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Research Article

Pancreatic Lipase-Catalyzed Hydrolysis of Esters of Hydroxymethyl Phenytoin Dissolved in Various Metabolizable Vehicles, Dispersed in Micellar Systems, and in Aqueous Suspensions

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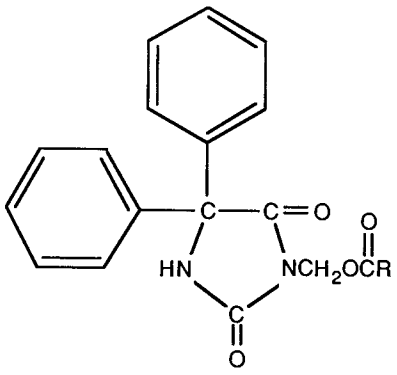
Lipase-catalyzed hydrolysis of fatty acid esters of 3-hydroxymethyl phenytoin was studied in various triglyceride and ethyl oleate emulsions, dispersed in micellar solutions, and suspended in an aqueous buffered solution. Phenytoin release from ethyl oleate emulsions of the prodrugs show apparent first-order kinetics with the pentanoate to nonanoate derivatives and sigmoidal kinetics with the long-chain fatty acid derivatives (stearate and oleate). A transition in the kinetic behavior, between the short- and the long-chain acyl prodrugs, was observed with the decanoate derivative. These observations are accounted for by a proposed kinetic model. Phenytoin release from the solid prodrugs follows zero-order kinetics and is independent of the total amounts of suspended material but directly proportional to the lipase concentration. Lipolysis of the solid suspended prodrugs was dependent on the length of the acyl side chain of the prodrug, with maxima for the pentanoate and the octanoate derivatives. The short-chain derivatives, acetate and propionate, as well as the long-chain prodrug, stearate, showed the slowest lipolysis rate when present as solid dispersions. The zero-order rate is qualitatively correlated with the melting point of the prodrugs. This result might be expected if the melting point is taken as a measure of the cohesivity or packing of the molecules at the surface of a crystal.

KEY WORDS: pancreatic lipase; lipase, phenytoin; prodrugs; prodrug hydrolysis; emulsions; micelles; lipolysis.

INTRODUCTION

Although the design of prodrugs to achieve various drug delivery goals has been extensively reviewed (1–5), good mechanistic investigations on enzyme-catalyzed prodrug reversion have been lacking. Most studies of ester prodrug cleavage have assumed that the hydrolysis step occurs via various esterases and/or proteases: ubiquitous enzymes found throughout various tissues and organs including the contents of the gastrointestinal tract. Phenytoin (1) (Scheme I), a high-melting point, low-water-soluble, and low-lipid-soluble drug, has shown erratic bioavailability after oral dosing (6–9). Although water-soluble prodrugs of phenytoin have shown less variable bioavailability after oral dosing (10), Yamaoka *et al.* (11) also found that 3-pentanoyloxymethylphenytoin, a low-melting point liposoluble prodrug of phenytoin, gave a superior oral availability of phenytoin compared to phenytoin itself when administered in



R	R
CH_3 (2)	C_7H_{15} (8)
C_2H_5 (3)	C_8H_{17} (9)
C_3H_7 (4)	C_9H_{19} (10)
C_4H_9 (5)	$C_{18}H_{35}$ (11, **Oleate**)
C_5H_{11} (6)	$C_{18}H_{37}$ (12, **Stearate**)
C_6H_{13} (7)	

Scheme I

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tributyrin to rats. In their study, Yamaoka *et al.* (11) explored the role of plasma esterases as possible sources of cleavage of various 3-acyloxymethyl prodrug derivatives of phenytoin. In the present study, the pancreatic lipase-catalyzed hydrolysis of various 3-acyloxymethyl prodrugs of phenytoin (**2–12**), dissolved in various metabolizable lipophilic vehicles, in micellar dispersions, and in the solid state, are explored. Hydrolysis of these prodrugs to phenytoin yields the corresponding carboxylic acid and 3-hydroxymethyl phenytoin (HMP). Under neutral conditions, the latter compound undergoes a fast and spontaneous decomposition to give phenytoin and formaldehyde (12,13).

MATERIALS AND METHODS

Chemicals

Tributyrin (Grade I; 99%; TB), trioctanoin (99%; TO), ethyl oleate (99%; EO), soybean oil, L-α-phosphatidylcholine (60% lecithin from egg yolk), anhydrous taurodeoxycholic acid (99%; sodium salt; TDC), tris(hydroxymethyl)aminomethane (Tris), and pancreatic lipase (Type II; crude preparation with 30% protein containing the lipase–colipase complex) were all obtained from Sigma Chemical Co., St Louis, Mo. Calcium chloride dihydrate and glycerol, spectral quality, were obtained from Matheson Coleman and Bell, Norwood, Ohio. All other chemicals were reagent grade. Phenytoin prodrugs, **2–12**, were better than 99% pure based on differential scanning calorimetry, C,H,N, and high-performance liquid chromatographic (HPLC) analysis (14). All solutions and buffers were prepared with double-distilled, deionized water.

Emulsions

A weighed amount of a phenytoin prodrug was dissolved in ethyl oleate, or a triglyceride, and mixed with a lecithin dispersion in water to give a crude emulsion containing, by weight, 10% oil, 1.2% lecithin, 2.25% glycerol, and water (phenytoin equivalents were kept constant at 22.3 m*M* in ethyl oleate unless indicated otherwise). These crude emulsions were then placed in an ice bath and sonicated (20 kHz, 350 W) with a Branson Model W-350 ultrasonifier for 6 to 8 min. The particle sizes of the emulsions kept under refrigeration for 7 to 8 months were determined at a later date when a Nicomp Model 370 submicron particle size analyzer (HIAC/Royco Instruments) became available and compared with the particle sizes of freshly prepared emulsions. Typical partical sizes of old ethyl oleate emulsions were 0.2 to 0.3 μm and did not differ more than 0.1 μm from those of the freshly prepared emulsions. Particle sizes of soybean oil, trioctanoin, and tributyrin emulsions were all about 0.2 ± 0.1 μm. The emulsions were kept at 4°C and used within 2 or 3 days from the date of preparation, although no decomposition was noted upon storage under refrigeration for several weeks.

