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PRODRUGS OF PEPTIDES AND PROTEINS FOR IMPROVED FORMULATION AND DELIVERY

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INTRODUCTION

This paper aims to review the existing literature on the use of prodrugs to circumvent some of the difficulties associated with the formulation, systemic delivery, and rapid clearance of peptide and protein therapeutic agents. Understanding the basic elements involved in effective delivery and formulation of peptides and proteins is both intellectually challenging and enormously important. The relatively recent evolution of recombinant DNA research and modern synthetic methodologies allow the biochemist and chemist to produce vast quantities of various peptides and proteins possessing pharmacological efficacy. However, the therapeutic potential of these compounds lies in our ability to design and achieve effective and stable delivery systems. The future challenge in biotechnology may not only be polypeptide cloning and synthesis but effective nonparenteral delivery of intact peptides and proteins to the systemic circulation and their site of action. Based on our current understanding of biochemical and physiological aspects of peptide and protein absorption and metabolism, it is difficult to conceive of efficient means of delivering of these agents through the use of conventional formulation technology, namely, simple tablets and capsules (1–2).

The main impediment to the use of peptides and proteins as potential therapeutic drugs is their inadequate and erratic oral absorption (3, 4). Most linear peptides undergo rapid and extensive metabolism by proteolysis in the

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gastrointestinal tract (5). After oral dosing, polypeptides are also subjected to presystemic clearance by the liver, which often leads to a lack of significant correlation between membrane permeability, absorption, and bioavailability. Many peptides are innately hydrophilic and of higher molecular weight than traditional drugs, which, in turn, diminishes their ability to passively penetrate biological barriers. In the case of proteins, crossing biological membranes may perturb the tertiary structure and thereby alter the pharmacological activity or toxicity profile of these agents.

The therapeutic usefulness of many peptides also suffers from the lack of prolonged half-life in the systemic circulation due to the presence of effective clearance mechanisms in the blood and in various highly perfused organs such as the liver, kidney, and lung. In addition to delivery problems, polypeptides may also manifest chemical and physical instability, which also further complicates the task of formulation (6).

The lack of patient compliance, the difficulties associated with parenteral delivery, and poor oral bioavailability have provided the impetus for exploring alternative routes to macromolecule delivery. These include routes such as pulmonary, ocular, nasal, rectal, vaginal, and transdermal (7–10). In absence of external stimuli to facilitate absorption, use of these alternative routes has had limited success. Various strategies have been implemented to promote bioavailability of polypeptides, including supplemental administration of protease inhibitors (11–13), use of absorption enhancers (14, 15), novel formulation strategies (16, 17), and irreversible (analogs) (18, 19) or reversible (prodrug) chemical modifications.

Prodrug strategies consist of a transient modification of the physicochemical properties of a given compound through chemical derivatization (20, 21). Such reversible chemical modification is designed to enhance chemical stability, alter aqueous solubility, or improve bioavailability while the inherent pharmacological properties of the parent drug remain intact. A prerequisite for success of the prodrug approach is the reliable conversion of prodrug to the parent drug through either enzymatic or nonenzymatic catalyzed reaction, once the barrier to delivery has been circumvented (Figure 1).

In using prodrugs to improve the formulation characteristics, transport properties, and metabolic stability of peptides, one must consider the factors influencing the chemical and physical stability of polypeptide as well as the basic elements governing the enzymatic and biological barriers. The reader should note that the existing literature on the prodrugs of polypeptides is quite limited and just evolving. In addition, a significant portion of the studies in this area are confined mainly to in vitro testing and evaluations. Often the chemically modified polypeptide, possessing improved in vitro hydrolytic stability and permeability, has not been shown to result in reliable systemic

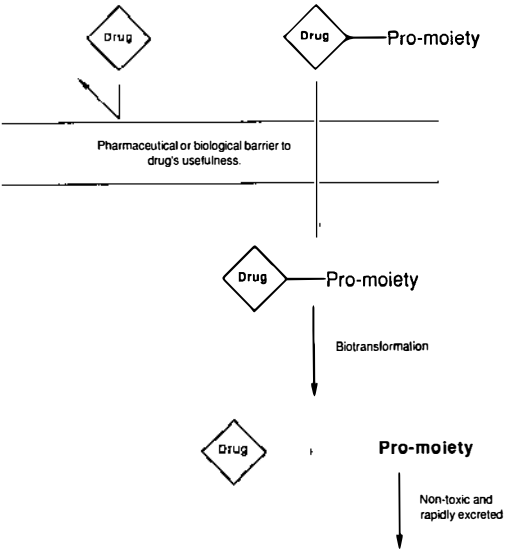

Figure 1 Schematic representation of the prodrug concept to circumvent various pharmaceutical and biological barriers to drug delivery.

delivery (18). Such observations suggest that in vivo studies are pivotal in evaluating the therapeutic potential of such prodrugs.

Our intent, therefore, is to discuss and evaluate the implication of various prodrug approaches applied to peptides and proteins. We hope that this review will encourage and guide appropriate research efforts in this area.

IMPROVEMENT OF THE PHYSICOCHEMICAL PROPERTIES OF POLYPEPTIDES FOR FORMULATION PURPOSES

Formulation of a drug entity that possess undesirable physicochemical properties, such as poor chemical stability, or low aqueous solubility, is a formidable task. These undesirable properties may be minimized or overcome via the use of prodrugs. Some of the pharmaceutical parameters modified by the prodrug approach are solubility, stability, crystallinity, and taste. An extensive review of the general application of the prodrug approach is beyond the scope of this paper but it has been reviewed elsewhere (See Refs. 20, 21). Discussed here is the potential of the prodrug approach in improving the physicochemical properties of polypeptides for formulation purposes.

The poor solubility of a drug molecule in an aqueous environment may

manifest itself by causing undesirable characteristics that often severely limit the therapeutic potential of the drug entity. These type of compounds often display erratic oral bioavailability and are difficult to formulate as aqueous parenteral dosage forms. Generally, this problem is not encountered for linear peptides since most are innately hydrophilic and possess adequate aqueous solubility, although some new experimental HIV-protease inhibitors are quite insoluble (22, 23). Many cyclic peptides are significantly less hydrophilic and possess lower aqueous solubility since the ionizable C- and N-terminus are capped upon cyclization. Such low aqueous solubility profile is often observed for cyclic peptides that only contain nonpolar amino acids (24–26).

The problems associated with a poor solubility profile may be improved by introduction of an ionizable moiety linked to derivatizable functionalities of the polypeptide. For example, Balkoves and co-workers synthesized a water-soluble phosphate monoester derivative of a lipopeptide by phosphorylation of the phenolic hydroxyl group of a homotyrosine residue (27). The prodrug exhibited excellent hydrolytic stability and had comparable in vivo activity to the parent cyclic peptide, indicating that the prodrug undergoes enzymatic hydrolysis to generate the parent drug. In addition to possessing improved aqueous solubility, the prodrug appeared to produce a more sustained drug level profile (28).

The N-alkylation of the peptide bond has been reported as an alternative approach in increasing the aqueous solubility of a polypeptide (25, 29). Replacement of the alanine or leucine residue by proline substantially improves the water solubility of a cyclic pentapeptide (25). The solubility enhancement has been attributed to the diminished structural regularity and lowered intermolecular hydrogen-bonding capability of the polypeptide. To our knowledge, the means to augment the aqueous solubility of a polypeptide through a selective and reversible N-alkylation of the amide bond has not been exploited. However, one may envisage the applicability of this concept in rational polypeptide prodrug design.

The thiol functionality of a polypeptide may also be a feasible site for prodrug modifications. Thiol prodrugs of conventional xenobiotics and peptide mimetic compounds have been prepared via a thioesterification, phosphorylation, or disulfide formation. These prodrugs are biologically converted to the parent thiol, thus restoring the pharmacological effect of the drug entity (30–32). Similar chemical modifications may be applied to polypeptides, to improve the aqueous solubility or minimize oxidative degradation of polypeptides. To our knowledge, this concept has not yet been extensively exploited for polypeptides.

Understanding the means to prevent nonenzymatic degradation of polypeptides under formulation conditions is also essential for their development as pharmaceutical products. Polypeptide degradation can be classified into

chemical or physical instability (33). Various approaches have been applied to chemically and physically stabilize polypeptides and are extensively reviewed elsewhere (34). One of the most prominent approaches in stabilizing polypeptides involves the irreversible chemical modifications of the primary structure using site-directed mutagenesis (35–37). However, such irreversible chemical modifications may alter the pharmacological activity of the polypeptides or may elicit an immunological response and, thus, is not considered as a prodrug approach.

