CHAPTER 12

Pancreatic Secretory Enzymes

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The human pancreas possesses an extraordinary capac-
ity to synthesize a wide range of products. It produces
and secretes more protein per gram of tissue than any
other organ and delivers between 6 and 20 g of digestive
enzymes and zymogens per day in an average volume of
2.5 liters of fluid. Because of the inaccessibility of fresh
human pancreatic tissue and juice, much of the present
knowledge of pancreatic secretory proteins has been
gained in studies with experimental animals. Although
extensive homology has been demonstrated between en-
zymes of various mammals, species differences often are
reflected in divergent biochemical properties of these
proteins, which in turn may affect their physiological or
pathophysiological role. The study of human enzymes
and zymogens is therefore of paramount importance for
the understanding of pancreatic function in health and
disease.

Many of the original studies carried out with duodenal
fluid or autopsy specimens were flawed because of con-
tamination with gastric, duodenal, and biliary secretions
and the rapid onset of autodigestion of autopsy material
and enzymes in duodenal fluid. More recent investiga-
tions are based on pure pancreatic secretions obtained
during surgery or by endoscopic cannulation of the main
pancreatic duct, procedures that permit isolation of na-
tive enzymes and zymogens and their study under con-
trolled conditions. Progress in this field also has been
facilitated by recent advances in isolation techniques
and the development of DNA sequencing and DNA li-
braries. Table 1 presents a summary of the most impor-
tant human pancreatic enzymes and zymogens that have
been isolated and characterized to date.

ISOLATION AND PROPERTIES OF
PANCREATIC ENZYMES (ZYMOGENS)

Serine Proteases

Trypsin, chymotrypsin, elastase, kallikrein, and others
are members of a group of enzymes termed "serine pro-
<table>
<thead>
<tr>
<th>Enzyme/zymogen</th>
<th>Function of enzyme</th>
<th>Molecular weight</th>
<th>pH optimum</th>
<th>pl</th>
<th>Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin(ogen) 1, anionic (EC 3.4.21.4)</td>
<td>Hydrolysis of proteins, Arg- and Lys-peptide bonds</td>
<td>Zymogens 25 kDa</td>
<td>7.5–8.5</td>
<td>4.7</td>
<td>–</td>
</tr>
<tr>
<td>Trypsin(ogen) 2, mesotrypsin</td>
<td></td>
<td>25 kDa</td>
<td>8.25</td>
<td>5.95</td>
<td>–</td>
</tr>
<tr>
<td>Trypsin(ogen) 3, cationic (EC 3.4.21.1)</td>
<td></td>
<td>23.4 kDa</td>
<td>7.5–8.5</td>
<td>6.6</td>
<td>–</td>
</tr>
<tr>
<td>Chymotrypsin(ogen) A (EC 3.4.21.1)</td>
<td>Hydrolysis of proteins, Phe–, Tyr–, Trp– peptide bonds</td>
<td>24 kDa</td>
<td>8</td>
<td>7.2</td>
<td>–</td>
</tr>
<tr>
<td>Chymotrypsin(ogen) B (EC 3.4.21.1)</td>
<td>Hydrolysis of proteins, Phe–, Tyr–, Trp– peptide bonds</td>
<td>27 kDa</td>
<td>8</td>
<td>7.2</td>
<td>–</td>
</tr>
<tr>
<td>(Pro) elastase 2 (pancreatopeptidase E) (EC 3.4.21.11)</td>
<td>Cleavage of bonds adjacent to aliphatic amino acids (elastin)</td>
<td>30.5 kDa</td>
<td>7.5–10.5</td>
<td>9.55</td>
<td>–</td>
</tr>
<tr>
<td>(Pro)protease E “(Pro) elastase 1”</td>
<td>Cleavage of aliphatic and hydroxyl amino acid residues</td>
<td>Enzyme 29 kDa</td>
<td>7.5–10.5</td>
<td>Zymogen</td>
<td>– (+)</td>
</tr>
<tr>
<td>Kalikreinogen</td>
<td>Cleavage of kininogen to kinin</td>
<td>Enzyme 35 kDa</td>
<td>8</td>
<td>Enzyme</td>
<td>3.9–4.1</td>
</tr>
<tr>
<td>(Pro)carboxypeptidase A 1</td>
<td>Cleavage of carboxy-terminal Phe, Tyr, Trp residues</td>
<td>Zymogen 45–47 kDa</td>
<td>7.5–8</td>
<td>4.9</td>
<td>–</td>
</tr>
<tr>
<td>(Pro)carboxypeptidase A 2</td>
<td>Cleavage of carboxy-terminal Phe, Tyr, Trp residues</td>
<td>Enzyme 34 kDa</td>
<td>7.5–8</td>
<td>4.9</td>
<td>–</td>
</tr>
<tr>
<td>(Pro)carboxypeptidase B 1</td>
<td>Cleavage of carboxy-terminal Arg, Lys residues</td>
<td>Zymogen 47 kDa</td>
<td>7.65</td>
<td>6.6</td>
<td>–</td>
</tr>
<tr>
<td>(Pro)carboxypeptidase B 2</td>
<td>Cleavage of carboxy-terminal Arg, Lys residues</td>
<td>Enzyme 35 kDa</td>
<td>7.1</td>
<td>6.6</td>
<td>–</td>
</tr>
<tr>
<td>(Pro)phospholipase A 2</td>
<td>Hydrolysis of sn-2-fatty acid esters of phosphoglycerides</td>
<td>Zymogen 14 kDa</td>
<td>6</td>
<td>Zymogen</td>
<td>–</td>
</tr>
<tr>
<td>(Pro)phospholipase A 2</td>
<td>Hydrolysis of sn-2-fatty acid esters of phosphoglycerides</td>
<td>Enzyme 14 kDa</td>
<td>7.5</td>
<td>Zymogen</td>
<td>–</td>
</tr>
<tr>
<td>Lipase (triacylglycerol hydrolase)</td>
<td>Hydrolysis of C1 and C3 glycerol ester bonds</td>
<td>Enzyme 48 kDa</td>
<td>8–9</td>
<td>5.8</td>
<td>+</td>
</tr>
<tr>
<td>(Pro)colipase I, II</td>
<td>Cofactor for lipase</td>
<td>9.8–10 kDa</td>
<td>5.8, 6.1</td>
<td>4.7</td>
<td>+</td>
</tr>
<tr>
<td>Carboxyl ester hydrolase (EC 3.1.1.1)</td>
<td>Hydrolysis of water-soluble &amp; insoluble esters</td>
<td>100 kDa</td>
<td>7.4, 8</td>
<td>4.7</td>
<td>+</td>
</tr>
<tr>
<td>Amylase (α-1,4-glucan-4-glucan hydrolase) (EC 3.2.1.1)</td>
<td>Hydrolysis of α-1,4-glycosidic bonds in starches</td>
<td>50 kDa</td>
<td>7.5–8</td>
<td>6.5–7.1</td>
<td>+</td>
</tr>
<tr>
<td>Ribonuclease (EC 3.1.27.5)</td>
<td>Hydrolysis of phosphate bonds in RNA</td>
<td>15 kDa</td>
<td>8.2</td>
<td>7.3–7.8</td>
<td>+ (–)</td>
</tr>
<tr>
<td>Deoxyribonuclease I</td>
<td>Hydrolysis of phosphate bonds in DNA</td>
<td>33–38 kDa</td>
<td>7–7.5</td>
<td>3.9–4.3</td>
<td>–</td>
</tr>
</tbody>
</table>

See also Scheele and Padfield (this volume).

Teases.'’ They are characterized by the reactivity of a serine residue at the active site of the enzyme. The hydroxyl group of the active serine is involved in rapid successive acylation and decylation steps, resulting in cleavage of a peptide bond of the substrate and simultaneous regeneration of the enzyme. This is achieved by a charge relay system that relies on His-57 to transfer protons from Asp-102 to Ser-195 (the amino acid numbers refer to the sequence of bovine chymotrypsin A). The mechanism of this catalysis has been studied extensively with chymotrypsin and is thought to be characteristic of all ‘‘serine proteases’’ (27). The members of this group exhibit considerable homology in their amino acid sequences and are classified as endopeptidases because they cleave natural substrates—proteins—at specific sites along the peptide chain. The remarkable differences in their specificity can be attributed to a few substitutions in the amino acid residues at the substrate binding site, but the location of surface loops of the molecule seems to be an equally important determinant of catalytic specificity (54). A common test for endopeptidases is inhibition of their active site by diisopropylfluorophosphate (DFP),
Trypsin plays a key role in the process of digestion. It is the trigger enzyme solely responsible for activating all other pancreaticzymogens (Fig. 1). Its precursor, trypsinogen, occurs in the form of several variants as do some of the other pancreatic zymogens. The major cationic form (trypsinogen-3)* is present in pancreatic juice of healthy individuals in a ratio of about 2:1 relative to the less prominent anionic form (trypsinogen-1). The purification and characterization of these zymogens by gel, ion exchange, and affinity chromatography, as well as their molecular properties, have been described principally by Guy et al. (52). Recently, the cDNAs encoding these two trypsinogens have been isolated from a human pancreatic DNA library and sequenced. The deduced amino acid sequences exhibit 89% homology and have the same number of amino acids (i.e., 247), including 15 amino acids for a signal peptide and 8 for an activation peptide. The two proteins have almost identical molecular weights: 25,002 daltons for the anionic and 24,930 daltons for the cationic trypsinogen (28), but they differ from one another immunologically (84). Enteropeptidase (enterokinase) activates both zymogens by cleaving the N-terminal octapeptide Ala-Pro-Phe-(Asp)\textsubscript{2}-Lys-, but trypsinogen-3, the less stable of the two variants, furnishes, in addition, the pentapeptide (Asp)\textsubscript{2}-Lys-. The tetraaspartyl group of the activation peptide is common to the trypsinogens of most other species: cow, pig, horse, goat, and so on.

A third, unique variant of trypsinogen, mesotrypsinogen (trypsinogen-2), has been isolated more recently by Rinderknecht et al. (140; cf. 152) from fresh human pancreatic juice. It has a molecular weight of about 25,000 daltons and represents less than 5% of the total potential trypsin activity. Activation with enteropeptidase yields the active enzyme, which, in contrast to anionic and cationic trypsin, is completely resistant to pancreatic secretory trypsin inhibitor, as well as most other biological trypsin inhibitors. It possesses a substrate specificity similar to that of the major pancreatic trypsins but differs from them immunologically. The physiological significance of this unusual enzyme has not been clarified to date.

In the past, the pancreas was thought to be the only tissue to produce trypsinogen(s). Recently, two trypsinogen variants have been isolated from cyst fluid of patients with ovarian cancer (71) and also from human colon carcinoma, fibrosarcoma, and leukemia cell lines (73). The purified trypsinogens, the anionic variant being the major component, are activated by enteropeptidase and yield an N-terminal activation peptide of 8 amino acids. They have molecular weights similar to those of their pancreatic counterparts: 28,000 daltons for the anionic and 25,000 daltons for the cationic form. They are immunologically indistinguishable from the pancreatic enzymes and have similar, but not identical, substrate specificities. Since tumor-associated trypsins are efficient activators of pro-urokinase, it has been suggested that they may take part in the protease cascade involved in tumor invasion of healthy tissue and degradation of extracellular matrix (72).
Activation of Trypsinogen

Autoactivation. Trypsinogens have a weak but inherent capacity to activate themselves (63). This conversion is accompanied by release of the activation peptide (see above) and a conformational change of the molecule. The rate of this activation increases rapidly as the trypsin formed begins itself to catalyze the activation process. Human trypsinogens, especially the cationic form, autoactivate much more readily than those of other species, for example, bovine trypsinogen (14,32). Autoactivation, as well as autodigestion, of trypsinogens to inert products is dependent on the pH and calcium concentration. Human cationic trypsinogen autoactivates spontaneously at a pH below 6, activation being most rapid at pH 5. The anionic trypsinogen shows little or no activation under these conditions. Calcium enhances activation of anionic trypsinogen but inhibits activation of the cationic variant. Calcium concentrations of 20 mM delay the onset of activation under all conditions (21,34). At pH 8, autodigestion predominates. It is enhanced by the absence of calcium, whereas calcium concentrations greater than 1 mM promote activation by stabilizing the trypsin configuration. In pure pancreatic juice, which in most cases has a pH higher than 8 and a calcium concentration less than 1 mM (131), degradation of zymogens to inert products is the main reaction (53).

Activation by Enteropeptidase (Enterokinase): the Pancreatic Enzyme Cascade. Autocatalytic activation of trypsinogens normally occurs only in vitro. In healthy subjects pancreatic juice entering the duodenum is devoid of proteolytic activity. Duodenal mucosa and fluid contain an enzyme, enteropeptidase (enterokinase), synthesized by the enterocytes of the proximal small intestine (56), whose sole function is the activation of trypsinogen to trypsin, the trigger enzyme for the "pancreatic enzyme cascade" (Fig. 1). It hydrolyzes the bond between Lys-Ile residues of the N-terminal part of the trypsinogen molecule, thereby releasing the activation peptide (see above) and active trypsin. It has been suggested by Maroux et al. (99) that the unique specificity of enteropeptidase may be due to an interaction of the enzyme with the (Asp)₄ sequence in the activation peptide of trypsinogen, and that the same sequence may prevent autoactivation of trypsinogen by an electrostatic mechanism. Enteropeptidase is a far more efficient enzyme in the activation process than trypsin. Observations with bovine trypsinogen indicate that the rate constant for enteropeptidase-catalyzed activation of the zymogen exceeds that of autoactivation by several orders of magnitude. Human enteropeptidase, a glycoprotein of molecular weight 316,000 daltons, has been purified and characterized by Grant and Hermon-Taylor (48). Its activity, which is optimal at pH 6 to 9, is enhanced by low levels of calcium and bile salts and reduced by increasing ionic strength. Optimal yields of trypsin are obtained at low concentrations of trypsinogen; high zymogen concentrations, especially at an alkaline pH, lead to partial autodigestion by the trypsin generated. A simple, highly sensitive, one-step assay for enteropeptidase has been described by Rinderknecht et al. (135).

Activation by Cathepsin B. Human trypsinoens-1 and -3 (and presumably trypsinogen-2) are also activated by the lysosomal hydrolase cathepsin B, which occurs in pancreatic tissue. Activation takes place between pH 2.5 and 4.0 and is optimal at pH 3.8. The rate of activation is the same for both major trypsinogen variants (34). The physiological and potential pathophysiological significance of these findings is discussed below.

Properties of Human Trypsin

Some of the properties of the human pancreatic trypsin variants are listed in Table 1. A comparison of additional physical and biochemical characteristics of the three trypsins has been reported by Rinderknecht et al. (140). Some differences between the main isoenzymes have already been pointed out in the preceding paragraphs. Their amino acid composition is quite similar, but the cationic variant has four additional Lys residues, one additional Met residue, and five disulfide bridges versus four in the anionic variant. The cationic variant also has fewer hydrophobic amino acids than the anionic variant (28). A characteristic difference between human and animal trypsins is the presence of only five (respectively, four) disulfide bridges in the human enzymes instead of six, as found in most other species (52). Although the specificity of the human pancreatic trypsins toward various substrates is very similar, their stabilities show considerable differences. Whereas the cationic variant is stable at pH 2, anionic trypsin and mesotrypsin are irreversibly denatured at this pH. At pH values above 7 and high concentrations, susceptibility to autodigestion (attack of the enzyme upon itself as a substrate) increases rapidly for all three variants, especially in the absence of calcium.

