

Perhaps the most characteristic histological alteration of acute pancreatitis is the development of focal areas of fat necrosis in the stromal, peripancreatic fat and fat depots throughout the abdominal cavity and at times in the pleura, pericardium, mediastinum (40), bone marrow (41), and brain. This necrosis is caused by the action of pancreatic lipase on the triglyceride of the fat storage cells. Fatty acids released by the enzymatic hydrolysis of neutral fat combine with calcium derived from blood and tissue fluid to form calcium soaps which are precipitated in situ. Liberated glycerol is reabsorbed. The replacement of neutral fat by calcium soaps and fatty-acid crystals, and the attendant inflammatory reaction, produce the characteristic gross and microscopical appearance of fat necrosis. When grossly visible, the lesions are small white, grey or yellowish plaques on the surface of the organs or peritoneum. On microscopical examination the vacuolated fat cells appear as shadowy outlines of cell membranes filled with pink, granular, opaque precipitate, corresponding to fatty acids. Depending upon the amount of calcium deposition, amorphous basophilic precipitate may be visible within the necrotic focus. Leukocytic infiltration is confined to the periphery of these areas and there is no white-cell aggregation in the central areas of fat necrosis.

Fat necrosis frequently occurs in and about the pancreas in the absence of haemorrhage or tissue digestion (34, 42), indicating that the mechanisms that protect against proteolytic activity are more effective than those acting to offset lipolysis. Lipase is secreted in the active form by acinar cells; Flexner (43) recovered this enzyme in areas of fat necrosis. Pancreatic juice of low enzyme concentration produces fat necrosis when it is

injected subcutaneously in the absence of haemorrhagic necrosis (7). Popper (44) produced extensive intra-abdominal fat necrosis experimentally in dogs by transecting the pancreatic duct and instituting early postoperative feeding to stimulate pancreatic flow in the peritoneal cavity.

D: PARENCHYMATOUS NECROSIS

Pancreatic necrosis in man presents as numerous yellow, grey, or black and red areas, according to the extent and the severity of the necrosis, infarction and haemorrhage which occur concomitantly but in different degrees in various zones of the organ. If the disease progresses without resolution, infection by intestinal bacteria produces suppuration and may result in pancreatic abscess formation.

Microscopically, various stages of cell disintegration may be seen. The cells are pale and their nuclei stain poorly. In more severely damaged regions all structure is lost in the stroma, acinar cells and islet tissue alike. The necrotic areas are demarcated from normal tissue by a zone of debris and a layer of inflammatory cells. Liquefaction of necrotic tissue, haemorrhagic collections and retention of pancreatic secretion result in the formation of cystic structures within the zones of demarcation. The histological reaction patterns of the canine pancreas to the injection of bile and trypsin under the experimental conditions described by Beck and his associates (45) were quite dissimilar. The reaction to the injection of bile was that of almost immediate (within five minutes) coagulation necrosis of a part of a lobule; although some oedema was seen in the septa, cellular inflammatory reaction was not a striking feature. The histological pattern in trypsin pancreatitis also was focal: the earliest response was a marked reactive hyperaemia with vasodilatation of the capillaries, followed by inter-

stitial haemorrhage; many of the acinar and islet cells in the involved areas had disappeared. The rapid cytolysis suggested liquefaction necrosis with loss of structure in the involved area.

The details of cell damage and cell death in spontaneous as well as in experimental pancreatitis are unknown, and the role of the exportable and intracellular pancreatic enzymes and their interrelationship is but poorly understood.

D: HAEMORRHAGE

The haemorrhagic component of acute pancreatitis may be minimal to extreme. In milder cases the interstitium is suffused with red blood cells and fibrin clots and, in severe instances, large areas of the pancreatic substance are converted into a blood clot. Arterial or venous thrombosis and erosion of blood vessels by activated pancreatic enzymes results in haemorrhage into the pancreas, the retroperitoneal tissues, and even into the bowel (31).

The first to describe necrosis of the vessel walls in experimental pancreatitis was Rosenbach (46), in 1911. Necrosis of the media in the smaller arteries, involving the entire circumference or limited to only a small portion was reported in 1932 by Lowenthal (47) in acute pancreatic necrosis in man. In 1936 Rich and Duff (11), in their excellent contribution, emphasized the importance of the vascular destruction, which they attributed to the action of trypsin; destruction of a segment of the vessel wall was found in the larger vessels, whereas the whole circumference was destroyed in the smaller ones. The first alterations in the media were always found in the outer layer, but in most cases the damage proceeded rapidly to involve the entire thickness of the vessel wall. These authors considered the "vascular lesion" to be a result of, or concomitant with, the pancreatic necrosis, but

recent experimental studies indicate that the vascular injury is the deciding factor for the type of lesion finally produced (12).

Nemir and associates (48-50) have shown that injection into the dog pancreas of whole blood that has been incubated with pancreatic enzymes will cause severe necrotizing pancreatitis, and concluded that this lesion results from the action of some toxic substance produced when blood is digested by pancreatic enzymes. Anderson (39, 52) and Anderson and Bergan (51) demonstrated the characteristic vascular injury in acute haemorrhagic pancreatitis, consisting of arteritis, phlebitis, thrombosis of venous channels, and destruction of small blood vessels. They believe that blood extravasated into the interstitium of the pancreas during an attack of acute pancreatitis may incubate with retained pancreatic enzymes and may produce a toxic agent which is responsible for the progression to pancreatic necrosis.

# CHAPTER III

## AETIOLOGY OF ACUTE PANCREATITIS

In the last fifty years, clinical and experimental studies have shown that the causes of acute pancreatitis are varied and that several factors may precipitate the disease; indeed, Sterling (53) has enumerated sixty-six possible aetiological factors. The literature on the subject is extensive and rather confusing, and it is timely to review certain pertinent concepts, based on clinical and experimental data.

### A: INFECTION

No specific infective factors have been identified from cases of acute pancreatitis, but its occasional association has been reported with mumps (54, 55), typhoid fever (56), scarlet fever (57), streptococcal food poisoning (58), dysentery (59), and Candida albicans infections (60); also, the beneficial effect upon co-existing pancreatitis of the eradication of biliary-tract disease (61, 62) strongly suggests an infective origin in such cases.

Blood-born infection as a cause of pancreatitis has been proved experimentally by transmission of Coxsackie virus in animals (63) and in man (60). However, bacteraemia has not been found in patients with acute pancreatic necrosis, and the clinical symptoms and findings suggest a toxaemia rather than a blood infection. Injections of bacterial suspensions into the pancreatic duct will induce pancreatitis (64); and the mere presence of bacteria in the duct markedly increases the severity of the disease when combined with other causative factors (65, 66, 67). Extracts of necrotic pancreas are exceedingly toxic when injected into the abdominal cavity or into the veins of experimental animals, but in vivo autolysis of pancreas from a foetus removed by Caesarean section is innocuous (71).

It seems that, in the majority of cases, infection as a primary factor is of minimal aetiological importance (68), although it may contribute to the severity of the disease. Several investigators (69, 69a, 69b) have shown the value of antibiotic therapy in bile-induced pancreatitis, and it is known that bacteria and their toxins may produce small-vessel thrombotic occlusions in the pancreas which may convert inflammation to tissue necrosis(70). One of the factors involved in the closed duodenal-loop preparation developed by Pfeffer et al. (72), which consistently produced a type of haemorrhagic pancreatitis, may have been the reflux of infected duodenal contents into the pancreatic ductal system. Pancreatitis did not develop with this preparation when the pancreatic ducts were ligated (73) or when gentian violet was instilled into the closed duodenal loop (74).

B: OBSTRUCTION, HYPERSECRETION, AND REFLUX OF BILE

The importance of a mechanical factor of obstruction at the papilla of Vater, with subsequent reflux of bile into the pancreatic ductal system, was first stressed by Opie (5-7), who propounded the 'common-channel' theory of pancreatitis in man. He and subsequent adherents of this concept suggested that it was the retrograde passage of bile into the pancreatic duct which initiated pancreatitis, either by activation of pancreatic enzymes or by the introduction of infection. It was soon realized, however, that considerable variability exists in the arrangement and communication between the major (Wirsung) and minor (Santorini) pancreatic ducts, and in the frequency of a 'common channel' that permits free association of bile and pancreatic secretion before discharge through the papilla into the duodenum (53).

Much has been written about the incidence of a common channel in man, the reports quoting figures of 20 to 80% (75-79). Precise studies in

which injection and dissection were carried out in unselected necropsy material showed a 'common-channel' configuration in 30 - 40% (80, 81). Janowitz and Dreiling (82) found evidence of a functional common channel in 67.2% of a large series of normal subjects, and Dragstedt et al. (83) stated that obstruction in the region of the papilla of Vater plays an important aetiological role in 60 - 70% of cases of acute pancreatitis.

Even when a common channel does exist, however, the actual reflux of bile into the pancreatic duct system depends upon the pressure gradients in the biliary and pancreatic tree. Harms (84) has shown in unanaesthetized dogs that at the height of digestion the secretory pressure in the pancreatic ducts is higher than in the biliary tree, permitting flow only from the pancreas to the biliary tract; and others (85-88) have found that secretory pressures in the pancreatic ducts are higher than those in the biliary tract after meals, after specific stimulation of pancreatic and biliary flow, or even in the resting state. That reflux can occur, however, is demonstrated by the fact that some segment of the main pancreatic duct may be visualized during T-tube cholangiography (89): retrograde filling of the pancreatic ductal system occurred in 18% of the human subjects in whom Mallet-Guy and Giuria (90) performed cholangiography during surgery, and in 35% in Ivy and Gibbs' series (65) during operation and postoperatively. Also, on rare occasions, Gaillard (91) has observed acute pancreatitis developing after T-tube cholangiography postoperatively. Elliot, Williams and Zollinger (92) have presented evidence which suggests that although, initially, pressure in the obstructed pancreatic duct of the dog exceeds that of the obstructed common bile duct, the two pressures approach one another after 24 hours' obstruction, so that regurgitation of fluid from bile duct to pancreatic duct is possible if a common channel exists. They also showed that bile or unincubated mixtures of bile and

pancreatic juice, infused at 40 mm. water pressure, enter the pancreatic ducts of dogs in only small quantities. When the bile is incubated with pancreatic juice or trypsin and then injected into the pancreatic duct at the same pressure it is readily accepted by the pancreas. Hermann and Knowles (93) united the biliary and pancreatic ductal systems of dogs into an obstructed common system and found that bile reflux occurs after 24 to 30 hours; bile flowed into the pancreatic duct and in some cases was found in pancreatic tissue at necropsy. Radiocinemanometric studies by Caroli et al. (94), during biliary-tract perfusion through a T-tube, showed that, at pressures of 250 - 300 mm. bile, reflux into the pancreatic duct occurred in a high percentage of cases; but they also maintained that sphincter spasm resulted in isolation of the biliary and pancreatic ducts, a situation that would mitigate against reflux and pancreatitis. This point has been stressed also by Paulino-Netto et al. (95), who used T-tube cholangiography to demonstrate the absence of spasm of the sphincter of Oddi in 15 cases of pancreatic ductal reflux. Considerable importance has been attached to the observation of Mann and Giordano (75) that during vomiting the pressure in the biliary tract may rise to 1000 mm. bile: any slight obstruction at the common outflow into the duodenum would cause regurgitation into the pancreatic duct.

From the preceding data it seems reasonable to assume that under normal conditions pancreatic ductal pressure is higher than biliary pressure, but that, when a common channel exists, obstruction at the ampulla of Vater, forceful contraction of the gall bladder or increase in intra-abdominal pressure may cause bile to be regurgitated in the pancreatic duct. The significance of bile reflux in pancreatitis has been challenged by experimental work in which the entire biliary flow was diverted under physiological pressures,

through the pancreatic ductal system in goats (108, 109) and in dogs (110, 111), without serious consequences. Thus, even accepting the hypothesis that bile is regurgitated into the pancreatic ductal system, its precise role in the development of acute pancreatitis is not easy to understand. When bile is injected under high pressure it infiltrates pancreatic tissues and exerts its toxic effect, and invariably leads to necrosis; when it is injected under low pressure, only small amounts enter the pancreas and no necrosis occurs (112). Thal (38) has demonstrated that the presence of bile in the interstitial tissues may initiate pancreatitis through a vascular effect, including stasis and arteriolar spasm.

Dragsted, Haymond and Ellis (83) pointed out that the direct cause of necrosis was the toxic property of bile salts; and they also expressed the view that bile does not activate trypsinogen. The latter view has been confirmed by others (111, 113), and some workers (114, 115) have shown also that trypsin is inhibited by bile. Dragsted and his group (83) drew attention to an increase in the toxicity of bile with the absolute content of bile salts, and it has been shown (116) that free bile acids are more toxic than conjugated. Conjugation with glycine and taurine occurs in the liver, and bile acids exist in the bile only in the conjugated form. Clostridia and enterococci, which may be present in bile when the biliary tract is infected, may effect deconjugation of bile acids (117, 118); thus, reflux into the pancreas of bile which contains free bile acids might give rise to acute pancreatitis (119).

Rich and Duff (7) have emphasized that the important factor predisposing to pancreatitis in humans in whom the anatomical common channel is present is the pancreatic-duct obstruction rather than bile regurgitation. Obstruction of the sphincter of Oddi at the ampulla of Vater may be due to spasm of the sphincter (96), calculus (5), oedema (97), fibrosis (98), hypertrophy (99),

and other causes, including tumour of the outflow tract. Although obstruction of the pancreatic duct or ductules by tumour, stone or epithelial metaplasia (7) may lead to pancreatitis, complete obstruction by carcinoma of the head of the pancreas usually does not, even when serum enzyme levels are elevated (100-102).

Experimental studies have further emphasized the significance of complete or partial obstruction of the pancreatic ducts, especially when it is combined with stimulation of the secretory activity or impairment of the blood supply (103, 104). Food, pilocarpine, acetyl choline, secretin, direct stimulation of the vagus (105) and mecholyl (106) have been used as secretory stimulants in dogs after ligation of the duct, and haemorrhagic pancreatitis has been induced in dogs by obstructing the duct intermittently during a four-day period while pancreatic secretion was stimulated by secretin and urocholin (107) and by anastomosing the pancreatic duct to the common bile duct before injecting morphine to induce spasm of the sphincter of Oddi (34).

In about 50% of human subjects a functioning accessory pancreatic duct may partly relieve obstruction of the main pancreatic duct. In a small percentage of cases, however, in whom the duct of Santorini is the only one functioning, acute pancreatitis often is fatal (13).

Simple pancreatic-duct obstruction in dogs usually results only in the development of high intraductal pressure which leads to oedema, congestion, and focal haemorrhage and subsequent atrophy of the gland (120, 121). Hong et al.(122) produced oedema, leukocytic infiltration and acinar degeneration by obstructing the main pancreatic duct in rabbits; and Zelander and his group (123) have shown, with electron microscopic studies on rat pancreas, that stasis resulting from occlusion of the outflow of pancreatic secretion for

2 - 4 days causes damage that varies more from one acinus to another than within them. In these latter studies the induced cytological events were a reorganization of the endoplasmic reticulum, reduction in the number of zymogen granules, mitochondrial swelling, increase in size of Golgi vacuoles surrounded by a thin rim of cytoplasm and, finally, occasional cell death and rupture of the acinus with leakage between the acinar lumen and the interacinar spaces.

C: VASCULAR FACTORS

For many years it has been considered that local vascular disturbances may initiate pancreatitis by interfering with the pancreatic circulation and may also accelerate pathology induced by other agents. Because of special interest in this particular aspect of the aetiology, significant contributions to the relevant literature will be reviewed.

1. Permanent Local Ischaemia

Pancreatic haemorrhagic infarcts but not pancreatitis were produced by injection of various substances (particles of wax, lycopodium powder, petroleum, and various types of oil) into pancreatic arteries, with or without subsequent ligation of the particular vessel, by Panum (124) as early as 1862, and subsequently by Lepine and others (125-128). Langerhans (129) was the first to report, in 1890, induction of acute pancreatitis by mass ligation of vessels to and from the pancreas with transection of the gland at one or more levels.

In recent years Block et al. (130) have shown that complete devascularization of large portions of rat pancreas causes ischaemic infarction and, occasionally, acute pancreatitis with parenchymatous and fat necrosis varied in severity according to the degree of arterial obstruction. Safadi et al. (131) induced haemorrhagic pancreatitis in dogs by injecting plastic micro-

spheres under controlled pressures into the pancreatic arteries; the most severe lesions developed with the smallest microspheres (8 to 20 μ.) when these were injected into the superior pancreatico-duodenal artery.

2. Transient Ischaemia

Blume (132) in 1897 was the first to report the development of local ischaemia and small foci of necrosis when digital pressure was applied to the pancreatic vessels in cats for 10 minutes, and Beneke (133), discussing these observations, remarked that the necrosis produced by vascular compromise underlay the specific autodigestion seen in acute pancreatitis. Popper et al. (103) failed to produce pancreatitis in dogs by ligation of the ducts combined with stimulation of the external secretion of the gland, but the addition of a clamp to the gastro-duodenal artery for 15 minutes, in the presence of oedema provoked by intraductal injection of normal saline, resulted in pancreatic necrosis in direct proportion to the degree of pre-existing oedema.

3. Disturbances of the Nervous Regulation of Blood Flow

Reboul (134) stimulated the left splanchnic nerve after ligation of the major duct in dogs and guinea pigs and produced changes of acute pancreatitis of slight to severe degree in 31%, and Longo et al. (135) obtained similar results. Menguy and his group (104) ligated both pancreatic ducts, or created local vascular disturbances, or combined both procedures, in 45 dogs and 13 cats: when both ducts were ligated in recently fed dogs, lesions of acute pancreatitis with necrosis developed; when ductal ligation was combined with ligation of major pancreatic arteries or veins, or with stimulation of the left splanchnic nerve, severe acute pancreatitis ensued; this did not develop with vascular disturbance alone, such as obstruction of pancreatic arteries or veins or simple stimulation of the left splanchnic nerve.

In discussing their results with stimulation of the left splanchnic

nerve, Menguy and his group admitted that they were at variance with earlier reports (134-137), but pointed out that comparison of the alternations they observed microscopically after nerve stimulation with photomicrographs published by Mallet-Guy and his associates (136) showed essentially similar changes: the difference was one of definition.

4. Vascular Spasm of Toxic Origin

Thal and Molestina (138) reported a method for producing regularly haemorrhagic pancreatitis by the injection of dilute staphylococcal toxin at a pressure of 20 mm. Hg in rabbits (in which they observed the sequence of events under direct vision) and in dogs; the ducts were ligated in each instance. The pancreatic necrosis so produced was the result of toxin-induced prolonged and profound vascular spasm in the interstitial blood vessels, with resultant suppression of pancreatic circulation. Animals which received toxin neutralized with specific antitoxin or heat-inactivated toxin did not develop pancreatitis.

5. Small-vessel Thrombosis

Experimentally induced increase in venous pressure does not always lead to acute pancreatitis. Thus, Couvelaire and Bargeton (139) produced venous stasis in the pancreas by injecting a 30% solution of sodium salicylate into a major pancreatic vein but failed to induce pancreatitis; and when Beck and Peterson (140) increased venous pressure by partial obstruction of the inferior vena cava in rats they produced only capillary congestion and atrophy of the glandular elements. On the other hand, Thal et al. (70) produced fulminating pancreatitis by intraductal instillation of 5 ml. of an E. coli suspension in a concentration of $10^6$ organisms per ml., when ductal obstruction was present; the characteristic feature of the experimental lesion was thrombosis of the capillaries and small venules. Also, extensive experimentally induced thrombosis of pancreatic veins has been shown to cause

a necrotizing form of pancreatitis in dogs (141) that is reminiscent of the 'wet gangrene' that develops in association with venous obstruction elsewhere.

With Pfeffer et al.'s closed duodenal-loop preparation (72), in which bile was completely eliminated by dividing and ligating the common bile duct, the ensuing acute pancreatitis was produced mainly by a vascular injury that consisted of congestion and thrombosis of capillaries and thin-walled veins. Hardaway and McKay (142) induced acute haemorrhagic necrosis in dogs by means of intra-aortic injection of incompatible (human) blood, producing a reaction that was associated with intravascular clotting in the pancreas as well as in other organs. The simultaneous occurrence of acute pancreatitis and lower-nephron nephrosis following transfusion of incompatible blood has been reported by Ackerman also (143); and Campbell (144), who induced intravascular haemolysis by injection of deoxycholate in the rat, produced a serious acute inflammatory lesion in the pancreas as well as a lower-nephron nephrosis. It is interesting to realize in the light of these considerations that, at necropsy, capillary and venous thromboses are consistently present in acute pancreatic necrosis in man (31).

6. Shwartzman and Arthus Sensitization Reactions

Fulminating haemorrhagic pancreatitis in rabbits and goats has been reported (145) after sensitization of pancreatic blood vessels to meningococcal or E. coli endotoxin which was injected into the pancreatic duct at pressures between 15 and 25 mm. Hg; this injection was followed by ligation of the duct and by intravenous injection of the same toxin 24 hours later. Pancreatic necrosis did not develop in any of the control experiments, in which the challenging intravenous dose of toxin was omitted. Capillary and venular hyaline thrombosis were demonstrated histologically in every case of pancreatitis, and it was suggested that in this local Shwartzman reaction the initial

lesion is vascular and the subsequent fulminating destruction is enhanced by the action of pancreatic ferments. It is interesting that recent studies (24) have shown that the local Shwartzman reaction is potentiated by trypsin and a certain degree of inhibition of the phenomenon is effected by crude soybean trypsin inhibitor.

Thal and Brakney (145) believe that, in pancreatitis in man, toxins or various micro-organisms present in bile or duodenal contents may sensitize the pancreatic blood vessels; the local reaction is readily provoked in such sensitized tissue by unrelated substances which may find access to the systemic circulation. Thal (146) subsequently suggested another method of producing both acute interstitial and acute haemorrhagic pancreatitis in the rabbit, mainly by producing the Arthus sensitizing reaction. Ductal injection of the antigen in highly sensitized rabbits after earlier intravenous and subcutaneous injections produced severe pancreatic necrosis. Histologically, the capillaries and venules in and around the areas of necrosis were occluded by hyaline or leukocytic-fibrin thrombi.

7. Vascular Injury of Toxic Origin

Nemir and associates (49, 50) mixed whole blood and pancreatic juice and incubated it for 24 - 28 hours at 37°C.; this material produced lethal necrotizing pancreatitis when injected into the main pancreatic duct of dogs, although the animals which received only one constituent survived. The mixture contained an abnormal haemin pigment, a haemochromogen-like substance, which was thought to result from red-cell haemolysis caused by bacteria. A similar haemin pigment has been found in the intestinal lumen of dogs which have strangulation obstruction (147, 148), in the plasma of dogs which have died of irreversible haemorrhagic shock (149), and in the peritoneal fluid of animals which have necrotizing pancreatitis (39). It is thought that this

lethal agent may contribute to the progression of pathology in acute pancreatitis as well as to the systemic toxicity of pancreatic necrosis.

Anderson and his group (150) produced a similar type of necrotizing pancreatitis by injecting an incubated mixture of autologous whole blood and sterile lyophylized trypsin into the pancreas of the dog; when aseptic precautions were employed to obtain autologous dog blood the material usually was sterile after 24 hours. In addition to parenchymal necrosis, they found oedema, leukocytic infiltration, fat necrosis, and a characteristic vascular lesion consisting of arteritis, phlebitis, and thrombosis of small arteries and veins. Anderson (52) later suggested that blood extravasated into the interstitium during an attack of acute pancreatitis may incubate with retained pancreatic enzymes and may produce a toxic agent which in turn damages the local blood supply and initiates pancreatic necrosis. However, he also stated that the mechanism responsible for the pancreatic haemorrhage is not clear, that the mode of action of the vasotoxic agent produced is unknown, and that the gross and microscopic features of human and experimental lesions have many characteristics of a hyperimmune reaction. The same author suggested recently (39) that partial venous-outflow occlusion may convert trypsin-induced pancreatic oedema into severe necrotizing pancreatitis; venous obstruction, he reasoned, prevents absorption of enzymes from the interstitial areas, where they may act upon extravasated fluids to produce substances which destroy pancreatic blood vessels and cause parenchymal necrosis.

8. Direct Effect of Proteolytic Enzymes on Blood Vessels

Rich and Duff (7) described what they believed to be a characteristic lesion of human and experimental pancreatitis, mainly a rapid necrosis of arteries and veins which is followed by either thrombosis or haemorrhage. They postulated that this vascular injury is due to trypsin, and showed that a

similar vascular lesion could be produced in vessels elsewhere in the body, e.g., in the subcutaneous tissue of dogs on injection of trypsin or activated pancreatic juice; inactivation of trypsin by heat destroyed its necrotizing power. They also advanced the concept that, in the majority of cases, escape of pancreatic juice into the interstitial tissues of the pancreas is caused by partial obstruction of the pancreatic outflow tract by epithelial metaplasia or by calculus material, with resultant destruction and rupture of ductules and acini behind the obstruction. However, Beck and his group (45) recently expressed the opinion that the vascular injury described by these authors was due to the presence of elastase in their trypsin preparations and in the activated pancreatic juice. Schneider et al. (12) have shown that pancreatic elastase injected into the pancreatic duct of dogs consistently induces haemorrhagic pancreatitis, with vascular changes identical to those of spontaneous pancreatitis in man. It has been shown also (112) that pancreatic-duct obstruction per se is insufficient to produce pancreatitis; ligation of the ducts merely leads to pancreatic oedema and ultimately to atrophy of the acinar tissue (121).

9. Role of the Bradykinin System

The extreme 'glassy' oedema of acute pancreatitis is seldom seen to a comparable degree in disease of any other viscus. The fluid is rich in protein and, in spite of the rapidity of its development and spread, there is little inflammatory cell reaction. The rapid accumulation of oedema within the confines of the pancreas may result in an impaired blood supply, since the swelling is restricted by the capsule.

The pain associated with pancreatitis is unduly severe. The ability of bradykinin to cause pain (151), vasodilatation (152), increased vascular permeability (153) and smooth-muscle stimulation suggests a role for this

polypeptide in acute haemorrhagic pancreatitis. The pancreas contains ample stores of trypsinogen and kallikreinogen; both these precursors, when activated, may release bradykinin and related polypeptides (155). Furthermore, the bradykinin precursor, bradykininogen, is a normal pseudoglobulin of plasma, lymph, and interstitial fluid, and as such is amply available to the pancreas (154). Bradykinin is a nonapeptide of known composition (156) and has been synthesized (157, 158); it is similar, if not identical, to kallidin, the product of activation by contact with plasma of kallikrein, a substance found in various glandular tissues. A substance that resembles bradykinin is a local mediator of functional vasodilatation of the submandibular gland (152, 159). A mildly acid pH reduces the rate of bradykinin inactivation and has no effect on the rate of its release (160). In view of the fact that a lowering of tissue pH has been reported in pancreatitis (161), the possibility exists that the onset of pancreatic inflammation may favour the local accumulation of bradykinin, which may then be operative in the pathogenesis of this disease; however, the evidence for such a role is inconclusive (162, 163).

Ryan et al. (164) recently advanced the concept of local release, local action and inactivation of bradykinin in the initial stages of acute pancreatitis, manifested by substantial loss of plasma bradykininogen and increased vascular permeability localized to the pancreas. The role of bradykinin and related vasoactive substances in the production of increased capillary permeability, oedema, and pain by local action, and in pancreatic shock by a systemic effect, possibly together with other humoral or neural mechanisms, awaits further investigation (165).

## D: METABOLIC AND NUTRITIONAL FACTORS

### 1. Alcoholism

Alcoholic intoxication frequently precedes or appears to precipitate

acute pancreatitis (166-169); and it is often associated with mild pancreatic oedema or subclinical acute pancreatitis, as evidenced by elevation of blood amylase (170, 171) and lactescence of blood serum (172). Since Friedrich's description of the "drunkard's pancreas" in 1878 (173), the frequent association of chronic alcoholism with chronic pancreatitis has been recognized (174, 175). The precise mechanism whereby alcoholic intoxication precipitates an attack of pancreatitis is not well understood. A direct toxic effect has been shown for methyl alcohol (176, 177, 179), but there is no evidence for such toxicity for the ethyl derivative (178). Doubilet (88) believes that ethyl alcohol, which has no effect on the normal pancreas, can cause acute pancreatitis in subjects who have had previous attacks, and attributes the sensitivity of the injured pancreas to a metabolic product which in turn acts directly on the pancreatic cell.

Despite the considerable evidence that alcohol may be a direct cause of pancreatitis, experimental data to date are inadequate for elucidation of the aetiological mechanism. Hong et al. (122) produced severe pancreatitis experimentally in rabbits by instilling alcohol into the main pancreatic duct after this had been obstructed. These findings are interesting when compared with the observation of Weiner and Tennant (180) who, during a careful study of a large number of patients with acute haemorrhagic pancreatitis, found that symptoms had developed in 66% after an 'alcoholic spree'. The effect of alcohol on pancreatic secretion has been studied by various physiologists. Bayliss and Starling (181), on the basis of their brilliant work on secretin in 1902, suggested that alcohol could stimulate the formation of this hormone by a direct action on the duodenal mucosa. Gizelt (182) made extensive studies in animals and showed that the effect on the pancreas was not due solely to the presence of alcohol in the duodenum and small intestine; he stimulated pancreatic secretion in animals by instilling alcohol into the isolated stomach or into the rectum, and suggested that the effects reflected a direct action

on the pancreas. Others (179), however, have shown that the intravenous administration of ethyl alcohol in man to a point of intoxication did not increase pancreatic secretion, and patients who had chronic pancreatitis and were so treated experienced no immediate or delayed effects.

Alcohol stimulates gastric secretion when given orally, intravenously or per rectum, or when perfused through an isolated loop of small intestine (182). Although there is a rise in pancreatic secretion after oral administration of ethyl alcohol (179), Brooks and Thomas (183) observed little stimulating effect of dilute ethyl alcohol upon pancreatic secretion when the solution was given intravenously or was instilled into the duodenum of dogs which had a chronic fistula. The local effect of alcohol on the gastric and duodenal mucosa may result in hyperacidity and duodenitis, with oedema of the papillae and spasm of the sphincter. Correlation of present-day concepts of the various effects of alcohol suggests that there is stimulation of pancreatic secretion by the acid-secretion mechanism against ductal obstruction (184).

2. Ethionine

In animals, d-l-ethionine ($\alpha$-amino-$\gamma$-ethyl butyric acid) has been given to provoke pancreatitis, because of its known ability to interfere with protein synthesis (185). The lesions produced varied from atrophy of the gland to severe pancreatitis with haemorrhagic necrosis, depending upon the dosage schedule (185-188). Ethionine is a metabolic competitor of methionine and its effects are prevented by the simultaneous administration of methionine but not cysteine or choline (186). Injection of a single dose of ethionine in dogs can produce a decrease in the amount of pancreatic enzymes entering the cannulated pancreatic ducts (189). It has been suggested (190) that the first effect on the pancreas after its administration is a decrease in the membrane permeability of the acinar cell to its enzymes, and that the basic cause of ethionine pancreatitis is an interference with enzyme extrusion at the

level of the acinar-cell membrane.

The ultrastructural changes of rat exocrine pancreas after brief exposure to d-l-ethionine (1-5 days) have been studied (191). The most significant changes were seen in the acinar cells; these comprised loss of the regular organization of endoplasmic reticulum, reduction in number of RNA particles, re-arrangement of mitochondrial inner membranes, degeneration of the Golgi apparatus, and appearance of zymogen granules of aberrant size and shape. No distinct changes were noted in the centro-acinar cells during the first three days of ethionine administration; these cells appear to be less susceptible to its action than are acinar cells, a fact related to the accepted view that centro-acinar cells take no part in the synthesis of pancreatic enzymes. Herman and Fitzgerald (192) have reported similar observations. Whether pancreatitis induced by methionine deficiency plays any part in pancreatitis in man is only speculative: it is conceivable that alcoholic or postoperative pancreatitis may be related to it (190).

3. Hyperparathyroidism

Acute pancreatitis has been noted to complicate hyperparathyroidism (193-195), cancer of the parathyroid glands (196-198), and removal of parathyroid adenoma (199). The relationship between parathyroid dysfunction and pancreatitis appears to be more than coincidental, and the latter condition has been termed a diagnostic clue to hyperparathyroidism. Mixter et al. (200) have reported the association of hyperparathyroidism with acute, postoperative, recurrent and chronic pancreatitis, with and without pain, and Jackson (201) described a family with hereditary hyperparathyroidism with pancreatitis that may have been hereditary in nature.

