Mechanism of Action of the Nonspecific Phosphomonoesterase from Potatoes*

Robert Y. Hsu,† W. W. Cleland, and Laurens Anderson

ABSTRACT: A procedure is described for the preparation, in one working day, of substantial quantities of the acid phosphomonoesterase of potatoes, purified *ca.* 100-fold over crude extract. A full kinetic study of the enzyme was made, with *p*-nitrophenyl phosphate and β-glycerophosphate as substrates. Linear competitive inhibition was observed with inorganic phosphate, and *p*-nitrophenol and glycerol showed linear noncompetitive inhibition with their respective phosphates. *p*-Nitrophenol showed hyperbolic uncompetitive inhibition with β-glycerophosphate.

This inhibition pattern establishes for potato phosphatase a mechanism of ordered release of products, alcoholic product first, inorganic phosphate second. Further analysis of the data shows that two enzyme–phosphate complexes are involved, the one isomerizing to the other. This mechanism may be general for phosphatases.

The mechanism of action of the phosphomonoesterases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.2), which include the specific and nonspecific phosphatases, has been a subject of interest for some time. Phosphomonoesterases have been shown to cleave their substrates at the P–O bond (Schmidt and Laskowski, 1961), and inorganic phosphate, always a product of the reaction, is a competitive inhibitor (Courtois and Lino, 1961). Detailed product-exchange and kinetic studies have led to the postulation of a mechanism for two of the specific phosphatases (Byrne, 1961), but similarly complete data have not been available for any of the nonspecific phosphatases.

When preparations of the acid phosphomonoesterase of potatoes having substantial specific activity became available as a result of work on another project, it was decided to subject this enzyme to a kinetic analysis by the methods recently developed by one of us (W. W. C.). Potato phosphatase is a fairly extensively investigated nonspecific phosphatase, and it has been obtained in a high degree of purity in the laboratories of Helferich and Bruck (1953) and Alvarez (Lora-Tamayo and Alvarez, 1959; Andreu *et al.*, 1960). Kinetic studies have been carried out by Jørgensen (1959), who drew some inferences regarding the mechanism of action, and by Andreu *et al.* (1960), Alvarez (1962), and Lora-Tamayo *et al.* (1962). The latter investigators have been particularly concerned with the effect of pH on the kinetics, and have tried to deduce the nature of the enzyme's active site from their data. In this paper we report data on product and alternate product inhibition in the hydrolysis of *p*-nitrophenyl phosphate and β-glycerophosphate by potato phosphatase. The results permit deductions concerning the nature and number of intermediate steps in the reaction, and the associated free energy changes.

Experimental Section

Materials. Potatoes were purchased from wholesale grocers. Two varieties, Kennebec and California-Long White Shafter, were used. Sodium *p*-nitrophenyl phosphate, obtained from the Sigma Chemical Company, was purified by the method of Bessey *et al.* (1946). The resulting solutions contained no detectable *p*-nitrophenol and less than 0.2% of the total phosphorus as inorganic phosphate. Ordinary sodium β-glycerophosphate·$5.5H_2O$ (*ca.* 25% α isomer) and sodium β-glycerophosphate·nH_2O containing only 0.02% α isomer were obtained from General Biochemicals, Inc., and the Fisher Scientific Co., respectively. The titers of all substrate solutions were checked by analysis for an appropriate product after complete enzymic hydrolysis.

Analytical Methods. Protein was estimated by the biuret method (Layne, 1957), after precipitation with trichloroacetic acid at 5% final concentration, or determined from the optical density at 280 mμ, using crystalline bovine serum albumin as the standard. Inorganic phosphate was determined either by the Fiske and Subbarow method (1925) or, in the presence of acid-labile phosphate esters, by the method of Lowry and Lopez (1946). Glycerol was analyzed by a modification of Lambert and Neish's (1950) periodate–chromotropic acid method.

Phosphomonoesterase activity was assayed with *p*-nitrophenyl phosphate as substrate. The assay mixture, total volume 1.0 ml, contained sodium acetate buffer, pH 5.0, final concentration 0.08 M, substrate

* From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin 53706. Supported by grants from the Carnation Company and from the Research Committee of the Graduate School (funds supplied by the Wisconsin Alumni Research Foundation). *Received August 30, 1965; revised November 11, 1965.*

† Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706.

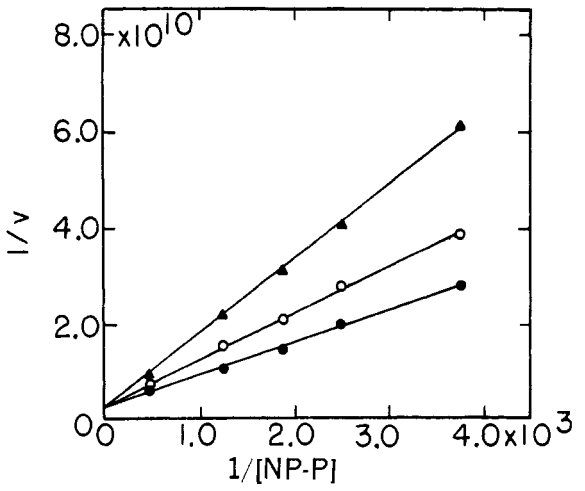


FIGURE 1: Double reciprocal plot of the inhibition of orthophosphate on *p*-nitrophenyl phosphate hydrolysis. Tris (chloride) was the buffer, final concentration 0.1 M. *p*-Nitrophenol was determined. P_i concentrations: ●, 0; ○, 1×10^{-4}; ▲, 3×10^{-4} M.