Kinetics

Experimental conditions for these studies were selected based on a detailed kinetic analysis of similar triglyceride emulsions in the absence of the prodrugs. Experimental details and results from those studies will be published elsewhere. Briefly, it was found that optimum conditions for lipase catalysis were closely related to physiological conditions found in the intestinal tract. Maximum lipase activity was observed in systems containing bile salts (TDC, 6.0 m*M*) and calcium ion (Ca^{2+}, 30 m*M*). Lecithin was selected as the emulsifier because it is a natural component of the bile and is necessary for the *in vivo* metabolism and/or absorption of lipids (15–17). The same general trends noted during the hydrolysis of triglycerides were also observed with ethyl oleate. Under optimum conditions long-chain triglycerides underwent lipolysis significantly faster than ethyl oleate.

An aliquot of emulsion (typically 500 μl) was mixed with measured volumes of 0.10 *M* $CaCl_2$, 30 m*M* TDC (prepared in 0.2 *M* Tris buffer, pH 8.5), and 0.20 *M* Tris buffer (pH 8.50) solutions to give the desired concentrations in a final volume of 5.0 ml. The final compositions of the various solutions are defined under Results. The temperature of the reaction was maintained at 25°C by a circulating water bath (Haake Model FE). The reaction was started with the addition of a lipase solution (30 to 200 μl, equivalent to 840 to 5600 U). Vigorous stirring of the reaction mixture was maintained during the kinetic run using a magnetic stirrer. The enzyme solution was prepared by dissolving 250 mg of the commercial preparation in 5.0 ml of cold water with stirring until all of the soluble material went into solution and separating by centrifugation (15 min using a benchtop centrifuge) the insoluble material.

Lipase activities were determined with TB and olive oil emulsions in the presence of 30 m*M* Ca^{2+}, pH 8.5, with and without lecithin. When lecithin was the emulsifier, the emulsions were prepared as indicated above; otherwise, the oil and water were mixed vigorously to form a crude emulsion and then sonicated as above. The latter emulsions were unstable and were used immediately after preparation. Lipase activity with olive oil, in the absence of lecithin and TDC, was 160 U/mg of protein (the lipase preparation used in these studies indicates that the lipase activity under similar conditions, pH 7.7, is 135 U/mg of protein). With a lecithin-stabilized olive oil emulsion the activities in the presence of 6.0 m*M* TDC were 1420 and 960 U/mg of protein at 37 and 25°C, respectively. With TB, in the absence of lecithin and TDC, the activity was 955 U/mg of protein at 37°C. The highest activities were obtained with lecithin-stabilized TB emulsions in the presence of 6.0 m*M* TDC. Activities at 37 and 25°C were 2600 and 1850 U/mg of protein, respectively. One unit is defined as the amount of lipase that liberates 1 μequiv of FA from a triglyceride (TG) per h at the temperature and pH specified. Lipase activities reported in this study refer to TB emulsions in the presence of 30 m*M* Ca^{2+}, 6.0 m*M* TDC, pH 8.5, at 25°C. Only freshly prepared enzyme solutions were used for these reactions.

At selected time intervals, 200-μl aliquots of the reaction mixture were withdrawn and added to 4800 μl of acetonitrile in order to quench the reaction. Proteins and other insoluble material were separated by centrifugation and the supernatant was assayed by HPLC. Control experiments, omitting the addition of lipase, were carried out in parallel. Similar experiments were carried out with weighed amounts of solid phenytoin prodrugs (5–25 mg solid) suspended in Tris buffer (0.180 *M*, pH 8.50) containing 30 m*M* $CaCl_2$ (re-

sulting in a final volume of 5.0 ml) at 25°C. Vigorous stirring was maintained in order to keep a homogeneous suspension of prodrug and phenytoin. Enough phenytoin was formed in these reactions to precipitate (water solubility ≈ 20 μg/ml or 8×10^{-5} *M*). Control experiments were carried out in the absence of lipase. Initial rates of ethyl oleate hydrolysis were measured potentiometrically with a Brinkmann pH-stat (Dosimat 665, pH meter Model 632, and Impulsomat 614). Tris buffer was replaced by a 0.2 *M* NaCl solution. All of the other conditions were the same.

Analytical Methods

HPLC analysis of phenytoin and prodrugs was carried out under isocratic conditions at room temperature. HPLC analysis used a cyano column (15 cm; 5-μm particle diameter), a Beckmann Model 110B pump, and a 20-μl injector. The mobile phase consisted of acetonitrile–water mixtures and was varied, 45:55 for phenytoin to 75:25 for the stearate prodrug, depending on the prodrug being studied. Flow rates were varied from 1.0 to 1.5 ml/min. Detection by UV at 214 nm (Kratos Spectroflow 757) was used for all compounds. Chromatograms were recorded with a Shimadzu C-R3A programmable integrator. Phenytoin and/or prodrug concentrations were calculated from standard curves which were obtained by dissolving the pure compounds in acetonitrile and analyzing under identical conditions. Peak area or peak height reproducibility was normally better than 3%.

