# On the Mechanism of Inhibition of Intestinal Alkaline Phosphatase by L-Phenylalanine

I. KINETIC STUDIES\*

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## SUMMARY

The degree of inhibition of rat intestinal alkaline phosphatase by L-phenylalanine was highly pH-dependent and varied from 0 to 66% within a pH range of 7.8 to 10.4, exhibiting a peak at pH 9.2 and 8.7 for phenylphosphate and  $\beta$ glycerophosphate, respectively.  $V_{\rm max}$  was also a function of pH with and without the inhibitor.

Rat intestinal alkaline phosphatase exhibited maximum enzyme activity at pH 9.8 and 8.8 with substrates, phenylphosphate and  $\beta$ -glycerophosphate, respectively, in presence of the noninhibitor, D-phenylalanine. The corresponding pH optima in the presence of L-phenylalanine inhibitor were 10.2 and 9.3, respectively. This shift in optimum pH by the inhibitor was observed in systems containing carbonatebicarbonate or borate buffers.

The Michaelis constant was pH-dependent. The Dixon plot ( $pK_m$  with respect to pH) showed one discontinuity at pH 8.6 for the free enzyme and another at pH 9.6 for the enzyme-phenylphosphate complex.

The values for the energy of activation for the enzymecatalyzed hydrolysis of phenylphosphate with and without L-phenylalanine were 18,000 and 6,000 calories per mole, respectively.

The inhibition was greatly dependent on substrate and inhibitor concentrations, and was of the "uncompetitive" type, because the double reciprocal plots of velocity and substrate concentrations in the presence of four different concentrations of L-phenylalanine were all straight lines parallel to those obtained without the inhibitor, in both the cases of phenylphosphate and  $\beta$ -glycerophosphate.

At this time, the kinetic data are interpreted as indicating either the formation of a thermodynamically stable enzymeinhibitor-substrate complex which, in effect, reduces the concentration of enzyme-substrate complex available to decompose into products or the production of a weakly dissociable enzyme-inhibitor-substrate complex. These interpretations are relevant to the explanation of the stereospecific, organ-specific inhibition of rat intestinal alkaline phosphatase by L-phenylalanine.

\* This investigation was aided by Grants CA-09734, K6-CA-18453, and CA-07538 of the National Institutes of Health, United States Public Health Service, and by Program Grants P-106, Organ-specific<sup>1</sup> and stereospecific inhibition of intestinal alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC. 3.1.3.1) by L-phenylalanine but not by its D isomer was first reported by Fishman *et al.* (1–6), and this led subsequently to the development of a convenient means of identifying and estimating alkaline phosphatase of intestinal origin in the presence of liver and bone components (6). Recent reports indicate that intestinal alkaline phosphatase is electrophoretically (3, 7), immunochemically (8), and chemically (9) different from its isoenzymes of liver and bone and that the concentration of the intestinal fraction of serum alkaline phosphatase may have some relation to blood group substances (10, 11), both of which may be genetically controlled (12). A plausible biochemical explanation of this phenomenon of organ-specific, stereospecific inhibition was the goal of the present studies.

This communication deals with enzyme kinetics. Among the new findings of considerable interest are the uncompetitive nature of the inhibition by L-phenylalanine, the dependence of this inhibition on pH, and the suggestion of the formation of an enzyme-inhibitor-substrate complex during the course of inhibition. The studies on the essential groups involved in both catalysis and inhibition and the mechanism arising out of these observations will be presented in a later paper.<sup>2</sup>

#### EXPERIMENTAL PROCEDURE

Materials and Methods—The highly purified specimen of alkaline phosphatase from rat intestine was prepared in this laboratory according to the method of Portmann (13) and Portmann, Rossier, and Chardonners (14). The enzyme preparation (0.7 mg of protein per ml) exhibited a specific activity of 54.0 units per mg of protein. The unit of enzyme is defined as that amount which hydrolyzes 1  $\mu$ mole of substrate per ml in 1 min at 37° in

<sup>2</sup> W. H. Fishman and N. K. Ghosh, in preparation.

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<sup>&</sup>lt;sup>1</sup> This term applies only for enzymes prepared from a particular group of organs and in this instance reflects the phenomenon in which L-phenylalanine inhibits preferentially alkaline phosphatase prepared from rat or human intestine but not appreciably the enzyme activity of liver, bone, kidney, lung, and spleen.

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50 mM carbonate-bicarbonate buffer, pH 9.8, and in 18 mM disodium phenylphosphate solution.