The properties of tumor-associated trypsin variants-1 and -2 are similar to, but not identical with, those of the corresponding pancreatic trypsinoens-3 and -1 (71).

Enzymatic Activity and Substrate Specificity

The enzymatic activity of the trypsins is highly specific and directed toward the carboxyl group of lysyl and arginyl residues in natural, as well as synthetic, substrates. The principle of the catalytic mechanism has been explained above, but specificity of action is determined by other features of the molecular structure as indicated above. The structure, catalytic mechanism, and substrate specificity of serine proteases are discussed in recent reviews (see references in ref. 16). Trypsins act over
Methods of Assay

The most widely used assays for trypsin are based on its esterase and amidase activities toward acyl-arginine esters and amides. Spectrophotometric, potentiometric, or titrimetric procedures utilizing N-benzoyl-L-arginine ethyl ester or p-toluensulfonfyl-L-arginine methyl ester as substrates are outlined in Colowick and Kaplan (22). In contrast to these substrates, which possess relatively low specificity for trypsin, N-benzoyl-D,L-arginine p-nitroanilide is a highly specific and sensitive amide substrate and is used in a colorimetric assay for this enzyme. An equally specific, but much more sensitive, fluorometric assay, capable of measuring nanogram quantities of trypsin, is based on N-carbenzoxy-glycyl-glycyl-L-arginine 2-naphthylamide as a substrate (cf. 141). Proteolytic activity of trypsin is determined most conveniently with Remazolbrilliant Blue hide (144), an insoluble collagen substrate labeled covalently with Remazolbrilliant Blue. The sensitivity of this colorimetric method, although not specific for trypsin, approaches that of the fluorometric assays.

Active Site Titration. The molarity of active trypsin in solution can be determined with specific, highly re-active acylation reagents that form relatively stable derivatives of the enzyme by reacting with the serine hydroxyl group at the active center and simultaneously releasing a chromophore or fluorophore, such as nitrophenol or 4-methylumbelliférole. The most widely used titrants are p-nitrophenyl-p'-guanidinobenzoate and 4-methylumbelliferone p-guanidinobenzoate (see ref. 22, p. 44).

Determination in Serum. A small amount of pancreatic enzymes is secreted normally into the bloodstream. In acute pancreatitis, serum concentrations increase markedly and therefore are useful parameters in the diagnosis of the disease. Because of the (presumably) uniquely pancreatic origin of trypsin—in contrast to amylase—radioimmunoassays have been developed for the determination of trypsin in serum (84). Several commercial diagnostic kits are available at present. They measure trypsinogen and trypsin bound to α1-proteinase inhibitor, but not trypsin complexed with α2-macroglobulin. More recently, an enzyme-linked immunoabsorbent assay (ELISA) (68) and a time-resolved immunofluorometric method (61) have been reported for the simultaneous determination of cationic and anionic serum trypsins. Although both trypsin variants were elevated in patients with pancreatitis, the increase in the concentration of the anionic form was far greater. Whereas the ratio of cationic/anionic trypsin in healthy individuals was about 1.3 (21/17 μg/liter), it decreased to about 0.4 (315/850 μg/liter) in patients with acute pancreatitis. A similar reversal of the ratio of cationic to anionic trypsin had been reported earlier by Rinderknecht et al. in pancreatic juice from alcoholics and patients with pancreatic disease (142, 143). It would appear that anionic trypsin is a more sensitive diagnostic parameter for pancreatic disease than the presently used cationic variant.

Trypsin Inhibitors

All trypsin variants are completely inactivated by diisopropylfluorophosphate (DFP) and, with the exception of mesotrypsin, by the specific trypsin inhibitor tosyllysine chloromethyl ketone (TLCK). A number of synthetic and peptide inhibitors is listed in Table 2. Some synthetic, wide-spectrum "trypsin inhibitors" are discussed below (see the section Other Trypsin Inhibitors). Pancreatic secretory trypsin inhibitor (PSTI) and plasma protease inhibitors are considered in separate sections (below).

Trypsin as Activator of Pancreatic Zymogens

Mention has already been made of the unique function of trypsin as activator of all other pancreatic zymogens, which is illustrated in Fig. 1. The dependence of each of these zymogens on a single activator, itself a precursor that normally is activated extrapancreatically, and the presence in pancreas of a specific trypsin inhibitor (PSTI) illustrate dramatically the intricate mechanisms designed by nature to prevent inappropriate release of active enzymes prior to their entry into the intestine. Although trypsin is capable of activating its own precursor, it is a far more efficient activator of other pancreatic zymogens. Observations made with the bovine enzyme and zymogen indicate that the rate constant for the activation of trypsinogen by trypsin is about three orders of magnitude lower than that for the activation of chymotrypsinogen (18).

Maximal activation of zymogens depends on a variety of factors, including concentration of zymogen, added trypsin, total protein, pH, temperature, and time of incubation. Optimal activation conditions are essential in order to obtain a linear response between the amounts of zymogen activated and enzyme activity generated. A careful study of the activation kinetics for guinea pig pancreatic zymogens has been published by Scheele et al. (153). Conditions (not necessarily optimal) for activa-


<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mesotrypsin</th>
<th>Cationic trypsin</th>
<th>Anionic trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human pancreatic trypsin inhibitor (PSTI) (Kazal type) (60×)(^a)</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Bovine pancreatic trypsin inhibitor</td>
<td>0</td>
<td>93</td>
<td>90</td>
</tr>
<tr>
<td>Trasyloc (Kunitz type) (200×)(^a)</td>
<td>0</td>
<td>50</td>
<td>92</td>
</tr>
<tr>
<td>Canine submandibular gland inhibitor (1×)(^a)</td>
<td>0</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>Chicken ovomucoid (2×)(^a)</td>
<td>0</td>
<td>0</td>
<td>99</td>
</tr>
<tr>
<td>α₁-Proteinase inhibitor (4×)(^a)</td>
<td>0</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Soybean inhibitor (60×)(^a)</td>
<td>0</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Lima bean inhibitor (2×)(^a)</td>
<td>5</td>
<td>68</td>
<td>98</td>
</tr>
<tr>
<td>Leupeptin (0.5 µg/ml)</td>
<td>95</td>
<td>89</td>
<td>97</td>
</tr>
</tbody>
</table>

**Synthetic inhibitors**

| p-Aminobenzamidine (1 mM)            | 99          | 100             | 100            |
| DFP (5 mM)                           | 99          | 100             | 100            |
| TLCK (3.5 mM)                        | 65          | 99              | 99             |
| TPCX (1.3 mM)                        | 7           | 0               | 0              |
| 
| HgCl₂ (1 mM)                         | 10          | 11              | 12             |
| EDTA (2 mM)                          | 45          | 50              | 65             |
| FOY, FOY-305, FUT-175 (see the section Other TrypsinInhibitors)         |             |                 |                |

\(^a\) Equal amounts (2 miU by NANA assay) of bovine trypsin, human cationic and anionic trypsins and mesotrypsin were used in the inhibition assays (total volume 1 ml). Multiples (given in parentheses) of the quantity of inhibitor necessary for complete inhibition of bovine trypsin were used in the experiments with the trypsins listed in the table (140).

The inhibition of individual human enzyme precursors are usually described along with their isolation and characterization. It must be realized, however, that the physiological environment of zymogen activation may differ considerably from the conditions, optimal or otherwise, established in vitro by various investigators. Thus autocatalytic or secondary cleavage of the initial enzyme may lead to less active species, as has been observed for bovine trypsin, and especially chymotrypsin, which is degraded to the well characterized α-, β-, γ-, and δ-chymotrypsins (27). However, no analogous study of the corresponding human enzymes has been reported to date, but recent observations point to the existence of degradation products of human cationic trypsinogen that may have physiological or pathophysiological significance (131) (see section Intrapancreatic Degradation of Zymogens).

**Chymotrypsinogen**

Extensive studies of chymotrypsinogen from other species (bovine, ovine) indicate that the human zymogen may also occur in two or more variants. The number of human chymotrypsinogens, however, is still a matter of some controversy (152). Earlier workers had reported isolation of two major forms of chymotrypsin with a high degree of immunological cross-reactivity from acid extracts of human pancreatic tissue, which they believed to originate from two separate zymogens. More recently, de Caro et al. (24) isolated a major, relatively acid-stable form of chymotrypsinogen A and a minor, acid-labile variant B by ion-exchange chromatography and electrophoresis of human pancreatic juice. The major form, representing about 70% of total activity, had a molecular weight of 24,000 daltons, a specific activity almost twice that of the minor form, and resembled bovine chymotrypsinogen A and B in most properties, including amino acid composition. The minor form B, representing about 7% of chymotrypsin activity, had a molecular weight of about 27,000 daltons and was immunologically different from the major variant. As it is known that activation of bovine chymotrypsinogen can give rise to several chymotrypsin species by cleavage of one or more peptide bonds of the zymogen, the two chymotrypsins from acid human pancreatic extracts described in earlier reports may well have been derived from the same major zymogen, which was termed chymotrypsinogen A by de Caro (24). Recently, Tomita et al. (177) isolated
the cDNA clone encoding human pre-chymotrypsinogen A from a human pancreas cDNA library and determined its nucleotide sequence. The deduced amino acid sequence of prechymotrypsinogen consisted of 263 amino acids, including 18 amino acids for a signal peptide and 15 amino acids for an activation peptide. Further analysis revealed that human genomic DNA carries at least two genes that are related to chymotrypsinogen.

Chymotrypsinogen is readily activated by small amounts of trypsin, but no activation peptide is released during the activation process, because the two fragments produced by cleavage of an Arg-Ile linkage are still bound together by a disulfide bridge. The activation peptide has been isolated after oxidation of the native enzyme. However, it had only 14 amino acid residues instead of 15 as indicated above. It lacked the carboxyl-terminal Arg, which was probably cleaved by carboxypeptidase B present in the activation mixture (179). In contrast to the bovine enzyme, human chymotrypsin, when subjected to autocatalytic cleavage, furnishes only inert degradation products.

**Enzymatic Activity, Substrate Specificity, and Methods of Determination**

The catalytic activity of chymotrypsin is directed specifically at peptide bonds involving the carboxyl group of aromatic amino acids (Phe, Tyr, Trp) and, to a lesser degree, Leu and Met within the protein substrate. The enzyme displays activity over a broad range of pH (pH 5–9), with an optimum at about pH 8. The assay methods most frequently used in the past are based on acetylsinopeptide C-terminal Arg, which was probably cleaved by carboxypeptidase B present in the activation mixture (179). In contrast to the bovine enzyme, human chymotrypsin, when subjected to autocatalytic cleavage, furnishes only inert degradation products.

**Active Site Titration.** The molarity of active chymotrypsin can be determined with such reagents as 2-hydroxy-5-nitro-so- touluenesulfonic acid sulfate or trans-cinnamoyl imidazole, which form slowly reversible, covalently bound derivatives with the active site, as described above for trypsin (see ref. 22, pp. 3–20).

**Determination in Serum.** A radioimmunoassay for this enzyme has been described by Geokas et al. (45). More recently, Carrere et al. (20) developed a noncom- petitive immunoassay for the two immunoreactive chymotrypsins in serum, that is, chymotrypsinogen and chymotrypsin-α1-proteinase inhibitor complex. The detection limit was 0.5 ng/ml.

**Chymotrypsin Inhibitors**

Chymotrypsin is completely inactivated by DFP and by the chymotrypsin-specific inhibitor tosyl-L-phenylalanine chloromethylketone (TPCK), both of which react irreversibly with the active center of the enzyme. Proline–valine peptide enol lactones with effective and selective chymotrypsin inhibitor activity have been reported recently by Reed et al. (127). Neither the bovine Kunitz-type trypsin inhibitor Trasylol (Aprotinin) nor human pancreatic trypsin inhibitor (PSTI) are capable of inhibiting human chymotrypsin. Reaction of the enzyme (zymogen) with plasma protease inhibitors is discussed below.

**(Pro)elastase**

Pancreatic elastases, like trypsin or chymotrypsin, are secreted as zymogens. Two variants, elastase 1 and elastase 2, have been isolated and characterized from the pancreas of numerous species. Most of the earlier work has been focused on porcine and rat pancreatic elastases. Largman et al. (82) in 1976 first reported the purification and characterization of two elastases, elastase 1 and elastase 2, from human pancreas. Human, rat, and porcine elastase 2 are cationic proteins and show over 80% homology. Rat and porcine elastases 1 also have cationic properties, whereas human "elastase 1" is an anionic enzyme and does not appear to be a counterpart of rat or porcine elastase 1 (cf. 174). The gene for human elastase 1, which corresponds to that of rat and porcine elastase 1, has been cloned and characterized by Tani et al. (174). However, this enzyme is not expressed in human adult pancreas, and Largman's human pancreatic "elastase 1" has now been identified as protease E (97), which itself is identical with the cholesterol-binding protein discovered by Szegoleit (173). Human protease E ("elastase 1") is therefore a new member of the serine protease family and is described below.

Human proelastase 2 occurs in two variants, proelastase 2A and proelastase 2B, and as a binary complex with procarboxypeptidase A1 (103). Of the two zymogens, only proelastase 2A has been isolated and purified to date. The primary structure of both elastases 2A and 2B has been elucidated by cloning cDNAs from a pancreatic cDNA library (36,64). Elastases 2A and 2B are synthesized in the pancreas at a ratio of about 4:1 as pre-proenzymes with 269 amino acids, including a signal peptide of 16 amino acids and an activation peptide of 12 amino acids. They are 90% homologous in their amino acid
sequences but differ considerably in their activation peptides. Four disulfide bonds are conserved in all members of the elastase family. In addition, two cysteine residues conserved in proelastase 2 are capable of forming an additional disulfide bond at positions -12 and 113. Thus proelastase 2, which is readily activated by small amounts of trypsin, furnishes active elastase with an activation peptide that remains attached to the mature enzyme by a disulfide bridge similar to that of chymotrypsin. Elastase 2A and 2B, like anionic trypsinogen, are irreversibly denatured at pH values below 2.5.

Pancreatic elastases have been implicated by various authors in emphysema, atherosclerosis, aging, and vascular injury in acute pancreatitis. However, more recent evidence (cf. 51,132) indicates that this role should more likely be attributed to leukocyte elastases rather than to the pancreatic enzyme. Although raised serum levels of immunoreactive forms of pancreatic elastase (i.e., zymogen and elastase-α1-proteinase inhibitor complex) are detected in acute pancreatitis, the catalytically active component in serum is probably a mixture of pancreatic and neutrophil elastases complexed with α2-macroglobulin. It is unlikely that this complex, with its greatly reduced enzymatic activity and very short halflife, plays a notable pathogenic role in the above conditions. Tissue damage is probably caused by excessive granulocyte activity, which includes in situ release of elastase, oxygen-derived free radicals, and a host of lysosomal enzymes and other factors (cf. 133). Human neutrophil elastase shows 36% homology with human pancreatic elastase 2. It is a glycoprotein with 218 amino acid residues and four disulfide bridges. It is more basic than the pancreatic enzyme, possesses 19 arginine residues, and differs immunologically from its pancreatic counterpart. Its primary structure has been determined by analysis of the cDNA sequence by Farley et al. (30), and three isozymes have been isolated that differ from one another in their carbohydrate chains (see references in ref. 16).

