ARZNEIMITTEL FORSCHUNG
DRUG RESEARCH

Einteilung dieses Jahrgangs
Subdivision of this annual volume

33. Jahrgang (1983)
Heft 1–12 und Sonderausgaben 1a, 2a, 4a, 7a und 9a

ARZNEIM.-FORSCH./DRUG RES.
EDITIO CANTOR AULENDORF

From the Department of Clinical Chemistry and Clinical Biochemistry in the Surgical Clinic of the University of Munich (FR Germany)

Biochemistry and Applications of Aprotinin, the Kallikrein Inhibitor from Bovine Organs

By H. Fritz and G. Wunderer[1])

Summary: *The basic proteinase inhibitor from bovine organs, aprotinin (active ingredient of Trasylol®) has been extensively studied with respect to its chemical, physical and biochemical properties and its inhibitory mechanism of action. It is widely used as a valuable tool for studying protein/protein interactions and protein conformation at the molecular level. There are numerous examples of the usefulness of aprotinin in biochemical and biomedical research. It has also become a valuable drug for the treatment of various diseases like, e.g. hyperfibrinolytic hemorrhage and traumatic-hemorrhagic shock.*

The purpose of this paper is threefold. It summarizes our present knowledge of the subject in various disciplines; it provides the active scientist with basic data for his experimental work; and above all it points the way to future directions of aprotinin research.

Zusammenfassung: *Biochemie und Anwendung des Kallikrein-Inhibitors Aprotinin aus Rinderorganen*

Der basische Proteinase-Inhibitor Aprotinin (Wirkstoff von Trasylol®) aus Rinderorganen wurde hinsichtlich seiner chemischen, physikalischen und biochemischen Eigenschaften sowie seiner Reaktionsweise mit Enzymen eingehend untersucht. Aprotinin dient heute allgemein als wertvolles Agens zum Studium der Wechselwirkungen von Proteinen und der Protein-Konformation auf molekularer Basis. In zahlreichen Anwendungsbeispielen in der biochemischen und biomedizinischen Forschung wurden mit Hilfe des Aprotinin richtungsweisende Erkenntnisse gewonnen. Aprotinin hat sich auch als potenter Wirkstoff zur Behandlung verschiedener Krankheiten erwiesen, so z. B. bei hyperfibrinolytischen Blutungen und beim traumatisch-hämorrhagischen Schock.

Der vorliegende Artikel dient einem dreifachen Zweck: Er faßt den gegenwärtigen Erkenntnisstand in den verschiedenen Fachdisziplinen zusammen, er bietet dem experimentell tätigen Wissenschaftler Basisdaten für seine Versuchsvorhaben und er gibt Hinweise für neue Zielsetzungen, die mittels Aprotinin angegangen werden können.

Key words: *Aprotinin, biochemistry, mechanism of action · Kallikrein, inhibition · Proteinase inhibitors, bovine · Trasylol®*

Contents

[1]) Present address: Dr. G. Wunderer, 1. Frauenklinik der Universität München (FR Germany).

1. Introduction: Discovery, distribution and isolation

Aprotinin[2] was independently discovered by Kraut et al. [1] as a kallikrein "inactivator" in bovine lymph nodes, and by Kunitz and Northrop [2] as a trypsin inhibitor in the bovine pancreas. Later, Werle et al. also found kallikrein-inhibiting activities in extracts of other bovine organs, such as the lung, parotid gland, spleen, liver, pancreas, and seminal vesicles. Lower activities were found in the thyroid gland, kidney and mucous membranes of the trachea and esophagus, and in extracts from the same organs of sheep and goat [3–6]. More recent investigations have shown that this kallikrein-inhibiting activity is due in most, if not all, cases to the presence of aprotinin or aprotinin-like inhibitors in these tissues [5–7].

In addition, aprotinin has been found in the following bovine organs or tissues: Ovary [8], heart, thyroid, posterior pituitary, and the rumen mucosa [9] as well as in cartilage and aorta [10]. Remarkably, the two latter tissues are known to be highly resistant to tumor invasion. Recently, Hochstrasser and Wachter discovered aprotinin-like inhibitors as components of the human and bovine inter-α-trypsin inhibitor [11] and in free form in bovine serum [12].

Of special interest, in view of its biological function [13] is the occurrence of aprotinin in mast cells, detected by the indirect immunofluorescence technique [13, 14], and its behavior as an intracellular compound [4, 5]. The mast cell origin of aprotinin is indicated also by a similar distribution pattern: organs known to contain high numbers of these connective tissue cells are also rich in aprotinin (especially lung, parotis and pancreas) and vice versa. Possibly, aprotinin is involved in the regulation of mast cell proteinase activities [13].

For commercial purposes, aprotinin is isolated by classical methods such as fractional precipitation, gel filtration and ion exchange chromatography, being extracted solely from bovine lung, pancreas and parotid glands [5]. These tissues contain approx. 1500 KIU (= biological kallikrein units) (lung) or 400 KIU (pancreas, parotid gland) per gram wet weight [4, 5]. For the rapid purification of aprotinin or aprotinin-like inhibitors on a laboratory scale in good yields (up to 90%), acidic extraction followed by affinity chromatography on water-insoluble enzyme derivatives (e.g. trypsin-Sepharose or trypsin-cellulose) is the method of choice [8, 15]. On the other hand, water-insoluble aprotinin derivatives are widely used for the isolation of proteinases (see section 6.1.).

[2] Aprotinin is also known as bovine pancreatic trypsin inhibitor (Kunitz) (BPTI) and trypsin-kallikrein inhibitor (TKI); it is the active ingredient of Trasylol®, a drug registered in the name of Bayer Leverkusen (FR Germany). In some countries Kallikrein is a registered trademark of Bayer.

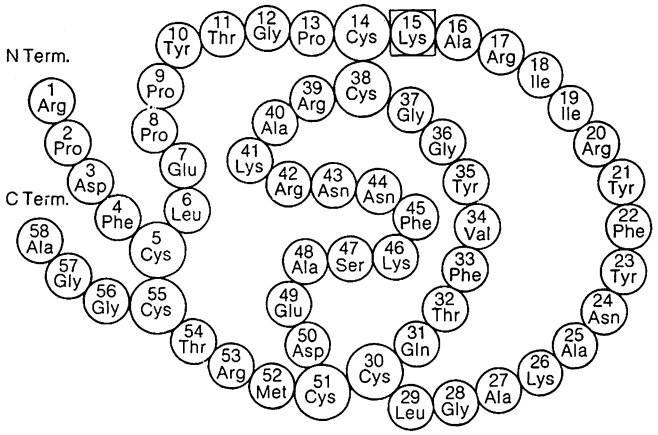


Fig. 1: Primary structure of aprotinin according to Kassell and Laskowski [17] and Anderer and Hörnle [18]. The inhibitor molecule consists of a single polypeptide chain of 58 amino acid residues cross-linked by 3 disulfide bridges.

2. Structure and physicochemical properties

2.1. Structural characteristics

Aprotinin, a polypeptide with a molecular weight of 6,512, is obtainable in crystalline form [2, 16]. It consists of 58 amino acid residues that are arranged in a single polypeptide chain. This chain is cross-linked by three disulfide bridges, one of which (Cys-14–Cys-38) is readily split by reducing agents. The primary structure of aprotinin is shown in Fig. 1 [5, 17, 18].

Information on the three-dimensional structure was obtained by X-ray crystallography, which revealed a pyriform molecule (Fig. 2) [19, 20]. The polypeptide chain is folded so that the hydrophobic radicals are concentrated in the interior of the molecule, whereas all of the hydrophilic radicals, except for the side chain Asp-43, are on the outside which is exposed to the aqueous environment. This arrangement results in a very compact tertiary structure and is mainly re-

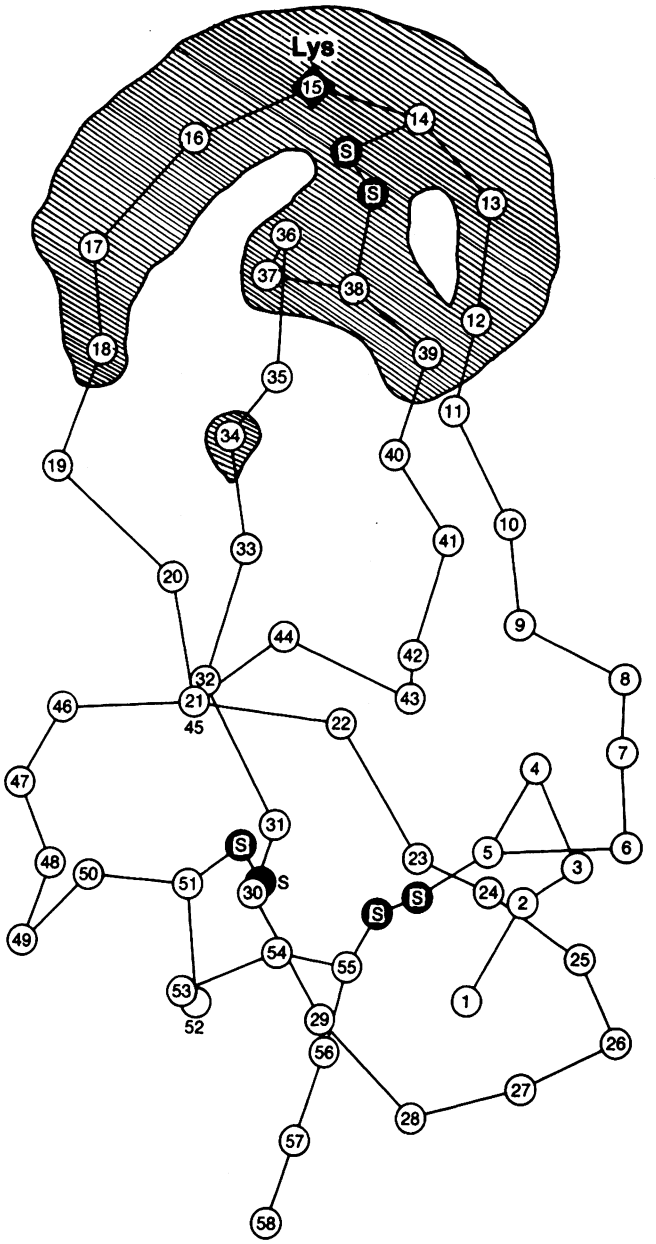


Fig. 2: Tertiary structure of aprotinin according to Huber et al. [19] and Deisenhofer and Steigemann [20]. The molecule has a length of about 29 Å, a diameter of 19 Å, and contains a double-stranded antiparallel β-sheet (from Ala-16 to Gly-36), twisted to form a right-handed double helix with 14 residues per turn. Amino acid residues included in the hatched area are in close contact with the enzyme in the trypsin-inhibitor complex. Residue number 19 is not included in this contact region, but its basic nature may be important for kallikrein inhibition in some cases; cf. Table 4 and Section 4.3.

sponsible for the remarkable stability of aprotinin against denaturation by high temperature, acids, alkalies and organic solvents or proteolytic degradation [21].

Other interesting features are: (i) The highly dipolar character of aprotinin due to concentration of the negatively charged radicals at one end of the molecule, viz. the bottom of the pear (see Fig. 2); and (ii) the strong basicity of the aprotinin molecule, with an isoelectric point close to 10.5 [21].

2.2. Stability, optical data and behaviour in dialysis

Aprotinin can be heated for a short time in dilute acid at 100 °C or in 2.5% trichloroacetic acid at 80 °C without losing any activity. It can be exposed to solutions with extreme pH-values ranging from 1 to 12.6; inactivation begins at pH 12.8 at room temperature [21]. These data were obtained by various methods, such as inhibition studies [21], NMR (nuclear magnetic resonance), and Raman measurements [22]. Aprotinin may be kept in a salt or buffer solution, e.g. 0.15 mol/l NaCl, at room temperature for at least 18 months without any loss of activity. The inhibitor is also stable and soluble in water, 70% (v/v) methanol, 70% (v/v) ethanol, and 50% (v/v) acetone [21].

Another outstanding property of aprotinin is its uncommon stability to proteolytic degradation by other proteinases [7, 21]. So far, only thermolysin has been found capable of digesting "native" aprotinin after heat labilization at 60–80 °C [23].

In a 1 mg/ml solution of aprotinin, absorption of A = 0.84 occurs at 280 nm (1 cm light path), relatively independently of pH [21]. In the presence of bactericidal agents, the inhibitor concentration should be optically determined at an alkaline pH. The absorption maximum of aprotinin is then shifted from 277 nm at pH 7 (specific absorbance: 0.89 cm^2/mg) to 297 nm in 0.1 N NaOH (specific absorbance: 1.32 cm^2/mg), thus differing from the absorption range of the commonly used preservatives (Arens, A., 1970, unpublished data).

Owing to its low molecular weight and high basicity, aprotinin penetrates or adheres to the commonly employed dialysis tubes. It is advisable, therefore, to use either acetylated material [24] or ultrafiltration membranes with an exclusion limit of 5000 daltons. Small quantities of aprotinin should be kept in suitable salt solutions (e.g. in volatile buffers) to avoid adsorption of the inhibitor on negatively charged surfaces during dialysis or filtration through columns (e.g. Sephadex).

3. Inhibitory characteristics and biological aspects

3.1. Units and assays

Aprotinin is often called a "broad-specificity inhibitor" as an expression of its ability to inhibit various proteinases such as those listed in Table 2. The "kallikrein inactivator unit" (KIU or KIE) is in worldwide use as a measure of aprotinin activity and is accepted as such [3–7]. One KIU is defined as that amount of aprotinin which decreases the activity of two biological kallikrein units (KU) by 50%. (One KU is defined as that amount of kallikrein which, when injected intravenously, causes the same decrease in dog carotid blood pressure as 5 ml of urine taken from 50 L, collected from healthy human individuals and dialyzed for 24 h against running tap water [3, 4]. This unit is also known as a Frey unit for FU [1, 3]).

As the biological assay is tedious to perform and has a high standard deviation, enzymatic assays with synthetic substrates are preferable. Compared with kallikrein, inhibition of trypsin by aprotinin (expressed in μmol of substrate turnover inhibited in unit time) proceeds much more rapidly and the resulting titration curve is linear over nearly the whole range, i.e. up to 90% inhibition of the applied amount of enzyme. Therefore, trypsin inhibition tests are advantageous and commonly used for the assay of aprotinin. The Enzyme Commission of the FIP (Fédération Internationale Pharmaceutique) recommends a titrimetric assay in which

Table 1: Conversion factors between the various units or inhibitor units (IU) that are recommended or used to express the inhibitory activity of aprotinin. Units or inhibitor units are defined as the reduction in substrate turnover, expressed in μmol per time interval, caused by aprotinin as compared with an inhibitor-free control sample.

Units wanted	Units given			
	KIU[a]) biol. assay	F.I.P. units[b]) (μmol BAEE/min)	μkat[c]) (μmol BAEE/s)	IU[d]) BAPA (μmol BAPA/min)
KIU[a])	× 1	× 30	× 1800	× 615
F.I.P. units[b])	× 1/30	× 1	× 60	× 20.5
μkat[c])	× 1/1 800	× 1/60	× 1	× 1/2.93
IU_{BAPA}	× 1/615	× 30/615 (= 1/20.5)	× 1800/615 (= 2.93)	1

[a]) "Kallikrein inactivator unit" based on a biologic assay, see text.
[b]) Units based on a titrimetric assay with BzArgOEt (BAEE) as (hog) trypsin substrate as recommended by the Enzyme Commission of the Fédération Internationale Pharmaceutique (F.I.P.); see text.
[c]) Units recommended by the NC-IUB (Nomenclature Committee of the International Union of Biochemistry), Eur. J. Biochem. 97, 319 (1979).
[d]) Inhibitor units based on a photometric assay with BzArgpNA (BAPA) as substrate and porcine trypsin as enzyme, see text.

residual trypsin activity is determined with BzArgOEt (N^{α}-benzoyl-L-arginine ether ester, also BAEE for short) as the substrate under conditions specified by the IUB (International Union of Biochemistry) [25, 26]. The conversion factors between the various recommended units are given in Table 1. Highly purified aprotinin free of water and salt ist found to have a specific activity of 7,150 ± 200 KIU/mg or 0.14 μg/KIU.

A photometric assay is also very useful for practical purposes. In this test, trypsin inhibition by aprotinin is determined by means of BzArgpNA (N^{α}-benzoyl-arginine-p-nitroanilide, also BAPA for short) as the substrate at 405 nm [21, 27]. The racemic substrate mixture D,L-BAPA (0.77 mmol/l) previously used in this assay should be replaced by L-BAPA (0.383 mmol/l). Inhibition of trypsin by the D-form and solubility problems frequently encountered with the mixture of the two enantiomers are then avoided [28]. The biological units (KIU) can be calculated from the inhibitor units that are obtained with porcine trypsin and L-BAPA as substrate by the equation: IU_{BAPA} (μmol/min) × 615 = KIU (see Table 1).