Two different buffers containing inorganic ions, carbonatebicarbonate, 50 mM (pH 9.2 to 10.6), and borate, 12.5 mM (pH 7.8 to 10.6), and not the usual organic buffers, were used in this study. Thus, Veronal buffer was excluded because it undergoes decomposition by heat even at the incubation temperature,  $37^{\circ}$ , and 2-amino-2-methyl-1,3-propanediol buffer was omitted because this di-alcohol could conceivably behave as a phosphoryl acceptor during enzyme estimations (5). Because in the present work the inhibition by an amino acid, L-phenylalanine, was to be studied, the introduction of another amino compound in the buffer medium was prohibited; hence, glycine, ethanolamine, tris(hydroxymethyl)aminomethane, and other such buffer reagents were eliminated. Ammonium buffer was avoided because it inhibits alkaline phosphatase (15).

The inhibitor, L-phenylalanine, and its enantiomorph, D-phenylalanine, were purchased from Calbiochem. Crystalline bovine serum albumin was obtained from Nutritional Biochemicals. *N*-Phenyl-*p*-phenylenediamine monohydrochloride used to prepare the *p*-semidine reagent was supplied by Eastman. Disodium phenylphosphate and  $\beta$ -glycerophosphate were obtained from Sigma and Heyden Newport Chemical Corporation, respectively.

The protein concentration was determined by the method of Lowry et al. (16). Phenol liberated from phenylphosphate was estimated by the diazo coupling method of Stolbach, Nisselbaum, and Fishman (17). The absorbance of the orange-pink color was read in either a Spectrosyn colorimeter at 500 m $\mu$  with a 20-mm light path or a Klett-Summerson photometer (filter No. 50). Lambert-Beer's Law was obeyed by the color reaction with the photometers used. Inorganic phosphate was determined by the method of Dryer, Tammes, and Routh (18) by reducing the phosphomolybdate by *p*-semidine reagent (containing a mixture N-phenyl-p-phenylenediamine and NaHSO3 as reducing of agents) and measuring the absorbance in a Klett-Summerson (filter No. 69) colorimeter. The reliability of the method was highly dependent on the concentration of trichloracetic acid so that if trichloracetic acid concentrations in the phosphate solutions were less than 5% or over 25% the color faded.

Enzyme Assay—The stock preparation of the enzyme was diluted either 1:500 or 1:1000 with 0.1% bovine serum albumin, the latter dilution being used when phenylphosphate was used as substrate and the former for  $\beta$ -glycerophosphate. Bovine serum albumin at this concentration stabilized the alkaline phosphatase but did not contribute any phosphatase activity (19, 20). The diluted enzyme solutions were prepared fresh just before the start of the experiments. Unless otherwise indicated, the assay was carried out as described below.

In each experiment, the test digest contained 5.0 mm L-phenylalanine and the control one, 5.0 mm D-phenylalanine. The results obtained with the latter were considered to be without the inhibitor, since the D isomer did not inhibit under the present experimental conditions. This procedure ensured identical conditions in test and control digests of the concentrations of buffer, substrate, enzyme, and pH except for the steric position of the chemical groups attached to the  $\alpha$  carbon in the particular phenylalanine. This experimental design made possible an unambiguous determination of a difference in the results between test and control digests.

Enzymatic hydrolysis of 18 mm phenylphosphate was initiated

5 40 80 120 160 200 0 TIME (MINUTES) FIG. 1. Time-activity curve with and without L-phenylalanine. Buffered 18 mm phenylphosphate substrate solution, pH 9.2, 15 ml, containing 5.0 mm L- or D-phenylalanine, was preheated in the thermostat at 37° for 5 min, and the reaction was initiated by adding 0.75 ml of diluted enzyme solution. Samples (1.55 ml) were withdrawn at zero time and at regular intervals up to 180 min and each sample analyzed for phenol. The amount (micromoles per ml) of phenol liberated during a particular interval was plotted against corresponding time (minutes).

by adding 0.05 ml of diluted enzyme solution to a preincubated (for 5 min) 1.5 ml of the incubation mixture, the pH of which had been adjusted to the requisite value by a pH meter. The digest was incubated for 15 min at  $37^{\circ}$  and the reaction was terminated by adding 1.5 ml of 1.5 M formaldehyde. The liberated phenol was measured according to Stolbach *et al.* (17).