The use of prodrugs to chemically or physically stabilize polypeptides has not been evaluated but is a potentially fruitful research area.

MINIMIZATION OF THE PROTEOLYTIC DEGRADATION OF POLYPEPTIDES

Two distinct classes of barriers—enzymatic inactivation and penetration—operate simultaneously to minimize the transport and absorption of polypeptides. The enzymatic barrier has been considered overriding in limiting the extent and the rate of polypeptide systemic availability (4). A significant research endeavor has recently been initiated to delineate the basic factors governing the absorption mechanism of polypeptides (38, 39), but considerably greater effort is needed. Our understanding of polypeptide metabolism, until recently, has been mainly distilled from nutritional studies (40–42). The human body is well equipped to digest and metabolize peptides and proteins into smaller fragments and amino acids. The digestive process is accomplished by various classes of peptidases and proteinases. Many such enzymes have been identified and, in a broad sense, they all catalyze the same reaction: hydrolysis of the peptide bond in polypeptides. Of course, these enzymes differ in substrate specificities and in their anatomical localization and distribution. The presence of peptidases and proteinases is required to sustain vital biochemical and physiological processes in the body. Almost every given tissue, therefore, contains considerable levels of these enzymes. The ubiquitous nature of these enzymes adds another dimension to the formidable problem of peptide and protein delivery.

The stomach contains a series of aspartic proteases called pepsin that are responsible for fragmentation of large proteins into smaller polypeptides. Pancreatic proteases such as chymotrypsin, trypsin, elastase, and carboxypeptidase A, which are mostly found in the small intestine, selectively catalyze degradation of polypeptides at specific amino acid residues. Proteases in the brush border and cytosol of the enterocytes mainly consist of aminopeptidase A and N, diaminopeptidase IV, endopeptidases 24.11, and angiotensin converting enzyme (ACE) (43–45). These enzymes are usually associated with the apical membrane with their active site in direct contact

with the extracellular environment (46). The brush border proteases are mainly involved in hydrolysis of tri- and tetrapeptides, whereas cytosolic proteases preferentially digest dipeptides compared to larger peptide fragments. Even if a given polypeptide is not subjected to hydrolysis in the gastrointestinal tract, liver clearance (first pass effect) may substantially reduce the apparent bioavailability of the peptide (47). Limited data on hepatic metabolism or extraction of polypeptides are available since most compounds have already been hydrolyzed well before reaching this organ. Nevertheless, the extent of hepatic metabolism in limiting the bioavailability of polypeptides may be much more important than was initially anticipated.

Alternative routes for delivery of polypeptides, other than oral, have also been exploited: these include the nasal, transdermal, ocular, buccal, pulmonary, rectal, and vaginal routes. However, the ubiquitous nature of peptidases limits the extent and the rate of polypeptide absorption via these routes as well as via the oral route. Nevertheless, with co-administration of enhancers and/or protease inhibitors, the systemic delivery of polypeptides by the nasal and pulmonary routes in particular appears tangible and may be promising (48).

Methods to diminish the proteolytic degradation of polypeptides through irreversible chemical modifications (analog design) have received considerable attention (18, 19). Despite prodigious effort, our understanding of the structural modification requirements to impart complete metabolic stability for peptide and peptide mimetic agents remains incomplete. The current literature on the prodrug approach in preventing or altering proteolysis is limited, mainly because research efforts have been targeted principally toward design and synthesis of prodrugs stable to the hydrolytic action of specific enzymes. Consequently, most of these prodrugs have been evaluated in vitro and their effectiveness in vivo has not been fully examined. We discuss some examples of prodrug approaches to metabolically stabilize specific sites in polypeptides. Some of these prodrug concepts were applied previously to nonproteogenic molecules possessing identical functional moieties and may not present a novel or specific prodrug concept developed especially for polypeptides.

Proteolysis may occur at N-terminus, C-terminus, or at distinct endo residues in a polypeptide. The mechanism by which catalytic enzymes appear to achieve biological specificity involves the use of various interactive mechanisms between the substrate and the enzyme such as hydrogen bonding, hydrophobic interactions, and electrostatic effects. A simple prodrug approach in preventing proteolysis may therefore disrupt the enzyme/substrate recognition process. This disruption may be achieved by reversible chemical modification of functional groups in the polypeptide that are close to the enzymatically labile site.

Various investigators have successfully designed and synthesized polypeptide prodrugs that are resistant to the proteolytic action of aminopeptidases by reversible chemical modification of the amino terminus. Amsbery & Borchardt (48) have developed a novel prodrug concept in which the amine functionality of the polypeptide is coupled to bioreversible derivatives of 3-(2′-hydroxy-4′-6′-dimethylphenyl)-3-dimethylpropionic acid (Figure 2, I), or 3-(3′,6′-dioxo-2′,4′-dimethyl-1′, 4′-cyclohexadiene)-3, 3-propionic acid (Figure 2, II). Under simulated physiological conditions the parent amine (RNH_2) is generated in a two-step process. The derivatized hydroxy amides (I or II) are initially converted to the hydroxy amide (III) by enzymatic catalysis (step 1), followed by the nonenzymatic lactonization of hydroxy amide, leading to the generation of the free amine (polypeptide) and the lactone (Figure 2, IV). The initial enzymatic steps (steps 1) may be catalyzed by hydrolytic or reductive mechanisms (49, 50). These investigators have successfully used the "trimethyl lock" concept (51, 52) to facilitate the rate of lactonization (step 2) under simulated physiological conditions to generate the parent amine. In addition to preventing proteolysis by aminopeptidases, this highly lipid soluble prodrug moiety may also improve the transport characteristics of the peptide by increasing its lipophilicity.

The enkephalins are rapidly hydrolyzed in vivo by two well-defined enzymes, neutral endopeptidase and aminopeptidase N. Rasmussen & Bundgaard (53) have developed aminopeptidase-resistant prodrugs of Leu-enkephalin and Met-enkephalin by condensing the enkephalins with various aldehydes and ketones to form a series of 4-imidazolidinone derivatives

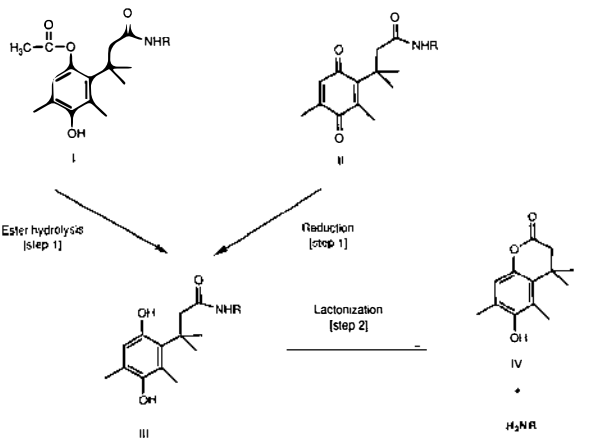

Figure 2 Proposed bioconversion mechanism for redox-sensitive and esterase-sensitive amide prodrug (49, 50).

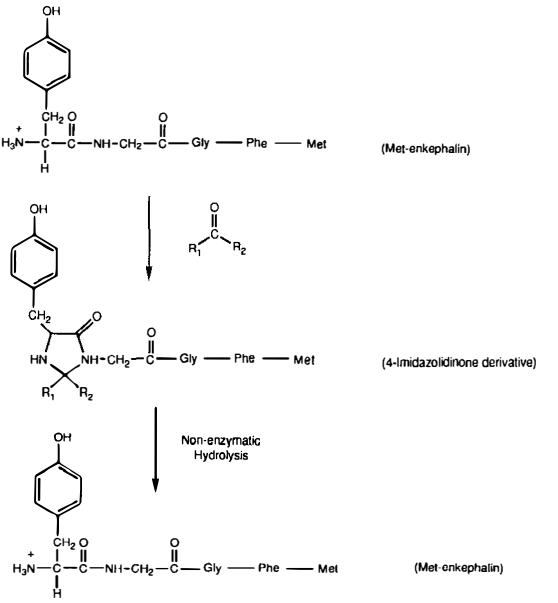

Figure 3 4-Imidazolidinone prodrugs of Met-enkephalin (53).