Fink and Finfrock (202) have stressed the possible relationship of pancreatitis to the syndrome of so-called acute parathyroid poisoning, and have

postulated a toxic action of parathormone on pancreatic tissue proper; and deposition of calcium has been found in areas of intra-abdominal fat necrosis in animals treated with high dosages of parathormone after pancreatic-duct ligation (203). It may be that the hypercalcaemia of hyperparathyroidism favours deposition of intraductal calculi in the pancreas, leading to obstruction and pancreatitis, although local thrombo-arteritis and necrosis of pancreatic tissue develop after administration of large doses of parathormone (204), an effect that may be due to the hormone itself or to the high levels of calcium. Haverback and his group (114) have demonstrated experimentally that the activation of trypsinogen to trypsin is controlled in a direct manner by the amount of calcium ion present, and they believe that this increase in trypsin activity may account for the association of pancreatitis and hyperparathyroidism. This concept is supported by several reports of pancreatitis with co-existent hypercalcaemia not due to parathyroid dysfunction (114, 205, 207).

4. Pituitary and Adrenal Hormones

Dreiling and his group (206) studied the effect of adrenocorticosteroids upon pancreatic secretion in man and found a reduction in volume, in bicarbonate, and in enzyme production -- changes attributed to acinar-cell damage. Specific tubular and acinar lesions can be produced by the administration of ACTH (208), growth hormone (209), deoxycorticosterone (210), cortisone (211, 212), and cortisone derivatives (208). Furthermore, hydrocortisone and ACTH aggravate the pancreatitis produced by stylomycin aminonucleoside (208). Also it has been shown that massive doses of cortisone result in interstitial inflammation and vacuolization of pancreatic acinar cells (213), and pancreatic necrosis in cortisone-treated children has been reported (214). The histological appearance of the pancreas of steroid-treated rabbits resembles that which develops after obstruction of the ducts, and it has been suggested that these

changes result from increased viscosity of pancreatic secretions (212). On the other hand, steroids have been given in a variety of forms of experimental pancreatitis and have been found to increase the survival rate (150, 215, 216). Anderson and his group (217) found that adrenocorticosteroids reduced mortality in dogs in which necrotizing pancreatitis was induced by intraductal injection of enzyme-digested autologous whole blood, and suggested their clinical use for brief periods of time early in the course of pancreatitis.

5. Other Metabolic and Nutritional Factors

Some rare causes of pancreatic inflammation will be reviewed briefly. The pancreatitis of essential hyperlipaemia has been attributed to fat embolism to the gland (218). Other workers (219) have found impaired release of the heparin-clearing factor and synthesis of abnormal chylomicrons during the interval between episodes of acute pancreatitis in cases of essential hyperlipaemia. Also, hyperlipaemia has been observed in acute pancreatitis (220), experimental corticosteroid pancreatitis (221), and pancreatitis complicating pregnancy (222).

Protein deficiency plays an important role in pancreatitis of nutritional aetiology, e.g., kwashiorkor (223) and other malnutrition states (224); tubular and glandular degeneration with fibrosis dominate the histological picture, and these conditions are not considered pertinent to this discussion.

E: INJURY TO THE PANCREAS

Acute pancreatitis may follow blunt abdominal trauma (225, 226), subtotal gastrectomy (227), biliary-tract surgery (228), sphincterotomy (229), and splenectomy (230); manipulation of the pancreas during surgery, injury to its ductal system, especially the duct of Santorini (231), interference with the pancreatic blood flow (232), chemical trauma and pre-existing pancreatic injury also may contribute to development of the disease. In addition, pancreatitis

may develop after surgical procedures far removed from that organ (233), especially after prostatic resection (234), and no satisfactory hypothesis has been advanced to explain this. Pancreatic necrosis has been observed after underwater blast injury (235) and electric shock in man (236) and in animals (237). Anderson and Bergan (238), who investigated the effect of trauma and its relationship to pancreatic inflammation, showed that crushing of the mid-body of the pancreas did not of itself produce extensive pancreatitis in the dog; when this injury was combined with duct obstruction, the changes were more pronounced; when both procedures were combined with exocrine stimulation, necrotizing pancreatitis developed in some of the animals.

F: MISCELLANEOUS FACTORS

Several substances have been shown to have a toxic effect on pancreatic parenchyma and to induce pancreatitis -- ferric oxide saccharate (239), stylomycin aminonucleoside (240), and chlorothiazide and its derivatives (241, 242), to name but a few. The disease sometimes develops after the administration of histamine, thiouracil, and other compounds (210). The ability of thiouracil to produce pancreatitis in man is intriguing, since propylthiouracil has been advocated in the treatment of this disease. Reid et al. (243) believe that this compound inhibits energy release and that, as a result, the pancreas is placed at rest when under the stress of inflammation. A milder form of bile pancreatitis has been observed in animals after administration of propylthiouracil (244, 245).

Accidental hypothermia due to exposure has been incriminated recently as a cause of acute pancreatitis (246, 247). Conversely, attempts have been made with hypothermia to reduce pancreatic activity and influence the course of experimental pancreatitis: pancreatic secretion in the dog after pilocarpine stimulation was reduced by local cooling (248), but the mortality rate in acute

pancreatitis induced by the Shwartzman reaction or by the intraductal injection of bile salts or duodenal juice was slightly increased when local or systemic hypothermia was applied. Enguist et al. (249) showed that the disease followed a milder course when hypothermia was instituted before the onset of pancreatitis and continued for 24 hours thereafter in dogs. The physiological effects of local or systemic cooling have been attributed to a direct action on metabolic rate and enzyme activity and to an indirect effect on blood flow.

# CHAPTER IV

## PANCREATIC ENZYMES: PHYSIOLOGICAL AND BIOCHEMICAL CONSIDERATIONS

"We may surely say of the advent of enzymes, as Hopkins said of the advent of life, that it was the most improbable and most significant event in the history of the Universe."

M. Dixon, and E.C. Webb, in: Enzymes; London; Longman's, Green, 1964, page 668.

### A: GENERAL DISCUSSION

In view of the fact that inappropriate release of enzymatic activity has been considered to be of great significance in the pathogenesis of acute pancreatitis, a brief review of the occurrence, structure and function of the exportable pancreatic enzymes and their precursors is considered appropriate at this point. Only a few of the intracellular pancreatic enzymes have been studied; their possible relationship to pancreatitis has been briefly discussed in Chapter I. A comprehensive discussion concerning pancreatic lipase is not considered pertinent in this study, despite its great significance in pancreatitis.

The volume of pancreatic juice may vary from 700 to 2000 ml. in a 24-hour period; the clean watery secretion contains minerals, enzymatic proteins, and a small amount of mucoid proteins (13). It has been said that a brilliant success of modern biochemistry has been to show that all or almost all proteins of bovine pancreatic juice are enzymes (250). The secretion of enzymes by the pancreas involves their synthesis within the acinar cells, their segregation as zymogen granules, and some process whereby they are discharged into the ducts

and the blood stream. With the exception of the lactating mammary gland, there is no secretory organ in the body which forms and secretes on demand such large quantities of protein (251). Cannulated steer pancreas, for instance, excretes an average of 1 g. protein per hour (252). The exocrine portion of the pancreas is metabolically more active in protein production than the liver and the reticulo-endothelial system combined, even though the former weighs less (253): the combined weight of the latter is estimated to be 4% of body weight, or 40 times that of the pancreas. Thus, the pancreas weighs only 1/40 as much as the organs which produce plasma proteins, but produces one-third as much protein (254). Hokin and Hokin (251) have shown that pancreatic slices, when incubated with labelled amino acids in vitro, produce enzyme proteins in a manner similar to that of the intact pancreas. Daly and Mirsky (255), who were among the first to use radioactively labelled amino acids to study pancreatic enzyme production in animals, showed that enzyme protein accounts for about 20% of the dry weight of the pancreas. A substantial part of this protein can be discharged following the injection of pilocarpine, but complete reconstitution of enzymes occurs within 6 hours (256).

Siekevitz and Palade (257, 258) have presented a morphological and cytochemical analysis of the acinar pancreatic cells of the guinea pig and have demonstrated that several of the enzymes occur in the microsomes and in microsomal fractions. It seems that the pancreatic cell produces protein in a fashion similar to that of other tissues, by the enzymatic assembly of free amino acids upon an RNA template within the microsomes (259). Warshawsky, Leblond and Droz (260), using radioautographic techniques, have further elucidated the question of how pancreatic enzymes are formed and extruded into the excretory ducts. Hansson (261) used labelled amino acids to study protein

synthesis in the pancreas of cats, mice, rats and guinea pigs and found that the organ showed a greater uptake of radioactivity than did other tissues; radioactivity was apparent in the pancreatic tissue as early as 5 minutes after isotope injection, with maximal activity at 30 minutes, and in the proteins of pancreatic juice in 40 to 80 minutes.

Recent experiments (262) with bovine pancreatic juice from a fistula revealed a spectrum of enzymes and zymogens which were separated and characterized quantitatively by chromatographic procedures. More than 70% of the total proteins of the juice comprised proteolytic enzymes in active or zymogen form. Quantitative distribution was as follows: trypsinogen 14%; chymotrypsinogen-A 16%, and -B 16%; procarboxypeptidase-A 19%; procarboxypeptidase-A + carboxypeptidase-B 7%; ribonuclease 2.4%; deoxyribonuclease 1.4%; amylase 2%; traces of lipase and 10% of an unidentified fraction. The enzymic composition of the zymogen granules is remarkably similar to that of pancreatic juice. Greene, Hirs and Palade (263) in a brilliant study by a combination of electron microscopy and cell fractionation procedures, demonstrated that the zymogen fraction of bovine pancreas has the exact enzymatic pattern of the juice. All of the protein in the pancreatic juice was recovered; recovery of the protein from granule preparations was only 10% less. However, despite this clear-cut demonstration of identity in protein composition of extracts of zymogen granules and pancreatic juice, these investigators suggest that further work is needed to prove or disprove the existence of a non-zymogen granule shunt in the pancreatic secretory process.

It is interesting that proelastase, which is known to occur in bovine pancreatic secretions (264-266) has not been identified with any of these protein components. Recently, elastolytic activity was demonstrated in pancreatic juice from an external pancreatic fistula (267). Some other enzymes

which have been ascribed to the pancreas in recent years are collagenase (29), pancrin (263), protaminase (269, 270) and insulinase (271). Leucine amino-peptidase is ubiquitous enzyme which has been identified in high concentration in pancreas and pancreatic juice as well as in liver, kidney, and small intestine (272, 273). Lecithinase-A, a phospholipolytic enzyme that splits one fatty acid of lecithin or cephalin and forms lysolecithin or lycocephalin, has been found in the duodenal contents of man (274). This enzyme is present in high concentrations in normal pancreatic tissue, and in pancreatitis the level in the serum increases in a manner similar to that of lipase and amylase (275). The proteolytic zymogens (trypsinogen, chymotrypsinogen-A and -B, procarboxypeptidase-A and -B) rather than the active enzymes are present in zymogen granule extracts and pancreatic juice (263). Lipase and collagenase are secreted in an active form. Recently the molecular structure of bovine trypsinogen, containing 229 amino-acid residues, has been proposed by Walsh et al. (276): this structure contains six disulfide cross-linkages and one amino-acid sequence containing two histidine residues, a cystine disulfide bridge, and a serine residue. This sequence, which is found in chymotrypsin also appears to be the site of the active centre. The molecular structure of bovine chymotrypsinogen-A has been outlined by Hartley (277); the zymogen contains 246 amino-acids and has a molecular weight of 25,000.

In recent years a great deal of work has been devoted to the understanding of physiological and biochemical mechanisms of exocrine pancreatic function; certainly, the close similarity in distribution of zymogens or enzymes, in the pancreatic juice, in zymogen granules, and in microsomes of the pancreatic acinar cells, is quite remarkable. Zymogens, and not active enzymes, are present in pancreatic juice; granule formation may not be the only essential part of the secretory process, as evidenced by the observation that, when the granule content of the cell is reduced to zero during prolonged

secretion, enzyme synthesis and secretion still occur (278).

B: ZYMOGEN ACTIVATION

The zymogen precursors of pancreatic proteolytic enzymes are synthesized within the gland but are converted into active enzymes after they have been secreted by the cells (279); this undoubtedly is a protective mechanism to prevent autodigestion of the tissues that produce them. The zymogens are present in pancreatic juice in soluble form, not as zymogen granules. They show their optimal stability at pH 5.5 and dissolve in a pH of 7 or higher (251). The studies of Palade and Siekevitz (280) indicate that zymogen granules are extruded, possibly denuded of their membrane, and dissolve completely on coming into contact with pancreatic juice (pH as high as 8.5). The conversion of a precursor into an active enzyme involves the splitting, by catalysis, of a specific peptide bond in the zymogen molecule, with or without the removal of free peptides. The process of activation appears to involve an unmasking of the active centre.

Trypsinogen, but not trypsin, is produced by the acinar cells and is secreted in the pancreatic juice. Under appropriate conditions, trypsinogen may be activated by trypsin itself -- the process may be autocatalytic. It can also be activated by enterokinase, which is the key enzyme in the activation of pancreatic zymogens under physiological conditions (279) and which is present in the intestinal secretion, and by a 'kinase' of penicillium notatum (281). The autocatalytic process of trypsinogen activation is complicated by an alternative reaction which converts trypsinogen into an inactive protein (282). The preponderance of one of these competing reactions depends upon pH and the presence of calcium ions. Haverback and his group (114), in their outstanding studies of trypsinogen activation, demonstrated that, in man,

pancreatic juice aspirated from the duct of Wirsung is inactive and contains substantial amounts of trypsin inhibitor as a safeguard to prevent production of free trypsin within the gland or the ductal system. No conversion of trypsinogen to trypsin occurred usually in a system that contained human or bovine trypsin in excess of the amount required for saturation of the inhibitor. These investigators emphasized that with a normal calcium ion concentration, little or no activation of trypsinogen by trypsin could occur in pancreatic juice; within limits, the greater the amount of calcium ion present the greater the activation, a fact that may explain the association of pancreatitis and hyperparathyroidism. Acidification of the pancreatic juice to a low pH did not activate trypsinogen, a point of great significance in acidosis (13). The actual process of trypsinogen activation by trypsin or the 'kinases' consists in the breaking of a peptide bond between lysine and isoleucine, with resultant production of trypsin and a hexapeptide and the unmasking of the active centre.

Both chymotrypsinogen-A (283) and -B (284) are activated by trypsin but not by chymotrypsin: therefore, the process is not autocatalytic. A special characteristic of the chymotrypsinogen--chymotrypsin system is the large number of the different active forms; most of them have been recovered in crystalline form.

The activation of procarboxypeptidase-A, a complex process which involves the splitting of many peptide bonds, is achieved by the combined action of trypsin and an endopeptidase which is a derivative of procarboxypeptidase-A (285). Procarboxypeptidase-B also is activated by trypsin. Elastase is present in a zymogen form in pancreatic juice in dogs and in fresh hog pancreatic extracts (268, 286); and trypsin or enterokinase appears to be capable of converting proelastase to active elastase. It seems that under physiological

conditions the pancreatic juice remains inactive until it comes into contact with enterokinase, when there is rapid activation of all zymogens. The chief function of enterokinase is to convert trypsinogen to trypsin, which then becomes the key for the activation of all other precursors. However, activation of the pancreatic complex of zymogens cannot be accomplished by only trivial amounts of enterokinase, since the small amounts of trypsin so produced are soon inactivated by the trypsin inhibitor which combines with the enzyme to form an inactive complex. The trypsin-inhibitor complex, and the inhibitor itself, have been recovered in crystalline form (287). The same inhibitor inhibits chymotrypsin to some extent. Other inhibitors of trypsin have been extracted from bovine (288) and human plasma (289).

It is interesting that collagenase, an enzyme which attacks native collagen at or near physiological pH, is found in the pancreatic juice in a form which requires no activation (30).

C: MECHANISM OF ACTION OF PANCREATIC PROTEOLYTIC ENZYMES

Proteolytic enzymes, in general, are highly selective in their catalytic effect, and require a specific chemical environment in order to catalyze the hydrolysis of a peptide bond. A typical protein molecule contains several hundreds of amino-acid residues in a linear sequence, with many peptide bonds. Trypsin and chymotrypsin cleave peptide bonds that occupy internal positions in the polypeptide chain, and they are referred to as endopeptidases (which break protein molecules into smaller fragments), in contrast to carboxy-peptidases (or exopeptidases, which attack the outermost peptide bonds), which remove terminal amino acids one at a time. It might be said that the endo-enzymes produce a large number of free ends at which the exo-enzymes can act.

Trypsin hydrolyzes peptide bonds whose carbonyl group (C=O) is contributed by an amino acid (e.g., arginine or lysine) that has a positively

charged side group (279). This limited specificity of trypsin was first demonstrated by Bergman and co-workers (290) and has been confirmed subsequently by others in studies with synthetic substrates and proteins. However, despite the high specificity exhibited by trypsin toward synthetic substrates and numerous polypeptides and proteins of known structure, several workers have observed tryptic hydrolysis of ester substrates which do not possess the usual specificity features (291); nevertheless, trypsin remains the most specific endopeptidase known at present. The specificity of chymotrypsin for hydrolysis of peptide bonds formed by the carboxyl groups of tyrosine, phenylalamine and tryptophan has been recognized for some time (292, 293); action on synthetic substrates of leucine (294) and methionine (295) also has been noted. When protein substrates or synthetic ester substrates are examined it is evident that a variety of bonds can be hydrolyzed by chymotrypsin. In one study (296) it was found that chymotryptic hydrolysis of α-benzoyl-L-arginine ethyl ester, a 'typical' trypsin substrate, occurred at a maximal rate which was 20% of that observed with trypsin.

Carboxypeptidases exclusively hydrolyze the last peptide bond in a polypeptide chain. Carboxypeptidase-B hydrolyzes most rapidly those bonds which are formed by terminal lysyl and arginyl residues, whereas carboxypeptidase-A hydrolyzes terminal bonds formed by a variety of aromatic, neutral or acidic amino acids (297). The methods for application of these peptidases to hydrolysis of proteins have been discussed in detail by Canfield and Anfinsen (298). Houck and Patel (299) have recently demonstrated that the alkaline extracts of porcine pancreas that they used in their experiments were capable of changing into dialyzable form about one-half the hydroxyproline content of native soluble collagen; the same extracts could also render soluble a dialyzable hydroxyproline from washed insoluble collagen. This collagenase

activity was not inhibited by EDTA, soybean trypsin inhibitor, or mercurobenzoate. No proteolytic activity upon denatured haemoglobin was observed.

Pancreatic elastase is discussed in detail in later sections.

# CHAPTER V

## THE QUESTION OF TRYPSINOGEN ACTIVATION IN ACUTE PANCREATITIS

"If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties."

Francis Bacon (1561-1626) in Advancement of Learning, I, 8.

Haverback (13) has stated that the question of whether trypsinogen becomes activated into trypsin within the pancreas in acute pancreatitis is still unanswered. Following the experiments of Polya (10) and Rich and Duff (11), in which evidence was obtained for a key role of trypsin in the production of parenchymatous necrosis, several workers have induced experimental pancreatitis in dogs by intraductal (11, 333), interstitial (300, 301, 304), or intra-arterial (302) injection of trypsin, in the last-named case after pancreatic-duct ligation for 24 hours.

Lampros et al. (303) fed dogs 250 mg. trypsin twice-daily and thereby increased by 20% their serum antitryptic activity, and suggested that increased antitryptic levels might confer protection against pancreatitis. Although a trypsin inhibitor and kallikrein inactivator isolated from the parotid gland of cattle by Frey et al. (155) has been shown by several workers (306-310) to play a protective role in experimental pancreatitis in dogs, such a beneficial effect has been questioned recently by Beck and his group (311), in whose experiments the severity of the pancreatic tissue reaction and the mortality rate were unaffected in a large number of dogs with bile- or trypsin-induced pancreatitis; and Creutzfeldt et al.'s experiments (312) yielded similar results in a well-controlled study with 300 rats. Despite the widespread use of this polypeptide in the treatment of acute pancreatitis in man,

its therapeutic value is uncertain (313).

It has long been assumed by many that the important factor in acute pancreatitis is the proteolytic action of trypsin; and the prominent feature of pancreatic necrosis -- namely, the destruction of vessel walls and associated haemorrhage, which also is attributed to tryptic activity -- is considered to be the strongest evidence implicating this enzyme in the pathogenesis of the disease (305). However, acceptance of this theory requires a clear understanding of the mechanism whereby trypsinogen is transformed into trypsin within the pancreas or pancreatic juice in pancreatitis, and of the ability of the enzyme to maintain, within its chemical environment, its proteolytic activity (314). Many investigators assume that conversion of trypsinogen to trypsin may be accomplished by a multiplicity of agents other than enterokinase, including bile, tissue fluids, and inflammatory exudates. In fact, during experiments in which various substances were added to pancreatic juice collected from the pancreatic duct of rats and dogs (rat hepatic bile, dog gall-bladder bile, 2% sodium cholate solution, inflammatory exudate, plasmin, thrombin, and streptokinase) only thrombin produced more increase in the rate of appearance of proteolytic activity than did pancreatic juice alone during 24-hours' observation (305). Also, the addition of human bile, a mixture of streptokinase and streptodornase, and calcium ion alone in concentrations of 10-200 mEq./litre did not activate trypsinogen in pancreatic juice (114).

As previously mentioned, the only intracellular mammalian enzyme that has been demonstrated to activate trypsinogen is the partially purified cathepsin-B (315, 316). In vitro, beef-spleen cathepsin-B, purified 200-fold, catalyzes activation of cystalline trypsinogen to trypsin as determined by the action of the active product on synthetic substrates and its inhibition by trypsin -- soybean inhibitor. Evidence has been presented recently that

endogenous trypsinogen in extracts of dog pancreas is activated under the same conditions by cathepsin-B-like enzymes; the activation is enhanced by cysteine and is inhibited by iodoacetic acid (15). This interesting study may have significant implications in clinical pancreatitis as well; zymogen granules that contain trypsinogen and that normally are protected from the action of intracellular hydrolases may come into contact with such enzymes under certain conditions, as in anoxia, ischaemia, and inflammation. The fact that zymogen granules have optimal stability at pH 5.5 and dissolve at a pH as high as 7.2 suggests that the pH of the acinar cell, at least in the neighbourhood of the zymogen granules, is near 5.5 and that the granules may become soluble when the pH becomes sufficiently alkaline (251). However, even if such activation of trypsinogen were granted, the presence of a potent trypsin inhibitor in tissue and in pancreatic juice would inhibit the activity of small amounts of enzyme; the same reasoning is applicable to the question of the existence of active trypsin in blood in acute pancreatitis (13). On the other hand, crystalline trypsin in excess of the amount required to saturate the inhibitor in pancreatic juice, usually does not catalyze conversion of trypsinogen into trypsin, human or bovine. It is conceivable that during acute pancreatitis a substantial decrease in the amount of pancreatic trypsin inhibitor (287) in pancreatic tissue could permit release of trypsin activity into the gland; however, recent studies (115) have shown that in canine bile pancreatitis the level of pancreatic trypsin inhibitor does not fall, although the serum trypsin inhibitor is said to decrease during acute pancreatitis (303, 317).

Recently, Haverback and his group (13, 361) have introduced an entirely new concept, that of protein-bound trypsin. It has long been presumed that trypsin which gains access to the blood is inhibited by the trypsin inhibitor which migrates with the $\alpha_1$-globulin fraction. However, a mechanism that involves the binding of trypsin and chymotrypsin by a macro-

globulin in the $\alpha_2$-globulin fraction of the serum permits both enzymes to maintain proteolytic activity despite the presence of inhibitors. The trypsin--macroglobulin complex retains 75% of the esterolytic and about 20% of the proteolytic activity of a similar amount of cystalline trypsin, but it is not inhibited by trypsin inhibitors in soybean, colostrum, or the $\alpha_1$-globulin fraction of human serum. The pancreatic trypsin inhibitor demonstrated by Kunitz and Northrop (287) and the inhibitor extracted from bovine parotid glands only partially inhibit the trypsin--macroglobulin complex. The $\alpha_2$-macroglobulin-binding protein has been purified and crystallized by Mehl and his associates (318). The implications of this intriguing concept remain to be established; whether trypsin bound or unbound or some other proteolytic enzyme is of significance in pancreatitis remains open to question (13).

Attempts to demonstrate changes in serum trypsin activity in acute pancreatitis have been frustrated by the presence of other proteolytic and antiproteolytic substances. The first synthetic peptide substrate to be used with apparent success in determining tryptic activity was benzoyl-arginine amide (319): increased hydrolysis was found, by serum of patients with acute pancreatitis or carcinoma of the pancreas. Benzoyl-arginine ethyl ester and tosyl-arginine methyl ester were introduced as substrates by other workers, some of whom (320-322) confirmed the presence of abnormal serum proteolytic activity in pancreatic disease, although others (323, 324) did not. Recently, further evidence has been presented that plasma hydrolysis of benzoyl-arginine amide represents the increased tryptic activity in blood that occurs in acute pancreatitis (325). However, all of these suggestive findings await confirmation by further refinements in methodology. As previously mentioned, most attempts to determine trypsin activity in homogenates of pancreatic tissue in experimental pancreatitis have been unsuccessful; this has been attributed by

some workers to the presence of the potent pancreatic trypsin inhibitor which is released during homogenization (257, 326). However, as previously indicated, Beck and his group (9) were unable to demonstrate either increase in tryptic activity or decrease in trypsinogen content in experimental bile pancreatitis in dogs.

Turner et al. (335) induced pancreatitis in dogs by temporary arterial occlusion and intraductal injection of autologous bile and noted a small increase in 'protease' activity in pancreatic tissue. Katz et al. (326) determined tryptic activity in aspirates of interstitial oedema fluid obtained with a No. 27-gauge hypodermic needle, thus avoiding the possibility of lysing viable cells and thereby releasing inhibitor, in dogs with bile-induced pancreatitis. The levels of trypsin-like activity in the aspirate rose rapidly, reaching maximal concentration in 45-60 minutes and then decreasing over the next three hours. The authors believe that, once activated, trypsin alone is incapable of destroying viable cells, and they attribute cytolysis to the effects of bile salts. Cell metabolism and cell-membrane structure constitute important safeguards against enzymatic digestion, as confirmed by results obtained in experiments performed by Becker and Wilde (327). In attempting to mimic in vitro the conditions that exist in pancreatitis they incubated rat pancreas with Krebs--Ringer solution at 37.5°C. at (the rather high) concentration of 1:1000 crystalline trypsin for 40 minutes: tissue structure was somewhat loosened but no gross destruction was seen. However, a combination of trypsin, 1:1000, and 3% sodium malonate resulted in complete destruction of the acinar epithelium. Thus, the hypothesis was advanced that the cause of tryptic pancreatitis is the result of a combination of tryptic activity in the gland with an acute reduction of metabolism in the pancreatic cell (328-332). However, even if one accepts some degree of tryptic activity in acute pancreatitis, two important points remain unanswered: (a) the identity of the factor(s) responsible for disruption of cell metabolism and activation

of trypsinogen stored in the zymogen granules, and (b) the factor(s) responsible for vascular destruction and gross interstitial haemorrhage that apparently operate in the progression to acute haemorrhagic necrosis.

The first point may relate to the action of the intracellular hydrolases known to occur in pancreatic tissue, which have been identified with the "lysosomal group of enzymes" (19) and which are capable of initiating cell death and of activating trypsinogen into trypsin (15) and, therefore, might set off the entire proteolytic mechanism. In this connection it is interesting to note that administration of a polypeptide (antiprotease from beef parotid gland) which possesses antiproteolytic activity inhibits regularly and almost completely the Shwartzman reaction induced by endotoxin or by polymorphonuclear granules (334). In regard to the second point, it is believed that the factor(s) responsible for vascular injury in acute pancreatitis are of great significance; whereas trypsin may account at least partially for an increase in capillary permeability (333) and also for vascular thrombosis (141), the striking change that occurs in the walls of vessels, especially the destruction of elastic tissue, remains to be accounted for. This particular elastolytic activity is responsible for the digestion of the elastic component of arterial and venous walls, with subsequent extensive interstitial haemorrhage, in pancreatitis.

From the preceding considerations it is reasonable to assume that trypsin action alone cannot explain the vascular destruction that occurs in acute pancreatitis. It is possible that elastase may be responsible for the vascular injury: this hypothesis appears even more attractive when one considers that trypsin, chymotrypsin and the intracellular cathepsins alone are incapable of digesting the insoluble protein of elastic fibres (469).

# CHAPTER VI

# THE ELASTASES

## A: INTRODUCTION

The term 'elastase' is used to describe any enzyme which will dissolve fibres of the insoluble protein elastin at or near physiological pH, and it is implicit in this definition that the elastic fibres used as substrate in the assay procedure should be undamaged and free from contamination by other substances.

Two dates are considered to be very important in the history of elastase research, namely 1904 and 1949. In 1904, Eijkman (336) discovered an elastolytic enzyme in Pseudomonas pyocyaneus and demonstrated elastolysis by bacterial action on media containing nuchal ligament, aortic wall, or lung. In 1949, Baló and Banga (337) reported the presence of a specific elastolytic enzyme, for which they coined the name elastase, in extracts of acetone-dried pancreas powder and fresh ox pancreas; it was first obtained in a crystalline form by Banga in 1952 (338), and in 1959 Mandl and Cohen (339) reported systematic screening of bacteria and moulds for elastase production.

Even before 1900 some observations concerning elastolysis were reported in the literature: the first mention of pancreatic elastase activity dates back to 1878, when Walchli (340) found that ligamentum nuchae elastin was digested by ox pancreas. Soon after, Pfeiffer (341) found that dried pancreas digests elastin from this source, and attributed the effect to trypsin. In 1896 Mall (342) stated that elastic fibres are rapidly dissolved by pancreatin, in contrast to white fibres (i.e., collagen) and reticulin, which are resistant to its action. Work on pancreatic elastase was neglected mainly because the elastolytic activity of pancreas was attributed to trypsin.

Even modern textbooks contain such statements as: "Elastin, unlike native collagen, is susceptible to tryptic digestion" (343), and: "Elastin differs from collagen in being digested by trypsin" (344). On the other hand, it is possible that during the early years elastase was not detected in the acid extracts from which other pancreatic enzymes were isolated, because of its acid lability (264).

Soon after the discovery of pancreatic elastase it was found that the enzyme contains at least two elements: a proteolytic component which is active by itself (elastoproteinase, $E_2$, or real elastase) and an apparently mucolytic component (elastomucoproteinase, $E_1$, or elastomucase) which shows only slight elastolytic activity but enhances the action of the proteolytic component when the two enzymes react simultaneously with elastin (345-347). The presence of a third component which is capable of suppressing the synergistic effect of elastomucase, called $E_2'$ because of its place in paper electrophoresis, has been reported by Czerkawski and Hall (348, 349); Loeven calls this element $E_1$-den (350).

Only one elastase preparation has been shown to be solely elastolytic and devoid of nonspecific proteolytic action -- the Flavobacterium elastase of Mandl and Cohen (351, 352). Although the concept of the dual nature of elastase is not accepted universally, and many of those who have studied the elastase--elastin system ignore or deny the existence of the mucolytic elastase component (264, 266, 353), in the past few years more and more the importance of its role has been demonstrated. And now the work of Saxl (354-356) on the 'clearing-factor' activity of elastomucase has raised the question of whether 'elastolipoproteinase' might be a more appropriate name for this enzyme.

The group of Hungarian workers who revived interest in pancreatic elastase (337) suggested that it might play a systemic role in the degradation

of elastic tissue in the arterial wall during the development of arteriosclerosis. Banga has mentioned recently (357, 358) that elastomucoproteinase has a specific substrate in the human aortic wall ( a mucolipoprotein) which probably is related to the pathogenesis of arteriosclerosis. Recently elastomucase was isolated from serum by Loeven (359).

Grant and Robbins (268, 286) have shown that elastase is present in zymogen form in dog pancreatic juice and fresh hog pancreatic extracts; trypsin or a duodenal factor appears to be able to convert this precursor to active elastase. In claiming elastolytic activity for an enzyme preparation or a pancreatic homogenate it is essential that the source and method of preparation of the substrate be specified, since there is a marked difference in rate of attack and optimal conditions with differently prepared substrates (352). The best source for enzyme studies is ligamentum-nuchae elastin, usually equine or bovine. A convenient substrate is the 'orcein-elastin', introduced by Sachar et al. (360), from equine ligamentum nuchae, which is impregnated with a specific dye and therefore lends itself to colorimetric determination. Because elastin resists denaturation, orcein-elastin is not digested by common proteolytic enzymes and remains a specific substrate for elastases.

