Inhibition of Pyrophosphatase Activity of Mouse Duodenal Alkaline Phosphatase by Magnesium Ions

By P. R. V. NAYUDU AND P. L. MILES

Department of Zoology, Monash University, Clayton, Vic. 3168, Australia

(Received 20 May 1969)

Duodenal alkaline phosphatase of juvenile (11-day-old) mice, like other non-specific alkaline phosphatases, has the ability to hydrolyse PP_i . When a constant $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio is maintained, plots of velocity as a function of PP_i concentration are consistent with Michaelis-Menten kinetics. Mg^{2+} activates pyrophosphate hydrolysis and maximal activity is obtained at a constant $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio of 0.66. At higher ratios there is strong inhibition. At constant concentrations of Mg^{2+} and increasing concentrations of PP_i , the velocity-substrate (PP_i) concentration plots show sigmoidal dependence. By assuming that the true substrate is $\text{MgP}_2\text{O}_7^{2-}$ complex, and using complexity constants, the concentrations of free Mg^{2+} , $\text{Mg}_2\text{P}_2\text{O}_7$ and $\text{MgP}_2\text{O}_7^{2-}$ were calculated in assay mixtures ranging in PP_i concentration from 0.1 to 2.5 mM and in total Mg^{2+} concentration from 0.6 to 2.6 mM. From these data, the concentrations of added Mg^{2+} and PP_i in the assay mixtures were selected so that the velocity could be measured (1) at three fixed concentrations of free Mg^{2+} ions with varied concentrations of $\text{MgP}_2\text{O}_7^{2-}$ and (2) at four fixed concentrations of $\text{Mg}_2\text{P}_2\text{O}_7$ with varied concentrations of $\text{MgP}_2\text{O}_7^{2-}$. Lineweaver-Burk and Hill plots from these data showed that the inhibition is caused by free Mg^{2+} ions, of a mixed type and consistent with Michaelis-Menten kinetics. The sigmoidal dependence observed between velocity and PP_i concentration at constant concentration of total Mg^{2+} is therefore not due to allosteric inhibition. It is due to a combined effect of (1) inhibition by free Mg^{2+} ions, (2) depletion of the true substrate, $\text{MgP}_2\text{O}_7^{2-}$, owing to the formation of $\text{Mg}_2\text{P}_2\text{O}_7$ and (3) the manner in which the concentrations of these three molecular or ionic species change when PP_i concentration is increased maintaining the total Mg^{2+} concentration constant.

Purified preparations of non-specific alkaline phosphatase (EC 3.1.3.1), from a variety of sources, have been shown to hydrolyse PP_i (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1966; Fernley & Walker, 1967; Sussman & Laga, 1968; Butterworth, 1968). Similarly duodenal alkaline phosphatase from 11-day-old mice has been found to manifest pyrophosphatase activity (P. L. Miles, unpublished work). It has also been observed that concentrations of Mg^{2+} ($\text{Mg}^{2+}/\text{PP}_i$ concentration ratio more than 1:1) that yield optimum phosphomonoesterase activity strongly inhibit hydrolysis of PP_i by mouse duodenal alkaline phosphatase. However, low concentrations of Mg^{2+} ($\text{Mg}^{2+}/\text{PP}_i$ concentration ratio less than 2:3) cause activation of the hydrolysis of PP_i . When assays were carried out at constant concentrations of total Mg^{2+} and increasing concentrations of PP_i , the inhibition at the lower PP_i concentrations was associated with a sigmoidal dependence of velocity on PP_i concentra-

tion, commonly a feature of allosteric interaction (Monod, Changeux & Jacob, 1963).