With the photometric assay (substrate L-BAPA) about 60–300 pmol aprotinin (in a given concentration of approximately 0.6–3 μmol/l) and with the titrimetric assay (substrate BAEE) up to twice this amount or concentration of aprotinin can be measured under the conditions given in the Appendix. Whereas the photometric assay is especially suitable for determination of aprotinin concentrations, the titrimetric assay is the method of choice if optimal conditions for enzyme catalysis are required. Principally, trypsin inhibition by aprotinin may be assayed also with more enzyme-specific chromatogenic ("chromogenic") or fluorogenic peptide substrates [29, 30]. However, due to the higher sensitivity (approx. 10- to 100fold) reached with such substrates, complex formation may proceed more slowly and the range of the linear part of the titration curve may be smaller, both effects being of disadvantage for practical purposes. The same holds true if instead of trypsin inhibition tissue kallikrein inhibition is determined by means of a chromogenic or fluorogenic peptide substrate, e.g. D-ValLeuArg-p-nitroanilide [31] or ProPheArg-4-methyl-coumaryl-7-amide [32]. A kallikrein inhibition assay (preferably with D-ValLeuArg-p-nitroanilide ≙ S-2266 [31]) may be advantageous, however, if aprotinin has to be quantified in tissue extracts or body fluids containing in addition naturally occuring trypsin inhibitors. In such cases the influence of the extracts on kallikrein activity also has to be considered and control samples with known aprotinin concentrations should be used as a reference standard (M. Jochum and H. Fritz, manuscript in preparation). Note, kallikrein inhibition alone

does not prove the presence of aprotinin in a test sample; for unequivocal identification of aprotinin at least the inhibition ratio towards its major target enzymes (trypsin, chymotrypsin, plasmin, plasma kallikrein and tissue kallikrein; cf. Table 2) has to be detected if specific immunological methods are not available.

In lieu of synthetic substrates, use may be made of the natural kallikrein substrates, the kininogens, for the assay of aprotinin activity based on kallikrein or trypsin inhibition [33]. When plasmin inhibition is assayed as described previously [34], 1 antiplasmin unit (APU) corresponds to 40 KIU of aprotinin. A radioimmunoassay technique developed by Fink and Greene permits specific detection of minimal quantities of aprotinin in biological fluids or tissue extracts [35].

3.2. Inhibitory specificity and biological aspects

The most striking feature of aprotinin is its broad inhibitory specificity. Besides trypsin and chymotrypsin, it strongly inhibits plasmin as well as kallikreins of different origin (Table 2) [3–7, 21].

In view of the therapeutic uses of aprotinin and its use in both clinical and experimental animal studies, its species specificity is of particular interest. Several investigators have found that various tissue kallikreins (from pancreas, submandibular glands, kidney or urine, and colon) as well as plasma kallikreins from man, pig and cattle are inhibited by aprotinin, whereas the corresponding guinea pig kallikreins are not inhibited [5–7]. Divergent results have repeatedly been obtained with mouse, dog and rat kallikreins [7]. Recently, however, it was shown that urinary kallikreins of the rat are very effectively inhibited by aprotinin [48].

The therapeutic uses of aprotinin are based on its inhibition of human trypsin, plasmin and plasma, tissue or urinary kallikrein. It should be noted that plasmin and tissue kallikreins are inhibited to a decidedly greater extend than the plasma kallikrein (Table 2).

The inhibition of human pancreatic proteinases by aprotinin is of special interest in connection with the therapeutic use of the drug Trasylol in acute pancreatitis. Human cationic as well as anionic trypsin is strongly inhibited by aprotinin [49, 50]. Human chymotrypsin II is not inhibited at all, while aprotinin appears to have a fairly low affinity for human chymotrypsin I and protease E [49]. Protease E is quite similar to porcine pancreatic elastase [51], but not to the "true" (i.e. elastin-digesting) human pancreatic elastase [52]. The affinity of aprotinin for human pancreatic kallikrein has not been quantitatively estimated. If the relation between tissue and urine kallikreins in man [44] is as close as that between tissue and urine kallikreins in the pig [53], aprotinin would have a rather strong affinity also for human pancreatic kallikrein [54] (Table 2). Remarkably, rat chymotrypsin, like human chymotrypsin II, is not inhibited by aprotinin [55].

Appreciable amounts of elastase and cathepsin G are released from human polymorphonuclear granulocytes during inflammations [56–58]. The affinity of aprotinin for these leukocytic neutral proteinases is therefore of considerable medical interest. Both elastase and cathepsin G have been shown to enter into specific reactions with aprotinin, but the dissociation constants K_i for the resultant enzyme-inhibitor complexes are quite high [46, 59–61] (Table 2). Cathepsin G seems to be inhibited by aprotinin to a very small extent [61]. Aprotinin-Sepharose nevertheless proved to be a very suitable affinity adsorbent for isolation of both elastase and cathepsin G (see section 6.1.).

Aprotinin has a relatively low affinity for the trypsin-like enzyme of spermatozoa, acrosin [62, 63]. Consequently, inhibition of this enzyme by aprotinin in vivo, hence prevention of fertilization, seems not to be practicable [64]. On the other hand, a trypsin-like or kallikrein-like proteinase of the rabbit blastocyst is strongly inhibited by aprotinin [65].

Interestingly enough, human urokinase, too, is inhibited by aprotinin, if rather weakly (Table 2; Dietl, T., Hugo, B., and Fritz, H., 1980, unpublished data). As urokinase may originate from plasminogen activators [66], there may also be a weak specific interaction between aprotinin and these tissue proteinases though this has not been reported thus far. Owing to the high affinity of aprotinin for plasmin, the inhibitor also reacts with the streptokinase-plasmin complex, progressively inhibiting its fibrinolytic activity, which is not inhibited by plasma proteinase inhibitors [41].

More recent observations indicate that aprotinin is also a strong inhibitor of the arginine esterases of growth factors [67, 68]. This is actually not surprising inasmuch as these proteinases show a striking similarity to the tissue kallikreins [69, 71]. The biological significance of this discovery is still unknown.

As for the substrate specificity of these proteinases, we may assume that the inhibitory specificity of aprotinin is mainly restricted to enzymes with trypsin-like substrate specificity and trypsin-like tertiary structures in the vicinity of the active site. Chymotrypsin seems to be an exception meeting the latter but not the former requirement. The same holds true for granulocytic elastase and cathepsin G. Of primarily theoretical interest are recent observations which suggest that aprotinin also forms equimolar complexes with enzymatically inactive anhydrotrypsin [36] and trypsinogen [37, 72], respectively.

Many proteinases and other enzymes that have been tested are not inhibited by aprotinin, viz. (porcine) pancreatic elastase, thrombin, enterokinase, subtilisin (Bacillus subtilis protease A and B), Aspergillus oryzae protease, papain, ficin, clostripain, thermolysin, pepsin, renin, angiotensin-converting enzyme, angiotensinase, carboxypeptidase A and B, ribonuclease, lysozyme, liver esterase, and ribosomal polypeptide chain elongation factors [7, 21].

Table 2: Dissociation constants K_i of enzyme-aprotinin complexes.

Enzyme	K_i (mol/l)	pH	Reference
Trypsin, bovine	6.0×10^{-14}	8.0	[36]
Anhydrotrypsin, bovine	$< 3 \times 10^{-13}$	8.0	[36]
Trypsinogen, bovine	1.8×10^{-6}	8.0	[37]
Chymotrypsin, bovine	9.0×10^{-9}	8.0	[36]
	6.0×10^{-9}	7.2	[38]
Plasmin, porcine	4×10^{-9} a)	7.8	[39]
human	2.3×10^{-10} a)	7.8	[40]
	1.0×10^{-9}	7.3	[41]
Kallikrein			
pancreatic, porcine	1×10^{-9} a)	8.0	[39]
pancreatic, porcine	1.3×10^{-9} a)	9.0	[42]
submand., porcine	1.6×10^{-9} a)	9.0	[42]
urinary, porcine	1.7×10^{-9} a)	9.0	[42]
plasma, porcine	1×10^{-7}	7.8	[43]
urinary, human	0.9×10^{-10}	8.0	[44]
plasma, human	3×10^{-8}	8.0	[45]
Elastase, human leukocytes	3.5×10^{-6} b)	8.0	[46]
Urokinase, human urine	8.0×10^{-6}	8.8	c)

a) Approximate values estimated from titration curves according to Green and Work [47].
b) Highly dependent upon ionic strength [46].
c) Dietl, T., Hugo, B., Fritz, H., unpublished data (1980).

3.3. Inhibition mechanism and kinetic constants

As a rule, the active site of the enzyme is blocked by complex formation with aprotinin (see section 4). If the esterolytic or amidolytic activity of the enzyme is inhibited as a result, its proteolytic activity is also inhibited, and vice versa [73]. In the case of components forming firm complexes, such as trypsin, plasmin or tissue kallikrein and aprotinin, the degree of inhibition is normally independent of the substrate used for the inhibition assay. However, if (1) the affinities of inhibitor and substrate for the enzyme are of similar magnitude [43] – the respective K_i and K_m values are suitable criteria – or (2) if the lifetime of the aprotinin-enzyme complex is short when compared with the length of the assay procedure [74], the observed degree of inhibition may depend on the substrate and assay conditions applied. Examples are (1) the system plasma kallikrein/BzArgOEt/aprotinin [43], and (2) the systems starfish trypsin 1/BzArgpNA

or 4-methylumbelliferyl p-guanidinobenzoate (GdnBzMum)/aprotinin [74]. Whereas no inhibition of starfish trypsin 1 by aprotinin is seen with BzArgpNA as the substrate, complex formation is clearly demonstrable by burst titration with GdnBzMum.

Inhibition by aprotinin is always competitive and reversible, as expressed by Equation (a) [75–78]:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \underset{k_C}{\overset{k_{-C}}{\rightleftharpoons}} X \underset{k_X}{\overset{k_{-X}}{\rightleftharpoons}} E + I^* \qquad (a)$$

where E is the proteinase; I and I* are the virgin (reactive-site Lys-15–Ala-16 bond intact) and modified (reactive-site bond hydrolyzed) inhibitor, respectively; L and X are loose, intermediate precomplexes preceding the stable complex C. (Other nomenclatures use k_3 for k_{-C}, k_{-3} for k_C, k_4 for k_{-X}, k_{-4} for k_X, and L* for X. However, as the nature of the complexes L and L* turned out to be quite different [77, 78], we prefer the nomenclature used in Eq. (a) above.)

In the first step from either side (k_1 or k_X), a fast second-order reaction, a relatively loose complex L (Michaelis-Menten complex) or X is formed, followed in the rate-determining step (k_2 or k_C), a slow first-order transition, by formation of the stable complex C. Rate constants (measured at pH 7.5 and 22.5 °C) for the various steps of the reaction of aprotinin with bovine trypsin (try) or bovine chymotrypsin (chy) are given by this system of equations [75–78]:

$$E + I \underset{2\times10^3-4\times10^4 \quad 4\times10^3-5\times10^4 \ s^{-1}}{\overset{\text{try} \quad \text{chy}}{\underset{5\times10^6-10^8 \quad 8\times10^6-10^8 \ M^{-1}s^{-1}}{\rightleftharpoons}}} L \underset{\sim 8\times10^{-8} \quad 9\times10^{-4} \ s^{-1}}{\overset{\text{try} \quad \text{chy}}{\underset{125 \quad 350 \ s^{-1}}{\rightleftharpoons}}} C \underset{\sim 1\times10^{-2} \quad 5\times10^{-4} \ s^{-1}}{\overset{\text{try} \quad \text{chy}}{\underset{3.3\times10^{-10} \quad 5\times10^{-9} \ s^{-1}}{\rightleftharpoons}}} X \underset{10^5 \quad 4\times10^3 \ M^{-1} \ s^{-1}}{\overset{\text{try} \quad \text{chy}}{\underset{0.35 \quad 0.26 \ s^{-1}}{\rightleftharpoons}}} E + I^*$$

The following rate constants have recently been reported [411] for the reaction of aprotinin with plasmin: $k_1 = 9 \times 10^5$ $M^{-1}s^{-1}$ and $k_2 = 1.4 \times 10^3$ s^{-1}. The difference in reaction rates may be illustrated by the fact that in the binding of aprotinin to α-chymotrypsin, the precomplex L, which has a life varying between 2×10^{-4} and 2×10^{-5} s, is destroyed 10 to 100 times before a single reaction to complex C takes place [76]. On the other hand, the rate constants k_2 and k_C for the monomolecular reaction which leads to C from the two sides, L and X, differ by a factor of 10^6 for bovine chymotrypsin [77].

Of primary interest for practical purposes are kinetic constants that describe overall reactions of a given system. Equation (b), a simplification of the enzyme-inhibitor interaction, may prove adequate [36, 75]:

$$E + I \underset{k_d}{\overset{k_{on}}{\rightleftharpoons}} C \underset{k^*_{on}}{\rightleftharpoons} E + I^* \qquad (b)$$

where k_{on} or k^*_{on} are the second-order rate constants of association (M^{-1} s^{-1}); k_d is the first-order rate constant of dissociation (s^{-1});

$$K_i = \frac{k_d}{k_{on}} = \frac{\text{conc. E} \times \text{conc. I}}{\text{conc. C}}$$

is the overall equilibrium (dissociation) constant (mol/l) for the liberation of the virgin inhibitor I, and K_I^* that for the formation of the modified inhibitor I*; $t_{1/2}$ is the half-life of dissociation of complex C. For definition and symbols, see [36, 75–77].

Typical kinetic constants for the reaction of aprotinin with proteinases are presented in Table 3. The association rate constants k_{on} given indicate that virgin aprotinin rapidly reacts with the proteinases to form a stable complex C, which means that the preincubation times ordinarily used in the inhibition assay (5–15 min before addition of substrate) are sufficient for complex formation to occur. The dissociation constants k_d, however, show significant differences between aprotinin-enzyme complexes, with half-lives ranging from 17 weeks (bovine trypsin) to 11 seconds (starfish trypsin 1). The very low k_d-value of the bovine trypsin-aprotinin complex is the main reason for the extremely high stability of this complex as reflected in the dissociation constant $K_i = 6 \times 10^{-14}$ mol/l, which is one of the lowest so far reported for a protein/protein interaction [36]. Although the equilibrium constant of the starfish trypsin 1-aprotinin complex is also rather low ($K_i = 1 \times 10^{-9}$ mol/l), it dissociates 10^6 times more rapidly ($t_{1/2} = 11$ s) than the former owing to the high dissociation rate constants $k_d = 6.5 \times 10^{-2} s^{-1}$ and $k_{-C} = 2 \times 10^{-3}$ s^{-1} as compared with $k_d = 6.6 \times 10^{-8}$ s^{-1} for the complex with bovine trypsin (see Table 3) [74, 79].

Another observation is of special interest in this connection. Ordinarily, the incubation of a proteinase inhibitor protein with catalytic quantities of a target enzyme results in specific hydrolysis of the reactive-site peptide bond so that an equilibrium is reached between the virgin and the modified inhibitor, as is shown in Eq. (a) and defined by Eq. (c) [73, 75].

$$K_{hyd} = \text{conc I*/conc I} \qquad (c)$$

In the case of aprotinin, cleavage of the reactive-site bond Lys-15–Ala-16 by commonly available serine proteinases is an extremely slow process (cf. the extremely low k_{-C} values for the complexes of aprotinin with trypsin and chymotrypsin in Table 3). It therefore remained undetected until recently, when an equilibrium constant K_{hyd} of 0.3–0.38 (at pH 5–7.5 [77]) could be established after nearly a year's incubation with plasmin [80]. The observation that rapid dissociation of proteinase-inhibitor complexes (high k_{-2} value) may be correlated with rapid hydrolysis of the reactive-site peptide bond (high k_{-C}) [81] prompted a search for proteinases suitable for the production of modified aprotinin. Trypsin 1 from the starfish Dermasterias imbricata proved to be well-suited for this purpose [74]. It catalyzes both the hydrolysis and the resynthesis of the reactive-site peptide

Table 3: Overall rate constants for formation and dissociation of enzyme-aprotinin complexes. For details and definition of constants, see text, especially equation (b) (K_i or K_I^*, k_{on}, k_d, k^*_{on}, $t_{1/2}$) and equation (a) (k_{-2}, K_{-C}). For definition, M = mol/l.