B: METHODS OF ASSAYING ELASTASE ACTIVITY

Most methods of assay in use at present are based on measuring the amount of the insoluble substrate that the enzyme renders soluble in a given time. This may be accomplished either by determining the loss in weight of the undigested elastin (337) or by deducing the amount of protein solubilized from the increased optical density at 280 mμ. (268), Folin phenol colour (361), biuret colour (346, 362), or refractive index (349). Nephelometric or colorimetric measurements of the decrease in turbidity (361) or colour released from

dyed elastin substrates (e.g., Congo-red elastin (266), azo-elastin (363), and orcein-elastin (360)) also indicate the amount solubilized. Lamy and his co-workers (364) and Czerkawski (365) have estimated the number of amino groups liberated either by ninhydrin formol titration or one of the many copper-phosphate methods; Czerkawski (365) has combined the two, using in addition a trichloracetic-acid precipitation stage to follow the relative rates of amino-group release and protein solubilization.

Sachar et al. (360) employed orcein-stained elastin as substrate and measured the amount of dye released after incubation with the enzyme. In some instances the dye has been measured actually in the filtered buffer solution, and in others (366) after extraction into butanol. Schneider et al. (367) measured elastolytic activity by using the ninhydrin reaction to determine the amount of amino acids and polypeptides released by enzymatic digestion of a uniform suspension of elastin. A much more sensitive method was devised by Sbarra et al. (368), who used an agar-plate procedure similar to that employed in bacteriological assessment of antibiotic activity.

Any one of the above methods under standard conditions yields fairly reproducible results, but comparison of results obtained by different methods reveals variation in ratios of activities even in different buffers of the same pH and ionic strength (361). A variety of elastase units have been proposed (349, 369-372) and most of them can be defined as the amount of elastase activity that dissolves 1 mg. elastin substrate under assay conditions; however, because of the great variability in these conditions, units obtained by different methods cannot be interconverted. Elastase activity has also been determined qualitatively by methods based on histological sections and electromicroscopic studies (337, 354). Despite the fact that the concept of a dual nature for pancreatic elastase has not received universal

approval, any statements concerning the quantitative measurement, or, indeed, the existence of elastase in a whole tissue or tissue extracts, must be taken as merely relating to gross elastolytic activity, without any classification of the enzyme's being intended.

In the discussion to follow, the term 'pancreatic elastase' denotes the elastolytic component of the overall elastolytic system (elastoproteinase); the mucolytic-lipolytic component will be referred to as such.

## C: PANCREATIC ELASTASE

### 1. Source

In the goosefish, Lophius piscatorius, in which islet tissue is anatomically separate from the acinar component, elastase-like activity occurs in the islets (373). Many workers have suggested, however, that elastase is a constituent of the exocrine secretion and that its function is mainly digestive. Elastase or an elastase precursor can be demonstrated in the pancreatic juice of the dog, cat, chicken and man (267, 286, 362, 374-376); the enzyme has been found in urine also (362, 377), which indicates its clearance through the kidney and should imply that elastase is present in the circulation at some stage in its active life.

Cohen et al. (362) and Kokas et al. (376) observed that the elastase secreted in the pancreatic juice could be increased by administration of secretin intravenously, hydrochloric acid intraduodenally, or pilocarpine subcutaneously; on the other hand, ligation of the pancreatic duct, with subsequent atrophy of acinar-tissue, produced reduction in pancreatic elastase to one-tenth its original amount (375). A very strong indication for the exocrine origin of elastase is found in some experiments (378, 379) in which the presence of elastase in the acini only was demonstrated with fluorescent antibody techniques.

Lamy and Lansing (380), using extraction techniques with the pancreatic tissue of Lophius piscatorius, demonstrated highly specific localization of the enzyme precursor -- the membrane of the acinar tissue that surrounds the islets. Elastase activity has not been reported from other sites, with one important exception: Core and Larkey (381) isolated from the aortic wall a polysaccharide fraction which had elastase activity on a stained elastin preparation. Very few data are available concerning the amount of elastase present in fresh normal pancreas or pancreatic juice.

Baló and Banga (337), in their original publication, stated that the amount of the enzyme is not always the same in human and animal pancreas, and since then a considerable interspecies difference has been reported. According to Lewis et al. (371), beef pancreas contains on average five times more than human, whereas Marrama et al. (382) found approximately the same amount in man, ox, and horse, but five times more in rat pancreas and not much less in pig and chicken. It has been said that the amount of the enzyme decreases with age and in association with arteriosclerosis (383). Lewis et al. (371) found no correlation between the amount of elastase in the pancreas and sex, age, or cause of death in 9 adult human beings.

2. The Elastase Precursor (Proelastase)

Grant and Robbins (268, 286) were the first to demonstrate in dog pancreatic juice and in fresh hog pancreatic extracts the presence of elastase in a zymogen form, proelastase, capable of effecting elastolysis only when activated by trypsin or by a duodenal factor, enterokinase. Lamy and Lansing (380), who studied proelastase, found that the proenzyme of the goose-fish is similar to that of the pig and the rat.

Partial purification of proelastase of hog pancreas and certain features of its activation were recently reported by Lamy and Tauber (431).

The authors postulated that trypsin activates proelastase very rapidly by breaking some specific and particularly exposed bond(s) in the zymogen molecule, with resultant formation of two fragments, one being active elastase and the other an inhibitor of trypsin that inactivates it rapidly. Since only native trypsin will activate proelastase, it is conceivable that cleavage of peptide bonds involving the carboxyl groups of lysine or arginine residues must take place. However, Lamy and Tauber found that chymotrypsin and the soybean--trypsin complex were inactive and DFP-trypsin (trypsin treated with the inhibitor diisopropyl-fluorophosphate), heat-denatured trypsin and trypsinogen had reduced ability to activate proelastase. The authors indicated that the validity of their hypothesis could be tested only after the preparation of pure proelastase has been achieved.

3. Isolation and Purification

All methods devised for the isolation of elastase start with a dried, defatted pancreas powder, usually hog, beef, or fresh porcine pancreas. Nearly all workers extract the enzyme with sodium-acetate buffer, pH 4.5 - 4.7; from this acetate extract the enzyme can be precipitated with ammonium sulphate, usually at 45% concentration, to obtain a crystalline though still impure preparation. The crystalline product is insoluble in water and electrophoretically inhomogeneous (347, 370, 371).

The first attempt to obtain a purer enzyme was made by Banga (338), and subsequent purification methods have included column electrophoresis and adsorption--elution techniques. The electrophoretically purified elastase is water-soluble, but even so it attacks a number of proteins (371). Using DEAE-cellulose, Lewis and Thiele (271) found that their elastase preparation contained five components, only the first being elastolytic. Dvonch and Alburn (370), using zone electrophoresis on starch, obtained two elastases, but it has been suggested that one may consist of elastomucase contaminated

with elastoproteinase (385). Lamy et al. (364), using adsorption on an Amberlite IRC-50 resin and subsequent elution with phosphate buffer, pH 6.0, obtained two well-separated elastolytic fractions; one contained most of the nonspecific proteolytic activity against casein and the other was poor in it.

Enzyme preparations rich in either the proteolytic or mucolytic elastase component and freed from all other contaminating proteins have been obtained by means of starch column electrophoresis (386); lyophilization and re-electrophoresis yielded pure elastoproteinase. Banga and Baló (387) separated elastoproteinase from elastomucase, by a modified starch-gel electrophoresis technique, and Loeven (385), using DEAE-Sephadex column chromatography, was able to separate the two components quantitatively. Despite the fact that elastase activities reported from different laboratories are difficult to compare, the enzyme preparations of Hall and Czerkawski (349), Loeven (385, 386), Banga and Baló (387) and Lewis et al. (264) appear to be electrophoretically pure. Purification of elastase by continuous paper electrophoresis was reported recently by Hormann and Fujii (388); such a method affords complete separation of the elastase-complex enzymes from inert proteins and from each other.

4. Properties

Lewis et al. (371) reported that electrophoretically purified pancreatic elastase has a sedimentation constant of 2.6 S, iso-electric point 9.5 $\pm$ 0.5, and molecular weight 25,000; this relatively low molecular weight was confirmed by Loeven (386), whereas Naughton and Sanger (266) calculated a molecular weight of 28,500 from specific radioactivity of purified diisopropoxy-$p^{32}$-phosphinyl elastase. Several authors have reported pH optima for elastoproteinase preparations which they regarded as purified: Sachar

et al. (360) pH 8.7-9.4, Rancati et al. (389) pH 8.2-9.7, Hall and Czerkawski (349) pH 8.45, Banga (358) pH 8.7, Lamy et al. (364) pH 9.2-9, and Loeven (350) pH 8.9-9.0.

Pepler and Brandt (390) were the first to comment on the stability of elastase, reporting that a 1% solution in distilled water remained stable for two to three days; the crystalline elastase of Lewis et al. (264, 371) retained its activity for 24 hours at 5°C., pH 4-12. Loeven (386) found that commercial and purified elastases retained their activity 100% for 3 months; he does distinguish, however, between elastoproteinase which is very stable and elastomucase which is rather unstable. On the other hand, Lamy et al. (364) found that stability depends upon the purity of the elastase sample, and Hormann and Fujii (388) reported that Ca ions stabilize elastase. Very little is known about the chemical composition of this enzyme. According to Czerkawski (365), elastase contains appreciable amounts of tryptophane (1.26), tyrosine (2.05), and hydroxyproline (2.2 moles/$10^4$ g.). Woessner (391), however recorded only 0.8 moles hydroxyproline; the ratio of tyrosine to tryptophane (1.6) was very different from that of other proteolytic enzymes, for which Beavan and Holliday (392) report a ratio of "between 3 and 4."

5. Toxicity and Antigenicity

Although several authors have used elastase in animal experiments, little is known about its pharmacological properties. Baló et al. (393) reported that the lethal dose for white rats was 25 - 30 mg. given in a single dose, although the same animals survived doses one-fifth this size when they were administered daily for considerable periods. When given the lethal dose, the rats collapsed within 50 minutes, with extreme dilatation of peritoneal capillaries and diapedesis bleeding. Elastase administered in smaller amounts daily for several weeks produced hyperplasia and hypertrophy of pancreatic

tissue, the elastase activity of which was markedly increased.

In subsequent studies, Borsy et al. (394) and Bagdy et al. (395) showed that the $LD_{50}$ for mice varies between 2,290 and 4,240 units/kg. The lungs of the animals, which died after a short time in convulsions, showed extreme hyperaemia, oedema, and increased weight. Intravenous administration of elastase in the cat and the rat resulted in transient hypotension, with low enzyme concentrations, which may be due to dilatation of peripheral blood vessels. On the other hand, in the isolated frog heart, elastase produced a transient rise in amplitude at low enzyme concentrations and diastolic standstill at higher doses; also, increased tonus of smooth muscle of frogs and contraction of rat uterus in oestrus has been observed. Unfortunately, it cannot be assumed that these pharmacological effects were directly associated with elastase itself, since the enzyme used in these early experiments was relatively impure. Kovacs and Bagdy (396) administered 10 - 500 mg elastase via the bronchoscope in rabbits and man, without any deleterious side effects. Only a few authors have studied the antigenicity of elastase.

Walford et al. (397) demonstrated that the anti-elastase found in the plasma of chickens which had received elastase injections precipitated elastase from solution quantitatively: this indicates the enzyme's dissimilarity from the serum inhibitor which, although it forms a complex with elastase, does so without precipitating it. The elastase antibody does not inhibit the elastolytic activity of the enzyme, a finding which is at variance with the observation of Tolnay and Bagdy (398), who found greater inhibition with rabbit antisera than with normal sera. This would suggest that enzymatic and antigenic reactions have distinct sites. The varied views of the antigenicity of elastase may be explained in the light of recent studies (378, 379), in

which a marked species specificity was shown in the antigen-antibody reactions of elastase.

6. Role of Elastase in the Blood-clotting Process.

Pancreatic elastase is not considered as a component of the clotting system normally; however, addition of small doses of the enzyme to normal human plasma appreciably shortens coagulation time and large doses completely inhibit coagulation (395, 399-401). Bagdy and co-workers (395, 399) suggest that the elastase reacts with prothrombin to form thrombin, a reaction which appears to be independent of the presence of platelets, thromboplastin, or calcium ions. On the other hand, according to Marrama et al. (401) the action of elastase occurs only in the presence of factor VII and is not of the thrombin type; large doses of elastase, they believe, inhibit blood coagulation by direct lytic effect on clotting factors.

Ferrari et al. (402) reported that the initial decrease in clearing factor following intravenous administration of elastase to atherosclerotic patients and the tendency toward hypercoagulability was in agreement with the effects of the enzyme in vitro. That elastase may, in fact, act on platelets, assisting the release of various platelet factors, was suggested by Hall and Wilkinson (403); carefully collected platelet-rich plasma responded to the addition of increasing concentrations of elastase over a range of 0 - 25 μg/ml. to give increasingly shorter decalcification times (404), but this response disappeared completely when the platelets were previously mechanically degraded. The authors advanced the hypothesis that elastase adsorbed on to the lipopolysaccharide-rich regions of arterial elastica, following local vascular damage, may bring about focal clotting and hence initiate thrombus formation in these regions.

7. Specificity

Lewis et al. (371) demonstrated that purified elastase could hydrolyze several other proteins, e.g., albumin, casein, fibrin, and haemoglobin. The enzyme does not attack native collagen and hair keratin, although denatured collagen (azocoll) is attacked by all known preparations. Elastase also cleaves prothrombin and fibrinogen (395), coagulates milk, and renders soluble the water-insoluble protein of the crystalline lens and lens capsule, which has some similarity to collagen in amino-acid composition (345). Walford et al. (397) found that elastase attacks casein and ovomucoid-$\beta$, but not the other egg proteins, chalazae, or ovomucoid-$\alpha$. Also, several synthetic substrates which contain tyrosine, phenylalanine, glutamic acid and aspartic acid are hydrolyzed (405, 406), and the Leu-Tyr bond between position 15 and 16 of the B chain of insulin is rapidly cleaved (266); slower splits in both the A and the B chain occurred at a wide variety of peptide bonds which involved neutral amino acids that have aliphatic side chains. However, a nonelastolytic insulinase has been isolated from a crude crystalline elastase preparation (271).

Using synthetic substrates, Naughton and Sanger (266) found, after column chromatography, that the purified elastase sample had 1-2% chymotryptic activity, thus accounting for its esterase activity. On the other hand, Partridge and Davis (407) believe that the proteolytic component of elastase has 2-3 times higher activity toward haemoglobin and other proteins, than trypsin, and Amati and Castelli (408) showed that elastase has a specificity of action against casein which is much wider than that of trypsin and chymotrypsin. Only the Flavobacterium elastase could be freed completely from non-specific proteolytic activity and was found to attack only elastin and none of the other proteins or synthetic substrates (351, 352). It seems that even highly purified pancreatic elastoproteinase preparations possess nonspecific activity. Furthermore,

the presence of a mucolytic elastase component, which shows only slight elastolytic activity has been demonstrated by three groups of workers: Hall and his co-workers in Leeds, Baló and Banga and their co-workers in Budapest, and Loeven in Leyden. The source of elastomucase is fresh pancreas or commercial pancreas powder, but nothing is known about its endocrine or exocrine origin, although Czerkawski (409) considered part of the elastolytic process to be mucolytic. Lipolytic activity of pancreatic elastase on elastic tissue has been reported by Lansing et al. (410), and by Labella (411), who found that lipid droplets were released during elastolysis. The lipolytic factor was shown by Hall (412) to be quite distinct from pancreatic lipase, and is said to have also a synergistic effect on elastoproteinase.

As early as 1954 Banga (436) reported that, through the interaction of elastomucase with serum, an antilipaemic factor is formed in tissues with high mucopolysaccharide levels (liver and blood). Saxl (354, 355) showed with biochemical and electron-microscope studies that elastomucase plays a part in the disruption of chylomicrons in tissue and also decreased the opacity of lipaemic serum. Hall (437) suggested that, by the interaction of elastomucase, $\alpha$- globulin and acid polysaccharide, lipoprotein lipase activity is produced, comparable to post-heparin lipoprotein lipase, in lipaemic serum. Thus, it seems that elastomucase exhibits lipoprotein lipase and mucase activity, as evidenced by the release of lipid droplets (410, 411) and acid polysaccharides bound to the elastic fibres, during elastolysis (347, 350). The name 'elastolipoproteinase' has been suggested instead of elastomucase, $E_1$, or elastomucoproteinase, on the basis of the (probably only apparent) dual nature of this component.

8. Elastolytic Activity of Other Enzymes

It has been demonstrated incontrovertibly since 1949 that carefully

purified trypsin is devoid of any elastolytic activity (337, 371, 390, 407, 413 - 416); earlier reports claiming elastolytic activity for trypsin were due to the use of impure enzyme that contained elastase (417-419) -- for example, an elastase content of about 3% was found by Lewis et al. (371) in Trypsin 1-300 (Nutritional Biochemical Co.); it is very difficult to free trypsin from contaminating elastase (363, 370). Crystalline chymotrypsin also is inactive toward elastin (264, 397, 370), and although some have reported an elastolytic effect of pepsin (363, 420) others have found it completely inactive (421, 416); the low pH of the reaction mixtures suggests, possibly, a nonspecific action of pepsin on acid-modified elastin. Some plant enzymes (ficin, papain, and bromelin) possess proteolytic and elastolytic activity (414, 416, 418); they require activation by KCN or cysteine. On the other hand, tissue cathepsins have no elastolytic activity in the presence or absence of cysteine (416). It would appear, then, that elastase is unique among proteolytic enzymes of animal tissue in that it is capable of taking solid elastin into solution at a rate comparable to that at which many soluble proteins are attacked by trypsin or pepsin. The reaction between elastase and its substrate is specific, in that other enzymes react more slowly or in quite different fashion.

9. Factors Affecting Elastase-activity Determinations: The Elastase Unit.

As previously mentioned, in almost all assay methods the amount of substrate which is made soluble by elastase is measured by determining the weight of the residual undigested elastin or of the amount of solubilized protein. Elastin from ligamentum nuchae or beef aorta is freed of collagen, other proteins and mucopolysaccharides by treatment with boiling NaOH or by autoclaving at 1-atmosphere pressure in distilled water or acetic acid. The lipids are extracted with a mixture of alcohol and acetone (410, 422) or an ethanol-ether mixture.

Criteria for the purity of elastin powder, a commonly used substrate, are amino-sugar and hydroxyproline content: total mucopolysaccharide content is indicated by the concentration of amino sugars (350). Insufficiently purified material may be protected by a surface coating of mucopolysaccharide or other components of the ground substance. Such preparations may be only slowly attacked by pure elastase but are rapidly attacked when the mucolytic component also is present (347).

Elastin preparations may be unduly slow in their reaction with elastase because of overheating or overdrying. Although alkali-treated elastin samples have similar susceptibility to elastase, there is some increase in activity as particle size decreases (346, 350); this partly explains the fact that enzyme activity is not constant when the same enzyme solution is tested against batches of elastin prepared at different times (350). Another reason for noncomparable results of elastase activity by various methods is that the velocity of elastolysis varied with the kind and ionic strength of buffer solutions with the same pH value (350, 361, 408). The influence of stirring on the enzyme reaction is not clear; Hall (346) observed that shaking may cause inactivation of the enzyme, whereas Sachar et al. (360) found that enzyme activity was increased up to 100% as a result of incubation with agitation. Incubation time may be as short as 20 minutes (360) or as long as 18 hours (346); Baló and Banga allowed 30 minutes (369), whereas Hall and Czerkawski proposed 3 hours (349), but obviously, long incubation times (12 - 18 hours) may result in low activity due to autohydrolysis of the enzyme. Also, variations in substrate concentration can result in marked alterations in the calculated number of elastase units in an elastase preparation (349).

Nearly all authors have defined an elastase unit (E.U.) as the amount of the enzyme necessary to dissolve 1 mg. elastin substrate under the conditions

of the assay method. Baló and Banga (369) and, later, Lewis et al. (371) defined an E.U. as the amount of elastase required to dissolve 1 mg. elastin in 0.5 hours; Thomas and Partridge (416) defined it as the amount which dissolves 50% of 20 mg. of elastin substrate in 30 minutes; and Loeven (350) expressed elastase activity in milligrammes of the enzyme needed to dissolve 50% of the 50 mg. of elastin originally present. Hall and Czerkawski (349) defined the E.U. as the amount of elastase that dissolved 1 mg. ox ligament in 3 hours; this amount of enzyme was chosen so that not more than 60% and not less than 20% of the initial substrate is hydrolyzed, since under these conditions the activity lies on the essentially linear central part of a sigmoid curve. Further complications in the choice of a satisfactory definition of the elastase unit arise from the length of incubation time, the use of buffer solutions of low ionic strength, and the fact that crude elastase preparations contain elastolipoproteinase, which can increase the elastase activity by 50 - 100% (347).

## D: ELASTASE INHIBITION

Studies during recent years of the mechanism by which various natural and synthetic compounds affect the course of elastolysis have increased overall knowledge of the reaction per se. The use of phosphonyl derivatives (266, 423) has demonstrated the type of reactive centre present in the enzyme, and the use of chelating agents has advanced the development of theories concerning the form of the enzyme substrate complex (424). The simplest form of elastase inhibition is that brought about by salts.

### 1. Inhibition by Salts

One of the characteristic properties of pancreatic elastase is the inhibition of its activity in the presence of common salt. Several authors have reported inhibition by saline in physiological or higher concentrations (364, 371, 398, 408, 416, 425); Lewis et al. (371) found 50% inhibition by 0.07 M NaCl

and similar results with KCl and $(NH_4)_2SO_4$; 0.2 M NaCl produces between 50 and 75% inhibition of elastolysis, depending on the method of estimation of elastase activity. The same author (371) compared the inhibition attained by 1% NaCl and 1% NaCN and believed it to be due to the Na ion, whereas Mandl and Cohen (351), who studied the inhibitory effect of NaCN on their Flavobacterium elastase, found it to be due entirely to the CN ion. These observations appear to be at variance with the studies of Winter and Frankel (425), who investigated the inhibition of the enzymatic activity brought about by salts with mono- and divalent ions, and found it to be of a nonspecific nature depending merely on total ionic strength. The Flavobacterium elastase differs from the pancreatic one in that, among other effects, the presence of 1% NaCl does not lower its activity (352). Of the divalent cations Ca, Cu and Mg, which strongly inhibit Flavobacterium elastase activity (352), only Cu also inhibits pancreatic elastase in a concentration $10^{-3}$M (371); the other cations inhibit partially when used at higher concentrations (364).

The effect of some electrolytes on both elastolytic and nonspecific proteolytic activities of elastase was compared by Amati and Castelli (408), who found that elastolytic activity could be reduced to zero while proteolytic activity was only slightly decreased. Diisopropylphosphofluoridate (DPF) completely inhibits pancreatic elastase at $10^{-4}$M (266, 416).

Calcium has been implicated in the action of pancreatic elastase; Hall (426) was able to demonstrate that those buffer solutions which contain salts capable of forming calcium complexes reduce the activity of the enzyme (e.g., citrate and phosphate buffers). Chelating agents inhibit the pancreatic elastase proportionally to their calcium chelate stability constants (427). Ethylene diamine tetra-acetic acid (EDTA) was thus found to be a powerful inhibitor; it has been suggested that its inhibitory effect occurs at the moment of interaction,

when fission of the elastin dimers takes place (428).

2. Pancreatic Elastase Inhibitor

In their original publications Baló and Banga (337, 429, 430) suggested the presence of an inhibitor in pancreatic extracts; they believed that, at low enzyme concentrations in the pancreas, elastase activity was demonstrated only after dialysis (383) and that the inhibitory action could be abolished only by means of acid extraction, dialysis, or ammonium sulphate precipitation of the pancreatic extract, with transformation of bound to free elastase. Lewis et al. (371) suggested that the inhibitory activity of pancreatic extracts was due to their high salt concentration; and Grant and Robbins (268) indicated that the inactivity of the aqueous extracts of pancreas was due to the presence of inactive zymogen rather than free enzyme.

Using starch column electrophoresis, Loeven (432) recently separated a non-dialyzable protein fraction which contained $IE_2$, an inhibitor for elastoproteinase; this inhibitor was present, in small amounts, in only about 40% of the enzyme batches obtained from fresh pancreas or commercial pancreas powder and was inactive against elastomucase. The possibility that a pancreatic elastomucase inhibitor also exists seems reasonable but is as yet unproved.

3. Elastase Inhibitors in Blood

Baló and Banga demonstrated a non-dialyzable inhibitor of pancreatic elastase in the serum of men, cattle, and rabbits (430), and other workers have observed this in guinea pigs, rats, mice, sheep, dogs, monkeys, horses and chickens (398, 433, 434); and Tolnay and Bagdy (398) reported 50 - 90% inhibition with 1:100 dilutions of human, cattle, rabbit and guinea pig serum. Banga et al. (435) have shown that serum contains 30% more inhibitory activity on elastase than does whole blood. According to Walford and Schneider (433), starch block electrophoresis showed the inhibitory agent to be at approximately

the $\alpha_1$-globulin-albumin junction of the curve of serum and it has been identified by Robert and Samuel (363) with the $\alpha$-lipoprotein fraction of serum. Fractionation with Cohn's Method 10 enabled Loeven (438) to find a strong inhibitor in fraction IV; and after repeated fractionation of serum proteins by column chromatography on DEAE-Sephadex the elastase inhibitor was identified in the $\alpha_2$-globulin fraction.

The suggestion has been made recently that some inhibitory activity in the serum is associated with the $\alpha_1$-glycoprotein orosomucoid (424); and Banga (439) found three inhibitory agents in the serum which corresponded to elastoproteinase, elastomucase, and collagen-mucoproteinase (which is said to remove a mucoprotein from collagen, leaving "metacollagen"). The inhibition of activity of porcine elastase by a specific antibody (anti-elastase $\alpha$-globulin) was studied recently by McIvor and Moon (378).

4. Inhibitor Levels in the Normal State and in Certain Pathological Conditions

Serum levels of elastase inhibitor have been studied in normal animals and man, in some selected disease states, in pregnancy, and in the ageing process, but no data have been obtained in acute pancreatitis.

The degree of inhibition of serum (or plasma or whole blood) is expressed in per cent according to the formula (438):

$$100 \times \frac{\text{(mg. elastin dissolved in the blank)} - \text{(mg. elastin dissolved in system containing the inhibitor)}}{\text{mg. elastin dissolved in the blank}}$$

The highest values were found in the mouse (413) and the lowest in the dog (28%), and about 50 - 65% for chicken, guinea pig, rat, monkey, sheep and rabbit. The sera of human males and females showed mean percentages of 55 and 66, respectively.

Walford and Schneider (433) reported that the level of the inhibitor is independent of age, and Hall (440) presented evidence for a slow decrease up to

the age of sixty, followed by a steeper rise thereafter; Petrolani (441, 442) found the inhibitory activity to increase progressively with age, whereas Salvini (443) reported a decrease parallel with advancing age.

Serum elastase inhibitor was significantly elevated during pregnancy in the human and in the rat, but not in the rabbit: the value rose from 26% in non-pregnant women to 59% in the third trimester (433, 434); this increase correlates with the incidence of dissecting aneurysms during pregnancy, which is highest just below the age of forty (397). The highest value (78%) was found three days after delivery; six weeks later values had returned to normal. Serum inhibitor levels in third-trimester pregnant women were three times higher than in non-pregnant women of the same age, but the percentage in placental cord blood was extremely low (444, 445). Hall et al. (446) reported that in Ehlers--Danlos syndrome the elastase inhibitor level was 50 - 100 times that of normal blood. The increased levels of elastase inhibitor in the disease states described by Petrolani (442), mainly diabetes, hepatic cirrhosis, and cardiovascular disease, had decreased markedly even two hours after the first injection of elastase. A slight increase in inhibitory activity was found also in the serum of patients with liver damage, nephrotic syndrome, dermatomyositis, and lupus erythematosus disseminatus (433).

5. Some Characteristics of the Serum Inhibitor

The serum inhibitor of elastase is a thermolabile macromolecular substance and is destroyed during precipitation by 2.5% trichloracetic acid. It is stable in buffer solutions of pH 6-10, but is rapidly destroyed at above pH 11. In the acid range its activity decreases much more slowly, but nearly linearly with decreasing pH values (438). At low temperatures (4°C.) the inhibitor is very stable; however, heating of the serum above 55°C. markedly reduces its activity (398, 433). The decrease in inhibition with length of

incubation has been attributed to a mild thermolability of the inhibitor at 37°C. (433). Robert and Samuel (447) mentioned the same effect - inhibition of elastolysis by serum -- but after 24 hours they obtained complete elastolysis. Loeven (438) has concluded from his experiments that, when inhibition is complete, it remains constant at 100% even after 3 days at 37°C. On the other hand, at a ratio of inhibitor:enzyme of < 1, inhibition decreases rapidly with length of incubation, due to continued elastolysis through free elastase.

6. The Similarity Between Elastase Inhibitor and Trypsin Inhibitor

Although Baló and Banga (430) pointed out in 1949 that the inhibitor of elastase that is found in the blood of man, rabbits and cattle is not the same as the serum trypsin inhibitor (367, 448) -- because the former is destroyed by 2.5% trichloracetic acid, whereas the latter can be purified in this way -- several workers have argued that they are similar or closely related. While investigating the inhibitory activity of various trypsin inhibitors on elastase, de Giuseppe and Castelli (449) found that the inhibitor present in the intestinal duct of an ascaride has a far greater effect than does pancreatic trypsin inhibitor; and Amati and Castelli (408) reported that the two elastase activities behaved differently with the pancreatic trypsin inhibitor. Furthermore while both the elastolytic and proteolytic activities could be completely inhibited by the ascaris trypsin inhibitor were not affected by ovomucoid or by soybean trypsin inhibitor. Wu and Laskowski (288) found that a purified crystalline acid-labile trypsin inhibitor from bovine plasma appeared to inhibit elastase in a stoichiometric manner, but the complex formed dissociated 100 times more readily than the inhibitor -- trypsin complex. The observation of Graham (450) that serum inhibition of elastase could be completely reversed by trypsin activity in a stoichiometric manner is remarkable.

Because chicken and human sera inhibit the elastin/elastase system but

have no effect on the casein/elastase system, Walford et al.(397) suggested that the inhibitor is active only against the elastolytic activity of the enzyme and not on the broad-spectrum proteolytic activity as measured against casein; this implies that serum contains a separate elastase inhibitor. Bagdy et al. (451, 452) believe that both an elastase inhibitor and trypsin inhibitors are present in the serum. A selective inhibition of elastolytic and non-specific proteolytic activities of elastase was reported recently by Walford and Kickhöfen (353); these authors found that the soybean trypsin inhibitor and the kallikrein inactivator (Trasylol) substantially suppressed the proteolytic activity of the enzyme (against fibrin, casein, and haemoglobin) but were without influence on its elastolytic properties.

E: THE MECHANISM OF ELASTOLYSIS

Since elastase was first characterized in pancreatic extracts by Baló and Banga (337) in 1949, numerous theories have been advanced to explain how it renders elastin soluble. Banga and co-workers (453 - 455) first suggested that the action of elastase was not one of proteolysis but rather of depolymerization, a fibrous elastin being transformed into globular elastin by the splitting of hydrogen bonds. This concept was supported by the facts that no free amino groups could be demonstrated during the elastolysis and that solubilized elastin did not show any double refraction under the polarizing microscope (453). The molecular weights of the two kinds of polypeptides produced during elastolysis in about equal amounts were calculated to be in the order of 2,000 and 20,000, respectively (435, 454).

However, the formation of free $\alpha$-amino groups was demonstrated by Partridge and Davis (407) with the fluorodinitrobenzene technique. The reaction of elastase with the A- and B-chains of insulin showed that the enzyme can attack a wide variety of peptide bonds that involve neutral amino acids that

have aliphatic side chains; and, since the elastin contains large amounts of neutral amino-acid residues, the elastolysis was explained in terms of this specificity (266). Some workers (457, 458) have indicated that the integrity of the elastic fibre might be associated with the presence of an essential polysaccharide, and others have suggested lipid as a nonprotein stabilizing factor (410, 411, 459). Both hypotheses necessitate the proposal of nonproteolytic roles for the enzyme. Lansing et al. (410) mentioned the liberation of lipids during elastolysis and suggested that elastin was a lipoprotein and elastase a lipolytic enzyme. Hall et al. (458) showed that relatively crude preparations of elastase brought elastin into solution, with simultaneous release of polysaccharide and acid. This attractive hypothesis was further supported by quantitative measurements of acid liberated during the first half of the dissolution of elastin in the experiments of Hall and Gardiner (460), which considered the acid to be an organically bound sulphate attached to acid polysaccharides.