It has been suggested that for Mg^{2+} -activated pyrophosphatases the true substrate is $\text{MgP}_2\text{O}_7^{2-}$ (Bloch-Frankenthal, 1954; Robbins, Stulberg & Boyer, 1955). Physicochemical studies have shown that PP_i readily forms complexes with Mg^{2+} (Lambert & Watters, 1957). Denberg & DeLuca (1968) were the first to suggest that, with enzymes that have substrates that are chelating agents and also require metal ions, anomalous results resembling allosteric behaviour of the enzyme could be obtained, as a result of formation of complex ions between substrate and metal ion. Butterworth (1968) demonstrated that the pyrophosphatase activity of pig kidney alkaline phosphatase manifests such a pseudo-allosteric effect in the presence of Mg^{2+} at a concentration in excess of that of PP_i . The apparent sigmoidal relation between velocity and total PP_i concentration at constant concentrations of total

Mg^{2+} observed with the pyrophosphatase activity of mouse duodenal alkaline phosphatase has been investigated in terms of absolute concentrations of free Mg^{2+} , $\text{MgP}_2\text{O}_7^{2-}$ and $\text{Mg}_2\text{P}_2\text{O}_7$ calculated by the use of the complexity constants (Lambert & Watters, 1957). The results show that the observed sigmoidal dependence is not related to allosteric behaviour based on co-operative interactions. At high concentrations of Mg^{2+} relative to PP_i , under which conditions inhibition is observed, kinetic data indicate that, of the two that are present, free Mg^{2+} ion and $\text{Mg}_2\text{P}_2\text{O}_7$, it is the former that causes the inhibition of the hydrolysis of the true substrate, $\text{MgP}_2\text{O}_7^{2-}$, in a manner consistent with Michaelis-Menten kinetics.

MATERIALS AND METHODS

Enzyme preparation. Duodenal alkaline phosphatase extraction from 11-day-old SWR/J mice and its purification by chromatography on DEAE-cellulose were described by Nayudu & Moog (1967). DEAE-cellulose column eluent was collected in 5ml. fractions, and those manifesting alkaline phosphatase activity were pooled and condensed by pressure dialysis. The condensed sample was chromatographed on a column (50cm. $\times$ 2cm.) of Sephadex G-150 and eluted by tris-HCl buffer, pH 8 at 4°. Every fraction eluted from DEAE-cellulose and Sephadex columns that showed alkaline phosphatase activity also manifested pyrophosphatase activity, suggesting that both enzymic functions were associated with the same enzyme. Protein determinations were made by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. After Sephadex chromatography, the enzyme activity was 100–120-fold higher per unit amount of protein as compared with the crude homogenate.

Inorganic pyrophosphatase assay. The assay was carried out by the method originally described by Berenblum & Chain (1938) and modified by Mazia, Chaffee & Iverson (1961). Freshly prepared buffer-substrate solution (2.5ml.) in tris-HCl buffer (0.1M-tris after adjustment to pH 9.2 with 1M-HCl) containing specified concentrations of $\text{Na}_4\text{P}_2\text{O}_7$ and MgCl_2 (A. R. grade; E. Merck A.-G., Darmstadt, Germany) was incubated in a water bath at 37° for 6min. There was no precipitation in the Mg^{2+} - PP_i solutions at the concentrations used in this study. The reaction was stopped 10min. after the addition of 0.5ml. of enzyme solution by the addition of 1ml. of 10% (w/v) trichloroacetic acid. Controls consisted of tubes in which trichloroacetic acid was added before the enzyme. A 1ml. portion of the substrate-enzyme-trichloroacetic acid solution was transferred to another test tube containing 0.25ml. of ammonium molybdate solution, mixed and followed by the addition of 2.0ml. of 2-methylpropan-1-ol. The test tubes were then shaken on a Vortex mixer. Then 1.5ml. of the supernatant 2-methylpropan-1-ol was carefully transferred to another tube containing 0.8ml. of acidified SnCl_2 and the two were mixed. A 1ml. portion of the blue supernatant layer of 2-methylpropan-1-ol was carefully transferred to another tube containing 4.5ml. of ethanol. The solution was mixed and read in a Klett-Summerson photoelectric colorimeter with a red filter

(660nm.). A standard calibration curve was obtained with known concentrations of KH_2PO_4 , treated by the above method. Enzymic activity is expressed as $\mu\text{moles of P}_i$ liberated/min./ $\mu\text{g. of protein}$.