0.01 M, and enzyme, diluted when necessary with 1% bovine serum albumin. After 10-min incubation at 37°, the reaction was stopped with 0.3 ml of 10% trichloroacetic acid (this step can be eliminated), and the samples were made alkaline with 1.0 ml of 0.2 N sodium hydroxide and diluted to 10 ml. The amount of *p*-nitrophenol released was determined at 400 mμ in an Evelyn colorimeter, with an instrument blank (*i.e.*, enzyme added last) for each enzyme fraction assayed. In this assay the substrate is saturating, and the amount of substrate cleaved is proportional to enzyme concentration or to time up to 3% hydrolysis.

In this paper, a phosphomonoesterase unit is defined as the amount of enzyme which releases 1 μg of phosphorus/hr under the above assay conditions. Specific activity is expressed in terms of units of enzyme activity/mg of protein. Division of our values by 1860 converts them to the proposed standard enzyme units (μmoles of substrate converted per minute).

Purification of Potato Phosphomonoesterase. The procedure used involved extraction with an acid buffer, followed by tannic acid fractionation (Helferich and Stetter, 1948) and selective heat denaturation. In a typical preparation, 1 kg of potatoes was diced and blended in 400-g portions in a 1-gal. Waring blendor for 1 min with 0.5 volume of 0.1 M sodium acetate buffer, pH 3.4. The pulp was squeezed through four layers of cheesecloth and fine particles were removed from the resulting initial extract by mixing the solution with 40 g of Celite and filtering it on a 9-cm Büchner funnel with suction. The volume of filtrate was 685 ml, the protein content 3200 mg, and the specific activity 560 units/mg.

This filtrate[1] was treated with a 5% (w/v) aqueous solution of tannic acid (stirring) until 0.9 mg of reagent/ml had been added. The resulting thick suspension was mixed with 40 g of Celite and filtered, and the filter cake was discarded. The filtrate assayed 520 mg of protein, specific activity 2100 units/mg. It was treated anew with tannic acid until the total added amounted to 1.5 mg/ml, and the suspension was again mixed with Celite (12 g) and filtered. The filtrate contained little activity, and was discarded. The filter cake was washed on the Büchner funnel successively with 150 ml of water, 300 ml of cold (−10°) acetone to remove tannic acid, and 300 ml of saturated ammonium sulfate solution to remove residual acetone. The enzyme was then eluted from the filter cake with 120 ml of 0.083 M Tris–chloride, pH 7.0. Elution may be accomplished by washing the cake on the funnel or by stirring it with portions of buffer in a beaker. The entire washing should be completed in 20–30 min. The eluate contained 185 mg of protein, specific activity 6900 units/mg.

The eluate (dilute, if necessary, to 1.5 mg of protein/ml) was adjusted to pH 4.3 with 1 M acetic acid and divided into five batches which were heated in a 60° water bath for 1 hr, cooled in an ice bath, and centrifuged at 2000*g* for 10 min. The combined supernatants contained 15 mg of protein, specific activity 65,000 units/mg. The over-all yield was thus 55%, the over-all purification factor 116.

Batches of up to 10 kg of potatoes can be worked up by the procedure just described, but further increase in scale is limited by both the blending and the heat denaturation steps. The preparation is simple and convenient, and can be made in 1 day. The final product is completely free of alkaline pyrophosphatase activity, assayed at pH 8.7 according to Naganna *et al.* (1955), and contains only traces of acid and alkaline phosphodiesterase activity (pH 5.0 and 8.7, *p*-nitrophenyl thymidine 5′-phosphate as substrate). It is also relatively free of proteolytic activity.

Kinetic Studies. All runs were carried out at 25° and pH 7. This pH is somewhat above the respective optima for the substrates used (*ca.* 5.4 for NP-P, 5.5–6.3 for G-P) (Alvarez, 1962), but usable rates were obtained without difficulty.[2] According to Alvarez's data, increasing the pH to 7 considerably increases the Michaelis constant for NP-P, but not for G-P. Maximum velocities for both substrates are about two-thirds as great as at the pH optima.

The high pH was chosen so that the release of *p*-nitrophenol could be followed continuously in a recording spectrophotometer. The Cary Model 11 was used. Reaction mixtures containing all components except enzyme in a total volume of 2.9 ml were preincubated for 10 min in a water bath at 25°. To start the reaction, 0.1 ml of dilute enzyme was added (890 units in 1% bovine serum albumin), and the solution

[1] If the protein concentration is appreciably over 5 mg/ml, the volume should be adjusted; if it is much less the amount of tannic acid needed to precipitate the inactive proteins should be determined experimentally.