(Figure 3). In the presence of purified aminopeptidases, human plasma, and rabbit intestinal homogenates, the 4-imidazolidinone derivatives (prodrugs) showed greater metabolic stability when compared to the parent enkephalin. These derivatives are converted to the parent enkephalin via nonenzymatic hydrolysis and the rates of conversion are significantly influenced by the steric characteristics of the 2-substituents on the terminal amino acid. Interestingly, the rate of conversion of these derivatives is significantly slower than the rate of systemic clearance of the parent peptides. The potential therapeutic or pharmacodynamics implication of slower prodrug reversion rate compared to systemic clearance of the enkephalin is unknown. The importance of the rate of bioconversion compared to the rate of parent peptide clearance is discussed in section V of this review.

Carboxypeptidase A is responsible for cleaving the C-terminal amino acid of polypeptides. For N-acyl dipeptides, the rate of carboxypeptidase A proteolysis of the C-terminal amino acid is significantly influenced by the nature of the neighboring amino acid (54, 55). Bundgaard & Rasmussen (56) have prepared a series of carboxypeptidase A-resistant peptide prodrugs derivatives. These N-α-hydroxyalkyl derivatives were prepared by hydrolysis or aminolysis of various N-acyl 5-oxazolidinones and were found to display

a much greater stability against the carboxypeptidase A than the parent peptide. The same concept may be applied to protect polypeptides against endopeptidases like chymotrypsin and trypsin by reversible alkylation of the endo peptide bond susceptible to enzymatic hydrolysis. Under physiological conditions, the N-α-hydroxyalkyl derivatives are nonenzymatically converted to the parent peptide (Figure 4) and the rate of hydrolysis depends on both steric and polar factors within the acyl and N-α-hydroxyalkyl moieties (56). The inherent chemical instability characteristics of these derivatives may present difficulties in achieving stable and reproducible formulations. In an attempt to alter the chemical reactivities of N-α-hydroxyalkyl prodrugs, further chemical modifications to form the N-acyloxyalkyl prodrugs were carried out (Figure 5). However, these prodrugs displayed chemical reactivities comparable to the N-α-hydroxyalkyl derivatives (57). The authors have attributed the lack of improved chemical stability of N-acyloxymethyl prodrugs to the occurrence of an alternative degradation mechanism involving a reactive N-acyliminium ion intermediate (57). The potential applicability of N-alkylated prodrugs of polypeptides has been extensively reviewed by Bundgaard (58).

α-Chymotrypsin is an endopeptidase that catalyzes the hydrolysis of peptide bonds in which the reactive carbonyl moiety belongs to the L-amino acids, tryptophan, tyrosine, phenylalanine, and, to a lesser degree, leucine and methionine. Since chymotrypsin is present in the gastrointestinal tract, stability to this enzyme may be critical for adequate oral absorption of polypeptides. The active site topology and substrate specificity of chymotrypsin have been investigated extensively (59, 60). Examination of the specificity

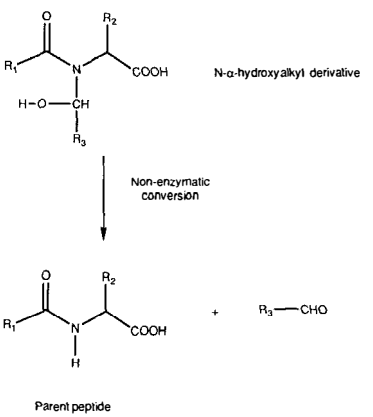

Figure 4 Hydrolytic bioconversion pathway for N-α-hydroxyalkyl prodrugs to the parent polypeptide (56).

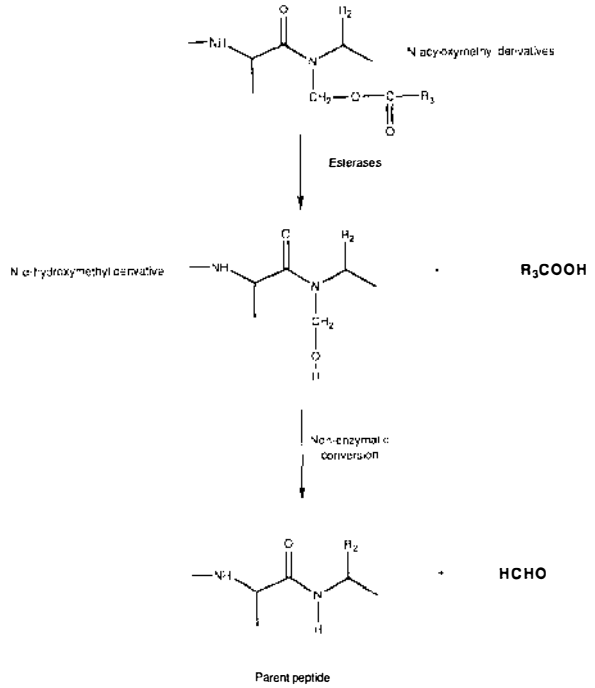

Figure 5 Proposed bioconversion pathway for N-acyloxymethyl prodrugs of polypeptides (57).

requirement reveals several interactions critical for efficient binding of a substrate to this enzyme. For substrates containing phenylalanine, these include a hydrogen bond between the α-NH and α-carbonyl of Phe and Ser-214 and Gly-139, respectively, and a van der Waals interaction between the benzyl side chain and the hydrophobic pocket adjacent to the active site of chymotrypsin (Figure 6). Modulation of any of these interactions may reduce the peptide affinity for the chymotrypsin and thus alter the kinetics of degradation.

The van der Waals interaction of the labile amino acid side chain with the hydrophobic pocket of chymotrypsin is essential for substrate recognition. Any significant alteration of the chemical structure, or reduction of conformational flexibility of the side chain of the labile amino acid in the peptide substrate will reduce the affinity of chymotrypsin for the substrate (polypeptide) (61). Accordingly, for tyrosine containing peptides, the derivatization of the phenolic hydroxyl with a prodrug moiety may decrease the van der Waals interaction between the tyrosyl side chain and the hydrophobic pocket of the enzyme, thus increasing proteolytic stability. For example, the esterification of phenolic hydroxyl group of a series of N-acetyl tyrosine esters or amides

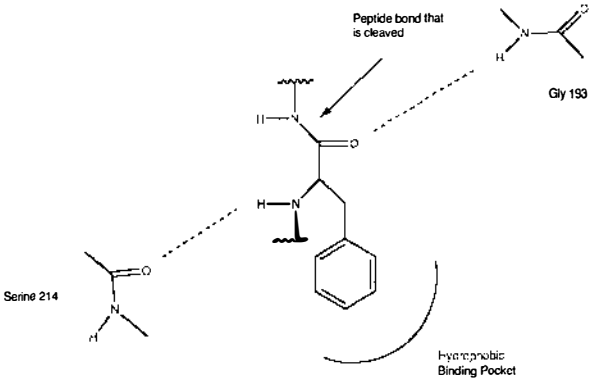

Figure 6 Critical interactions of phenylalanine-containing peptide substrate with the enzyme chymotrypsin.

has led to development of chymotrypsin-stable derivatives (62, 63). However, as in other peptide analogs (18), simple proteolytic resistance toward chymotrypsin may not result in adequate oral absorption. Consequently, in vivo bioavailability studies are essential for adequate evaluation of this prodrug concept.

Thyrotropin-releasing hormone (TRH, PGlu-L-His-L-Pro-NH2) modulates the synthesis and secretion of thyrotropin from the anterior pituitary gland. The therapeutic potential of this peptide is severely compromised by its rapid clearance and its poor permeability across biological membranes, including the blood-brain barrier (64, 65). Various N-alkyloxycarbonyl prodrugs of TRH have been prepared by acylation of the imidazole group of the histidine residue (66, 67). Although these prodrugs have displayed greater stability in plasma compared to the parent TRH, they manifested hydrolytic susceptibility to other proteolytic enzymes. The in vivo consequences of these changes have not been explored. Interestingly, the N-octyloxycarbonyl TRH, in spite of its improved metabolic stability and lipophilicity, did not enhance the transport properties of TRH across Caco-2 cells (68).

ENHANCING THE MEMBRANE PERMEABILITY OF POLYPEPTIDES

Understanding the mechanism of permeability of polypeptides to biological membranes is critical both in the design of new polypeptides and their modification as prodrugs. In general, molecules penetrate biological barriers via transcellular and/or paracellular pathways. The transcellular penetration may occur via passive diffusion through the lipid membranes, carrier-mediated active transport, and/or facilitated transport. Historically, penetration through

the lipid barrier of cell membranes has been the generally accepted pathway for the solute transport across biological membranes. However, the implications of paracellular and carrier-mediated transport processes have recently been exploited (69, 70). Biological membranes possess specific chemical and physical characteristics that tend to exclude highly polar and large macromolecules. Numerous physicochemical properties such as octanol-water partition coefficient, ionization state, protein binding, and adopted conformation have been used to predict the route and the extent by which molecules cross biological barriers (71, 72).