Enzymatic Activity and Substrate Specificity

Elastases are unique among the proteases in that they are capable of hydrolyzing the scleroprotein elastin. In contrast to trypsin and chymotrypsin, elastases, including human pancreatic elastase 2 and leukocyte elastase display a relatively low specificity toward different peptide bonds but have a preference for peptides with allphatic side chains. Their activity spans a wide pH range with an optimum at pH 7.5 to 10.5. Porcine elastase 2 and probably the human enzyme as well, in contrast to neutrophil elastase, possess a calcium-binding loop in their structure that stabilizes the molecule but does not affect its enzymatic activity. Elastases also have esterase and amidase activity toward synthetic substrates such as N-acetyl-(L-alanyl)-p-nitroanilide (cf. 141), which are used in titrimetric and colorimetric assays of the enzyme. The most sensitive and specific substrate for determining elastolytic activity is elastin labeled covalently with Remazol-brilliant Blue (RBB-elastin) (145). Interestingly, human pancreatic elastase possesses only about one-fifth to one-third the activity of the porcine enzyme toward elastin.

Determination in Serum. As pointed out previously, increased levels of serum elastase can be detected in acute pancreatitis and cancer of the pancreas. A radioimmunoassay for “elastase 1” has been described by Geokas et al. (44) and a RIA-kit is available from Abbott Diagnostics, Wiesbaden, Germany. These assays measure “proelastase 1” and “elastase 1” complexed with α1-proteinase inhibitor, but not enzyme complexed with α2-macroglobulin. It should be noted that the assays, in reality, determine protease E concentrations, since elastase 1, as mentioned above, is not expressed by human pancreas. Determination of serum granulocyte elastase seems to be more helpful in the diagnosis and prognosis of acute pancreatitis than assays of the pancreatic enzyme (51).

Elastase Inhibitors

Like other serine proteases, elastases are inactivated by DFP. Because of the putative role of elastase in emphysema and obstructive lung disease, much effort has been devoted to the synthesis of elastase inhibitors. They include peptide chloromethyl ketones and fluoroketones; heterocyclic compounds, such as isoucomarins; benzoxazinones; pyrones; and cephalosporin esters. Most of them inhibit pancreatic as well as neutrophil elastase (cf. 16). Pancreatic elastase is inhibited by canine submandibular gland inhibitor, eglin c (a polypeptide inhibitor from the leech), partially inhibited by soybean trypsin inhibitor, but not inhibited by Trasylol (Aprotinin) or pancreatic secretory trypsin inhibitor (PSTI). Like trypsin and chymotrypsin, it is inhibited by α1-proteinase inhibitor, and its proteolytic, but not esterolytic and amidolytic, activity is abolished by complex formation with α2-macroglobulin.

(Pro)protease E, “(Pro)elastase 1”

Protease E is a serine protease with a molecular weight of 29,000 daltons, first isolated and characterized by Mallory and Travis (97) and Largman et al. (82). It is present in human pancreatic juice as a proenzyme in the form of a binary complex with procarkoxypeptide A. A glycosylated, truncated form of protease E lacking the N-terminal valine-valine residues also occurs as a binary complex with procarkoxypeptide A (104). Shirasu et al. (161) found two isozymes of protease E in human pancreatic juice. One of them appeared to be glycosylated and, in contrast to the other, did not hydrolyze
elastin. Tani et al. (174) have isolated two different, but closely related, cDNAs encoding proteases E from a human pancreatic cDNA library. The deduced amino acid sequence of the active enzyme consists of 242 amino acids, preceded by a signal peptide and propeptide of 28 amino acids. The amino acid homology between the two enzymes is 93%. The substrate binding region of human protease E is highly homologous to those of porcine and rat elastase 1, but the sequence outside the substrate binding site is less than 50% conserved (159). In contrast to the elastases, protease E is an anionic protein with a net charge of −6 versus a net charge of +8 for the elastases. Its isoelectric point is about 5. Although the enzyme clearly possesses the catalytic potential of an elastase, the factors responsible for its inability to bind to the surface of elastin have not yet been elucidated. As mentioned above, protease E was found to be identical with the cholesterol-binding protein described by Szegoelet (173). According to this author, 1 mol of "protein" is capable of binding 24 mol of cholesterol. It has been postulated that the enzyme may play a role in intestinal transport and metabolism of cholesterol.

**Enzymatic Activity and Substrate Specificity**

Like other protease zymogens, pro-protease E is readily activated by trypsin. The active enzyme displays selective activity toward alanine, isoleucine, valine, and hydroxyamino acids and complements the activity of chymotrypsin, which is directed against aromatic amino acids, leucine, and methionine. According to some authors (cf. 97), it possesses little or no elastolytic activity, whereas Largman et al. (82) found that it hydrolyzed native and dyed elastins, but at a greatly reduced rate compared with porcine elastase. It also had general proteolytic activity, as demonstrated by its ability to hydrolyze RBB hide. Like the elastases, protease E hydrolyzes synthetic ester and amide substrates and can be assayed with N-acetyl-(alanyl), methyl ester, N-tert-butyloxycarbonyl-L-alanine p-nitrophenyl ester, or succinyl-(alanyl) 2-p-nitroanilide (82).

A radioimmunoassay kit for serum protease E ("elastase 1") is available from Abbott Diagnostics, Wiesbaden, Germany. It measures zymogen, zymogen-α1-proteinase inhibitor complex, and active enzyme complexed with this inhibitor, but not that complexed with α1-macroglobulin.

Protease E is not inhibited by Trasylol (Aprotinin), but its proteolytic activity is completely abolished by α1-proteinase inhibitor and α1-macroglobulin.

**Kallikrein(ogen)**

Kallikreinogen is a minor component of pancreatic juice and constitutes less than 0.04% of its secretory proteins. It is a glycoprotein of a highly anionic character. It has a molecular weight of about 30,000 to 50,000 daltons, depending on its carbohydrate content, and it has been isolated and purified by Terashima et al. (175) by ion-exchange and affinity chromatography. The zymogen is labile and activates spontaneously during isolation. The enzyme is stable at pH 8 but is rapidly inactivated at pH 2.5. The carbohydrate parts of the molecule are relatively rich in biantennary complex type sugar chains. Sequence analysis of cloned cDNA has indicated an amino acid sequence of 238 amino acids for the enzyme, preceded by a signal peptide and a pro-fragment of 24 amino acids (39). Pancreatic kallikrein mRNA is also expressed in the kidney and submandibular gland, and enzymes from these sources differ in their carbohydrate content but cross-react immunologically with pancreatic kallikrein. Serum kallikrein appears to differ immunologically from the glandular kallikreins.

**Enzymatic Activity and Substrate Specificity**

The physiological function of the enzyme in pancreatic secretion is not clear, but it has been suggested that it may be involved in the activation of proinsulin (115). Its main function appears to be the release of kinin from kinogen (α1-globulin in plasma). This activity can be determined by measuring the isotonic contraction of guinea pig ileum in tyrode solution after calibration with bradykinin. When injected intravenously, as little as 1 μg of enzyme produces measurable hypotension in experimental dogs. In acute pancreatitis, serum prokallikrein levels are decreased and kallikrein activity increased. When pigs with experimental pancreatitis were pretreated with high doses of methylprednisolone, these changes were greatly reduced and all animals survived (1).

Kallikrein also has esterase, but not amidase, activity. It has been assayed traditionally with such trypsin substrates as N-benzoyl-L-arginine ethyl ester or N-tosyl-L-arginine methyl ester. A more specific and sensitive substrate, prolyl-phenylalanyl-arginine α-naphthyl ester, has been described by Fujii et al. (37). It permits detection of as little as 0.001 kU. Kallikrein possesses negligible activity toward such protein substrates as casein or RBB hide. It is inhibited by DFP, but not by the trypsin inhibitor TLCK, pancreatic secretory trypsin inhibitor (PSTI), or trypsin inhibitors of plant origin. However, Trasylol (Aprotinin) is capable of inhibiting not only esterase activity but also the pharmacological activity of the enzyme. The human enzyme requires about five times more inhibitor for effective inhibition than does porcine kallikrein. In contrast to most known serine proteases, porcine kallikrein, which has been studied far more extensively than the human enzyme, does not form a complex with α1-macroglobulin, or if so, at an extremely slow rate. In view of the extraordinary pharmacological potency of human kallikrein when injected
into the circulation, it can be assumed that it does not differ from the porcine enzyme in its behavior toward this plasma inhibitor.

**Exopeptidases**

This group of enzymes is characterized by the ability to cleave carboxy- or amino-terminal amino acid residues from proteins or peptides. Although pancreatic juice contains both types of exopeptidases, it is unlikely that aminopeptidases are part of the complement of digestive enzymes packaged in zymogen granules. Available evidence points to a lysosomal origin for these enzymes, and for this reason they will be included in the section on pancreatic lysosomal hydrolases.

**(Pro)carboxypeptidase A**

In contrast to the serine proteases, carboxypeptidases are true metalloenzymes, containing one atom of zinc at the active site of the enzyme. The zinc atom has a dual role in catalysis, both helping to position the peptide carboxyl group for efficient nucleophilic attack and helping to enhance its reactivity. Zinc may be replaced by a number of other metals, such as Co, Cu, Ni, and Mn, with varying effects on enzyme activity. Like the serine proteases, pancreatic carboxypeptidases occur as inactive precursors that are activated by trypsin. Human carboxypeptidase has been obtained in several forms. Kim and White (67), in 1971, first isolated four closely related variants from pancreatic juice. More recently, Pascual et al. (118) reported the isolation of three forms of the enzyme, A1, A2, and A3, from pancreatic extracts by high performance liquid chromatography (HPLC). The A1 and A2 forms occur as monomers, while the A3 form is obtained as a binary complex with pro-protease E (cf. the section Protease E, above). A fourth form, a binary complex with proelastase 2, was isolated recently from human pancreatic juice by Moulard et al. (103) (cf. the section Elastase, above). The binary complexes of procarboxypeptidase A are quite stable but dissociate following activation of the zymogen with trypsin. The reported molecular weight of the active enzymes (A2 form and A3 protomer, respectively) is 33,000, that of the A1 form is 31,000 daltons. The isoelectric point for A1 and A3 is 4.9, that for A2 is 5.1. The N-terminal sequence of the A1 and A3 forms exhibit striking homology, whereas that of the A2 form shows marked differences. There is considerable homology too between the human sequence and those of rat, pig, and cattle. Both the A1 and A3 forms are activated readily by small amounts of trypsin. The activation process of the A2 form is more rapid than that of the A1 and A3 forms, but the yield of active enzyme is lower. There are differences too in the thermal stability of the three carboxypeptidases. The A1 and A3 forms of the active enzyme are almost completely inactivated by heating at 60°C and pH 8 for 10 min, whereas the A2 form retains most of its activity under these conditions and, in this respect, resembles carboxypeptidases B1 and B2.

The physiological significance of the propensity of procarboxypeptidases to associate with the zymogens of protease E and elastase 2 is not clear at present, but it has been postulated that it could improve the overall digestive action of the active forms by virtue of their complementary specificities on alimentary proteins.

**Enzymatic Activity and Substrate Specificity**

The enzymatic properties of carboxypeptidase A have been studied most extensively with the bovine and porcine enzymes. The close similarities of their structural and biochemical characteristics to those of the human enzyme also extend to the enzymatic properties of the latter. Carboxypeptidase A catalyzes the hydrolysis of carboxy-terminal amino acids from protein and peptide substrates and acts in concert with the endoproteases chymotrypsin and protease E. The rate of hydrolysis is optimal when the carboxyl terminus is an aromatic amino acid. The pH optimum for the human enzyme is 7.5 to 8.0. Activity falls off sharply at salt concentrations below 0.15 M NaCl and increases slowly at higher salt concentrations. Carboxypeptidase A also possesses esterase activity toward such synthetic depsipeptides as Bz-Gly-Gly-O-Phe. It shares with the bovine and porcine enzymes certain kinetic abnormalities toward such substrates, probably a reflection of active site features common to all three.

The most convenient assay methods are based on hippuryl-l-phenylalanine or N-carbo-β-naphthoxy-l-phenylalanine and monitor hydrolysis titrimetrically or spectrophotometrically (122) and fluorometrically, in the case of the latter substrate (cf. 67).

**Determination in Serum.** Enzymatic assays for serum carboxypeptidase A have been developed by Peterson et al. (121) and, more recently, by Brown et al. (19). The enzyme is not detectable in the sera of healthy individuals, but it occurs in the sera of patients with acute pancreatitis.

**Inhibitors of Carboxypeptidase A**

The most effective inhibitors of carboxypeptidases A are such metal chelators as 1,10-phenanthroline, 2,2'-dipyridyl, β-phenylpropionic acid, and indole acetic acid. There is no evidence that plasma protease inhibitors are capable of inhibiting carboxypeptidase A activity, but the enzyme has been shown to bind loosely to α2-macroglobulin (121).
(Pro)carboxypeptidase B

The presence of procarboxypeptidase B in human pancreatic juice has been demonstrated by Scheele et al. (152), who used a two-dimensional isoelectric focusing/gel electrophoresis technique to separate two variants of this zymogen in pancreatic secretions in 4 of 10 subjects investigated. While the carboxypeptidase B1 zymogen occurred in all individuals, the B2 form was detected in only four of them. The appearance of a second variant was considered by the authors to be an expression of genetic polymorphism. Pascual et al. (118) have isolated and purified both human procarboxypeptidase B1 and B2 from pancreatic extracts by anion-exchange HPLC. The B1 and B2 forms have identical molecular weights of 47,300 daltons. The isoelectric points are 6.6 for the B1 form and 7.1 for the B2 form. These data are in general agreement with those deduced by Scheele et al. (152) from their electrophoretic results. Procarboxypeptidases B1 and B2, like the A forms, contain one atom of zinc. The active enzymes are obtained by incubation with trypsin. The activation processes for the B forms are similar and resemble those of the A1 and A3 forms. However, generation of maximum activity requires the addition of relatively large amounts of trypsin (proenzyme/trypsin 4:1). This contrasts with the homologous procarboxypeptidases B from other species, which are easily and rapidly activated by small amounts of trypsin (118). The N-terminal sequences of the two B forms are identical over the first 28 residues and show marked similarities to those of the A forms. Active carboxypeptidases B1 and B2 have a molecular weight of 35,500 daltons. Both B forms show considerable resistance to denaturation at 60°C and pH 8, similar to the A2 form. Three active B forms of the enzyme have also been isolated from pancreatic juice by Broderick et al. (17). One of them appeared to be an artifact arising from prolonged storage of the pancreatic juice. Incubation with trypsin yielded an enzyme composed of two polypeptide chains of 24,000 and 9000 daltons. The native enzyme was more basic than that produced by trypsin digestion but, immunologically, they were indistinguishable from each other.

Enzymatic Activity and Substrate Specificity

Carboxypeptidase B is defined as an enzyme that is capable of rapidly hydrolyzing peptide bonds to release carboxyl-terminal arginine and lysine from protein and peptide substrates and that acts in concert with the endopeptidase trypsin. The most widely used assay for carboxypeptidase B is the spectrophotometric method based on hippuryl-L-arginine (cf. 17). The pH optimum in this assay is 7.6. NaCl concentrations greater than 0.3 M enhance peptidase activity. Like other pancreatic zymogens, procarboxypeptidase B is secreted in minute amounts into the peripheral circulation. A radioimmunoassay for the zymogen has furnished evidence for elevated concentrations in serum of patients with acute pancreatitis (46).