[2] Abbreviations used: P_i, inorganic phosphate; NP-P, *p*-nitrophenyl phosphate; G-P, β-glycerophosphate; NP-OH, *p*-nitrophenol; G-OH, glycerol.

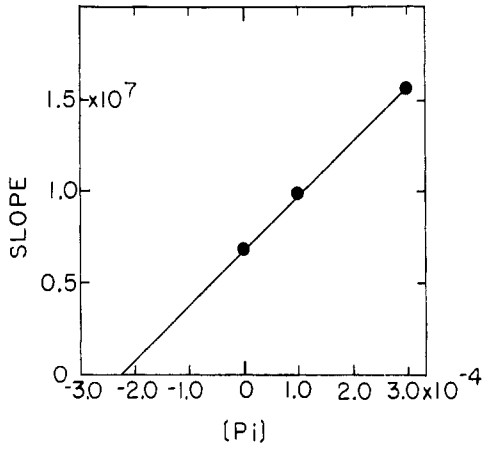


FIGURE 2: Replot of Figure 1. Slopes *vs.* [P_i].

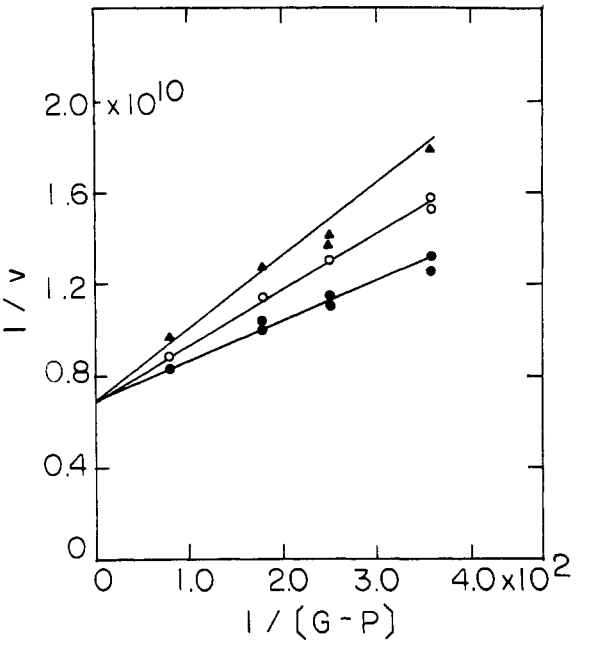


FIGURE 3: Double reciprocal plot of the inhibition of orthophosphate on β-glycerophosphate hydrolysis. Tris (chloride) was the buffer, final concentration 0.1 M. Orthophosphate was determined. P_i concentrations: ●, 0; ○, 1.02×10^{-4}; ▲, 2.04×10^{-4} M.

was mixed and immediately poured into a 1-cm cuvet which was placed in the sample compartment. Velocities were computed from the slopes of the initial, linear portions of the recorder traces, using absorbancy values determined on standard solutions of *p*-nitrophenol in the buffers employed.

When phosphate or glycerol were the products determined, a thermally equilibrated mixture of buffer, substrate, and inhibitor (4.85 ml) and 0.15 ml of enzyme (1335 units) were mixed and incubated for 5 min. Usually duplicate reaction tubes and one zero-time tube were included for each determination. The reaction was stopped by the addition of 0.5 ml of 40% trichloroacetic acid, the precipitated protein was centrifuged off, and suitable aliquots of the supernatant liquid were taken for the determination of the product formed. Ordinary sodium β-glycerophosphate was used in following the release of phosphate from this substrate, but when glycerol was the product of interest the material free of α isomer was employed, since the α isomer interferes with the periodate assay. In the experiment on the *p*-nitrophenol inhibition of β-glycerophosphate hydrolysis, the set of tubes for each inhibitor concentration was incubated and then analyzed for glycerol immediately, before the next set was run, to avoid any acid-catalyzed conversion of the unreacted substrate to the α isomer.

Each experiment (all determinations involving one substrate–inhibitor combination) was repeated until random errors were minimized, as judged by the deviations of the points from straight lines in the reciprocal plot. The best plots are presented here. The recorded kinetic constants were calculated from these plots.

Results and Discussion[3]

The rate data obtained by varying the concentration of each of the two substrates, alone and in the presence of the respective products of the reactions, are shown in Figures 1, 3, 4, and 5. Table I lists the Michaelis constants and other pertinent kinetic constants which could be derived from the data. It may be seen that the inhibition caused by inorganic phosphate is competitive with both *p*-nitrophenyl phosphate (Figure 1) and β-glycerophosphate (Figure 3) as substrates. This inhibition is linear as shown by a replot (Figure 2) of the slopes from Figure 1 *vs.* phosphate concentration. A replot (not shown) of the slopes from Figure 3 is also linear. The inhibition constants determined from the horizontal intercepts of these replots are nearly identical (Table I). Figure 4 shows the noncompetitive inhibition caused by *p*-nitrophenol with *p*-nitrophenyl phosphate as substrate, and Figure 5 shows the similar noncompetitive inhibition caused by glycerol with β-glycerophosphate as substrate. Both of these inhibitions are linear; replots of both slopes and intercepts are straight lines. The inhibition constants are given in Table I.