The classical treatment of relating membrane permeability to octanol-water partition coefficients in describing the biological transport phenomena for numerous polypeptides has been inadequate (68, 73, 74)). In particular, Conradi et al (74) demonstrated a lack of obvious correlation between the transport properties through Caco-2 cells and octanol-water partition coefficient for a homologous series of peptides. On the other hand, a good correlation was observed between the number of possible hydrogen-binding sites in a given peptide series (obtained using the Stein model (75)) and their permeability coefficient across Caco-2 cell monolayer. The decrease in the number of hydrogen-bonding sites accompanied by an increase in the permeability coefficient was attributed to the energy required to disrupt hydrogen bonds for a solute to undergo the phase transition from an aqueous to more lipid environment. However, such correlations have been demonstrated for only a limited number of neutral peptides of limited length. In this particular study only metabolically stable D-amino acid peptides were investigated. For ionic species (polypeptides containing ionizable side chains or an unprotected C- or N-terminus), electrostatic interactions may become dominant and thus minimize the contribution of hydrogen-bonding interactions to the overall permeability.

Wright & Diamond (76) showed that the permeability of a tertiary amide across rabbit gall bladder epithelium was significantly higher than the structurally equivalent molecule containing a secondary amide. Conradi and co-workers further examined this concept by sequential methylation of the amide nitrogens for a series of tetrapeptide analogues and demonstrated a substantial increase in permeability with each methyl group added (77). The modest perturbation of the octanol-water partition coefficient by N-methylation of the peptide bone could not account for the apparent permeability enhancement. However, the improvement in the permeability was well correlated with the variation in the number of possible hydrogen bonding sites in the peptide. N-Methylation is not generally considered a reversible chemical modification. Some investigators are currently exploring strategies to reduce the hydrogen-bonding potential of polypeptides via reversible chemical modifications (77). Until additional experimental data are generated, we

cannot definitively generalize the applicability of relating the number of hydrogen bonding potential to the permeability of polypeptides.

The ionic state of a solute depends on the pKa of the ionizable moieties and the pH of the environment in which the molecule finds itself. Therefore, as it relates to membrane transport, it is imperative to know the pKa of any ionizable moiety in a molecule and the pH at the interphase. Traditionally, pKa values have been measured in an aqueous environment, although the pKa of an ionizable moiety in a molecule may be altered in a less aqueous environment (78). The 4-imidazolidinone prodrugs (Figure 3) of the amino terminus of model peptides (53) described earlier provide an example of reversible chemical modification in which the pKa of the derivatized moiety is significantly altered. 4-Imidazolidinone derivatives are much weaker bases (pKa $\approx$ 3.0) than the parent peptide (pKa $\approx$ 7–8) and result in the shift of pKa value by 4–5 pKa units. The pKa of primary amines may also be altered by formation of enaminone prodrug derivatives (79). Such chemical modifications in the ionic character of the peptide at physiological pH result in increased hydrophobicity of the molecule that could positively impact its permeability profile across various biological membranes.

Since it is not easy to mimic the in vivo environment, it is difficult to assign the relative importance of the preferred conformational state of isolated peptide to its permeability characteristics. Small linear peptides exist as an ensemble of many conformers in solution and if a preferred peptide solution conformation is determined by the use of spectroscopic techniques, it represents a dynamic average of various conformers (80–82). Additionally, it is impossible to unambiguously identify the conformer(s) responsible for the transport property of the peptide. Nonetheless, various investigators have attempted to correlate the permeability of peptides to their preferred aqueous solution conformation (83–86). If a given peptide can adopt a conformation such that the hydrophobic side chains are placed on the exterior portion of the molecule and the hydrophilic components are buried in the interior, then the molecule is, a priori, likely to interact favorably with the membrane and thus enhance passive transport. In addition to the aqueous solution conformation, the preferred peptide conformation should be examined in an environment with lipid membrane-like characteristic and at an interface between an aqueous and lipid membrane surface. For example, functional signal peptide OmpA adopts different secondary structures depending on the solvent environment, concentration of the signal peptide, and available membrane surface area (84). Therefore, the preferred aqueous solution conformer may not represent the conformational characteristics of a peptide at the membrane surface or when inserted within the membrane. Based on the above observations, in the absence of in vivo or in vitro transport studies, a simple extrapolation of aqueous

solution conformation to the permeability potential of small peptides must be viewed cautiously.

Chemical modification of polypeptides to improve lipophilicity or reduce the number of hydrogen-bonding sites may also alter their preferred solution conformation. These conformational changes may affect the permeability of the peptide and must be considered as additional modulating factors. To our knowledge, modifying the physicochemical properties of a peptide by conformational perturbation via a prodrug approach has not been exploited. However, this concept could well be applicable in rational polypeptide prodrug design.

It has been demonstrated that the intestinal transport of some di- and tripeptides is carrier mediated (87–89). Many orally active peptide mimetic agents, such as a number of penicillins and cephalosporins, take advantage of these carriers (90). Enalapril and a number of ethyl ester prodrugs of other ACE inhibitors, which are structurally comparable to small di- or tripeptides, may also owe their reasonable oral availability, in part, to these or other still unidentified carriers (91, 92). The di- and tripeptide carriers are less structurally stringent than amino acid carrier systems (70). For example, Hu et al (93) have synthesized a series of dipeptidyl prodrugs of α-methyldopa possessing high affinity for the peptide carrier system. In situ intestinal studies revealed that these dipeptidyl prodrugs had significantly increased permeabilities relative to the parent compound, which is poorly permeable even though it is L-amino acid-like (93). Amidon et al have recently conducted systemic bioavailability studies in rats, comparing the availability of α-methyldopa from α-methyldopa and its prodrug α-methyldopa-L-phe. The prodrug showed a bioavailability of 90% compared to 30% for α-methyldopa (G. L. Amidon, personal communication).

Proteins may also cross biological membranes by carrier-mediated processes (94). The ability of the toxic protein, Ricin, to cross cell membranes is attributed to the binding of Ricin B chain to a surface binding site followed by internalization (95). Upon entering the cell, the active component, the Ricin A chain, is liberated where it exerts its toxicological effects. Therefore, Ricin behaves essentially as a prodrug of Ricin A chain. Poznansky & Juliano (96) have reviewed the use of monoclonal antibody-Ricin A chain conjugates for selective delivery of this peptide toxin. Similar scenarios can be envisaged where other prodrugs of polypeptides, including proteins, could be delivered via this or similar carrier-mediated mechanisms.

IMPROVING THE PHARMACOKINETIC PROFILE OF POLYPEPTIDES

The ultimate goal in designing a drug delivery system is to maximize the therapeutic efficacy and minimize the undesirable side effects associated with a given drug therapy. Achieving this goal requires a comprehensive under-

standing of the pharmacokinetic and pharmacodynamic behavior of the compound in the intended pathological state. Since it is usually impossible or impractical to determine the drug concentration at the active site, it is generally assumed that the plasma concentration correlates by some algorithm with the drug concentration at its site of action. Generally, the drug must reach and sustain sufficiently high concentrations at the site of action to elicit a desired therapeutic effect over the period of therapy. Conversely, a spike of drug concentration in the plasma or possibly tissues may produce adverse effects.

A problem in evaluating the pharmacokinetic and metabolic characteristics of many polypeptides is the lack of specific and sensitive analytical procedures (97, 98). Many standard analytical methods (such as immunoassays, bioassays, radiolabeled molecules, etc) used in studying the pharmacokinetics of polypeptides do not provide an absolute identification and quantitation of the analyte and its metabolites, complicating the interpretation of the pharmacokinetics and the correlation to its pharmacodynamics. Additionally, the pharmacological activities of polypeptides sometimes involve complex feedback interactions. Such phenomena further complicate and cloud our understanding of the correlations between the pharmacokinetics and pharmacodynamics of these compounds.

Reflecting the inherent difficulties of conventional analytical methods, data on the clearance mechanisms of polypeptides in vivo are relatively insubstantial. These limited pharmacokinetic investigations have revealed that most polypeptides lack prolonged half-lives as compared to more conventional xenobiotics (99–101).