RESULTS

Hydrolysis of Phenytoin Prodrugs in Ethyl Oleate Emulsions

In ethyl oleate emulsions the hydrolysis rates of phenytoin prodrugs (22 m*M* in ethyl oleate) varied depending on the length of the acyl side chain and, to a lesser extent, the oil-to-prodrug ratio (Table I). A continuous decrease in reaction rate as a function of methylene groups in the fatty acid side chain was observed for derivatives **5** through **12**. The time dependencies for the disappearance of prodrugs **5–8**, or the appearance of phenytoin, are relatively well described by first-order kinetics (Figs. 1a and b), while the dependencies for the oleate (**11**) and stearate (**12**) prodrugs are distinctively sigmoidal (Fig. 1d). Changes in the **12**-to-ethyl oleate ratio (12 to 44 m*M* **12** in ethyl oleate) did not modify the general shape of the time profile. However, an increase in the **12** concentration was manifested by a moderate increase in the lag time for its own hydrolysis (Table I). The time dependencies for **9** and **10** cleavage show a gradual change in their kinetic behavior from that of the lower-chain analogues to the sigmoidal one of the stearate and oleate derivatives (Fig. 1c). Hydrolysis of prodrugs **2–4** could not be studied in this system because they were insoluble in ethyl oleate at the concentration range employed (5 to 10 mg/ml of ethyl oleate) (11). In all cases, prodrug disappearance corresponded unequivocally with the appearance of phenytoin. This indicates that hydrolysis is the only decomposition pathway and that no other degradation products are formed. In most cases, the time dependence for ethyl oleate hydrolysis, as determined by HPLC, was followed to completion and found to be relatively independent of the presence of prodrug (Table II). These results were confirmed following the time dependence of hydrolysis with a pH-stat apparatus. Hydrolysis rates of ethyl oleate were approximately zero order for over 60% reaction (Table II) and showed little dependence on the total amount of emulsion present in the reaction mixture. As a consequence, it was found that hydrolysis of the prodrugs could precede, follow, or take place at about the same rate as the hydrolysis of ethyl oleate (Fig. 2).

Table I. Observed Pseudo First-Order Rate Constants, *k*, for Phenytoin Release from Its Various Prodrugs in Ethyl Oleate Emulsions in the Presence of Lipase[a]

Prodrug	Solubility in ethy oleate (mg/ml)[b]	$(k \pm SD) \times 10^3$ (min^{-1})	$t_{1/2}$ (min)
5	40.8	11.7 ± 1.4[c]	5.9
5	40.8	15.1 ± 1.2	4.6
6	19.9	6.9 ± 0.5	10.1
7	29.4	6.2 ± 0.5	11.3
8	105.0	4.3 ± 0.5	16.0
9	54.5	1.3 ± 0.1	55.5
10	123.0		110[d]
11			180[d]
12 (12)[e]			150[d]
12 (22)[e]			155[d]
12 (44)[e]			205[d]

[a] Reactions carried out in the presence of 30 m*M* Ca^{2+}, 6.0 m*M* TDC, and 5600 U lipase (200 μl, 3 mg protein). T = 25°C, pH 8.5.
[b] From Ref. 11.
[c] Calculated from the disappearance of **5**. All other rate constants based on phenytoin appearance.
[d] Values represent the observed time for the appearance of 50% of the maximal expected phenytoin concentration.
[e] The values in parentheses refer to the concentration (m*M*) of prodrug in ethyl oleate. If not specified, the prodrug concentration in ethyl oleate was 22 m*M*.

Hydrolysis of Phenytoin Prodrugs Emulsified with Triglycerides

A few representative phenytoin prodrugs were emulsified in representative short-chain (tributyrin), medium-chain (trioctanoin) and long-chain (soybean oil) triglycerides. In soybean oil, phenytoin release from **5** showed apparent first-order kinetics. However, this same prodrug displayed sigmoidal kinetics when dissolved in either tributyrin or trioctanoin (Fig. 3). A sigmoidal time profile was also observed with trioctanoin emulsions containing **8**. Phenytoin release was not detected during hydrolysis of various triglyceride emulsions containing the stearate prodrug (**12**). It was noted that **12** was concentrated continuously in the tributyrin phase of the tributyrin emulsion as the oil was depleted by the action of lipase. The prodrug precipitated and could be recovered when all the tributyrin had been consumed. During hydrolysis of **5** in the tributyrin emulsion, phenytoin release slowed down and stopped before reaching 100% reaction. This was due to the fact that all the tributyrin had been consumed. However, precipitation of **5** was not observed because a large fraction of **5**, over 80%, had already hydrolyzed (Fig. 3).

