The *in Vitro* Enzymic Labilities of Chemically Distinct Phosphomonoester Prodrugs

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The kinetics of decomposition of phosphomonoesters of hydroxymethyl-5,5-diphenylhydantoin (**1**), estrone (**2**), 17β-testosterone (**3**), 1-phenylvinyl alcohol (**4**), and 17α-testosterone (**5**) were studied in rat whole blood at 25 and/or 37°C. As the acidity of the leaving hydroxyl group of the phosphomonoester increased, there was a tendency for the rate of hydrolysis to increase, except for the anomalous behavior of **4**, which was consistent with its relative rate of hydrolysis in aqueous solutions (1). In addition, the kinetics of hydrolysis of **1–5** and *p*-nitrophenyl phosphate (*p*-NPP) were studied in the presence of isolated alkaline phosphatases from a variety of sources. The initial rate of production of 17α- and 17β-testosterone from their respective phosphate esters (**5** and **3**), in the presence of human placental alkaline phosphatase, revealed that **3** was hydrolyzed 5.3-fold more rapidly than **5**. This difference in reactivity might have been the result of differences in the stereochemical and/or steric nature of the two isomers. For *p*-NPP, **1**, **2**, and **4**, the k_{cat} and k_{cat}/K_m values determined in the presence of the various alkaline phosphatases showed little variation, whereas for **3**, the catalytic constants, k_{cat} and k_{cat}/K_m, were found to be dramatically less than those found for *p*-NPP, **1**, **2**, and **4**. This suggested that the reaction steps, involving the noncovalent binding of the phosphomonoester to the enzyme and/or the nucleophilic displacement of the leaving alcohol of the phosphomonoester by the reactive amino acid residue of the enzyme, might have been less favorable in the case of **3**, where the carbon atom of the ester linkage was secondary and was associated with a rigid ring system.

KEY WORDS: alkaline phosphatase; phosphate esters; hydrolysis; prodrugs.

INTRODUCTION

One solubilization technique, which has been exploited to enhance the aqueous solubility of a drug which contains a hydroxyl group or of a drug where a hydroxyl group can be attached via a spacer group, is the introduction of an ionizable moiety, such as the phosphate group. Improvements in the aqueous solubility via a prodrug approach may dramatically affect the delivery properties and the *in vivo* behavior of the drug.

To be useful as a promoiety, the phosphoryl functionality must improve the aqueous solubility of the drug, and the phosphomonoester prodrug must possess adequate chemical stability in the desired formulation. In addition, an equally important determinant is the biologic lability of the phosphate promoiety of a given prodrug. This new derivative must readily breakdown in the body to form the parent drug. Therefore, a thorough understanding of the biologic lability of phosphate monoesters is essential in optimizing their abilities to function as prodrugs. The biologic labilities of a number of phosphomonoester prodrugs have been studied in humans, including the phosphate esters of dexamethasone (2–5), estramustine (6), methylprednisolone (7,8), clindamycin (9,10), vidarabine (11), triamcinolone (12), and hydroxymethyl-5,5-diphenylhydantoin (13,14).

This manuscript deals with the hydrolytic reactivities of the phosphate monoesters of hydroxymethyl-5,5-diphenylhydantoin (**1**), estrone (**2**), 17β-testosterone (**3**), and 1-phenylvinyl phosphate (**4**) in the presence of rat whole blood and in the presence of isolated alkaline phosphatases from a variety of sources. The latter ester, **4**, is the enol phosphate of acetophenone and is presented here as a model compound. In the above compounds, phosphorylation occurs at a primary, an aromatic, a secondary, and an enolic hydroxyl group. Therefore, these studies should reveal if the structural environment of the derivatized hydroxyl group has any influence on the enzyme-catalyzed pathway, and ultimately, this should prove useful in predicting with which types of hydroxyl-containing compounds the phosphoryl promoiety can be exploited for maximal therapeutic gain.