Carboxypeptidases B1 and B2, like the A forms, are inhibited by metal chelators but are not affected by plasma protease inhibitors.

Other Digestive Enzymes

(Pro)phospholipase A2

Phospholipases constitute a family of enzymes that hydrolyze phosphoglycerides at different sites (98). Phospholipase A2 (phosphatide 2-acylhydrolase) specifically cleaves the sn-2-fatty acyl group (sn = stereospecific number) from phosphoglycerides. Phospholipases A2 have been isolated and purified from many sources. Two general structural patterns have been identified and named group I and group II. Group I consists of the pancreatic enzyme and those enzymes from venoms of elapid (cobras and coral snakes) and hydrophid (sea) snakes. This group of enzymes is characterized by a disulfide link between the Cys-11 and Cys-77 residues. Group II—which includes crotalid viper (e.g., rattlesnake) venom and intestinal and platelet phospholipases A2—lacks Cys-11 but possesses a short carboxyl-terminal extension ending in a cysteine residue, which is linked to Cys-50.

In contrast to group II phospholipases A2, which are secreted in the active form, human pancreatic phospholipase A2 occurs as a zymogen. It has been isolated from pancreatic juice by Figarella et al. (33). It is a relatively small molecule, with a molecular weight of about 14,000 daltons, being similar to prophospholipase A2 of other species, and constituting less than 1% of the total protein of this secretion. The enzyme possesses seven disulfide bonds, which confer to it a high degree of stability. Thus it is not denatured by 8 M urea, 5 M guanidine HCl, prolonged heating at 85°C in 2% sodium dodecyl sulfate, or treatment with acid. The zymogen is a basic protein with a pl of 7.5. It is activated by small amounts of trypsin, but conversion to the active enzyme is less rapid than that of zymogens from other species. A heptapeptide is cleaved from the N terminus during activation, and a conformational change takes place, giving rise to the formation of an interface recognition site for specific interaction with certain lipid–water interfaces. Note that the human and equine zymogens are also activated by thrombin. The active enzyme has been isolated and studied by Grataroli et al. (50), and its primary structure has been established by Verheij et al. (183). More recently, Seihamer et al. (156) obtained a cDNA clone from human lung that encoded a 126-residue peptide virtually
identical with pancreatic phospholipase A₂. The deduced amino acid sequence differed from that reported by Verheij by only five residues and probably represents the correct structure for human pancreatic phospholipase A₂. The human sequence is highly conserved and shows 84% homology with canine, 89% with porcine, and 78% with rat pancreatic enzyme, but only about 50% with other, nonpancreatic phospholipases A₂.

Interest in phospholipases A₂ has been greatly stimulated by the discovery that they play a crucial role not only in normal cellular function but also in pathological processes. They have been implicated in the pathogenesis of cardiovascular, pulmonary, and gastrointestinal disorders, including pancreatitis. However, as will be shown below, it is leukocyte and not pancreatic phospholipase A₂ that is involved in the pathogenesis of these conditions. Leukocyte phospholipases A₂ are secreted as functionally active enzymes and belong to group II. Only one of them, platelet phospholipase A₂, has been purified and sequenced so far (76). It is a molecule consisting of 124 amino acid residues that shows features common to all known phospholipases A₂. It is a highly basic protein with a pI > 10.5 and a pH optimum of 8 to 10. An up-to-date review on phospholipases, their structure, properties, and functions has been published by Mansbach (98).

**Enzymatic Activity and Substrate Specificity**

Phospholipase A₂ hydrolyzes the sn-2 fatty acyl ester bond of 1,2-diacyl-sn-glycerophosphocholines, resulting in the production of highly cytotoxic lysocompounds (e.g., lyssolecithin from lecithin) capable of disrupting cell membranes by their detergent action. The enzyme acts on lipid–water interfaces, that is, on substrates present as micelles, as molecular dispersed solutions, or monomolecular surface films. Its potency depends on a variety of factors, such as substrate packing density, nature of the head group of the substrate, and temperature. For a detailed discussion of this topic see Mansbach (98). There are three important portions of the enzyme that have been identified: the active site, the interface recognition site, and the Ca-binding loop. The architecture of the active site is already present in the zymogen, which is capable of hydrolyzing monomeric substrates at about half the rate of the free enzyme. The enzyme has an absolute requirement for Ca ions, but its activity decreases with increasing Ca concentrations and at 0.4 mM Ca²⁺, it possesses only about 25% of its optimum activity (50). The pH optimum of the human enzyme appears to be closer to 6 than 8, the pH optimum for the porcine enzyme (50).

In contrast to the snake phospholipases A₂, human pancreatic phospholipase A₂ cannot penetrate and hydrolyze biological membranes, and pancreatic acinar cells are quite resistant to its action (57). The pancreatic enzyme has a minimal effect on red cells, whereas phospholipase A₂ from snake venom readily hydrolyzes them. Likewise, it is virtually unable to attack rat diaphragmatic muscle, which is easily hydrolyzed by venom phospholipase A₂. This remarkable impotence of pancreatic phospholipase A₂ toward intact cell membranes is another example of the numerous protective mechanisms that safeguard the pancreas from injury by inappropriately activated enzymes. Despite this handicap, pancreatic phospholipase A₂ is capable of digesting phospholipids in the intestinal lumen. This can be explained by a decrease in the surface pressure of the lipid emulsion present after a meal, brought about by the relatively high concentration of bile acids in the intestinal lumen. It is of interest to mention here that pancreatic phospholipase A₂ can be converted to a highly penetrating, membrane-bound form by site-specific ε-NH₂ monoacylation (182). Phospholipase A₂ performs another function in digestion: the preparation of the lipid–water interface for the action of pancreatic lipase. Thus neutral lipids surrounded by phosphatidylcholine are poorly hydrolyzed by lipase. Following phospholipolysis by phospholipase A₂, neutral lipid digestion is greatly accelerated. Phospholipase A₂ activity in the past has been determined by potentiometric titration of the fatty acids produced by its action on egg yolk emulsions (cf. 33), or turbidimetrically by the clearing action of lysolecithin, the product of phospholipase A₂ action on a lecithin emulsion. More convenient and specific methods are described below.

**Determination in Serum.** Small amounts of (pro)phospholipase A₂, like other pancreatic zymogens and enzymes, escape into the peripheral circulation. Elevated serum levels of phospholipase A₂ have been observed in acute pancreatitis. However, in this, as well as in such other conditions as septicemia, trauma, and burns, leukocytes contribute markedly to the serum concentration of active phospholipase A₂. A time-resolved fluoroimmunoassay, specific for pancreatic phospholipase A₂, measuring both zymogen and active enzyme (107), demonstrated elevated serum levels during the first week of pancreatitis, similar to other enzyme assays, but gave no useful information on the severity and prognosis of the disease. By contrast, a test kit (NEFA-CTest, Wako, Japan) that measures enzymatic activity only, clearly separated edematous from acute necrotizing pancreatitis. Furthermore, a photometric assay of active phospholipase A₂ in a patient with a duodenal ulcer and pancreatic involvement exhibited two markedly different, postoperative time courses when run at pH 6.5 and 8. Readings at pH 6.5 (pancreatic enzyme) increased to a maximum on day 2 and thereafter declined until the patient's death. Conversely, enzyme levels obtained at
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pH 8 (granulocyte phospholipase A₂) remained elevated until day 5, when they increased dramatically until the patient's demise on day 7 (58).

Phospholipase A₂ Inhibitors

Phospholipase A₂ is inhibited by ethylenediaminetetraacetate (EDTA) or high Ca concentrations. Inhibitors, such as aristolochic acid, manoolide, and retinoids, also have a direct effect on the enzyme itself. Another type of inhibitor, p-bromo-phenacyl bromide or 1-bromo-2-octanone, acts as an active site titrant, probably reacting irreversibly with a histidine residue at the active site (50). Some antimalarials, flavonoids, nonsteroidal anti-inflammatory drugs (indomethacin), xylocaine, and chlorpromazine also inhibit the enzyme, most likely by competitive inhibition of the interface binding (cf. 98). When administered to piglets with experimental pancreatitis, the latter drug caused marked improvement in the survival rate (106). Two proteins that were recently isolated from human monocytes—lipocortin I and lipocortin II—strongly inhibit pancreatic and leukocyte phospholipases A₂, as well as phospholipases C and D (cf. 98). Lipocortin I with a molecular weight of 36 kDa is identical with the cytoskeletal protein calpain II, while lipocortin II is the same as calpain I. The latter has been cloned (77) and, as a monomer, has a molecular weight of 38 kDa. These proteins exert their inhibitory action by binding directly to phospholipids, barring the enzyme access to its substrate. Binding is dependent on the presence of Ca. It is of interest to note that lipocortins can be induced by corticosteroids.

Lipase

The hydrolysis of dietary long-chain triacylglycerols in the human digestive tract is performed by at least three different lipases: a gastric, an intestinal, and a pancreatic lipase (see Gorelick et al., this volume). The pancreatic enzyme, which is secreted as a fully active enzyme, is by far the most important in the digestion of fat. Human lipase has been isolated and purified from pancreatic juice by de Caro et al. (23). It occurs in the form of two isoenzymes with a molecular weight of about 48,000 daltons. The isoelectric points of the two enzymes (5.8 and 5.85) suggest that one might be derived from the other by deamidation (hydrolysis of the carboxamide group of a glutamine residue to a carboxyl group). Lipase is a glycoprotein that consists of a single polypeptide chain of 449 amino acids (189). It shows extensive homology with the porcine enzyme and cross-reacts immunologically with lipase of six different mammalian species. However, in contrast to ovine and bovine lipases, only the human and porcine enzymes are glycosylated. A cDNA encod-

ing human pancreatic lipase has been isolated by Lowe et al. (96). The deduced amino acid sequence consists of 465 residues, including a 16 amino acid signal peptide. Human hepatic and lipoprotein lipases share extensive homology with the pancreatic enzyme. Structural investigations of human lipase by x-ray crystallography (189) provide evidence that Ser-152 is the nucleophile residue essential for catalysis and is part of an Asp-His-Ser triad, which is chemically analogous to, but structurally different from, that in the serine proteases. The putative hydrolytic site is covered by a surface loop that undergoes reorientation during interfacial surface activation, a characteristic of lipolytic enzymes acting on water-insoluble substrates.

Enzymatic Activity and Substrate Specificity

The activity of lipases is restricted to hydrophobic–hydrophilic interfaces and depends on the presence of substrates in micellar or emulsified form. The chemical structure of the substrate is therefore of crucial importance for the formation of optimal interfaces. Triglycerides with ester chain lengths of 4 to 10 carbon atoms represent particularly suitable substrates that form stable, finely dispersed emulsions, even at high concentrations. Ester bonds in the 1 and 3 positions of triglycerides are hydrolyzed first. However, lipase acts on all fatty acid esters, as long as they form emulsions and thus hydrophobic–hydrophilic interfaces. When the substrate is associated with bile salts and phospholipids, which is the case in vivo, lipase is inactive or inhibited. The presence of colipase (see below) restores lipolytic activity, but triacylglycerol digestive products associated with emulsified lipid also play an important role in the restoration of this activity (100). A detailed discussion of the catalytic activity and mechanism of activation of pancreatic lipase is presented in a review by Antonov et al. (5).

Simple fatty acid esters cannot be used for the specific determination of lipase because they are hydrolyzed also by other esterases. The most widely used assay has been the titrimetric determination of fatty acids liberated by the action of the enzyme on emulsions of olive oil (cf. 23). An optimized turbidimetric method has been described by Tietz et al. (176).

Determination in Serum. As noted already for other pancreatic enzymes, a low concentration of lipase is present normally in the blood circulation. The marked increase of serum lipase in acute pancreatitis has long been used as a diagnostic criterion in this disease. More convenient and sensitive methods for the determination of serum lipase have been developed recently. A lipase immunoassay has been described by Rizzotti et al. (147) and a highly sensitive, fluorometric assay, 50 times more sensitive than the turbidimetric method, has been developed by Salvayre et al. (149).
As pointed out previously, lipase is inhibited by bile acids and phospholipids. It is also inhibited by the potent lipase inhibitor tetrahydrolipstatin (Hoffmann-La Roche, Nutley, NJ) and is inactivated by phenylmethanesulfonyl fluoride, a reagent that reacts irreversibly with the active center of serine proteases.

*Pancreatic Fatty Acid Ethyl Ester Synthase*. A 52-kDa enzyme has been isolated recently and purified to homogeneity from human pancreas by Riley et al. (130). It is structurally related if not identical with triglyceride lipase and catalyzes the synthesis and hydrolysis of ethyl oleate from oleic acid and ethanol at rates of 2400 nmol/mg/hr and 30 nmol/mg/hr, respectively. The enzyme is present in the pancreas at 0.6 to 1.2 mg/g tissue and has a pronounced substrate specificity for unsaturated octadecanoic acids such as oleic, linoleic, and linolenic acids. Similar rates of ethyl ester synthase activity were observed with commercial porcine triglyceride lipase. The purified synthase also hydrolyzes triglycerides and this activity is stimulated by colipase and inhibited by sodium chloride.

The high level of pancreatic fatty acid ethyl ester synthase activity exhibited by lipase, which is synthesized in high concentrations within the cell and has no absolute requirement for an effector such as bile salts or colipase in this nonoxidative pathway for ingested ethanol, may be an important factor in the altered lipid metabolism and pathogenesis of alcoholic pancreatitis.

*(Pro)colipase*

Colipase is a protein cofactor essential for the efficient digestion of dietary fat by lipase as indicated earlier. In contrast to lipase, it is secreted as a zymogen that is hydrolyzed by trypsin to generate active colipase. It is present in the pancreas of all vertebrates tested so far. The cDNA sequence and deduced amino acid sequence of human procolipase have been reported recently by Lowe et al. (95) and Renaud and Dagorn (128). The complete preprocolipase comprises 17 amino acid residues for a prepeptide, 95 residues for procolipase, including a propeptide of 5 residues. The predicted amino acid sequence agrees completely with the sequence obtained by chemical methods (170). The molecule is cross-linked by four disulfide bridges and has a molecular weight of about 9800 to 10,000 daltons. Colipase is a glycoprotein and has been reported to occur in the form of two isoenzymes of identical amino acid sequence. However, it is likely that one of the variants is derived from the other by deamidation (170).

*Enzymatic Activity and Mode of Action*

Hydrolysis of triglycerides by lipase is strongly inhibited by bile salts present in the duodenum, and colipase seems to be the only agent known to counteract this inhibition. Lipase, as pointed out previously, possesses affinity only toward hydrophobic phases and cannot act on hydrophilic substrates on its own. Colipase has strong affinity for bile salt micelles and other polar interfaces. It is thought that it may form a ternary complex with bile salts and lipase through which it could provide an anchor for lipase on more hydrophilic surfaces, thus opening the range of substrates for the latter enzyme. Ca ions stimulate cofactor activity by removing fatty acids and preventing a shift of the pH optimum to lower values. The importance of mono- and diacylglycerols in the interaction of lipase and colipase has been mentioned already (100).

Colipase can be assayed by its activating effect on lipase in an emulsion of olive oil containing sodium deoxycholate, CaCl₂, and NaCl. An enzyme-linked immunosorbent assay (ELISA) has been developed recently by de la Fournier et al. (26). The useful range of the assay is 2 to 20 ng/ml, about 1000 times less than the amount that can be detected titrimetrically. Under standard conditions, the method did not distinguish between zymogen and enzyme. The inclusion of the detergent Tween 20 in the coating of the immunoplates with antigen prevented the binding of the zymogen to the plate and permitted selective assay of the active enzyme.