In 1957, using paper electrophoresis, Hall (347) was able to separate completely the different components of the elastase complex; he showed that, if both enzyme preparations contain up to 10% of each other, the activity of the elastolytic component of elastase is much higher than that of the elastomucase, even though the ratio of polysaccharide to protein liberated is twice as great in the case of elastomucase. The importance of elastomucase in the over-all reaction of elastolysis has been investigated in detail by Loeven (350). The opinion that one component of elastase reacts with the polysaccharide moiety, and another with the protein moiety of elastin, was suggested by Walford et al. (397), who estimated hexose and hexosamine content in the elastolyzates of elastin pretreated in various ways. Saxl (355, 356), using light and electron microscopy, studied the effect of both elastase components on

human aortic tissue, from a structural point of view, and demonstrated release of metachromatic material by elastomucase action on the inner layers.

F: ENZYME KINETICS IN ELASTOLYSIS

The kinetics of the enzymatic dissolution of elastin are complex, and the elastolytic reaction commonly is characterized by a lag phase or a markedly sigmoid time course (349, 423). Under certain conditions the dissolution of elastin is no longer linear, which contributes to the difficulty of determining accurately the specific activity of enzyme samples. According to Sachar et al. (360), a zero-order reaction is followed when less than 35% of the 20 mg. elastin present in the reaction mixture has been rendered soluble; however, others (371, 375) have cited 75%. The purity of the enzyme preparation and the pretreatment and particle size of elastin also affect the linear relationship between enzyme concentration and amount of substrate rendered soluble. Loeven (385) has suggested that linearity is possible only in the presence of elastomucase; on the other hand, Lamy et al. (364) showed that the activity time curves (followed by three types of assay) showed zero-order reactions up to a certain concentration of elastase. Depending upon ratio of substrate to enzyme concentration or upon type of buffer solution used, it was possible to obtain a zero-order reaction, a first-order, or an undetermined type of reaction that started slowly and accelerated with time.

Various authors (266, 349, 361) have confirmed the presence of a lag phase during which, with low concentration of elastase, no elastolysis occurs. This phase is thought to arise from the cross-linked nature of elastin (465); when elastin is attacked by the enzyme, a finite number of peptide bonds must be broken and a finite time must elapse before release of a soluble fragment occurs (442-444).

Enzymatic hydrolysis appears to proceed at the surface of the fibres or microfibrils, and the rate of reaction is dependent upon particle size (350, 386). The reaction commonly starts slowly and later accelerates, in a sigmoid-type curve. However, if the same reaction is carried out at a constant pH with an automatic titrator, usually there is no lag phase and the release of $\alpha$-amino and carboxyl groups follows a logarithmic course. The initial phase of the reaction with pancreatic elastase exhibited zero-order kinetics in the experiments of Lamy et al. (364), with Tris (hydroxymethyl-aminomethane) as buffer.

Hall and Czerkawski (349) distinguished between solubilizing action, during elastolysis, resulting in the formation of $\alpha$-elastin (real elastolysis), and pure hydrolysis (involving the splitting of $\alpha$-elastin into smaller peptides with an approximate molecular weight of 600). The same authors (461, 462) stated that the reaction between elastase and elastin may be divided into at least three separate phases which together result in the conversion of solid elastin of high molecular weight into soluble degradation products. The three phases are:

1. An initial, adsorptive phase, during which the enzyme is adsorbed on to the available side-chain carboxyl groups of elastin, with resultant formation of the enzyme--substrate complex, fission of bonds, and loosening of structure with marked decrease in rigidity of the elastic fibres. This could be called preliminary reaction, and it would correspond to the slow phase of the elastolysis time curve (349).

2. A second, disruptive phase, in which disruption of solid elastin, giving rise to soluble $\alpha$-elastin, is brought about by fission of a limited number of peptide bonds (407). The $\alpha$-elastin produced at this stage is considered to be identical in properties to similar material that results from chemical degradation (442, 445, 446). Under normal conditions digestion does not stop at this stage (the solubilization step), the production of $\alpha$-elastin being followed immediately

by its further degradation during the third phase.

3. In the third, final part of the over-all reaction, considerable amounts of amino groups are liberated, showing that here also the system is proteolytic; this is maintained until all solid elastin has been dissolved, and has been named the homogeneous reaction. The kinetics of the homogeneous reaction between elastoproteinase and soluble $\alpha$-elastin were studied by Hall and Czerkawski (461) during controlled elastolysis in the presence of dodecyl sulphate, which agent inhibits conversion of high-molecular-weight $\alpha$-elastin into lower-molecular-weight $\beta$-elastin. These authors confirmed the empirical relationship in the homogeneous reaction between elastase and $\alpha$-elastin (409) by a theoretical equation (461).

## G: REACTIVE SITES IN ELASTASE AND IN ELASTIN

During investigation of the amino-acid sequence around the reactive serine residue of some pancreatic endopeptidases it was found (423, 448) that the active centre in elastase is GlyAspSerGly, a sequence identical with that determined for trypsin and chymotrypsin (467, 468).

That proline--glycine sequences must be relatively frequent in elastin was shown by Mandl (469) from an analysis of Flavobacterium elastase digests of elastin. Grant and Robbins (268) pointed to at least two distinct active sites in elastase, namely a combining and a catalytic site. Hall (424), who studied the inhibitory effect of human plasma on elastase, maintained that there may be two active centres on the enzyme molecule; and Walford and Kickhöfen (353) made the same suggestion, based on their studies of selective inhibition of the elastolytic and nonspecific proteolytic properties of elastase.

The free side-chain carboxyl groups of glutamic and aspartic acids are essential for the absorption of elastase to elastin and for the subsequent

reaction, according to Hall and Czerkawski (462), who studied the effect of various chemical agents on elastin. Elastase and elastin possibly are united by calcium, which is held to both proteins by chelate bonds (426); it is possible also that both elastin and elastase exist as dimers, in which adjacent chains are linked together through the donation of electrons from COOH and OH or $NH_2$ groups to form the coordination shell of a shared calcium atom (428).

H: PRODUCTS OF ELASTOLYSIS

Very little is known about the products of the elastolytic elastin breakdown. Partridge and Davis (407) determined the free N-terminal amino-acid groups liberated, and found (moles/100,000 g.) glycine (55.9), alanine (42.5), leucine + isoleucine (13.4), valine (4.1), glutamic acid (3.1) and aspartic acid (2.9); C-terminal residues were not determined. Electron microscopy showed that the smallest particles observed at the end of the process of elastolysis were of the order of 200 A diameter (446): it appears likely that these are still much larger than the ultimate particles of molecular weight 600 which would be visible by electron microscopy.

I: FATE OF THE ENZYME DURING ELASTOLYSIS

The activity/time curve of the elastolytic reaction is not linear over its whole length; in addition to the lag period (266, 361) there is a reduction in activity, toward the end of the reaction, which may be due to actual destruction of the enzyme or to its adsorption on to some nonactive site. At least part of the decrease in the elastolysis/time curve may be due to involvement of the enzyme in the homogeneous reaction (463); the remainder may result from autodigestion.

A freeze-dried preparation of elastase can be maintained for several months at $4^{\circ}C$. without loss of activity, but in solution its stability is not

great. When a solution of elastase is kept at 37°C., two phenomena occur; a decrease in activity and an increase in free amino groups present in solution. It is possible, though, that elastin may protect the enzyme from itself by providing a more natural substrate. This has been shown to occur in preparations of elastolipoproteinase, which is subject to even more rapid autodigestion (466).

# CHAPTER VII

# ELASTIC TISSUE

## A: INTRODUCTION

The specific elements of connective tissue, called elastic fibres, are characterized by their high refractivity, their homogeneous filament-like or ribbon-like character, and their easy stretching when wet. In a broad sense, the protein of the elastic fibre is among the most chemically inert in the body, and its resistance to acid and alkaline hydrolysis approaches that of the keratins (470). It is impossible to bring elastin into solution except by the addition of hydrolytic reagents or elastase, both of which can rupture peptide bonds. Purified elastin is pale yellow in colour and has characteristic bluish fluorescence in ultraviolet light. Elastic tissue is generally distributed throughout the mammalian body, in those sites in which elasticity is required, such as vessel walls, lungs, ligaments, and dermis. In all of these sites elastic fibres are associated with collagen, and both fibrous elements are surrounded by an amorphous material known as ground substance. Semiquantitative values of the amount of elastin present in these tissues may be obtained by visual estimates, using one of the specific stains for elastica. However, chemical methods have almost completely replaced subjective assessments; they are based on the apparent resistance of elastin to chemical attack. Such methods, in which usually hot acid or alkali is used removes the majority of extraneous nonelastic staining material without degrading the elastic fibres themselves (471, 472). Discovery of the enzymes of the elastase complex has greatly facilitated structural studies of elastic tissue.

## B: MORPHOLOGY

A typical elastic fibre is a yellow branching structure which is the

unfixed state demonstrates a high degree of elasticity when teased between needles. Under the microscope, elastic fibres from the ligamentum nuchae of the ox are short, smooth, rod-like, and almost circular on cross-section (470). The arterial and venous elastic formations are very different in character; they are closely associated with collagen and polysaccharide. Also, smooth muscle cells are present in varying amounts in arteries of different kinds. Ishida (472), who studied the elastic fibres of veins, suggests that the shape of the fibre depends upon its size: thin fibres are circular and thicker ones are lenticular on cross-section. The structure of arterial elastica differs from that of the veins in that the elastica-staining material is not restricted to the fibrous form; in fact, the flattened ribbons apparent in vein walls may represent a half-way stage between true fibres of circular cross-section and the sheet-like structure present in the artery. Gillman et al. (474) reported that the elastin of the vascular elastic membranes differs tinctorially, morphologically and histochemically from that found in other organs. The elastic fibres seen in spreads of fresh tissue or in histological sections are highly refractile and have sharply defined margins when examined with a polarizing microscope, and they are isotropic in their relaxed condition.

C: ULTRASTRUCTURE

Rhodin and Dalhamn (475) were the first to examine elastic tissue with the electron microscope, using thin sections; they observed a two-phase system and suggested that the central portion consists of a bundle of 'fibrils' which are closely packed at the centre but which become sparser toward the periphery. They also pointed out that, at the outside, but possibly penetrating between the more loosely packed fibrils, lies an amorphous layer (elastomucin). However, the fibrils may represent arrays of individual micelles, since X-ray

diffraction (464, 476) and electron diffraction (356) studies show no true fibrillar structure. Cox and Little (477) were unable to demonstrate any internal structure, but More et al. (478) believe that this homogeneity may only apply to mature elastic tissue and not to immature fibres. Saxl (356) and Charles (479) observed osmophilic striations in elastic tissue which may be true fibrils or may represent mechanical alignment of lipoprotein molecules. The identification of polysaccharide in electron micrographs is difficult and can be accomplished only by inference.

D: ELASTIC FIBRES DURING ELASTOLYSIS

Using light and electron microscopy, Dempsey and Lansing (480) observed the process of elastolysis in detail. Early in digestion, when the glassy refractility of the fibres is lost and small indentations begin to appear, the smooth cylindrical structure appears as a pair of loosely twisted fibrils; each fibril in turn splits into two more. The process proceeds by fragmentation and dissolution of small fibrils. With the electron microscope, this fraying and splitting could be followed until small, rather short fibrils of about 180 Å diameter remained, and these dissolved completely after longer incubation time.

E: HISTOCHEMISTRY

Elastin, in contrast to collagen, has a very low content of amino acids with charged side chains, and its affinity for dyes with a predominantly acid or basic fraction is low, regardless of the pH of the solution. Elastic fibres stain selectively with orcein and Weingert's resorcin-fuchsin; they also take up a number of others, such as Verhoeff's haematoxylin, Nile-blue sulphate, basic fuchsin, osmic acid, and Sudan black (480). Mallory's aniline blue stains collagen blue and elastic tissue red. Combined stains for demonstrating elastin,

reticulin and collagen in the same tissue have been developed (481). The affinity of elastic fibres for orcein is unaffected by pH over a fairly wide range, but above 8.5 the staining fails; acid--alcohol solutions produce the best results. It seems that ionic forces are not critically involved in the staining of elastic fibres with orcein, and the absorption is due possibly to secondary short-range forces not involving a difference in net charge (482). The fibres show a loss of elastica-staining power if they are damaged by exposure to elastase: Partridge and Davis (465) showed that such exposure results in production of new $\alpha$-amino and $\alpha$-carboxyl groups before the occurrence of complete dissolution, which may inhibit the uptake of dyes because of alteration in charge distribution.

F: CHEMICAL COMPOSITION OF ELASTIN

Elastin can be prepared from fresh tissues by procedures which destroy other components (410). Inorganic constituents, such as calcium and phosphorus, vary with the age of the subject and the anatomical source of the sample. Purified elastin contains no detectable sulphur (480). The nitrogen content is 15.6 - 16% in ligamentum nuchae and about the same in elastin from blood vessels. Complete hydrolysis of elastin from various sources indicates a varying amino-acid composition (480). The identity of polysaccharide and lipid associated with elastin has not been fully elucidated, and much study recently has been devoted to attempts to identify the pigment responsible for the yellow color of elastic fibres. Labella (411) suggests that several non-porphyrin pigments are present, some of them fluorescent.

G: ORIGIN OF ELASTIC TISSUE AND ITS RELATIONSHIP TO COLLAGEN

The existence of a special tissue cell which is capable of forming elastic fibres is a matter of controversy. Lansing (484) has stated that with the exception of the post-partum uterine artery there is little evidence that

elastic fibres can regenerate in adult animal tissues. Massive formation of elastica-staining material in breast cancers treated with oestrogens has been seen and elastica-staining fragments are apparent in the human foetus in the third month of pregnancy (485). The formation of collagen fibres *in vivo* results from the activity of fibroblasts, but no similar 'elastoblast' has been recognized.

Because collagen and elastin are intimately related, it has been suggested that collagen and elastin may be formed in natural tissue from the same mixture of soluble peptide precursors, or even that preformed collagen may be converted to elastin (486) or elastin to collagen. Burton *et al.* (486) showed that collagen, after treatment with alkaline buffers or pancreatic enzymes, could give rise to material which had some of the staining and histological characteristics of elastin. Since reliable amino-acid analyses for collagen (487, 488) and elastin (407, 489) are available, a direct comparison of composition is feasible. Both proteins have unusual and highly characteristic amino-acid compositions, but, except for a preponderance of glycine and proline, the two proteins have little in common. On the other hand, although native collagen is resistant to the action of elastase, denatured collagen is dissolved by the enzyme about 10 times faster than is elastin itself (454). Grant and Alburn (490) pointed out that collagenase does not attack elastic fibres, although recently submicroscopic structural damage has been observed with the polarizing microscope (491).

Final solution of the problems raised by the concept of Burton *et al.* (486) must await establishment of the route of elastin biosynthesis.

## H: ELASTIC TISSUE IN ARTERIES AND VEINS

Harkness *et al.* (492) studied changes in elastin content along the length of the aortic system. The ratio of elastin: elastin + collagen in dogs

remained roughly constant at 50 - 60% from the aortic valve to within 5 cm. of the diaphragm. The proportion of elastin decreased and remained at about 25 - 30% throughout the abdominal aorta and the iliac and femoral arteries. Collagen and elastin formed approximately 50% of the dry weight of all arteries, although the proportion was somewhat higher in the smallest.

The arterial wall consists of three coats: the innermost is the tunica intima, then the tunica media, and, the outermost, the tunica adventitia. Most arteries, including the arterial branches entering the pancreatic parenchyma, are of the distributing type. Characteristically (493), the intima is relatively thin, with a well-developed internal elastic lamina that consists of a single thick layer of elastic fibres: on section it presents a wavy appearance. The media is a thick coat of smooth muscle, collagen, and elastic fibres: and the adventitia consists mainly of elastic but contains collagen fibres also. The elastic fibres are condensed to form the external elastic lamina. In arterioles (arteries of less than 100 $\mu$ diameter) there is an internal elastic lamina, a media consisting of smooth muscle, and a mixture of collagen and elastic fibres in the adventitia. The internal lamina becomes thinner with decrease in size of the arterioles and is absent from the smallest. Small-vein walls also consist of three layers: the intima comprises endothelium which rests on a poorly defined internal elastic membrane, and the media, which is thinner than in arteries consists mainly of collagenic connective tissue.

Movat and Fernando (494) recently emphasized the importance of small venules (the smallest vessels of the venous side of the circulatory system) in the study of inflammation. Migration of leucocytes and exudation of plasma occurs through the walls of venules in the inflammatory process. Their walls contain no elastic fibres and their structure has been best studied by the electron microscope.

## CHAPTER VIII

## THE ROLE OF PANCREATIC ELASTASE IN THE PATHOGENESIS OF ACUTE PANCREATITIS

The only available study concerning the role of elastase in acute pancreatitis is that of Schneider *et al.* (12), which has been mentioned previously. After investigating the lesions caused by pancreatic elastase on arterial walls *in vitro* and by arterial perfusion *in vivo*, these workers explored the ability of the enzyme to initiate haemorrhagic pancreatitis in dogs. In 14 animals the pancreatic duct was cannulated and 75 mg. crystalline elastase (P-II fraction, prepared by the method of Grant and Robbins (268) yielding 160 elastase units/mg.) was infused in a 15-ml. solution in distilled water at a pressure of 40 - 60 cm. water. Infusion was completed in 15 - 30 minutes. The animals were killed 5 hours afterwards.

All of the 14 dogs developed marked changes in the pancreas consistent with haemorrhagic pancreatitis. Microscopical examination revealed that the pancreatic parenchyma was extensively involved, with haemorrhages and large numbers of polymorphonuclear leucocytes. The vascular damage comprised partial or complete digestion of arteries, with loss of elastic fibres and necrosis of the media. Fresh thrombi were found in many of the medium-sized veins; no mention was made, however, of changes in their walls or concerning elastolytic activity in extrapancreatic tissues. The authors believe that the vascular lesions they observed in these animals are identical with those that occur in haemorrhagic pancreatitis in man.

These findings are significant only in the sense that a relatively large dose of elastase (75 mg. x 160 = 12,000 elastase units) was capable of inducing pancreatitis following its intraductal injection. However, this study

is of limited value in elucidating the pathogenesis of experimental and clinical pancreatitis, for the following reasons.

It is known that acute pancreatitis may be induced in a variety of animals and by many kinds of chemical substances injected into the pancreatic duct. The fact that intraductal administration of elastase, an enzyme capable of dissolving the insoluble protein of elastic fibres and of digesting several other proteins, results in pancreatitis is not surprising. Of considerable importance, however, are the natural stores of proelastase in the pancreas and the degree and mode of activation of the proenzyme in the course of experimental pancreatitis; information concerning these points is not available. Furthermore, if such an activation of proelastase is granted, elastolysis will depend upon the amount of locally available pancreatic elastase inhibitor. Determination of the inhibitory activity of pancreatic tissue extracts in the course of experimental pancreatitis has never been attempted.

# PART II

# EXPERIMENTAL SECTION

# PART TWO : EXPERIMENTAL SECTION

## CHAPTER IX

## GENERAL PLAN OF INVESTIGATION

1. The first objective of this study is the development of a relatively simple, reliable colorimetric method suitable for quantitative determinations of proelastase and free elastase in extracts of fresh pancreatic tissue and other biological materials. A restricted set of conditions will be observed, to obtain a single relationship between the amount of elastin dissolved and the elastolytic activity determined (zero-order kinetics). A proelastase and free-elastase unit will be defined under the conditions of the assay.

2. Proelastase and free and total elastase determinations in extracts of fresh pancreatic tissue obtained from the three anatomical parts of the pancreas of normal fasting mongrel dogs will be carried out in an attempt to establish their 'normal' mean values in canine pancreas.

3. Determinations of proelastase and free and total elastase will be performed in fresh human pancreas obtained immediately after death from renal-homotransplantation donors.

4. Proelastase and elastase determinations will be made in extracts of pancreatic tissue of fasting mongrel dogs after the induction of pancreatitis by intraductal injections of autologous bile or trypsin, to compare proenzyme and free-enzyme changes occurring in the three anatomical parts of the organ during the course of acute pancreatitis.

5. Elastica stains on histological sections of pancreatic tissue of dogs with bile- or trypsin-induced pancreatitis will be studied, to obtain evidence for the release of elastolytic activity (loss of stainable characteristics or dissolution of elastic fibres in intralobular and interlobar vessels).

6. Studies will be made of induced parenchymatous necrosis in the dog's submaxillary gland (which does not contain elastase), by intraductal injection of autologous bile or trypsin, followed by elastica stains of the necrotic tissue, in an attempt to establish the concept that dissolution of elastic tissue, the sine qua non of vascular necrosis, cannot occur in the absence of elastase.

7. The inhibitory activity of pancreatic and mandibular tissue extracts will be determined on an elastase/elastin--orcein system, to determine the levels of activity in 'normal' and necrotic tissue.

8. The effect of crystalline elastase on fresh oxygenated pancreatic slices incubated under optimal conditions, followed by elastica stains of the tissue in order to evaluate the in vitro digestion of elastic fibres and compare it with the in vivo occurring changes in the course of bile or trypsin pancreatitis.

9. Induction of pancreatitis in the fasting mongrel dog by the intraductal injection of crystalline pancreatic elastase and histological sections with elastica stains for evaluation of elastic tissue destruction in vivo.

The overall objective of this investigation consists of an attempt to delineate the role of pancreatic elastase in regard to the distinct vascular injury of acute pancreatitis.

The vascular lesion which includes destruction and thrombosis of small vessels leading to interstitial haemorrhage and parenchymatous necrosis is considered to be of crucial importance in the progression of pathology in this disease.

# CHAPTER X

## A NEW COLORIMETRIC METHOD FOR MEASURING ELASTASE ACTIVITY

### A: INTRODUCTION

It was puzzling to the early investigators that elastase activity could be demonstrated only with difficulty, if at all, in fresh extracts of pancreatic tissue, although it was readily extracted from dried, defatted pancreas powder. The problem was not solved until, in 1955, Grant and Robbins (286) showed that homogenates of fresh hog pancreas contained elastase in an inactive zymogen form which they named proelastase. They also found that trypsin or a duodenal factor was capable of converting this precursor to active elastase. The activation could be blocked by pancreatic and soybean trypsin inhibitors, indicating that the duodenal factor probably is enterokinase, which converts the inactive trypsinogen into trypsin which, in turn, activates proelastase. Recently, 40-fold purification of proelastase from fresh hog pancreas was achieved (168), and it was found that approximately 1.1 µg. of trypsin is needed to activate each proelastase unit.

All assay methods for elastolytic activity are based on measuring the amount of insoluble substrate (elastin) which is solubilized in unit time. Sachar *et al.* (360) employed orcein-stained elastin as substrate and measured the amount of dye released after incubation with pancreatic extracts, a method which has been adopted by others (266), using Congo-red-stained substrate.

A method has been devised in this laboratory by which, under certain conditions, free elastase, proelastase and total elastase content of pancreatic extracts can be separately and quantitatively determined.

### B: PRINCIPLE

The elastolytic activity of pancreatic extracts is measured by a

modification of Sachar's colorimetric method, based on solubilization of an elastin--orcein substrate. The unit of elastase is considered to be the amount of activity that renders soluble 1 mg. elastin in 30 minutes under the conditions of the assay. Total elastase and free elastase are determined separately in two different aliquots from the same extract. For the estimation of free elastase activity an aliquot of fresh pancreatic extract is incubated, immediately after it has been homogenized, with the elastin--orcein substrate; the dye released is determined colorimetrically.

For estimation of total elastase, crystalline trypsin is added to an aliquot from the same pancreatic extract, effecting activation of proelastase; this is followed by incubation of the aliquot with the orcein--elastin substrate, dye release, and colorimetric reading. Subtraction of the free- from the total-elastase value yields the amount of proelastase.

C: PREPARATION OF SUBSTRATE

1. Reagents

a) Elastin: a purified preparation from equine ligamentum nuchae, prepared by a modification (410) of the method of Lowrey et al. (471) was obtained from Worthington Biochemical Corporation, Freehold N.J.

b) N-butanol, reagent grade.

c) Ethanol, 95%.

d) Ethanol, 70%.

e) Acetone, reagent grade.

f) HCl conc., reagent grade.

2. Methods

Elastin powder is suspended in an orcein solution comprising 1 g. orcein in 100 ml. 70% ethanol to which 1 ml. HCl conc. has been added; 20 g.

elastin is suspended in each 100 ml. of this solution. The solution is stirred constantly while standing overnight at room temperature. Then the stained elastin is washed separately with large volumes of 70% ethanol, with constant stirring overnight, until the colour of the washings is constant as determined colorimetrically at 525 mμ. This is followed by repeated washing with 95% ethanol for three weeks, until the washings are colour-free. Finally, the stained elastin is washed with acetone; the air-dried residue is ground in a mortar and stored refrigerated. (Samples of this substrate incubated in an enzyme-free system, as described in Methods (E), gave no colour change in the supernatant fluid).

D: MATERIALS

De-ionized water is used throughout the procedure and assay.

1. Tris buffer

1/5 M Tris (hydroxymethyl aminomethane), pH 8.8, was prepared as follows.

Solution A. - 12.1 g. Tris (primary standard grade; obtained from Fisher Scientific Co.) is dissolved in 500 ml. water.

Solution B. - 0.2 N HCl.

The buffer is prepared by mixing 250 ml. solution A with 41 ml. solution B and diluting to 1000 ml. with water; this buffer hereafter will be referred to as 'Tris'. It has the advantages that it does not precipitate Ca salts, is low in price, and is stable at room temperature of > 3 months (495).

2. Phosphate buffer

1/15 M phosphate buffer, pH 6.0, is prepared as follows.

Solution A. - Dissolve 9.08 g. $KH_2PO_4$ in 1000 ml. water.

Solution B. - Dissolve 11.88 g. $Na_2PO_4.2H_2O$ in 1000 ml. water.

The buffer solution is made by mixing 87.7 ml. of solution A with

12.3 ml. solution B.

3. Trichloracetic-acid solution (50%)

4. N-butanol, reagent grade.

5. Trypsin solution (0.1%)

Dissolve an appropriate amount of 2 X crystallized lyophilized trypsin (Frank W. Horner, Ltd.: 'Parenzymol') in Tris buffer. The solution is prepared freshly each time for use.

6. Pancreatic elastase

2 X crystallized pancreatic elastase in water suspension (Worthington Biochemical Corp.), prepared from porcine pancreas (371).

7. Pancreatic extract

A 5% (1:20) extract is prepared by homogenizing 1 g. fresh pancreas in 19 ml. ice-cold Tris buffer in a VirTis No-45 Homogenizer (Research Equipment, Gerdiner, N.Y.) for 5 minutes, under ice. Then the homogenate is centrifuged for 20 minutes at $0^{\circ}$ C. at 10,000 gravity. The supernatant fluid is decanted into an ice-chilled 250 ml. Erlenmeyer flask; 1 ml. is used for assaying elastolytic activity.

E: METHODS

The method for determination of free and total elastase and of proelastase in biological material is as follows.

1. Duplicate 25-mg. samples of orcein--elastin are placed in 1.5 X 10 cm. test tubes which have been numbered: $F_{10}^{A}$ and $F_{40}^{B}$ = 40 minutes' reaction; $T_{10}^{A}$ and $T_{10}^{B}$ = total elastase activity after 10 minutes' reaction; $T_{40}^{A}$ and $T_{40}^{B}$ = 40 minutes' reaction.

2. 2. ml. Tris buffer is added to the 'F' series of tubes.

3. 1 ml. Tris buffer is added to the 'T' series of tubes, followed by 1 ml. 0.1% trypsin in Tris buffer solution.

4. 1 ml. pancreatic extract is added to the reaction mixture in each tube.

5. The tubes are sealed with rubber stoppers, and are placed in a rack in a water-bath, at 37° C., with intermittent shaking. Incubation times: 10 and 40 minutes for each couple in the 'F' and 'T' series.

6. 2 ml. phosphate buffer is added to the tubes at the end of the intervals, as noted in the previous paragraph; then the tubes are shaken and cooled in an ice-bath, to stop the reaction.

7. The tubes are centrifuged for 10 minutes at 2000 r.p.m.

8. 4 ml. supernatant fluid is decanted into 5-ml. cellulose-nitrate tubes, with the addition of 1 ml. 50% trichloracetic-acid solution for protein precipitation.

9. 5 ml. N-butanol is added as the extracting medium, and then the tubes are capped.

10. The resultant purplish colour is extracted by shaking the tubes vigorously for 15 seconds.

11. The tubes are centrifuged for 15 minutes at 10,000 gravity. A white precipitate is seen at the interphase; the aqueous phase is cloudy but colourless.

12. The upper (alcohol) phase containing the dye is transferred with a Pasteur pipette into the micro cells designed for the Fisher electro-photometer, and reading is performed at 525 mμ., with N-butanol as the blank. These samples remain clear and unchanged thereafter, even if they are stored at 4° C. for up to 24 hours.

F. STANDARD CURVE

Duplicate 5 mg. increments of elastin--orcein substrate (5 - 25 mg.)

are placed in 1.5 x 10 cm. test tubes, with 2 ml. Tris buffer and 1 ml. crystalline elastase solution (100 units elastase/ml.). The tubes are sealed with rubber stoppers and placed in a rack in a water-bath at 37° C., with intermittent shaking, for 5 hours. (Since an excess of enzyme is added to the tubes, all of the substrate should be in solution at the end of the incubation period.) 2 ml. phosphate buffer is added to each tube, followed by shaking. The remainder of the procedure, for extracting dye and recording optical density (O.D.), is as described before (Procedure, 7 - 12). By plotting the O.D. against mg. substrate, the standard curve is obtained; from this the coversion table can be deduced.

G: CALCULATION OF ACTIVITY

1. Elastase unit: One unit of elastase activity is defined as that amount of enzyme which will render soluble 1 mg. elastin--orcein substrate in 30 minutes (between the 10- and the 40- minute reaction times).

2. Free elastase activity is determined from the corrected O.D.; the latter is the difference between the reading at 40 minutes (mean of two) minus the 10-minute reading (mean of two) i.e., $F_{40} - F_{10}$. The amount of elastin--orcein rendered soluble, corresponding to the particular O.D. reading, is read in the standard-curve or conversion table. This value, multiplied by a factor of 20, yields elastolytic activity (units/g. tissue).

3. Total elastase activity is calculated from the difference in O.D. between the 40- and 10-minute tubes of the 'T' series (corrected O.D., $T_{40} - T_{10}$). Total elastolytic activity/g. tissue is determined as described for free elastase.

4. Proelastase unit: One unit of proelastase activity is defined as the amount of the proenzyme that, when completely activated by trypsin, will render soluble 1 mg. elastin--orcein substrate in 30 minutes, between the 10-

and 40-minute reaction times. The activity of proelastase/g. tissue is obtained by subtracting the units of free elastase from those of total elastase.

## H: KINETICS OF ELASTASE/ELASTIN--ORCEIN INTERACTION

The amount of elastin--orcein rendered soluble in a given time (in this case, 30 minutes) is directly proportional to the enzyme concentration only if the reaction follows zero-order kinetics: the rate of elastolysis is proportional to the enzyme concentration but is independent of the initial amount of elastin--orcein used. To establish the set of conditions under which this occurs, a series of kinetic curves was devised, at constant elastin--orcein concentration (25 mg.) and with extracts of normal pancreas of 5% and 10% dilution, containing unknown amounts of elastase.

### 1. Activity time curve

Determination of free and total elastase were carried out as previously outlined with duplicate tubes containing the elastin--orcein substrate and Tris buffer, with 1 ml. 5% or 10% pancreatic extract for determination of free elastase and with added trypsin for total elastase. The tubes were withdrawn from the water-bath at 10-minute intervals for 2 hours (timed by stop-watch); each time the reaction was stopped by the addition of phosphate buffer. The results were recorded as time in minutes *vs.* optical density at 525 mμ. in the electrophotometer and mg. elastin--orcein rendered soluble.