MATERIALS AND METHODS

Materials

Phenytoin and 17α-epitestosterone were obtained from Sigma Chemical Company (St. Louis, MO), and **1** was synthesized by the procedures described by Varia *et al.* (15). Estrone and 17β-testosterone were obtained from Aldrich Chemical Company (Milwaukee, WI). Compound **4** was synthesized by the procedures described previously (1). Alkaline phosphatases from human placenta (Type XVII), from canine intestine (Type X), and from calf intestine (Type I) were obtained from the Sigma Chemical Co., St. Louis, MO. The disodium salt of *p*-nitrophenyl phosphate (*p*-NPP) was obtained from the Aldrich Chemical Co., Milwaukee, WI. It was used both as a reference compound and to standardize the activity of the stock enzyme solutions. The water was deionized and charcoal filtered prior to distillation from an all glass still. All other chemicals were of reagent or analytical grade and were used without further purification.

Analysis

The HPLC system and the pH-measuring apparatus were the same as described elsewhere (1). The chromatographic conditions and retention characteristics for all of the phosphomonoesters are shown in Table I. The hydrolysis of *p*-NPP was studied by following the production of the product *p*-nitrophenol at 410 nm with a Cary 118 spectrophotometer. This wavelength was chosen because *p*-NPP did not have a detectable absorbance, and *p*-nitrophenol had a molar absorptivity of $1.71 \times 10^4\ M^{-1}\ cm^{-1}$. Elemental analyses of carbon and hydrogen were performed by the Department of Medicinal Chemistry at the University of Kansas. All melt-

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Table I. Chromatographic Conditions and Retention Volumes for the Phosphomonoester Prodrugs and the Respective Parent Compounds

Compound	Mobile phase (v/v)	λ (nm)	Retention volume (ml)[a]
1	45% methanol/55% aqueous[b]	214	5.7
Phenytoin	"	"	11.7
2	40% acetonitrile/60% aqueous[c]	220	3.6
Estrone	"	"	8.8
3 (and 5)	60% methanol/55% aqueous[b]	240	5.7
Testosterone	"	"	12.8
4	95% acetonitrile/5% aqueous[b]	240	5.1
Acetophenone	"	"	27.4

[a] At an eluent flow rate of 1.5 ml/min.
[b] 25 m*M* KH_2PO_4, pH 4.0.
[c] 15 m*M* NaH_2PO_4, 15 m*M* tetrabutylammonium phosphate, pH 6.0.

ing points were determined with a capillary melting-point apparatus and were uncorrected. Thin-layer chromatography (TLC) was performed on silica gel (Kiesel 60 F_{254}), and the compounds of interest were visualized by short-wave UV detection.

Synthesis of 2 and 3

Freshly distilled phosphoryl chloride (2 mmol) was added with stirring to a solution of the given drug (1 mmol) in anhydrous pyridine, which was maintained at 0°C. The reaction was allowed to warm to room temperature and to proceed until TLC showed that no starting material was present (4–10 hr). Ice water was then added to the mixture, which was stirred for an additional hour, followed by evaporation under reduced pressure. The residual liquid was alkalinized with 1 *N* NaOH and extracted with an equal volume of ether.

For 2, the aqueous phase was then acidified with 1 *N* HCl and extracted with ether; the ethereal layer was dried over anhydrous sodium sulfate. This mixture was filtered, then evaporated *in vacuo* to give the free acid (43% yield; mp 208–213°C). For 3 (and its isomer 5), the aqueous phase was acidified with 1 *N* HCl, and the resulting precipitate was filtered and dried to give the free acid (37% yield; mp 135–140°C).

In both cases, the corresponding disodium salts were formed by mixing methanolic NaOH (2.1 molar equivalent) and the respective free acid. This was followed by the addition of ether until precipitation was complete. The disodium salts were collected by filtration, purified by recrystallization, and dried *in vacuo*.

Elemental analysis. For 2, Anal. ($C_{18}H_{21}O_5PNa_2 \cdot 2H_2O$). Calc: C, 50.24; H, 5.86. Found: C, 50.30; H, 5.51. For 3, Anal. ($C_{19}H_{27}O_5PNa_2 \cdot 2H_2O$). Calc: C, 50.89; H, 6.97. Found: C, 50.48; H, 6.58.