Our values for the Michaelis constants for *p*-nitrophenyl phosphate and β-glycerophosphate at pH 7 agree satisfactorily with the results of Alvarez (1962) (interpolated): 1×10^{-2} for NP-P at 38°; 3×10^{-3} for G-P at 20°. It is of interest that at pH 7 and 25° the two substrates have nearly the same K_a, but the dissociation constants of the enzyme–substrate complexes (the K_{ia} values in Table I, last column) differ by a

[3] The kinetic nomenclature and mathematical symbolism used in this paper are those of Cleland (1963a,b). The reader is cautioned to remember that the symbol P stands for the first product released by the enzyme, not for inorganic phosphate.

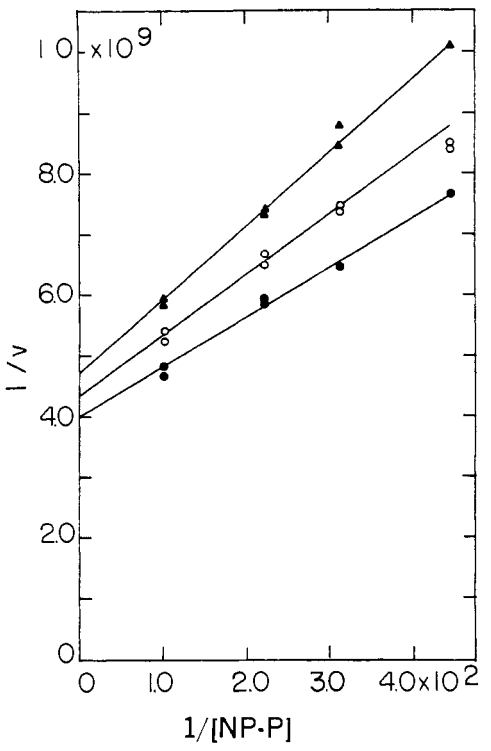


FIGURE 4: Double reciprocal plot of the inhibition of *p*-nitrophenol on *p*-nitrophenyl phosphate hydrolysis. Tris (chloride) buffer was used, final concentration 0.1 M. Orthophosphate was determined. *p*-Nitrophenol concentrations: ●, 0: ○, 0.01; ▲, 0.02 M.

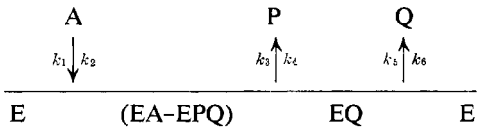
factor of two, β-glycerophosphate being more strongly bound.

Order of Release of Products. The linearity of reciprocal plots of $1/v$ *vs.* $1/A$ shows that the enzyme follows the simple rate law

$$v = \frac{VA}{K_a + A} \qquad (1)$$

where V is maximum velocity, K_a is the Michaelis constant, and A is substrate concentration. It is thus clear that one molecule of the substrate combines with the enzyme to give a complex which then breaks down with release of products. Mechanisms in which two or more molecules of substrate participate in the reaction at one active center give more complex rate laws.

The products could be released in a definite order, or in random order. For the simple mechanism



(2)

where P is the first product released and Q the second, the rate equation can be written (Cleland, 1963a)

TABLE I: Kinetic Data. Summary of the Data Obtained with Potato Acid Phosphomonoesterase at pH 7.0 and 25°.[a]

Substrate	Inhibitor	Product Determined	Type of Inhibition	Michaelis Constant, K_a (M)	Inhibition Constants		
					K_i Slope (M)	K_i Intercept (M)	Other (M)
NP-P	P_i	NP-OH	Linear competitive	2.5×10^{-3}	$K_{iq} = 2.4 \times 10^{-4}$		
NP-P	NP-OH	P_i	Linear noncompetitive	2.0×10^{-3}	$\frac{K_{ia}K_{ip}}{K_{ia}} = 0.04$	$K_{ip} = 0.12$	$K_{ia} = 6.0 \times 10^{-3}$
G-P	P_i	P_i	Linear competitive	2.5×10^{-3}	$K_{iq} = 2.5 \times 10^{-4}$		
G-P	G-OH	P_i	Linear noncompetitive	3.3×10^{-3}	$\frac{K_{ia}K_{ip}}{K_{ia}} = 8.5$	$K_{ip} = 8.0$	$K_{ia} = 3.1 \times 10^{-3}$
G-P	NP-OH	G-OH	Hyperbolic uncompetitive	2.7×10^{-3}		$K_{irr} = 0.02$	$\frac{K_bK_{ir}}{K_{ib}} = 0.03$