Concentrations of molecular and ionic species derived from Mg^{2+} and PP_i in assay mixtures. The stability constants given by Lambert & Watters (1957) are as follows:

$$K_{\text{MgP}_2\text{O}_7^{2-}} = \frac{[\text{MgP}_2\text{O}_7^{2-}]}{[\text{Mg}^{2+}][\text{P}_2\text{O}_7^{4-}]} = 10^{5.4}$$

$$K_{\text{Mg}_2\text{P}_2\text{O}_7} = \frac{[\text{Mg}_2\text{P}_2\text{O}_7]}{[\text{Mg}^{2+}][\text{MgP}_2\text{O}_7^{2-}]} = 10^{2.4}$$

These values were used to calculate the absolute concentrations of free Mg^{2+} , $\text{MgP}_2\text{O}_7^{2-}$ and $\text{Mg}_2\text{P}_2\text{O}_7$ in solutions of Mg^{2+} and PP_i .

RESULTS

Fixed $\text{Mg}^{2+}/\text{PP}_i$ concentration ratios. Plots of v (initial velocity) against s (PP_i concentration) with and without added Mg^{2+} and Lineweaver-Burk plots of the same data are presented in Fig. 1. When Mg^{2+} was added, the $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio was kept constant at 1:2, 2:3 and 1:1. It is observed that added Mg^{2+} caused increased activity, and maximal activity was obtained with a $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio of 2:3. A further increase of Mg^{2+} to give a $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio of 1:1 caused a substantial decrease in activity. The shape of the plots of v against s appears to be a rectangular hyperbola in each case. The apparent K_m values were determined from the double-reciprocal plots (Fig. 1). The K_m (PP_i) without added Mg^{2+} was $0.14 \times 10^{-3}\text{M}$ and increased to $0.62 \times 10^{-3}\text{M}$ at the $\text{Mg}^{2+}/\text{PP}_i$ concentration ratios 2:3 and 1:1. The V_{max} values were calculated from the intercepts of the ordinate in the double-reciprocal plots and the highest value was obtained for the $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio 2:3. Replotting the data from Fig. 1 in the form of $\log[(V_{\text{max}} - v)/v]$ against $-\log[\text{PP}_i]$ (Hill, 1910) gave linear plots with a slope of 1, confirming the conformity with Michaelis-Menten kinetics.

Fixed Mg^{2+} concentrations. When the concentration of added Mg^{2+} was fixed and the concentration of PP_i varied, the $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio decreased as the concentration of PP_i increased. Plots of v against s (PP_i) and Lineweaver-Burk plots at four different fixed concentrations of Mg^{2+} are presented in Fig. 2, alongside the control plot, for which a constant $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio of 2:3 was maintained. The highest initial velocity at any given concentration of PP_i was obtained when the $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio was 2:3. Also, when the Mg^{2+} concentration was kept constant, the highest initial velocity was attained at that PP_i concentration at which the $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio fell to 2:3. At lower concentra-

Fig. 1. (a) Effect of PP_1 concentration, s , on pyrophosphatase activity, v , determined without added Mg^{2+} (■) and at three different constant $\text{Mg}^{2+}/\text{PP}_1$ concentration ratios (□, 1:1; ○, 2:3; ●, 1:2). (b) Lineweaver-Burk plots of the data from (a).

Fig. 2. (a) Effect of increasing PP_1 concentration, s , on pyrophosphatase activity, v , determined at $\text{Mg}^{2+}/\text{PP}_1$ concentration ratio 2:3 (○) and at constant concentrations of total Mg^{2+} (■, 0.66 mM; △, 1.32 mM; ▲, 1.98 mM; ●, 2.64 mM). (b) Lineweaver-Burk plots of the data from (a).

tions of PP_1 at which the $\text{Mg}^{2+}/\text{PP}_1$ concentration ratio was greater than 2:3, there was an inhibition of pyrophosphatase activity, which had the following characteristics: (a) increasing concentrations of Mg^{2+} shifted the plots to the right, indicating that, for a given concentration of PP_1 , the extent of inhibition increased with the Mg^{2+} concentration; (b) velocity as a function of PP_1 concentration showed sigmoidal dependence at fixed concentrations of Mg^{2+} , suggesting an allosteric type of inhibition based on co-operative interaction. The double-reciprocal plots (Fig. 2b) showed that standard Michaelis-Menten kinetics did not prevail at fixed Mg^{2+} concentrations.