Hydrolysis of Phenytoin Prodrugs in Micellar Solutions

Micellar solutions of taurodeoxycholic acid were used

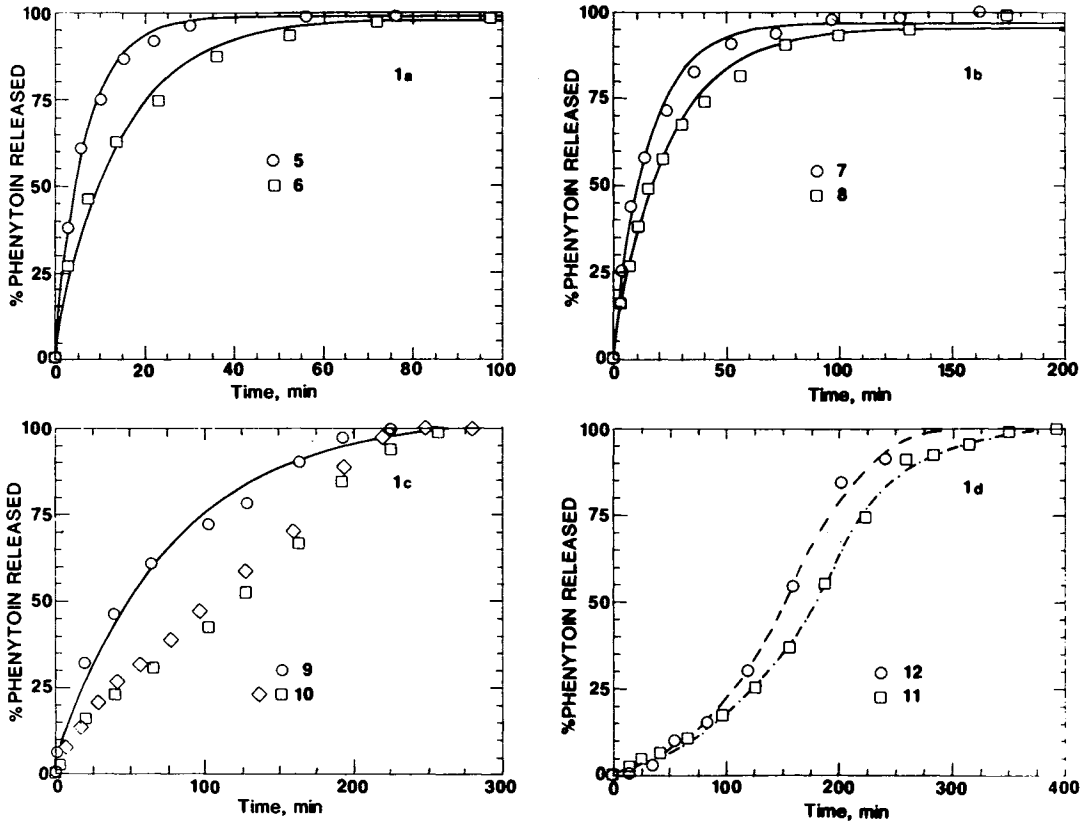


Fig. 1. Time dependencies for the appearance of phenytoin during lipolysis of prodrugs 5–12 dissolved in ethyl oleate (1%; 22 μ*M* prodrug in ethyl oleate) and emulsified in the presence of lecithin. Reactions carried out in Tris buffer (0.12 *M*, pH 8.5) in the presence of 30 m*M* Ca^{2+}, 6.0 m*M* TDC, and 5600 U lipase (200 μl, 3 mg protein) at 25°C. The solid lines on the dependencies of 5 through 9 were calculated from the parameters obtained when the data were analyzed by a first-order kinetics expression. Two data sets for the hydrolysis of **10** are presented (c) to show that the complex kinetic behavior observed with this prodrug is reproducible.

to dissolve the phenytoin prodrugs. Aqueous solubility was improved in the micellar medium (18). Phenytoin release from prodrugs 5 and 8 in the presence of pancreatic lipase followed apparent first-order kinetics (Table III). Hydrolysis rates in micellar solutions were faster than in buffered aqueous solutions in the absence of the bile salt. However, the overall hydrolysis rates were modest compared with phenytoin release rates when the prodrug was presented to lipase as an oily solution dispersed in water (Fig. 4). In the presence of phospholipids (emulsion conditions but without an oil), the time dependencies for phenytoin release from prodrugs 5 and 8 showed an initial "burst" followed by an apparent first-order dependence (Fig. 4).

Hydrolysis of Phenytoin Prodrugs in Suspension

Suspensions of phenytoin prodrugs were incubated with pancreatic lipase at pH 8.50, and the progress of hydrolysis was determined by measuring the amount of liberated phenytoin. Bile salts and phospholipids were absent to prevent complicating factors such as micellar solubilization of the prodrug (15–22). Thirty millimolar calcium ion was added to promote lipase activity (23,24,36). In all cases, phenytoin release followed apparent zero-order kinetics (Fig. 5), and for a given prodrug, the hydrolysis rate was independent of the amount of suspended material. Hydrolysis rates were much slower than with the emulsions and were followed for only 10 to 50% reaction. Some curvature in the time profiles started to develop after 6 to 8 hr. This was suspected to arise from partial enzyme denaturation. Indeed, 10 to 20% loss of enzyme activity had been observed after 24 hr. The dependence of the zero-order rate on the number of carbon atoms in the alkyl portion of the acyl side chain is indicated in Fig. 6 and in Table IV. A striking feature of this dependence is the presence of two maximums, prodrugs 5 and 8, as indicated in Fig. 6. Both 2 and 12 reacted at less than one-tenth of the rate for 8, while 6, at the bottom of the valley between 5 and 8, reacted at only one-third of the maximum rate. The magnitude of the zero-order rate constants was proportional to the enzyme concentration (Table IV). The reproducibility, as indicated by the standard deviations in Table IV, of these reactions was compromised by the difficulty encountered during the collection of aliquots of the suspended material. The compounds, being hydrophobic, were poorly wetted and tended to "cling" to either plastic or glass.

DISCUSSION

The rates of esterase-catalyzed ester hydrolysis generally follow a parabolic relationship when plotted against the acyl-group alkyl-chain length. Short and long acyl side-chain esters are generally cleaved slowly, while medium-length

Table II. Pseudo Zero-Order Rate Constants (Initial Rates) for Pancreatic Lipase-Catalyzed Lipolysis of Ethyl Oleate as a 1% Emulsion (10% Emulsion Diluted 1:10) in the Absence and Presence of Various Phenytoin Prodrugs[a]

Prodrug[b]	Initial rate (μmol/min)	Corr. coef. (lin. reg.)	Percentage reaction[c]
—	1.35 ± 0.20	—	—
5	1.35 ± 0.10	0.968	65
6	0.92 ± 0.01	0.999	77
8	1.19 ± 0.64	0.990	65
10	1.26 ± 0.07	0.986	61
10	0.75 ± 0.01	0.999	90
12 (12)[d]	0.98 ± 0.05	0.989	64
12 (22)[d]	1.05 ± 0.02	0.999	59
12 (44)[d]	0.92 ± 0.04	0.991	63
Mean	1.08 ± 0.20		

[a] Reactions in the presence of 30 m*M* Ca^{2+}, 6.0 m*M* TDC, and 5600 U lipase (200 μl, 3 mg protein).
[b] Ethyl oleate in the presence of the indicated phenytoin prodrug.
[c] The percentage reaction refers to the extent of reaction up to which the disappearance of ethyl oleate was linear. Data in this interval were used to calculate the zero-order rate constant (initial rate). Reaction rates were determined by HPLC and/or pH-stat.
[d] Values in parentheses refer to the concentration of prodrug (m*M*) in ethyl oleate. If not specified, the prodrug concentration in ethyl oleate was 22 m*M*.