[a] Kinetic constants were determined from the reciprocal plots in Figures 1, 3, 4, 5 and 6, or from replots of the slopes and intercepts *vs.* inhibitor concentration.

$$v = \frac{VA}{K_a + A + \frac{K_{ia}P}{K_{ip}} + \frac{K_aQ}{K_{iq}} + \frac{K_aPQ}{K_pK_{iq}} + \frac{AP}{K_{ip}}} \quad (3)$$

where K_{ia} is the dissociation constant of A ($= k_2/k_1$), K_{ip} and K_{iq} are inhibition constants for P and Q, and K_p is the Michaelis constant for P in the reverse reaction. This rate equation holds as long as the reaction is far enough from equilibrium for the rate of the back reaction to be negligible. The form of the equation is unchanged by isomerization of the (EA–EPQ) or EQ complexes, although isomerization of the free enzyme would add AQ and APQ terms to the denominator.

If the reaction is run with P, but not Q, present, eq 3 in reciprocal form becomes:

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}P}{K_aK_{ip}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{P}{K_{ip}}\right) \quad (4)$$

Thus the first product released should give linear noncompetitive inhibition, with slope and intercept inhibition constants equal to K_aK_{ip}/K_{ia} and K_{ip}, respectively. When the reaction is run in the presence of Q, but not P, the rate equation is:

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{Q}{K_{iq}}\right)\left(\frac{1}{A}\right) + \frac{1}{V} \quad (5)$$

The second product released should thus give linear competitive inhibition with an inhibition constant equal to K_{iq}. If there is isomerization of free enzyme, however, and an AQ term is present in the denominator of eq 3, then the equation corresponding to 5 will also predict linear noncompetitive inhibition, and the behavior of the two products will be the same.

If the products are released in random order, eq 3 has additional terms in both the numerator and denominator and in the general case both products will give noncompetitive inhibition in which the intercepts are a hyperbolic function of inhibitor concentration and the slopes are a more complex function (Cleland, 1963b). However, if in such a mechanism the rate limiting step is the conversion of an (EA) into an (EPQ) complex, and the release of products is very rapid, then the steps for release of products will be very nearly at equilibrium, and under these conditions the products will both give linear competitive inhibition.

A mechanism in which no order of product release is specified, and phosphate forms a dead end complex with the free enzyme, was considered by Jørgensen (1959) to be consistent with his kinetic and product-exchange data on potato phosphatase. This author found competitive inhibition by phosphate, but did not succeed in demonstrating any kind of inhibition by the alcoholic product (phenol). The data obtained in the present work show that with two different substrates, the alcoholic product gives linear noncompetitive inhibition while phosphate gives linear competitive inhibition. These data are consistent with ordered release of products, with the alcohol released first and phosphate last. The identical inhibition constant for phosphate with the two substrates used also supports this view. Random release of products is definitely ruled out.

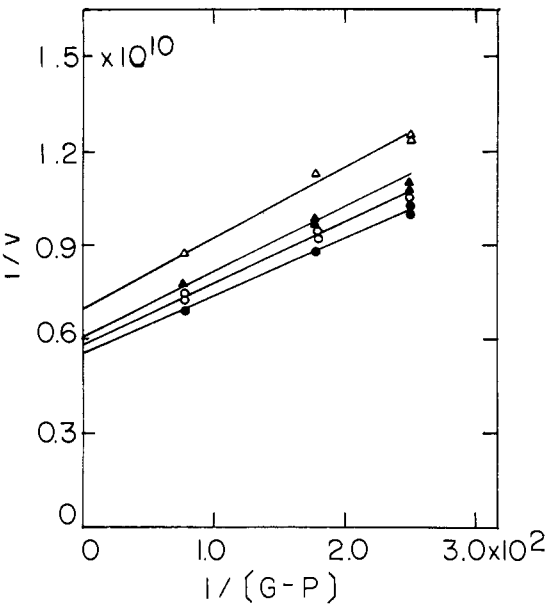
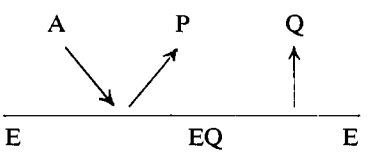


FIGURE 5: Double reciprocal plot of the inhibition of glycerol on β-glycerophosphate hydrolysis. Tris (chloride) buffer was used, final concentration 0.1 M. Orthophosphate was determined. Glycerol concentrations: ●, 0; ○, 0.5; ▲, 1.0; △, 2.0 M.

However, in addition to the simple mechanism in which there is ordered release of the alcohol and then phosphate, several mechanisms involving additional dead end inhibitions by the products can give the observed inhibition pattern. If the order of release were first alcohol, then phosphate, and if the alcoholic product also could combine with free enzyme, there would be an additional effect on the slopes of the reciprocal plots to give noncompetitive inhibition where the intercepts were a linear but the slopes a parabolic function of inhibitor concentration. If in such a case the slope inhibition constant for the normal product inhibition were larger than both the intercept inhibition constant and the inhibition constant for the dead end inhibition, then the parabolic nature of the slope replot might be difficult to see and would escape detection. The calculation of values of K_{ia} from the ratio of slope and intercept inhibition constants according to eq 4 would then be invalid.