*Carboxyl Ester Hydrolase (EC 3.1.1.1), also known as Carboxyl Ester Lipase, Lyso phospholipase (EC 3.1.1.5), and Cholesterol Ester Hydrolase (Sterol Ester Acylhydrolase, EC 3.1.1.13)*

Pancreatic carboxyl ester hydrolase has an unusually broad substrate specificity. This has led to considerable confusion among investigators of the pancreas. Enzymes corresponding to carboxyl ester hydrolase isolated from pancreas of different species have often been named after the type of substrate used for the determination of their respective activities, for example, nonspecific lipase, carboxyl ester lipase, cholesterol ester hydrolase, sterol ester hydrolase, and lyso phospholipase (phospholipase A₁). However, all available evidence suggests that these activities originate in one and the same functional entity (see references in ref. 109). The human enzyme was first isolated and purified from fresh human pancreatic juice by Lombardo et al. (91). More efficient and practical procedures have been published more recently by Abouakil et al. (2) and Wang (184). The enzyme is a glycoprotein with a molecular weight of 105,000 daltons. Its primary structure is similar if not identical with that of the human milk-bile salt-stimulated lipase whose amino acid sequence has been deduced from the sequenced cDNA by Nilsson et al. (109). The two enzymes seem to have identical protein cores starting with a 23-residue leader peptide, which is continued by 722 amino acid residues.
Both are characterized by an unusually high proline content (13%) and cross-react immunologically. The somewhat higher molecular weight of the "milk-lipase" (125,000 daltons) may be due to a different pattern of glycosylation. However, the different molecular weights of carboxyl ester hydrolases from different species are not solely due to differences in glycosylation (the rat enzyme is not glycosylated!), but instead reflect variable numbers of an 11-amino acid repeat (109) in their structure. Both carboxyl ester hydrolase and milk–bile salt–stimulated lipase are new members of the superfamil

Enzymatic Activity and Substrate Specificity

Carboxyl ester hydrolase, contrary to lipase, hydrolyzes a great variety of lipid substrates. In vitro it hydrolyzes not only mono-, di-, and triacylglycerols, but also cholesteryl and retinyl esters and lysophosphatidylglycerols. Moreover, it hydrolyzes emulsified, micellar, and soluble substrates at similar rates. Bile salts are a prerequisite for hydrolysis of emulsified long-chain triacylglycerols (octanoyl groups and longer). They act as activators by inducing an enzyme–bile salt–substrate ternary complex. At the same time they serve as fatty acid acceptors to relieve product inhibition. By contrast, lipase (in the absence of colipase) is inhibited by bile salts and strongly binds to lipids and artificial surfaces, whereas carboxyl ester hydrolase is only weakly activated by interfaces. Hydrolysis of short-chain lecitins and lysolecithins has been studied mostly with bovine pancreatic lysophospholipase. However, the finding that lysolecithin hydrolysis is inhibited by deoxy- and taurocholate indicates that the primary function of this enzyme may not be the hydrolysis of lysolecithins (25,181). A study of the relative roles of carboxyl ester hydrolase and lipase in the digestion of lipids by Lindstrom et al. (89) indicates that both enzymes are needed for hydrolysis of cholesteryl and retinyl esters present in the physicochemical form of dietary fat during digestion, that is, in phospholipid-covered triacylglycerol oil emulsion droplets. Lipase hydrolyzes the triacylglycerol oil phase, which leads to the transfer of lipids from the oil phase to bile salt–lipolytic product aggregates in the aqueous phase. The main function of carboxyl ester hydrolase under physiological conditions probably is to hydrolyze retinyl and cholesteryl esters.

Kyger et al. (78) recently reported that a 100-kDa cholesterol esterase, like pancreatic fatty acid ethyl ester synthase mentioned previously, exhibits bile salt dependent as well as independent synthetic pathways for cholesteryl esters.

Carboxyl ester hydrolase can be determined with p-nitrophenyl acetate or vinyl 8-phenyloctanoate as substrates. An assay utilizing fluorescent esters of pyrene-2,3-dicanoic or -butanoic acid and cholesterol has been described recently by Negre-Salvayre et al. (105). The pH optimum was 7.5 to 8.0 and maximal stimulation was obtained with 5 mM sodium cholate.

Determinant in Serum. An enzyme-linked immunosorbent assay for serum carboxyl ester hydrolase has been developed by Blind et al. (15). The test exhibited a high sensitivity for acute pancreatitis (98%) and was capable of distinguishing between the interstitial and necrotizing form of the disease. Serum levels after the second day of admission remained high in patients with necrotizing pancreatitis, whereas those in patients with interstitial pancreatitis decreased notably during the following 10 days.

Immunohistochemical studies in acute pancreatitis revealed interstitial localization of carboxyl ester hydrolase around necrotic pancreatic lobules and areas of fat necrosis, a staining pattern similar to that of other lipolytic enzymes, but different from that of trypsin and chymotrypsin (4). This observation suggests a combined action of carboxyl ester hydrolase and lipase in the production of fat necroses in acute pancreatitis.

α-Amylase

Human amylase occurs in pancreatic tissue and secretion, parotid glands, saliva, serum, urine, and possibly in lung, liver, and genital tissue. α-Amylase also has been found in normal as well as malignant thyroid tissue (parotid type, AMY 1), breast carcinoma (pancreatic type, AMY 2), and lung carcinoid tissue (AMY 2B; different from AMY 1 and AMY 2) (60,193). Pancreatic amylase (AMY 2) is by far the most important. Like other pancreatic enzymes, it plays a vital role in human nutrition and, because of its ready detection in serum, has long been the most widely used biochemical criterion in the diagnosis of pancreatic disease. α-Amylase has been isolated from human pancreatic juice, purified and characterized by Stievel and Keller (171). It is a glycoprotein with a reported molecular weight of 50,000 to 56,000 daltons and an isoelectric point of 7.1. It consists of a single chain of amino acids and contains carbohydrate in a ratio of 1 mol/mol enzyme. By contrast, one of the two parotid amylase isoenzymes (AMY 1) seems to be non-glycosylated (171). The amino acid sequences of both,
the pancreatic and the parotid enzymes, have been deduced from the cDNAs isolated from a cloned cDNA library (190). The pancreatic α-amylase AMY 2 consists of a 511-residue preamylase polypeptide with an amino-terminal signal peptide of 15 amino acid residues. The structure of the pancreatic amylase isoenzyme AMY 2A, according to Wise et al. (190), differs from the latter by 17 amino acid positions. AMY 2B from lung carcinoma tissue differs from pancreatic α-amylase by only 6 amino acid positions (193). Whereas the parotid amylases are extremely stable at low temperature, pancreatic amylases lose activity even when stored at −20°C. They are also more readily denatured at elevated temperature than the parotid enzymes. This and other properties that distinguish the two amylases from one another (below) indicate a less compact configuration for the pancreatic enzyme. Human pancreatic amylase possesses an SH group that is essential for activity and appears to be more accessible in the pancreatic than in the parotid enzyme. Differences between the very similar gene structures of AMY 1 (parotid) and AMY 2 (pancreatic) have been reported by Horii et al. (59). A comparison between the complete amino acid sequence of porcine and human pancreatic α-amylase and location of the five disulfide bridges has been presented by Pasero et al. (119).

Many studies using chromatography, electrophoresis, and isoelectric focusing techniques have shown the presence of multiple isoamylase forms in pancreatic and parotid tissue and fluid as well as in serum and urine (148). However, evidence has been accumulating suggesting the existence of only one genetically determined form of pancreatic α-amylase and one form of parotid amylase, which are immunologically similar but differ chemically from each other (93) (see, however, ref. 190). It has been shown conclusively that additional isoamylases are formed by post-translational changes, probably by oxidative deamidation. Warshaw and Lee (185) demonstrated by electrophoresis of serum that the main peak (80–90% of total enzyme) of three pancreatic isoamylases decreased successively during 6 hr incubation at 37°C, whereas the two minor peaks exhibited simultaneous dramatic increases. However, genetic polymorphism of human pancreatic α-amylase has been reported by Kompf et al. (74), but the frequency of the additional AMY alleles was insignificant (0.048–0.001).

Enzymatic Activity and Substrate Specificity

The main function of pancreatic α-amylase is to catalyze the hydrolytic degradation of starch (amylose and amylpectin from plant sources) and glycogen (from animal sources). α-Amylase hydrolyzes the branched-chain amylpectins at the more central 1,4-glycoside linkages to produce short-chain polysaccharides termed dextrans. An intestinal enzyme, 1,6-oligo-glucosidase then splits the 1,6-linkages of the dextrans and permits the action of α-amylase to continue to form maltotetrose, maltohexaose, maltose, and isomaltose. Finally, the disaccharides are cleaved to glucose by intestinal maltase. α-Amylase hydrolyzes starch by multiple attack through cleavage of several bonds during the lifetime of the enzyme–substrate complex (171). Amylases from different species display different degrees of multiple attack toward substrate. The different action patterns appear to depend on the number of —SH groups in the enzyme. Thus porcine α-amylase with two —SH groups/enzyme molecule exhibits a higher degree of multiple attack than the human enzyme with only one —SH group. The action of α-amylase is dependent on the presence of Ca²⁺ and Cl⁻. A chloride concentration of 10 mM appears to be essential for optimum hydrolytic activity. The pH optimum for the enzyme is 7.5 to 8.0. It is inhibited by Cu²⁺ and other heavy metals. Iodoacetate also has an inhibitory effect, particularly after removal of Ca²⁺ from the molecule by EDTA.

Determination in Serum and Urine. The importance of amylase in the diagnosis of pancreatic disease has generated volumes of literature on methods for its determination, probably reflecting the lack of a perfect test. Common procedures are based either on the disappearance of starch (amyloclastic methods) or the appearance of reducing sugars (saccharogenic methods). A third, colorimetric method, probably the most convenient and sensitive test, utilizes an insoluble starch substrate, covalently labeled with a dye, which is solubilized by the action of amylase (146). The discovery of a wheat protein that selectively inhibits parotid amylase has led to the development of an assay specific for pancreatic α-amylase (111). A commercial isoamylase assay combines the two principles (Phadebas, Pharmacia Diagnostics, Piscataway, NJ). An alternative, rapid, and simple method that uses monoclonal antibodies that specifically inhibit salivary amylase has been evaluated by Steen et al. (167), and a radioimmunoassay for pancreatic α-amylase has been described by Fujita et al. (38). The capabilities and advantages of electrophoretic methods for the specific determination of pancreatic α-amylase have been reviewed by Mifflin et al. (101) and reviews on the clinical significance of isoamylase determinations have been presented by Skrha and Stepan (163) and Pieper-Bigelow et al. (123).

Pancreatic Ribonuclease

Pancreatic ribonucleases (RNases) from species other than humans have been studied extensively, partly because of their ready availability and partly because of their unusual stability. RNase occurs in pancreatic tissue and secretion (65, 178), but its physiological function in this fluid is a matter of conjecture. The low level of en-
zyme present in human pancreatic tissue (<5 μg/g tissue compared with 1200 μg/g in bovine pancreas) suggests that the enzyme may be vestigial in nonruminant vertebrates, where it is no longer needed for recovery of phosphorus from bacterial RNA, as is the case in ruminants.

Human RNase has been isolated from pancreatic tissue and characterized by Weickman et al. (186). Its complete amino acid sequence has been determined by Beintema et al. (11), and its molecular weight is estimated to be 15,000 daltons. It occurs in two forms, one of which is glycosylated and has a high content of fucose. Pancreatic-type RNase also occurs in serum, urine, and seminal plasma. Urinary RNase, a protein consisting of 128 amino acid residues, according to Beintema et al. (9), differs from pancreatic RNase only in the presence of an additional threonine residue at the C terminus and in its higher degree of glycosylation. Sequence data on the major RNase in seminal plasma revealed no differences between it and the major urinary enzyme, suggesting that the structural differences between the secretory RNases from human pancreas, urine, and seminal plasma must originate from organ-specific post-translational modifications of the one primary gene product. A blood vessel-inducing protein—angiogenin—obtained from a human tumor cell line also has shown considerable sequence homology with pancreatic ribonuclease, which includes all three catalytic residues, His-12, Lys-41, and His-119 (157). However, it does not hydrolyze the customary RNase substrates [poly(C), poly(U)], but it does cleave 28S and 18S RNA. This finding may bear importantly on the physiological function of angiogenin.

Enzymatic Activity and Substrate Specificity

Secretory RNases from different organs, including human pancreatic RNase, have very similar amino acid compositions. They contain many positively charged amino acids near the amino terminus and several negatively charged ones at the carboxyl terminus. Such RNases have a high degree of activity against double-stranded RNA. They exhibit optimum activity at pH about 8 and avidly cleave the synthetic substrate polycytidylic acid. By contrast, the nonsecretory RNases (from liver, kidney, spleen, and leukocytes) have a low preference for polycytidylic acid and hydrolyze RNA most effectively at pH 6.5 to 7.0. Activity is measured by the formation of perchloric acid-soluble nucleotides from wheat germ RNA or polycytidylic acid (cf. 186) or by radioimmunoassay (164).

Pancreatic RNase is inactivated by treatment with iodoacetate at pH 5.5, which carboxymethylates the imidazole ring of a critical histidine residue. Protein inhibitors of RNase have long been known to be present in mammalian tissues, for example, human placenta. Placental RNase inhibitor is a labile, sulphhydryl-dependent protein with a molecular weight of about 51,000 daltons. It is extremely potent (Kᵢ = 0.3 nm) and inhibits not only pancreatic RNase but also the enzymatic as well as biological activity of angiogenin (158).

Determination in Serum and Urine. It has been suggested that serum and urinary RNases may be of pancreatic origin and that elevated levels of these enzymes may be diagnostic of pancreatic disease, particularly cancer of the pancreas. However, initially promising results reported by Reddy et al. (126) could not be confirmed. Both secretory and nonsecretory ribonucleases occur in body fluids, including urine and serum (cf. 10), and it has been shown conclusively by Abramson et al. (3) that the increase in serum RNase does not necessarily originate from the pancreas, because serum RNase in patients with cancer of the pancreas increased to even higher levels after pancreatectomy and remained elevated for more than 3 weeks postoperatively.

Pancrætic Deoxyribonuclease I (DNase I)

DNase I is present in pancreatic tissue as well as in fluid (94,178). Its physiological function in pancreatic secretion, as in the case of RNase, is a matter of speculation. Interest in this enzyme stems from the detection of DNases in serum and urine, similar to those found in pancreas, and the possibility that they may serve as diagnostic markers in pancreatic disease (94,192). Funakoshi et al. (41) reported that DNase I output in duodenal juice after stimulation with secretin–cholecystokinin was much lower than normal in patients with chronic pancreatitis or cancer of the pancreas. However, a recent study of 117 patients with pancreatic and nonpancreatic disease by Basso et al. (8) concluded that serum DNase is an even less satisfactory index of pancreatic disease (including cancer of the pancreas) than other pancreatic enzymes.