Proteolysis is well recognized as a general mechanism by which exogenously administered peptides and proteins are catabolized. For many larger polypeptides (proteins), this process appears to be initiated via receptor-mediated endocytocis followed by degradation in the lysosomes (102–104). It is well established that the liver and kidney are the most important organs in clearing polypeptides and proteins from the circulation (105, 106). The larger polypeptides and proteins cleared via this mechanism possess distinct recognition markers that subject them to specific receptor-mediated uptake. Additionally, the physical and chemical characteristics such as size, charge, state of glycosylation, also influence the rate at which individual polypeptides are cleared.

The lack of a prolonged biological half-life for many peptides requires multiple or high dosing to maintain the drug concentrations above the required therapeutic level. In addition to being inconvenient and impractical, multiple bolus dosing (as opposed to infusion studies) may lead to drastic fluctuations of the plasma levels, which can also result in greater toxicities. Strategies such as sustained release delivery systems, reversible and irreversible chemical modifications have been tried to extend the lifetime of polypeptides in systemic circulation. Liposomes and microsphere technologies are capable of providing

sustained-release polypeptide delivery by altering the input rate of the peptide. Both can suffer, however, from rapid extraction by the liver and both have other practical and therapeutic shortcomings (107, 108). Alternatively, polypeptide degradation and metabolism, or output rate, may be modulated by modifying the polypeptide primary structure or conformation so that their susceptibility to the existing degradation pathways are altered. Various chemical modifications have been exploited to enhance the biological half-life of peptides and proteins (109). The primary structure can be modified through site-directed mutagenesis or by chemical derivatizations of the polypeptide with a nonproteogenic moiety. Such chemical derivatizations include PEGylation (110), glycosylation (111), and acylation (112). These chemical modifications have led to the development of polypeptides with extended biological half-lives and an improved immunological profile. Some of these derivatives behave as analogs whereas others might possess prodrug characteristics.

The prodrug concept has been successfully applied to numerous fibrinolytic enzymes to improve their clinical profile. Fibrinolytic enzymes have been primarily used in treating thrombosis and embolisms (113). The clinical use of fibrinolytic enzymes, as with most other polypeptides, is hampered by their rapid metabolism and clearance from the systemic circulation. In addition, these compounds elicit hemorrhagic complications. Fibrinolytic enzymes are rapidly cleared from the systemic circulation by many pathways, one of the most important being the interaction between endogenous plasma inhibitors and the active site of the enzyme, which results in total loss of biological activity (114, 115). Chemical modification of the active site blocks the physiological clearance mechanism involving the endogenous plasma inhibitors and ameliorates the pharmacokinetics and clinical characteristics of these compounds. Active-site acyl derivatives of a number of therapeutic fibrinolytic enzymes such as streptokinase-plasmin activator complex (116), urokinase (117), plasmin (112), and tissue-type plasminogen activator (118) have been synthesized. These derivatives possess prodrug-like characteristics in which the pharmacological activity is restored upon deacylation of the enzyme through an intramolecular mechanism (112). These acyl derivatives display prolonged pharmacological activity and act as a reservoir for the parent enzyme. As a result of this chemically driven sustained delivery, administration of acyl derivatives of fibrinolytic enzymes presents a distinct clinical advantage over the administration of the parent enzyme by minimizing the peripheral side effects.

The rate of deacylation is influenced by the nature of the parent enzyme and the chemical stability of the derivatized acyl group. Many acyl derivatives with varying deacylation rates have been prepared and used to modulate and control the pharmacokinetic profile of exogenously administered fibrinolytic enzymes. The simplest way of enunciating this concept is depicted by Figure

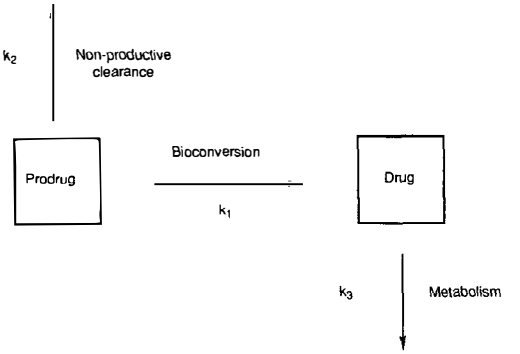

Figure 7 Simplified schematic representation of the kinetics phenomena modulating the extent and input rate from a prodrug precursor.

7. Upon rapid intravenous administration of the prodrug, the plasma profile of the parent drug is modulated by three distinct kinetic processes k_1, k_2 and k_3 (Figure 7), where k_1 is the rate process by which the parent drug is generated from the prodrug precursor, k_2 is the rate process by which the prodrug is nonproductively cleared and k_3 represents the rate process for clearance of the parent drug from circulation. Obviously, for the prodrug concept to be successful, k_2 must be small or insignificant as compared to k_1.

It is informative to hypothetically examine the concentration profile for the parent polypeptide in three different cases (A, B, C), in which the value of k_1 is varied while the value of k_3 is kept constant. In this simulation, k_2 is assumed to be negligible and the plasma half-life of the parent polypeptide is assumed to be only 10 min while the bioconversion half-lives for the prodrug to the drug in cases A, B, and C are assumed to be 1 min, 25 min, and 120 min, respectively. Figure 8 displays the concentration profile for the parent polypeptide after rapid intravenous injection of prodrugs A, B, and C. As illustrated, if the rate of bioconversion is reasonably fast, the prodrug system will generate a concentration profile almost identical to bolus intravenous injection of the parent drug (prodrug system A). Such an input rate results in a rapid spike in the plasma concentration of the polypeptide, with possible undesirable side effects. An optimal pharmacokinetic profile may be obtained when the rate of bioconversion and the rate of clearance of the parent polypeptide (Prodrug system B) are in balance. However, if the bioconversion rate is too slow, the plasma concentration of the parent peptide remains low and possibly ineffective (prodrug system C).

In theory, however, the shortcomings of prodrug system C could be circumvented by administration of a higher dose of prodrug C. It would appear from this last simulation (prodrug C) that the prodrug has produced a longer

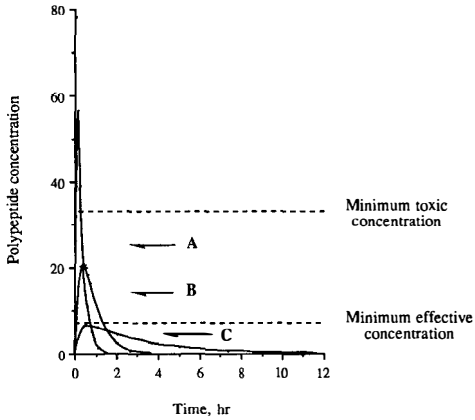

Figure 8 The concentration time course for the parent polypeptide following the rapid intravenous administration of various prodrug systems possessing different rates of bioconversion. See the schematic illustration in Figure 7.

apparent half-life for the parent drug. The actual half-life of the parent peptide is not extended and the longer apparent half-life is the result of what has been termed "flip-flop" kinetics where the longer apparent half-life is actually representative of the "input " rate rather than the "output" rate (119). The increase in the residence time of the prodrug in the body may be accompanied by its (prodrug) greater susceptibility to alternative routes of clearance including nonproductive metabolic degradation. Albert (120) summarized this concept when he stated that "although a detailed knowledge of permeability and enzymes can assist a designer in finding pro-agents....(they) will have in mind an organism's normal reaction to a foreign substance is to burn it up for food".

A greater understanding of the basic elements involved in the clearance mechanisms of polypeptides is vital for the rational development of prodrugs for improving their pharmacokinetic and pharmacodynamic properties. For example, by unraveling the mechanism of receptor-mediated clearance at the molecular level, it is possible to envisage the design of prodrugs of proteins with diminished affinity for the uptake receptors and consequently, prolonged biological half-lives.

CONCLUSION

In summary, this review, although not comprehensive, has attempted to highlight and assess examples of the prodrug approach applied to peptide and proteins, especially those that relate to metabolism, transport, and chemically

driven controlled release of polypeptides. The importance of in vivo studies in more thoroughly evaluating the potential of these prodrugs has been stressed throughout this paper. We hope this review will stimulate more extensive research into the use of prodrugs for improved formulation, delivery and pharmacokinetic profile of peptides and proteins.