Two other mechanisms which also give the competitive–noncompetitive product inhibition pattern are ones in which the steady-state concentrations of central complexes are very low and not kinetically important:



(6)

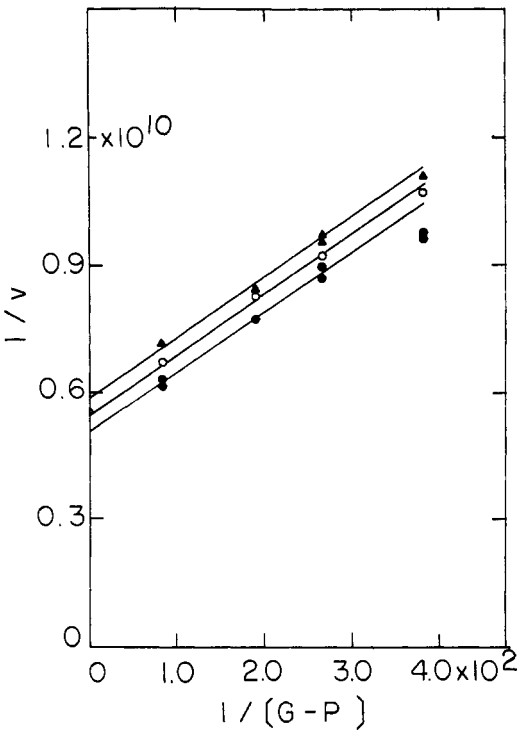
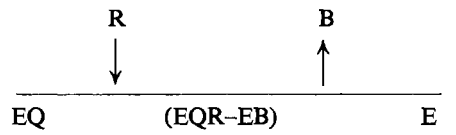


FIGURE 6: Double reciprocal plot of the inhibition of *p*-nitrophenol on β-glycerophosphate hydrolysis. Veronal (sodium) buffer was used, final concentration 0.025 M. Glycerol was determined. *p*-Nitrophenol concentrations: ●, 0; ○, 6.25 × 10^{-3}; ▲, 1.875 × 10^{-2} M.

Mechanism 2 reduces to 6 if k_3 is much larger than both k_5 and k_4. Mechanism 6, as written, predicts competitive inhibition by both products, but if P reacts with EQ to give a dead end (EPQ) complex, it becomes a noncompetitive inhibitor, and if there is isomerization of free enzyme (that is, the form reacting with A is different from that resulting from the dissociation of the EQ complex) then Q becomes a noncompetitive inhibitor. (Note that the product giving competitive inhibition would then be the first one released.) The former mechanism with a dead end (EPQ) complex is indistinguishable by any method from mechanism 2 and will not be further discussed, since if an (EPQ) complex is present, it would seem much more reasonable for it to be part of the reaction pathway than to be a dead end complex.

Alternate Product Inhibition. As described above, the direct product inhibition data do not by themselves rule out the possibility that part of the slope effect in the noncompetitive inhibition is caused by dead end combination with free enzyme, or the possibility of a mechanism without central complexes but with isomerization of free enzyme. Both of these possibilities can be tested by an alternate product inhibition study, in which the hydrolysis of one substrate is measured in the presence of inhibitory concentrations of an alcohol which would be a product if another substrate were used. In the present work, β-glycerophosphate was used as substrate and *p*-nitrophenol as inhibitor. The reactions occurring are the sum of mechanism 2 with A = β-glycerophosphate and P = glycerol, and mechanism 7



(7)

where R is *p*-nitrophenol and B is *p*-nitrophenyl phosphate.

Two rate equations can be derived for this situation, one for each of the principal hydrolysis products (Cleland, 1963a). When the product measured is phosphate (Q) the rate equation is

$$\frac{1}{\left(\frac{dQ}{dt}\right)} = \frac{K_a}{V}\left(1 + \frac{K_{ib}R}{K_bK_{ir}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{R}{K_{irr}}\right) \quad (8)$$

where K_b and K_{ib} are the Michaelis and dissociation constants for *p*-nitrophenyl phosphate, K_{ir} is the same constant as K_{ip} in eq 3 and 4, and K_{irr} is a new kinetic constant. Similarly, when glycerol (P) is determined, the rate equation is:

$$\frac{1}{\left(\frac{dP}{dt}\right)} = \frac{K_a}{V}\left(\frac{1}{A}\right) + \frac{1}{V}\left(\frac{1 + \frac{R}{K_{irr}}}{1 + \frac{K_{ib}R}{K_bK_{ir}}}\right) \quad (9)$$

As with eq 4, these equations are written assuming zero initial concentration of Q and no formation of complexes of the type EP and ER. Since eq 8 is identical in form with eq 4, measurement of phosphate release should show linear noncompetitive inhibition by *p*-nitrophenol, as in experiments with either of the substrates and the *corresponding* alcohol as inhibitor. But when glycerol release (P) is measured, hyperbolic uncompetitive inhibition is expected (eq 9). However, if complexes of the type EP or ER are formed, the inhibition by *p*-nitrophenol would be noncompetitive rather than uncompetitive. Uncompetitive inhibition by *p*-nitrophenol of the appearance of glycerol is thus evidence against the formation of alcohol–enzyme complexes in kinetically important amounts.