Plots of v against total Mg^{2+} concentration (Fig. 3) at four fixed concentrations of PP_1 show that addi-

tion of Mg^{2+} activated the pyrophosphatase activity, but once the amount of Mg^{2+} equalled or exceeded the PP_1 concentration the metal ion was strongly inhibitory. It is seen from Fig. 3 that the plots of velocity against total Mg^{2+} concentration also presented a sigmoidal dependence, implying allosteric inhibition. However, when the inhibition results of Fig. 2 were plotted according to the Hill equation [the activity at optimum $\text{Mg}^{2+}/\text{PP}_1$ concentration ratio (2:3) being substituted for V_{max} , in the expression $(V_{\text{max}} - v_1)/v_1$ and v_1 is the velocity of the inhibited reaction] in the form $\log[(V_{\text{max}} - v_1)/v_1]$ against $-\log i$ (total Mg^{2+} concentration), curves were obtained that were not useful for the estimation of a slope.

By using the complexity constants determined by

Fig. 3. Effect of increasing concentration of total Mg^{2+} on pyrophosphatase activity, v , at four constant concentrations of PP_i (●, 0.1 mM; □, 0.6 mM; △, 1.2 mM; ▲, 1.8 mM).

physical methods (Lambert & Watters, 1957), the absolute concentrations of Mg^{2+} , $MgP_2O_7^{2-}$ and $Mg_2P_2O_7$ in the buffer-substrate-enzyme solutions of assays presented in Fig. 2 were calculated. The results showed that at a fixed concentration of added Mg^{2+} (1) the concentration of $MgP_2O_7^{2-}$ increases, (2) the concentration of free Mg^{2+} decreases and (3) the concentration of $Mg_2P_2O_7$ increases to a maximum and decreases again, as the concentration of PP_i increases (Table 1).

Assays at predetermined concentrations of free Mg^{2+} or $Mg_2P_2O_7$ and increasing concentrations of $MgP_2O_7^{2-}$. It was therefore decided to obtain information on enzyme activity at fixed concentrations of (1) free Mg^{2+} or (2) $Mg_2P_2O_7$, at increasing concentrations of the true substrate, $MgP_2O_7^{2-}$. By using the complexity constants, the absolute concentrations of free Mg^{2+} , $MgP_2O_7^{2-}$ and $Mg_2P_2O_7$ were calculated for PP_i concentrations ranging from 0.09 to 2.4 mM with added magnesium chloride concentrations ranging from 0.66 to 2.64 mM (Table 1). From these values, the amounts of magnesium chloride to be added to known amounts of PP_i could be obtained to yield (1) varied concentrations of $MgP_2O_7^{2-}$ at three fixed concentrations of free Mg^{2+} and (2) varied concentrations of $MgP_2O_7^{2-}$ at four fixed concentrations of $Mg_2P_2O_7$ (Table 1). Buffer-substrate solutions were then prepared to give the chosen values of free Mg^{2+} or $Mg_2P_2O_7$ and assays carried out.

The effect of increasing the concentration of free

Table 1. Concentrations of Mg^{2+} , $MgP_2O_7^{2-}$ and $Mg_2P_2O_7$ in mixtures containing varied concentrations of magnesium chloride and PP_i