Kinetic Procedures

In Rat Whole Blood

The kinetics of hydrolysis of 1–5 were determined in rat whole blood. Seven milliliters of fresh, rat (Sprague–Dawley), whole blood, containing sodium heparin as an anticoagulant, was equilibrated in a 25 or a 37°C water bath. The reaction was initiated by the addition of 80 μl of an aqueous solution of either the disodium salt of 1 (2.26×10^{-2} *M*), 2 (1.82×10^{-2} *M*), 3 (1.86×10^{-2} *M*), 4 (2×10^{-2} *M*), and 5 (1.86×10^{-2} *M*).

The addition of the given phosphomonoester was followed by vortexing for 30 sec. At appropriate time intervals, 0.5 ml of the blood was removed and centrifuged for 2 min, whereupon 100 μl of the resulting plasma was added to 200 μl of acetonitrile. This mixture was vortexed for 1 min, followed by centrifuging for 2 min. The resulting supernatant was directly injected onto the analytical HPLC column.

Apparent first-order rate constants were obtained by following the disappearance of the peak heights of the respective phosphomonoesters with time by HPLC. The reactions were followed for at least two half-lives.

In the Presence of Isolated Enzymes

Cuvettes filled with 3-ml aqueous solutions of *p*-NPP (3×10^{-3}–8.1×10^{-5} *M*), containing 1.0 m*M* $ZnCl_2$, 1.0 m*M* $MgCl_2$, and 0.1 *M* glycine (pH 10.4 with sodium hydroxide), were equilibrated at 25°C in the thermostated cell holder of the spectrophotometer. The reaction was initiated by the addition of 60 μl of a stock enzyme solution (1.02 mg/10 ml of reaction media). Only freshly prepared enzyme solutions were used even though these stock solutions were found to be 95% active following storage for 1 week at 4°C. The initial rates were determined by following the production of the parent compound, *p*-nitrophenol, at 410 nm with time. The experimentally observed molar absorptivity of 1.71×10^4 M^{-1} cm^{-1}, determined in reaction media devoid of phosphatase, was used to convert the observed *p*-nitrophenolate absorbance to concentration units. The molar absorptivity was consistent with the value determined previously (16).

Volumetric flasks filled with 5-ml aqueous solutions of 1 (1×10^{-2}–8×10^{-5} *M*), 2 (5×10^{-3}–1.35×10^{-4} *M*), 3 (1.41×10^{-3}–3.81×10^{-5} *M*), and 4 (3.40×10^{-3}–9.07×10^{-4} *M*) containing 1.0 m*M* $ZnCl_2$, 1.0 m*M* $MgCl_2$, and 0.1 *M* glycine (pH 10.4), were equilibrated at 25°C in a temperature-controlled water bath. For 1, 2 and 4, the reactions were initiated by the addition of 100 μl of a stock enzyme solution (1.02 mg/10 ml of reaction media). For 3, the reaction was initiated either by the addition of 200 μl of a stock enzyme solution (100 mg/ml of reaction medium for calf intestine alkaline phosphatase and 80 mg/ml for canine intestine alkaline phosphatase) or by the addition of 100 μl of a stock enzyme solution (40 mg/ml of reaction medium for human placental alkaline phosphatase). Although the hydrolysis of 3 was carried out at a different enzyme concentration than that of the other phosphomonoesters, no attempt was made to determine if the initial rates increased proportionally with increases in the enzyme concentration.

The initial rates were determined by following the appearance of the peak heights of the corresponding parent compounds with time by HPLC. These peak heights were converted into concentration units by interpolating from the appropriate standard curves, which were generated from samples of the parent compounds dissolved in methanol.

Peak reproducibility was normally better than 3%. No hydrolysis was observed in control experiments which were carried out in parallel in reaction media devoid of phosphatase, except in the case of **4**, where the non-enzyme-catalyzed pathway accounted for <2% of the total dephosphorylation reaction in the presence of human placental alkaline phosphatase. Generally, the kinetics were followed for ≤10% reaction.