The results obtained by measuring glycerol release during the hydrolysis of β-glycerophosphate in the presence of *p*-nitrophenol (Figure 6) indicate that the inhibition was uncompetitive, and the variation of vertical intercept with inhibitor concentration (Figure 7) was consistent with the hyperbolic curve predicted by eq 9. Thus, it can be concluded that the inhibition of the reaction by its alcoholic products is due to the reversal of the step (EA–EPQ) $\rightleftharpoons$ EQ + P in the hydrolysis sequence. These results also rule out the mechanism without central complexes in which there is an isomerization of free enzyme and the order of release of the

products is the opposite of that so far deduced. In this mechanism the alternate product (*p*-nitrophenol) would give linear noncompetitive inhibition regardless of the product measured.

A further test of the validity of eq 9 is that the inhibition constant K_bK_{ir}/K_{ib}, calculated for *p*-nitrophenol from the alternate product inhibition data, should be the same as the slope inhibition constant from eq 4 for the product inhibition of *p*-nitrophenol *vs.* *p*-nitrophenyl phosphate. The inhibition constants from the hyperbolic inhibition are not easily obtained from a direct plot of intercepts *vs.* R (Figure 7), but can be obtained by plotting $1/(\text{intercept}_I - \text{intercept}_0)$ *vs.* $1/R$ according to eq 10

$$\frac{1}{(\text{intercept}_I - \text{intercept}_0)} = V\left(\frac{1}{\frac{1}{K_{irr}} - \frac{K_{ib}}{K_bK_{ir}}}\right)\left(\frac{1}{R}\right) + V\left(\frac{\frac{K_{ib}}{K_bK_{ir}}}{\frac{1}{K_{irr}} - \frac{K_{ib}}{K_bK_{ir}}}\right) \quad (10)$$

where intercept_I and intercept_0 (which is also $1/V$) are the vertical intercepts of the lines in Figure 6 in the presence and absence of inhibitor. (This replot, not shown, is merely a normal reciprocal plot of the hyperbola in Figure 7, after moving up the horizontal axis of that figure so that its curve goes through the origin.) The inhibition constants obtained from the replot, and given in Table I, are calculated by eq 11 and 12:

$$K_{irr} = \frac{\text{slope of replot}}{V + \text{vertical intercept of replot}} \quad (11)$$

$$\frac{K_bK_{ir}}{K_{ib}} = \frac{-1}{\text{horizontal intercept of replot}} \quad (12)$$

The value for K_bK_{ir}/K_{ib} (0.03 M) agrees well with the value (0.04 M) obtained for the same constant ($K_a \cdot K_{ip}/K_{ia}$) by product inhibition.

In sum, mechanism 2 is the simplest mechanism (*i.e.*, the one with the fewest steps) incorporating the features which can be inferred by an examination of the inhibition patterns of potato phosphatase. The pattern of competitive inhibition by phosphate and noncompetitive inhibition by the alcoholic product is the same as that shown by liver glucose 6-phosphatase and liver phosphoserine phosphatase, the two specific phosphatases examined by Byrne and co-workers (Byrne, 1961). Likewise, mechanism 2 is essentially the same as the "minimal mechanism" proposed by Byrne (1961) for the specific phosphatases.

Inadequacy of the Simple Mechanism. If the simple mechanism (mechanism 2) correctly represents the reaction catalyzed by potato phosphomonoesterase, the expression for the over-all equilibrium constant is

$$K_{eq} = \left(\frac{k_1}{k_2}\right)\left(\frac{k_3}{k_4}\right)\left(\frac{k_5}{k_6}\right) \quad (13)$$

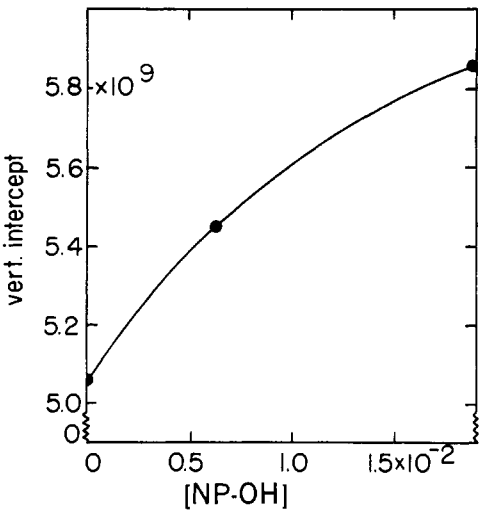


FIGURE 7: Replot of Figure 6. Vertical intercepts *vs.* *p*-nitrophenol concentration.