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Literature Cited

1. Sanders, L. M. 1990. Drug delivery system and routes of administration of peptide and protein drugs. *Eur. J. Drug Metab. Pharmacokinet.* 15:95–102
2. Davis, S. S. 1986. Advanced delivery systems for peptides and proteins-pharmaceutical considerations. In *Delivery Systems for Peptide Drugs,* ed. S. S. Davis, L. Illum, E. Tomlinson, pp. 1–21. New York: Plenum
3. Humphrey, M. J., Ringrose, P. S. 1986. Peptides and related drugs: A review of their absorption, metabolism and excretion. *Drug Metab. Rev.* 17: 283–310
4. Lee, V. H. L., Yamamoto, A. 1990. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Deliv. Rev.* 4:171–207
5. Wiedhaup, K. 1981. The stability of small peptides in the gastrointestinal tract. In *Topics in Pharmaceutical Sciences,* ed. D. D. Breimer, P. Speiser, pp. 307–24. Amsterdam: Elsevier
6. Wang, Y. J., Pearlman, R., eds. 1992. Stability and characterization of protein and peptide drugs. Case histories. In *Pharmaceutical Biotechnology,* Vol. 5. New York/London: Plenum. In press
7. Zhou, X. H., Li Wan Po, A. 1991. Peptide and proteins drugs: II. Non-parenteral routes of delivery. *Int. J. Pharm.* 75:117–30
8. Su, K. S. E. 1986. Intranasal delivery of peptides and proteins. *Pharm. Int.* 7:8–11
9. Saffran, M., Bedra, C., Kumar, S., Neckers, D. C. 1988. Vasopressin: A model for the study of effects of additives on the oral and rectal administration of peptide drugs. *J. Pharm. Sci.* 77:33–38
10. Angust, B. J., Rogers, N. J., Shefter, E. 1988. Comparison of nasal, rectal, buccal, sublingual and intramuscular insulin efficacy and the effects of the a bile salt absorption promoter. *J. Pharmacol. Exp. Ther.* 244:23–27
11. Hussain, M. A., Shenvi, A. B., Rowe, S. M., Shefter, E. 1989. The use of α-aminoboronic acid derivatives to stabilize peptide drugs during their intranasal absorption. *Pharm. Res.* 6: 186–89
12. Lee, V. H. L. 1990. Protease inhibitors and penetration enhancers as approaches to modify peptide absorption. *J. Control. Release* 13:213–23
13. Ziv, E., Lior, O., Kidron, M. 1987. Absorption of protein via the intestinal wall. A quantitative model. *Biochem. Pharmacol.* 36:1035–39
14. Chiou, G. C. Y., Chuang, C. Y. 1989. Improvement of systemic absorption of insulin through eyes with absorption enhancers. *J. Pharm. Sci.* 78:815–18
15. Van Hoogdalem, E. J., Heijligers-Feijen, C. D., deBoer, A. G., Verhoef, J. C., Breimer, D. D. 1989. Rectal absorption enhancement of des-en-kephalin-γ-endorphin (DEγE) by medium-chain glycerides and EDTA in conscious rats. *Pharm. Res.* 6:91–95
16. Hutchinson, F. G., Furr, B. J. A. 1990. Biodegradable polymer system for the sustained release of polypeptides. *J. Control. Release* 13:279–94
17. Damge, C., Michel, C., Aprahamian,

M., Couvreur, P., Devissagute, J. P. 1990. Nanocapsules as carriers for oral peptide delivery. *J. Control. Release* 13:233–39

18. Plattner, J. J., Marcotte, P. A., Kleinert, H. D., Stein, H. H., Greer, J., et al. 1988. Renin inhibitors. Dipeptide analogues of angiotensinogen utilizing a structurally modified phenylalanine residue to impart proteolytic stability. *J. Med. Chem.* 31:2277–88
19. Morley, J. S. 1980. Structure-activity relationships of enkephalin-like peptides. *Annu. Rev. Pharmacol. Toxicol.* 20:81–110
20. Higuchi, T., Stella, V. J. 1975. *Prodrugs as Novel Drug Delivery Systems.* Washington, DC: Am. Chem. Soc.
21. Bundgaard, H. 1985. *Design of Prodrugs.* New York: Elsevier
22. Young, S. D., Payne, L. S., Thompson, W. J., Gaffin, N., Lyle, T. A., et al. 1992. HIV-1 protease inhibitor based on hydroxyethylene dipeptide isosteres: An investigation into the role of the P1′ side chain on structure-activity. *J. Med. Chem.* 35:1702–9
23. Tucker, T. J., Lumma, W. C., Payne, L. S., Wai, J. M., de Solms, S. J., et al. 1992. Series of potent HIV-1 protease inhibitors containing a hydroxyethyl secondary amine transition state isostere: Synthesis, enzyme inhibition, and antiviral activity. *J. Med. Chem.* 35:2525–33
24. Ismailos, G., Reppas, C., Dressman, J. B., Macheras, P. 1991. Unusual solubility behavior of cyclosporin A in aqueous media. *J. Pharm. Pharmacol.* 43:287–89
25. Ishikawa, K., Fukami, T., Nagase, T., Fujita, K., Hayama, T., et al. 1992. Cyclic pentapeptide endothelin antagonists with high ETA selectivity. Potency-and solubility-enhancing modifications. *J. Med. Chem.* 35:2139–42
26. Gordee, R. S., Zeckner, D. J., Howard, L. C., Alborn, W. E., Debono, M. 1988. Anti-candida activity and toxicology of LY121019, a novel semisynthetic polypeptide antifungal antibiotic. *Ann. NY Acad. Sci.* 544:294–301
27. Balkovec, J. M., Black, R. M., Hammond, M. L., Heck, J. V., Zambias, R. A., et al. 1992. Synthesis, stability, and biological evaluation of water-soluble prodrugs of a new echinocandin lipopeptide. Discovery of a potential clinical agent for the treatment of systemic candidiasis and pneumocystis carinii pneumonia (PCP). *J. Med. Chem.* 35:194–98
28. Hajdu, R., Sundelof, J. G., Bartizal, K., Abruzzo, G., Trainor, C., et al. 1991. Comparative pharmacokinetics in four animal species of L-688,786 and its water-soluble prodrug L-693,989. *Proc. ICAAC, 31st, Chicago, IL,* Sept. 29–Oct. 2, Abstr. 209
29. Blaakmeer, J., Tijsse-Klasen, T., Tesser, G. I. 1991. Enhancement of solubility by temporary dimethoxybenzyl-substitution of peptide bonds. *Int. J. Pept. Protein Res.* 37:556–64
30. Fournié-Zaluski, M. C., Coric, P., Turcaud, S., Lucas, E., Nobel, F., et al. 1992. 'Mixed-inhibitor-prodrug' as a new approach toward systemically active inhibitors of enkephalin-degrading enzymes. *J. Med. Chem.* 35:2473–81
31. Fournié-Zaluski, M. C., Coric, P., Turcaud, S., Bruetschy, L., Lucas, E., et al. 1992. Potent and systemically active aminopeptidase N inhibitors designed from active site investigation. *J. Med. Chem.* 35:1259–66
32. Swynnerton, N. F., McGovern, E. P., Mangold, D. J., Nino, J. A., Gouse, E. M., Fleckenstein, L. 1983. HPLC assay for S-2-(3-aminopropylamino) ethylphophorothioate (WR-2721. in plasma. *J. Liq. Chromatogr.* 6:1523–34
33. Manning, M. C., Patel, K., Borchardt, R. T. 1989. Stability of protein pharmaceuticals. *Pharm. Res.* 6:903–18
34. Ahern, T. J., Manning, M. C. eds. 1992. Stability of protein pharmaceuticals: Chemical and physical pathways of protein degradation. In *Pharmaceutical Biotechnology,* Vol. 2. New York/London: Plenum. In press
35. Alber, T. 1989. Mutational effects on protein stability. *Annu. Rev. Biochem.* 58:765–98
36. Alber, T., Wozniak, J. A. 1985. A genetic screen for mutations the increased the stability of phage T4 lysozyme. *Proc. Natl. Acad. Sci. USA* 82:747–50
37. Hampsey, D. M., Das, G., Sherman, F. 1988. Yeast iso-1-cytochrome c: Genetic analysis of structural requirements. *FEBS Lett.* 231:275–83
38. Friedman, D. I., Amidon, G. L. 1991. Oral absorption of peptides: Influence of pH and inhibitors on the intestinal hydrolysis of Leu-enkephalin and analogues. *Pharm. Res.* 8:93–96
39. Friedman, D. I., Amidon, G. L. 1989. Intestinal absorption mechanism of dipeptide angiotensin converting enzyme inhibitors of the lysyl-proline type: lisinopril and SQ 29,852. *J. Pharm. Sci.* 78:995–98