RESULTS AND DISCUSSION

Hydrolysis in Rat Whole Blood

The kinetics of decomposition of **1–5** were studied in rat whole blood at 25 or 37°C. As can be seen in Fig. 1, good first-order kinetics were obtained in all cases; **4** was not included in this plot due to its relatively rapid rate of decomposition compared to the other phosphate esters. The hydrolysis of these phosphomonoesters in rat plasma was not attempted since **1** was previously shown to be fairly stable in plasma (15). The slow hydrolysis in plasma was attributed to the fact that phosphatases are associated primarily with the red blood cells of whole blood (17–19).

The apparent-first-order rate constants, along with the corresponding half-lives, for the decomposition of the phosphomonoesters in rat whole blood are given in Table II. The decomposition half-life observed with **1** at 37.0°C was in good agreement with the value at 30 min found previously (15), and the hydrolysis rates were found to vary in the following manner: **4** > **2** > **1** > **3** and **5**. The enol of acetophenone, the leaving group of **4**, was found to have a pK_a value of 10.34 as the oxygen acid (20); estrone, the leaving group of **2**, was found to have a value of 10.26 (21); hydroxymethylphenytoin, the leaving group of **1**, was found to have a value of 12.6 (22); and testosterone, the leaving group of **3** and **5**, was estimated to have a value in the range of 15–17 based on the values found for simple alcohols (23). Hence, as the acidity of the leaving group of the phosphomonoester substrate increased, there was a tendency for the rate of hydrolysis to increase, except for the anomalous behavior of **4**.

By HPLC, the decompositions of **1** and **4** apparently resulted in only the formations of their respective parent compounds, phenytoin and acetophenone. This finding was consistent with the occurrence of a predominant dephosphorylation reaction over the observed time frame. In contrast, the steroid phosphate monoesters (**2**, **3**, and **5**) were subject to other degradation reactions, in addition to the dephosphorylation reaction, as evidenced by the appearance of non-parent-compound peaks on the HPLC chromatograms and by the eventual decrease in the parent compound (testosterone and estrone) peaks with time. However, the dephosphorylation reaction appeared to be the predominant early reaction.

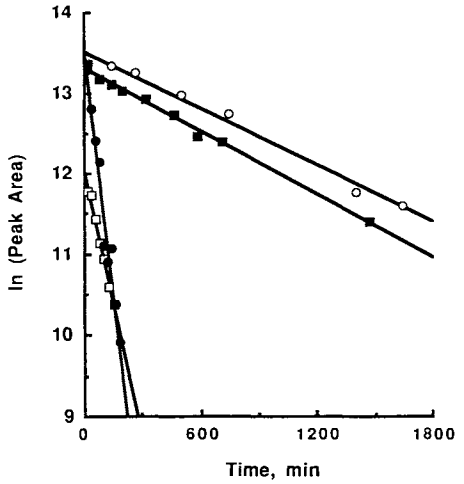

Fig. 1. Decomposition of the phosphomonoesters in rat whole blood: **1** (●), **3** (○), and **5** (■) at 37°C and **2** (□) at 25°C.

Table II. Kinetics of Phosphomonoester Decomposition in Rat Whole Blood and the pK_a Values of the Respective Leaving Alcohol Groups

Compound	pK_a	Temp. (°C)	$k_{obs} \times 10^3$ (min^{-1})	$t_{1/2}$ (min)
1	12.6	25.0	5.78	120[a]
		37.0	20.1 (±1.6)	35
2	10.26	25.0	9.96 (±0.43)	70
		37.0	—	<15
3	15–17	37.0	1.30 (±0.04)[b]	533
4	10.34	25.0	81.3 (±3.1)	9
5	15–17	37.0	1.17 (±0.06)[b]	592

[a] Data taken from Ref. 15.
[b] The difference between these two values was not statistically different at the 95% confidence limit.

An *F* test (24), performed on the apparent first-order, decomposition rate constants of **3** and **5**, revealed that there was no statistically significant difference between these two values using 95% confidence limits. This tended to suggest that the involved enzymatic system(s) did not significantly interact more favorably with one of the isomers. However, the involvement of more than one degradation pathway complicated the drawing of any useful conclusions from the data about the possible stereoisomeric preference of the associated phosphatase(s).