 $-\Delta$ , with inhibitor.

without inhibitor;  $\triangle$ -

In the case of  $\beta$ -glycerophosphate, the digest (4.0 ml) contained 9 mM substrate, 50 mM carbonate-bicarbonate buffer of the desired pH, and 1.0 ml of diluted enzyme. After incubation for 30 min at 37°, 30% trichloracetic acid (1.0 ml) was added to arrest the enzyme reaction and also to precipitate the protein. The mixture was centrifuged for 15 min and the inorganic phosphate content in 2 ml of the supernatant solution was determined (18).

The percentage of inhibition of alkaline phosphatase by Lphenylalanine was calculated with the formula

$$\%$$
 inhibition  $= \frac{D-L}{D} \times 100$ 

in which L was the activity measured in the presence of 5.0 mM L-phenylalanine and D in the presence of the same concentration of D-phenylalanine.

#### RESULTS

Rate of Hydrolysis of Substrate—The time-activity curves with and without the inhibitor (Fig. 1) were linear for the first 40 min with phenylphosphate and for the first 90 min for  $\beta$ -glycerophosphate (1), the reactions observing zero order kinetics. The extent of hydrolysis of the substrates by the enzyme was kept within 15% of its initial molar concentration during these time intervals. The initial velocity rates were measured with the use of the 15-min point for phenylphosphate and the 30-min 24





FIG. 2. Intestinal alkaline phosphatase activity with phenylphosphate (micromoles of phenol per ml released in 15 min) as a function of pH;  $\triangle - - \triangle$ , carbonate buffer in the presence of Lphenylalanine;  $\bigcirc - - \bigcirc$ , carbonate buffer in its absence;  $\square - - \square$ , borate buffer with the inhibitor;  $\bullet - - \bullet$ , borate buffer without the inhibitor. The inhibitor concentration used was 5 mM.



FIG. 3. Dependence of enzyme activity on pH with  $\beta$ -glycerophosphate as substrate;  $\bigcirc - - \bigcirc \bigcirc$ , without inhibitor;  $\bigtriangleup - - \bigtriangleup$ , with 5 mm inhibitor. The activity is expressed in micromoles of phosphate liberated in 30 min under the assay conditions described in the text (carbonate buffer).

point for  $\beta$ -glycerophosphate in order to compute the data presented in Figs. 2 to 9. The agreement in the values of the initial velocities of the reaction with one preparation and same concentrations of the enzyme, substrate, buffer, and pH on 12 different occasions was within experimental error, and statistically the coefficient of variation did not exceed 6.5%.

Influence of pH on Enzyme Activity and Inhibition-Enzyme

activities in the presence and absence of L-phenylalanine in carbonate-bicarbonate and borate buffers were plotted against the corresponding pH in Fig. 2, phenylphosphate serving as substrate. The optimum pH without the inhibitor is 9.8, whereas in the presence of inhibitor it is 10.2. As in the present study, pH 9.8 was the optimum for alkaline phosphatase from calf intestinal mucosa (21), human sera (22, 23), and rat intestine (13).

The optimum pH for  $\beta$ -glycerophosphate without and with the inhibitor are 8.8 and 9.3, respectively (Fig. 3). As in this study, different pH optima for phenylphosphate and  $\beta$ -glycerophosphate were also observed by other workers (14, 24).

The shift in the optimum pH towards the alkaline range observed in the presence of L-phenylalanine for both substrates is reminiscent of a similar shift recorded for another amino acid inhibitor, L-histidine (25). Also potassium thiocyanate, a noncompetitive inhibitor for fumarase, shifts the optimum pH toward the alkaline range (26).

The percentage of inhibition by 5.0 mm L-phenylalanine was plotted against pH in Fig. 4 and shows a peak at pH 9.2 with phenylphosphate and at 8.7 with  $\beta$ -glycerophosphate

Inhibition as Function of Concentration of L-Phenylalanine— Lineweaver-Burk (27) plots of 1/v with respect to 1/S with fixed amounts of the inhibitor for phenylphosphate and  $\beta$ -glycerophosphate are shown in Figs. 5 and 6, respectively. These reciprocal plots are all straight lines parallel to those obtained in the absence of inhibitor. These results conform to those expected in the case of the infrequently encountered category of uncompetitive inhibition, an example of which is the inhibition of arylsulfatase by cyanide and hydrazine (28). Another term for this phenomenon is "coupling inhibition" (29).