Complex with	Conditions		Rate constants and half-lifes							Reference
	pH	T (°C)	K_i(M)	K_I^*(M)	k_{on} ($M^{-1}s^{-1}$)	k_d (s^{-1})	k^*_{on} ($M^{-1}s^{-1}$)	k_{-C} (s^{-1})	$t_{1/2}$[b]	
Trypsin, bovine	8	25	6×10^{-14}		1.1×10^6	6.6×10^{-8}			17 weeks	[36]
	8	21	$\sim 1\times10^{-13}$		2×10^5	1×10^{-7}[a]				[75]
	7.5	22.5	$\sim 3\times10^{-13}$	$\sim 1\times10^{-13}$	3×10^5	$\sim 8\times10^{8}$[a]	3×10^3	3.3×10^{-10}		[78]
	7.5	21				8×10^{-8}		8.7×10^{-10}		[79]
Anhydrotrypsin	8	25	$< 3\times10^{-13}$		7.7×10^5	$< 2\times10^{-7}$			> 5 weeks	[36]
Chymotrypsin, bovine	8	25	9×10^{-9}		1.1×10^5	1×10^{-3}			12 min	[36]
	7.5	22.5	2×10^{-9}	7.6×10^{-10}	6×10^5	9×10^{-4}[a]	8.5	5.4×10^{-9}		[77]
	7.5	21				9×10^{-4}[a]		1.4×10^{-8}		[79]
Trypsin 1, starfish	7.8	21	1×10^{-9}			6.5×10^{-2}[a]		2×10^{-3}	11 sec	[74, 79]

[a] k_{-2} in equation (a), normally practically identical with k_d.

[b] Half-life of dissociation. The half-life of association ($t_{1/2}^{ass}$) is concentration-dependent. For the reaction of excess native aprotinin with trypsin (1×10^{-7} mol/l) $t_{1/2}^{ass} \sim 6.3$ s, and with chymotrypsin (1×10^{-7} mol/l) $t_{1/2}^{ass} \sim 63$ s; for the reaction of modified aprotinin (I*) the corresponding values are $t_{1/2}^{ass} \sim 39$ min (trypsin) and $t_{1/2}^{ass} \sim 9.5$ days (chymotrypsin), respectively, at the same enzyme concentration. (Calculated from the values given in Table 3 by F. Fiedler, Munich).

bond approximately 10^6 times more rapidly than bovine trypsin [79]. Incubation of aprotinin with 0.2 mol% of trypsin 1 at 21 °C at a pH between 6.2 and 7.8 resulted in an equilibrium constant K_{hyd} close to 1, corresponding to equimolar amounts of virgin and modified inhibitor in the incubation mixture. This constant increased exponentially with increasing pH ($K_{hyd} \sim 2.4$ at pH 8.5 and $K_{hyd} \sim 4.5$ at pH 8.9) [79]. At pH 8.2, the equilibrium was reached after 4.6 days, starting either from virgin or from modified aprotinin.

Besides the decidedly higher (approx. 10^4 times) dissociation rate (see k_d in Table 3) of the aprotinin-chymotrypsin complex as compared with the aprotinin-trypsin complex, there are striking differences in the global association rates for the formation of the stable complex C from either virgin or modified aprotinin (Table 3). The reaction of the modified inhibitor with chymotrypsin and trypsin proceeds much more slowly (by a factor of 10^5 and 10^2, respectively) than with virgin aprotinin ($k^*_{on} = 8.5$ and 3×10^3 M^{-1} s^{-1} vs $k_{on} \sim 6 \times 10^5$ and 3×10^5 s^{-1}, respectively) [77, 78]. This is of practical significance in that the two inhibitors may be quantitatively estimated in mixtures preincubated for different lengths of time. Whereas, for example, 99.9% of native aprotinin but less than 0.1% of modified aprotinin complexes with chymotrypsin, under suitable conditions, within 1 min, both inhibitor forms are totally complexed with trypsin after 2.5 h of preincubation [79].

The following observations are also of general interest: (1) Any chemical modification of the ion pair formed between Lys-15 of aprotinin and Asp-177 of trypsin in the specificity pocket results in a significant increase (10^3 times or more) in the dissociation rate of the complex [36]. The marked difference between the stability of the trypsin-aprotinin complex and that of the chymotrypsin complex (see Table 3) is mainly due to the additional, stabilizing interactions afforded by the specificity pocket for Lys-15 [36, 77]. (2) The formation of a very tight complex between aprotinin and anhydrotrypsin (Table 3) clearly shows that the alcohol side chain of Ser-183 of trypsin plays no role in the stabilization of the complex [36]. (3) The absolute values of free energy of association are very high for the firm complexes, e.g. $\Delta G^\circ_a = -18.1$ and -11 kcal/mol for the complex with trypsin and chymotrypsin, respectively, whereas the enthalpic values (ΔH°) are near zero (25 °C, pH 8.0) [36].

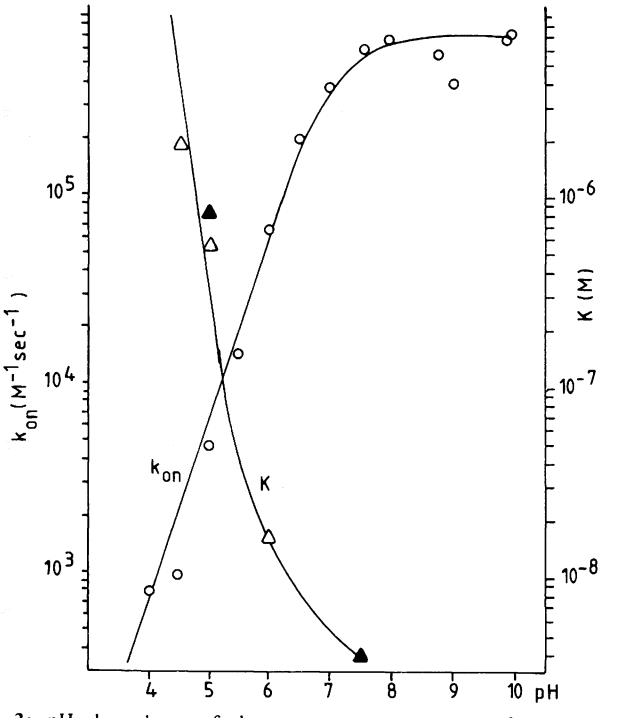


Fig. 3: pH dependence of the apparent rate constant of association k_{on} (**left ordinate**) and of the dissociation equilibrium constant K_i (**right ordinate**) of the system aprotinin/α-chymotrypsin. Taken from Engel et al. [76].

The pH of the medium has a significant effect on the rate and equilibrium constants shown in Table 3 and 2 and on K_{hyd}. Normally, the equilibrium constants K_i and K_{hyd} as well as the dissociation rate constants k_d and k_{-C} are lowest in the neutral and slightly alkaline pH range (pH ~ 7–9), whereas the association rate constants k_{on} and k^*_{on} then have the highest values. Accordingly, complex formation is strongly favored at the optimal pH of the proteinases. As the pH of the medium decreases, K_i and k_d or k_{-C} increase sharply, whereas k_{on} or k^*_{on} decrease significantly so that dissociation of the complex is promoted in acidic media. The K_i of the pig pancreas kallikrein-aprotinin complex, for example, increases by a factor of 10^3 from pH 7.8 to 5; at pH 4 the complex is completely dissociated [4]. The K_i of the bovine trypsin-aprotinin complex increases approximately 10^8 times from pH 8 to pH 3 as estimated from data given in Refs. [36, 75]. A more specific example is given in Fig. 3, which clearly shows the marked change in the association rate constant k_{on} and in the dissociation equilibrium constant K_i with increasing pH for the aprotinin-chymotrypsin system [76].

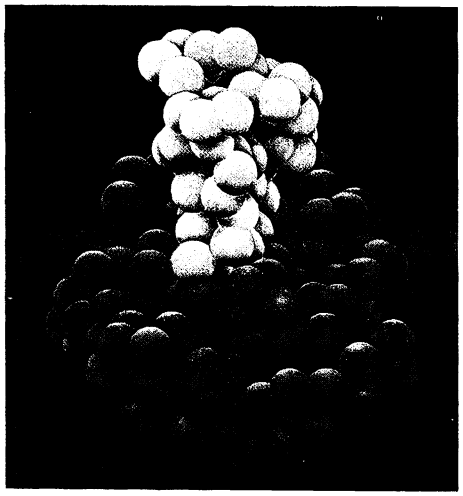
4. Molecular mechanism of inhibition

4.1. Formation and structure of aprotinin-proteinase complexes

Our knowledge about the motive forces responsible for the formation of a firm complex of a proteinase and a protein-type inhibitor is based chiefly on the results of X-ray crystallographic studies [82–88] as well as NMR (section 6.2.) [89], circular dichroism [90], and kinetic studies [36, 37, 75–80]. Complex formation is generally associated with: (1) A perfect fit of the reactive-site inhibitor residue (a lysine or arginine residue in the case of trypsin, plasmin and kallikrein) in the specificity pocket of the enzyme; (2) formation of a tetrahedrally oriented intermediate product by action of the nucleophilic oxygen of the alcohol group of the enzymatic active-site serine residue upon the carbonyl group of the lysine or arginine residue of the reactive peptide bond of the inhibitor; and (3) stabilization of the adduct by additional reactions in the vicinity of the specificity pocket and reactive-site bond residues, respectively.

The extended contact area existing between the two polypeptide segments of the inhibitor (Posns. 12–18 and 34–39) and the various peptide segments of trypsin (Posns. 39–42, 57–60, 96–99, 151, 175, 189–195, 214–216; cf. [83, 84]) is shown schematically in Fig. 4 and, in greater detail for the aprotinin molecule, in Fig. 2. Remarkably, 14 out of 58 amino acid residues in aprotinin, i.e. 24% of the total number of residues in the molecule, and 24 out of the 224 residues of trypsin (i.e. 10.7%) are tightly packed in a density comparable to that found in the interior of globular protein regions [91]. Consequently, numerous contacts (12 hydrogen bonds, a salt bridge, more than 200 van der Waals interactions, and 8 intermolecular water molecules [83, 84, 92]) contribute to the appreciable gain in free energy, the driving force for complex formation, so that an extremely high free energy of association of about 18 kcal/mol and an association constant K_a ($K_a = 1/K_i$) of 1.6×10^{13} l/mol result [36]. This high yield of free energy in the complexation is possible because the reactive inhibitor domain of unbound aprotinin already has a structure that almost perfectly complements the contact region at the surface of trypsin so that only a minimal adaptation of the two molecules is necessary [87]. This is the principal difference from the reaction of an enzyme with a normal substrate chain in which many degrees of freedom need to be immobilized to permit optimal interaction with the catalytic site of the enzyme.

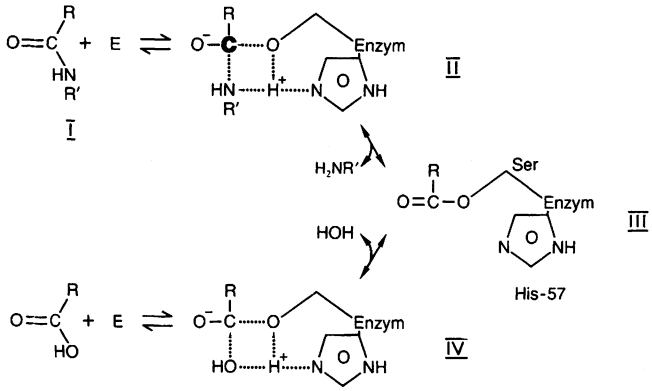
Enzyme-catalyzed peptide bond hydrolysis involves formation of an acyl-enzyme intermediate (III) via a covalent tetrahedral adduct (II) before the hydrolysis can be completed with the aid of a water molecule (IV) (see Scheme 1 [87, 93]). The reaction is initiated by action of the nucleophilic oxygen atom of the enzyme's serine residue on the carbonyl carbon atom of the scissile peptide bond (I) as soon as the



Figg. 4: Schematic representation of the numerous contacts formed in a proteinnase-aprotinin complex between the amino acid residues of the inhibitor (appprox. 24% of all residues) and the enzyme (approx. 11% of all residues). Thue key area of the inhibitor fits nearly perfectly into the lock area of the enzynme. Painted by A. Hermann according to a space filling α-carbon plot calculated by W. Bode and R. Huber. The X-ray structure of porcine pancreatic kallikrein and its complex with aprotinin has been elucidated very recently, seee: Bode, W., Chen, Z., Adv. Expt. Med. Biol. (Kinins-III, Part A) **156**, 289 (19983).

subbstrate is exactly aligned towards the catalytic residues. In thee case of the aprotinin-enzyme complex, the peptide carboonyl carbon (medium-faced, Scheme 1) is only turned in thee direction of the tetrahedral configuration; in other wcords, in the stable complex, an intermediate state between thee initial Michaelis-Menten complex (not shown in Scheme 1)) and the tetrahedral transition state is frozen, presumably owwing to the optimal fit which this conformation offers both prroteins without any large expenditure of energy [87]. Cleavaage of the scissile bond is then hindered by the tight packingg of the catalytic residues, especially His-57, so that traansfer of the proton from His-57 to HN-Ala-16 of the leavingg group, a necessary step for acyl-enzyme formation (see II anad III in Scheme 1), is prevented. Although complex formaation is attended by slight distortions in the inhibitor bindingg segments, the reactive-site peptide bond of aprotinin remaains intact, therefore. This recently developed concept [87, 922] provides a rational explanation for the low dissociation rattes of most of the aprotinin-proteinase complexes (see sectioon 3.3.).

In view of the gain in free energy resulting from the numerouas contacts between enzyme and inhibitor it is not surprisingg that, contrary to previous assumptions, complex formatioon need not always be preceded by formation of a tetrahedral or acyl-enzyme intermediate. In fact, the enzymaticallly inactive anhydrotrypsin likewise forms a tight complex wiith aprotinin (Table 2). The structural geometry of this



Schheme 1: Enzyme-catalyzed peptide bond (I) hydrolysis involves formation of aan acyl-enzyme intermediate (III) via a covalent tetrahedral adduct (II) beforee hydrolysis proceeds to completion with the aid of a water molecule (IV). Moodified according to Blow [93] and Huber and Bode [87].

complex is very similar to that of the trypsin-aprotinin complex [86]. The same is true of the aprotinin-trypsinogen complex [88]. It appears that enough energy is gained during contact of the complementary regions to force the disordered, enzymatically inactive conformation of trypsinogen into the ordered high-energy state of the active enzyme [90]. Most remarkably, this complex is able to catalyze resynthetis of modified (reactive-site peptide bond hydrolyzed) aprotinin [94].

The relatively low affinity of aprotinin for chymotrypsin as compared with trypsin (Table 2) is mainly a reflection of the poor fit of the inhibitor Lys-15 residue in the specificity pocket of this enzyme [82]. The strong interaction of this residue with the specificity pocket of trypsin, especially between the Lys-15 ammonium group and the carboxylate group of Asp-189 at the base of the pocket, contributes appreciably to the free energy that is gained during formation of the aprotinin-trypsin complex [83, 84, 87].

4.2. Analogs of aprotinin formed by enzymatic and chemical modification

There is now general agreement that aprotinin is a single-headed inhibitor having the Lys-15 residue in its active center. The location of the reactive-site bond within the sequence is generally demonstrated: (1) By limited proteolysis in a slightly acidic solution, which results in an equilibrium between the modified (hydrolyzed reactive peptide bond) and virgin inhibitor (reactive peptide bond intact); and (2) by resynthesis of the virgin inhibitor from the modified inhibitor under appropriate conditions [73, 75, 95]. In the case of aprotinin, however, limited proteolysis of the Lys-15–Ala-16 bond by bovine trypsin could originally be effected only after selective reduction of the disulfide bridge Cys-14–Cys-38 [96, 97]. After reoxidation, the modified inhibitor could be reconverted to virgin aprotinin by means of trypsin, α-chymotrypsin, plasmin or pancreatic kallikrein, and the localization of the reactive peptide bond could thus be demonstrated also by the conventional route [96].

It has been shown in the meantime that catalytic amounts of trypsin, chymotrypsin or plasmin establish a true thermodynamic equilibrium between virgin and modified aprotinin [80]. The modification reaction proceeds very slowly, however. With plasmin, for example, the equilibrium concentration between virgin and modified aprotinin is reached, under optimal conditions, after 300 days, starting from either the virgin or the modified inhibitor. In the cases of trypsin and chymotrypsin the reaction is even slower. This also shows, however, that aprotinin essentially reacts like other proteinase inhibitor proteins [73]: its reactive-site bond Lys-15–Ala-16 is hydrolyzed by catalytic amounts of proteinases, and the hydrolyzed bond is subject to thermodynamically controlled resynthesis [80].