The rate of hydrolysis in rat whole blood (or in human whole blood) should underestimate the *in vivo* rate of hydrolysis. For example, dexamethasone phosphate was cleaved 25 times faster following the intravenous administration to man than it was in whole blood (3), whereas **1**, which showed no observable cleavage in human whole blood (15), was dephosphorylated *in vivo* with a conversion $t_{1/2}$ of about 8 min (13,14). Two related factors would contribute to a more rapid *in vivo* rate of hydrolysis. First, the blood has been found to have a relatively low activity of alkaline and acid phosphatases compared to the activity found in other tissues such as the kidney, brain, liver, lung, and heart (25–27). Second, in addition to the acid phosphatases associated with the red blood cells, *in vivo* the given phosphomonoester would be accessible to phosphatases associated with these other tissues.

An approximation of the extent of the rate enhancement in rat whole blood relative to in aqueous solution can be made. At pH 7.6 and in 0.05 *M* Tris buffer, the apparent half-life for the degradation of **1** at 25°C was about 3.4×10^7 min. In contrast, the half-life determined for the decompo-

sition of 1 in rat whole blood at 25°C was about 120 min (15). A comparison of these two half-lives revealed a 22.8×10^5-fold enhancement in the degradation rate in rat whole blood relative to in aqueous buffered solution. Based on their comparable aqueous solution stabilities, similar rate enhancements would be expected for 2 and 3. However, for 4, the buffer-independent, dephosphorylation half-life at 25°C was about 4.58×10^3 min (1). In contrast, the half-life determined for the decomposition of 4 in rat whole blood at 25°C was 9 min. A comparison of these two half-lives revealed a 5.1×10^2-fold enhancement in the degradation rate of 4 in rat whole blood relative to that in aqueous solution.

When compared to the behavior in aqueous solutions, the decomposition kinetics determined in rat whole blood suggested that the phosphomonoesters studied were substrates for at least one enzyme-catalyzed dephosphorylation pathway (although this was less apparent for 4 than for 1). Also, the observation of excellent first-order kinetics revealed that the apparent K_m values for the involved enzymes were greater than the initial concentrations of the various phosphomonoesters.

Hydrolysis in the Presence of Isolated Enzymes

As discussed above, 3 and 5 degraded at rates in rat whole blood, which statistically were not significantly different. To determine if one of these two stereoisomers was preferentially hydrolyzed by alkaline phosphatase, a similar study was performed in the presence of isolated alkaline phosphatases. The use of an isolated phosphatase should negate the contribution of multiple, enzyme-catalyzed, nondephosphorylation pathways to the degradation rate, and it should also eliminate any potential problems which may arise due to the differing activities, on the two isomers, of the various phosphatases of rat whole blood.

Figure 2 shows the initial rate of production of 17α- and 17β-testosterone, under the exact same reaction conditions, from 4.23×10^{-4} *M* of their respective phosphomonoesters in the presence of human placental alkaline phosphatase at 25°C. From the slopes of these plots, it was found that 3 was hydrolyzed 5.25-fold more rapidly than 5, under initial-rate conditions. This difference in reactivity might be the result of differences in the stereochemical and/or steric nature of the two isomers. Both of these factors may affect the interaction of the phosphomonoester substrate with the enzyme. Sterically, for 17β-testosterone phosphate the presence of the β-methyl group at position 18 may result in an enhancement in the hydrolysis rate due to a steric acceleration effect since both the phosphate group and the adjacent methyl group are on the same side of the molecule. In addition, the stereochemical differences between the 17β- and the 17α-testosterone phosphate isomers may play an important role. This was found to be the case for the alkaline phosphatase catalyzed hydrolysis of phosphomandelic acid, where the L-(+)-isomer was preferentially hydrolyzed relative to the D-(−)-isomer (28).