Love and Hewitt (94) in 1979 reported the isolation and purification of the major form of DNase I from human serum and pancreatic tissue and compared the properties of the two enzymes. The two DNases exhibit essentially the same physical and enzymatic characteristics and thus may originate from the same source. Their molecular weight is estimated to be about 33,000 to 38,000 daltons and they have isoelectric points in the range of 3.9 to 4.3. Optimal activity at pH 7 is dependent on the presence of Ca²⁺ and Mg²⁺. A DNase I, with virtually identical properties, has also been isolated from duodenal juice by Funakoshi et al. (40), and a DNase I strongly resembling pancreatic DNase has been isolated from urine, purified and characterized by Yasuda et al. (192). It is a glycoprotein and consists of multiple forms with different isoelectric points.

Both serum and pancreatic DNases are endo-DNases by virtue of their ability to react with covalently closed
circular PM2 DNA. A highly sensitive assay using closed circular DNA from the marine bacteriophage PM2 has been described by Love and Hewitt (94) and additional methods are referred to in their article. A radioimmunoassay for human serum and urinary DNase has been developed by Miyachi et al. (102). G-actin, a naturally occurring DNase inhibitor, did not affect immunoreactivity.

DNase I is inhibited 50% by the presence of 10^{-4} M Zn, by 165 mM NaCl, and by 2-mercaptoethanol in the presence of calcium chelators. A highly specific DNase inhibitor has been isolated from pancreatic juice and identified with actin of skeletal muscle by Funakoshi et al. (42). The secretin–cholecystokinin-stimulated output of this inhibitor was significantly reduced in patients with chronic pancreatitis.

**PROTECTION OF THE PANCREAS FROM INAPPROPRIATE ACTIVATION OF ZYMOGENS**

It is generally believed that premature intrapancreatic activation of zymogens by trypsin may occur in the early phases of acute pancreatitis, and that subsequent inflammatory and necrotic changes in the pancreas may be the result of autodigestion of the organ by its own, inappropriately activated enzymes. The crucial event in this process would be the activation of trypsinogen to trypsin and the release of a cascade of free enzymes from their inactive zymogens. This was generally thought to occur intraductally by autoactivation of trypsinogen (34,63) during stasis of secretions behind a duct obstruction, or by enteropeptidase entering the pancreatic duct system by biliary–pancreatic reflux (49). A different mechanism of intrapancreatic activation of zymogens was suggested by Steer et al. (168). Cytological studies at the ultrastructural level in experimental pancreatitis demonstrated co-localization of zymogens and lysosomal enzymes within large intracytoplasmic vacuoles, and concurrent biochemical tests indicated activation of trypsinogen by the lysosomal hydrolyase cathepsin B in the acid environment of the vacuole. These findings were thought to reflect the initial phase of potentially uncontrolled zymogen activation and subsequent autodigestion of the organ. This and other hypotheses are discussed elsewhere in this volume.

The pancreas is protected from the potentially destructive action of its own digestive enzymes by a formidable array of barriers (cf. 131,132).

1. Enzymes and zymogens are synthesized as inactive presecretory proteins.
2. They are sequestered by lipoprotein membranes as zymogen granules within the acinar cell.
3. Zymogens require a trigger enzyme (trypsin) for activation, which itself is released extrapancreatically from its zymogen—trypsinogen.
4. Intrapancreatic release of trypsin can activate an enzyme(s) that readily degrades trypsinogen and other zymogens (131).
5. The pancreas contains an inhibitor capable of trapping up to 20% of the potentially available trypsin activity.
6. Lysosomal hydrolases, normally present in zymogen granules, can digest zymogens and enzymes either within the zymogen granule or colocalized with zymogens in cytoplasmic vacuoles when normal secretion of granule contents is impaired or blocked (see below).
7. Pancreatic acinar cells are remarkably resistant to the action of trypsin, chymotrypsin, and pancreatic phospholipase A_{2}.

Protective mechanisms 1 and 2 are dealt with elsewhere in this volume; mechanism 3 has been discussed under the section Trypsinogen (above) and mechanisms 6 and 7 will be discussed under the section Pancreatic Lysosomal Enzymes. The following paragraphs therefore focus on mechanisms 4 and 5. Since plasma protease inhibitors play a vital role in the defense of the organism against the effects of pancreatic as well as other proteases released into the circulation during pancreatitis, a brief description of these inhibitors is included.

**Intrapancreatic Degradation of Zymogens**

An additional agent, complementary to the pancreatic secretory trypsin inhibitor (PSTI), for the protection of the pancreas from prematurely activated enzymes has been described by Rinderknecht et al. (140). Whereas trypsin, liberated intrapancreatically, is rapidly inactivated by PSTI at neutral pH, mesotrypsin (trypsin-2) activity is not affected by this inhibitor. Extremely small amounts of mesotrypsin under conditions prevailing in pancreatic juice (pH > 7 and low calcium concentration, <1 mM) are capable of initiating rapid degradation rather than activation of trypsinogen and removing the source of the potential trigger enzyme for the pancreatic enzyme cascade along with other zymogens before the supply of PSTI is exhausted by complex formation with free enzyme (134,140). Thus incubation of pancreatic juice with mesotrypsin (600 ng/ml incubation mixture) at 37°C for 30 min degraded about 50% of trypsinogen, chymotrypsinogen, proelastase, and PSTI to inert products without apparently causing any activation of trypsinogen (PSTI was not inactivated by liberated trypsin but was degraded by mesotrypsin!). However, zymogen degradation may be more complex as indicated by recent evidence that pancreatic juice contains an enzyme or zymogen, different from the known pancreatic proteases, that might be activated by traces of trypsin or mesotrypsin and cause the destruction of zymogens (134).
Trypsin Inhibitors

The kinetics of enzyme inhibition are determined by a variety of factors, including the type of interaction between the enzyme and inhibitor, concentration of reactants, pH, and reaction time. Inhibition can be irreversible or reversible, and in the latter case may be competitive, noncompetitive, or of a mixed type. Reaction between enzyme and inhibitor can be instantaneous or require an extended period of time. The effectiveness of an inhibitor is normally expressed by the kinetic constant \( K_i \), which is the reciprocal of the enzyme–inhibitor affinity. (For a comprehensive treatment of this topic, the reader is referred to ref. 155). Protease inhibitors described in this chapter cover a variety of types, and their mode of action is mentioned along with their chemical and physical properties.

Pancreatic Secretory Trypsin Inhibitor (PSTI)

Isolation and Characterization

Pancreas and pancreatic fluid from mammalian species, including human, contain a basic polypeptide, pancreatic secretory trypsin inhibitor (PSTI), which inhibits trypsin by forming an inactive, relatively stable complex with the enzyme. It is termed "Kazal" pancreatic inhibitor after Kazal, who first isolated this substance from bovine pancreas (cf. 125). It should not be confused with the "Kunitz" bovine pancreatic trypsin or kallikrein inhibitor (Trasylol, Aprotinin), which occurs mainly in pancreas, lung, and other tissues of ruminant species. However, contrary to common belief, Kunitz-type trypsin inhibitor does occur in humans as well. It has been isolated from serum, purified and characterized by Fioretti et al. (35). Its chemical, immunological, and functional properties are very similar to those of the bovine "Kunitz" inhibitor (Trasylol). Human PSTI (Kazal type) has been isolated from pancreatic tissue and fluid and characterized by Pubols et al. (125), and its amino acid sequence and primary structure have been reported by Bartelt et al. (7). The pure polypeptide, a heat- and acid-stable linear structure of 56 amino acids with three disulfide bridges, was obtained as five chromatographic forms by gel permeation, ion-exchange chromatography, and electrophoresis, and had a molecular weight of 6242 daltons. All five forms had an identical amino acid composition and inhibitor activity. Available evidence suggested that deamidation and probably formation of \( \beta \)-aspartylpeptide bonds (125) may account for the multiple forms.

Immunoreactive PSTI has also been detected in human gastric mucosa, and a form of PSTI is synthesized by rat liver. The purified human inhibitor from gastric mucosa consists of 56 amino acids and is identical in its chemical and biological properties with the inhibitor from human pancreas (160). An inhibitor similar or identical with PSTI has been identified in urine and sera of patients with ovarian cancer, and very high concentrations of this inhibitor in association with two trypsinogen isoenzymes (see above) have been found in extracts of ovarian tumors (169). Interestingly, copious amounts of PSTI were found also in the serum-free culture medium of an established human pancreatic adenocarcinoma cell line. This supports the view that elevated serum levels of PSTI in cancer-bearing patients may be due to secretion of this peptide by the tumor cell per se (113), but contrasts sharply with the consistently below normal PSTI levels reported by Rinderknecht et al. (143) in pancreatic juice of patients with cancer of the pancreas. An extrapancreatic origin (liver?) of serum PSTI is suggested by the finding that PSTI is an acute-phase reactant whose serum concentrations increase remarkably in response to surgical stress, severe infections, pancreatitis, and most types of cancer at advanced stages. It is noteworthy that the magnitude of this increase is far greater than that of other known acute-phase reactants (114).

There is marked homology of human, bovine, porcine, and ovine PSTIs, but homology is not restricted to mammalian PSTIs and extends in varying degrees to other proteins widely distributed in nature, for example, inhibitors from boar seminal plasma, dog submandibular gland, Streptomyces alboegriseus, and epidermal growth factor. The observation that PSTI stimulates DNA synthesis in human fibroblasts and growth of endothelial cells further emphasizes that PSTI may play a more general and fundamental physiological role than previously thought.

Mode of Action and Specificity

PSTI possesses a reactive site, -Lys-Ile-, which serves as a specific target substrate for trypsin (7). Incubation of equimolar quantities of enzyme and inhibitor rapidly (in less than 3 min) produce a stable complex with a covalent bond between the catalytic serine residue of the enzyme and the lysine carboxyl group of the reactive site of the inhibitor. However, prolonged incubation of the complex results in hydrolysis of the reactive-site peptide bond, regeneration of the enzyme, and the production of a modified inhibitor molecule (temporary inhibition) (85). By the same specific interactions, the inhibitor also forms a complex with trypsinogen, which, however, dissociates more readily than that with the enzyme. Inhibition of trypsin is linear with increasing PSTI concentrations until about 90% of the enzyme activity has been abolished. A low level of trypsin activity persists even when the inhibitor is present in a large excess, due to the high rate of dissociation of the enzyme–inhibitor com-
plex. Homologous bovine or porcine PSTIs do not effectively inhibit human trypsins. Unlike the Kunitz-type inhibitor (Trasylol, Aprotinin), PSTI does not inhibit kallikrein or chymotrypsin, and it is completely ineffective in abolishing mesotrypsin activity (140).

**Assay of PSTI**

Inhibitor activity can be determined by measuring residual trypsin activity after incubation of a standard solution of trypsin with PSTI (125,141), or by radioimmunoassay (69). As mentioned previously, PSTI serum concentrations are elevated in a variety of severe diseases and can provide useful information in the diagnosis and prognosis of pancreatic disease (69).

**Other Trypsin Inhibitors**

Protease inhibitors, and in particular trypsin inhibitors, are widely distributed in nature and have been studied extensively. In addition, a large number of synthetic inhibitors have been prepared. Table 2 lists some of the most commonly used inhibitors of trypsin and gives a comparison of their relative effectiveness against the three human pancreatic trypsin variants.

**Synthetic Protease Inhibitors: FOY, FOY-305, and FUT-175**

Prompted by the notion that acute necrotizing pancreatitis results from autodigestion of the pancreas by its own, prematurely activated proteases, intensive efforts have been made to find inhibitors of these enzymes for potential therapeutic use. Several promising agents have been described recently: ethyl-p-(6-guanidinoheanoyloxy)-benzoate methanesulfonate (gabexate mesilate or FOY), N,N-dimethylcarbamoylmethyl-4-[(4-guanidino- benzoyloxy)-phenylacetate methanesulfonate (camostate mesilate or FOY-305), and 6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate (nafamostate mesilate or FUT-175). These synthetic, low-molecular-weight compounds inhibit a broad spectrum of serine proteases, including trypsin, chymotrypsin, elastase, kallikrein, plasmin, thrombin, Clr and Cl esterases, and also to a lesser extent phospholipase A2 (see references in refs. 117 and 172). When given prophylactically in secretagogue- or diet-induced acute pancreatitis in experimental animals (81,117,172,191), they significantly reduced serum amylase levels and improved the survival rate of the animals, but showed no favorable effect when administered therapeutically. Oshio et al. (117) showed that both FOY and FOY-305, prevented cathepsin B redistribution to the more zymogen granule-enriched fraction—characteristic in both secretagogue- and diet-induced acute pancreatitis—indicating that a protease sensitive to these inhibitors is involved in this phenomenon. However, as FOY, but not FOY-305, prevents vacuole formation and increase in soluble amylase activity in the “diet” model (117), the mechanism by which these agents exert their protective effect remains unclear.

**PLASMA PROTE(IN)ASE INHIBITORS**

Small amounts of pancreatic enzymes and zymogens are discharged normally into the bloodstream. During pancreatitis the flow of such enzymes into the peripheral circulation increases dramatically. The potentially disastrous effects of these enzymes on the organism are kept in check by a number of protease inhibitors present in normal plasma. Four of at least seven such inhibitors are capable of binding and inactivating pancreatic proteases. They are listed in Table 3.

**α1-Proteinase Inhibitor (α1-Antitrypsin)**

This protein is by far the most copious plasma protease inhibitor on a molecular basis (Table 3) and accounts for about 90% of the inhibitor capacity of plasma. It also is the most studied because its genetic deficiency strongly predisposes individuals homozygous for its Z allele to emphysema and liver disease (120). The complete DNA sequence for α1-proteinase inhibitor has been published by Long et al. (92). The inhibitor is a sensitive,

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Source</th>
<th>mg/100 ml</th>
<th>Molecular weight</th>
<th>Carbohydrate content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic secretory trypsin</td>
<td>Pancreatic juice</td>
<td>5–10</td>
<td>6200</td>
<td>—</td>
</tr>
<tr>
<td>inhibitor</td>
<td>PSTI</td>
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<tr>
<td>α1-Proteinase inhibitor (α1-antitrypsin)</td>
<td>Plasma</td>
<td>140–460</td>
<td>50,000–55,000</td>
<td>12</td>
</tr>
<tr>
<td>α2-Macroglobulin (α2-M)</td>
<td>Plasma</td>
<td>190–390</td>
<td>720,000</td>
<td>8</td>
</tr>
<tr>
<td>Inter-α1-trypsin inhibitor</td>
<td>Plasma</td>
<td>20–70</td>
<td>160,000–200,000</td>
<td>8.4</td>
</tr>
<tr>
<td>α1-Antichymotrypsin</td>
<td>Plasma</td>
<td>14–35</td>
<td>65,000–68,000</td>
<td>27</td>
</tr>
</tbody>
</table>


heat-labile 55,000-dalton glycoprotein consisting of a single chain of 394 amino acids and three asparagine-linked complex carbohydrate side chains. The molecule has a globular shape and three internal salt bridges. It occurs in two major isoforms in serum, depending on the presence of a bi- or triantennary configuration for the carbohydrate side chains. The isoelectric point of these isoforms is 4.4 to 4.6. The active site (P residue) is methionine. The protein is synthesized mainly in the liver but is also expressed in monocytes and macrophages where synthesis is regulated directly by neutrophil elastase. Restriction length and DNA sequence analysis have now made it possible to identify more than 75 allelic variants. The most common normal variant is designated M, the one exhibiting the highest isoelectric point Z.