Concn. of $MgCl_2$ (mM)	Concn. of PP_i (mM)	0.09	0.1	0.12	0.15	0.2	0.3	0.55	0.6	0.75	0.9	1.2	1.3	1.5	1.8	2.1	2.4
0.66	Mg^{2+}	0.56	0.55	0.53	0.5	0.44	0.33	0.1	0.05								
	$MgP_2O_7^{2-}$	0.08	0.09	0.11	0.13	0.18	0.27	0.54	0.59								
	$Mg_2P_2O_7$	0.01	0.01	0.01	0.02	0.02	0.03	0.01	0.01								
	Combination chosen for assay presented in				Fig. 4			Fig. 4									
1.32	Mg^{2+}	1.21	1.19	1.17	1.13	1.07	1.00	0.68	0.6	0.5	0.36	0.1					
	$MgP_2O_7^{2-}$	0.07	0.07	0.09	0.11	0.15	0.24	0.46	0.5	0.66	0.82	0.16					
	$Mg_2P_2O_7$	0.02	0.03	0.03	0.04	0.05	0.06	0.09	0.1	0.09	0.08	0.04					
	Combination chosen for assay presented in					Fig. 5	Fig. 4		Fig. 5	Fig. 4		Fig. 4					
1.98	Mg^{2+}	1.85	1.84	1.8	1.77	1.7	1.59	1.26	1.2	1.0	0.88	0.58	0.5	0.32	0.1		
	$MgP_2O_7^{2-}$	0.05	0.06	0.07	0.09	0.12	0.2	0.38	0.42	0.55	0.7	1.0	1.11	1.34	1.73		
	$Mg_2P_2O_7$	0.04	0.04	0.05	0.06	0.08	0.1	0.17	0.18	0.2	0.2	0.19	0.16	0.07			
	Combination chosen for assay presented in			Fig. 5			Fig. 5			Figs. 4 and 5	Fig. 5	Fig. 5	Fig. 4		Fig. 4		
2.64	Mg^{2+}	2.5	2.48	2.45	2.41	2.33	2.2	1.84	1.78	1.58	1.36	1.0	0.96	0.77	0.5	0.3	0.1
	$MgP_2O_7^{2-}$	0.04	0.04	0.05	0.07	0.1	0.15	0.3	0.34	0.44	0.52	0.82	0.92	1.13	1.47	1.85	2.27
	$Mg_2P_2O_7$	0.05	0.06	0.07	0.08	0.1	0.15	0.25	0.26	0.31	0.38	0.38	0.33	0.37	0.33	0.25	0.13
	Combination chosen for assay presented in	Fig. 5				Fig. 5					Fig. 5	Figs. 4 and 5	Fig. 5	Fig. 5	Fig. 4		Fig. 4

Fig. 4. Lineweaver-Burk plots showing the reciprocal of velocity against the reciprocal of concentration of $\text{MgP}_2\text{O}_7^{2-}$, the true substrate, at three different concentrations of free Mg^{2+} (●, 0.1 mM; ▲, 0.5 mM; ■, 1.0 mM). The absolute concentrations of free Mg^{2+} and $\text{MgP}_2\text{O}_7^{2-}$ were obtained by the use of complexity constants (Table 1).

Fig. 5. Lineweaver-Burk plot showing the reciprocal of velocity against the reciprocal of concentration of $\text{MgP}_2\text{O}_7^{2-}$, the true substrate, at four constant concentrations of $\text{Mg}_2\text{P}_2\text{O}_7$ (○, 0.05 mM; □, 0.1 mM; △, 0.2 mM; ●, 0.38 mM). The absolute concentrations of $\text{MgP}_2\text{O}_7^{2-}$ and $\text{Mg}_2\text{P}_2\text{O}_7$ were obtained by the use of complexity constants (Table 1).

Fig. 6. Hill plot related to the inhibition of pyrophosphatase activity by free Mg^{2+} ions at four different concentrations of $\text{MgP}_2\text{O}_7^{2-}$, the true substrate (●, 0.1 mM; □, 0.2 mM; ○, 0.3 mM; ■, 0.9 mM). The value chosen for $V_{\text{max.}}$ was the velocity at the optimum $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio 2:3, at which there can be no free Mg^{2+} ions. v_1 represents the inhibited velocity in the presence of free Mg^{2+} ions. The slope of the plot is 1, indicating that the inhibition is consistent with Michaelis-Menten kinetics.

Mg^{2+} ions on the velocity is presented in Fig. 4, which is a plot of $1/v$ against $1/[\text{MgP}_2\text{O}_7^{2-}]$ at three different fixed concentrations of free Mg^{2+} . It is observed that free Mg^{2+} acted both as a competitive and non-competitive inhibitor, and the kinetics of the inhibition were consistent with the Michaelis-Menten equation. A similar plot of $1/v$ against $1/[\text{MgP}_2\text{O}_7^{2-}]$ at four different concentrations of $\text{Mg}_2\text{P}_2\text{O}_7$ resulted in one straight-line plot (Fig. 5), showing that $\text{Mg}_2\text{P}_2\text{O}_7$ had no effect on the velocity and increasing its concentration did not inhibit the reaction. After this evidence that free Mg^{2+} is the inhibitor had been obtained, the apparent suggestion of allosteric interaction was tested by using Hill plots. The activity at optimum $\text{Mg}^{2+}/\text{PP}_i$ concentration ratio of 2:3 was substituted for $V_{\text{max.}}$ in the expression $(V_{\text{max.}} - v_1)/v_1$. The activity in the presence of free Mg^{2+} was used as v_1 . The plots of $\log[(V_{\text{max.}} - v_1)/v_1]$ against $-\log i$ (i being the concentration of free Mg^{2+} ions) are presented in Fig. 6. The slope for the four different concentrations of $\text{MgP}_2\text{O}_7^{2-}$, the true substrate, was precisely 1. Treatment of the enzyme with 3M-urea or pre-heating the enzyme at 55° for 30 min. did not affect the shape of the plots presented in Fig. 2.