The kinetic data of the ensuing studies were analyzed with the Lineweaver–Burk equation (29),

$$\frac{e}{v_0} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}} \qquad (1)$$

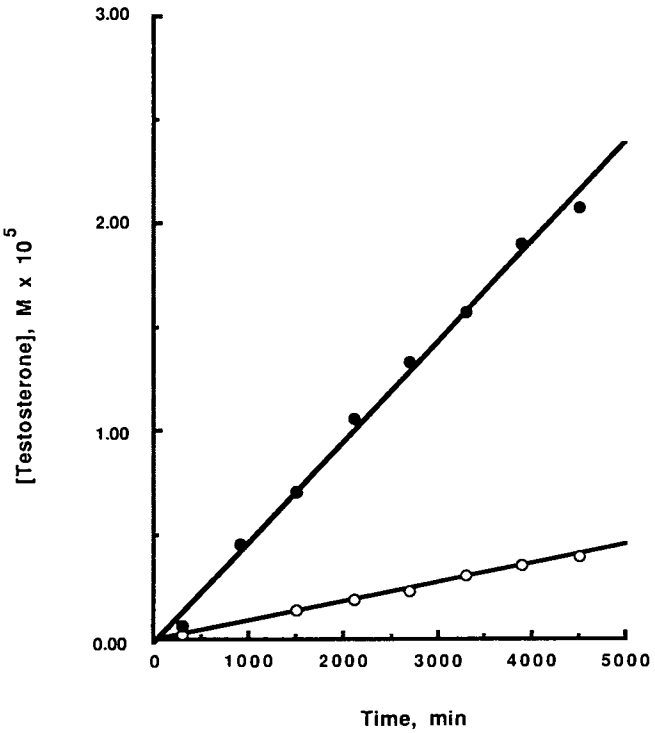

Fig. 2. Rate of production of 17α-testosterone (●) and 17β-testosterone (○) from their respective phosphomonoester prodrugs (4.23×10^{-4} *M*) in the presence of human placental alkaline phosphatase (2×10^{-3} mg/ml) at 25°C.

since the resulting double-reciprocal plots appeared to be reasonably linear. In Eq. (1), e represents the total concentration of the isolated enzyme, v_0 is the initial rate of dephosphorylation, S is the initial phosphomonoester substrate concentration, K_m is the Michaelis constant, and k_{cat} is the maximal dephosphorylation rate constant for saturating substrate concentrations for a given enzyme concentration. Since the e term is expressed as milligrams of the purified enzyme per milliliter of reaction medium and not as the molar concentration of the enzyme, the resulting k_{cat} and k_{cat}/K_m values, unlike the K_m value, represent apparent catalytic constants. These isolated constants should prove useful for comparative purposes for the hydrolytic studies performed in the presence of an enzyme from a given source (where e represents the same quantity).

The range of substrate concentrations used in the isolated enzyme studies was limited by two factors: solubility and assay sensitivity. The upper limits for substrate concentrations were set by the aqueous solubilities of the respective parent compounds, whereas the lower limits were defined by the sensitivity of the associated HPLC assay procedure. The kinetic parameters were determined from Lineweaver–Burk plots [Eq. (1)]. The errors associated with the catalytic constants, k_{cat} and K_m, were larger, whereas the catalytic parameter, k_{cat}/K_m, had a smaller associated error since it was determined from the initial slopes.

Representative Lineweaver–Burk plots are shown in Fig. 3, which depicts the hydrolysis of 1, 2, and *p*-NPP in the presence of human placental alkaline phosphatase at 25°C

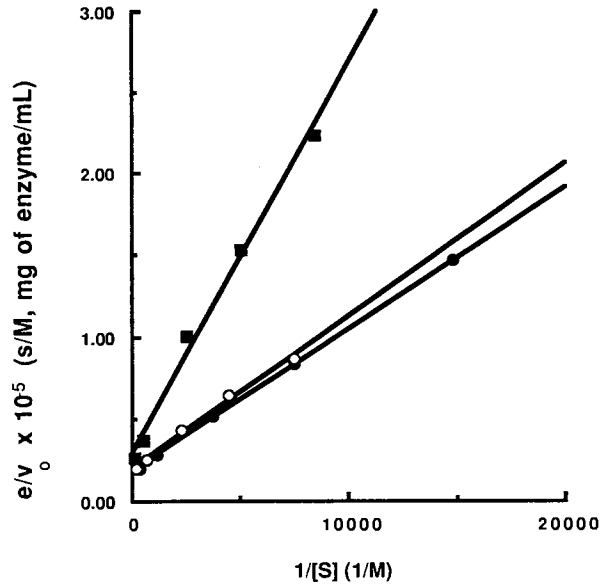

Fig. 3. Lineweaver–Burk plots for the hydrolysis of 1 (■), 2 (○), and *p*-NPP (●) in the presence of human placental alkaline phosphatase (2×10^{-3} mg/ml) in pH 10.4 glycine buffer at 25°C.