$\alpha_1$-Proteinase inhibitor forms inactive complexes with a wide variety of proteases, including trypsin, chymotrypsin, and elastase, as well as granulocyte and microbrial proteases. The reaction with these enzymes occurs in an equimolar ratio and is rapid in most cases: less than 7 sec for trypsin but may take hours for others (e.g., kalirein). During this reaction a peptide bond adjacent to the reactive-site methionine of the inhibitor is cleaved by the protease with a simultaneous, irreversible structural rearrangement of the inhibitor molecule. The fact that DFP-treated proteases fail to react with inhibitor suggests that the serine residue of the active site of the enzyme is involved in this reaction. The inhibitor-protease complex is covalently stabilized and resistant to dissociation by denaturing agents. Complex formation releases a 4000-dalton carboxy-terminal fragment that possesses a high degree of chemotactic activity (see references in ref. 120) and clings tenaciously to the parent protein. Recent evidence indicates that not only active proteases, but also their precursors [e.g., chymotrypsinogen (45) and proelastase 2 (83)], are capable of forming complexes with $\alpha_1$-proteinase inhibitor. $\alpha_1$-Proteinase inhibitor-bound enzymes or zymogens are totally inactive, even toward small, synthetic substrates. However, they are capable of dissociating and re-equilibrating with other protease inhibitors, for example, $\alpha_2$-macroglobulin. The in vivo half-life of $\alpha_1$-proteinase inhibitor complexes is relatively short, for example, about 3.5 hr for $\alpha_1$-proteinase inhibitor trypsin–complex, versus about 5 days for the inhibitor protein itself. $\alpha_1$-Proteinase inhibitor shares structural and functional characteristics with many other protease inhibitors such as antithrombin III, $\alpha_2$-antichymotrypsin, C1 inhibitor, $\alpha_2$-antiplasmin, plasminogen-activator inhibitor, and ovalbumin (120).

$\alpha_1$-Proteinase inhibitor diffuses into most tissue and body fluids. It is an acute-phase reactant and its plasma concentration can increase three- to fourfold during the inflammatory process. A major physiological function of $\alpha_1$-proteinase inhibitor is the control of neutrophil elastase, one of the critical agents in the pathogenesis of emphysema. The kinetics of association of neutrophil elastase and $\alpha_1$-proteinase inhibitor are more favorable by several orders of magnitude than those of any other serine protease. It is hypothesized that an imbalance of functional $\alpha_1$-proteinase inhibitor (as in individuals homozygous for the Z allele) and elastase in the local environment of the lung may result in destructive lung disease. However, other factors are capable of modifying net functional activity of $\alpha_1$-proteinase inhibitor. Thus neutrophils and macrophages can release oxidants that inactivate the reactive-site methionine of the inhibitor. They also secrete a thiol protease—cathepsin L—that is capable of cleaving and inactivating the inhibitor protein.

$\alpha_1$-Proteinase inhibitor may have functional activities other than inhibition of serine proteases. The carboxy-terminal fragment released during complex formation with a protease possesses chemoattractant activity equivalent to that elicited by formyl-methioninyl-leucyl-phenylalanine and may also be involved in the regulation of the synthesis of $\alpha_1$-proteinase inhibitor in human monocytes and macrophages. Reviews, including numerous references, have been presented by Perlmuter and Pierce (120) and Travis and Salvesen (180).

$\alpha_2$-Macroglobulin ($\alpha_2$-M)

Human $\alpha_2$-macroglobulin is a sensitive, heat- and acid-labile plasma glycoprotein (cf. Table 3). Relatively high concentrations of $\alpha_2$-M have also been found in lymph and colostrum. The major source of $\alpha_2$-M are hepatocytes, but other cells (e.g., macrophages) also synthesize and secrete this protein. It is a large molecule with a molecular weight of 720,000 daltons and a carbohydrate content of 8%. It occurs as a tetramer of four identical peptide chains linked by disulfide bonds and strong noncovalent interactions. Upon denaturation the subunits dissociate into pairs of chains, and into single chains following reduction of the six disulfide bridges (180). The primary structure (complete amino acid sequence) of the molecule has been determined by Sottrup-Jensen (165,166). Structural comparisons revealed that $\alpha_2$-M belongs to a superfamily of proteins that includes complement factors C3 and C4 and “pregnancy associated protein.”

The principal biological functions of $\alpha_2$-M are its unique nonspecific protease-binding activity and its more recently recognized interaction with a variety of cytokines. $\alpha_2$-M inhibits endopeptidases from all four mechanistic classes (serine, thiol, aspartic, metallo). The nature of this inhibition has been the subject of numerous investigations. It is generally assumed that proteases react with $\alpha_2$-M by splitting a specific peptide bond within a small "bait" region near the center of each subunit. The sites of proteolytic cleavage have been mapped
extensively and generally conform to the primary substrate specificity of the protease involved (165). Cleavage of the specific peptide bond is accompanied by a rapid conformational change of the molecule, which results in a nondissociable complex by trapping of the protease. This reaction can be very rapid (instantaneous for neutrophil elastase, less than 2 sec for trypsin) or require longer periods of time (several hours for plasmin and thrombin). The entrapped proteases may bind covalently to the α2-M molecule (through an internal reactive thiol ester of α2-M) or noncovalently. One or two molecules of protease react with one molecule of α2-M, depending on the association rate and the size of the protease. The α2-M–protease complex is often referred to as the “activated form” of α2-M. Activated α2-M forms exhibit increased mobility during electrophoresis and are also called “fast α2-Ms” versus slow, native α2-M.

Proteases bound to α2-M are virtually inactive toward large protein substrates but retain considerable activity (50–80%) toward small peptides and synthetic substrates. Furthermore, they can still be inhibited by low-molecular-weight inhibitors such as DFP, PSTI, and Trasylol, but not by the larger soybean or lima bean inhibitors. It is therefore clear that the active site of the protease is still open, but that the conformational changes of the α2-M molecule have rendered the enzyme inaccessible to any but low-molecular-weight substances.

Much interest has been focused on the physiological importance of α2-M relative to other plasma protease inhibitors. Addition of a small amount of protease such as trypsin to human serum results in rapid partitioning of the enzyme between α2-M and α1-proteinase inhibitor. For trypsin the ratio is 85:15 (83), for elastase about 60:40 (44), and for chymotrypsin 10:90 (45). α2-M complexes, in contrast to α1-proteinase inhibitor adducts, do not dissociate and appear irreversible. For this reason, incubation of α2-M with PSTI–trypsin ultimately results in transfer of all trypsin to α2-M. In vivo this process is greatly exaggerated, since α2-M complexes are rapidly cleared from the bloodstream by the reticuloendothelial system. The half-life of such complexes is only about 10 min. Clearly, one of the major physiological functions of α2-M is the clearance of noxious proteases from the circulation. α2-M complexes may also play a role in the activation of enzymes and hormones and their clearance. Whereas α1-proteinase inhibitor–trypsin complexes are totally inactive, α2-M–trypsin shows some highly selective proteolytic activity. Thus it is capable of activating trypsingen and chymotrypsinogen, converting proinsulin to desalanyl-insulin, hydrolyzing angiotensin, vasopressin, and parathyroid hormone, converting cholecystokinin 39 to cholecystokinin 33 and fibrinogen to fibrin, and mimicking the action of thrombin (55, 137). However, the physiological or pathophysiological significance of the proteolytic activity displayed by these complexes is uncertain. Elevated serum levels of α2-M–protease complexes in patients with pancreatitis have been reported (6, 86), but the increase in these complexes did not correlate with the severity of the disease.

Studies over the past 10 years have shown that a second essential function of α2-M is the regulation of activity, distribution, and clearance of many cytokines (62, 80). The role of some of these cytokines in the pathogenesis and prognosis of acute pancreatitis is discussed below. α2-M has been identified as a carrier of many important cytokines [transforming growth factors, tumor necrosis factor-α (TNF-α), platelet derived growth factor, interleukin-1β (IL-1β), interleukin-6 (IL-6), nerve growth factor]. Although the nature of the binding mechanism is poorly understood, there are two distinct modes of interaction between α2-M and cytokines. Some cytokines, such as platelet derived growth factor, IL-6, and nerve growth factor, bind to native α2-M. Such complexes retain much of their biological activity. Other cytokines (e.g., TNF-α, IL-1β and transforming growth factors-β1 and -β2) bind preferentially to α2-M–protease complexes (“activated α2-Ms”). Interestingly, the extent of cytokine binding often varies with the protease involved in complex formation with α2-M. Thus α2-M–plasmin shows normal or enhanced TNF-α binding capacity whereas α2-M–trypsin or α2-M–thrombin bind less or not at all. α2-M complex formation with proteases exposes a latent receptor-binding domain on the α2-M molecule, which is recognized by specific α2-M–cell-membrane-associated receptors on hepatocytes, fibroblasts, macrophages, and so on. The receptor molecule on these cells is a glycoprotein with a molecular weight of 440,000 (see references in ref. 80). “Activated α2-M”–cytokine-receptor complexes are internalized by endocytosis. Clearance of these complexes from the circulation, principally by hepatocytes, is rapid (similar to “activated” α2-M alone), but the fate of the cytokines is as yet undetermined. However, it should be noted that such complexes can also be cleared locally by macrophages and other cells expressing α2-M receptors. It has been proposed (80) that active cytokines and α2-M–protease complexes are secreted, activated, or otherwise delivered locally in the environment of tissue damage or inflammation. Ongoing research in this rapidly developing field may lead to strategies for preventing the dangerous and often lethal effects of excess cytokines such as TNF-α and IL-1. Recent reviews of this topic have been published by LaMarre et al. (80) and James (62); see also Feinman (31).

Inter-α1–Trypsin Inhibitor (ITI)

This minor plasma inhibitor (Table 3) is a glycoprotein with a molecular weight of about 160,000 to 200,000 daltons and a carbohydrate content of 8.4%. It is
synthesized in the liver and consists of three different subunits, a light chain (L) \( (M = 26,000 \text{ daltons}) \) and two different heavy chains \( (M = 78,000 \text{ and } 85,000 \text{ daltons}) \) (43). It has been suggested that the chains are covalently cross-linked by a carbohydrate bridge containing chondroitin sulfate (112), but the exact mode of the linkage remains to be elucidated. The inhibitor (ITI) forms readily dissociable complexes with a variety of serine proteases (e.g., trypsin, chymotrypsin, elastase, plasmin, granulocyte cathepsin G). The inhibitory capacity of the molecule resides within the light chain (L), which contains two tandem Kunitz-type inhibitory domains. Trypsin and chymotrypsin react at one of the domains whereas elastase and cathepsin G react at the other (112). The reaction with a protease can lead to an inhibitor complex or to proteolysis of the inhibitor. Both reactions occur simultaneously, but the inhibitory activity of ITI is not lost since proteolysis yields acid-stable fragments (ITI-L) that retain inhibitor activity. Acid treatment of serum yields similar fragments (ITI-L) that are immunologically indistinguishable from inhibitors (UTIs) excreted in urine. UTIs have a molecular weight of 22,000 to 68,000 daltons, depending on the carbohydrate content.

The physiological function of ITI is uncertain. None of the human proteases tested have an inhibitor constant \( K_i \) low enough to be physiologically relevant, but evidence has been obtained that ITI might act as a shuttle by transferring proteases from ITI complexes to other plasma protease inhibitors, modulating their distribution and facilitating clearance (124). It is interesting to note that ITI-L is an acute-phase reactant comparable to C-reactive protein. Elevated plasma levels have been observed in myocardial infarction, inflammatory disease, and cancer. By contrast, the plasma concentration of immunoreactive ITI is not affected by these conditions (112), suggesting that the synthesis of heavy chains is not influenced by disease.

\( \alpha_1 \)-Antichymotrypsin

Like inter-\( \alpha_1 \)-trypsin inhibitor, \( \alpha_1 \)-antichymotrypsin (Table 3) is a minor plasma inhibitor. It occurs in many tissues and virtually all body fluids (e.g., serum, urine, seminal fluid, uterine and amniotic fluid, synovial fluid in rheumatoid arthritis, bronchial lavage fluid). It is a glycoprotein with a molecular weight of 65,000 to 68,000 daltons and a carbohydrate content of about 27%. The 16 to 28 amino acid sequence of \( \alpha_1 \)-antichymotrypsin is homologous with the 33 to 45 amino acid sequence of inter-\( \alpha_1 \)-trypsin inhibitor. The two inhibitors also share the same unusual amino-terminal arginine. The reactive site of the inhibitor is a Leu-Ser bond. \( \alpha_1 \)-Antichymotrypsin is perhaps the most specific plasma protease inhibitor known, since it controls specifically the activity of chymotrypsin and the chymotrypsin-like activity of leukocyte cathepsin G. It forms equimolar complexes with these proteases and this interaction is accompanied by the formation of a modified, inactive inhibitor. Complexes of inhibitor and protease are labile and dissociate spontaneously (79).

The physiological function of \( \alpha_1 \)-antichymotrypsin is uncertain, but it is unlikely that it is involved in controlling pancreatic chymotrypsin released into the circulation since no chymotrypsin-\( \alpha_1 \)-antichymotrypsin complex can be detected in serum after addition of the enzyme. However, it is one of the fastest changing acute-phase reactants along with C-reactive protein. Increases in serum concentrations can be observed in inflammatory diseases and when any kind of tumor is present. It has been suggested that it may be an excellent marker for tumor progression since it lacks phenotypic variation and has a nonspecific elevation response parallel to tumor progression (cf. references in ref. 12).

**Pancreatic Exocrine Lysosomal Enzymes**

In view of the extraordinary capacity of the pancreas to synthesize a diversity of products, it is not surprising that this organ is also a rich source of lysosomal enzymes. Both lysosomal and digestive enzymes (zymogens) are synthesized on ribosomes attached to the rough endoplasmic reticulum, and both contain signal sequences that mediate their transport to the Golgi apparatus, where they are sorted and packaged: lysosomal enzymes into lysosomes, and digestive enzymes and zymogens into zymogen granules (see Scheele, *this volume*). However, recent cytological investigations at the ultrastructural level have shown conclusively that the sorting mechanism for lysosomal hydrolases and secretory proteins is incomplete (187) and that lysosomal enzymes are normally present in condensing vacuoles and zymogen granules in acinar cells of healthy individuals.

The physiological rather than pathological nature of colocalization of lysosomal and digestive enzymes in acinar cells has also been indicated by studies on pancreatic juice from healthy volunteers and alcoholics. Rinderknecht et al. (142) showed that sequential stimulation of the pancreas with secretin and cholecystokinin resulted in parallel release of digestive and lysosomal enzymes (e.g., arylsulfatase, \( N \)-acetyl-\( \beta \)-D-glucosaminidase, \( N \)-acetyl-\( \beta \)-D-galactosaminidase, \( \alpha \)-L-fucosidase) into the pancreatic secretions. The secretory patterns of the two types of enzymes were indistinguishable from each other, as illustrated in Fig. 2. Some lysosomal enzymes, such as \( \beta \)-D-glucuronidase, \( \alpha \)-D-glucosidase, and leucine naphthylamidase, did not show the striking response to cholecystokinin stimulation, and cathepsin B, although present in pancreatic tissue, was not found in normal
juice. This may be due to low concentrations and the great instability of these enzymes in the highly alkaline pancreatic secretions, or different locations in the acinar cell. However, pancreatic disease, as will be shown below, can increase this response dramatically (see Fig. 4).