Two representative curves are shown in Figure 1: they were obtained with 10% and with 5% pancreatic extract. A linear relationship between time and the amount of substrate rendered soluble under the condition of the 30-minute assay (between 10 and 40 minutes incubation time) was obtained. This corresponds to solubilization of 72% of the 25 mg. elastin--orcein substrate in the case of the 10% extract and to 32% of the substrate for the 5% extract. In

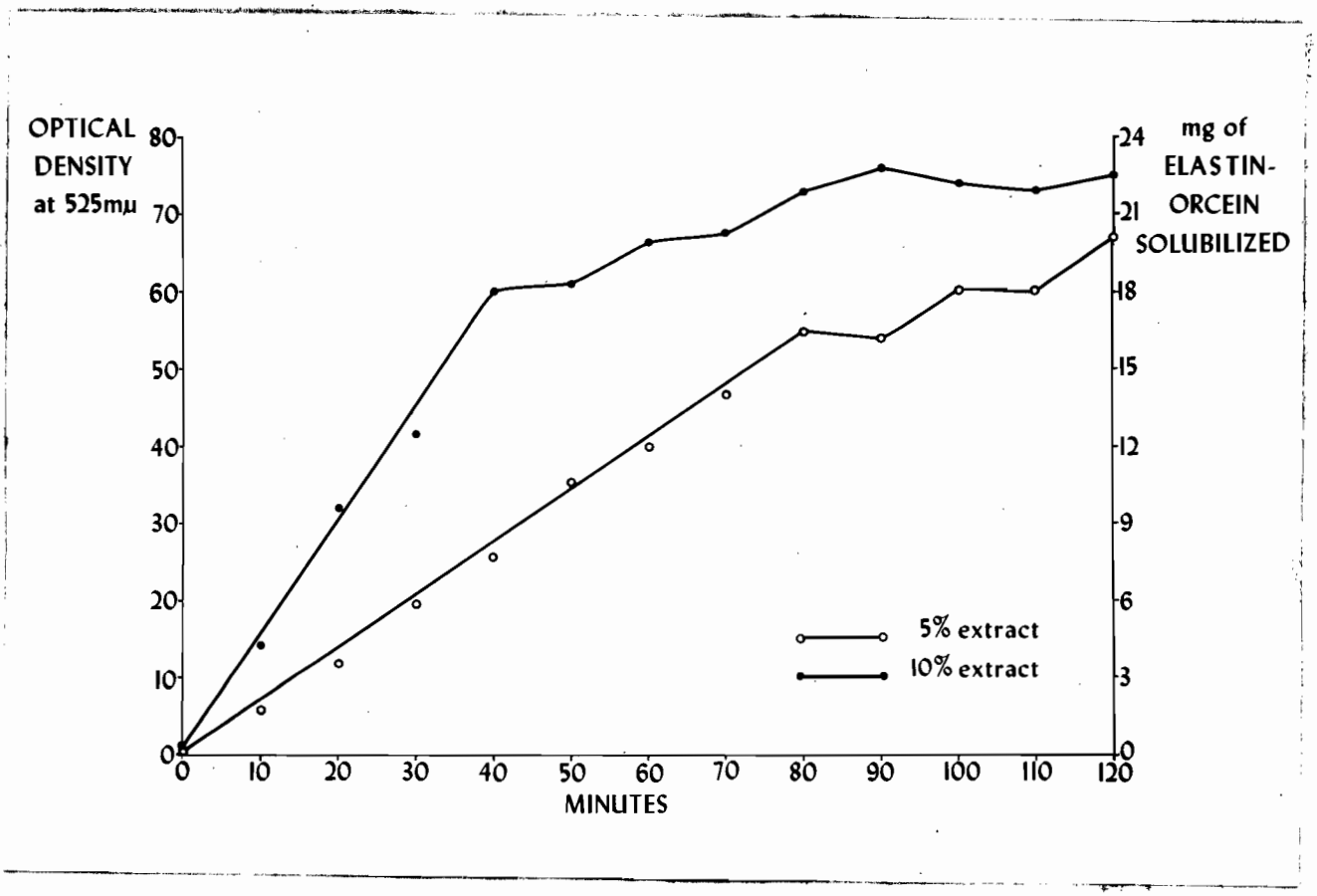


FIGURE 1. Elastolysis of elastin--orcein after incubation with 5% and 10% pancreatic extract, of unknown elastase content, as a function of time.

both cases the determinations of 160 - 180 units of elastolytic activity could be obtained, an amount that is considered satisfactory for the purpose of this study. Total elastolytic activity/g. tissue in canine pancreas was found to exceed the above range in very few cases.

The values of free elastase in the elastolysis time curves showed a fast initial rise during the first 10 minutes and then, almost uniformly, remained at that level thereafter.

2. Dilution curve

Determinations of total elastolytic activity were carried out, with duplicate tubes containing 25 mg. elastin--orcein substrate, Tris buffer, and the following amounts of 10% or 5% pancreatic extract (extract of normal canine pancreas with added trypsin): 0.3, 0.6, 0.9, and 1.2 ml., with an incubation time of 40 minutes (30 minutes assay, extending between the 10 and 40 minute interval). The results were plotted as mg. elastin--orcein rendered soluble vs. the dilutions of the pancreatic extract. Again, a linear relationship was observed between the substrate and the dilution of the pancreatic extract of unknown enzyme content up to the point where approximately 50% of the elastin--orcein substrate was rendered soluble.

3. Thirty-minute assay

The reasons for determining the rate of elastolysis between the 10- and 40-minute intervals are as follows:

a) To allow for complete activation of the proenzyme by trypsin.

b) To avoid the initial slow phase of the elastase/elastin--orcein interaction (adsorption phase).

c) To avoid colour interference related to the nature of the pancreatic extracts. In view of the fact that necrotic tissue containing substantial amounts of bile and blood will be used for elastolytic-activity determinations,

their colour interference was nullified by subtracting the 10-minute from the 40-minute reading. Colour increase observed in the material incubated between the 10- and 40-minute reaction times represents only orcein release. To further reduce the colour interference of the pancreatic extracts, the 5% dilution of the extract was used in all subsequent determinations.

I: ERROR OF THE METHOD

Free and total elastase, and proelastase content were determined in five samples of pancreatic tissue which had been obtained from adjacent parts of the head (right branch) of the pancreas of three normal dogs. Separate extracts were prepared and were analyzed for enzyme activity as previously described. Analysis of variance of the three sets of 15 data provided coefficients of variation. The coefficients express the error variation as a percentage of the mean, and were as follows:
total elastase, 10.81%; free elastase, 42.03%; and proelastase, 13.53%. Thus, the error variation for the determinations in each case has been expressed on the basis of a mean of 100 throughout. The relatively high error variation of free elastase determinations is due probably to the uniformly low values obtained, so that a minute colour change is magnified and expressed as a high percentage of error.

J: DISCUSSION

A critical evaluation of this new colorimetric method for determining elastolytic activity in biological materials will be attempted later in the general discussion. However, the following points are of particular interest.

Several factors have been considered significant in attempting to demonstrate a linear relationship between enzyme concentrations of the material in question and the amount of substrate rendered soluble.

1. Pretreatment of elastin -Alkali-treated elastin, which was used as

substrate in this method, has been found to be superior to the acid-treated material; Loeven (385) demonstrated a linear elastolysis time curve which continued until about 95% of the alkali-treated elastin had been dissolved by elastoproteinase.

2. Constant pH, with high-ionic-strength buffers.- The Tris buffer (0.2 M, pH 8.8) employed here is considered suitable for the purpose of this study. Many workers have used buffer solutions of low ionic strength to obtain higher activities. Czerkawski (365) suggested that, rather than sacrifice reproducibility due to large fluctuations in pH, it would be better to use more strongly buffered reaction mixtures, with their unavoidable lowering of activity.

3. Presence of elastomucase.- Loeven (385) suggested that linearity in the elastolysis time curve is possible only in the presence of elastomucase, which, although itself elastolytically inert, can enhance the activity of the elastolytic component. The whole pancreatic extracts used in this study undoubtedly contain elastomucase.

4. Particle size of substrate.- The elastolytic reaction appears to proceed on the surface of the fibres or microfibrils and the reaction rate is dependent upon particle size (350). A very fine size was achieved by careful grinding in a mortar, during the preparation of the elastin--orcein substrate.

5. The lag phase.- It has been suggested that the slow initial phase of the elastolysis time curve makes it difficult to identify the rate of elastolysis with the classically defined "initial reaction velocity" (462, 462). The rate of elastolysis in the method used here is determined during the 10- to 40-minute reaction times, for this and other reasons previously discussed.

By strictly observing the above criteria, and by using a 5% dilution of whole pancreatic extract, it is thought that determination of elastolytic activity (expressed in units/g. tissue) closely approaches the type of reaction

that occurs between an enzyme and its soluble substrate. As for the incubation time -- which might be considered relatively short by some authors (462, 463) -- irrespective of the time of incubation, the percentage of total hydrolysis rather than actual weight of elastin dissolved is assayed whenever colour release or other indirect measurements are compared with standard curves.

# CHAPTER XI

## DETERMINATION OF ELASTASE IN NORMAL CANINE PANCREAS

### A: INTRODUCTION

It has been mentioned (Chapter VI, C, 1) that relatively few data are available concerning the amount of elastase present in fresh pancreas of animals or man. It has been shown (375) that the normal elastase activity (elastase units, E.U.) in rabbit pancreas amounts to about 29 E.U./g. tissue, and in the rat it has been cited as about 193 E.U./g. (393) and 75 E.U./g. (362). A wide range of values for pancreatic elastase in various animals has been found and is said to be highest in the rat and lowest in the ox (382). However, because of differences in methods for elastase determination it is difficult to compare values. The influence of corticosteroids on pancreatic elastase activity in the rat was studied by Marrama et al. (496); they found that administration of small doses of cortisone and prednisolone decreased the enzyme activity, but larger doses of prednisone increased the yield of elastase.

The determination of proelastase and of free and total elastase in the pancreas of normal mongrel dogs is of great significance in this study, for the subsequent appreciation of changes in the proenzyme and free enzyme in experimentally induced pancreatitis.

### B: ANATOMY OF CANINE PANCREAS

A brief description of the anatomy of the pancreas in the dog will follow.

The pancreas is a well-defined structure (497). V-shaped, it consists of two long branches which meet at an acute angle behind the pylorus (this section will be referred to as 'the body'). The right branch (or 'head') is

enclosed by the mesoduodenum and extends backwards above the first part of the duodenum, below the caudate lobe of the liver and the right kidney, ending usually a short distance behind the latter. The left branch (or 'tail') passes to the left and backwards between the surface of the stomach and the transverse colon, ending at the anterior pole of the left kidney. Usually there are two pancreatic ducts: the smaller enters the second part of the duodenum by itself or in association with the common bile duct; the larger main duct opens into the bowel 3-5 cm. caudally to the first duct (498).

C: MATERIALS AND METHODS

Eighteen male and female normal mongrel dogs, each weighing 10-18 kg., were used. They fasted overnight and were killed by intravenous injection of 20 ml. 6% pentobarbital. The abdomen was opened immediately and the pancreas was removed; it was divided into the three parts (head, body, and tail) and placed at once on dry ice. Enzymatic determinations were carried out soon thereafter. One gramme of tissue was taken from each of the three parts of the frozen pancreas, and determinations were carried out separately for free elastase, total elastase, and proelastase, with the method previously described (Chapter X).

D: RESULTS

The values obtained in the pancreas of the 18 dogs are expressed as means $\pm$ S.E., for free elastase, proelastase, and total elastase, in the three anatomical parts of the organ (Table 1).

An analysis of variance showed that no significant difference exists between zones, for all values obtained; however, the difference was highly significant from one dog to another for proelastase and for total-elastase values ($P < 0.001$).

**Table 1**

**FREE ELASTASE, PROELASTASE AND TOTAL ELASTASE IN NORMAL CANINE PANCREAS :**

**MEAN VALUES FROM 18 DOGS, UNITS/Gm. TISSUE ± S.E.**

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 4.1 ± 0.89 | 6.15 ± 1.27 | 6.24 ± 1.28 | – |
| **Proelastase** | 104.99 ± 18.30 | 110.14 ± 19.12 | 108.18 ± 18.29 | – |
| **Total Elastase** | 109.09 ± 18.81 | 116.30 ± 19.01 | 114.42 ± 18.39 | – |

– No significant difference between zones ($P > 0.05$)

## E: EVALUATION OF RESULTS AND DISCUSSION

In this part of the study it has been shown (Table 1) that the negligible amounts of free elastolytic activity that can be detected in normal dog pancreas possibly can be accounted for by the error inherent to the method, which was found to be substantial (42.03%) for free elastase (Chapter X). On the other hand, the biochemical error for proelastase was much smaller (13.53%).

It can be seen that dog pancreas contains considerable amounts of the proenzyme which, in some of the animals, was as high as 240 units/g. tissue. The most striking phenomenon, however, was the variation in proelastase content per gramme of pancreatic tissue from one dog to another.

The fact that elastase is an enzyme with a dual nature may possibly influence determinations of proelastase content in that the activity of elastoproteinase may be enhanced by up to 100% by the elastomucase present in the pancreatic extract (345, 347, 359). The ratio of the elastolytic to the mucolytic elastase component in normal dog pancreas in unknown, and variations in it may substantially influence quantitative determinations of elastolytic activity in the extracts of a given pancreas. Furthermore, it is possible that there may be subtle variations in the amount of pancreatic elastase inhibitor in different dogs which also may influence determination of elastolytic activity in the pancreatic extracts. In most of the dogs tested, however, ample stores of proelastase existed, and it is interesting to investigate the fate of the proenzyme in the course of experimental pancreatitis.

# CHAPTER XII

## DETERMINATION OF ELASTASE IN NORMAL HUMAN PANCREAS

### A: INTRODUCTION

There has been some uncertainty as to the elastase content of human pancreas. Some authors (371, 383) have reported finding the enzyme in human pancreatic extracts, but others (458) have found none. Since these investigators assayed for elastase and not for proelastase, it is conceivable that any activity detected would be very small indeed. However, Lamy and Tauber (431), who were unable to detect any proelastase in the pancreas of man and rabbit, demonstrated the proenzyme in hogs, rats, and goosefish. They attributed the lack of elastase activity in man to the presence of a potent pancreatic elastase inhibitor or to the inability of bovine trypsin to activate human proelastase. It is noteworthy that considerable elastase activity has been found recently in the pancreatic secretion of a man with an external pancreatic fistula, (267).

The determination of elastase activity in extracts of human pancreas is fraught with difficulties. Post-mortem tissue specimens probably are unsuitable if obtained several hours after death, because of early autolysis and subsequent bacterial contamination, and surgical specimens from a normal human pancreas are rarely available. However, in the course of this study, fresh pancreatic tissue was obtained during operation or immediately after the death of kidney homotransplantation donors; in the latter case the organ could be totally removed a few minutes after the donor's death.

### B: MATERIALS

1. Pancreatic tissue. - Three surgical specimens consisting of the tail of the pancreas, and the whole organ from six kidney homotransplantation

donors, were obtained. All were placed in dry ice immediately after their removal from the body, and elastase determinations were performed soon thereafter: when only the pancreatic tail was available, one sample of tissue was taken and immediately processed for enzyme determinations; when the whole pancreas was removed, separate tissue samples were obtained from the head, body, and tail, and elastase activity was determined in three separate extracts.

2. Crystalline trypsin. - (Parenzymol; F.W. Horner, Ltd., Montreal) was used for activation of proelastase.

C: METHODS

Free elastase, total elastase and proelastase were determined with the colorimetric method, using an elastin--orcein substrate, as described in Chapter X, E.

D: RESULTS

The average free and total elastase and proelastase content in the three surgical specimens from the tail of the pancreas were as follows

- Free elastase 2.80 units/g. tissue
- Total elastase 13.53 " " "
- Proelastase 10.73 " " "

The values for the six pancreases obtained from kidney homotransplantation donors are shown in Table 2. An analysis of variance for the values obtained from the three anatomical parts of the pancreas showed no significant difference ($P > 0.05$).

E: EVALUATION OF RESULTS AND DISCUSSION

In this part of the study it has been shown that, under the conditions of this method, small amounts of elastolytic activity were found in fresh human pancreas. The highest values obtained for proelastase were in the range

**Table 2**

## FREE ELASTASE, PROELASTASE AND TOTAL ELASTASE IN NORMAL HUMAN PANCREAS

## MEAN VALUES FROM SIX SUBJECTS, UNITS/$G_m$TISSUE ± S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 7.97 ± 1.47 | 6.83 ± 1.17 | 7.73 ± 2.48 | – |
| **Proelastase** | 0.77 ± 2.59 | 3.0 ± 2.72 | 3.67 ± 2.14 | – |
| **Total Elastase** | 8.73 ± 1.65 | 9.83 ± 2.96 | 11.40 ± 2.35 | – |

– No significant difference between zones ($P > 0.05$)

of 18 units/g. tissue in one of the surgical specimens and 14.8 units/g. in tissue from a kidney homotransplantation donor; these subjects were 37 and 38 years old, respectively. The low values for proelastase could be related to the following factors.

The surgical material obtained in the course of partial pancreatectomy was subjected to a great deal manual handling and its blood supply was compromised; the state of relative or complete anoxia may have existed in the part removed for 90 minutes or longer. The effect of anoxia, mechanical manipulation and general anaesthesia on the zymogen content of pancreas is unknown but certainly cannot be beneficial. On the other hand, the kidney homotransplantation donors usually were comatose for about a week before death, a condition marked by hypotension, respiratory failure, and superimposed infection, e.g., pneumonia, requiring a multiplicity of medications. The effect of anoxia, hypotension, negative nitrogen balance, concomitant infection and of extensive drug therapy on the pancreatic zymogens cannot be assessed.

Other possible factors to explain the low elastase activity of human pancreas under the conditions of this study are the possibility that a potent elastase inhibitor exists in the human pancreas or that human trypsin has a specific ability to activate human proelastase, which is lacking in bovine trypsin (431). It is true that activation of human proelastase by bovine trypsin has been achieved by others (267), in pancreatic juice from a pancreatic fistula, but here again it may be that human trypsinogen is first activated into active trypsin, followed by proelastase activation. One might suggest that enterokinase may be a good activator, although this enzyme requires an optimum pH of 5.8 and a long incubation time, both of which are at variance with optimal conditions for elastase determination. Long incubation times (10-12 hours) give low values for elastase activity because of autohydrolysis of this enzyme

(461 - 463). It seems that the addition of an excess amount of human crystalline trypsin should be the ideal way to obtain quantitative data concerning the proelastase content of human pancreas. Therefore, the results of this study concerning the determination of proelastase in pancreas of man under the conditions of the method employed must be considered inconclusive.

# CHAPTER XIII

## INTRAPANCREATIC ELASTOLYTIC ACTIVITY IN BILE-INDUCED ACUTE EXPERIMENTAL PANCREATITIS IN DOGS

### A: INTRODUCTION

The inability of trypsin and chymotrypsin to digest elastic tissue has been discussed in Chapter VI, C, 8; and the specificity of elastase in effecting dissolution of the insoluble protein of elastic fibres and its ability to induce acute haemorrhagic pancreatitis when injected into the pancreatic duct of dogs have been dealt with in Chapter VI, C, 7 and Chapter VIII respectively. It has been shown (Chapter XI, C, 2) that ample stores of proelastase exist in normal canine pancreas; no studies of the fate of the proenzyme during experimental pancreatitis have been published.

It is possible in parenchymatous necrosis, that proelastase may be activated with resultant release of free elastase activity in the pancreatic parenchyma. Such a mechanism could explain the vascular injury and the progression of pathology in acute pancreatitis. Data reported in this chapter concern the release of intrapancreatic elastase activity in bile-induced pancreatitis and the inhibitory activity of pancreatic extracts, on an elastase/elastin--orcein system *in vitro*.

### B: MATERIALS AND METHODS

1. Bile

10 ml. bile was obtained by puncture of each dog's gall bladder.

2. Elastase

Twice-crystallized pancreatic elastase was purchased from Worthington Biochemical Corp., Freehold, N.J., for use in the studies of the pancreatic elastase inhibitor *in vitro*. The enzyme has an activity (elastase units, E.U.) of 962.5 E.U./ml. (35 E.U./mg. protein; 27.5 mg. protein/ml. of suspension).

3. Experimental Animals

Thirty-four mongrel dogs of both sexes (weight, 10-18 kg.) were anaesthetized, and pancreatitis was induced. The dogs were divided into three groups, according to the duration of pancreatitis.

Group I: 9 dogs; killed at five minutes.
Group II: 17 dogs; killed at 60 minutes.
Group III: 8 dogs; killed at 3 hours.

4. Induction of Pancreatitis

The dogs, which had fasted for 20 hours, were anaesthetized with an intravenous injection of 6% sodium pentobarbital, 1 ml./5 lb. body-weight. Using sterile technique, the abdomen was opened and approximately 10 ml. bile was aspirated via a 22-gauge needle from the gall bladder. The second part of the duodenum was opened by a longitudinal incision, approximately 8 cm. long, on the antimesenteric border. The contents of the bowel were swabbed off and the opening of the main pancreatic duct was identified. The duct was cannulated with a polyethylene tube (size, PE 50/S 12) that was connected to the syringe that contained the aspirated bile. The tube was passed 8-10 cm. into the duct and bile (0.5 ml./kg. body-weight) was injected under 250 mm. Hg pressure monitored by a modified mercury manometer. The polyethylene tube was removed and the intestinal wall was sutured. At 5 minutes (Group I), 60 minutes (Group II), and 3 hours (Group III), the dogs were killed by the intravenous injection of 20 ml. 6% sodium pentobarbital.

5. Classification of gross pathology

Immediately after the dogs were killed, the pancreas was removed and inspected; the gross pathology was graded from 0 to 4 according to the degree of oedema, fat necrosis and haemorrhage. After grading the pancreas was prepared for enzyme and dry weight determinations and histological studies.

6. Enzyme Assays

Samples of pancreatic tissue, weighing approximately 10 g. were taken from each of the three portions (head, body, and tail) and immersed in dry ice for enzyme studies. Quantitative determinations of free and total elastase and proelastase in the three parts were carried out by the colorimetric method described in Chapter X. Identical determinations were made in ascitic fluid obtained from dogs with 60-minute or 3-hour pancreatitis immediately before they were killed. The ascitic fluid (1 ml., 5% dilution) was treated exactly the same way as the equivalent amount of pancreatic extract.

7. Determination of Dry Weight

Samples of pancreatic tissue weighing approximately 1 g. were obtained from the three parts of each pancreas and from immediately adjacent to sites from which tissue for enzyme studies was taken; they were dried for 24 hours at 100°C. and then re-weighed. The dry weight corresponding to 1 g. fresh pancreatic tissue was calculated in this way.

8. Staining Procedures

Preparation of tissue - Samples taken from the involved part of the pancreas, corresponding approximately to Grade 3 changes, were preserved in Bouin's fixative for histological studies. They were dehydrated and embedded in paraffin. Sections at different levels from each of the blocks were stained with Verhoeff's haematoxylin alone or in combination with Masson's trichrome.

Verhoeff's elastin stain (499) was used, which stains elastic fibres (brilliant blue-black), nuclei (blue to brownish black), collagen (red), other tissue elements (yellow). The elastic fibres are stained by the iron--haematoxylin--iodine component of this solution. The stain gives excellent results when fresh solutions are used, and even the smallest fibres are clearly visible under high power. Visual appreciation is enhanced by the sharp contrast in colour,

vivid colour transparencies can be obtained, and the slides are said to be unusually durable (414).

A combination of Verhoeff's elastin (499) and Masson's trichrome (500) stain was used also, as suggested by Clermont (456); by this means both elastic fibres and collagen tissue are clearly and distinctly visualized in the same sections. This combination stains nuclei (brown), elastic fibres (black), collagen (green), and cytoplasm (orange red).

9. Inhibition Studies

The elastolytic activity of 15 units of twice-crystallized pancreatic elastase on 25 mg. of elastin--orcein substrate was determined in three series of duplicate tubes designated A, B, and C. The A series contained the enzyme--substrate mixture, with Tris buffer (pH 8.8), in a total volume of 5 ml. (used as the blank). The B series contained enzyme--substrate mixture, 1 ml. 5% extract from the least-involved part of the pancreas (head), and Tris buffer, in a total volume of 5 ml. The C series contained the same amount of enzyme and substrate in the same total volume, with 1 ml. 5% pancreatic extract from the most-involved part of the pancreas (tail). Aliquots from the same primary homogenized extracts, which were used for enzyme determinations in the head and tail, were used in the B and C series, respectively. The degree of inhibition in the series B and C was calculated against the blank and expressed as a percentage by application of a formula (Chapter VI, D, 3) given by Loeven (438), which is the same as that used for the serum elastase inhibitor.

## C: EXPERIMENTS AND RESULTS

1. Grading of Macroscopic Changes

In almost all cases (5, 30-minute and 3-hour pancreatitis) the pancreas showed grade 3-4 pancreatitis in the tail (left branch), grade 1-2 in the body (central part) and grade 0-1 in the head (right branch).

2. Enzyme Assays

Group I: 5-minute bile pancreatitis. The 27 tissue samples which were analyzed were obtained from the head, body and tail of the pancreas of 9 dogs in which bile pancreatitis of five-minutes' duration had been induced. Mean values for free elastase, proelastase and total elastase (units/g. tissue $\pm$ S.E.) are given in Table 3. No significant difference in free elastase content was observed between the least involved (head) and the most-involved part (tail) of the pancreas, although a significant difference for total elastase and proelastase values ($P < 0.001$) was found.

Group II: 60-minute bile pancreatitis. The 51 tissue samples which were analysed were obtained from the head, body and tail of the pancreas of 17 dogs in which bile pancreatitis of 60-minutes' duration had been induced. Mean values $\pm$ S.E. (units/g. tissue) for free elastase, proelastase and total elastase are given in Table 4. No significant difference in free elastase content was found between head and tail of the pancreas; a significant difference ($P < 0.01$) was found, however, for proelastase and total elastase.

Group III: 3-hour bile pancreatitis. The 24 tissue samples which were analyzed were obtained from the head, body and tail of the pancreas of 8 dogs in which bile pancreatitis of 3-hours' duration had been induced. Mean values $\pm$ S.E. (units/g. tissue) for free elastase, proelastase and total elastase are shown in Table 5. There was a significant difference in free elastase content between the most-involved part (tail) and the least-involved part (head), ($P < 0.05$). Also significant was the difference between head and tail for proelastase and total elastase ($P < 0.01$).

3. Effect of Dry-Weight Changes on Proelastase Determinations

When it became evident that the proelastase content is significantly reduced in the involved part of the organ in acute bile pancreatitis, it was considered necessary to establish whether this decrease was true or only apparent

Table 3

## ELASTOLYTIC ACTIVITY IN THE PANCREAS OF 9 DOGS WITH BILE-INDUCED PANCREATITIS OF 5 MINUTES DURATION : MEAN VALUES IN UNITS/Gm. TISSUE ± S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 4.93 ± 1.68 | 10.72 ± 1.50 | 7.04 ± 1.10 | – |
| **Proelastase** | 130.09 ± 24.24 | 89.39 ± 20.99 | 33.58 ± 17.10 | + |
| **Total Elastase** | 135.02 ± 23.76 | 99.48 ± 21.80 | 38.27 ± 17.58 | + |

– No significant difference in head vs. tail ($P > 0.05$)

+ Significant difference ($P < 0.05$)

**Table 4**

## ELASTOLYTIC ACTIVITY IN THE PANCREAS OF 17 DOGS WITH BILE PANCREATITIS OF 60 MINUTES DURATION : MEAN VALUES IN UNITS/Gm. TISSUE ± S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 3.18 ± 1.03 | 4.04 ± 1.09 | 2.86 ± 0.76 | – |
| **Proelastase** | 75.15 ± 16.62 | 50.70 ± 16.84 | 24.72 ± 11.50 | + |
| **Total Elastase** | 83.25 ± 18.41 | 48.99 ± 16.74 | 29.29 ± 9.98 | + |

– No significant difference in head vs. tail ($P > 0.05$)

+ Significant difference ($P < 0.05$)

Table 5

ELASTOLYTIC ACTIVITY IN THE PANCREAS OF 8 DOGS WITH BILE-INDUCED PANCREATITIS OF 3 HOURS DURATION : MEAN VALUES IN UNITS/Gm. TISSUE ± S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 6.1 ± 1.2 | 6.3 ± 1.2 | 11.3 ± 1.0 | + |
| **Proelastase** | 92.61 ± 27.80 | 66.40 ± 25.72 | 2.38 ± 3.59 | + |
| **Total Elastase** | 98.70 ± 27.31 | 72.72 ± 24.21 | 13.70 ± 1.06 | + |

+ Significant difference between zones ($P < 0.05$)

due to dilution of the proenzyme in the increased bulk of oedematous and haemorrhagic tissues. The dry weight produced by 1 g. wet weight in the three zones of the pancreas was determined in 9 dogs with 5-minute, 13 dogs with 60-minute, and 8 dogs with 3-hour pancreatitis. A significant difference in dry weight between the least-involved (head) and the most-involved part (tail) ($P < 0.05$) occurred in all groups, but covariance analysis with the corresponding values of proelastase revealed that the latter values maintain their significant difference in all three types. Therefore, the reduction in proelastase content in the most-involved part in experimental bile pancreatitis cannot be attributed to concomitant oedema and dilution by tissue fluids and blood, but is due to an actual decrease in the amount of proenzyme.

4. Inhibitory Activity of Pancreatic Extracts on Elastase in Vitro

The inhibitory activity of 60 pancreatic extracts obtained from the least-involved (head) and the most-involved part (tail), of the pancreas was determined for 26 dogs: 9 with 5-minute, 9 with 60-minute, and 8 with 3-hour pancreatitis. The degree of inhibition for head and tail, calculated as % inhibition against the blank, is shown in Table 6. Extracts of pancreas in which pancreatitis had been induced for 5 and for 60 minutes possessed little inhibitory activity, whereas those in which pancreatitis had been induced for 3 hours yielded slightly higher values. A moderate decrease in percentage inhibition was found with extracts from the most involved part in 60-minute pancreatitis. The reverse was true with extracts from 5-minute and 3-hour pancreatitis. However, an analysis of variance between the values for inhibitor activity of extracts obtained from head and tail showed no significant difference ($P > 0.05$) in all three types of bile pancreatitis.

5. Ascitic Fluid

Six samples of ascitic fluid were analyzed. No free or total elastase

**Table 6**

## THE INHIBITORY ACTIVITY OF PANCREATIC EXTRACTS ON ELASTASE IN VITRO : MEAN INHIBITION % FOR HEAD AND TAIL OF PANCREAS IN 30 DOGS WITH BILE PANCREATITIS

| | No. of Dogs | Head | Tail | Significant Difference |
|---|---|---|---|---|
| **Five Minutes** | 9 | 0.6 | 2.1 | – |
| **Sixty Minutes** | 9 | 6.0 | 3.0 | – |
| **Three Hours** | 8 | 13.8 | 12.8 | – |

– No significant difference between zones ($P > 0.05$)

activity was found in any sample of 60-minute (3 dogs) or 3-hour (3 dogs) pancreatitis.

6. Effect of Bile

The effect of canine bile on proelastase and the elastin--orcein substrate was tested in a series of in vitro experiments. It was shown that bile does not activate or destroy proelastase, does not interfere with its activation by trypsin and does not inhibit or initiate elastolysis.

7. Pathology

Five Minutes

Gross appearance:- Immediately after the injection of bile, profound swelling, streaky yellowish in appearance, was observed in the tail and part of the body of the pancreas, and during the next five minutes the involved area became fairly well delineated and areas of dark brown discolouration appeared. The pancreatitis usually involved the tail and part or all of the body, whereas the head showed slight or no involvement.

Microscopical features:- The parenchyma exhibited focal coagulation necrosis, with marked oedema of the septa and no inflammatory reaction. The blood vessels were dilated and engorged. The arteries were well maintained, whereas the veins showed a modest focal loss of definition and of staining of their elastic fibres.

Sixty Minutes

Gross appearance:- The pancreas was engorged, appearing brownish-black, with a friable and mushy consistency. Blackish areas resembling blood clot could be seen at times in the most-involved part (tail). The body was swollen and soft and the head relatively intact. Small amounts of ascites were seen.

Microscopical features:- There was some margination and emigration of polymorphonuclear cells. The arteries were well maintained, although there was

some loss of elastic tissue in their adventitiae. The changes in the veins were more pronounced, occasional veins showing complete dissolution of elastic components over segments of varying extent. Thrombosis of medium sized and small veins. Minimal extravasation of red blood cells (Fig. 2 and 3).

Three Hours

Gross appearance:- This was almost the same as in the one-hour experiment, with a moderate amount of dark red ascites.

Microscopical features:- Interstitial oedema and vasodilatation persisted. The inflammatory response was still moderate. The internal elastic lamina of the arteries was well maintained, although there was wide-spread loss of elastic tissue in the adventitia. The changes in the veins were more pronounced than in the one-hour pancreatitis. Thrombosis of veins and minimal extravasation of red blood cells (Figure 4).