The inhibition was found to be a function of substrate and inhibitor concentrations. Thus, under the present experimental conditions, the percentage of inhibition changes from 30 at 0.05 mM phenylphosphate to 70 at 1.0 mM at pH 9.2 with 5.0 mM L-phenylalanine. With 10 mM inhibitor and 1 mM phenylphosphate, the inhibition attained 82% at the same pH.

Values for  $K_m - K_m$  values were obtained from the experimental data (not shown) and from Figs. 5 and 6 and are listed in Table I, which shows that the values of the Michaelis constants and of  $V_{\max}$  are decreased by the inhibitor. From similar Lineweaver-Burk plots, Morton (21) reported a  $K_m$  value of 0.96 mm for calf intestinal alkaline phosphatase acting on phenylphosphate at pH



FIG. 4. Effect of pH on L-phenylalanine (5 mM) inhibition with phenylphosphate and  $\beta$ -glycerophosphate as substrates:  $\bigcirc - \bigcirc$ , phenylphosphate;  $\triangle - - \triangle$ ,  $\beta$ -glycerophosphate.

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9.8 and Ross, Ely, and Archer (24) reported a  $K_m$  value of 3.0 mM for rat intestinal alkaline phosphatase cleaving  $\beta$ -glycerophosphate at pH 8.8. Moss and King (30) determined  $K_m$  of a variety of human tissues at their respective optimum pH in the presence of Mg<sup>++</sup>. The values for bone, liver, kidney, and intestine were 0.124, 0.07, 0.1, and 0.098 mM, respectively. The  $K_m$  value reported (22) for human serum alkaline phosphatase with phenylphosphate at its optimum pH, 9.8, was 0.645 mM and that for calf duodenal alkaline phosphatase (14) was 5.8 mM for  $\beta$ -glycerophosphate and 1.1 mM for phenylphosphate. These previous data agree fairly well with the data in Table I. Our  $K_m$  value with  $\beta$ -glycerophosphate was 2.3 mM.

Influence of pH on  $K_m$ —In a separate series of experiments, the initial velocity of hydrolysis in the absence of any phenylalanine was measured as a function of pH in order to determine the pK values of the dissociable groups in the enzyme and the enzymesubstrate complex. The pH range of the incubation mixture was 7.8 to 10.6 and phenylphosphate was used as substrate. The Michaelis constants were computed by the Lineweaver-Burk method and expressed as  $pK_m$  ( $-\log_{10} K_m$ ) in a plot against pH (Fig. 7). The plot of  $pK_m$  against pH, when analyzed according to Dixon and Webb (31), shows that the free enzyme has a pK of 8.6 while the enzyme-substrate complex has a pK of 9.6. The curves obtained in the present study are similar to those reported by Anagnostopoulos and Matsudaira (15) in their work on human placental alkaline phosphatase. The pK of phenyl-



FIG. 5. Lineweaver-Burk plots  $(1/v \ versus \ 1/S)$  at different concentrations of the inhibitor, L-phenylalanine, with phenylphosphate as substrate. The different concentrations of substrate were incubated with the same concentration of the enzyme at  $37^{\circ}$  for 15 min at pH 9.2 and the amount of liberated phenol in micromoles per ml per min was computed and used as the velocity of hydrolysis (v). The concentrations of the inhibitor, L-phenylalanine, viz. 5.0, 7.5, 10.0, and 15.0 mM, are shown over the linear plots obtained.



FIG. 6. Double reciprocal plots of the velocity against  $\beta$ -glycerophosphate concentration at different inhibitor concentrations (2.5, 3.8, and 5.0 mM) indicated above each curve. The amount of phosphate in micromoles per ml released per min at pH 8.7 was measured and this value was accepted as the velocity.

#### TABLE I

## Apparent Michaelis constant with and without inhibitor

 $K_m$  values were determined in presence of 5.0 mm L- or D-phenylalanine with phenylphosphate as substrate. Each value was checked by duplicate experiments.

	Michaelis constants				
рн	Without inhibitor (d)	With inhibitor (L)			
		M			
8.9	0.00018	0.0000526			
9.1	0.00017	0.000077			
9.3	0.000625	0.0003			
9.5	0.00125	0.00154			
9.7	0.00286	0.00154			
9.9	0.0025	0.00154			
10.3	0.00286	0.00154			

phosphate, 5.7 (32), is far distant from the values obtained here and becomes thereby eliminated as a source of discontinuity in the  $pK_m$ -pH curve at pH values 8.6 and 9.6, which are presumably caused by the ionizable groups (31, 33) in the enzyme protein.