Trypsin 1 from the starfish Dermasterias imbricata was recently found to provide easy access to modified aprotinin [74, 79]. In contrast to starfish trypsin 2 which, in common with bovine trypsin, forms a very long-lived complex with aprotinin, starfish trypsin 1 splits aprotinin rapidly and with high specificity at the reactive-site Lys-15–Ala-16 bond. Although starfish trypsin 1 also forms a relatively tight complex with aprotinin ($K_i \sim 1 \times 10^{-9}$ mol/l), this complex dissociates much more quickly ($t_{1/2} = 11$ s) than the bovine trypsin-aprotinin complex ($t_{1/2} = 10^7$ s). The rates of reactive peptide bond hydrolysis show a similar difference: starfish trypsin 1 splits the Lys-15–Ala-16 bond a million times faster than does bovine trypsin [79].

The modified inhibitor with the hydrolyzed Lys-15–Ala-16 bond was used to replace the Lys-15 residue with other amino acids [96, 98]. As expected, replacement of Lys-15 by arginine did not affect the inhibitory properties of aprotinin, whereas enzymatic replacement of this basic residue by phenylalanine or tryptophan produced a significant increase in affinity for chymotrypsin and a decrease in affinity for trypsin. Of the various aprotinin derivatives (modified also at the β/γ-carboxyl groups of the aspartic and glutamic acid

Table 4: Reactive site residues of structurally related trypsin-chymotrypsin inhibitors of the aprotinin (Kunitz) type. ++, strong inhibition ($K_i \leq 10^{-8}$ mol/l); +, weaker inhibition ($K_i \geq 10^{-8}$ mol/l); –, no inhibition; ∅, not tested (known); ITI, inter-α-trypsin inhibitor.

Inhibitor source	Position of residues										Inhibition of			Reference
	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_{23}'	P_{24}'	Plasmin	Kallikrein of		
												Tissue	Plasma	
Bovine organs:	13	14	15	16	17	18	19[a])	20[a])	38	39				
aprotinin	Pro	CYS	Lys	Ala	Arg	Ile	Ile	Arg	CYS	Arg	++	++	+	[17, 18]
Sea anemones, 5 II	Pro	CYS	Arg	Ala	Arg	Phe	Pro	Arg	CYS	Arg	++	++	+	[102]
Snails, K	Pro	CYS	Lys	Ala	Ser	Phe	Arg	Gln	CYS	Arg	++	+	∅	[103]
Bovine serum, BI-8	Pro	CYS	Lys	Ala	Ala	Met	Ile	Arg	CYS	Arg	++	+	+	[12]
Russel's viper, II	Arg	CYS	Arg	Gly	His	Leu	Arg	Arg	CYS	Gly	+[e])	+[e])	+[e])	[104]
Cobra, NNV II[b])	Leu	CYS	Lys	Ala	Arg	Ile	Arg	Ser	CYS	Gly	∅	++	∅	[42, 105]
Cobra, HHV II[c])	Leu	CYS	Lys	Ala	Tyr	Ile	Arg	Ser	CYS	Gly	++[e])	++	∅	[42, 105]
Cow colostrum	Pro	CYS	Lys	Ala	Ala	Leu	Leu	Arg	CYS	Gln	++	–	–	[106]
Human ITI-14-2	Pro	CYS	Arg	Ala	Phe	Ile	Gln	Leu	CYS	Gln	+[f])	–[f])	∅	[11]
Bovine ITI-14-2	Pro	CYS	Arg	Ala	Phe	Ile	∅	∅	CYS	Lys	+[f])	–[f])	∅	[12]
Soybean, Kunitz[d])	Ser	Tyr	Arg	Ile	Arg	Phe	Ile	Ala			++	–	++	[107]

[a]) Not involved in the contact with trypsin in the complex.
[b]) Naja nivea venom.
[c]) Hemachatus haemachatus venom.
[d]) Not structurally related.
[e]) Estimated from titration curves or author's classification (no quantitative data available, see references).
[f]) Hochstrasser, K., unpublished data (1981).

residues) described recently [99], those with a hydrophobic amino acid residue in position 15 are interesting inasmuch as they show high affinity for human granulocytic elastase [100]. It is noteworthy that substitution of Lys-15 by glycine did not entail a significant decrease in the affinity of aprotinin for porcine pancreatic kallikrein [100].

The recently accomplished total synthesis of aprotinin opens the way to a detailed study of the effect of amino acid exchanges on the inhibitory properties of this molecule [101]. The results of studies on the synthesis of aprotinin, aprotinin analogs, and polypeptide models of the reactive center of aprotinin have lately been reviewed [7].

4.3. The structural basis of kallikrein inhibition

While the tertiary structure of the active site of the kallikreins remains unknown[3]), insight into the structural basis for kallikrein inhibition by aprotinin can be gained only by comparison with the inhibitory specificities and reactive-site sequences of structurally homologous inhibitors (Table 4). Crystallographic studies have revealed that the amino acid residues of aprotinin that are in most intimate contact with trypsin in the complex comprise, in addition to Cys-14 and Cys-38, the "specificity pocket" residue Lys-15, Ala-16, and the basic residues Arg-17 and Arg-39 (Fig. 2) [83, 84]. In view of the inhibitory specificities of structurally homologous inhibitors it is evident that the relatively strong reaction of aprotinin with the kallikreins is due to the basic character of the residues in positions 17 and 39 [108].

Replacement of both basic amino acid residues in these positions by neutral residues completely abolishes the affinity of an aprotinin-type inhibitor for both tissue and plasma kallikreins (cf. cow colostrum inhibitor). If, however, a basic residue is present in position 19 (cobra HHV II), kallikrein-inhibiting activity is displayed. It appears, then, that in an aprotinin-type inhibitor a basic amino acid residue is required in one or more of these positions – 17, 19, 39 – to produce enough energy for complex formation with kallikrein.

This hypothesis is supported by recent studies of the structure/function relation in an aprotinin-type inhibitor from bovine serum [12] and of the trypsin-inhibiting aprotinin-type domains (ITI-14-2) derived from human and bovine inter-α-trypsin inhibitor [11, 12] (Table 4). The inhibitor from bovine serum bearing arginine in position 39 is a fairly potent inhibitor of tissue kallikrein, whereas the inhibitory domain ITI-14-2 of the human inter-α-trypsin inhibitor has no basic residue in any of the above-cited positions and so fails to inhibit tissue kallikrein. The ITI-14-2 domain derived from the bovine inter-α-trypsin inhibitor, on the other hand, bears a basic residue in position 39, lysine, but does not inhibit tissue kallikrein, either (Table 4). In the latter case, however, a large residue (e.g. tryptophan) in position 19 may prove a steric hindrance to complex formation.

The affinity of an aprotinin-type inhibitor for plasmin (or trypsin), as contrasted with kallikrein, is not significantly affected by such replacements of amino acids at the reactive site. The inhibition of plasma kallikrein and, notably, tissue kallikrein evidently requires additional structural features in specific subsite positions. This is true because of the more restricted substrate specificity of the kallikreins as compared with trypsin or plasmin. We may justifiably infer that the active sites of any of the kallikreins that form complexes with aprotinin are very similar. A close similarity between porcine and human tissue kallikrein or urinary kallikrein has in fact been reported recently [44]. Yet the decidedly lower affinity between aprotinin and the kallikreins when compared with trypsin (Table 2) indicates that the fit of the complementary contact regions in the aprotinin-kallikrein complex is less than perfect, or that the contact area differs markedly from that of the aprotinin-trypsin complex. This is true at least for contact in the specificity pocket – replacement of Lys-15 by glycine did not significantly affect the affinity of aprotinin for tissue kallikrein [100].

Kallikrein inhibitors as well as kallikreins are known to occur in snake venoms [104, 105]. Furthermore, snake toxins may be structurally related to aprotinin without possessing inhibitory activity against known proteinases [109]. The significance of these observations is as yet unclear.

5. Special properties relevant to administration of aprotinin

5.1. Interaction with glycoproteins and mucopolysaccharides

The relatively high basicity of aprotinin, which is due to its high isoelectric point of 10.5 [21], seems to be chiefly responsible for two special features of this inhibitor molecule: (1) Its binding to acidic glycoproteins or mucopolysaccharides including heparin [110, 111], and (2) its "fixation" and degradation in the kidney following therapeutic administration [112–114]. Aprotinin derivatives of lower basicity [96, 112] and aprotinin-type inhibitors from sea anemones [115] and snails [96, 116] as well as other trypsin inhibitors of si-

[3]) The X-ray structure of porcine pancreatic kallikrein and its complex with aprotinin has been elucidated recently, see: Bode, W., Chen, Z., Adv. Expt. Med. Biol. (Kinins-III, part A) **156**, 289 (1983).

millar molecular weight but lower basicity [112] are excreted in the urine.

5.2. Immunogenicity

Aprotinin-specific antibodies have not so far been observed in human sera during or after aprotinin treatment [117]. Aprotinin thus appears to be a comparatively weak immunogen.

Guinea pigs can, however, be sensitized to it by a special immunization program [117]. Aprotinin-precipitating antisera have also been obtained in rabbits by immunization with complete and incomplete Freund's adjuvant. These antisera were identified by immunodiffusion and immunoelectrophoresis (Eben, A. and Truscheit, E., 1964; data published by Haberland [117]). Other immunization methods for aprotinin were described recently [10, 13, 14]. Aprotinin-specific antibodies were also obtained when high-molecular weight cross-linked aprotinin served as the immunogen [118].

Aprotinin-specific antisera produced according to Eben and Truscheit [117] have been used in immunofluorescence studies [114] and in radioimmunoassays [35, 114]. These antisera were found capable of neutralizing almost completely the trypsin-inhibiting activity of aprotinin when gelatin [118], casein or BzArgOEt (Truscheit, E., unpublished observation) was used as substrate.

5.3. Elimination after administration

Serum or plasma levels of aprotinin obtained after i.v. injection in animals and humans decline rather rapidly owing to distribution of the inhibitor in the extracellular fluid and subsequent accumulation, particularly in the kidney [7, 119–121]. The following human serum levels were measured, for example, per Milliliter after i.v. administration of a single bolus of 500,000 or 50,000 KIU [121]: 0.25 h: 50, 5; 0.5 h: 35, 3.5; 1 h: 25, 2.5; 2 h: 17, 1.7; 4 h: 10, 1. The half-life of elimination changed from approximately $t_{1/2}$ = 0.7 h 1 h after injection to $t_{1/2}$ = 7 h 12 h after injection. About 80% of the aprotinin dose is found in the rat kidneys after 4 h [119]; aprotinin is taken up by the epithelial cells of the proximal tubules [113, 120]. It appears that aprotinin is almost entirely metabolized in the kidney lysosomes [120] inasmuch as biologically active aprotinin is not normally excreted in the urine. Only 1.5% of 1,000,000 KIU, for example, was found in human urine a short time (1.5 h) after i.v. injection [119]. Aprotinin that is covalently bound to soluble polysaccharides is more slowly eliminated from the circulation and is excreted with the urine without accumulating in the renal tissue [122].

Although the toxicity of aprotinin is extremely low – the LD_{50} for mice and rats is 2.5×10^6 KIU/kg and that for rabbits 0.5×10^6 KIU/kg – rapid i.v. injection of large doses should be avoided, for the high basicity of aprotinin may cause liberation of histamine and lead to an anaphylactoid reaction.

5.4. Effects on cellular function

In view of the widespread use of aprotinin as a proteinase inhibitor, recent observations of additional biological effects of this inhibitory polypeptide merit special attention. Aprotinin has been found to influence the response of body cells such as leukocytes, platelets, and macrophages to various stimuli as well as the activities of membrane-associated enzymes. We know little, however, about the mechanisms of these actions of aprotinin. These effects are briefly discussed in section 6.5.

6. Biochemical and biomedical applications

6.1. General applications

Aprotinin has proved to be a valuable tool in biochemical and biomedical research and in routine procedures used in these areas. It is used extensively for the following purposes:

6.1.1. Proteinases inhibited by aprotinin can easily be purified by affinity chromatography with insolubilized aprotinin derivatives [7], e.g. aprotinin-Sepharose [123] and aprotinin-cellulose [15]. Besides trypsin [123–125], chymotrypsin [123], pancreatic elastase [52], and plasmin or plasmin fragments [126, 127], plasma and tissue kallikreins [7, 44, 128, 132], as well as granulocytic elastase and cathepsin G [133, 134] have been successfully isolated by this means under appropriate conditions. Aprotinin-Sepharose has also been used for purification of a hormone-degrading enzyme [135].

6.1.2. Aprotinin-sensitive enzymes can be crystallized as aprotinin-proteinase complexes either for purposes of purification [2] or for X-ray crystallography [82–84]. For the latter purpose, complexes of aprotinin with inactive enzymes such as anhydrotrypsin [86] and trypsinogen [88] have been crystallized as well.

6.1.3. Aprotinin is widely used as a molecular weight marker (M.W. approx. 6500) in gel permeation chromatography [136] and SDS electrophoresis [137] (see e.g. Product Profile 6040SA from Bethesda Research Lab., Rockville, USA).

6.1.4. Kallikrein activity in a mixture of esterases and proteinases can be identified by specific inhibition of the kallikrein with aprotinin. Thus, the presence of aprotinin (10 KIU/ml, i.e. 2×10^{-7} mol/l) in the assay kit for determination of kallikrein activity in human urine by use of a synthetic substrate permits a clear distinction to be made between substrate cleavage induced by kallikrein and by urokinase [138].

6.1.5. Aprotinin is frequently used to solve a common problem in biochemical and biomedical research: how to prevent proteolytic degradation of proteins or polypeptides, enzymes, proenzymes, preproteins, etc. in biological fluids or tissue extracts and during purification. Aprotinin is used for the following purposes, for example: (a) Suppression of proenzyme activation during purification of kininogens [139, 141], and preparation and assay of plasma samples used for determination of fibrinopeptide A or B [142]; (b) prevention of breakdown of proteohormones such as glucagon during the preparation and assay of samples to be used in radioimmunoassays [143–146]; (c) preservation of protease-sensitive plasma factors such as clotting factor VIII (antihemophilic factor) [147] and fibronectin [148] during their isolation, storage and analysis. Aprotinin concentrations used for these purposes ranged from 100 KIU/ml (i.e. approx. 2×10^{-6} mol/l) to 1600 KIU/ml (i.e. approx. 3×10^{-5} mol/l); in most cases 500 KIU/ml (approx. 1×10^{-5} mol/l) should be sufficient.

6.1.6. Aprotinin may be used to terminate lysis caused by proteinases in tissue culture studies (see section 6.3.) and in experiments in which only a limited proteolysis is required. Examples are inhibition of kinin-liberating enzymes in samples subjected to biological assays (by 1650 KIU/ml) [149], and preparation of defined molecular forms of plasmin [150, 151].

6.2. Tool for protein conformation and protein-protein interaction studies

Aprotinin, a typical globular protein, has been used in numerous studies on the fundamental aspects of protein conformation [22, 152–155]. These have included theoretical and experimental studies of the internal mobility of proteins, protein folding, and surface interactions with solvents, and they have led to a novel description of globular protein conformation by a dynamic grouping of rapidly interconverting molecular structures [22, 152, 153]. It is especially interesting that the average solution conformation of aprotinin closely matches the conformation of its crystalline structure: "Hydrophobic clusters form stability domains" in the interior of the inhibitor "which function as pillars for the architecture of the protein molecule" [153]. In these studies, furthermore, nuclear magnetic resonance was used with advantage for the conformational description of aprotinin analogs adapted to mechanistic studies of proteinase-inhibitor interactions [156] and to studies of the state of catalytic groups in proteinases, proenzymes, proteinase inhibitors and their complexes [157, 158]. Extensive knowledge of the pro-

perties of aprotinin also permits its application to the solution of as yet unsolved problems, such as e.g. a molecular explanation for the dramatic differences in the dissociation rates of the complexes of aprotinin with either bovine trypsin or crayfish trypsin 1 despite the excellent affinity for aprotinin shown by both enzymes (see section 3.3.) [74, 79].

The significance of aprotinin ("E. Werle's kallikrein inactivator") as a model compound in biochemistry and biophysics has been further outlined recently by Huber, R., Adv. Expt. Med. Biol. (Kinins-III, Part A) **156**, 29 (1983).

6.3. Tissue culture and organ preservation

When present in cell culture media in concentrations ranging from 300 KIU/ml (approx. 6×10^{-6} mol/l) to 10,000 KIU/ml (approx. 2×10^{-4} mol/l), aprotinin exerts a variety of effects:

(1) It delays the degradation of proteohormones such as substance P [159] and insulin [160, 161]. Prevention of insulin degradation by aprotinin accounts for the observed increase in the absorption of subcutaneously injected insulin in the presence of the proteinase inhibitor [162]. Aprotinin may thus be successfully used to treat insulin-resistant diabetes [163].

