not by the second, there is insufficient data to say which is correct. In either case, the peptide bond is broken via acid catalysis by Tyr 248. If an acyl enzyme were formed, the subsequent attack by $H_2O$ would be aided by the proximity of the Tyr 248 conjugate base.

### 5.4.2. *Leucine aminopeptidase*

Although it is thought of as the 'classic' N-terminal exopeptidase, there is relatively little information available on leucine aminopeptidase (LAP). This is due partly to difficulty in identifying aminopeptidase obtained from different sources and partly to its size. It was originally isolated from porcine kidney [90], but many studies have been done on samples extracted from bovine eye lens [91, 92].

The molecular weight of the enzyme is around $3 \times 10^5$ g/mole. The porcine kidney sample is thought to consist of four subunits [93], whereas the bovine eye lens sample consists of 10 subunits [94]. Low-angle X-ray diffraction studies done on the eye lens sample indicate that the enzyme is shaped like a hollow cylinder with inside diameter of about 32 Å [95]. No efforts to investigate the detailed tertiary structure have been made and, since efforts to determine end groups have been unsuccessful, the primary and secondary structures are completely unknown. However, Himmelhoch [96] has found that the enzyme contains approximately one atom of Zn per subunit.

Unlike CPA, the tertiary structure of LAP is stabilized by the Zn ions: once the Zn has been removed, catalytic activity is lost permanently. However, the Zn can be replaced with other ions [97]. LAP is strongly activated by $Mn^{2+}$ and $Mg^{2+}$, inhibited by $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Pb^{2+}$, and unaffected by $Ca^{2+}$, $Co^{2+}$ and $Ni^{2+}$. The Zn (or other metal ion) has been shown to play an essential role in the catalytic process but to be unessential for substrate binding [98].

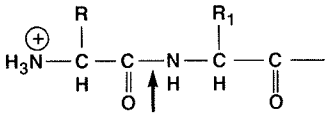
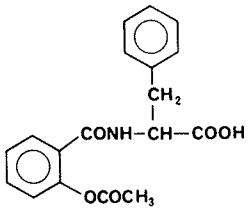


Fig. 12. LAP catalyzes the hydrolysis of the indicated peptide bond.

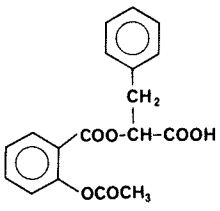
The primary action of LAP is indicated in Figure 12. It can also function as an esterase but at about 10% of the rate for the analogous peptide [97]. There are two absolute requirements for potential LAP substrates [97]: the N-terminal residue must be of the L configuration and the terminal $-NH_2$ groups must be free (i.e., not acylated). In addition to peptides and esters, amides have long been known to be good substrates for LAP (e.g., L-leucine-amide [99]). Almost any N-terminal L-amino acid (or glycine) will be released at a measurable rate, although substrates with a hydrophobic R-group are best. Generally, substrates wherein the N-terminal residue is Leu, norLeu or norVal are hydrolyzed the fastest, although L-Ala-L-leucinamide is the best substrate known. Compounds having an N-terminal D-amino acid residue are

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

kinetic parameters for carboxypeptidase A hydrolysis of aspirin phenylalamine (I) and aspirin phenyllactic acid (II).




I




II

Isolation and identification of carboxy and aminopeptidase from enterocyte brush borders and cytosol have shown different though somewhat overlapping specificities. It appears that these exopeptidases function at the surface or interior of the absorptive cell to further reduce small oligopeptides released by pancreatic protease action. Their radial distributions appear to be geared to couple small peptide and amino acid intestinal transport. Tetrapeptide activity is associated exclusively with the brush border membrane, tripeptide activity is distributed about equally between membrane and cytosol, while enzymes that cleave dipeptides are more prevalent in the cytosol than brush border [101]. Axial distributions appear to be fairly even over the entire small intestinal length and less subject to species, age, and nutritional input parameters than is alkaline phosphatase. However, this may be attributed to the fact that isoenzymes and specific peptidases have not been well characterized with respect to their association with particular axial regions [102]. Intestinal absorption of L-lysine-*p*-nitroanilide, L-alanine-*p*-nitroanilide and glycine-*p*-nitroanilide was studied in the presence of competitive inhibitors in perfused rat intestine in order to establish the coupled brush border hydrolysis and transport [103]. This study has shown that the peptidase in the brush border region that serves as the hydrolysis site requires a free $\alpha$-amino group (an aminopeptidase).