and pH 10.4. The catalytic constants, generated from the initial-rate data in the presence of all three alkaline phosphatases and a linear regression analysis of Eq. (1), are listed in Table III, where n represented the number of different initial substrate concentrations used (or the number of initial-rate experiments performed with a given substrate) to generate the catalytic constants.

Equation (1) represents a simplified kinetic expression, where k_{cat} and K_m are complex rate constants. A ratio of these catalytic constants should prove useful for comparative purposes. Under initial-rate conditions, the parent alcohol or phenol, R_1OH, should be formed to a small extent. Therefore, assuming the reaction step in Fig. 4 involving the $k_4[R_1OH]$ term can be omitted, k_{cat}/K_m can be described by

$$\frac{k_{cat}}{K_m} = \frac{k_3 k_1}{k_3 + k_2} \tag{2}$$

where the kinetically important reaction steps are formation of the noncovalent complex between the phosphomonoester substrate and the alkaline phosphatase, $R_1OP \cdot E$, and formation of the phosphoryl-enzyme intermediate, E-P (or, alternatively, dephosphorylation of the phosphomonoester).

For *p*-NPP, **1**, **2**, and **4**, the k_{cat} values determined in the presence of the various alkaline phosphatases showed little variation. Similarly, for *p*-NPP, **1**, and **2**, the k_{cat} values determined in the presence of calf intestine and canine intestine alkaline phosphatases showed little variation. This finding of comparable rate constants was not unexpected since the maximum velocities (and hence, k_{cat} values) for alkaline phosphatase-catalyzed reactions have been found to be essentially independent of the acidity of the leaving alcohol or phenol group, over a wide pK_a range (30). This was consistent with the fact that the rate-limiting step, under alkaline reaction conditions, involved dephosphorylation of the noncovalent phosphoenzyme intermediate (k_7 in Fig. 4). Likewise, the k_{cat}/K_m values, determined in the presence of the alkaline phosphatases from the various sources, showed little variation. Recalling Eq. (2), this finding suggested that for *p*-NPP, **1**, and **2**, the rates of binding of these substrates to the phosphatases and the rates of the ensuing phosphorylation/dephosphorylation steps were similar. The similarity in the binding steps was expected since the active site of the enzyme was known to interact with the phosphoryl moiety, leaving the remainder of the substrate molecule exposed to the enzyme surface, during hydrolysis (31).

In contrast, for **3**, these same kinetic constants, k_{cat} and k_{cat}/K_m, were found to be dramatically less than those found for the other phosphomonoesters studied, consistent with the decomposition kinetics observed in rat whole blood (Ta-

Table III. Lineweaver–Burk Generated Kinetic Constants for Alkaline Phosphatase-Catalyzed Hydrolysis of the Phosphomonoesters at pH 10.4 and 25°C[a]

Compound	pK_a	Source	$K_m \times 10^4$ (M)	$k_{cat} \times 10^7$ (sec^{-1})	$k_{cat}/K_m \times 10^2$ ($M^{-1}\ sec^{-1}$)	n
p-NPP	7.14	a	4.66 ± 0.24	538 ± 20	11.5 ± 0.14	5
		b	12.3 ± 1.0	92.5 ± 6.7	0.752 ± 0.007	5
		c	15.9 ± 1.4	319 ± 30	2.01 ± 2	4
1	12.6	a	8.07 ± 1.89	338 ± 60	4.18 ± 0.22	5
		b	163 ± 46	526 ± 150	0.322 ± 0.0002	3
		c	62.5 ± 7.1	710 ± 80	1.13 ± 0.003	4
2	10.26	a	4.66 ± 0.58	497 ± 10	10.7 ± 0.49	5
		b	20.8 ± 5.5	148 ± 30	0.713 ± .034	4
		c	36.1 ± 4.0	414 ± 40	1.15 ± 0.01	4
3	15–17	a	0.63 ± 0.18	0.093 ± 0.014	0.015 ± 0.002	4
		b	22.1 ± 13.7	0.019 ± 0.011	[8.6 ± 0.5 × 10^{-7}]	3
		c	52.5 ± 43.2	0.13 ± 0.11	[2.5 ± 0.1 × 10^{-6}]	3
4	10.34	a	3.77 ± 0.43	755 ± 20	20.1 ± 1.8	4