(2) It preserves the integrity and so extends the lifes of cells and cell membranes from kidney [164] and cerebellum [165].

(3) A dose-dependent inhibition of DNA, RNA and protein synthesis has been observed in stimulated mous lymphocytes at aprotinin concentrations between 0.3×10^{-7} and 2.5×10^{-7} mol/l [166].

(4) If applied in relatively small amounts (10 to 500 KIU/ml), it prevents toxic products released from disintegrating cells from damaging intact targets [167].

(5) It inhibits the uptake of adenosine by cardiac cells; this can be important for improved perfusion of the myocardium in vivo [168].

Aprotinin has also been reported to have an advantageous effect on organ preservation. For kidney preservation, aprotinin (500–2000 KIU/kg body weight) was injected into animals immediately before removal of the organ, which was then incubated in an aprotinin-containing medium prior to transplantation [169, 170]. A protective effect of aprotinin on lung tissue in vitro has also been described recently [171].

6.4. Inhibition of (transformed) cell growth

Several groups have investigated the possible usefulness of aprotinin as an immunotherapeutic agent in cancer. It was found to inhibit the growth of malignant transformed cells (at lower doses) but also the growth of normal cells (at higher doses), aprotinin being bound and endocytosed by the tumor targets [172, 173]. Aprotinin administered by various routes depressed the growth and invasiveness of solid tumors in animals [174]. Recent studies indicate that this therapeutic effect of aprotinin may be mediated by improvement in the host's immune response to tumors [175]. It was noted, for example, that bolus injection of a high dose of aprotinin in both human subjects and animals led to a significant improvement of the in-vitro lymphocyte response, and this in turn resulted in reduction of the tumor-induced lymphocyte suppression [175]. Aprotinin is apparently able to improve cell-mediated immunity by direct action on the lymphocyte (see section 6.5.), and the effect is stronger in cancer patients than in normal subjects. However, not all studies have confirmed that aprotinin can inhibit tumor growth and invasiveness. The conflicting results reported by different authors may be due to a dose and species dependence of the effect.

6.5. Effects on leukocytes and macrophages

Aprotinin affects various enzyme systems of the leukocytes and the response of lymphocytes. It inhibits, though rather weakly, a basophil kallikrein-like enzyme that is released (together with histamine) from human peripheral leukocytes upon stimulation with IgE [176, 177]. By means of aprotinin-Sepharose, leukocytic proteinases generating factors from complement C5 that are chemotactic for leukocytes and tumor cells can be removed from the incubation medium [178]. With the use of this affinity sorbent, a kallikrein-like enzyme was recently isolated from the cytosol of polymorphonuclear granulocytes [179]. This enzyme seems to be different from the kininogenase that is believed to be present in the lysosomal lysate of the same cells [180].

Aprotinin can either increase (at low doses) or inhibit (at higher doses) the response of peripheral lymphocytes to various stimuli but it is toxic to unstimulated cells in culture [181, 182]. Remarkably, it binds to the plasma membrane of both peripheral blood lymphocytes and polymorphonuclear leukocytes [181]. Endocytosis of aprotinin was demonstrable in the latter case. It is therefore conceivable that aprotinin influences cell function by inhibiting (in secondary lysosomes) neutral proteinases that are normally released extracellularly to potentiate lymphocyte stimulation.

Leukocytic cathepsin D releases sizable amounts of leukokinins, i.e. potent permeability agents, from a precursory protein, leukokininogen, which is found in ascites fluid produced in neoplastic diseases [183]. Cathepsin D can act upon this substrate only after conversion of a prosubstrate to leukokininogen, a process mediated by a trypsin-like enzyme. This proteinase, too, is effectively inhibited by aprotinin [184].

During macrophage-tumor interaction, aprotinin can inhibit the cytolysis of the neoplastic targets by activated macrophages if present in a concentration of 750 KIU/ml (approx. 1.5×10^{-5} mol/l) [185]. The cytolytic activity was found to be generated by a specific proteinase that is secreted by the activated macrophages [186].

6.6. Blood preservation and inhibition of platelet aggregation

Platelet aggregation (the second phase, associated with release of serotonin) is very effectively inhibited by aprotinin concentrations between 0.4×10^{-5} and 1×10^{-5} mol/l [187–191]. The spontaneous formation of microaggregates in stored blood is thus prevented, clotting factors are protected, and fibrinolytic activity is depressed, at least for 5 days. Under transfusion conditions, platelet function is immediately restored so that the patient's coagulation status remains unchanged [187, 189]. Furthermore, a bolus injection of 20,000 KIU/kg body weight of aprotinin can lessen the tendency to aggregation ordinarily observed after major surgery [192].

6.7. Wound healing

It has been reported that aprotinin reduces the development of adhesions and of secondary necrosis after operations [193, 194]. The inhibitor is also used as an additive to adhesive fibrin ("fibrin glue") for adapting tissues and sealing bleeding areas with fibrin, preventing its dissolution before tissue repair has set in [195–197].

6.8. Tool for studies of muscle metabolism and renal function

Glucose uptake by working human muscle cells is significantly reduced after aprotinin administration (bolus injection of 500,000 KIU/10 min) but returns to normal upon concomitant administration of bradykinin (13 ng/min) [198–200]. High doses of aprotinin (125,000 KIU/kg i.v.) also delayed appreciably the metabolic rehabilitation of rat skeletal muscle during recirculation following ischemia [201]. A rational explanation for these observations is the involvement of kallikrein or a kallikrein-like enzyme in the mediation of insulin action on glucose uptake by skeletal muscle by way of kinin liberation [200].

The administration of aprotinin to volume-expanded rats leads to a significant reduction of glomerular filtration rate, clearance, urine volume, and urinary immunoreactive PGE_2 [202]. This finding suggests that the kallikrein-kinin system may contribute to changes in renal function during extracellular volume expansion. Aprotinin is therefore a valuable tool in studies on the involvement of the kallikrein-kinin

system in the body's regulatory mechanisms. Besides kallikreins and kininogenases, however, other enzyme systems may be affected by aprotinin. An example is the human kidney aminopeptidase of the brush border membrane. This enzyme is activated by aprotinin in its membrane-bound form but inhibited by it after solubilization [203].

6.9. Effects on fertilization

Proteases play a significant role in the fertilization process [204, 205]. The trypsin-like acrosomal proteinase acrosin, for example, assists the spermatozoon in penetrating the zona pellucida of the ovum; trophoblast and uterine proteinases aid in implantation of the blastocyst in the uterus. Proteinase inhibitors could therefore be utilized to prevent conception. Recent investigations have shown that in-vivo use of aprotinin for the blockade of acrosin is difficult because of the high concentration (approx. 1×10^{-2} mol/l) that is needed if the inhibitor is to penetrate the sperm head membranes [644]. In rabbits, however, implantation could by prevented with much lower concentrations of aprotinin when this was introduced into the cavum uteri [206]. This contraceptive effect of aprotinin may result from inhibition of a trypsin-like or kallikrein-like proteinase of the rabbit trophoblast that plays a role in the initiation of embryo implantation in the uterus [65].

Infusion of aprotinin in pregnant rats led to a reduction of uterine motility and a significant prolongation of parturition by comparison with saline-infused controls [207]. Aprotinin is of benefit in cases of menorrhagia and intramenstrual spotting due to the use of intrauterine devices [208]. Repeated injections into the uterine cavity stop the bleeding and markedly reduce inflammatory reactions including pain. Prior intravaginal administration of aprotinin has been reported to preserve the integrity of cells in vaginal smears [209].

6.10. Experimental shock

Aprotinin has been widely used in experimental studies of shock and shock lung and in the treatment of these conditions in humans [210–212]. The underlying rationale stems from the finding that kinins and polypeptides are released from hypoxic and damaged tissue by the action of proteinases including the kallikreins [211–213]. Such peptides can intensify the inflammatory response via several routes and may prove toxic to the heart and lungs [212, 214]. Further, aprotinin has been found capable of aborting hyperfibrinolysis and of reducing the disseminated intravascular coagulation which is frequently associated with the shock syndrome [213, 215, 216]. While a protective effect of aprotinin on animals in experimental shock has been observed in most of these studies, a beneficial effect of aprotinin treatment was not seen in other studies [217, 218]. That conflicting results should have been obtained is not surprising considering the diffferences in experimental approach and the varying aprotinim doses used by different workers. There is growing evidence from both experimental and clinical studies that the therapeutic effectiveness is dependend on administration of a high dosage of aprotinin in an early phase of the disease as shown e.g. convincingly for bile-trypsin induced pancreatitis in dogs [219].

Recently refined techniques have made it possible to study in greater detail the mechanism of trypsin inhibition by serum inhibitors and additionally injected aprotinin in dogs with trypsin-induced shock [220]. In the absence of aprotinim administration, infusion of trypsin led to irreversible shock as soon as the α-macroglobulins were saturated, while free α_1-antitrypsin was still present in excess. The drop in blood pressure was correlated with kininogen consumption, reflecting the release of highly vasoactive kinins [221]. In the presence of aprotinin, however, trypsin infusion could be comtinued beyond saturation of the alpha-macroglobulins without causing irreversible hypotension or significant kininogen consumption [220, 221]. Evidently, aprotinin, unlike α_1-antitrypsin, can protect the animal against the irreversible shock that is produced in control animals after consumption of the α-macroglobulins. Aprotinin-trypsin complexes and α-macroglobulin-trypsin-aprotinin complexes were in fact demonstrable in the aprotinin-treated animals. Apparently, trypsin is even transferred from the trypsin-α_1-antitrypsin complex to free aprotinin.

These studies have made it clear that a powerful exogenous proteinase inhibitor can, at the very least, delay serious clinical complications when the inhibitor reserve of the body itself has been exhausted. That this can occur under pathological conditions has been shown in experiments concerned with pathobiochemical processes secondary to experimental induction of pancreatitis in dogs [222]. Besides a considerable increase of trypsinogen levels in the circulation, α_1-antitrypsin-trypsin complexes were detected. Such complexes can cause irreversible hypotension when infused into dogs depleted of α-macroglobulins that have not been treated with aprotinin [220]. Remarkably, the distribution of trypsin between α_2-macroglobulin, aprotinin and α_1-antitrypsin in human serum samples in vitro [223] is similar to that found in dog plasma specimens following trypsin-induced shock.

6.11. Effect on virus replication and bacterial growth

According to evidence presented recently, virus replication is regulated by cell-specific proteases [224, 225]. Proteinase inhibitors should consequently be able to interfere with virus replication and infectiosity. Aprotinin has in fact been shown to increase the survival rate in swine fever disease [226]. This disease is linked to the formation of a chymotrypsin-like proteinase by the virus-infected cells [227]. It is possible that aprotinin affects this disease by inhibiting such a virus-associated proteinase. Inhibition of bacterial growth by aprotinin has also been reported [228]. In these studies aprotinin proved to be effective both in in vitro conditions and in vivo.

7. Clinical use

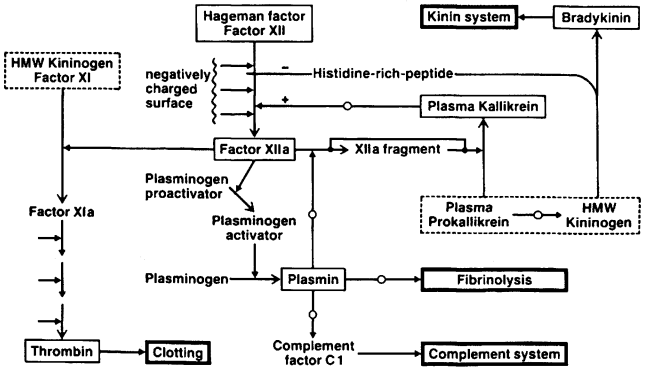
7.1. General aspects

Indications for therapeutic or prophylactic use of aprotinin have been studied intensively. Aprotinin administration is recommended e.g. as part of the therapeutic regimen in acute pancreatitis, various states of shock syndroms, and hyperfibrinolytic hemorrhage [229–236]. However, the attempt to demonstrate clinical effectiveness by prospective controlled clinical trials has proved to be extremely difficult and has been successful only for some of the suggested indications, despite of sound biochemical rationale. This may be largely due to problems in clinical methodology. For acute pancreatitis, as an example, several clinical double-blind trials yielded conflicting results. The statistically significant reduction in mortality observed in one study by Trapnell et al. [229] could not be confirmed by others [237, 238], leaving the question unsolved whether aprotinin is effective only in special types of the disease. On the other hand, activation of trypsin, chymotrypsin and elastase in acute pancreatitis has been demonstrated by detection of complexes of these enzymes with α_1-antitrypsin in peritoneal exudates [239] as well as in the circulation [240, 241]. In addition, most of the α_2-macroglobulin present in the exudate was found to be complexed with proteinases [239]. These findings provide a clear biochemical rationale for the therapeutic use of suitable proteinase inhibitors even though the benefit to the patient may be hard to prove statistically because of the complexity of the disease.

In traumatic-hemorrhagic shock prospective clinical trials with reference either to mortality [236] or post-traumatic pulmonary insufficiency [230] have clearly demonstrated clinical effectiveness. Patients may be expected to benefit most if aprotinin is given in an early phase of this disease [236].

7.2. Interaction with plasmin and plasma kallikrein

Among the numerous biological effects of aprotinin, its inhibition of plasmin and plasma kallikrein is of special interest.



Scheme 2: Solid-phase activation of the intrinsic coagulation pathway: interactions between clotting, fibrinolysis, complement, and the kinin-generating system [242, 243].
Plasma kallikrein accelerates activation (+, positive feedback), whereas the histidine-rich peptide, cleaved off from high-molecular weight (HMW) kininogen by plasma kallikrein, inhibits activation (–, negative feedback). HMW kininogen binds factor XI and plasma prokallikrein (both occur in plasma associated with it) via the histidine-rich peptide to the negatively charged surface, an exposed collagen fiber or basal membrane, lipopolysaccharides, glass, kaolin, etc. Aprotinin can inhibit the action of both plasmin and plama kallikrein, as indicated by circles in the pathways.

How these enzymes interact with other coagulation factors or fibrinolytic factors is shown diagrammatically in Scheme 2 [242, 243].

Since aprotinin has a significantly greater affinity for plasmin (see Table 2), it should be possible to regulate the desired effect, viz. inhibition of either fibrinolysis or blood clotting, by commensurate dosing of the inhibitor [244]. This presupposes knowledge of the actual concentrations of all the reactants in relation to time, under in-vivo conditions, and these concentrations cannot at present be determined because of the lack of appropriate methods. They can, however, be calculated for systems containing only plasminogen, plasma prokallikrein, and aprotinin [245]. In this case aprotinin will be bound to liberated plasmin up to equimolar concentrations inasmuch as the dissociation constant of this complex is far below the in-vivo plasmin/plasminogen concentration. The in-vivo concentration of plasma prokallikrein, however, approaches the K_i-value of the aprotinin-kallikrein complex. Therefore, a large molar excess of aprotinin is needed to obtain complete inhibition of liberated plasma kallikrein. This can be simulated in a test system measuring contact activation of the coagulation cascade, which is stimulated by plasma kallikrein. Strong inhibition is obtained only at aprotinin concentrations ranging from 250 to 500 KIU/ml, equivalent to a five to tenfold molar excess of aprotinin over plasma prokallikrein resp. kallikrein [245, 246]. For complete inhibition of plasmin in the pure system, an aprotinin concentration of 125 KIU/ml is sufficient provided all of the plasminogen is transformed into plasmin [245]. Smaller quantities of aprotinin should suffice under in-vivo conditions since small amounts only of the proenzymes are activated ordinarily and most of the liberated proteinases are rapidly inhibited and eliminated by the natural inhibitors, which are present in molar excess over the proenzymes/enzymes [247].

It is noteworthy that aprotinin also effectively inhibits – in addition to plasmin – the plasmin-streptokinase complex which is an intermediate in plasminogen activation during thrombolytic therapy with streptokinase [41]. Even though the inhibition of this complex proceeds comparatively slowly (at 1 μmol/l aprotinin, the association half-life is 250 s; $k_1 = 1.1 \times 10^4$ l mol^{-1} s^{-1} and $k_2 = 1.1 \times 10^3$ s^{-1}; cf. section 3.3.), aprotinin may be used to control bleeding complications following thrombolytic therapy with streptokinase [41].

8. Synopsis

The data and observations we have cited show that we already have detailed knowledge of the chemical, physical and biochemical properties of aprotinin and its inhibitory mechanism of action. Aprotinin is readily available in pure form and is therefore widely used in various research disciplines and routine assays. It has also become a valuable drug for the treatment of a variety of diseases. The information about functional aspects of aprotinin remains scanty, however.