## 6. Alkaline phosphatases

The mammalian alkaline phosphatases are distributed among various tissues of the body. Examples of tissue exhibiting alkaline phosphatase activity are: the intestinal mucosa, placenta, kidney, bone, liver, lung and spleen [104]. Alkaline phosphatases may not be as readily useful as targeted reconversion site for prodrugs as the hydrolytic enzymes of the GI tract. Fishman [105] has investigated the organ-specific behavior of rat alkaline phosphatases toward a variety of compounds.

A typical alkaline phosphatase (e.g., human placental alkaline phosphatase) has a molecular weight around 125,000 g/mole and contains approximately 3 moles of

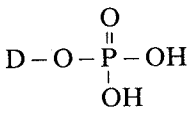
DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

phosphatase activity, while calcium, sucrose and alcohol intake have been correlated with reduced activities [120, 121].

There is also considerable evidence that intestinal alkaline phosphatases exist as several isoenzymes [122]. In this regard, it has been shown that the ileal enzyme is precipitated less easily than duodenal enzyme by mixed anti-phosphatase, L-phenylalanine inhibition is more marked for duodenal than jejunal enzyme, heat stability is greatest for duodenal alkaline phosphatase, and the phenylphosphate to beta-glycerophosphate activity ratio is reversed from duodenum (high ratio) to ileum. It is believed this enzyme functions in the small intestine for absorption of phosphate from dietary phosphates, but much uncertainty remains concerning its physiologic function.

### 6.1. Prodrug Considerations

Since phosphatase activity is distributed widely in mammalian tissues, phosphate monoester prodrugs are not restricted to oral dosage forms. However, for planned and specific targeting using phosphate monoesters, alkaline phosphatase in the intestine is the enzyme of choice. Since, in general, P-N bonds are not hydrolyzed, a free hydroxyl group in the drug moiety would be required. Prodrugs of the form



would be potential phosphatase substrates.

The concept of alkaline phosphatase hydrolysis coupled with facilitated transport has been studied recently [123] using the 21-phosphate ester of hydrocortisone. Bioavailability experiments in beagle dogs were done along with the parent drug, succinate and lysinate esters in equivalent molar doses. The phosphate derivative, which was shown previously to have high rat intestinal wall permeability in the upper intestine, showed high early peak plasma concentration which dropped off rapidly, leading, however, to a somewhat reduced bioavailability for this prodrug. The bioavailability results are shown in Figure 14. The fact that intestinal alkaline phosphatase distribution in dog is predominantly in the upper small intestine and that the ionized prodrug is not well absorbed passively accounts for the data.

These results indicate that prodrugs targeted for intestinal alkaline phosphatase potentially can improve oral drug delivery. For stable phosphate derivatives, axial enzyme distribution in the particular species under consideration will determine the relative success for these prodrugs.

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

'half-molecules' has been observed in several laboratories (see, Refs. 132, 133). Krish [124] has reviewed studies of the amino acid composition of various esterases. The B-esterases, which have received far more attention than the other two classes, are phosphorylated by inhibitors such as diethyl *p*-nitrophenyl phosphate at a serine residue. This fact has enabled the determination of the amino acid sequence in the vicinity of the 'active serine' for a number of different carboxylesterases [124].

Esterases catalyze the hydrolysis of esters according to the reaction:

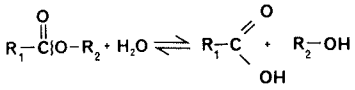


Table 11 lists a few of the many compounds known to be esterase substrates. Although some labile ester substrates are also hydrolyzed by several endopeptidases (e.g., α-chymotrypsin) and other enzymes, these catalytic rates are, typically, 4 to 5 orders of magnitude less than those of 'true' esterases [138, 139, 145]. For example, using *p*-nitrophenyl butyrate as the substrate, Stoops et al. [138] reported that, at pH 8.1, $K_m = 2.3 \times 10^{-4}$ M and $k_{cat} = 3.72 \times 10^2$ sec$^{-1}$ for pig liver carboxylesterase. For the same substrate under similar conditions, Cane and Wetlaufer [146] found $k_{cat} = 7.7 \times 10^{-3}$ sec$^{-1}$ for α-chymotrypsin. Also, like α-chymotrypsin, liver and kidney esterases have been shown to catalyze the hydrolysis of several amino acid esters [132, 134, 138] at comparable rates. For the carboxylesterase- and α-chymotrypsin-catalyzed hydrolysis of L-tyrosine ethyl ester, Stoops et al. [138] found $k_{cat}$ values of 71 and 39 sec$^{-1}$, respectively. However, in contrast with the peptidases, peptides are not hydrolyzed by porcine liver carboxylesterase [134], although some amides have been found to be poor substrates. With $K_m = 2.5 \times 10^{-3}$ M and $k_{cat} = 0.1$ sec$^{-1}$, phenacetin was typical of the several amide substrates studied by Franz and Krisch [131].