The value of  $K_m$  with p-phenylalanine as judged by the data in Table I is different from that without it (Fig. 7). A plot (not shown) of  $pK_m$  against pH with p-phenylalanine indicates pK values of the groups (9.1 for the free enzyme and 9.75 for enzymesubstrate complex) slightly shifted towards the alkaline side which suggests the attachment of this p enantiomorph to some groups in the enzyme involved in the catalysis.

The slope of the line in Fig. 7 is 1.7. Motzok (34) recorded slopes of 0.8 and 1.3 with chicken-plasma alkaline phosphatase. Nonintegral slopes were also observed by Lazdunski and Ouellet (35) and Fernley and Walker (36) in similar studies on alkaline phosphatase. Thus, the statement that the  $pK_m$ -pH curve will always have an integral slope of 1.0 with alkaline phosphatase



FIG. 7. Plot of  $pK_m(-\log_{10} K_m)$  against pH without phenylalanine.  $K_m$  was determined by the Lineweaver-Burk method by plotting the reciprocal velocity against corresponding reciprocal phenylphosphate concentrations as in Figs. 5 and 6 but in the absence of phenylalanine. Either carbonate-bicarbonate or borate buffer of the same molar concentration (50.0 mM) was used.



FIG. 8. Plots of  $V_{\max}$  against pH with  $(\Box - \Box)$  and without (D)  $(\bigcirc - \bigcirc)$  5 mM inhibitor, L-phenylalanine.  $V_{\max}$  is expressed as micromoles per ml per min of phenol liberated from phenyl-phosphate.

(37) has not applied in the subsequent studies cited. Nevertheless, if the 1.7 slope obtained under the present experimental conditions is interpreted as 2.0, the inference, according to Dixon (37) would be that rat intestinal alkaline phosphatase gains two negative charges during enzyme-substrate interaction.

Influence of pH on  $V_{max}$ —Maximal initial velocities were determined by extrapolating Lineweaver-Burk curves (not shown) to infinite substrate concentration. These values were correlated with pH in the presence of D- and L-phenylalanine, with phenylphosphate as substrate (Table II). Two resultant symmetrical bell-shaped curves (Fig. 8) resemble those of fumarase (26, 38) which were interpreted mathematically to yield two dissociation constants.

Studies<sup>2</sup> with group-specific reagents have suggested the presence of amino, sulfhydryl, and metal sites.

Effect of Temperature on  $V_{max}$  and Determination of Activation Energy—The maximal velocity,  $V_{max}$ , for the hydrolysis of phenylphosphate by rat intestinal alkaline phosphatase was determined with the use of carbonate-bicarbonate buffer, pH 9.2, and a substrate concentration range of 0.1 to 3.0 mM at 22, 27, 32, and 37° (Table III). The logarithm of  $V_{max}$  was plotted against the reciprocals of the absolute temperature and the energy of activation was calculated according to the following relation deduced from the equation of Arrhenius (39).

$$\log_{10} V_{\rm max} = A - \frac{E}{2.303 \ RT}$$

in which A is a constant factor concerning probability of reaction



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FIG. 9. Plots of  $\log_{10} V_{\text{max}}$  against 1/T for calculating the activation energy with and without 5 mm L-phenylalanine.  $V_{\text{max}}$  is expressed as micromoles of phenol per ml liberated from phenyl-phosphate per min.  $\bullet$ , experimental points with 5 mm D-phenylalanine;  $\blacktriangle$ , those with 5 mm L-phenylalanine.

TABLE II V<sub>max</sub> as function of pH

	$V_{\max}^a \times 94$		
рН	Without inhibitor (D)	With inhibitor <sup>b</sup> (L)	
8,00	0.043	0.028	
8.35	0.068	0.032	
8.80	0.087	0.042	
9.30	0.240	0.110	
9.50	0.338	0.130	
9.70	0.337	0.147	
10.25	0.146	0.080	

<sup>a</sup> Micromoles of phenol per ml liberated from phenylphosphate per min.

<sup>b</sup> L-Phenylalanine (5.0 mM).