The following aspects are of special interest for future research: (1) The molecular basis for the pharmacologic effects of aprotinin not associated with proteinase inhibition, including its interactions with membranes; (2) evaluation of optimal dosage for interfering either with the coagulation/kinin-liberating or the fibrinolytic pathway in vivo; (3) evaluation of optimal dosage for inhibition of tissue kallikreins and/or other proteinases such as trypsins and chymotrypsins liberated during local inflammatory processes; (4) the functional significance of the occurrence of aprotinin in the highly specialized tissue mast cells throughout the bovine organism; and (5) the functional significance of the aprotinin-like inhibitor that is present in bovine serum and of the aprotinin-type polypeptide domains in the inter-α-trypsin inhibitor. Elucidation of the last two questions is likely to open additional avenues of aprotinin research and future medical applications.

9. Appendix on aprotinin applications

9.1. General comments

9.1.1. Availability

Aprotinin is available as a sterile solution in ampoules (Trasylol®) from Bayer and from FBA-Pharmaceuticals. For research purposes it may also be obtained in lyophilized form. It is identical with BPTI (basic pancreatic trypsin inhibitor), PTI (pancreatic trypsin inhibitor (Kunitz)), kallikrein inhibitor from bovine organs, etc. [21]. Aprotinin preparations may contain ATPase inhibitors [248] but the Trasylol commercially available has been free of this agent for several years now (A. Arens, personal communication, 1980).

9.1.2. Storage

Aprotinin may be stored in lyophilized form below 4 °C for an "unlimited" time without losing its inhibitory activity. Aprotinin solutions in salt-buffer media, pH 5–8, are stable at least for one month at 4 °C and below –20 °C for years; the same holds true for open ampoules. During storage in solution, bacterial growth should be prevented by addition of preservatives such as sodium azide, benzyl alcohol, pentachlorophenol, thiomersal, and antibiotics. However, a possible direct pharmacologic effect of the preservative has to be taken into account when biological studies are performed. For biochemical assays preservatives need not be added.

Aprotinin solutions of pH < 4 and > 9 should be used within a few hours. Direct exposure of aprotinin solutions to sunlight, UV-light and reducing agents should be avoided.

9.1.3. Dialysis, ultrafiltration and chromatography

Aprotinin penetrates readily ordinary dialysis tubes but not acetylated ones. In the absence of a suitable salt concentration (below approx. 0.1 mol/l NaCl) it may be bound to negatively charged surfaces of cell membranes and solid supports, thus giving rise to unspecific effects or loss of aprotinin (e.g. in gel permeation chromatography or ultrafiltration).

9.1.4. Interaction with heparin

Under in-vitro conditions heparin may associate with aprotinin, causing turbidity or precipitation. This can be avoided by increasing the salt concentration of the solution. As a precaution, aprotinin should not be used in chromogenic substrate assays in the presence of heparin, or mixed with heparin for blood sample collection and infusion solutions. Under in-vivo conditions such an interference between aprotinin and heparin is not to be expected because of the low aprotinin concentration in circulating blood. On the other hand, the combined use of aprotinin and heparin may have a beneficial therapeutic effect [249].

9.1.5. Immunoassays

In immunodiffusion experiments the antibody should be applied to the gel about 4 h before aprotinin because of the rapid diffusion rate of the latter. Possible adsorption of aprotinin on agarose loaded with negatively charged groups should be borne in mind.

9.1.6. Inhibition studies

In inhibition assays preincubation of aprotinin with the enzyme for 5 min (with kallikrein for 30 min) at the optimum pH of the pro-

teinnase is generally sufficient to achieve complex formation. As a lonnger incubation does not normally affect aprotinin because of its stabbility against proteolytic degradation, the consistency of the degreee of inhibition reached after 5 min should be checked for a longer timne when an unknown proteinase is used for the first time. Aprotininn-proteinase complexes dissociate at alkaline pH (> 10) and in acididic solution (pH < 5, chymotrypsin and kallikrein; pH < 3, tryypsin and plasmin).

9.11.7. Use in biological studies

At t present it is not feasible to recommend precise aprotinin concentrattions sufficient for totally inhibiting proteinases in biological fluiids and tissue extracts. The optimum aprotinin amount needed muust be determined individually in each case. However, 1×10^{-5} mool/l (approx. 500 KIU/ml) of aprotinin should be sufficient in moost cases.

Approtinin solutions should be tested for their inhibitory activity beforre use. A simple trypsin inhibition assay with the substrates BzAArgpNA (L-BAPA) [27, 28] or BzArgOEt [33] (possibly in combinnation with alcohol dehydrogenase and NAD [250]) is suitable for thiis purpose.

9.22. Inhibition assays

9.22.1. Photometric assay

A ı modification of the originally described trypsin DL-BAPA test [211, 27] has been reported. In this assay aprotinin inhibits the trypsin-ı-catalyzed hydrolysis of Nα-benzoyl-L-arginine-p-nitroanilide (BzzArgpNA or L-BAPA), which is followed photometrically at 4055 nm. One trypsin unit (U_{BAPA}) corresponds to the hydrolysis of 1 μμmol substrate per min, i.e. a ΔA_{405}/min of 3.32 for a 3 ml volume at aa 1 cm light path. One inhibitor unit (IU_{BAPA}) decreases the activity ı of two trypsin units by 50%, which corresponds arithmetically to the ɔ inhibition of 1 U_{BAPA} of trypsin. The specific inhibitor activity of aaprotinin is given in IU_{BAPA}/mg polypeptide.

Reaagents

Subbstrate: 50 mg L-BAPA · HCl in 100 ml distilled water

Bufiffer: 0.2 mol/l triethanolamine · HCl/NaOH, 0.02 mol/l $CaCl_2$, pH 7.8

Tryypsin: 10 mg hog trypsin (⩾ 2 U_{BAPA}/mg) or 18 mg bovine trypsin (⩾ 1.2 U_{BAPA}/mg) dissolved in 100 ml 0.0025 n HCl, stored at 4 °C

Inhhibitor: 10–15 mg aprotinin dissolved in 1000 ml buffer or approx. 80 KIU/ml (in the case of bovine trypsin approx. 130 KIU/ml)

Proocedure

Plaace thermostated (25 °C) 3-ml cuvettes in a (spectro) photometer. Pippet in the following order: 0.1 ml trypsin solution, 1.8 ml buffer, 0.1 ml inhibitor solution, each prewarmed to 25 °C; mix with a plastic ɔ spatula and pre-incubate the mixture for 3 min. Start L-BAPA hyddrolysis by adding 1 ml of substrate solution, mix well and record the ɔ linear (!) increase in absorbance at 405 nm for at least 5 min. Reppeat or make a parallel determination with buffer instead of inhibitoor solution ("reference sample").

Caldculation

1 mnU_{BAPA} of trypsin corresponds to a ΔA of 0.00332/min (reference sammple). Reduction of the trypsin activity by 50% corresponds to 0.5 mIU_{BAPA} aprotinin. Calculate the specific activity in IU_{BAPA}/mg inhibbitor. Biological units (KIU) of aprotinin may be calculated from the ɔ IU_{BAPA} by the following equation: $IU_{BAPA} \times 615 = KIU$.

If boovine trypsin (⩾ 1.2 U_{BAPA}/mg) is used in the assay instead of hogg trypsin, KIU are calculated by: $IU_{BAPA} \times 1025 = KIU$.

9.22.2. Titrimetric assays

Thee proposed method is a modification of the test described previouusly [25, 26]. In this assay, aprotinin inhibits the trypsin-cataalyzed hydrolysis of Nα-benzoyl-L-arginine ethyl ester (BzArgOEtt, BAEE), and this is followed by titration of the liberated carboxyyl groups (BzArgOH) with an automatic titrator or manually. Onee F.I.P. unit (U_{FIP}) of trypsin corresponds to the hydrolysis of 1 μrmol of substrate per min under the given conditions. One U_{FIP} of aprcotinin is defined as the quantity of inhibitor which inhibits two U_{FIP} of trypsin by 50%. 1 U_{FIP} of aprotinin corresponds to 30 KIU aprcotinin, 1 mg pure aprotinin to approximately 238 U_{FIP} or 7143 KIU.

Reaagents

Borrate buffer: Dissolve 572.2 mg of disodium tetraborate-10-hyddrate and 2.94 g of calcium chloride-2-hydrate in approx. 900 ml distt. water. Adjust the pH to 8.00 (25 °C) with 0.1 N HCl, fill up with dist. water to a 1000 ml final volume, and check the pH again.

Trypsin solution: Dissolve approx. 20 mg trypsin (from pigs with a minimal activity of 45 U_{FIP}/mg) in 10 ml 0.001 N HCl, store at 0–4 °C. This solution must be prepared daily or kept frozen until used.

Substrate: Dissolve 68.56 mg BAEE · HCl in 10 ml dist. water, store at 0–4 °C. This solution must be prepared daily or kept frozen until used.

Equipment

pH Meter with an accuracy of 0.02 pH units; glass and calomel electrodes; closable titration vessel with temperature-controlled jacket; magnetic stirrer; temperature-controlled heating unit for the reaction vessels (25 ± 0.1 °C); 0.5 ml microburettes; in addition, for automatic titration a control unit for pH state titration, a 0.25 ml motor burette, and a recorder. Complete titration units are supplied by several companies, e.g. Radiometer, Copenhagen, and Metrohm, Herisau.

Procedure

Preincubation samples

Use an aprotinin solution containing approx. 1000 KIU/ml. Prepare the following mixtures in 5 ml tubes:

(a) 3.9 ml borate buffer, 0.1 ml trypsin solution (reference sample);
(b) 3.5 ml borate buffer, 0.2 ml trypsin solution, 0.3 ml aprotinin solution (inhibitor test sample).

Close the tubes, shake and incubate for 10 min at 25 °C.

Automatic titration: Fill 1.8 ml of borate buffer and 0.2 ml of substrate solution into the titration vessel and stir for 5 min to maintain constant temperature. Adjust the pH to 8.00 by addition of 0.02 N NaOH and add quickly 0.2 ml of either sample (a) or (b). Maintain the pH between 7.95 and 8.05 by continuous addition of 0.02 N NaOH. Record the NaOH consumption for at least 6 min.

Manual titration: Fill 9.0 ml of borate buffer and 1.0 ml of substrate solution into the titration vessel and stir as for 5 min to maintain constant temperature. Adjust the pH to 8.00 by addition of 0.1 N NaOH and add quickly 1.0 ml of either sample (a) or (b); at the same time start a stopwatch. Maintain the pH between 7.95 and 8.05 by continuous addition of 0.1 N NaOH. Record the NaOH consumption for at least 6 min.

Repeat each test at least once.

Calculation of units

Determine the NaOH consumption per min graphically from the titration curve (aut, automatic titration; man, manual titration):

$$U\ (\mu mol/min) = \frac{V_{aut} \times 20}{t} \text{ or } \frac{V_{man} \times 100}{t}$$

U = μmol NaOH consumed per min;
V = volume of NaOH consumed during test;
t = test time in min.

Calculate the biological units by the formula:

$$KIU/ml = \frac{(2\ U_{ref} - U_{inh}) \times 4 \times F \times 30}{0.3 \times c}$$

U_{ref} = activity of reference sample;
U_{inh} = activity of inhibitor test sample;
F = dilution factor;
c = volume in ml of pre-incubation sample (a) or (b), cf. Procedure.

For each U_{ref} and U_{inh}, mean values of 4 determinations should be used.

The numerical proportion of the values U_{ref} and U_{inh} should be between 0.8 and 1.2.

10. References

[1] Kraut, H., Frey, E. K., Werle, E., Hoppe-Seyler's Z. Physiol. Chem. **192,** 1 (1930) – [2] Kunitz, M., Northrop, J. H., J. gen. Physiol. **19,** 991 (1936) – [3] Frey, E. K., Kraut, H., Werle, E., eds., Kallikrein-Padutin, pp. 157–168. F. Enke Verlag, Stuttgart (1950) – [4] Frey, E. K., Kraut, H., Werle, E., eds., Das Kallikrein-Kinin-System und seine Inhibitoren, pp. 114–142. F. Enke-Verlag, Stuttgart (1968) – [5] Vogel, R., Trautschold, I., Werle, E., eds., Natural Proteinase Inhibitors, pp. 76–95. Academic Press, New York (1968) – [6] Vogel, R., Werle, E., in: Bradykinin, Kallidin and Kallikrein – Handb. Exp. Pharm., Vol. 25, Erdös, E., Wilde, A. F., eds., pp. 213–249. Springer-Verlag, Berlin (1970) – [7] Vogel, R., in: Bradykinin, Kallidin and Kallikrein – Handb. Exp. Pharm., Vol. 25 Suppl., Erdös, E. G., ed., pp. 163–225, Springer-Verlag, Berlin (1979) – [8] Chauvet, J., Acher, R., FEBS Lett. **23,** 317 (1972) – [9] Wilusz, T., Wilimowska-Peilic, A., Mejbaum-Katzenellenbogen, W., Acta Biochim. Polon. **20,** 25 (1973) – [10] Rifkin, D. B., Crowe,