acyl side-chain esters are more rapidly hydrolyzed (11,23). The results obtained with **5** through **12**, a series of phenytoin prodrugs in ethyl oleate, are consistent with those observations. To facilitate the comparison of results, all prodrug solutions in ethyl oleate were studied at the same concentration, molar fraction of prodrug, X_{PD} = 0.0072, and molar fraction of ethyl oleate, X_S = 0.9918. The low aqueous solubilities of the prodrugs guarantee that equivalent phenytoin molar concentrations in ethyl oleate were maintained in all the emulsions (22 m*M*; approximately 6 to 12 mg prodrug/ml ethyl oleate). Since the molar fraction of ethyl oleate represented more than 99% of the overall composition of the lipophilic substrates in the emulsion, the prodrug concentration in the oil would be expected to have little effect on the emulsion properties. Therefore, binding of the lipase/colipase complex to the surface of the emulsion and other physical steps can be assumed to be independent of the nature of the included prodrug. Consistent with this hypothesis is the fact that initial rates for ethyl oleate hydrolysis are essentially the same in the presence or absence of prodrug in this vehicle (Table II). A reasonable mechanistic scheme for hydrolysis of the prodrugs dispersed in the oils, assumes that

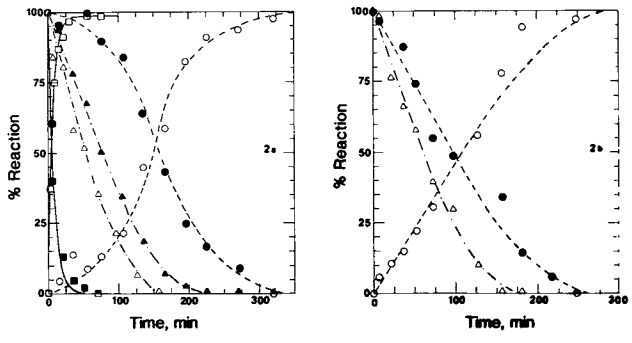


Fig. 2. (a) Time profiles for the disappearance of prodrugs **5** (■) and **12** (●) and ethyl oleate [containing **5** (△) and **12** (▲), respectively] and for the appearance of phenytoin [from **5** (□) and **12** (○), respectively] during lipolysis of prodrugs dissolved in ethyl oleate (1%; 22 μ*M* prodrug in ethyl oleate) and emulsified in the presence of lecithin. (b) Time profiles for the disappearence of prodrug **10** (●) and ethyl oleate (△) and for the appearance of phenytoin (○) during lipolysis of **10**. Prodrug **10** lipolysis takes place at about the same rate as ethyl oleate lipolysis.

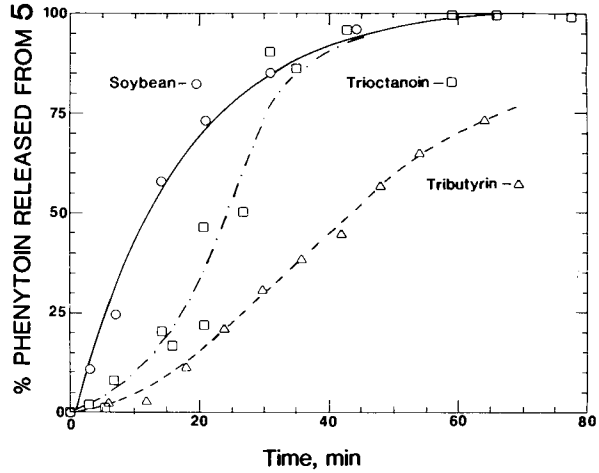


Fig. 3. Time dependencies for phenytoin appearance during lipolysis of **5** dissolved in different triglycerides (soybean oil, ○; trioctanoin, □; and tributyrin, △) and emulsified in the presence of lecithin. Reactions carried out in Tris buffer (0.12 *M*, pH 8.5) in the presence of 30 m*M* Ca^{2+}, 6.0 m*M* TDC at 25°C and 1400 U lipase (50 μl, 0.75 mg protein) for soybean oil and trioctanoin and 840 U lipase (30 μl, 0.45 mg protein) for tributyrin.

Table III. Pseudo First-Order Rate Constants for Phenytoin Release from Prodrugs **5** and **8** When Present in Triglyceride Emulsions and Dispersed in Micellar Solutions of TDC[a]

Prodrug/vehicle	$(k \pm SD) \times 10^2$ (min^{-1})	$t_{1/2}$ (min)
5/soybean	23.2 ± 5.3	3.0
5/trioctanoin	—[b]	7[c]
5/tributyrin	—[b]	13[c]
5/TDC	2.1 ± 0.2	33
5/TDC + lecithin	—[d]	25[c]
8/trioctanoin	—[b]	30[c]
8/TDC	0.5 ± 0.1	139
8/TDC + lecithin[e]	—[d]	55[c]

[a] Reactions carried out in the presence of 22 m*M* prodrug in triglyceride (1%), 30 m*M* Ca^{2+}, 6.0 m*M* TDC. *T* = 25°C, pH 8.5. Rate constants and $t_{1/2}$ were normalized to the same lipase concentration, 5600 U lipase (3 mg protein).
[b] Sigmoidal kinetics were observed.
[c] Values represent the time for the appearance of 50% of the maximal expected phenytoin.
[d] Time dependencies for phenytoin appearance showed a "burst."
[e] A lecithin suspension was added to give a final concentration of 0.12%. This concentration is equivalent to the lecithin present in the emulsion systems.