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TABLE III Maximal velocities at different temperatures and activation energies

	$V_{\rm max}  imes 10^3  imes 94$		$Log_{10} [V_{max} \times 10^3 \times 94)]$		Activation energy	
T <sup>a</sup>	Without inhibitor (D)	With in- hibitor (L)	Without inhibitor	With inhibitor	Without inhibitor (D)	With inhibitor (L)
	µmoles/ml/min		-			
310	182	76	2.2601	1.8808		
305	171	55	2.2330	1.7404		
300	144	38	2.1584	1.5798		
295	113	22	2.0531	1.3424	6,000	18,000

<sup>a</sup> Temperature in the absolute scale.

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 $\dot{b}c$ 

and molecular collision, R, the universal gas constant, and T, the absolute temperature. The values for  $V_{\max}$  necessary for the evaluation of the energy of activation (Fig. 9) (Table III) were obtained by the extrapolation of Lineweaver-Burk plots (not shown) which were previously computed from initial velocity data. The detailed data and plots, however, are not included, for brevity.

The energy of activation (E) can be calculated from the slope of the linear plots of  $\log_{10} V_{\text{max}}$  against 1/T given in Fig. 9 where the enzyme-catalyzed hydrolytic cleavage of phenylphosphate was determined with and without 5.0 mm L-phenylalanine (without = 5.0 mm p-phenylalanine). The value for the activation energy in the presence of L-phenylalanine is three times higher than that in its absence. Hence, L-phenylalanine appears to increase the requirement in the activation energy (40) necessary for the reaction and thus makes less facile the cleavage of the substrate.

### DISCUSSION

The present investigation offers a number of points of interest. Intestinal alkaline phosphatase activity in the presence of L-phenylalanine shifts its optimum pH towards the alkaline range and the inhibition is pH-dependent. The extent of inhibition of the enzyme by L-phenylalanine is likewise greatly dependent on substrate concentration. It is also observed that L-phenylalanine lowers the Michaelis constant with concomitant lowering of  $V_{\text{max}}$ . The energy of activation (E) in the presence of the inhibitor is nearly three times greater than the corresponding value in its absence. The Dixon plot  $(pK_m \text{ against } pH)$  indicates two points of discontinuity, one at pH 8.6 and the other at pH 9.6 which may be attributed to dissociable groups in the enzyme protein. The maximal initial velocity  $(V_{max})$  is also pH-dependent. Finally, the double reciprocal plots of velocity against substrate with and without the inhibitor provide the conclusion that the inhibition of rat intestinal alkaline phosphatase by L-phenylalanine is of the "uncompetitive" type.

A reasonable simplified physical interpretation (28, 31) of the kinetic data is that the inhibitor (I) interacts primarily with the enzyme-substrate complex (ES) to form an inactive thermodynamically stable enzyme-inhibitor-substrate (EIS) complex in the scheme

$$E + S \rightleftharpoons ES + \text{products}$$
  
 $+$   
 $I$   
 $\downarrow$   
 $EIS$ 

The inhibition would be a consequence then of a reduction in the amount of active ES complex.

However, data of the type presented are subject to more than one interpretation according to Frieden (41) who has offered a general kinetic treatment. Thus, his Mechanism I lists four steps, each with the corresponding dissociation constant, K, and decompositions of ES and EMS to products with their rate constants  $k_5$  and  $k_6$ 

$$E + S \rightleftharpoons ES \left( ES \xrightarrow{k_5} E + P \right) \tag{1}$$

$$E + M \rightleftharpoons EM$$
 (2)

$$ES + M \rightleftharpoons EMS \left( EMS \xrightarrow{k_6} EM + P \right)$$
(3)

$$EM + S \rightleftharpoons EMS$$
 (4)

in which S is the substrate, E, the enzyme, and M, the modifier which is defined as binding to a site other than the active site of the enzyme.

Parallel curves of 1/v against 1/S are also characteristic of Frieden's "limiting Case 3," in which  $k_6 = 0$  and  $K_2 = \infty$ ; and of his "nonlimiting Case 1" which can occur in any set of circumstances in which  $K_2/K_3 = k_5/k_6$ . In the latter situation when  $k_6 < k_5$ , the enzyme binds the modifier more firmly in the presence of substrate than in its absence (inhibition). A choice between these various alternatives will have to await additional experimentation.

An activating effect of p-phenylalanine on intestinal alkaline phosphatase is seen under some circumstances which fits Frieden's "nonlimiting Case 3" and this enters into the explanation of the observation that the pH dependence of  $K_m$  is quite different in the absence or presence of p-phenylalanine. (Compare Table I to Fig. 7.) However, these two sets of data were not designed to evaluate the effects of D-phenylalanine per se, which are now receiving separate attention.

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