Identification and Determination of Lysosomal Enzymes

The origin and identity of these acid hydrolyses in pancreatic secretions were suggested by their instability in alkaline medium, their acid pH optima, their substrate specificities, and secretory patterns. Definite proof of their identity has been furnished more recently by immunocytochemical methods (187). The use of highly sensitive and specific substrates (142), which on hydrolysis release fluorophores such as 4-methylumbelliferone or naphthylamine, permits rapid determination of numerous enzymes in microliter quantities of pancreatic juice and monitoring of their secretory kinetics following pancreatic stimulation by secretin and cholecystokinin (Fig. 2). Although these methods measure enzymatic activity and not enzyme concentration, available data suggest that N-acetyl-β-D-glucosaminidase may be the most prominent of the secreted acid hydrolyses, followed by N-acetyl-β-D-galactosaminidase, α-L-fucosidase, arylsulfatase, β-D-glucuronidase, and α-D-glucosidase. The concentrations of these hydrolyses appear to be low in comparison with those of digestive enzymes such as trypsin or amylase. Cathepsin B, as pointed out previously, could not be detected in normal pancreatic juice but was present in pancreatic secretions of a patient with cancer of the pancreas (139).

Leucine amidase occupies a unique position among the lysosomal hydrolyses in pancreatic juice. Unlike the others, it does not have an acid pH optimum but acts optimally at about pH 7. It is not strictly a leucine-specific peptidase but hydrolyzes other N-terminal residues, including aromatic and basic amino acids. Sidorowicz et al. (162) have studied the degradation of 15 biologically active peptides, such as somatostatin, secretin, angiotensin, and l-lysylbradykinin, by an "alanine aminopeptidase" from extracts of human pancreas that most likely is identical with the above "leucine aminopeptidase."

Physiological and Potential Pathophysiological Role of Pancreatic Lysosomal Enzymes

Most of the lysosomal hydrolyses found in pancreatic juice have an acid pH optimum and are rapidly inactivated at the strongly alkaline pH of this secretion. They are unlikely therefore to play a role in the digestion of food in the intestinal lumen. A more plausible explanation for their presence in pancreatic tissue and juice is suggested by one of their primary physiological functions, namely, intracellular digestion and disposal of ex-
cessive or degraded cellular constituents (150). As synthesis of secretory proteins in pancreatic acinar cells proceeds at an unusually high rate, a commensurate complement of lysosomal enzymes would appear to be a prerequisite for the protection of the cell from the potentially dangerous accumulation of digestive enzymes andzymogens as has been observed ("exit block") in experimental (70,151,168) as well as human pancreatitis (188).

The protective role of lysosomal hydrolases in pancreatitis is illustrated by the formation of large, Golgi-derived vacuoles containing both lysosomal and digestive enzymes, and fusion of lysosomal bodies with zymogen granules resulting in large autophagosomes (188). Moreover, increased diversion of lysosomal hydrolases to autophagosomes and to zymogen granules has been observed in humans (see references in refs. 187 and 188) as well as experimental pancreatitis (116). Figure 3A-C illustrates progressive loss of electron-dense material (digestion?) in zymogen granules, starting at the periphery and moving toward the center ("fried-egg"-like structures, arrows) in experimental pancreatitis in mice (Fig. 3A) (90), in acute human pancreatitis (Fig. 3B) (188), and in exocrine pancreatic insufficiency in CBA/J mice (Fig. 3C) (29). Although the mechanism of this process remains obscure, digestion of the granule contents by lysosomal enzymes is suggested by (a) the acid pH of the interior of this organelle (108), which severely restricts or precludes autodigestion of pancreatic digestive enzymes, (b) by the increase in the inegransular concentration of lysosomal hydrolases in the early phases of pancreatitis (see references in refs. 187 and 188), and (c) by the advance of the presumed digestive process from the periphery—the preferential location of lysosomal hydrolases (110)—toward the center of the granule. However, in the absence of experimental proof, other explanations must be considered. Thus perturbation of the granule membranes (as indicated by their increased fragility in pancreatitis) may impair the hydrogen pump that maintains an acid environment within the granule, resulting in an increase of the inegransular pH, autoactivation of trypsinogen, triggering of the pancreatic enzyme cascade, and subsequent autodigestion of pancreatic enzymes. Alternatively, condensation of secretory proteins may be less efficient during pancreatitis, due either to perturbation of the zymogen granule membranes or the condensation process itself. During the fixation process, Na, K, and fluid may move into these granules and solubilize the poorly condensed proteins at the periphery. A study of the inactivation of pancreatic zymogens and enzymes by lysosomal hydrolases, and of the autoactivation of trypsinogen and activation of other zymogens at pH 4 to 5, may furnish answers to some of these questions.

By contrast, a pathogenic role for lysosomal enzymes in acute pancreatitis has been suggested by Figarella et al. (34) and Steer et al. (168). Ultrastructural studies of acinar cells of experimental animals with acute, diet-, or secretagogue-induced pancreatitis (see references in ref. 188) demonstrated fusion of lysosomes with zymogen granules in large, intracytoplasmic vacuoles. Subcellular fractionation and analysis of the zymogen granule/vacuole fraction revealed the presence of significant amounts of active serine proteases as well as fragments of degraded enzymes (13,168), providing evidence of intracellular activation of trypsinogen by cathepsin B (cf. 34,88). These findings were believed to reflect the initial phase of potentially uncontrolled zymogen activation (cf. 66) and subsequent autodigestion of the pancreas by its own prematurely activated digestive enzymes. However, there are a number of observations that are difficult to reconcile with this concept. (a) The greatly increased fragility of zymogen granule membranes in pancreatitis makes it almost impossible to ascertain that trypsinogen activation in the zymogen granule/vacuole fraction is not, at least in part, an artifact due to experimental manipulation (pH, Ca concentration, etc.). (b) It is unknown at present whether cathepsin B or trypsin are capable of activating zymogens other than trypsinogen at the acid pH < 5 within these organelles (cf. 88,108). It should be kept in mind too that cathepsin B is capable not only of activating trypsinogen but also of degrading this zymogen, as well as its free enzyme. Moreover, at a higher pH (approaching 7) pancreatic secretory trypsin inhibitor (PSTI) will begin to neutralize trypsin (up to 20% of potentially available) and effectively stop activation of all zymogens. (c) Most importantly, acinar cells are remarkably resistant to proteolytic enzymes and in the absence of bile resist even the action of phospholipase A2. Thus degranulated acinar cells in the vicinity of fat necroses retain their structure, and acinar necroses, according to Schmitz-Moormann (154), seem to be due to local ischemia rather than autodigestion. Although several investigators have observed discharge of zymogen granules into the interstitial space, mostly in close proximity to fat necroses, no immunoreactive trypsin could be found in the interstitics (cf. 188). In addition, α1-proteinase inhibitor, present in most tissues and body fluids (120), might effectively neutralize extracellular serine proteases. This may also explain the virtual absence of active proteases in pancreatic extracts of animals or humans with acute pancreatitis (cf. references in refs. 132 and 133). (d) Finally, CBA/J mice with pancreatic insufficiency die of the effects of malnutrition and not of acute necrotizing pancreatitis despite the fact that most of the trypsin and chymotrypsin in the zymogen granule fraction apparently is present in the active form (29).

In view of these findings and observations, the traditional hypothesis that acute necrotizing pancreatitis results from autodigestion of the pancreas by its inappropriately activated proteolytic enzymes is no longer tenable and should be reformulated.
FIG. 3. A: Loss of peripheral density in zymogen granules of acinar cells of the pancreas of a mouse fed dL-ethionine with a choline-deficient diet for 3 days. ×9075. (From ref. 90, with permission.) B: Focal breakdown of zymogen granules in human acute pancreatitis. Loss of electron density of the core proceeding from the periphery to the center. ×9100. (From ref. 188, with permission.) C: Initial cytopathology observed in CBA/J mouse acinar cells. Digestion of zymogen granules progressing from the periphery to the center. ×12,200. (From ref. 29, with permission.)
This does not imply that pancreatic enzymes are irrelevant in the pathogenesis of pancreatitis. Thus trypsin is capable of activating the kinin system and other cascades involved in the inflammatory process (87), and pancreatic lipase and carboxyl ester hydrolase, which need no activation and are not inhibited by protease inhibitors, undoubtedly are responsible for fat necroses, the earliest visible lesion in pancreatitis. It has been hypothesized that neutrophils and macrophages that rapidly invade the border between fat necroses and healthy tissue may be the initiators of the often fatal outcome of the severe form of the disease (132,133). According to this concept excessive stimulation of leukocytes by a severe initial insult will activate these cells to produce massive amounts of oxygen-derived free radicals, induce overt secretion of a large number of lysosomal enzymes, induce toxic levels of inflammatory mediators such as tumor necrosis factor (TNF), IL-1, platelet activating factor, and leukotrienes, damage adjacent acinar cells, and exacerbate the initial injury. Finally, their normally protective action, now turned against the host, may result in lethal multiple organ failure as observed not only in fatal pancreatitis but also in adult respiratory distress syndrome (ARDS), sepsis, and severe burns.

PANCREATIC SECRETORY PROFILES IN HEALTH AND DISEASE

Knowledge of the secretory profiles of pancreatic exocrine products is important to the understanding of physiological as well as pathophysiological processes in the pancreas. While earlier studies had to contend with the gastric, duodenal, and biliary contaminants of duodenal aspirates, the recent technique of endoscopic cannulation of the pancreatic duct now provides ready access to pure pancreatic juice for the study of pancreatic function in health and disease. Such studies have included (a) simultaneous determinations of a number of enzymes and other constituents in minute-to-minute collections of pancreatic secretion after sequential stimulation of the pancreas with secretin and cholecystokinin (141); (b) two-dimensional isoelectric/sodium dodecyl sulfate
(SDS) gel electrophoresis of a single specimen of pancreatic juice (152); and (c) high performance liquid chromatography of pancreatic juice (47). While methods (b) and (c) provide an overall picture of most of the secretory proteins and their quantitative relationships in a single specimen, method (a) furnishes quantitative information on individual enzymes and other constituents as well as their secretory kinetics in response to hormones, drugs, or pathological processes in the pancreas. Most of the data published to date have been obtained by this method.

**Secretory Profiles in Healthy Human Subjects**

Pancreatic secretory profiles of normal healthy volunteers obtained by endoscopic cannulation of the main pancreatic duct and sequential stimulation of the pancreas with i.v. secretin (1 clinical unit/kg) and cholecystokinin (1 Ivy dog unit/kg) have been described by Rinderknecht et al. (141) and are illustrated in Fig. 2. The initial high concentrations of protein and enzymes (trypsin, chymotrypsin, elastase, and amylase) during the first 3 to 5 min probably represent a wash-out phenomenon before secretory products reach a steady baseline. Cholecystokinin (CCK) elicits a striking, approximately 10-fold increase in total protein and enzyme concentration. The half-life of this effect is between 73 and 90 sec for protein and enzymes, but only 64 sec for trypsin inhibitor (PSTI). It should be noted that CCK-stimulated secretion of total protein and individual enzymes, as well as the half-life of this effect, are roughly parallel. By contrast, secretion of trypsin inhibitor is not parallel with that of the enzymes listed, and it responds to CCK with an almost 20-fold increase in concentration, and a half-life of this effect of only 64 sec. Nonparallel secretion of amylase and chymotrypsin also has been reported by several investigators (see references in ref. 141). The mechanisms underlying these discrepancies are not yet fully understood.

The secretory profiles of some lysosomal enzymes—arylsulfatase, N-acetyl-β-D-glucosaminidase, N-acetyl-β-D-galactosaminidase, α-L-fucosidase, and α-D-mannosidase—are virtually indistinguishable from those of the digestive enzymes (Fig. 2). Others, like leucine amidas and α-D-glucosidase, show profiles that lack the marked increase in activity following CCK stimulation. The reason for this difference remains to be clarified (Fig. 4).

**Chronic Alcoholism**

Chronic alcoholism is a well-recognized factor in the etiology of pancreatitis. Comparison of pancreatic secretory profiles of chronic alcoholics with those of healthy nonalcoholic individuals shows remarkable abnormalities (142,143), some of which are encountered also in profiles of patients with pancreatitis: (a) increased protein concentration and output following CCK stimulation; (b) striking, selective increase (over twofold) of trypsinogen secretion; (c) consistent reversal of the normal ratio of cationic/anionic trypsinogen from about 2 to 0.5 [it is of interest to note that a similar rate reversal of cationic/anionic trypsinogen after dietary manipulations in rats has been observed by Scheele (this volume)]; (d) striking increase in the trypsinogen/PSTI ratio, (e) some increase in lysosomal hydrolase concentration and marked response of glucuronidase and leucine amidase to CCK; and (f) parallel secretion of PSTI, protein, and enzymes, versus nonparallel secretion of PSTI in nonalcoholics.

**Pancreatitis**

Pancreatic secretory profiles of patients with acute or chronic pancreatitis cover a wide range, depending on the type and stage of the disease (129,143). Digestive enzymes tend to be in the low normal, while lysosomal hydrolases are in the high normal range or exceed it. Small amounts of free proteolytic activity may appear intermittently and the normal ratio of cationic/anionic trypsinogen (about 2) begins to decrease to 1 or less. The most marked changes are seen in chronic pancreatitis, particularly in the final stages of the disease, in patients with pancreatic insufficiency. Digestive enzyme activity is minimal, response to CCK virtually absent, the ratio of cationic/anionic trypsinogen <! 1, and lysosomal hydrolases, including glucuronidase and leucine amidase, dominate the profile. Pancreatic pathology is reflected dramatically in the increasing ratios of lysosomal to digestive enzymes (143). Such ratios are independent of biological variations between individuals and unrelated to protein concentrations in the samples investigated.

**Pancreatic Cancer**

The most marked secretory abnormalities are observed in the profiles of patients with cancer of the pancreas. Because the disease usually is far advanced when diagnosed, pancreatic secretory profiles are difficult to obtain. In many cases the pancreas has become almost nonfunctional and responds neither to secretin nor CCK. In other cases the pancreatic duct is obstructed by the tumor and cannot be cannulated. Where functional tissue still exists and the duct is patent, digestive enzymes are greatly diminished, as is the flow rate. Evidently tumor cells do not synthesize pancreatic enzymes nor do they secrete juice. In nine cases of pancreatic cancer investigated by Rinderknecht et al. (143) protein and enzyme concentrations were below the normal or in the
lowest range of normal levels, but values for PSTI were consistently below the normal range. Cationic/anionic trypsinogen ratios were reversed (<1) and about half the samples collected contained traces of free proteolytic activity. By contrast, lysosomal enzymes remained in the normal range or exceeded it. The tumor seemed to have diametrically opposite effects on digestive and lysosomal enzymes, as illustrated in Fig. 4. The pathological shift in the secretory enzymes is perhaps reflected even more dramatically in the increase of the ratios of lysosomal/digestive enzymes. Almost all of 10 such ratios—for example, β-D-glucuronidase/trypsin, α-D-glucosidase/chymotrypsin, leucine, amidase/elastase—were far above the normal average values. Only three of 25 patients with chronic pancreatitis exhibited secretory patterns similar to those of the cancer patients. It is of interest to note that pancreatic secretory profiles of hamsters receiving a single injection of bis-2-(oxopropyl)-N-nitrosamine to induce pancreatic cancer exhibited similar changes in the ratios of lysosomal/digestive enzymes even before histologically recognizable tumors could be detected (138). It is therefore likely that such changes may also occur early in the development of the human disease. Their detection by a noninvasive technique might lead to new strategies for early detection of this deadly malignancy.

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