D: EVALUATION OF RESULTS AND DISCUSSION

In this part of the study it has been clearly demonstrated that in bile-induced pancreatitis in dogs a significant decrease in proelastase content/g. of tissue occurred in the most-involved part (tail) of the pancreas as compared with the least-involved part (head). Proelastase values in the body (central part) showed a moderate decrease. The lowest mean values for the proenzyme were always found in the most-involved part of the pancreas, in which Grade 3-4 changes were observed and tissue obtained from areas closely adjacent showed histological signs of elastolysis in the 60-minute and 3-hour pancreatitis. The decrease in dry weight/g. of tissue followed the same pattern, with the lowest mean values in the most-involved part of the organ, but covariance analysis with corresponding proelastase values showed that the decrease in the latter is real and cannot be attributed to dilution by oedematous fluids.

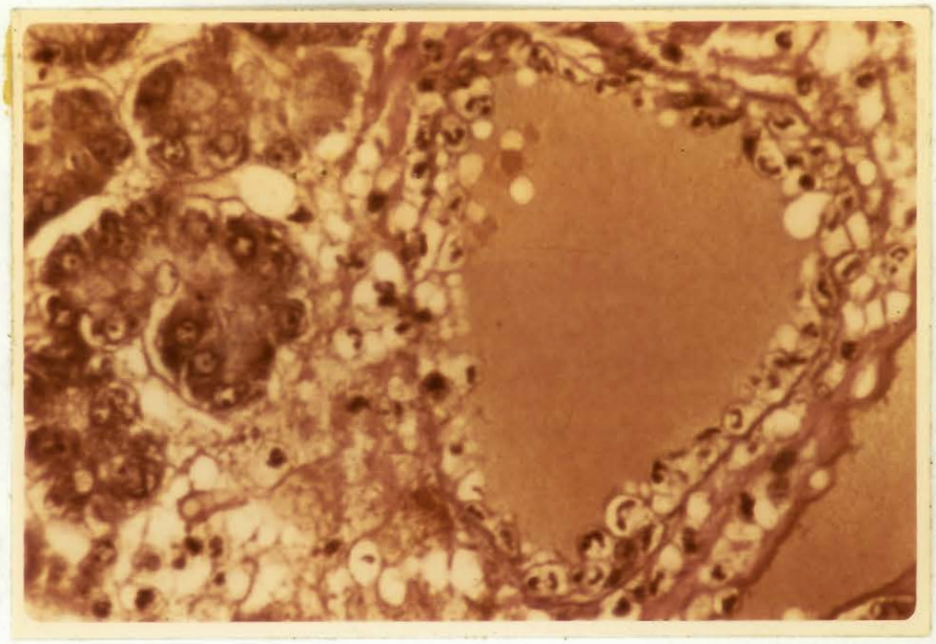

FIGURE 2: Bile Pancreatitis in 60 Minutes:
Venule showing segmental destruction of wall, adjacent to an area of parenchymal necrosis. Margination and early migration of polymorphs. (Verhoeff's H. x 250).

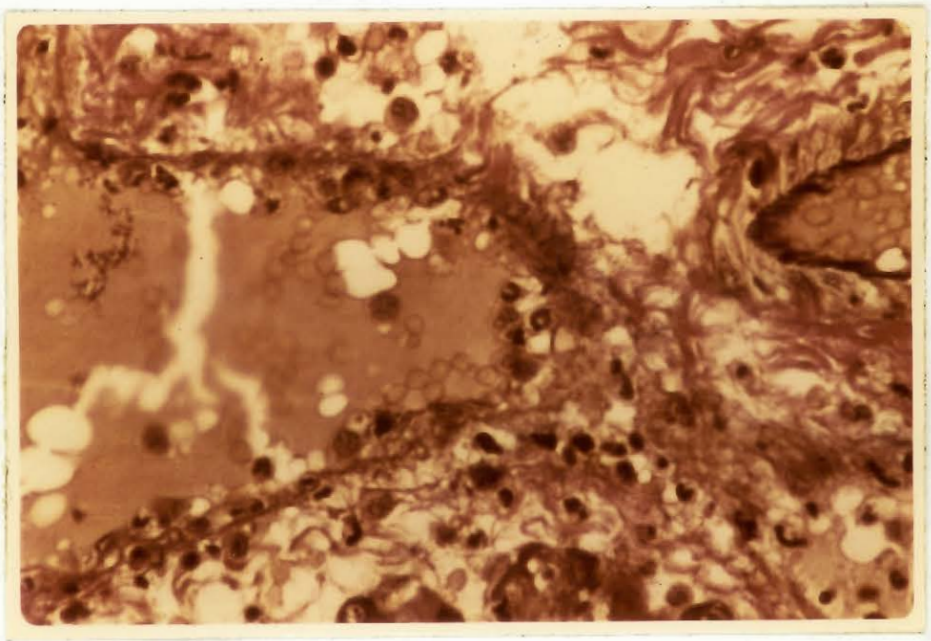

FIGURE 3: Bile Pancreatitis in 60 Minutes:
Venules showing disruption of wall over a short segment. Loss of definition of elastic tissue in venule and in adventitial coat of arteriole. Internal elastic lamina of arteriole well maintained. Early acute inflammatory changes (Verhoeff's H. x 250).

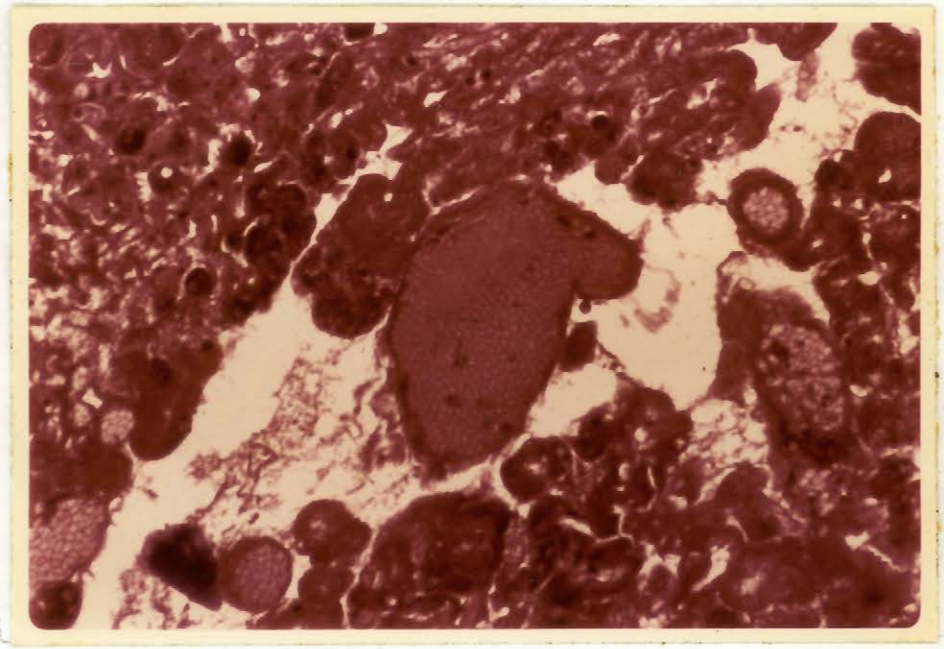

FIGURE 4: Bile Pancreatitis in 3 Hours.
Venule showing segmental loss of elastic tissue. Arteriole well maintained. Coagulation necrosis of parenchyma. (Verhoeff's H. x 200).

On the other hand, mean values of the free enzyme showed a significant difference between tail and head only in the 3-hour pancreatitis. Nevertheless, free elastolytic activity of extracts of the most-involved part of the pancreas in all three types of bile pancreatitis was low or negligible. The colorimetric method used for the determination of elastolytic activity - despite its specificity - is characterized by an error of 13.53% for proelastase and 42.03% for free elastase. However, the histological evidence of elastolysis in the involved tissue, consisting in loss of definition and staining power and dissolution of elastic fibres, attests to the local release of elastase, implying proelastase activation.

The mechanism of this activation in the course of bile pancreatitis is not clear; it is known that bile does not activate trypsinogen (115) and there is no reason to believe that it activates proelastase either. It may be that an intracellular enzyme (cathepsin B ?) liberated during bile-induced parenchymatous necrosis activates proelastase directly or, less probably, indirectly by activating small amounts of trypsinogen (315, 316). The details of such a mechanism, which required substantial shifts of intracellular pH, are not known, and no attempt was made in this study to delineate the mode of activation of proelastase in bile-induced parenchymatous necrosis. It was shown by the in vitro experiments that canine bile does not activate proelastase, does not affect its activation by trypsin, and does not inhibit or initiate elastolysis.

The fact that no increase in free elastolytic activity in pancreatic extracts from the involved part of the pancreas could be found is intriguing, and no attempt was made to investigate the ultimate fate of the enzyme except to demonstrate that no activity was demonstrable in the ascitic fluid that accumulated in 60-minute and 3-hour pancreatitis.

However, the following possibilities exist:

1. That part of the free enzyme enters the blood stream via the venous or lymphatic system.

2. That the enzyme combines with its pancreatic inhibitor in vivo and during homogenization and, therefore, cannot be detected. Also, the serum inhibitor from extravasated blood may contribute to this effect.

3. That almost all free elastase is bound to the elastic and other protein components of pancreatic parenchyma and cannot be found in supernatant fluid after centrifugation.

4. It may be that the part of the enzyme which is not adsorbed on its substrate is subject to autodigestion. The activity of elastase at 37° C. is said to decrease rapidly, with concomitant increase in free amino groups in the solution (365).

Despite the fact that the mechanism of proelastase activation and the fate of the enzyme in the course of bile pancreatitis are not clear, there is ample histological evidence of elastase activity, consisting in loss of staining power and dissolution of elastic fibres, accompanying the coagulation necrosis of the parenchyma. Gross interstitial bleeding does not occur, however, and thrombosis of small blood vessels prevails. It seems that medium-sized and small veins are the first vascular structures subject to injury. It is conceivable that destruction of vessel walls and thrombosis leads to anoxia and stasis, which propagate tissue necrosis. On the other hand, the inhibitory capacity of pancreatic extracts was small, a finding which is in agreement with data of other authors (432) and which indicates, possibly, that no substantial inhibition of elastolytic activity in vivo should be anticipated in the early stages of bile pancreatitis.

The enzymatic, histological and inhibition studies reported here strongly suggest an important role for elastase in the production of vascular injury and progressive tissue destruction in bile pancreatitis, and are in agreement with previous work in this laboratory that indicated the inability of a potent trypsin inhibitor from beef parotid gland (which does not inhibit elastolysis) to influence tissue destruction in this experimental disease (311).

# CHAPTER XIV

## INTRAPANCREATIC ELASTOLYTIC ACTIVITY IN TRYPSIN-INDUCED ACUTE EXPERIMENTAL PANCREATITIS IN DOGS

### A: INTRODUCTION

It has been shown that, if certain conditions concerning dosage and injection pressure are observed, the injection of trypsin into the pancreatic duct of dogs results in acute pancreatitis (45). It has been shown also that trypsin rapidly activates proelastase into free elastase (431) and thus may initiate release of elastolytic activity early in the course of trypsin-induced pancreatitis.

Quantitative determinations of proelastase and free elastase in trypsin-induced experimental pancreatitis and histological evidence (with elastica stains) of elastic-tissue destruction have never been published. In this part of the study, changes relating to free elastase and its proenzyme, as well as to the elastic-tissue in the walls of the intrapancreatic vessels, have been investigated; the inhibitory activity of pancreatic extracts on elastase in vitro will be reported also.

### B: MATERIALS

1. Trypsin - A crystalline trypsin preparation (Parenzymol; F.W. Horner, Ltd., Montreal) was used. Solutions were prepared with the manufacturers' solvent, which comprises 5% denatured gelatin, 0.09% methyl-paraben, and 0.01% propylparaben, in distilled water.

2. Twice-crystallized pancreatic elastase (Chapter XIII, B) was used in studying the inhibitory activity of pancreatic extracts.

3. Pancreatitis was induced under anaesthesia with pentobarbital sodium in 35 mongrel dogs of both sexes, weighing 10-18 kg., by injecting 50 mg. of a freshly prepared trypsin solution under 250 mm. Hg pressure into the pancreatic

duct. The dogs were divided into three groups, according to the duration of pancreatitis.

Group I: 14 dogs; killed at 5 minutes.

Group II: 17 dogs; killed at 60 minutes.

Group III: 4 dogs; killed at 3 hours.

C: METHODS

The induction of pancreatitis, grading of gross pathology, staining of histological sections, enzyme assays in pancreatic extracts and ascitic fluid, inhibition studies and dry weight determinations, were carried out in a way identical to that described for bile-induced pancreatitis (Chapter XIII)

D: EXPERIMENTS AND RESULTS

1. Grading of Macroscopic Changes

In all cases (5, 30 minutes and 3 hours pancreatitis) grade 3-4 pancreatitis developed in the tail (left branch) of the pancreas, grade 2-3 in the body (central part) and grade 0-1 in the head (right branch).

2. Enzyme Assays

a) Group I: 5-minute trypsin pancreatitis. The 42 tissue samples which were analyzed were taken from the head, body and tail of the pancreas of 14 dogs with trypsin-induced pancreatitis of 5 minutes' duration. Free elastase, total elastase and proelastase values, expressed in units/g. tissue $\pm$ S.E., are shown in Table 7. No significant difference in free elastase content was found between the least-involved (head) and the most-involved part (tail) of the pancreas. There was a significant difference, however, in the amount of proelastase and total elastase in the head and the tail ($P < 0.05$).

b) Group II: 60-minute trypsin pancreatitis. The 51 pancreatic tissue samples that were analyzed were obtained from the three parts of the pancreas of 17 dogs with trypsin pancreatitis of 60 minutes' duration. Mean values $\pm$ S.E.,

**Table 7**

## ELASTOLYTIC ACTIVITY IN THE PANCREAS OF 14 DOGS WITH TRYPSIN-INDUCED PANCREATITIS OF 5 MINUTES DURATION : MEAN VALUES IN UNITS/Gm. ± S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 9.93 ± 0.88 | 10.26 ± 1.00 | 10.49 ± 3.75 | – |
| **Proelastase** | 74.06 ± 22.50 | 69.73 ± 22.15 | 42.27 ± 16.50 | + |
| **Total Elastase** | 84.01 ± 22.56 | 79.27 ± 22.40 | 52.76 ± 16.61 | + |

– No significant difference in head vs. tail ($P > 0.05$)

+ Significant difference ($P < 0.05$)

in units/g. tissue, for free elastase, proelastase and total elastase are shown in Table 8. There was a significant difference in free elastase content between head and tail ($P < 0.05$) as well as for proelastase and total elastase ($P < 0.01$).

c) Group III: 3-hour trypsin pancreatitis. The 12 tissue samples were obtained from the three areas of pancreas of four dogs which had been killed three hours after the onset of trypsin-induced pancreatitis. Mean values $\pm$ S.E. in units/g. tissue for free elastase, proelastase and total elastase are shown in Table 9. In this group, a significant increase of free elastase was noted in the most-involved (tail) as compared with the least-involved part (head) of the pancreas. Significant changes in proelastase and total elastase content was observed in the tail vs. the head of the pancreas.

3. Effect of Dry-Weight Changes on Proelastase Determinations

Dry-weight determinations were carried out, with the method described for bile pancreatitis (Chapter XIII, C), to assess the significance of the decrease in proelastase content in the most-involved (tail) as compared with the least-involved part (head) of the pancreas. Covariance analysis of the dry weights for head, body and tail, and the corresponding values for proelastase content in the pancreas of 14 dogs in Group I, 13 dogs in Group II and 4 dogs in Group III, showed that the significant decrease in the proenzyme is real and not just apparent. The reduction in proelastase content in the tail cannot be explained only on the basis of dilution in oedematous tissues.

4. Inhibitory Activity of Pancreatic Extracts on Elastase in Vitro

The inhibitory activity of 60 pancreatic extracts obtained from the least-involved (head) and the most-involved part (tail) of the pancreas of 30 dogs was determined. The number of dogs with 5- and 60-minute and three-hour pancreatitis, and the degree of inhibition by extracts obtained from the head and tail, calculated in percentage of inhibition against the blank, are shown in Table 10. A moderate decrease in percentage inhibition was found with extracts

**Table 8**

## ELASTOLYTIC ACTIVITY IN THE PANCREAS OF 17 DOGS WITH TRYPSIN-INDUCED PANCREATITIS OF 60 MINUTES DURATION : MEAN VALUES, IN UNITS/Gm. S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 5.45 ± 1.05 | 5.04 ± 1.16 | 3.95 ± 1.98 | + |
| **Proelastase** | 82.86 ± 13.72 | 63.59 ± 12.22 | 37.80 ± 12.17 | + |
| **Total Elastase** | 99.78 ± 19.10 | 57.85 ± 12.12 | 34.16 ± 12.17 | + |

+ Significant difference in head vs. tail ($P < 0.05$)

Table 9

## ELASTOLYTIC ACTIVITY IN THE PANCREAS OF 4 DOGS WITH TRYPSIN-INDUCED PANCREATITIS OF 3 HOURS DURATION : MEAN VALUES, UNITS/Gm. ± S.E.

| | Head | Body | Tail | Significant Difference |
|---|---|---|---|---|
| **Free Elastase** | 6.9 ± 2.14 | 7.4 ± 1.36 | 17.85 ± 3.65 | + |
| **Proelastase** | 130.10 ± 45.51 | 106.25 ± 44.99 | 0.40 ± 4.73 | + |
| **Total Elastase** | 137.0 ± 45.94 | 113.6 ± 43.64 | 18.25 ± 7.97 | + |

+ Significant difference in head vs. tail ($P < 0.05$)

**Table 10**

## THE INHIBITORY ACTIVITY OF PANCREATIC EXTRACTS ON ELASTASE IN VITRO : MEAN OF PERCENTAGE INHIBITION FOR HEAD AND TAIL OF PANCREAS OF 30 DOGS WITH TRYPSIN PANCREATITIS

| | No. of Dogs | Head | Tail | Significant Difference |
|---|---|---|---|---|
| **Five Minutes** | 14 | 8.7 | 12.1 | – |
| **Sixty Minutes** | 12 | 9.4 | 6.0 | – |
| **Three Hours** | 4 | 13.7 | 15.7 | – |

– No significant difference between zones ($P > 0.05$)

from the most-involved part in 60-minute pancreatitis. The reverse was true with extracts from 5-minute and 3-hour pancreatitis. However, an analysis of variance between the values for inhibitory activity of head and tail showed no significant difference ($P > 0.05$) for all three durations of trypsin-induced pancreatitis.

5. Ascitic Fluid

Analysis was made of 4 samples of ascitic fluid, 2 from dogs with 60-minute and 2 from dogs with 3-hour pancreatitis. No elastase activity was found in any sample.

6. Effect of Crystalline Trypsin on the Elastin--Orcein Substrate

Increasing amounts of trypsin (0.5, 1, 2, 4 and 5 mg.) were added to a series of duplicate tubes, each of which contained 25 mg. of elastin--orcein substrate in Tris buffer (pH 8.8) in total volume 3 ml. No release of dye was observed after 40 minutes' incubation in a water-bath at 37°C. with intermittent shaking.

7. Pathology

Five Minutes

Gross appearance:- Immediately after the injection, pancreatic swelling was visible mainly in the tail and part of the body. In some areas a dark red discolouration occurred among areas of glassy oedema, but the head and part of the body remained normal. No plaques of fat necrosis or ascites were seen.

Microscopical features:- There was focal liquefaction necrosis, scattered foci of interstitial haemorrhage and widespread oedema of the interlobular septa. Small foci of fat necrosis were seen. The blood vessels were dilated and showed some margination of polymorphs. Small arteries were well maintained. There was some loss of definition and of staining of the elastic fibres in veins.

Sixty Minutes

Gross appearance:- The tail of the pancreas appeared swollen and mushy,

with wide blackish areas which resembled blood clot. The head of the organ was largely intact; the body was oedematous, with red streaks and small black areas of haemorrhage. A moderate amount of serosanguineous ascites was present.

Microscopical features:- The liquefaction necrosis was more extensive, as was the interstitial oedema and haemorrhage. The arteries showed loss of elastic tissue in the adventitia. The changes in the veins were more pronounced, with complete loss of elastic tissue and destruction of the vein wall. Intraluminal thrombosis of arteries and veins was seen (Fig. 5 and 6).

Three Hours

Gross appearance:- By this time, more bloody, brownish ascites had accumulated, and the involved part of the pancreas resembled a large blood clot, more or less sharply demarcated from uninvolved parts of the organ.

Microscopical features:- Marked interstitial haemorrhage was seen, with extensive liquefaction necrosis. The internal elastic lamina of small arteries appeared destroyed, and in some places the walls of companion veins had completely disappeared. 'Ghost' veins could be seen. Thrombosis was seen in arteries and veins alike (Fig. 7 and 8).

## E: EVALUATION OF RESULTS AND DISCUSSION

In this part of the study it has been shown that, in trypsin-induced acute pancreatitis in dogs, a significant decrease in proelastase content occurred in the most-involved (tail) as compared with the least-involved part (head) of the pancreas. A less pronounced decrease in the proenzyme was found in the body (central part). The lowest values for proelastase were always found in the tail of the pancreas, in which Grade 3-4 changes were regularly seen, and tissue sections from adjacent areas showed signs of vascular destruction, including elastolysis, in 60-minute and 3-hour pancreatitis. The decrease in dry weight/g. tissue followed the same pattern, with the lowest mean values found in the most-

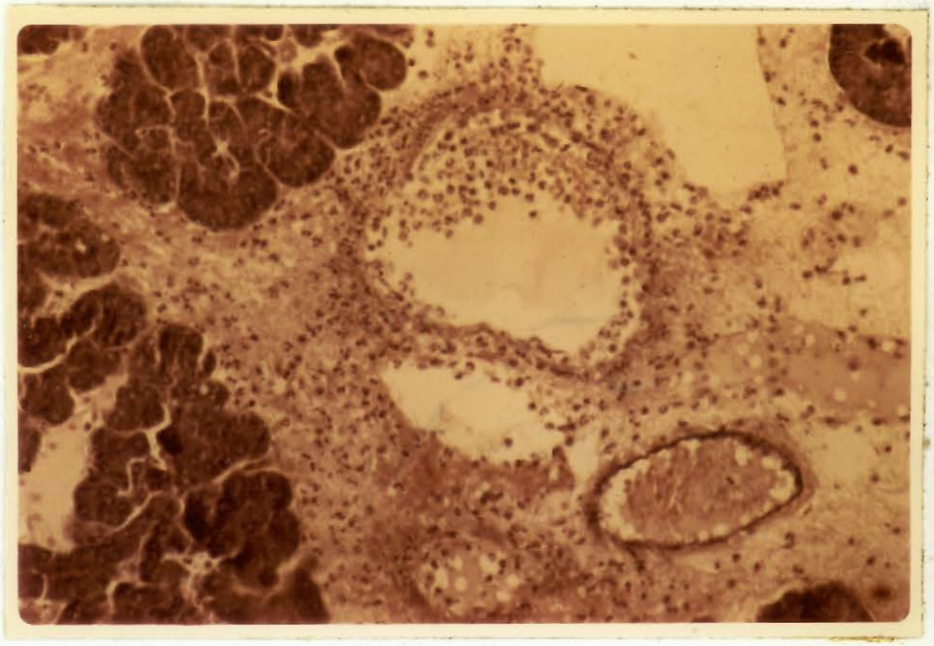

FIGURE 5: Trypsin Pancreatitis in 60 Minutes:
Widespread disappearance of elastic tissue in the walls of two venules, loss of elastic tissue in adventitia of neighbouring arteriole. Internal elastic lamina maintained. Interstitial edema with marked early acute inflammatory changes. (Verhoeff's H. x 100).

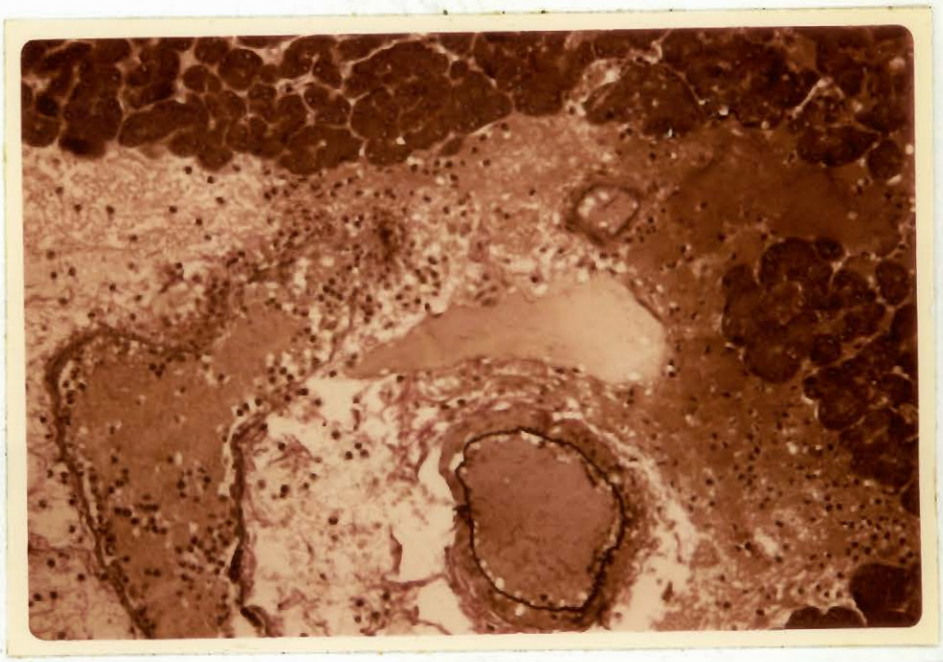

FIGURE 6: Trypsin Pancreatitis in 60 Minutes:
Disruption and destruction of wall of venule. Two neighbouring arterioles show some loss of elastic staining in adventitial and medial coat, but with sharply preserved internal elastic lamina. Interstitial edema and acute inflammation. (Verhoeff's H. x 250).

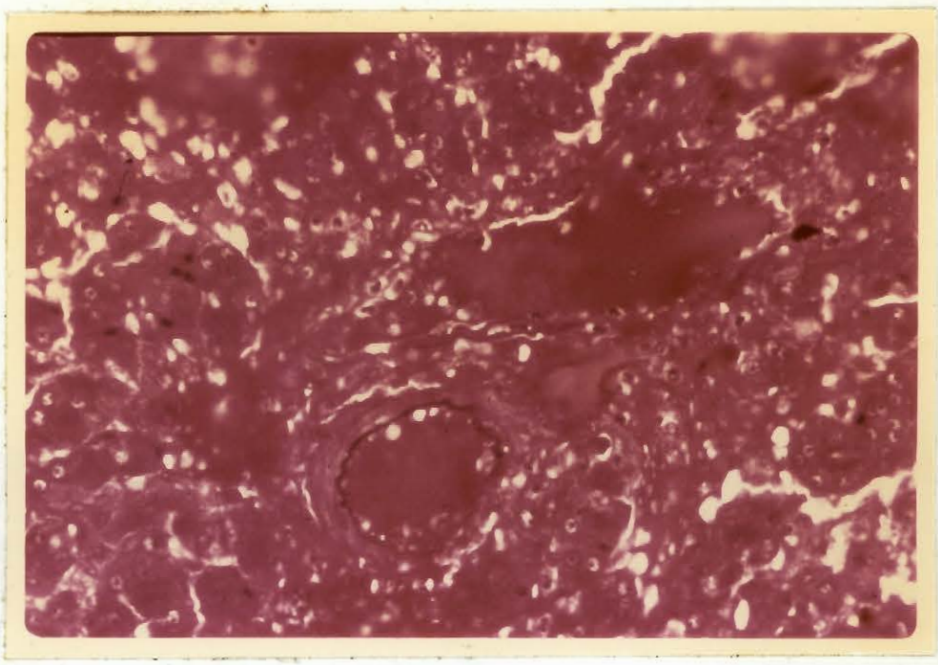

FIGURE 7: Trypsin Pancreatitis in 3 Hours:
Complete dissolution of wall of venule. Arteriole showing loss of elastic tissue in adventitia and media and in a broad segment of the internal elastic lamina. (Verhoeff's H. x 250).

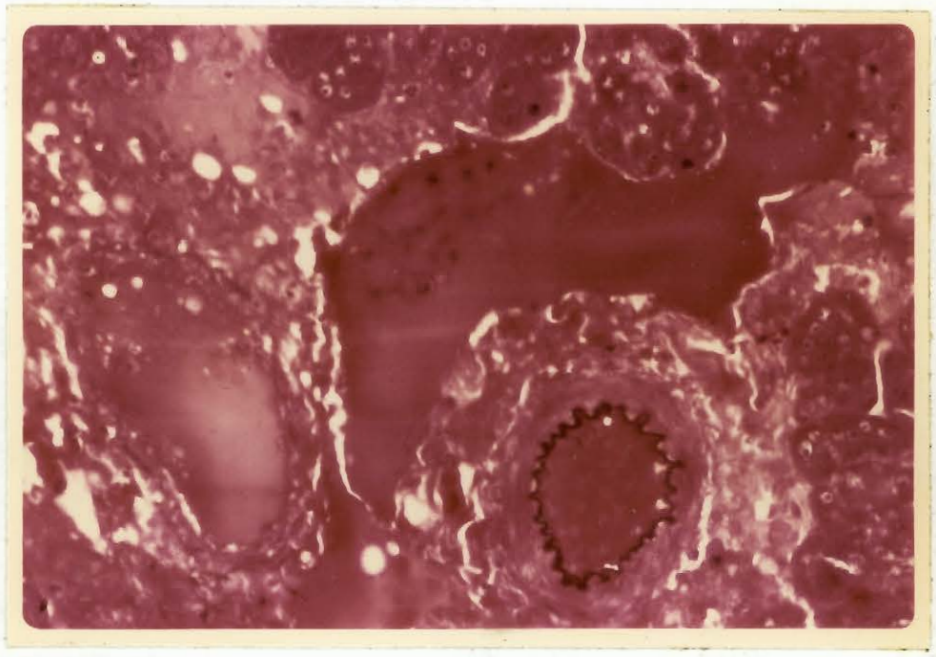

FIGURE 8: Trypsin Pancreatitis in 3 Hours:
Complete dissolution of elastic tissue in wall of venule and in adventitia and media of adjacent arteriole. Preservation of internal elastic lamina. Marked interstitial edema. (Verhoeff's H. x 250).

involved part of the organ, but covariance analysis with the corresponding proelastase values confirmed that changes are real and cannot be attributed to dilution. It appears that in bile- or trypsin-induced pancreatitis there is a progressive decrease of proelastase content which is proportional to the degree of involvement of pancreatic parenchyma and to the length of time since the induction of pancreatitis. It is possible that complete activation of the proenzyme in the most-involved part of the pancreas occurs between 60 and 180 minutes, since no measurable amounts of proelastase could be detected at the end of three hours. On the other hand, a slight increase in free elastase was found in the most-involved part of the pancreas only in 3-hour pancreatitis. Lack of substantial increase in free elastase levels in trypsin pancreatitis can be attributed possibly, to the same factors enumerated in the discussion of bile induced disease. No elastolytic activity was detected in the ascitic fluid of dogs with 60-minute or 3-hour pancreatitis. The inhibitory activity of pancreatic extracts was low, and no significant difference was found between the most-involved and the least-involved part in 5-, 60-minute and 3-hour disease.

Trypsin is the physiological activator of proelastase; it is known that elastase is not susceptible to trypsin, since it can be liberated without loss of activity by trypsin from its inactive proenzyme and from combination with serum inhibitor (450). The histological findings of severe necrosis of the walls of arteries and veins, including dissolution of elastic fibres, were more severe than in bile pancreatitis of equal duration. Here again the small veins appear to be subject to early destruction. The liquefaction necrosis produced by trypsin is characterized by profound interstitial bleeding, in contrast with bile-induced coagulation necrosis where bleeding is minimal. The evidence suggests that proelastase is activated by trypsin <u>in vivo</u>, with resultant severe vascular injury, including destruction of arteries and veins, with

dissolution of elastic tissue, interstitial haemorrhage and thrombosis.

In attempting to explain the severity of vascular injury in trypsin- as contrasted to bile-induced pancreatitis, the following points may be of significance: it has been shown that the activity of elastase in vitro can be enhanced by the addition of trypsin in the reaction mixture, and the inhibitory action of serum on elastase may be completely reversed by the action of trypsin in a stoicheiometric manner (450).

# CHAPTER XV

## ABSENCE OF ELASTOLYSIS IN THE CANINE MANDIBULAR GLAND IN BILE- OR TRYPSIN-INDUCED PARENCHYMATOUS NECROSIS

### A: INTRODUCTION

It has been mentioned in Chapter VI, C, 1 that elastased activity has never been found in any site other than the pancreas, with one exception, the aortic wall; this was found to contain a polysaccharide fraction that was capable of releasing dye from a stained elastin preparation (381). This fact, coupled with the specificity of elastase in effecting dissolution of elastic fibres at physiological pH, initiated this part of the study, which reports induction of parenchymatous necrosis in the mandibular gland of the dog by intraductal injection of autologous bile or trypsin. In this organ, which does not contain elastase, any vascular injury that might accompany parenchymatous necrosis should not include dissolution of elastic tissue, or even alterations in its staining characteristics.