DISCUSSION

It has been suggested that the true substrate for several pyrophosphatase enzymes is the complex ion $\text{MgP}_2\text{O}_7^{2-}$ (Bloch-Frankenthal, 1954; Pynes & Younathan, 1967; Butterworth, 1968). It has also been demonstrated by physicochemical methods

that in aqueous solutions containing Mg^{2+} and PP_1 the formation of complex ions is instantaneous (Rogers & Reynolds, 1949; Lambert & Watters, 1957). Inhibition results at constant $\text{Mg}^{2+}/\text{PP}_1$ concentration ratios obtained in this investigation of pyrophosphatase activity of mouse duodenal alkaline phosphatase appear to be consistent with the concept of $\text{MgP}_2\text{O}_7^{2-}$ being the true substrate. Addition of Mg^{2+} to PP_1 increases the activity and maximal activity is obtained at the $\text{Mg}^{2+}/\text{PP}_1$ concentration ratio 2:3. Further addition of Mg^{2+} causes inhibition. When the $\text{Mg}^{2+}/\text{PP}_1$ concentration ratio is less than 2:3, the activity could be limited by the relatively low concentrations of the true substrate, $\text{MgP}_2\text{O}_7^{2-}$. At concentration ratios greater than 1:1 the inhibition could be brought about by one or more of the molecular species, namely Mg^{2+} ion and $\text{Mg}_2\text{P}_2\text{O}_7$, that appear in increasing amounts as the ratio increases. The fact that optimum activity is obtained at the ratio 2:3 suggests that even the minute quantities of either Mg^{2+} ion or $\text{Mg}_2\text{P}_2\text{O}_7$ at equimolar concentrations of Mg^{2+} and PP_1 cause considerable inhibition. At fixed concentrations of Mg^{2+} the inhibition is greater at the higher Mg^{2+} concentration. Plots of both v against [total PP_1] at constant Mg^{2+} concentrations and of v against [total Mg^{2+}] at fixed PP_1 concentrations show sigmoidal dependence. This apparently suggests that co-operative interaction between PP_1 and Mg^{2+} causes an allosteric effect. However, when the absolute concentrations of $\text{MgP}_2\text{O}_7^{2-}$, free Mg^{2+} and $\text{Mg}_2\text{P}_2\text{O}_7$ were calculated in the assay mixtures that yielded the sigmoidal data, it was found that all three molecular species change in concentration from one concentration of PP_1 to another. With a truly allosteric enzyme the sigmoidal dependence is due to a shift in the ratio of two conformational states of the enzyme (Monod, Wyman & Changeux, 1965). In addition, the concentration of the inhibitor must remain constant at increasing concentrations of substrate. The plots of v against [PP_1] at four different constant concentrations of Mg^{2+} do not fulfil this stipulation of constant inhibitor concentration.

When separate assays were carried out at previously determined concentrations of total Mg^{2+} and PP_1 , with the free Mg^{2+} concentration kept constant at three different values, it is seen that free Mg^{2+} acts as both a competitive and a non-competitive inhibitor (Fig. 4). The reciprocal plot at four different fixed concentrations of $\text{Mg}_2\text{P}_2\text{O}_7$ fits one straight line, indicating that $\text{Mg}_2\text{P}_2\text{O}_7$ has no inhibitory effect on hydrolysis of PP_1 . The inference is that free Mg^{2+} is the true inhibitor. However, the formation of $\text{Mg}_2\text{P}_2\text{O}_7$, an enzymically inert complex, would deplete the concentration of the substrate, $\text{MgP}_2\text{O}_7^{2-}$, and the resultant lowering of activity at subsaturation concentrations of sub-

strate would be superimposed on the decrease in activity due to inhibition by free Mg^{2+} .