40. Matthews, D. M. 1975. Intestinal absorption of peptides. *Physiol. Rev.* 55: 537–607
41. Adibi, S. A. 1971. Intestinal transport of dipeptide in man: Relative importance of hydrolysis and intact absorption. *J. Clin. Invest.* 50:2266–75
42. Adibi, S. A., Morse, E. L. 1977. The number of glycine residues which limit intact absorption of glycine oligopeptides in human jejunum. *J. Clin. Invest.* 60:1008–16
43. Kenny, A. J., Maroux, S. 1982. Topology of microvillar membrane hydrolases of kidney and intestine. *Physiol. Rev.* 62:91–128
44. Ward, P. E., Sheridan, M. A. 1983. Immunoelectrophoretic analysis of renal and intestinal brush border converting enzyme. *Biochem. Pharmacol.* 32:265–74
45. Tobey, N., Haizers, W., Yeh, R., Huang, T.-I., Hoffner, C. 1985. Human intestinal brush border peptidases. *Gastroenterology* 88:913–26
46. Hussain, M. M. 1985. Reconstitution of purified dipeptidyl peptidase IV. A comparison with aminopeptidase N with respect to morphology and influence of anchoring peptide on function. *Biochim. Biophys. Acta* 815:306–12
47. Krieter, P. A., Olins, G. M., Verret, S. P., Durley, R. C. 1989. In vivo metabolism of atrial natriuretic peptide: Identification of plasma metabolites and enzyme responsible for their generation. *J. Pharmacol. Exp. Ther.* 249:411–17
48. Edgington, S. M. 1991. The anatomy of access: peptide drug delivery. *Bio/Technology* 9:1327–31
48. Amsberry, K. L., Borchardt, R. T. 1990. The lactonization of 2′-hydroxyhydrocinnamic acid amides: A potential prodrug for amines. *J. Org. Chem.* 55:5867–77
49. Amsberry, K. L., Borchardt, R. T. 1991. Amine prodrugs which utilize hydroxy amide lactonization. I. A potential redox-sensitive amide prodrug. *Pharm. Res.* 8:323–30
50. Amsberry, K. L., Gerstenberger, A. E., Borchardt, R. T. 1991. Amine prodrugs which utilize hydroxy amide lactonization. II. A potential esterase-sensitive amide prodrug. *Pharm. Res.* 8:455–61
51. Milstien, S., Cohen, L. A. 1972. Stereopopulation control. I. Rate enhancement in the lactonization of o-hydroxyhydrocinnamic acids. *J. Am. Chem. Soc.* 94:9158–65
52. Caswell, M., Schmir, G. L. 1980. Formation and hydrolysis of lactone of phenolic acids. *J. Am. Chem. Soc.* 102:4815–21
53. Rasmussen, G. J., Bundgaard, H. 1991. Prodrugs of peptides. 15. 4-Imidazolidinone prodrug derivatives of enkephalins to prevent aminopeptidase-catalyzed metabolism in plasma and absorptive mucosae. *Int. J. Pharm.* 76:113–22
54. Hanson, H. T., Smith, E. L. 1948. The application of peptides containing β-alanine to the study of the specificity of various peptidases. *J. Biol. Chem.* 175:833–48
55. Snoke, J. E., Neurath, H. 1949. Structural requirements of specific substrate for carboxypeptidases. *J. Biol. Chem.* 181:789–802
56. Bundgaard, H., Rasmussen, G. J. 1991. Prodrugs of peptides. 9. Bioreversible N-α-hydroxyalkylation of the peptide bond to effect protection against carboxypeptidases or other proteolytic enzymes. *Pharm. Res.* 8:313–22
57. Bundgaard, H., Rasmussen, G. J. 1991. Prodrugs of peptides. 11. Chemical and enzymatic hydrolysis kinetics of N-acyloxymethyl derivatives of a peptide-like bond. *Pharm. Res.* 8:1238–42
58. Bundgaard, H. 1992. Means to enhance penetration. (1) Prodrugs as means to improve the delivery of peptide drugs. *Adv. Drug Deliv. Rev.* 8:1–38
59. Steitz, T. A., Henderson, R., Blow, D. M. 1969. Structure of crystalline α-chymotrypsin. III. Crystallographic studies of substrates and inhibitors bound to the active site of α-chymotrypsin. *J. Mol. Biol.* 46:337–48
60. Bender, M. L., Killheffer, J. V. 1973. Chymotrypsins. *Crit. Rev. Biochem.* 1:149–99
61. Konno, S., Stammer, C. H. 1978. The synthesis of N-benzyloxycarbonyl-L-prolyl-dehydrophenylalanyl-LL-histidyl-L-leucine. *Int. J. Pept. Protein Res.* 12:222–31
62. Kahns, A. H., Bundgaard, H. 1991. Prodrugs of peptides. 14. Bioreversible derivatization of the tyrosine phenol group to effect protection of tyrosyl peptides against α-chymotrypsin. *Int. J. Pharm.* 76:99–112
63. Kundu, N., Roy, S., Maenza, F. 1972. Esterase activity of chymotrypsin on oxygen-substituted tyrosine substrate. *Eur. J. Biochem.* 28:311–15
64. Griffiths, E. C. 1987. Clinical application of thyrotropin-releasing hormone. *Clin. Sci.* 73:449–57
65. Hichens, M. 1983. A comparison of thyrotropin-releasing hormone with analogs: influence of disposition upon

pharmacology. *Drug Metab. Rev.* 14: 77–98
66. Møss, J., Buur, A., Bundgaard, H. 1990. Prodrugs of peptides. 8. In vitro study of intestinal metabolism and penetration of thyrotropin-releasing hormone (TRH) and its prodrugs. *Int. J. Pharm.* 66:183–91
67. Møss, J., Bundgaard, H. 1990. Prodrugs of peptides 7. Transdermal delivery of thyrotropin-releasing hormone (TRH) via prodrugs. *Int. J. Pharm.* 66:39–45
68. Lundin, S., Møss, J., Bundgaard, H., Artursson, P. 1991. Absorption of thyrotropin-releasing hormone (TRH) and a TRH prodrug in human intestinal cell line (Caco-2). *Int. J. Pharm.* 76: R1–R4
69. Nellans, H. N. 1991. Mechanism of peptide and protein absorption. Paracellular intestinal transport: modulation of absorption. *Adv. Drug Deliv. Rev.* 7:339–64
70. Adibi, S. A., Kim, Y. S. 1981. Peptide absorption and hydrolysis. In *Physiology of Gastrointestinal Tract,* ed. L. R. Johnson, p. 1073. New York: Raven
71. Lein, E. J. 1981. Structure-activity relationships and drug disposition. *Annu. Rev. Pharmacol. Tox.* 21:31–61
72. Burton, P. S., Conradi, R. A., Hilgers, A. R. 1991. Transcellular mechanism of peptide and protein absorption: passive aspects. *Adv. Drug. Deliv. Res.* 7:365–86
73. Ho, N. F. H., Day, J. S., Barsuhn, C. L., Burton, P. S., Raub, T. J. 1990. Biophysical model approaches to mechanistic transepithelial studies of peptides. *J. Control. Release* 11:3–24
74. Conradi, R. A., Hilgers, A. R., Ho, N. F. H., Burton, P. S. 1991. The influence of peptide structure on the transport across Caco-2 cells. *Pharm. Res.* 8:1453–60
75. Stein, W. D. 1967. The molecular basis of diffusion across cell membranes. In *The Movement of Molecules Across Cell Membranes,* pp. 65–125. New York: Academic
76. Diamond, J. M., Wright, E. M. 1969. Molecular forces governing non-electrolyte permeation through cell membranes. *Proc. R. Soc. London Ser. B* 172:173–316
77. Conradi, R. A., Allen, R. H., Ho, N. F. H., Burton, P. S. 1992. The influence of peptide structure on transport across caco-2 cells. II. Peptide bond modification which results in improved permeability. *Pharm. Res.* 9: 435–39
78. Mason, R. P., Rhodes, D. G., Herbette, L. G. 1991. Reevaluating equilibrium and kinetic binding parameters for lipophilic drugs based on a structural model for drug interaction with biological membranes. *J. Med. Chem.* 34:869–77
79. Naringrekar, V. H., Stella, V. J. 1990. Mechanism of hydrolysis and structure-stability relationship of enaminones as potential prodrugs of model primary amines. *J. Pharm. Sci.* 79:138–46
80. Vida, J. A., Gordon, M. 1984. Conformationally directed drug design. *ACS Symp. Ser.* 251. Washington, DC: Am. Chem. Soc.
81. Kessler, H., Griesinger, C., Wagner, K. 1987. Peptide conformations. 42. Conformation of side chains in peptides using heteronuclear coupling constants obtained by two-dimensional NMR spectroscopy. *J. Am. Chem. Soc.* 109: 6927–33
82. Kim, Y., Prestegard, J. H. 1990. Demonstration of a conformational equilibrium in acyl carrier protein from spinach using rotating frame nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc.* 112:3707–9
83. Briggs, M. S., Cornell, D. G., Dluhy, R. A., Gierasch, L. M. 1986. Conformations of signal peptides induced by lipids suggest initial steps in protein export. *Science* 233:206–8
84. McKinght, C. J., Stradley, S. J., Jones, J. D., Gierasch, L. M. 1991. Conformational and membrane binding properties of signal sequence are largely unaltered by its adjacent mature region. *Proc. Natl. Acad. Sci. USA* 88:5799–803
85. Hoyt, D. W., Gierasch, L. M. 1991. Hydrophobic content and lipid interactions of wild-type and mutant OmpA signal peptides correlate with their in vivo function. *Biochemistry* 30:10155–63
86. Bruch, M. D., Gierasch, L. M. 1990. Comparison of helix stability in wild-type and mutant LamB signal sequences. *J. Biol. Chem.* 265:3851–58
87. Matthews, D. M., Payne, J. W. 1980. Transmembrane transport of small peptides. *Curr. Top. Membr. Transp.* 14: 331–425
88. Addison, J. M., Burston, D., Dalrymple, J. A., Matthews, D. M., Payne, J. W., et al. 1975. A common mechanism for transport of di- and tri-peptides by hamster jejunum in vitro. *Clin. Sci. Mol. Med.* 49:313–22
89. Addison, J. M., Burston, D., Payne, J. W., Wilkinson, S., Matthews, D.