Other authors (11) have injected trypsin solution or pancreatic juice into the subcutaneous tissue of dogs, with resultant oedema and haemorrhage at the site of injection, a finding which now has been attributed to elastase activity (45). On the other hand, gall-bladder bile injected subcutaneously produced oedema but no haemorrhage (11).

In this chapter the lack of elastolysis in the mandibular gland of dogs, in the presence of parenchymatous necrosis induced by the injection of bile or trypsin, and the inhibitory activity of extracts from normal and from necrotic mandibular tissue on an elastase/elastin--orcein system *in vitro* will be reported.

### B: MATERIALS

Bile - 5 ml. autologous bile was obtained by puncture of the dog's own gall

bladder.

Trypsin - 25 mg. crystalline trypsin (the same preparation as was used for inducing trypsin pancreatitis, Chapter XIV, B, 1) dissolved into 5 ml. of the manufacturer's solvent was used.

Experimental animals - Nine mongrel dogs of both sexes, each weighing 10-18 kg., were anaesthetized by intravenous administration of sodium pentobarbital (1 ml. 6% solution/5 lb. body-weight). Parenchymatous necrosis of the right mandibular gland was induced by intraductal injection of bile or trypsin.

Elastase - 15 units of twice-crystallized pancreatic elastase (Worthington Biochemical Corp., Freehold, N.J.) was used for determination of inhibitory activity of mandibular tissue extracts from normal (left) and necrotic (right) glands.

## C: METHODS

### 1. Enzyme Assays

Determinations of proelastase and free and total elastase were carried out with the same colorimetric method used for the pancreatic tissue studies (Chapter XI, E.). Three 5% extracts were used: (a) from the normal left gland, and (b) and (c) from two adjacent parts of the necrotic (right) mandibular gland.

### 2. Inhibition Studies

The inhibitory activity of mandibular gland extracts on an elastase/elastin--orcein system *in vitro* was determined with the method outlined in Chapter XIII, B, 9. The three sets of duplicate tubes were designated (a) blank, (b) extract of normal (left) gland, and (c) extract of necrotic (right) gland. Aliquots from the same primary extracts used for enzyme determinations were utilized for the study of inhibitory activity.

3. Staining

The combined stain of Verhoeff's haematoxylin and Masson's trichrome, as described in Chapter XIII, B, 8, was used for the histological sections.

4. Canine Mandibular Gland

Gross anatomy - The mandibular gland usually is larger than the parotid. In large dogs it is about 5 cm. long X 3 cm. wide. It is rounded in outline, pale yellow in colour, and is enclosed in a fibrous capsule. Its upper part is covered by the parotid gland, but otherwise, it is superficial, and is palpable in the angle of junction of the jugular and external maxillary veins. The mandibular duct leaves the deep face of the gland, passes along the surface of the occipito-mandibularis and styloglossus, and opens into the mouth on a very indistinct papilla near the frenum linguae (498).

Histology - The mandibular gland is similar to the pancreas in the general plan of construction but differs in its secretory functions. It is a mixed gland, delivering both serous and mucous secretion, because it contains a combination of serous and mucous units. The combination consists of mucous units capped by cresent shaped aggregations of serous cells called serous demilunes (half moons). In the serous cells the nuclei are rounded and situated near the bases of the cells; in mucous cells they are flattened and crowded against cell bases. Connective-tissue septa divide the gland into lobes and lobules. The interlobar ducts, from which the interlobular ducts branch off, entering the lobules, are situated among dense connective tissue in the interlobar spaces. An arteriole and two or more venules accompany the interlobar ducts. The elastic tissue in the walls of the vessels can be clearly visualized with an elastica stain.

5. Induction of Parenchymatous Necrosis

In bile-induced necrosis of the mandibular gland, first the abdomen

of the anaesthetized dog was opened and 5 ml. bile was aspirated by puncturing the gall bladder; after the aspiration of bile the abdomen was sutured. In trypsin-induced parenchymatous necrosis a 5 ml. freshly prepared trypsin solution was used. The outer surface of the right mandibular gland was exposed by dividing the skin, subcutaneous muscle, and covering fascia. The anterior surface of the gland was identified and separated from the adjacent sublingual gland. The mandibular duct was separated from the surrounding connective tissue and cannulated with a polyethylene tube (size PE 50/S 12) which was secured in place with double silk ligature when it had been passed about 3-4 cm. into the duct. Five ml. of autologous bile or of 25 mg. crystalline-trypsin solution was injected, under 250 mm. Hg pressure; this was monitored with the modified mercury manometer that was used for injection of the pancreas (Chapter XIII, B, 4). After injection the tube was removed.

Autologous bile was injected into the right mandibular gland of five dogs; three of these were killed (by intravenous injection of 20 ml. 6% sodium pentobarbital), at 5 minutes, one at 60 minutes, and one at 3 hours. Trypsin was injected into the right mandibular gland of four dogs; one of these was killed at 5 minutes two at 60 minutes, and one at 3 hours. The gland was removed and sliced longitudinally and the gross appearance of the parenchyma was recorded. One half of the right gland was placed in Bouin's fixative for histological examination, and the other in dry ice. Subsequently, the normal (left) gland was removed and placed in dry ice. Enzyme and inhibition studies were made on extracts of both glands soon thereafter.

## D: RESULTS

### 1. Enzyme Determinations

No proelastase was found in extracts of normal or necrotic gland; the negligible amount of free elastolytic activity that was noted was well within the range of error of the colorimetric determination.

2. Inhibitory Activity of Mandibular-gland Extracts on Elastase in Vitro

Normal glands - Extracts of the normal mandibular glands of the 9 dogs showed a mean inhibitory activity of 96.6% on the elastase/elastin--orcein system. Necrotic glands - Extracts of the necrotic mandibular glands of the same 9 dogs showed a mean inhibitory activity of 93.7%. An analysis of variance showed a significant difference between the mean inhibitory activity of extracts from the normal and necrotic glands ($P < 0.05$). However, covariance analysis of the values for inhibitory activity and the dry weights of mandibular tissue was not carried out.

3. Pathology

Gross appearance:- Immediately after injection the gland was considerably swollen, to about twice normal size, in all cases. At five minutes the parenchyma appeared pale yellow but without haemorrhage after both bile or trypsin injection. At 60 minutes and three hours the gland was oedematous and softer than the normal with a small amount of brownish mucous containing fluid extruding from its surface. Here again there was a brown-yellow appearance of the parenchyma without gross evidence of haemorrhage.

Microscopical features:-

Five Minutes:- Necrosis of mucous and serous cells was seen after injection of bile; following trypsin injection, these cells appeared blurred, with frequent loss of nuclei. The interlobar and interlobular spaces appeared wider than normal, with the septa maintaining almost normal staining characteristics. No destruction of vessel walls was seen in bile- or trypsin-induced necrosis, and no elastolysis.

Sixty Minutes:- More pronounced necrosis of both types of parenchymatous cells (mucous and serous demilunes) occurred with marked oedema of the interlobar and interlobular spaces, which were filled with fibrinoid material.

The septa lost their staining power completely. The overall picture was that of coagulation necrosis, with loss of nuclei and preservation of cell outlines induced by the injection of bile. In trypsin-induced necrosis, there was disintegration of cells and slight infiltration by inflammatory cells, but the septa maintained almost normal staining capacity. In both bile- and trypsin-induced necrosis, however, there was no vascular injury and no evidence of elastolysis (Fig. 9 and 10).

Three Hours:- There was advanced coagulation necrosis of both cell types, widening of the septa, but still no vascular destruction and no elastolysis after injection of bile. Following trypsin injection, advanced parenchymat ous necrosis developed, with moderate inflammatory cell infiltration, widening of septa, and, again, no vascular destruction or elastolysis. There was no loss of staining power of elastic tissue (Fig. 11 and 12).

## E: EVALUATION OF RESULTS AND DISCUSSION

In this series of experiments it was shown that the intraductal injection of bile or trypsin in the mandibular gland of the dog produced parenchymatous necrosis of mucous and serum cells but no vascular destruction and no dissolution of elastic fibres. This finding is in striking contrast with changes in bile- or trypsin-induced pancreatitis of the same duration (60 minutes and 3 hours), in which vascular destruction and elastolysis regularly occurred. Two points are of particular interest -- the absence of elastase in the mandibular gland and the strong inhibitory activity of mandibular extracts against elastase *in vitro*. In these circumstances, even if elastase were present in mandibular tissue it is doubtful whether any degree of elastolytic activity would have developed. On the other hand, it has been shown that bile or trypsin alone is unable to effect vascular destruction. The strong

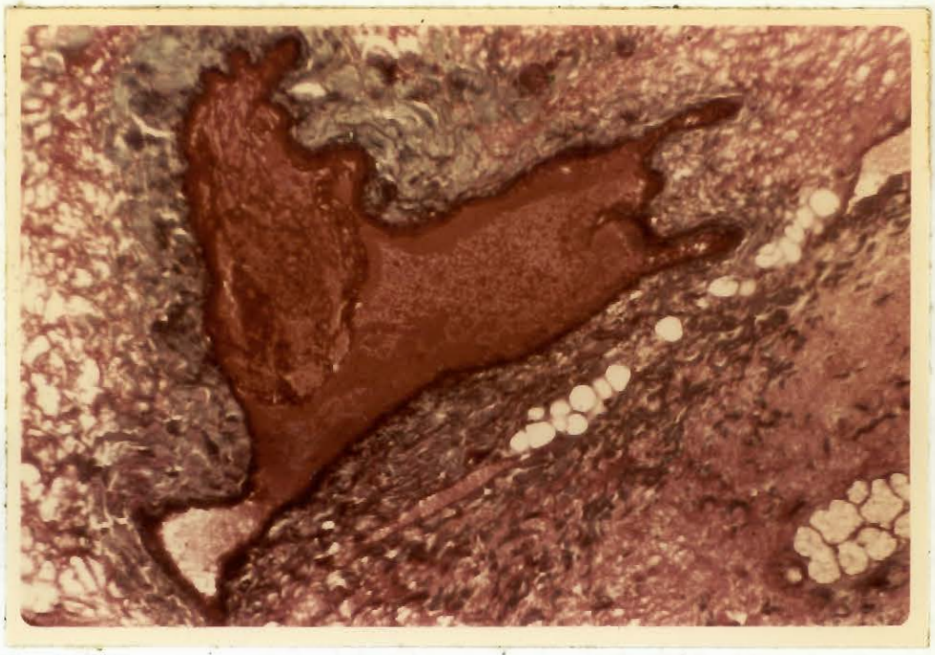

FIGURE 9: Mandibular Gland, Bile Induced Parenchymatous Necrosis in 60 Minutes: Medium size interlobar venule retains normal complement of elastic tissue. An island of mucus glands shows loss of nuclei, though cells persist as "ghosts". (Combined Verhoeff's H. and Masson's T. x 50).

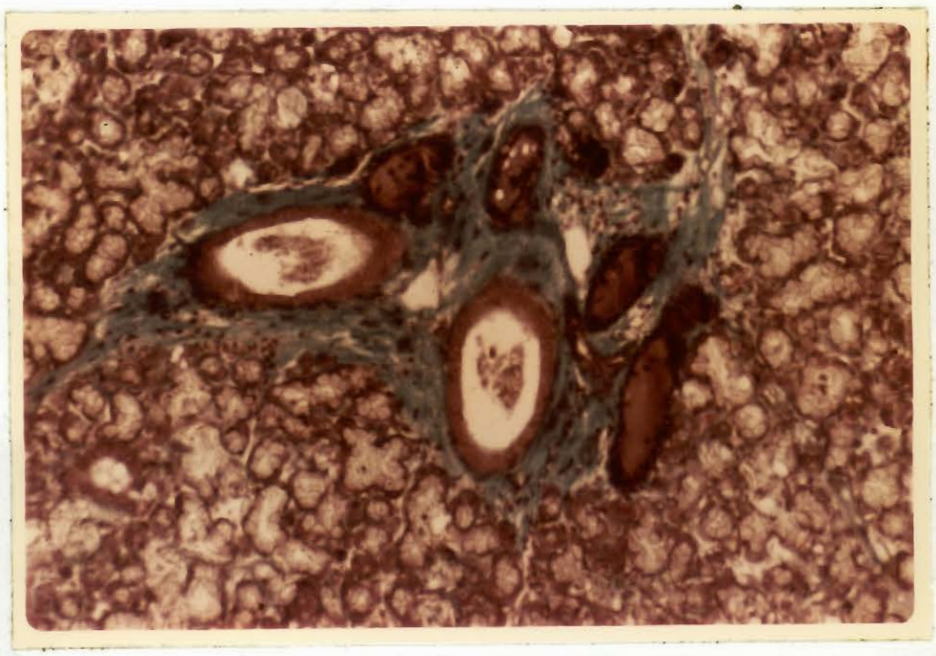

FIGURE 10: Mandibular Gland, Showing Trypsin Induced Parenchymatous Necrosis at 60 Minutes: Ducts well maintained; arterioles and venules retain full complement of elastic tissue; focal, early necrosis of individual acini or small groups of acini. (Combined Verhoeff's H. and Masson's T. x 50).

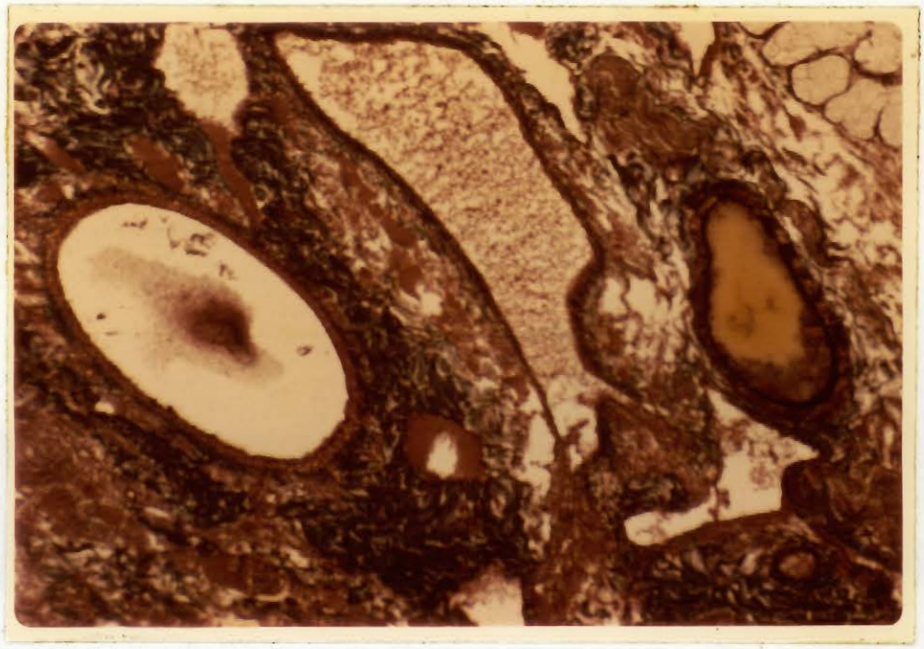

FIGURE 11: Mandibular Gland, Showing Bile Induced Parenchymatous Necrosis at 3 Hours: Elastic tissue of arteriole and venules is well maintained; large duct intact; loss of nuclei in parenchyma; persistence of cell and acinar outlines. (Combined Verhoeff's H. and Masson's T. x 100).

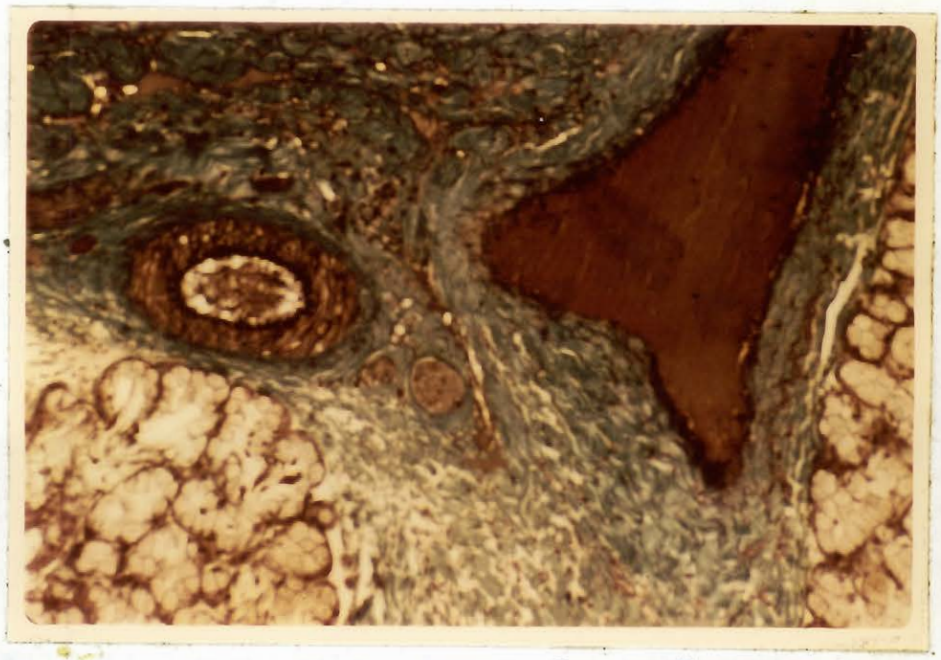

FIGURE 12: Mandibular Gland, Showing Trypsin Induced Parenchymatous Necrosis at 3 Hours: Arteriole and venule well maintained; parenchymal necrosis more advanced. Minor early inflammatory changes. (Combined Verhoeff's and Masson's T. x 100).

inhibition of elastolysis by extracts of mandibular gland, as contrasted with the minimal inhibition by pancreatic extracts on the same elastase/elastin--orcein system, possibly underlines the significance of the pancreatic elastase inhibitor in the pathogenesis of vascular injury in acute pancreatitis.

# CHAPTER XVI

## THE EFFECT OF ELASTASE ON PANCREATIC TISSUE SLICES

### A: INTRODUCTION

The use of the light microscope in the study of the effect of elastase on intact elastic tissue was introduced the same year as the discovery of the enzyme (337). It was found that when elastic tissue is exposed to elastase its staining capacity is reduced or totally absent, depending upon length of incubation with the enzyme. Others (12) have studied the effect of elastase on the arterial wall *in vitro* and by arterial perfusion *in vivo*. In this study the effect of twice-crystallized pancreatic elastase on the elastic tissue of the walls of arteries, veins and ducts of pancreatic tissue slices has been investigated. Histological evidence of the selective destruction of elastic fibres and the nonspecific proteolytic activity of elastase on the pancreatic parenchyma will be reported in this chapter.

### B: MATERIALS AND METHODS

#### 1. Elastase

Twice-crystallized pancreatic elastase (Worthington Biochemical Corp., Freehold, N.J.) prepared by the method of Lewis *et al.* (371) was used. The same preparation was employed in determinations of the inhibitory activity of pancreatic and mandibular-gland extracts (Chapters XIII, B, 9; XIV, B, 2; and XV, B, ) and for the induction of elastase pancreatitis (Chapter XVII, B, 1).

#### 2. Experimental Animals

Two mongrel dogs were killed by intravenous injection of 20 ml. 6% sodium pentobarbital. The abdomen was opened at once and the whole pancreas was removed, placed in crushed ice, and taken to the laboratory, where it was immediately sliced and processed for the incubation studies.

3. Incubation Technique

a) Preparation of tissue - Portions of the pancreas were placed on a petri dish filled with crushed ice. The pancreatic tissue was cut, using a Stadie - Riggs tissue slicer (483), and weighed on a torsion balance; slices weighing 148-240 mg. were placed in 25 ml. Erlenmeyer flasks containing appropriate media, as indicated below, and chilled in crushed ice. After addition of the slices, the vessels were gassed with $O_2$ for $2\frac{1}{2}$ minutes and then incubated at 37°C. in a shaker bath for the indicated period of time.

b) Types of Experiments -

Experiment No. 1. - The incubation media was a $Ca^{++}$-free Krebs - Ringer solution containing NaCl (145 mM), KCl (5.8 mM) $KH_2PO_4$ (1.4 mM), and $MgSO_4$ (1.4 mM). The medium was buffered at pH 8.8 with 140 mM Tris (hydroxymethyl-amino-methane) to final volume 3 ml.; incubation time was 40 minutes. The elastase content of the flasks was nil, 24.5 units, 77 units, and 210 units, in flasks labelled a, b, c and d, respectively. Experiment 2. - The incubation medium consisted of 275 mM isotonic sucrose in place of the Krebs - Ringer solution, 100 mM. Tris buffer (pH 8.8), 18 mM sodium malonate where indicated, and the following amounts of enzyme: nil, 24.5, 77, 210, 140 and 140 units + 18 mM Na malonate, in flasks labelled a, b, c, d, e and f, respectively. Final volume in all cases was 3 ml., and incubation time was 40 minutes. In both experiments the reaction was terminated by removing the flasks from the bath and cooling them in crushed ice. The slices were removed with forceps, placed in Bouin's fixative, and prepared for histological staining.

4. Staining

Verhoeff's haematoxylin was used for staining of tissue from experiment No. 1 (Chapter XIII, B, 8) and a combination of Verhoeff's and Masson's stains was employed in the tissue from experiment No. 2 (Chapter XIII, b, 8).

D: RESULTS

1. Experiment No. 1

Gross appearance

There was good preservation of tissue in the control slices. The incubation fluid was clear. There was increasing loosening of tissue and of turbidity of fluid in slices b, c and d.

Histology

a) Control - No elastolysis was seen. The arteries contained a thick, wavy, continuous internal elastic lamina, and finer discrete elastic fibres scattered throughout the media and adventitia. The veins contained one or more fine continuous elastic laminae in their walls, and there were small autolytic foci in the parenchyma, in the centre of the slices (Fig. 13).

b) 24.5 elastase units (E.U.)- The elastic tissue was maintained in the arterial and duct walls, but that of the veins showed loss of definition and of intensity of staining. There were areas of autolysis in the centre of the slices (Fig. 14).

c) 77 E.U. - Dissolution of elastic fibres and loss of staining power was observed. There was some autolysis in the centre and necrosis of the parenchyma in the periphery of the slices (Fig. 15).

d) 210 E.U. - No elastic tissue could be identified in the walls of arteries, veins, or ducts. Moderate parenchymal necrosis had occurred throughout (Fig. 16).

2. Experiment No. 2

Gross appearance

Good preservation of tissue was observed in the control slices and no turbidity was seen in the incubation fluid. Increasing turbidity of the medium and loosening of tissue was seen in slices b, c, d, e and f.

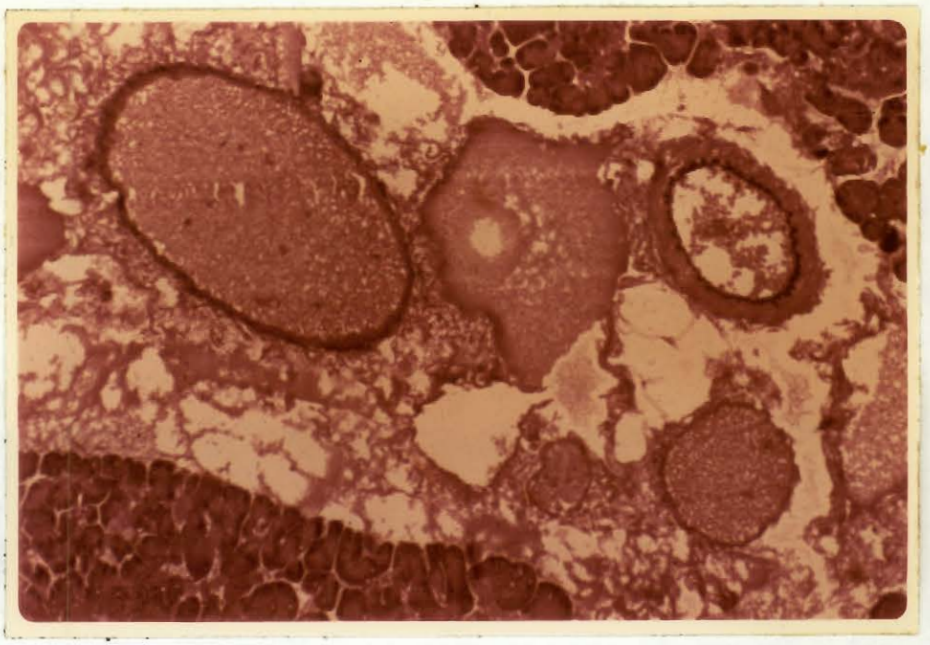

FIGURE 13: Dog Pancreas in Krebs-Ringer Solution and Tris Buffer at 37°C. for 40 Minutes: Arteriole and venules appear intact; they retain a sharply staining normal complement of elastic tissue. (Verhoeff's H. x 80).

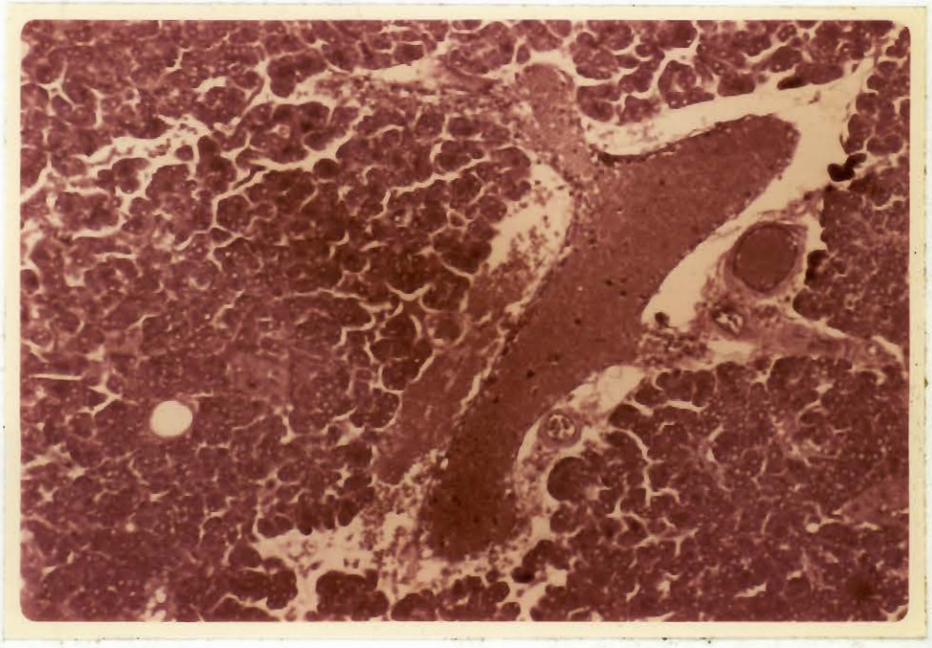

FIGURE 14: Dog Pancreas in 24.5 Units Elastase in Krebs-Ringer Solution and Tris Buffer at 37°C. for 40 Minutes: Arteriole showing loss of intensity of staining in internal elastic lamina. Venule showing widespread loss of definition and staining of elastic component. (Verhoeff's H. x 80).

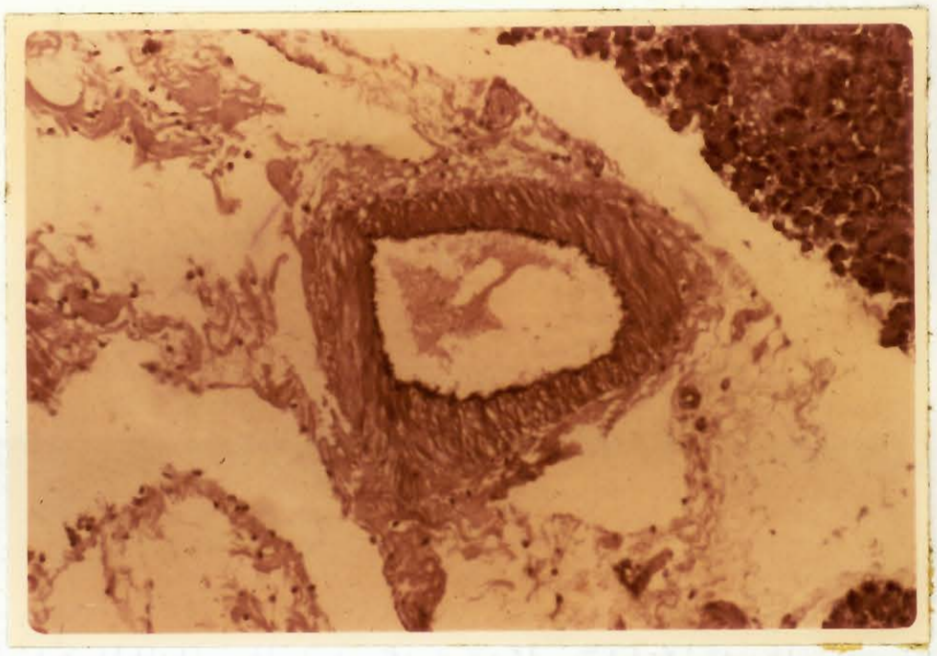

FIGURE 15: Dog Pancreas in 77 Units Elastase, in Krebs-Ringer Solution and Tris Buffer at 37°C. for 40 minutes: Arteriole showing loss of elastica in media and adventitia, with attenuation and focal disruption of internal elastic lamina. (Verhoeff's H. x 80).

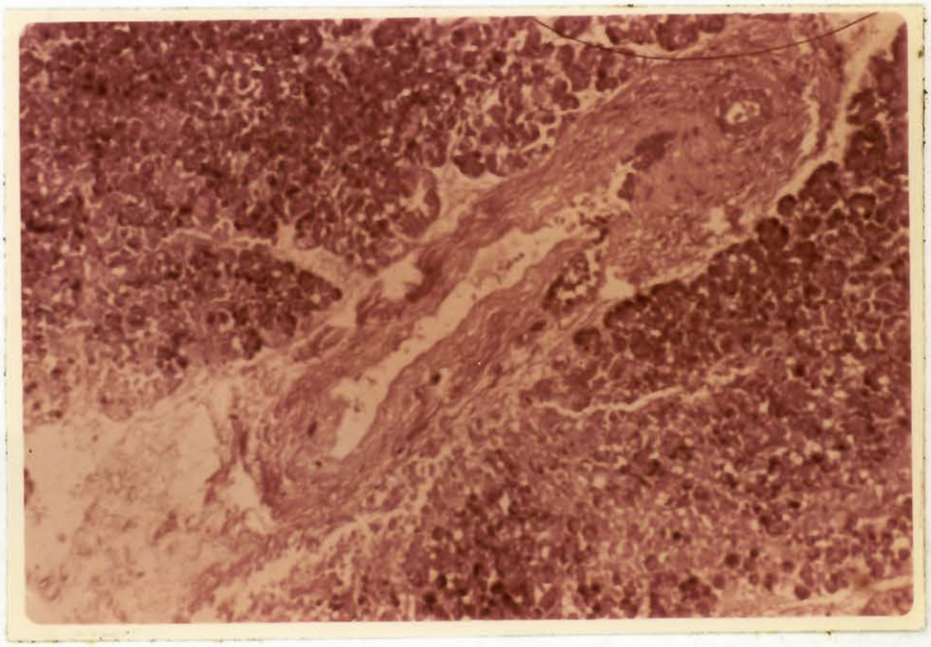

FIGURE 16: Dog Pancreas in 210 Units Elastase, in Krebs-Ringer Solution and Tris Buffer at 37°C. for 40 Minutes: Complete loss of elastic fibers in periductal connective tissue. Autolytic parenchymal changes. (Verhoeff's H. x 80).

Histology

a) Control - No elastolysis was seen in these sections. Minimal autolysis of pancreatic parenchyma was observed, however, in the centre of the lobules.

b) 24.5 E.U. - A moderate degree of necrosis of the parenchyma was seen, especially in the peripheral parts of the lobules, and there was a substantial degree of dissolution of elastic fibres, more pronounced than in sections (b) of experiment No. 1.

c) 77 E.U. - Necrosis of parenchyma and dissolution of elastic fibres had occurred in vessel and duct walls, with fragmentation of internal elastic lamina of arteries. The latter changes were more pronounced than in sections (c) of experiment No. 1.

d) 210 E.U. - Pronounced necrosis of pancreatic parenchyma and complete dissolution of elastic fibres were observed.

d) 140 E.U. - Dissolution of parenchyma, with almost complete digestion of elastic fibres had taken place. The walls of vessels and ducts were still surrounded by a rim of collagen tissue.

f) 140 E.U. + 200 mM sodium malonate - Advanced dissolution of parenchyma and complete digestion of elastic fibres had occurred. The walls of arteries, veins and ducts were completely digested.