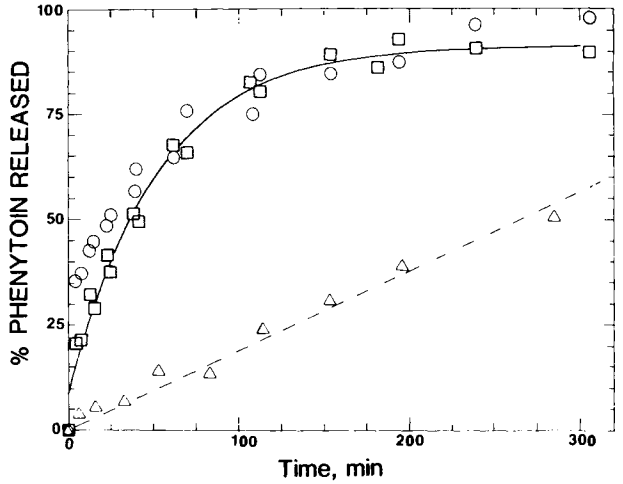


Fig. 4. Appearance of phenytoin during lipolysis of 5 dispersed in micellar solutions with and without lecithin and in a buffered aqueous solution. Reactions carried out in Tris buffer (0.12 *M*, pH 8.5) in the presence of 30 m*M* Ca^{2+}, 6.0 m*M* TDC, and 5600 U lipase (200 μl, 3 mg protein) at 25°C. Reaction in the presence of TDC and 0.12% lecithin, 0.22 m*M* 5 (○). Reaction in the presence of TDC, 0.22 m*M* 5 (□). Lipolysis of 5 dissolved in the Tris buffer solution in the absence of TDC (△). A saturated solution of 5 was used for this experiment. During lipolysis of 5 in suspension, phenytoin production (see Fig. 5) corresponding to the same amount as produced from 5 in solution took only 50 min.

lipase binding to the oil globule is independent of the prodrug and that turnover of the substrate is statistically controlled by the concentrations of oil and prodrug at the interface ($K_S = K_{PD}$; Scheme II). Since events after substrate binding to the active site, including various chemical steps, are probably not rate determining in these systems (23–25), this model would predict that depletion of the vehicle (ethyl oleate or the other triglycerides) and the phenytoin prodrug should parallel each other and should display the same kinetic behavior regardless of the prodrug. The observations in the present study do not support this model ($K_S \neq K_{PD}$; Scheme II). Indeed, the observed dependency between hydrolysis rates and the length of the acyl side chain of the prodrug

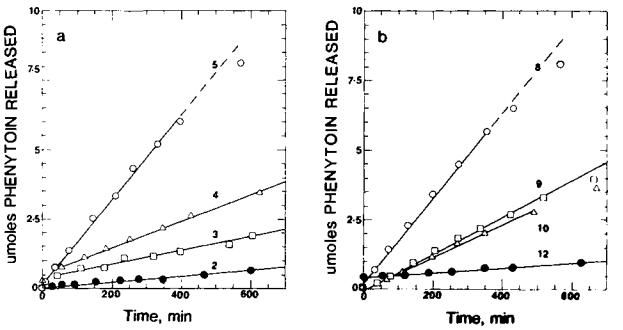


Fig. 5. Observed time dependencies for phenytoin release from various solid prodrugs suspended in Tris buffer (0.14 *M*, pH 8.5). Reactions carried out in the presence of 30 m*M* Ca^{2+} and 5600 U lipase (200 μl, 3 mg protein) at 25°C. Solid lines were calculated from the slopes and intercepts of linear least-squares regressions of the data. When curvature was apparent, data points in that interval were not included for the linear regression (normally after 8 to 10 hr).

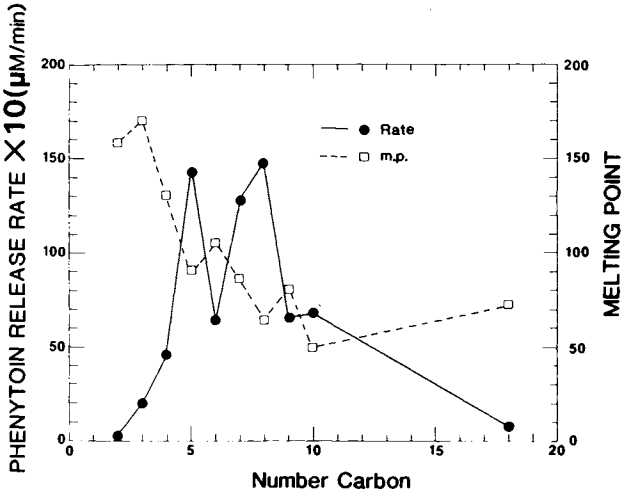
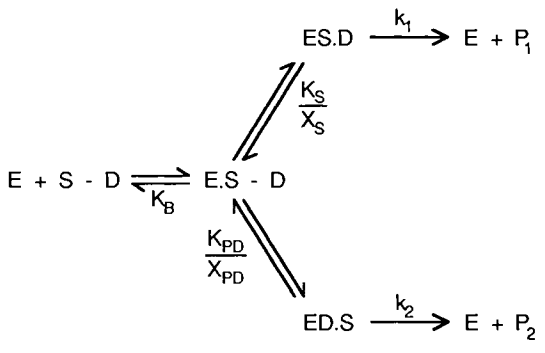


Fig. 6. Dependencies of the pseudo zero-order rates of lipolysis of prodrugs 2–12 from aqueous suspensions of the prodrugs in relation to the melting points of the prodrug and the number of carbons in the acyl side chain of the prodrug. Reaction conditions as indicated in Table IV.

indicates that bound enzyme at the interface can discriminate between substrates depending on their structures and physicochemical properties. As a result, the time profiles changed from apparent first-order dependencies for the short-chain prodrugs to sigmoidal dependencies for the long chain prodrugs. For example, approximately 85% of the ethyl oleate remained unchanged when about 90% of 5 was already hydrolyzed. At the other extreme, 90% of ethyl oleate was consumed when over 50% of 12 still remained intact (Fig. 2). A kinetic model that takes into account these observations is summarized in Scheme II.