They also noted that procaine was an equally poor ester substrate of porcine kidney carboxylesterase, having $K_m$ and $k_{cat}$ values of $4 \times 10^{-4}$ and 0.1 sec$^{-1}$, respectively. Levy and Ocken [135] have distinguished three groups of carboxyl ester substrates: (1) unsubstituted monocarboxylate esters (which are hydrolyzed with the highest relative velocities), (2) substituted monocarboxylate esters, and (3) dicarboxylate diesters and substituted diesters, of which only one ester group is hydrolyzed. Charged compounds are extremely poor substrates [134, 135]. Hofstee [136] has found that the reactivity of fatty acid esters of *m*-hydroxybenzoic acid increases as the acyl chain length is increased. Chain length effects have been discussed in more detail by Dixon and Webb [137], who reported the reactivity and affinity (defined as the inverse of $K_m$) of 33 esters (in which both the acyl and alkyl chain lengths ranged between $C_1$ and $C_9$) with respect to horse liver carboxylesterase. They concluded that the alkyl and acyl chain length effects are largely independent. Both the affinity and reactivity increase as either chain is lengthened to about $C_4$ to $C_6$. Fur-

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

ther increase in the alkyl chain length results in a decrease in both the affinity and reactivity, suggesting that the alkyl binding site is only large enough to accomodate a butyl to hexyl chain. Further increase of the acyl chain length produces the same decline in reactivity but a sharp rise in affinity, suggesting the existence of a secondary acyl binding site which leads to non-optimal orientation at the esteratic site. Similarly, branched-chain substrates exhibit higher affinities but lower reactivities [137]. Esterases are also capable of hydrolyzing thioesters [121, 122] and aromatic amides, including acetanilide [129, 132, 134] and phenacetin, at reduced rates. Lastly, carboxylesterases can also act as transferases as was first observed by Bergmann and Wurzel [140] in 1953.

Kinetic studies on mammalian carboxylesterases consistently report the pH optimum to be in the range of 7.5 – 9.0 [124]. It is generally agreed that, like other serine hydrolases (e.g., $\alpha$-CT), carboxylesterase catalysis proceeds through an acyl-enzyme intermediate [141]. Deviations from normal Michaelis-Menten kinetics attributable to substrate activation have been observed by several workers (see, e.g., Refs. 138 – 142). The details of the catalytic mechanism are still unknown, although the participation of a histidine residue has been discussed [137, 144, 145] but not verified.

## 7.2. Lipases

The lipases are a distinct subset of the large family of esterase enzymes. In mammals, lipases are found in the digestive tract, in such tissues as the heart, brain, muscle, arteries, kidney, adipose tissue and serum, and have also been identified in milk [147]. Their primary function is the metabolism of triglycerides.

Most of the lipase found in the GI tract is produced in the pancreas. The lipase isolated from lyophilized aqueous extracts of fresh porcine pancreas was found to be almost entirely in the form of a high-molecular-weight complex of enzyme and lysolecithin-rich micelles [148]. Treatment of this complex with organic solvents produced enzyme molecules weighing approximately 48,000 g/mole [149]. The amino acid compositions of several lipases are known [149, 150], but there is no information on their structure. The observation that the lipase activity of porcine pancreatic extracts is significantly depressed upon chromatography [151] or gel filtration [152] pointed to the existence of a pancreatic colipase, which subsequently was isolated and purified [153]. It was found to be a small protein (molecular weight, about 8,000 g/mole) which binds to the lipase in a 1:1 molar ratio [153] and increases the lipase activity when bile salts are present [154, 155].

Lipases are best distinguished from the other carboxylesterases by the physical state of the substrate. Unlike other esterases, lipases do not catalyze the hydrolysis of dissociated (i.e., totally solvated) substrate molecules. Lipases generally act at hydrophobic interfaces or on emulsified particles. However, some lipases are known to hydrolyze micelles [156, 157] and the minimum extent to which substrate

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

DOCKET ALARM

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

**LAW FIRMS**
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

**FINANCIAL INSTITUTIONS**
Litigation and bankruptcy checks for companies and debtors.

**E-DISCOVERY AND LEGAL VENDORS**
Sync your system to PACER to automate legal marketing.