D: EVALUATION OF RESULTS AND DISCUSSION

In this study, fresh pancreatic tissue slices were well oxygenated to secure respiration in the pancreatic cell - a prerequisite for preservation of its natural resistance to proteolytic activity and avoidance of the autolysis that follows disruption of the normal equilibrium between synthetic and catabolic activities (14).

In experiment No. 1, an attempt was made to maintain isotonicity, and a chemical environment as nearly physiological as possible on the one hand and an optimal pH for elastase activity on the other. It was realized, however, that some degree of elastase inhibition by the salts of the Ringer's solution was unavoidable. In experiment No. 2, sucrose solution was used to maintain isotonicity and to avoid the inhibitory effect of salts: this theoretical assumption was substantiated by the histological findings. The dissolution of elastic fibres seen in the slices incubated in media that contained sucrose were more advanced than were the changes seen in slices incubated in media that contained the same concentration of elastase (experiment No. 1). Also, the parenchymatous changes in the (f) slices in experiment No. 2, which contained sodium malonate, a potent inhibitor of succinic dehydrogenase, were much more severe than in (e), which contained the same concentration of elastase units but no inhibitor. Sections from slices (b) and (c) of experiment No. 1 showed changes closely resembling the vascular injury observed in sections from bile- or trypsin-induced pancreatitis.

In both experiments the elastolytic and nonspecific proteolytic activity of pancreatic elastase are evidenced by the selective destruction of elastic fibres and the dissolution of pancreatic cells. Some degree of inhibition of elastolysis by the salts of the Ringer's solution in experiment No. 1 and potentiation of cytolysis by sodium malonate in experiment No. 2 have been observed. On the basis of this study it is reasonable to assume that the release of elastase during bile- or trypsin-induced pancreatitis effects destruction of elastic tissue in the walls of arteries and veins and also contributes to some extent to the development of parenchymatous necrosis.

# CHAPTER XVII

## INTRAPANCREATIC ELASTOLYTIC AND NONSPECIFIC PROTEOLYTIC ACTIVITY IN ELASTASE-INDUCED ACUTE PANCREATITIS IN THE DOG

### A: INTRODUCTION

Acute haemorrhagic pancreatitis in dogs, induced by the intraductal injection of a solution of electrophoretically purified pancreatic elastase in a relatively large dose (75 mg. of protein = 12,000 elastase units), has been reported (12); the findings of these workers have been discussed in Chapter VIII. In fact that pancreatitis was induced by injection of a substantial dose of this enzyme, which is characterized by potent specific elastolytic and broad nonspecific proteolytic activities, should come as no surprise. The authors focussed their attention on the changes in arterial walls and, as far as small veins were concerned, reported only fresh intraluminal thrombi. In our study on bile- or trypsin-induced pancreatitis (Chapters XIII and XIV) it was shown that the medium-sized and small veins were the vascular structures that appeared to be most vulnerable to the action of elastase.

In this study, acute pancreatitis was induced in dogs by intraductal injection of a relatively small dose of pancreatic elastase; observations of the type of parenchymatous necrosis and of vascular injury will be reported.

### B: MATERIALS AND METHODS

#### 1. Elastase

Twice-crystallized pancreatic elastase (Chapter XIII, B, 2) was used, in a dose of 1925 E.U. in 10 ml. distilled water. The enzyme solution was kept at $0^{\circ}$ - $4^{\circ}$ C. and was warmed to near body temperature immediately before use.

#### 2. Experimental Animals

Two mongrel dogs weighing 15 kg. each were anaesthetized by the intra-

venous injection of 1 ml. 6% sodium pentobarbital solution per 5 lb. body-weight; pancreatitis was induced with the same technique as in the experiments in which bile or trypsin was injected. Injection pressure was monitored with the modified mercury manometer at 250 mm. Hg. Both animals were killed at 60 minutes by the intravenous administration of 20 ml. 6% pentobarbital solution. The pancreas was removed immediately, the macroscopic appearance graded from 0 to 4 (Chapter XIII, B, 4), and specimens corresponding to Grade-3 changes were obtained and placed in Bouin's fixative for histological studies.

3. Staining

Verhoeff's haematoxylin and Masson's trichrome stains were combined for the histological sections (Chapter XIII, B, 8).

C: RESULTS

Gross appearance

Immediately after the injection of elastase, the tail and part of the body showed oedema and small punctate haemorrhages. Gradually the pancreas became more congested, and confluent haemorrhages appeared on its surface, but the head of the organ remained intact. At the end of 60 minutes haemorrhagic pancreatitis had developed, accompanied by the accumulation of a small amount of serosanguineous ascites. When the pancreas was removed, the observed changes were graded as 0, 2 and 4 for the head, body and tail, in both cases.

Microscopical features

There was severe oedema and haemorrhage in the interlobular septa; in some places the haemorrhage was dissecting and destroying the parenchyma. Elastase-induced parenchymatous necrosis closely resembled the necrosis induced by trypsin at three hours. The blood vessels in the interlobar septa

and their intralobular branches were dilated and packed with sludged red cells; fresh thrombi could be seen in medium-sized veins. The inflammatory response was focal and minimal. All veins showed some alteration in their elastic component, varying from loss of definition and staining intensity to destruction and complete disappearance of broad segments of the vessel wall. The arteries showed loss of adventitial elastic tissue and in some cases there was loss of staining and destruction of the internal elastic lamina. The elastic tissue of the duct walls was relatively well maintained (Fig. 17, 18, 19 & 20).

D: EVALUATION OF RESULTS AND DISCUSSION

It has been shown that the injection of bile (Chapter XIII) or trypsin (Chapter XIV) into the pancreatic duct of dogs, at pressure of 250 mm. Hg, regularly induces acute pancreatitis, with resultant release of elastolytic activity in the involved part of the organ, as indicated by the reduction in proelastase content per g. tissue and histological evidence of elastolysis.

In this part of the study, pancreatic elastase itself, injected at the same pressure and in a relatively moderate dose (1925 E.U.), induced acute pancreatitis which was characterized by liquefaction necrosis of the parenchyma and vascular destruction which included dissolution of elastic fibres. Such an effect on the pancreatic tissue is the result of both the elastolytic and the nonspecific proteolytic activity of elastase. The degree of activation and release, if any, of other proteolytic enzymes during the process of elastase-induced pancreatitis is not known. However, of considerable significance is the fact that elastase alone produced at 60 minutes a pronounced parenchymatous and blood-vessel destruction and thrombosis which resembled trypsin-induced pancreatitis at three hours. The striking features of the elastase-induced

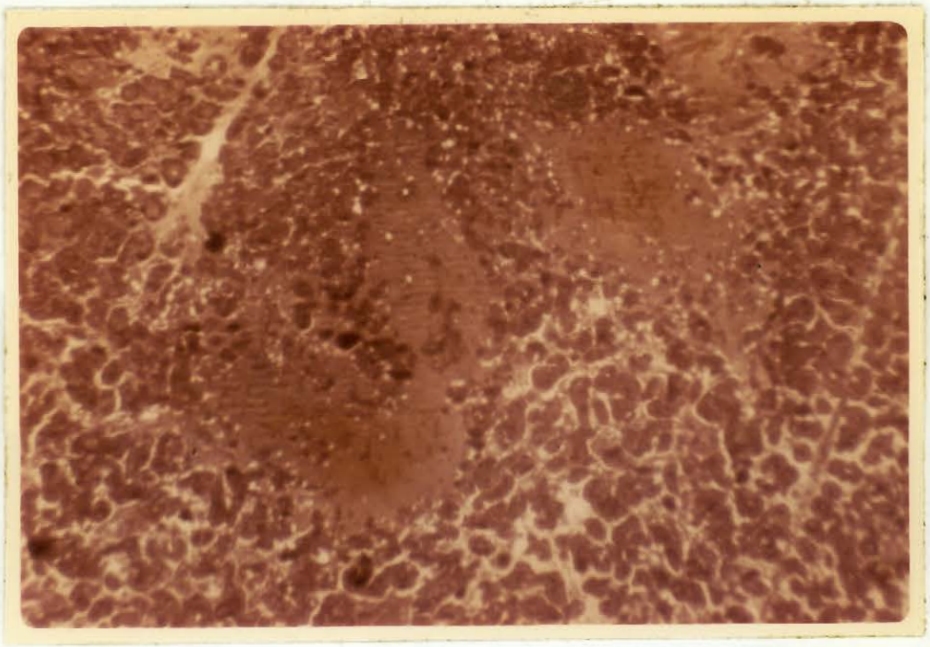

FIGURE 17: Elastase Pancreatitis in 60 Minutes: Focal hemorrhagic necrosis of parenchyma. (Combined Verhoeff's H. and Masson's T x 100).

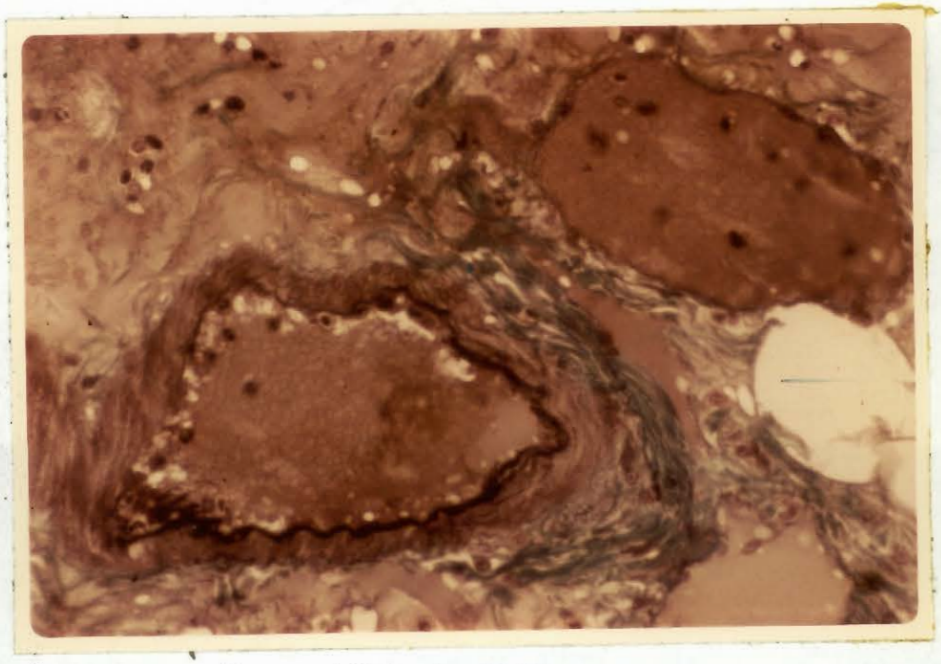

FIGURE 18: Elastase Pancreatitis in 60 Minutes: Arteriole showing segmental attenuation of internal elastic lamina, with complete disappearance in a restricted portion. Loss of elastic tissue in adventitia. Wall of venule shows lack of definition of elastic tissue with focal disruption. Minor early acute inflammatory changes. (Combined Verhoeff's H. and Masson's T. x 100).

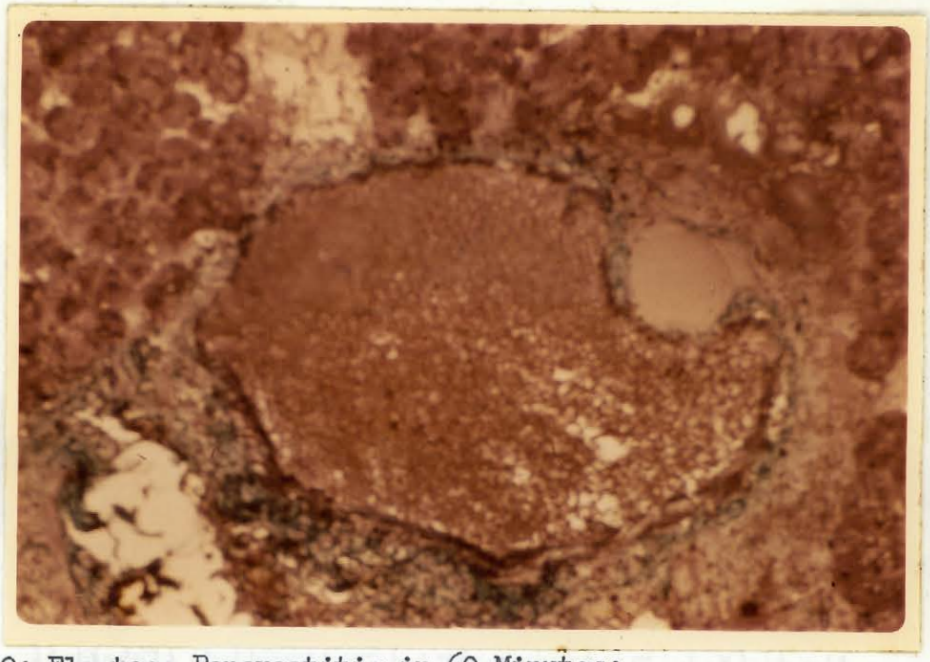

FIGURE 19: Elastase Pancreatitis in 60 Minutes:
Venule showing segmental attenuation of wall, with focal loss of staining and disruption of elastic component. Adjacent parenchyma showing early necrotic changes. (Combined Verhoeff's H. and Masson's T. x 100).

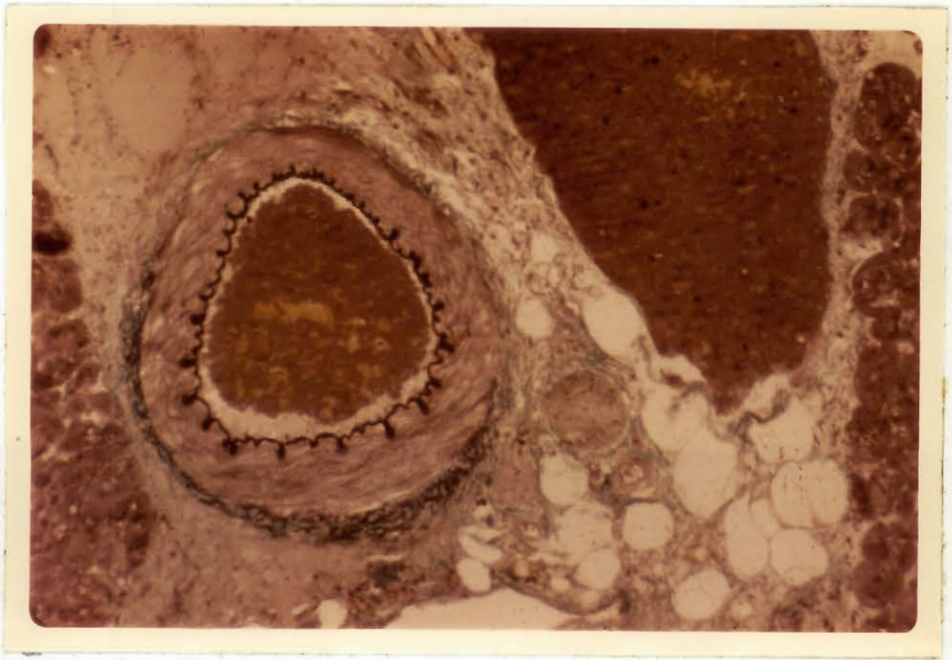

FIGURE 20: Elastase Pancreatitis in 60 Minutes:
Venule wall showing complete loss of elastic tissue. Arteriole showing a well maintained internal elastic lamina and segmental loss of elastic component of adventitia adjacent to involved venule. (Combined Verhoeff's H. and Masson's T. x 100).

pancreatitis were haemorrhage and oedema in the interstitial spaces, necrosis of the parenchyma, destruction of vessel walls, especially of medium sized and small veins, and thrombosis; the vascular lesions were also reminiscent of those seen in clinical haemorrhagic pancreatitis (31). These findings, coupled with results of the in vitro studies (Chapter XVI), suggest that pancreatic elastase plays an important, if not the major role, in production of the vascular injury that occurs in the acute experimental pancreatitis in dogs that results from injection of bile, or trypsin.

# CHAPTER XVIII

## G E N E R A L  D I S C U S S I O N

The release of inappropriate enzymatic activity into the pancreas constitutes the most plausible mechanism concerned in the pathogenesis of acute pancreatitis, and has been held responsible for both the parenchymatous necrosis and the distinct vascular injury, including thrombosis and destruction of blood vessels. The distributing intrapancreatic arteries and veins, up to their smallest ramifications, contain a modest amount of elastic tissue. The specificity of elastase in effecting digestion of elastic tissue and other proteins brings this particular enzyme, from the host of pancreatic peptidases, into sharp focus. However, exploration of the role of elastase in regard to the vascular injury of acute pancreatitis required assessment of the natural stores of its zymogen in the pancreas of the dog and investigation of the fate of the proenzyme and the behaviour of the free enzyme in the modified chemical environment of experimentally induced pancreatic inflammation and necrosis. Thus, the development of a reliable colorimetric method suitable for quantitative determination of proelastase and free and total elastase in pancreatic tissue extracts and other biological materials, and the definition of an appropriate elastase unit, were considered of great significance in this study. However, certain difficulties had to be overcome. The most important problem was the complexity of the elastase/elastin interaction (350, 364, 460, 461, 462), stemming from the fact that the initial slow phase of the elastolysis time curve (due to the adsorption of the enzyme on the substrate) is exactly the reverse of the phenomenon encountered in other enzyme systems, in which a small substrate molecule is adsorbed on to the surface of a colloidally dispersed enzyme. Preparation and particle size of elastin, and an optimal and

constant pH for elastase, secured with a suitable buffer, were most important. Therefore, strict criteria were established for determination of the rate of elastolysis between the 10- and 40-minute reaction times and in a relatively low enzyme concentration (with the 5% pancreatic extract), to achieve a linear elastolysis-time curve (349). It is felt that this objective was largely achieved by the method described. The error for proelastase and total-elastase estimations is relatively small, but that for free elastase is considerable, because of the small amounts measured and their considerable fluctuation in different parts of the same pancreas.

Colorimetric methods using a dyed elastin substrate have been criticized as representing a reaction far removed from normal conditions (349); it has been said that the points of attack of elastase on powdered elastin may be quite dissimilar from those of fresh tissue. On the other hand, in a molecule with so few polar groups as elastin, sites of dye attachment may be intimately related to those at which the enzyme becomes attached. It seems that staining of elastin with resorcinol-fuchsin, for example, inhibits elastase attack on whole tissue but not on powdered material. Another point of criticism is that, because of the dual nature of elastolysis (solubilization and homogeneous reaction), only a portion of the whole enzyme can be regarded as being available for the measurable solubilization reaction (384). Measurement of the release of amino groups as practised by Lamy et al. (364), which takes into account both of these reactions, simplifies the system in one respect but complicates it also, since the two reactions do not take place at equal rates (384). Furthermore, it has been said that estimations of elastolytic activity in whole-pancreas extracts apparently are higher than their elastase content would warrant, because of the synergistic effect of the mucolytic component (347). However, the colorimetric method used in this study has been found satisfactory

for determinations of the elastolytic activity of pancreatic extracts, not only of normal but of diseased tissue also. Excessive colour interference in extracts of pancreatic tissue is avoided to a great extent by the 30-minute assay (10- to 40-minute reaction time).

Whereas a significant variation in proelastase content was found to occur from one normal dog to another, no significant difference was observed in the three anatomical parts of the same pancreas, and negligible or no free elastase activity was detected. As far as is known, this is the first time that a systematic evaluation of the proelastase content of canine pancreas has been undertaken; no similar data exist in the literature.

Determinations of elastolytic activity in human pancreas obtained during surgery or from kidney homotransplantation donors immediately after death have been considered inconclusive for the reasons enumerated in the discussion in Chapter XII, E. The findings are to some extent in agreement with those of Lamy and Tauber (431), who found no activity in human pancreas but gave no details of how the tissue was obtained.

The identification of intrapancreatic elastolytic activity in the course of bile- or trypsin-induced pancreatitis at 5, 60 and 180 minutes in a sizable number of dogs was considered to be of great significance in this study.

The decreasing gradient in proelastase content in the three parts of canine pancreas, corresponding roughly to their degree of involvement, with the lowest values always observed in the most-involved (tail) portion, constitutes an important finding, especially when coupled with the histological evidence of dissolution of elastic fibres of vessel walls in the same area. This fact is in striking contrast with data obtained previously in the same laboratory, concerning the fate of trypsinogen in the course of bile or trypsin pancreatitis induced by the same method. No decreasing gradient (head --- tail) was found

for trypsinogen (115), a fact which indicates that the two zymogens, as determined in fresh pancreatic extracts, behave differently, at least in the initial stages of acute pancreatitis.

Despite the fact that a significant decrease in proelastase content was uniformly observed in extracts from the most-involved part as compared with the least-involved part of canine pancreas (at 5, 60 and 180 minutes) in bile- and trypsin-induced pancreatitis, a concomitant modest increase in free elastolytic activity was found only in the extracts from pancreatic tails of dogs suffering from trypsin pancreatitis of three hours' duration. On the other hand, no identifiable elastolytic activity could be found in the ascitic fluid which accumulated by 60 and 180 minutes in bile- or trypsin-induced disease. The several possibilities regarding the fate of free elastase after its release into the pancreatic parenchyma have been enumerated in Chapter VIII, D. It is possible that part of the enzyme may enter the general circulation via the venous or lymphatic systems but the determination of elastolytic activity in the serum or plasma has never been achieved, due possibly to the presence of powerful inhibitors for both elastase components (438, 439).

Solution of this problem must await refinements in methodology - elastase determination in blood, preferably from the portal vein, during experimental pancreatitis. It seems plausible also that part of the enzyme is adsorbed on the elastic tissue of vessel and duct walls and the proteinaceous component of pancreatic parenchyma and is not detectable in supernatant fluid after centrifugation. However, irrespective of the ultimate fate of the free enzyme, the dissolution of elastic fibres in the vessel walls that has been demonstrated with the elastica stains undoubtedly represents elastolysis subsequent to proelastase activation. The mechanism of activation of the

proenzyme in the necrotic parenchyma in bile pancreatitis is not clear: whereas bile does not activate trypsinogen (115) or proelastase, it may be that cathepsin-B liberated in the course of cytolysis is responsible for this effect (315, 316). It is noteworthy that any remaining proelastase which has been extracted from the tail of the pancreas is easily activated in vitro with an excess of crystalline trypsin during the enzymatic studies on tissue obtained from both bile- and trypsin-induced pancreatitis of 5 and 60 minutes' duration. However, extracts of the most-involved part of the pancreas from 3-hour bile- or trypsin-induced disease yield no measurable amounts of proelastase.

Another interesting finding relating to the activity of liberated elastase into the pancreas in the course of induced parenchymatous necrosis is the low inhibitory activity of pancreatic extracts from the head and tail of the organ, on the elastase/elastin--orcein system in vitro. This finding, which is in agreement with data from another laboratory (432), possibly implies that no substantial interference in intrapancreatic elastase activity released in the course of acute pancreatitis should be anticipated. A decrease of inhibitory activity in the most-involved part of the pancreas in bile- or trypsin-induced pancreatitis was not observed. In previous studies in this laboratory, it has been shown that the pancreatic trypsin inhibitor behaves similarly in canine bile-induced pancreatitis (115).

Intrapancreatic elastolysis followed a pattern of increasing severity (loss of staining power and definition in five-minute, and destruction of elastic tissue and vessel walls in 60-minute and 3-hour pancreatitis). The early vascular destruction and digestion of elastic fibres attests to the destructive capacity of elastase liberated in the interstitial tissues. The

vascular injury concomitant with the liquefaction necrosis of trypsin pancreatitis is more pronounced than that seen in bile-induced coagulation necrosis, despite the fact that elastolysis is observed in both; whereas thrombosis of vessels prevails in the latter, interstitial haemorrhage is the main characteristic of the former. This striking difference in regard to the vascular component of pancreatic necrosis is quite remarkable and supports earlier data from this laboratory (115) that have emphasized the dissimilarity of the histopathology in bile- and trypsin-induced disease under identical experimental conditions.

The role of elastase in the production of the distinct vascular injury of acute pancreatitis is further supported by the studies on parenchymatous necrosis of the mandibular gland of the dog induced by the injection of bile or trypsin. No elastolytic activity could be detected in extracts of normal or necrotic tissue and no signs of elastolysis were seen in histological sections.

An objective evaluation of the elastolytic and nonspecific proteolytic activities of a crystalline elastase preparation was obtained with the in vitro studies on pancreatic tissue slices. The severity of the selective destruction of elastic fibres and dissolution of parenchyma parallelled the concentration of elastase in the incubation media. More-pronounced changes in elastic tissue were observed when sucrose was substituted for salts, to maintain isotonicity, a finding related possibly to the inhibitory activity of the latter.

The ability of a modest dose of elastase to induce acute haemorrhagic pancreatitis when injected into the pancreatic duct of the dog has been tested, and the histological sections that show the effects of elastase in vivo have demonstrated its ability to effect vascular injury and parenchymatous destruc-

tion which is reminiscent of the liquefaction necrosis of trypsin pancreatitis. The fact that this preparation of elastase is electrophoretically inhomogeneous (371), approaching possibly the activity of fresh pancreatic tissue extracts, makes the observed changes more meaningful. The histological findings in elastase pancreatitis are comparable to those reported by Schneider et al. (12), who injected a larger dose of an electrophoretically purified preparation.

In attempting to establish a common denominator in the histopathological changes observed in bile-, trypsin- or elastase-induced pancreatitis, it seems that the dissolution of elastic fibres and the early destruction of medium-sized and small veins is the most striking finding. The method used in this study for the induction of acute pancreatitis, consisting of the intraductal injection of materials under relatively high pressure, has been shown to be very satisfactory; pancreatic necrosis was regularly produced in these and in previous studies (115). However, no attempt was made to duplicate the clinical picture of acute pancreatitis, but only to accelerate and magnify the progress of the disease, providing an experimental model suitable for enzymatic and histological studies. The available evidence strongly suggests an important role of pancreatic elastase in the pathogenesis of the vascular injury of acute experimental pancreatitis in dogs. It seems that vascular destruction and thrombosis (403) can easily be attributed to elastase, without incriminating a hyperimmune reaction (52) or unidentified toxins (39, 48, 49, 50-52). However, the role of elastase in effecting parenchymatous necrosis in the course of bile- or trypsin-induced pancreatitis cannot be assessed. On the other hand, as seen in the histological sections, the elastic tissue of vessel walls is closely associated with collagen, and the concomitant

destruction of this component in acute pancreatitis must be accounted for. Elastase is unable to digest native collagen but is capable of hydrolysing its denatured form (268, 371, 469) at a rate 10 times faster than elastin digestion (454). It is not known whether such denaturation occurs during pancreatic necrosis, which renders collagen suitable for digestion by elastase. The opinion that release of collagenase activity (which could account for digestion of collagen) occurs in acute pancreatitis (30), while plausible, needs further documentation. Although the significance of elastase in clinical pancreatitis cannot be deduced from these studies, the following facts are considered of some significance. A modest amount of elastic tissue is present in the walls of intrapancreatic blood vessels and ducts of man (493), and pro-elastase has been shown to exist in pancreatic secretion (267). Certain difficulties concerning the determination of proelastase in fresh and suitable human pancreatic tissue must be overcome before an accurate determination of the proenzyme content can be accomplished; also, the development of a method for elastase determination in the peripheral blood, obtained during the early stages of acute pancreatitis in man, will be of value: it seems that such a method will soon be in use (501). Studies on the serum elastase inhibitors in acute pancreatitis may shed some light on the possible significance of an enzyme/inhibitor interplay in the pathogenesis of the disease. The release of intra-pancreatic elastolytic activity, with resultant pancreatic haemorrhage and necrosis, may explain the inability of the trypsin inhibitor from beef parotid gland (155) to modify the course of the disease (311, 313, 478).

## SUMMARY

1. A brief outline of the possible mechanism of tissue destruction in acute pancreatitis, as it appears when viewed in the light of newer concepts of cell structure and function - the expression of inappropriate enzymatic activity leading to cell degeneration and necrosis - has been presented. The inadequacy of existing information concerning the autolytic and heterolytic concepts of cell injury has been discussed and some pertinent points relating to the complex enzymatic mechanisms involved in acute pancreatitis have been enumerated.

2. The histopathological features of acute pancreatitis, ranging from oedema to parenchymatous and fat necrosis and haemorrhage, and the several aetiological factors that can precipitate the disease in clinical as well as in experimental conditions, have been reviewed from the extensive literature on the subject, with special emphasis on the vascular injury and its pathogenesis.

3. Certain aspects of enzyme synthesis, secretion, and activation, and the mechanism of action of exportable pancreatic proteolytic enzymes have been discussed. The literature on pancreatic elastase, as related to its origin, physical constants, secretion, purification, activation, specificity, and the mechanism and kinetics of elastolysis, has been reviewed. The structure, composition, histochemistry and distribution of elastic tissue as a component of vessel walls have been outlined. The scanty information regarding the role of pancreatic elastase in the pathogenesis of acute pancreatitis has been critically reviewed.

4. A new reliable colorimetric method for quantitative determination of elastase activity in biological materials has been developed. Determinations of free and total elastase and proelastase in normal pancreatic tissue of mongrel

dogs were performed; ample stores of proelastase and no free elastase were found. The proelastase and free elastase content of fresh human pancreas was found to be low or negligible.

5. Significant decrease in the amount of proelastase was found in the most-involved part (tail) of the pancreas of mongrel dogs as compared with the least-involved part (head), in the course of bile- or trypsin-induced experimental pancreatitis, at 5, 60 and 180 minutes. Histological evidence of elastolytic activity in the diseased pancreas, consisting of loss of staining power and dissolution of elastic fibres in the walls of medium-sized and small veins and arteries, was obtained by means of elastica stains. The inhibitory activity of pancreatic extracts on the elastase/elastin--orcein system in vitro was low, and no decrease of this activity occurred in the most-involved part of the pancreas in bile- or trypsin-induced pancreatitis.

6. In the course of parenchymatous necrosis induced in the mandibular gland of the dog by injection of bile or trypsin, no vascular destruction or dissolution of elastic fibres was found at 60 and 180 minutes. However, there was a pronounced inhibitory activity of mandibular gland extracts, from normal or necrotic tissue, on elastase in vitro.

7. Pancreatic tissue slices exposed under optimal conditions to a solution of crystalline pancreatic elastase showed selective dissolution of elastic fibres in the vessel and duct walls, and parenchymatous destruction of increasing severity, depending upon the enzyme concentration.

8. The intrapancreatic elastolytic and nonspecific proteolytic activity of pancreatic elastase was observed in histological sections of dog pancreas at 60 minutes in elastase-induced acute pancreatitis.

9. The proelastase - elastase enzyme system appears to be of

considerable significance in the pathogenesis of the distinct vascular injury observed in acute experimental pancreatitis induced by bile or trypsin in the dog. The possible role of this enzyme system in clinical pancreatitis has been discussed.

## CLAIM OF ORIGINALITY

To the best knowledge of the author the following are new contributions of this study:

1. The development of a colorimetric method for quantitative determination of free and total elastase and proelastase in biological materials; under a strict set of conditions, zero-order kinetics are followed up to a certain amount of elastase activity.

2. For the first time, a systematic quantitative determination of proelastase free and total elastase content of normal dog pancreas has been accomplished.

3. The same statement holds concerning quantitative determinations of free and total elastase and proelastase in bile-induced pancreatitis, and in three anatomical parts of dog pancreas.

4. For the first time, also, proelastase and free and total elastase have been quantitatively determined in the course of trypsin-induced pancreatitis in dogs.

5. A correlation of the studies in (3) and (4) with the histological evidence of elastolysis by elastica stains has been achieved for the first time.

6. This study is the first to be reported in which parenchymatous necrosis has been induced, by the injection of bile or trypsin, in the mandibular gland of the dog, and in which the absence of elastolysis has been demonstrated.

7. The effect of pancreatic elastase on pancreatic tissue slices <u>in vitro</u> and the demonstration of vascular and parenchymatous injury has also been achieved for the first time.

8. This author believes with reasonable certainty that the release of elastase activity is responsible for the vascular injury observed in the course of bile- or trypsin-induced acute experimental pancreatitis in the dog.

"It is scant modesty for man, even if he is 'the highest vertebrate' to presume that he can predict the cosmic plan on the intensity of his joy and pain, or cement the stars together with even his highest aspirations."

Homer Smith. From Fish to Philosopher; Boston; Little, Brown, 1953.

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