Scheme II

Two possible enzyme–substrate complexes in the active site, one with the vehicle (ES.D) and one with the prodrug (ED.S), derive from the same preceding intermediate E.S-D. The intrinsic dissociation constants from these complexes, K_S and K_{PD}, respectively, are normalized by taking into account the molar fraction of each substrate in the oil globule to correct for the statistical probability of encountering a molecule of ethyl oleate or of prodrug. This normalization assumes, however, that the prodrug concentration in the bulk of the oil is proportional to the interfacial concentration. For simplicity, the kinetic analysis assumes a proportionality constant of unity. For the model depicted in

Table IV. Calculated Pseudo Zero-Order Rate Constants [Micromoles of Acid (FA) Released per Minute] for Phenytoin Release from Solid Prodrugs When Subjected to the Action of Pancreatic Lipase, 5600 U (200 μl, 3 mg Protein), in the Presence of 30 m*M* Ca^{2+}, at 25°C, pH 8.5

Prodrug	Rate (±SD) × 10^3 (μmol FA/min)	Corr. coef.	m.p. (°C)	Average rate (±SD) × 10^3 (μmol FA/min)	Percentage error
2	0.89 ± 0.03	0.985	158.1	0.25 ± 0.04[a]	14.3
	0.64 ± 0.04[b]	0.984			
3	2.09 ± 0.12	0.967			
	2.42 ± 0.11	0.986	170.6	2.0 ± 0.2[a]	11.7
	0.27 ± 0.03[b]	0.957			
4	4.29 ± 0.13	0.995			
	4.90 ± 0.05	0.999	131.1	4.6 ± 0.4	9.3
5	7.09 ± 0.10[c]	0.998			
	14.17[d]				
	14.60 ± 0.25	0.997			
	14.43 ± 0.36	0.994			
	13.89 ± 0.09	1.000	91.0	14.3 ± 0.3	2.2
6	5.80 ± 0.08	0.998			
	6.99 ± 0.17	0.996			
	6.49 ± 0.08	0.999	105.6	6.4 ± 0.6	9.3
7	13.85 ± 0.24	0.998			
	14.23 ± 0.45	0.992			
	10.28 ± 0.17	0.998	86.4	12.8 ± 2.2	17.0
8	8.21 ± 0.24[c]	0.994			
	16.42[d]				
	11.69 ± 0.10	0.999			
	15.06 ± 0.42	0.995			
	16.00 ± 0.36	0.998	65.2	14.8 ± 2.1	14.5
9	6.49 ± 0.09	0.998			
	6.67 ± 0.09	0.999	80.9	6.6 ± 0.1	1.9
10	8.83 ± 0.11	0.999			
	5.82 ± 0.10	0.998			
	5.71 ± 0.11	0.998	49.8	6.8 ± 1.8	26.1
12	0.73 ± 0.03	0.850			
	0.87 ± 0.06	0.975	72.6	0.8 ± 0.1	12.5

[a] Corrected rates for the water-catalyzed hydrolysis of the prodrug in the absence of lipase. This correction was necessary for 2 and 3 only.
[b] Water-catalyzed hydrolysis of prodrug in the absence of lipase.
[c] Reactions carried out with one-half of lipase concentration (2800 U, 100 μl, 1.5 mg protein).
[d] Calculated zero-order rate constant from footnote *c* assuming 5600 U.

Scheme II, the steady-state rates for ethyl oleate, V_1, and phenytoin prodrug hydrolysis, V_2, under rapid equilibrium kinetics, are given by Eqs. (1) and (2), where e_o represents the total enzyme concentration.

$$V_1 = \frac{k_1 e_0 [S - D]}{([S - D] + K_B)\left(\frac{K_S}{X_S} + \frac{[S - D]}{[S - D] + K_B} + \frac{K_S X_{PD}}{K_{PD} X_S}\right)} \quad (1)$$

and

$$V_2 = \frac{k_2 e_0 [S - D]}{([S - D] + K_B)\left(\frac{K_{PD}}{X_{PD}} + \frac{[S - D]}{(S - D] + K_B} + \frac{K_{PD} X_S}{K_S X_{PD}}\right)} \quad (2)$$

When the overall oil concentration, as present in the oil globule containing the prodrug, is larger than the dissolution constant, K_B, from the enzyme–substrate complex E.S-D ($[S - D] \gg K_B$), Eqs. (1) and (2) simplify to Eqs. (3) and (4).

$$V_1 = \frac{k_1 e_0}{1 + (K_S/X_S) + (K_S X_{PD}/X_S K_{PD})} \quad (3)$$

and

$$V_2 = \frac{k_2 e_0}{1 + (K_{PD}/X_{PD}) + (K_{PD} X_S/K_S X_{PD})} \quad (4)$$

Since the molar fraction of prodrug is negligible with respect to the molar fraction of ethyl oleate ($X_{PD} \ll X_S$) and since X_S can be written in the form $X_S = S/(S + PD)$, where S and PD are the oil and prodrug concentrations, respectively, Eq. (3) can be further simplified to Eq. (5), which describes the zero-order time dependency for ethyl oleate hydrolysis at the beginning of the reaction, that is, before the oil composition is altered substantially due to lipolysis. Equation (5) also indicates that no lag phase with respect to P_1, the product arising from ethyl oleate hydrolysis, should be observed.

$$V_1 = \frac{k_1 e_0}{K_S + 1} \quad (5)$$

When the top pathway in Scheme II is less important (the affinity for the prodrug is much greater than for the vehicle; $K_S \gg K_{PD}$), Eq. (4) can be simplified to Eq. (6), which

holds as long as the vehicle concentration S remains approximately constant. Equation (6) can be integrated to a familiar first-order dependency ([PD] = [PD]exp($-k_2e_0t/S$). This situation can be seen experimentally during hydrolysis of prodrugs **5–8** and, to a lesser extent, with prodrug **9**. Of course, apparent first-order behavior with respect to phenytoin appearance (P_2) under these conditions is also predicted, in agreement with some of the observations shown in Fig. 1.

$$V_2 = \frac{-d[\mathrm{PD}]}{dt} \approx \frac{k_2e_0[\mathrm{PD}]}{S} \tag{6}$$

On the other hand, when the top pathway becomes more important (the affinity for the vehicle is greater than for the prodrug) and/or decomposition of ED.S (k_2 pathway) is much slower than that of ES.D (k_1 pathway), the prodrug is continuously concentrated in the oil. Its hydrolysis does not become kinetically significant until its mole fraction X_{PD} has increased to a certain value. The time required to deplete enough vehicle, so as to reach the critical mole fraction, should be related to the experimentally observed lag time for phenytoin appearance. In these cases, one can assume that the prodrug acts as a competitive inhibitor sequestering or binding a fraction of the enzyme, thus retarding the hydrolysis of the vehicle and the buildup of the critical mole fraction, X_{PD}.