R. M., Hoppe-Seyler's Z. Physiol. Chem. **358,** 1525 (1977) – [11] Wachter, E., Hochstrasser, K., Hoppe-Seyler's Z. Physiol. Chem. **360,** 1505 (1979) – [12] Hochstrasser, K., Wachter, E., FEBS Lett. **119,** 58 (1980) – [13] Fritz, H., Kruck, H., Rüsse, J., Liebich, H. G., Hoppe-Seyler's Z. Physiol. Chem. **360,** 437 (1979) – [14] Shikimi, T., Kobayashi, T., J. Pharm. Dyn. **3,** 400 (1980) – [15] Fritz, H., Brey, B., Müller, M., Gebhardt, M., in: Proc. Int. Res. Conf. on Proteinase Inhibitors, Munich, Nov. 1970, Fritz, H., Tschesche, H., eds., pp. 28–37, W. de Gruyter, Berlin (1971) – [16] Schultz, F., Naturwiss. **13,** 338 (1967) – [17] Kassell, B., Laskowski, M., Biochem. Biophys. Res. Commun. **20,** 463 (1965) – [18] Anderer, F. A., Hörnle, S., J. Biol. Chem. **241,** 1568 (1966) – [19] Huber, R., Kukla, D., Rühlmann, A., Steigemann, W., in: Proc. Int. Res. Conf. on Proteinase Inhibitors, Fritz, H., Tschesche, H., eds., pp. 56–64, W. de Gruyter, Berlin (1971) – [20] Deisenhofer, J., Steigemann, W., in: Proteinase Inhibitors – Bayer Symposium V, Fritz, H., Tschesche, H., Greene, L. J., Truscheit, E., eds., pp. 484–496. Springer Verlag, Berlin (1974) – [21] Kassell, B., Meth. Enzym. **19,** 844 (1970) – [22] Wüthrich, K., Wagner, G., J. Mol. Biol. **130,** 1 (1979) – [23] Kassell, B., Wang, T.-W., in: Proc. Int. Res. Conf. on Proteinase Inhibitors, Munich, Nov. 1970, Fritz, H., Tschesche, H., eds., pp. 89–94. W. de Gruyter, Berlin (1971) – [24] Craig, L. C., King, P., Meth. Biochem. Anal. **10,** 175 (1962) – [25] F.I.P. Commission, 3rd report, J. Mond. Pharm. **1,** 33 (1968) – [26] Ruyssen, R., Lawers, A., Pharmaceutical Enzymes, pp. 227–241. Story-Scientia, Gent, Belgium (1978) – [27] Fritz, H., Trautschold, I., Werle, E., in: Methoden der Enzymatischen Analyse, Bergmeyer, H. U., ed., pp. 1105–1122. Verlag Chemie, Weinheim/Bergstr. (1974) – [28] Klockow, M., Kontakte (Merck) **1,** 7 (1974) – [29] Witt, I., New Methods for the Analysis of Coagulation Using Chromogenic Substrates. Walter de Gruyter, Berlin–New York (1977) – [30] Iwanaga, S., Morita, T., Kato, H., Harada, T., Kimura, T., Sakakibara, S., Adv. Expt. Med. Biol. (Kinins-II) **120A,** 147 (1979) – [31] Amundsen, E., Pütter, J., Friberger, P., Knos, M., Larsbraten, M., Claeson, G., Adv. Expt. Med. Biol. (Kinins-II) **120A,** 147 (1979) – [32] Kato, H., Adachi, N., Iwanaga, S., Abe, K., Takada, K., Kimura, T., Sakakibara, S., J. Biochem. **87,** 1127 (1980) – [33] Trautschold, I., in: Bradykinin, Kallidin and Kallikrein – Handb. Exp. Pharm., Vol. 25, Erdös, E., Wilde, A. F., eds., pp. 52–81. Springer Verlag, Berlin (1970) – [34] Remmert, L. F., Cohen, P. P., J. Biol. Chem. **181,** 431 (1949) – [35] Fink, E., Greene, L. J., in: Proteinase Inhibitors – Bayer Symposium V, Fritz, H., Tschesche, H., Greene, L. J., Truscheit, E., eds., pp. 243–249, Springer Verlag, Berlin (1974) – [36] Lazdunski, M., Vincent, J.-P., Schweitz, H., Peron-Renner, M., Pudles, J., in: Proteinase Inhibitors – Bayer Symposium V, Fritz, H., Tschesche, H., Greene, L. J., Truscheit, E., eds., pp. 420–431. Springer Verlag, Berlin (1974) – [37] Vincent, J.-P., Lazdunski, M., FEBS Lett. **63,** 240 (1976) – [38] Bösterling, B., Engel, J., Hoppe-Seyler's Z. Physiol. Chem. **357,** 1297 (1976) – [39] Fritz, H., Schult, H., Meister, R., Werle, E., Hoppe-Seyler's Z. Physiol. Chem. **350,** 1531 (1969) – [40] Kalckreuth, W., Das Verhalten von Humanplasmin gegen Proteaseinhibitoren – ein Beitrag zur Differenzierung zwischen Humanplasmin und Humanserumkallikrein. Ph.D. Thesis, Medical Faculty of the University of Munich, FR Germany (1972) – [41] Wiman, B., Thromb. Res. **17,** 143 (1980) – [42] Dietl, T., Huber, C., Geiger, R., Iwanaga, S., Fritz, H., Hoppe-Seyler's Z. Physiol. Chem. **360,** 67 (1979) – [43] Wunderer, G., Kummer, K., Fritz, H., Hoppe-Seyler's Z. Physiol. Chem. **353,** 1646 (1972) – [44] Geiger, R., Stuckstedte, U., Fritz, H., Hoppe-Seyler's Z. Physiol. Chem. **361,** 1003 (1980) – [45] Heber, H., Geiger, R., Heimburger, N., Hoppe-Seyler's Z. Physiol. Chem. **359,** 659 (1978) – [46] Lestienne, P., Bieth, J. G., Arch. Biochem. Biophys. **190,** 358 (1978) – [47] Green, N. M., Work, E., Biochem. J. **54,** 347 (1953) – [48] Chao, J., Margolius, H. S., Biochem. Pharmacol. **28,** 2071 (1979) – [49] Mallory, P. A., Travis, J., Am. J. Clin. Nutrition **28,** 823 (1975) – [50] Figarella, C., Negri, G. A., Guy, O., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 213–222, Springer Verlag, Berlin (1974) – [51] Mallory, P. A., Travis, J., Biochemistry **14,** 722 (1975) – [52] Ohlsson, K., Ohlsson, A.-S., Hoppe-Seyler's Z. Physiol. Chem. **357,** 1153 (1976) – [53] Fritz, H., Fiedler, F., Dietl, T., Warwas, M., Truscheit, E., Kolb, H. J., Mair, G., Tschesche, H., in: Kininogenases – Kallikrein 4, G. L. Haberland, J. W. Rohen, T. Suzuki, eds., pp. 15–28. Schattauer Verlag, Stuttgart (1977) – [54] Amouric, M., Figarella, C., Hoppe-Seyler's Z. Physiol. Chem. **361,** 85 (1980) – [55] Esparza, I., Brock, J. H., Comp. Biochem. Physiol. **61B,** 347 (1978) – [56] Havemann, K., Janoff, A., Neutral Proteases of Human Polymorphonuclear Leukocytes. Urban & Schwarzenberg, Baltimore/Munich (1978) – [57] Egbring, R., Schmidt, W., Fuchs, G., Havemann, K., Blood **49,** 219 (1977) – [58] Fritz, H., Ciba Foundation **75,** 351 (1980) – [59] Starkey, P. M., Barrett, A. J., Biochem. J. **155,** 265 (1976) – [60] Marossy, K., Hauck, M., Elödi, P., Biochim. Biophys. Acta **615,** 237 (1980) – [61] Starkey, P. M., Barrett, A. J., Biochem. J. **155,** 273 (1976) – [62] Fritz, H., Förg-Brey, B., Fink, E., Schiessler, H., Jaumann, E., Arnhold, M., Hoppe-Seyler's Z. Physiol. Chem. **353,** 1007 (1972) – [63] Zaneveld, L. J. D., Schumacher, G. F. B., Tauber, P. F., Propping, D., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 136–146. Springer Verlag, Berlin (1974) – [64] Schill, W.-B., Feifel, M., Fritz, H., Hammerstein, J., Int. J. Androl. **4,** 25 (1981) – [65] Denker, H. W., Fritz, H., Hoppe-Seyler's Z. Physiol. Chem. **360,** 107 (1979) – [66] Schleuning, W.-D., Granelli-Piperno, A., in: Biological Functions of Proteinases, H. Holzer, H. Tschesche, eds., pp. 171–185. Springer-Verlag, Berlin (1979) – [67] Au, A. M.-J., Dunn, M. F., Biochemistry **16,** 3958 (1977) – [68] Wilson, W. H., Shooter, E. M., J. Biol. Chem. **254,** 6002 (1979) – [69] Bradshaw, R. A., Grant, G. A., Thomas, K. A., Eisen, A. Z., in: Protides of the Biological Fluids, Vol. 28, H. Peeters, ed., pp. 119–122. Pergamon Press, Oxford (1980) – [70] Bothwell, M. A., Wilson, W. H., Shooter, E. M., J. Biol. Chem. **254,** 7287 (1979) – [71] Fiedler, F., Fritz, H., Hoppe-Seyler's Z. Physiol. Chem. **362,** 1171 (1981) – [72] Colomb, E., Figarella, C., Guy, O., Biochim. Biophys. Acta **570,** 397 (1979) – [73] Laskowski, M., Jr., Sealock, R. W., in: The Enzymes, P. D. Boyer, ed., 3rd ed., pp. 375–473, Academic Press, New York (1971) – [74] Estell, D. A., Laskowski, M., Jr., Biochemistry **19,** 124 (1980) – [75] Finkenstadt, W. R., Hamid, M. A., Matthis, J. A., Schrode, J., Sealock, R. W., Wang, D., Laskowski, M., Jr., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 389–411, Springer Verlag, Berlin (1974) – [76] Engel, J., Quast, U., Heumann, H., Krause, G., Steffen, E., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 412–419. Springer Verlag, Berlin (1974) – [77] Quast, U., Engel, J., Steffen, E., Tschesche, H., Kupfer, S., Eur. J. Biochem. **86,** 353 (1978) – [78] Quast, U., Engel, J., Steffen, E., Tschesche, H., Kupfer, S., Biochemistry **17,** 1675 (1978) – [79] Estell, D. A., Wilson, K. A., Laskowski, M., Jr., Biochemistry **19,** 131 (1980) – [80] Tschesche, H., Kupfer, S., Hoppe-Seyler's Z. Physiol. Chem. **357,** 769 (1976) – [81] Laskowski, M., Jr., Kato, I., Leary, T. R., Schrode, J., Sealock, R. W., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 597–611, Springer-Verlag, Berlin (1974) – [82] Blow, D. M., Wright, C. S., Kukla, D., Rühlmann, A., Steigemann, W., Huber, R., J. Mol. Biol. **69,** 137 (1972) – [83] Huber, R., Kukla, D., Steigemann, W., Deisenhofer, J., Jones, A., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp 497–512. Springer Verlag, Berlin (1974) – [84] Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., Steigemann, W., J. Mol. Biol. **89,** 73 (1974) – [85] Janin, J., Sweet, R. M., Blow, D. M., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 513–520, Springer Verlag, Berlin (1974) – [86] Huber, R., Bode, W., Kukla, D., Kohl, U., Ryan, C. A., Biophys. Struct. Mechanism **1,** 189 (1975) – [87] Huber, R., Bode, W., Acc. Chem. Res. **11,** 114 (1978) – [88] Bode, W., Schwager, P., Huber, R., J. Mol. Biol. **118,** 99 (1978) – [89] Richarz, R., Tschesche, H., Wüthrich, K., Biochemistry **19,** 5711 (1980) – [90] Bode, W., J. Mol. Biol. **127,** 357 (1979) – [91] Janin, J., Chothia, C., J. Mol. Biol. **100,** 197 (1976) – [92] Bode, W., in: The Physiological Inhibitors of Coagulation and Fibrinolysis, D. Collen, B. Wiman, M. Verstraete, eds., pp. 5–16, Elsevier/North-Holland Biomedical Press, Amsterdam (1979) – [93] Blow, D. M., Acc. Chem. Res. **9,** 145 (1976) – [94] Tschesche, H., Kupfer, S., Hoppe-Seyler's Z. Physiol. Chem. **358,** 316 (1977) – [95] Kowalski, D., Leary, T. R., McKee, R. E., Sealock, R. W., Wang, D., Laskowski, M., Jr., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 311–324. Springer Verlag, Berlin (1974) – [96] Tschesche, H., Jering, H., Schorp, G., Dietl, T., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 362–377. Springer Verlag, Berlin (1974) – [97] Jering, H., Tschesche, H., Eur. J. Biochem. **61,** 443 (1976) – [98] Jering, H., Tschesche, H., Eur. J. Biochem. **61,** 453 (1976) – [99] Wenzel. H. R., Tschesche, H., Hoppe-Seyler's Z. Physiol. Chem. **364,** 345 (1980) – [100] Wenzel, H. R., Tschesche, H., Angew. Chem. Int. Ed. **20,** 295 (1981) – [101] Tan, N. H., Kaiser, E. T. Biochemistry **16,** 1531 (1977) – [102] Wunderer, G., Béress, L., Machleidt, W., Fritz, H., Meth. Enzymol. **45,** 881 (1976) – [103] Dietl, T., Tschesche, H., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 254–264, Springer Verlag, Berlin (1974) – [104] Iwanaga, S., Takahashi, H., Suzuki. T., Meth. Enzymol. **45,** 874 (1976) – [105] Hokama, Y., Iwanaga. S., Tatsuki, T., Suzuki, T., J. Biochem. **79,** 559 (1976) – [106] Čechová, D., Meth. Enzymol. **45,** 806 (1976) – [107] Koide, T., Ikenaka. T., Eur. J. Biochem. **32,** 417 (1973) – [108] Fritz, H., in: Chemistry and Biology of the Kallikrein-Kinin-System in Health and Disease, J. J. Pisano, K. F. Austen, eds., pp. 181–193. U.S. Government Printing Office, Washington, D.C. (1976) – [109] Joubert, F. J., Taljaard, N., Hoppe-Seyler's Z. Physiol. Chem. **361,** 661 (1980) – [110] Heine, H., Förster, F. J., Neufahrt, A., Med. Welt **27,** 1774 (1976) – [111]