If the metal activator combines with PP_1 to form the true substrate, $\text{MgP}_2\text{O}_7^{2-}$, it is postulated that the combination of the enzyme with the substrate will take place through the metal atom (Dixon & Webb, 1964). In such a case the free metal ion could compete with the true substrate for the enzyme. The observation that free Mg^{2+} acts as both a competitive and a non-competitive inhibitor is therefore consistent with the concept of $\text{MgP}_2\text{O}_7^{2-}$ being the true substrate. With truly allosteric enzymes the Hill plots obtained from the inhibition data should yield straight lines, with slopes greater than 1, since the slope, n , represents an 'interaction coefficient', which in turn is related to the number of ligand-binding sites on the enzyme (Frieden, 1967). The Hill plot (Fig. 6) for duodenal pyrophosphatase utilizing free Mg^{2+} concentrations for the inhibitor yielded a slope of 1, confirming that the inhibition in this case is not based on co-operative interactions. Both heat and urea treatments also confirmed the absence of any allosteric effects.

The apparent sigmoidal dependence of velocity against PP_1 concentration seen in Fig. 2 is therefore not a case of allosteric behaviour. The part of the sigmoid curve representing strong inhibition at low PP_1 concentrations corresponds to high concentration of the inhibitor, Mg^{2+} , at which a considerable fraction of the substrate, $\text{MgP}_2\text{O}_7^{2-}$, is lost owing to $\text{Mg}_2\text{P}_2\text{O}_7$ formation. The segment of the curve that shows steep acceleration of velocity coincides with rapidly falling concentrations of both free Mg^{2+} and $\text{Mg}_2\text{P}_2\text{O}_7$. Thus the sigmoidal dependence appears to be due to a combined effect of inhibition by free Mg^{2+} and substrate depletion due to $\text{Mg}_2\text{P}_2\text{O}_7$ formation and the manner in which the concentrations of these two moieties change when PP_1 concentrations are increased in maintaining constant total Mg^{2+} concentration.

We acknowledge the help given by Mrs Pam Hillard with the assays and Mrs Dru. B. Troon with the drawings.

REFERENCES

- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
- Bloch-Frankenthal, L. (1954). *Biochem. J.* **57**, 87.
- Butterworth, P. (1968). *Biochem. J.* **110**, 671.
- Cox, R. P. & Griffin, M. J. (1965). *Lancet*, ii, 1018.
- Denburg, J. & DeLuca, M. (1968). *Biochem. biophys. Res. Commun.* **31**, 453.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 437. London: Longman, Green and Co.
- Fernley, H. N. & Walker, P. G. (1967). *Biochem. J.* **104**, 1011.
- Frieden, C. (1967). *J. biol. Chem.* **242**, 4045.
- Hill, A. V. (1910). *J. Physiol.* **40**, 4r.
- Lambert, S. M. & Watters, J. I. (1957). *J. Amer. chem. Soc.* **79**, 5606.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mazia, D., Chaffee, R. R. & Iverson, R. M. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 788.
- Monod, J., Changeux, J. & Jacob, F. (1963). *J. molec. Biol.* **6**, 306.
- Monod, J., Wyman, J. & Changeux, J. (1965). *J. molec. Biol.* **12**, 88.
- Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1966). *Biochem. J.* **99**, 19P.
- Nayudu, P. R. V. & Moog, F. (1967). *Biochem. Genet.* **1**, 155.
- Pynes, G. D. & Younathan, E. S. (1967). *J. biol. Chem.* **242**, 2119.
- Robbins, E. A., Stulberg, M. P. & Boyer, P. D. (1955). *Arch. Biochem. Biophys.* **54**, 215.
- Rogers, L. B. & Reynolds, C. A. (1949). *J. Amer. chem. Soc.* **71**, 2081.
- Sussman, H. H. & Laga, E. (1968). *Biochim. biophys. Acta*, **151**, 281.