In general, Eq. (4) can be rewritten as Eq. (7), where the constants A and B are related to k_2, e_0, K_S, and K_{PD}. When the vehicle concentration remains approximately constant, as occurs when $K_S \gg K_{PD}$, Eq. (7) reduces to Eq. (6), *vide supra,* and can be integrated. More often, if the concentration of the vehicle changes as a function of time, Eq. (7) cannot be integrated unless such a time dependence is inserted into Eq. (7).

$$V_2 = \frac{A[\mathrm{PD}]}{BS + [\mathrm{PD}]} \tag{7}$$

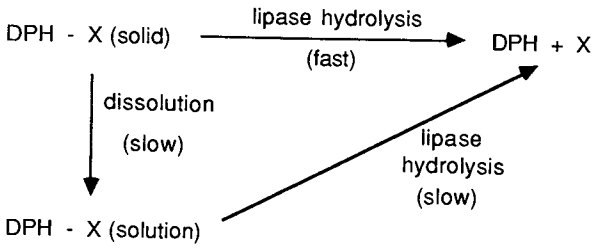
As an approximation, Eq. (7) was used to simulate the time dependencies for phenytoin release when the disappearance of the vehicle was linear with time ($S = S_0 - kt$). These simulations, by numerical integration of Eq. (7), showed that sigmoidal kinetics are possible under these conditions. The data obtained with prodrugs **10–12** were not fitted to this model because the time dependencies for ethyl oleate were not known during the complete course of the reaction.

To check if Scheme II is a reasonable representation for the lipolysis of prodrug and vehicle, it seems possible to alter the partition ratio of E.SD into ES.D and ED.S by selecting oils of different reactivities to lipase. As the oil becomes more reactive, the bottom pathway in Scheme II becomes less significant at the beginning of the reaction. In this way, it should be possible to find a set of lipophilic vehicles where the time profiles for prodrug hydrolysis should change from apparent first-order kinetics to a sigmoidal dependence. This hypothesis was confirmed when three triglycerides of varying reactivity were studied. In the case of soybean oil, the least reactive triglyceride selected, the hydrolysis of **5** still shows apparent first-order kinetics. In trioctanoin and tributyrin, more reactive triglycerides under the experimental conditions employed, the presence of a lag time during **5** lipolysis is observed. Results consistent with the model depicted in Scheme II were also obtained with **8** and **12**. The latter compound was expected to show a substantial lag time. Instead, phenytoin release was not detected at all. The difference in affinity and reactivity between the triglyceride and the prodrug favors the former compound to the extent that almost all of the oil is consumed before any prodrug hydrolysis can take place. The increased concentration of **12** in water promotes its precipitation. Regardless of the system employed, **5** appears to be the most reactive of all prodrugs. It is interesting to note that the rate of hydrolysis of these prodrugs in plasma reaches its maximum value with **5** (11). One is tempted to suggest that in plasma these lipophilic prodrugs might partition into chylomicrons and/or other lipoproteins which can provide an interface for lipoprotein lipase (26) which could act efficiently on them.

From these observations, it can be concluded that it is possible to find the right "match" for a given prodrug so as to retard or accelerate its hydrolysis rate and therefore control, to some extent, its biological release characteristics. Equally important, it shows that lipophilic vehicles are not simple innocuous carriers but can control the fate of the prodrug (27–30). Prodrug hydrolysis in a micellar phase represents a special case of heterogeneous catalysis where the "micelle" provides the support or interface for the enzyme (31–33). Observations from different groups have lead to the conclusion that a lipase–micelle complex behaves as a single catalytic unit (34–36). As expected, the lipase-catalyzed hydrolysis of phenytoin prodrugs in solution was greatly enhanced by taurodeoxycholate micelles. However, these hydrolysis rates were slower than those observed from the emulsions where a distinct interface for lipase was present.

The hydrolysis rate of a molecularly dispersed solution of **5** in the presence of lipase was very low when compared to any of the other experimental conditions explored here. As the concentration increased above the solubility limit, a dramatic increase in the reaction rate was observed (Fig. 4). This behavior is typical for enzymes catalytically active at an interface (23–25) and suggested that the solid material can provide an adequate anchoring surface for the enzyme.

Lipase-catalyzed hydrolysis of solid prodrugs has been reported (37–40). A particularly relevant example is the lipase-catalyzed hydrolysis of two polymorphs of chloramphenicol palmitate at substantially different rates (37–39), where the difference in surface zeta potential can be related to their reactivities (41). Even if a solid prodrug provides a suitable interface for the enzyme, the crystal structure and the packing of the molecules in a unit cell should be responsible for the relative reaction rate. In this regard, if the melting point is taken as a measure of the strength of the packing in the crystal lattice, some general correspondence between melting point and prodrug hydrolysis rate in the solid state should be observed. Such a general trend was confirmed experimentally (Table IV and Fig. 6). Of course, contributions to the rate from other factors such as the intrinsic affinity of the enzyme for the substrate are not considered. Therefore a quantitative correlation was not attempted. Scheme III shows a mechanistic model for this situation relative to our results.



Scheme III

Observe that one can envision this overall process as a lipase-catalyzed dissolution of phenytoin. The top pathway involves the formation of phenytoin as a result of direct attack of lipase on the solid material. Our results suggest that under the conditions employed here, this is the most important pathway for the appearance of phenytoin from aqueous suspensions of the prodrugs in the presence of pancreatic lipase. However, in the presence of bile salts, where dissolution can be enhanced by micellar solubilization (18–22) before lipolytic action, the lower pathway in Scheme III might be relevant. Since mixed micelles and triglycerides may be part of the relevant dissolution medium for lipophilic compounds in the intestinal lumen, especially if the drug is administered with meals, this bottom pathway is suspected to be important under physiological conditions. If so, different oral bioavailabilities should be observed depending on whether a solid prodrug is administered in a fasting or nonfasting animal, a fact that has been confirmed experimentally for some of the phenytoin prodrugs studied here (42).

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