Stoddart, R. W., Kiernan, J. A., Histochemie **34,** 275 (1973) – [112] Werle, E., Trautschold, I., Haendle, H., Fritz, H., Ann. N. Y. Acad. Sci. **146,** 464 (1968) – [113] Just, M., Naunyn-Schmiedebergs Arch. Pharmacol. **287,** 85 (1975) – [114] Just, M., Török, P., Habermann, E., in: Kininogenases – Kallikrein, G. L. Haberland, J. W. Rohen, eds., pp. 123–127, F. K. Schattauer Verlag, Stuttgart (1973) – [115] Wunderer, G., Kummer, K., Fritz, H., Béress, L., Machleidt, W., in: Proteinase Inhibitors – Bayer Symposium V, H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, eds., pp. 277–281, Springer Verlag, Berlin (1974) – [116] Tschesche, H., Dietl, T., Meth. Enzymol. **45,** 772 (1976) – [117] Haberland, G. L., in: Synthetic and Natural Proteinase Inhibitors: Basic and Clinical Aspects. Int. Symp. in Tokyo. Nov. 20th 1967, pp. 47–55. Japanese Society of Medical Sciences, Tokyo (1967) – [118] Anderer, F. A., Hörnle, S., Ann. N. Y. Acad. Sci. **146,** 381 (1968) – [119] Fritz, H., Oppitz, K.-H., Meckl, D., Kemkes, B., Haendle, H., Schult, H., Werle, E., Hoppe-Seyler's Z. Physiol. Chem. **350,** 1541 (1969) – [120] Habermann, E., Arndts, D., Just, M., Räker, K.-O., Török, P., Med. Welt **24,** 1163 (1973) – [121] Kaller, H., Patzschke, K., Wegner, L. A., Horster, F. A., Eur. J. Drug Metab. Pharmacokin. **2,** 79 (1978) – [122] Larionova, N. I., Sakharov, I. Y., Kazanskaya, N. F., Zhuravlyov, A. G., Vladimirov, V. G., Tolstich, P. I., in: Enzyme Engineering Future Directions, L. B. Wingard, Jr., I. V. Berezin, A. A. Klyosov, eds., pp. 241–255, Plenum Press, New York (1980) – [123] Johnson, D. A., Travis, J., Anal. Biochem. **72,** 573 (1976) – [124] Temler, R. S., Kägi, J. H. R., Enzyme **22,** 249 (1977) – [125] Johnson, D. A., Biochim. Biophys. Acta **452,** 482 (1976) – [126] Fritz, H., Brey, B., Schmal, A., Werle, E., Hoppe-Seyler's Z. Physiol. Chem. **350,** 617 (1969) – [127] Chauvet, J., Acher, R., in: Protides of the Biological Fluids, Vol. 28, H. Peeters, ed., pp. 399–403, Pergamon Press, Oxford (1980) – [128] Lemon, M., Fiedler, F., Förg-Brey, B., Hirschauer, C., Leysath, G., Fritz, H., Biochem. J. **177,** 159 (1979) – [129] Oza, N. B., Ryan, J. W., Biochem. J. **171,** 85 (1978) – [130] Ole-MoiYoi, O., Spragg, J., Austen, K. F., J. Immunol. **121,** 66 (1978) – [131] Ole-MoiYoi, O., Spragg, J., Austen, K. F., Proc. Soc. Natl. Acad. Sci. USA **76,** 3121 (1979) – [132] Amouric, M., Figarella, C., Hoppe-Seyler's Z. Physiol. Chem. **360,** 457 (1979) – [133] Baugh, R. J., Travis, J., Biochem. **15,** 836 (1976) – [134] Saklatvala, J., Barrett, A. J., Biochim. Biophys. Acta **615,** 167 (1980) – [135] Koch, Y., Baram, T., Hazum, E., Fridkin, M., Endocr. Res. Commun. **4,** 247 (1977) – [136] Andrews, P., Meth. Biochem. Analysis **18,** 1 (1970) – [137] Weber, K., Osborn, M., in: The Proteins I, R. L. Hill, C. L. Boeder, H. Neurath, eds., pp. 179–223, Academic Press, New York (1976) – [138] Amundsen, E., Pütter, J., Friberger, P., Knos, M., Larsbraten, M., Claeson, G., Adv. Expt. Med. Biol. **120A,** 83 (1979) – [139] Komiya, M., Kato, H., Suzuki, T., J. Biochem. **76,** 811 (1974) – [140] Hamberg, U., Elg, P., Nissinen, E., Stelwagen, P., Int. J. Peptide Res. **7,** 261 (1975) – [141] Adam, A., Damas, J., Schots, C., Heynen, G., Franchimont, P., in: Biochemical and Biological Applications of Isotachophoresis, A. Adam, C. Schots, eds., pp. 47–62. Elsevier Scientific Publishing Company, Amsterdam (1978) – [142] Schramm, W., Drost, W., Schmidt, M., Borlinghaus, P., Marx, R., in: Fibrinogen, Fibrin und Fibrinkleber. Fibrinogen, Fibrin and Fibrin Clue. K., Schimpf, ed., pp. 391–397, F. K. Schattauer Verlag, Stuttgart (1980) – [143] Eisentraut, A. M., Whissen, N., Unger, R. H., Am. J. Med. Sci. **255,** 137 (1968) – [144] Besser, G. M., Orth, D. N., Nicholson, W. E., Byyny, R. L., Abe, K., Woodham, J. P., J. clin. Endocr. **32,** 595 (1971) – [145] Nars, P. W., Stahl, M., Dambacher, M., Baumann, J., Girard, J., Experientia, **28,** 213 (1972) – [146] Zyznar, E. S., Life Sci. **28,** 1861 (1981) – [147] Beck, E. A., Bachman, P., Barbier, P., Furlan, M., Thromb. Haemostasis **35,** 186 (1976) – [148] Yamada, K. M., Olden, K., Nature **275,** 179 (1978) – [149] Mann, K., Geiger, R., Werle, E., Adv. Expt. Med. Biol. **70,** 65 (1979) – [150] Summaria, L., Spitz, F., Arzadon, L., Boreisha, I. G., Robbins, K. C., J. Biol. Chem. **251,** 3693 (1976) – [151] Suensen, E., Lützen, O., Thorsen, S., in: Protides of the Biological Fluids, Vol. 28, H. Peeters, ed., pp. 383–386. Pergamon Press, Oxford (1980) – [152] Wagner, G., Wüthrich, K., J. Mol. Biol. **134,** 79 (1979) – [153] Wagner, G., Tschesche, H., Wüthrich, K., Eur. J. Biochem. **95,** 239 (1979) – [154] Creighton, T. E., Kalef, E., Arnon, R., J. Mol. Biol. **123,** 129 (1978) – [155] Creighton, T. E., J. Mol. Biol. **144,** 521 (1980) – [156] Richarz, R., Tschesche, H., Wüthrich, K., Eur. J. Biochem. **102,** 563 (1979) – [157] Porubcan, M. A., Neves, D. E., Rausch, S. K., Markley, J. L., Biochemistry **17,** 4640 (1978) – [158] Markley, J. L., Biochemistry **17,** 4648 (1978) – [159] Johnson, A. R., Boyden, N. T., in: Kininogenases – Kallikrein 4, G. L. Haberland, J. W. Rohen, T. Suzuki, eds., pp. 113–118. F. K. Schattauer, Stuttgart, New York (1977) – [160] Matas, A. J., Sutherland, D. E. R., Steffens, M. W., Najarian, J. S., Surgery **80,** 183 (1976) – [161] Offord, R. E., Philippe, J., Davis, J. G., Halban, P. A., Berger, M., Biochem. J. **182,** 249 (1979) – [162] Berger, M. H., Cüpper, J., Halban, P. A., Offord, R. E., Diabetes **29,** 81 (1980) – [163] Müller, W. A., Taillens, C., Léreret, S., Berger, M., Philippe, J., Halban, P. A., Offord, R. E., Lancet I, 1245 (1980); Freidenberg, G. R., White, N., Cataland, S., O'Dorisio, T. M., Sotos, J. F., Santiago, J. V., New England J. Med. **305,** 363 (1981) – [164] Davis, H., Gascho, C., Kiernan, J. A., In Vitro **12,** 192 (1976) – [165] Davis, H., Gascho, C., Kiernan, J. A., Acta neuropath. **32,** 359 (1975) – [166] Higuchi, S., Ohkawara, S., Nakamura, S., Yoshinaga, M., Cell. Immunol. **34,** 395 (1977) – [167] Bereiter-Hahn, J., Virchows Arch. B Cell Path. **23,** 265 (1977) – [168] Mustafa, S. J., Biochem. Pharm. **28,** 340 (1979) – [169] Guthrie, R., Rizzi, G. T. A., McCabe, R. E., Jr., Am. J. Surg. **112,** 835 (1966) – [170] Godfrey, A. M., Salaman, J. R., Transplantation **25,** 167 (1978) – [171] Hoyer, J., Garbe, L., Delpierre, S., Prieur, A., Macquart-Moulin, G., Noirclerc, M., Respiration **39,** 323 (1980) – [172] McIlhinney, A., Hogan, B. L. M., Biochem. Biophys. Res. Commun. **60,** 348 (1974) – [173] Thomson, A. W., Tweedie, D. J., Pugh-Humphreys, R. G. P., Arthur, M., Brit. J. Cancer **38,** 106 (1978) – [174] Back, N., Steger, R., Eur. J. Pharmacol. **38,** 313 (1976) – [175] Freeman, J. G., Aprotinin in malignancy. M. D. thesis, University of Newcastle upon Tyne, England (1980) – [176] Newball, H. H., Berninger, R. W., Talamo, R. C., J. Clin. Invest. **64,** 457 (1979) – [177] Newball, H. H., Talamo, R. C., Lichtenstein, L. M., J. Clin. Invest. **64,** 466 (1979) – [178] Orr, F. W., Varani, J., Kreutzner, D. L., Senior, R. M., Ward, P. A., Am. J. Pathol. **94,** 75 (1979) – [179] Coblyn, J. S., Austen, K. F., Wintroub, B. U., J. Clin. Invest. **63,** 998 (1979) – [180] Wasi, S., Movat, H. Z., Pass, E., Chan, J. Y. C., in: Neutral Proteases of Human Polymorphonuclear Leukocytes, K. Havemann, A. Janoff, eds., pp. 245–260. Urban & Schwarzenberg, Baltimore-Munich (1978) – [181] Thomson, A. W., Pugh-Humphreys, R. G. P., Tweedie, D. J., Horne, C. H. W., Experientia **34,** 528 (1978) – [182] Freeman, J. G., Latner, A. L., Shenton, B. K., Turner, G. A., Venables, C. W., Brit. J. Cancer **38,** 636 (1978) – [183] Greenbaum, L. M., Semente, G., Grebow, P., Roffman, S., Adv. Exp. Med. Biol. (Kinins-II) **120 B,** 205 (1979) – [184] Greenbaum, L. M., Am. J. Path. **68,** 613 (1972) – [185] Adams, D. O., J. Immunol. **124,** 286 (1980) – [186] Adams, D. O., Kao, K.-J., Farb, R., Pizzo, S. V., J. Immunol. **124,** 293 (1980) – [187] Harke, H., Gennrich, M., Anaesthesist **29,** 266 (1980) – [188] Harke, H., Stienen, G., Rahman, S., Flohr, H., Anaesthesist **31,** 165 (1982) – [189] Harke, H., ed., Massivtransfusionen. Hämostase und Schocklunge. Springer Verlag, Berlin (1982) – [190] Aoki, N., Naito, K., Yoshida, N., Blood **52,** 1 (1978) – [191] Fürstenberg, H. S., Anaesthesist **14,** 109 (1965) – [192] Ketterl, R., Haas, S., Heiss, A., Fritsche, H.-M, Lechner, F., Kienzle, H., Blümel, G., Med. Welt **33,** 480 (1982) – [193] Mooney, R. A. H., J. Int. Med. Res. **4,** 360 (1976) – [194] Mazykant, L. I., Zaets, T. L., Dolgina, M. I., Kotkina, T. I., Nosova, I. M., Kaem, R. I., Panova, Yu. M., Kerova, A. I., Bull. Exp. Biol. Med. **84,** 1257 (1977) – [195] Schimpf, K. I., ed., Fibrinogen, Fibrin und Fibrinkleber. Fibrinogen, Fibrin and Fibrin Glue, pp. 199–296. F. K. Schattauer Verlag, Stuttgart (1980) – [196] Stemberger, A., Fritsche, H.-M., Primbs, P., Blümel, G., Med. Welt **29,** 720 (1978) – [197] Matras, H., Jesch, W., Kletter, G., Dinges, H. P., Wien. Klin. Wschr. **90,** 419 (1978) – [198] Dietze, G., Wicklmayr, M., FEBS Lett. **74,** 205 (1977) – [199] Dietze, G., Wicklmayr, M., Böttger, I., Mayer, L., Adv. Expt. Med. Biol. **120A,** 511 (1979) – [200] Dietze, G., Wicklmayr, M., Böttger, I., Schifmann, R., Geiger, R., Fritz, H., Mehnert, H., Agents & Actions **10,** 335 (1980) – [201] Molzberger, H., Heugel, E., Isselhard, W., Arzneim. Forsch./Drug Res. **28 (I),** 394 (1978) – [202] Kramer, H. J., Moch, T., von Sicherer, L., Düsing, R., Clin. Sci. **56,** 547 (1979) – [203] Scherberich, J. E., Mondorf, W., Arzneim.-Forsch./Drug Res. **30 (I),** 487 (1980) – [204] Morton, D. B., in: Proteinases in Mammalian Cells and Tissues, A. J. Barrett, ed., pp. 445–500. North Holland Publ. Comp., Amsterdam (1977) – [205] Zaneveld, L. J. D., Polakoski, K. L., Schumacher, G. F. B., in: Proteases and Biological Control, E. Reich, D. B. Rifkin, E. Shaw, eds., pp. 683–706. Cold Spring Harbor Laboratory, Cold Spring Harbor (1975) – [206] Denker, H.-W., ed., Adv. Anat. Embryol. Cell Biol. **53,** Fasc. 5 (1977) – [207] Whalley, E. T., Riley, A. J., J. Reprod. Fert. **55,** 377 (1979) – [208] Tauber, P. F., Herting, W., Zaneveld, L. J. D., Propping, D., Ludwig, H., in: Human Fertilization, H. Ludwig, P. F. Tauber, eds., pp. 261–270. G. Thieme Publ., Stuttgart (1978) – [209] Janousek, F., in: Neue Aspekte der Trasylol-Therapie, Vol. 4, G. L. Haberland, P. Huber, P. Matis, eds., pp. 93–98. F. K. Schattauer Verlag, Stuttgart–New York (1970) – [210] Back, N., Wilkens, H., Steger, H., Ann. N. Y. Acad. Sci. **146,** 491 (1968) – [211] Glenn, T. M., Herlich, B. L., Lefer, A. M., Arch. Int. Pharmacodyn. Ther. **203,** 292 (1973) – [212] Araki, H., Lefer, A. M., Arch. int. Pharmacodyn. **241,** 316–323 (1979) – [213] Haberland, G. L., Klin. Wschr. **56,** 325 (1978) – [214] Clowes, G. H. A., MacNicol, M., Voss, H., Altug, K., Savaris, C., in: New Aspects of Trasylol Therapy, W. Brendel, G. L. Haberland, eds., Vol. 5, pp. 209–222, F. K. Schattauer, Stuttgart–New York (1972) – [215] Ambrus, J. L., Ambrus, C. M., Stutzman, L., Schimert, G., Jacobsen, R., Schor, J. M., Jainchill, J. L., in: Chemical Control of Fibrinolysis – Thrombolysis, J. M. Schor, ed., pp. 153–203. Wiley-Inter-

science, New York (1970) – [216] Markwardt, F., in: Fibrinolytics and Antifibrinolytics, Hdb. Expt. Pharmacol., Vol. 46, F. Markwardt, ed., pp. 787–509. Springer Verlag, Berlin (1978) – [217] Smith, J. A. R., Norman, J. N., Resuscitation **6,** 249 (1978) – [218] Bottoms, G. D., Coppoc, G. L., Roesel, O. F., Wilcock, B., Weirich, W., Am. J. Vet. Res. **39,** 1023 (1978) – [219] Imrie, C. W., Mackenzie, M., Digestion **22,** 32 (1981) – [220] Balldin, G., Ohlsson, K., Hoppe-Seyler's Z. Physiol. Chem. **360,** 651 (1979) – [221] Balldin, G., Gustafsson, E.-L., Ohlsson, K., Eur. Surg. Res. **12,** 260 (1980) – [222] Borgström, A., Ohlsson, K., Hoppe Seyler's Z. Physiol. Chem. **361,** 625 (1980) – [223] Balldin, G., Ohlsson, K., Olsson, A.-S., Hoppe Seyler's Z. Physiol. Chem. **359,** 691 (1978) – [224] Korant, B. D., in: Proteases and Biological Control, E. Reich, D. B. Rifkin, E. Shaw, eds., pp. 621–644. Cold Spring Harbor Laboratory, Cold Spring Harbor (1975) – [225] Klenk, H. D., Bosch, F. X., Garten, W., Kohama, T., Nagai, Y., Rott, R., in: Biological Functions of Proteinases, H. Holzer, H. Tschesche, eds., pp. 139–149. Springer Verlag, Berlin (1979) – [226] Korn, G., Berl. Münch. Tierärztl. Wschr. **90,** 461, 469 (1977) – [227] Korn, G., Lorenz, R. J., Tierärztl. Umschau **34,** 587 (1979) – [228] Gastaldi, G., Mattiello, A., Pathologica **63,** 223 (1971) – [229] Trapnell, J. E., Rigby, C. C., Talbot, C. H., Duncan, E. H. L., Brit. J. Surg. **61,** 177 (1974) – [230] McMichan, J. C., Rosengarten, D. S., Philipp, E., Circulatory Shock **9,** 107 (1982) – [231] Auer, L. M., Marth, E., Heppner, F., Holasek, A., Acta Neurochir. **49,** 207 (1979) – [232] Popov-Cenić, S., in: Proteases – Antiproteases in Clinical Practice, Int. Symposium Cavtat/Dubrovnik, 9th and 10th May 1980, pp. 15–26. Excerpta Medica, Amsterdam (1981) – [233] Schneider, B., Schnells, G., Trentz, O., Tscherne, H., Rev. Int. Serv. Santé Armées Terre Mer Air **52,** 251 (1979) – [234] Sher, G., Am. J. Obstet. Gynecol. **129,** 164 (1977) – [235] Graeff, H., Kuhn, W., eds., Coagulation Disorders in Obstetrics. Pathobiochemistry, Pathophysiology, Diagnosis, Treatment. Thieme Verlag, Stuttgart–New York (1980) – [236] Schneider, B., Arzneim.-Forsch./Drug Res. **26,** 1606 (1976) – [237] Imrie, C. W., Benjamin, S., Ferguson, J. C., McKay, A. J., Mackenzie, I., O'Neill, J., Blumgart, L. H., Brit. J. Surg. **65,** 337 (1978) – [238] Medical Research Council Multicentre Trial, Gut **21,** 334 (1980) – [239] Balldin, G., Ohlsson, K., Surgery **85,** 451 (1979) – [240] Borgström, A., Ohlsson, K., Hoppe Seyler's Z. Physiol. Chem. **359,** 677 (1978) – [241] Borgström, A., Kukora, J., Ohlsson, K., Hoppe Seyler's Z. Physiol. Chem. **361,** 633 (1980) – [242] Kaplan, A. P., Meier, H. L., Yecies, L. D., Heck, L. W., in: Chemistry and Biology of the Kallikrein-Kinin-System in Health and Disease, J. J. Pisano, K. F. Austen, eds., pp. 237–254, U.S. Government Printing Office, Washington D.C. (1976) – [243] Kato, H., Han, Y. N., Iwanaga, S., Hashimoto, N., Sugo, T., Fuji, F., Suzuki, T., in: Kininogenases – Kallikrein 4, G. L. Haberland, J. W. Rohen, T. Suzuki, eds., pp. 63–72. F. K. Schattauer Verlag, Stuttgart (1977) – [244] Fritz, H., in: Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, J. F. Davidson, R. M. Rowan, M. M. Samama, P. C. Desnoyers, eds., pp. 285–290. Raven Press Publ., New York (1978) – [245] Philipp, E., in: Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, J. F. Davidson, R. M. Rowan, M. M. Samama, P. C. Desnoyers, eds., pp. 291–295. Raven Press Publ., New York (1978) – [246] Blombäck, B., Blombäck, M., Olsson, P., Thromb. Diath. Haemorrh. **18,** 190 (1967) – [247] Fritz, H., Truscheit, E., Fink, E., Fed. Proc. **38,** 2753 (1979) – [248] Hagiwara, H., Hasebe, H., Hirose, S., Inada, Y., FEBS Lett. **111,** 87 (1980) – [249] Zimmermann, W. E., Vogel, W., Mittermayer, Ch., Walter, F., Kuner, E., Schäfer, H., Birzle, H., in: New Aspects of Trasylol Therapy, Vol. 5, W. Brendel, G. L. Haberland, eds., pp. 141–163. F. K. Schattauer, Stuttgart – New York (1972) – [250] Fiedler, F., Meth. Enzymol. **45,** 289 (1976)

Acknowledgements

The authors are very grateful to Drs. A. Arens, G. Arndt, E. Philipp, G. Reinhardt, E. Schnabel, F. Schumann, H. Schutt and E. Truscheit of Bayer, Wuppertal/Leverkusen, FR Germany, for intensive discussions and valuable suggestions during preparation of the manuscript and its critical revision. They would also like to thank Mrs. Hilde Gerstenberger for careful and efficient assistance.

The conversion factors between BAPA units and KIU (see Table 1 and sections 3.1 and 9.2) have been measured resp. calculated by A. Arens, Bayer, Wuppertal/Leverkusen.

The space filling α-carbon plot of the proteinase-aprotinin complex has been calculated by W. Bode and R. Huber, Max-Planck-Institut, Munich, FR Germany. The authors appreciate the cooperation of these colleagues very much.

For the authors: Prof. Dr. H. Fritz, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nußbaumstraße 20, D-8000 München 2 (FR Germany)