[a] The error limits are standard errors. Source of the enzyme: a (from human placenta), b (from canine intestine), and c (from calf intestine). The bracketed values in the k_{cat}/K_m column represent the actual values and are not $\times 10^{-2}$.

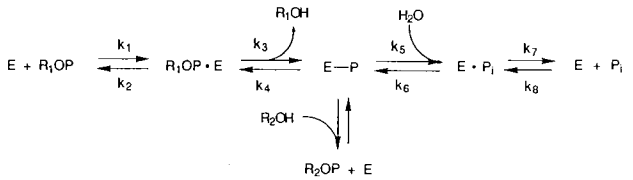

Fig. 4. Kinetic scheme for alkaline phosphatase catalyzed hydrolysis of a phosphomonoester substrate.

ble II). Recalling Eq. (2), at least two possibilities could account for the smaller k_{cat}/K_m values observed with **3**. First, the binding step (k_1 in Fig. 4) may be dramatically slower, and/or second, the nucleophilic-displacement step (k_3 in Fig. 4) may be dramatically slower.

Supporting the latter contention was the fact that for a series of simple alkyl phosphates, the phosphatase-catalyzed reaction was shown to be highly dependent on the steric factors associated with the leaving hydroxyl group (32). The k_{cat}/K_m values for this series of esters was found to decrease as the degree of alkyl substitution at the carbon atom of the ester linkage increased: methyl > ethyl > *n*-butyl > isopropyl > *t*-butyl. This decreased rate was attributed to a less favorable nucleophilic attack, by the reactive amino acid residue of the enzyme on the phosphomonoester substrate, as the steric hindrance increased. Based on this reasoning and for the phosphomonoesters in this study, the rate should be slowest for **3** (and **5**), where the carbon atom of the ester linkage was secondary.

The hydrolytic reactivities of the monoanionic and dianionic species of a wide variety of physicochemically distinct phosphomonoesters were readily predicted from Brønsted relationships having the pK_a of the leaving hydroxyl group as the dependent variable (33). However, the hydrolytic reactivities of these same monoesters, in the presence of alkaline phosphatase, were no longer correlated by a single free energy relationship which allowed for accurate predictions of these reactivities. Hall and Williams (34) found that initial-rate *Escherichia coli* alkaline phosphatase-catalyzed hydrolysis was best described by two Brønsted relationships, which correlated $\log(k_{cat}/K_m)$ and the pK_a of the leaving group. One relationship described the hydrolytic behavior of simple aryl monoesters, and the other relationship described the hydrolytic behavior of simple alkyl monoesters. It was also noted that the reactivities of alkyl phosphates having aryl or large lipophilic side chains fell somewhere in between these two limiting relationships.

For *p*-NPP, **1**, **2**, and **4**, a poor correlation was found to exist between $\log(k_{cat}/K_m)$ and the acidity of the respective leaving groups. However, as the acidity of the leaving group increased, there was a tendency for the enzyme-catalyzed hydrolysis rate to increase, except for the apparent anomalous behavior of **4**. One possible explanation for the unexpected behavior of **4** was that the enzyme-catalyzed hydrolysis may be activated by metal-ion addition to the olefinic bond, which would facilitate the reaction in an analogous manner to protonation of this bond (1). This metal-ion activation may lower the effective pK_a of the leaving group thereby enhancing the rate of hydrolysis. Previously, metal-ion catalysis was observed in the hydrolysis of phosphoenolpyruvate (35).

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