M. 1975. Evidence for active transport of tripeptides by hamster jejunum in vitro. *Clin. Sci. Mol. Med.* 49:305–12

90. Nakashima, E., Tsuji, A., Kagatani, S., Yamana, T. 1984. Intestinal absorption mechanism of amino-β-lactam antibiotics. III. Kinetics of carrier-mediated transport across the rat small intestine in situ. *J. Pharm. Dyn.* 7:452–64
91. Hu, M., Amidon, G. L. 1988. Passive and carrier-mediated intestinal absorption compenents of captopril. *J. Pharm. Sci.* 77:1007–11
92. Friedman, D. I., Amidon, G. L. 1989. Passive and carrier-mediated intestinal absorption components of two angiotensin converting enzyme (ACE) inhibitor prodrugs in rats: Enalapril and Fosinopril. *Pharm. Res.* 6:1043–47
93. Hu, M., Subramanian, P., Moseberg, H. I., Amidon, G. L. 1989. Use of the peptide carrier system to improve the intestinal absorption of L-α-methyldopa: Carrier kinetics, intestinal permeabilities, and in vitro hydrolysis of dipeptidyl derivatives of L-α-methyldopa. *Pharm. Res.* 6:66–70
94. van Deurs, B., Hansen, S. H., Olsnes, S., Sandvig, K. 1993. Protein uptake and cytoplasmic acess in animal cells. Biological barrier to protein delivery. In *Pharmaceutical Biotechnology,* ed. K. L. Audus, T. J. Raub, Vol. 4. New York/London: Plenum Press. In press
95. Raub, T. J., Audus, K. L. 1990. Adsorptive endocytosis amemberane recycling by cultured primary bovine brain microvessel endothelial cell monolayers. *J. Cell. Sci.* 97:127–38
96. Poznansky, M. J., Juliano, R. L. 1984. Biological approaches to the controlled delivery of drugs: A critical review. *Pharmacol. Rev.* 36:277–336
97. Gosling, J. P. 1990. A decade of development of immunoassay methodology. *Clin. Chem.* 36:1408–27
98. Gloff, C. A., Benet, L. Z. 1990. Pharmacokinetics and protein therapeutics. *Adv. Drug Deliv. Rev.* 4:359–86
99. Kuo, B.-S., Kusmik, W. F., Poole, J. C., Elsea, S. H., Chang, J., Hwang, K.-K. 1992. Pharmacokinetics evaluation of two human epidermal growth factors (hEGF51 and hEGF53) in rats. *Drug Metab. Dispos.* 20:23–30
100. Bartocci, A., Mastroginannis, A., Migliorati, G., Stockert, R. J., Wolkoff, A. W., Stanley, E. R. 1987. Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc. Natl. Acad. Sci. USA* 84:6179–83
101. Blick, M., Sherwin, S. A., Rosenblum, M., Gutterman, J. 1987. Phase I study of recombinant tumor necrosis factor in cancer patients. *Cancer Res.* 47: 2986–89
102. Hilaire, R. J. S., Hradek, G. T., Jones, A. L. 1983. Hepatic sequestration and biliary secretion of epidermal growth factor: Evidence for a high capacity uptake system. *Proc. Natl. Acad. Sci. USA* 80:3791–801
103. Sugiyama, Y., Hanano, M. 1989. Receptor-mediated transport of peptide hormones and its importance in the overall hormone disposition in the body. *Pharm. Res.* 6:192–202
104. Baumann, G., Vance, M. L., Shaw, M. A., Thorner, M. O. 1990. Plasma transport of human growth hormone in vivo. *Endocrinology* 71:470–73
105. Schiff, J. M., Fisher, M. M., Jones, A. L., Underdown, B. J. 1986. Human IgA as a heterovalent ligand: Switching from the asialoglycoprotein receptor to secretory component during transport across the rat hepatocyte. *J. Cell Biol.* 102:920–31
106. Kim, D. C., Sugiyama, Y., Sawada, Y., Hanano, M. 1992. Renal tubular handling of p-aminohippurate and epidermal growth factor (EGF) in filtering and non-filtering perfused rat kidney. *Pharm. Res.* 9:271–75
107. Ishii, Y., Aramaki, Y., Hara, T., Tsuchiya, J., Fuwa, T. 1989. Preparation of EGF labeled liposomes and their uptake by hepatocytes. *Biochem. Biophys. Res. Commun.* 160:732–36
108. Pitt, C. G. 1990. The controlled parenteral delivery of polypeptides and proteins. *Int. J. Pharm.* 59:173–96
109. Knadker, M. P., Ackermann, J. E., Coutant, J. E., Hurst, G. H. 1992. Metabolism of the anticoagulant peptide, MDL 28,050, in rats. *Drug. Metab. Dispos.* 20:89–95
110. Ho, D. H., Brown, N. S., Yen, A., Holmes, R., Keating, M., et al. 1986. Clinical pharmacology of polyethylene glycol-L-asparaginase. *Drug Metab. Dispos.* 14:349–52
111. Rademacher, T. W., Parekh, R. B., Dwek, R. A. 1988. Glycobiology. *Annu. Rev. Biochem.* 57:785–838
112. Markwardt, F. 1989. Acyl-enzymes as prodrugs. *Pharmazie* 44:521–26
113. Duckert, F. 1979. Thrombolytic therapy in myocardial infarction. *Prog. Cardiovasc. Dis.* 21:342–50
114. Wiman, B., Collen, D. 1978. On the kinetics of the reaction between anti-

plasmin and plasmin. *Eur. J. Biochem.* 84:573–78
115. Christensen, U., Clemmensen, I. 1977. Kinetic properties of the primary inhibitor of plasmin from human plasma. *Biochem. J.* 163:389–91
116. Smith, R. A. G., Dupe, R. J., English, P. D., Green, J. 1981. Fibrinolysis with acyl-enzyme: a new approach to thrombolytic therapy. *Nature* 290:505–7
117. Stürzebecher, J., Kroneck, I., Klöcking, H.-P. 1986. Untersuchungen zur fibrinolytischen Aktivitat von Acyl-urokinase. *Folia Haematol. (Leipzig)* 113:137–43
118. Stürzebecher, J., Richter, M., Markwardt, F. 1987. Stable acyl-derivatives of tissue-type plasminogen activator. *Thromb. Res.* 47:699–703
119. Notari, R. E., Huang, M.-Y., Byron, P. R. 1978. Calculation of optimum pharmacokinetic drug supply rates for maximum duration during multiple dose therapy by prodrug administration. *Int. J. Pharm.* 1:233–47
120. Albert, A. 1965. *Selective Toxicity.* New York: Wiley. 3rd ed.