and the rate constants are related to the kinetic constants of eq 3 as follows (Cleland, 1963a):

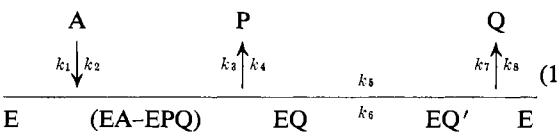
$$K_{ia} = \frac{k_2}{k_1}\ ;\ K_{ip} = \frac{k_3 + k_5}{k_4}\ ;\ K_{iq} = \frac{k_5}{k_6}$$

Combination of these relationships gives the inequality:

$$K_{eq} \leqq \left(\frac{1}{K_{ia}}\right)(K_{ip})(K_{iq}) \quad (14)$$

Values for K_{ia} (for *p*-nitrophenyl phosphate and β-glycerophosphate), K_{ip} (for *p*-nitrophenol and glycerol), and K_{iq} (for phosphate) have been calculated from the experimental data (Table I), and if mechanism 2 is fully valid, substitution of these values into inequality 14 should give reasonable limiting values for K_{eq}. The actual computation gives, for the hydrolysis of *p*-nitrophenyl phosphate, $K_{eq} \leqq 4.8 \times 10^{-3}$ M, and for the hydrolysis of glycerophosphate, $K_{eq} \leqq 0.65$ M. These values are much too small (see next paragraph), showing that mechanism 2 is too simple.

If the mechanism is expanded to include isomerization of the enzyme–phosphate complex EQ to a second complex EQ′



(15)

the rate is still given by eq 3, but now:

$$K_{eq} = \left(\frac{k_1}{k_2}\right)\left(\frac{k_3}{k_4}\right)\left(\frac{k_5}{k_6}\right)\left(\frac{k_7}{k_8}\right) \quad (16)$$

The relations to the kinetic constants are

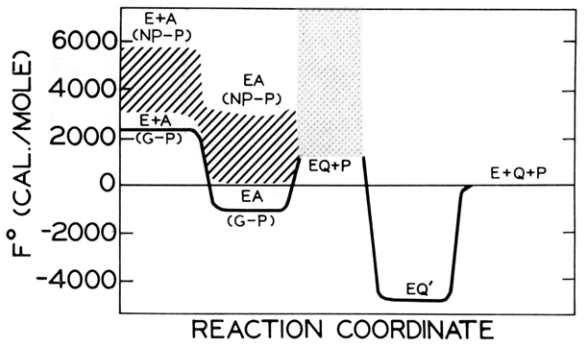


FIGURE 8: Ideal free energy diagram.

$$K_{ia} = \frac{k_2}{k_1}\ ;\ K_{ip} = \frac{k_3}{k_4} + \frac{k_5(k_3 + k_7)}{k_4(k_6 + k_7)}\ ;\ \text{and}$$

$$K_{iq} = \frac{k_5 k_7}{(k_5 + k_6)k_8}$$

and if, in eq 16, k_1/k_2, k_3/k_4, and k_7/k_8 are replaced by $1/K_{ia}$, K_{ip}, and $(k_5 + k_6)K_{iq}/k_5$ respectively, then:

$$K_{eq} \leqq \left(\frac{1}{K_{ia}}\right)(K_{ip})\left(\frac{k_5}{k_6}\right)\left(\frac{k_5 + k_6}{k_5}\right)(K_{iq}) \qquad (17)$$

Because it is not entirely in terms of kinetic constants, inequality 17 cannot be tested in the same way as inequality 14. However, if inequality 17 is rearranged to

$$\frac{k_5}{k_6} \geqq \frac{K_{eq}K_{ia}}{K_{ip}K_{iq}} - 1 \qquad (18)$$

limiting values of k_5/k_6 can be calculated. The free energy of hydrolysis of β-glycerophosphate is estimated at -2400 cal/mole (K_{eq} = 58 M) at pH 7 (Meyerhof and Green, 1950), and that for *p*-nitrophenyl phosphate, while it has not been determined, presumably lies between -3000 (K_{eq} = 158 M) and -6000 cal/mole ($K_{eq} = 2.5 \times 10^4$). If these three values of K_{eq} are successively substituted into inequality 18, along with appropriate values for K_{ia}, K_{ip}, and K_{iq} as before, one arrives, respectively, at $k_5/k_6 \geqq 90$, $\geqq 3.3 \times 10^4$, and $\geqq 5.2 \times 10^6$. Since the two substrates must necessarily yield the same enzyme–phosphate complexes, the first value, based on glycerophosphate, may be discarded, and the second, corresponding to the lower figure for ΔF° (NP-P), may be taken as a lower limit for k_5/k_6. Thus the postulated isomerization step must have an equilibrium constant $\geqq 3.3 \times 10^4$, corresponding to a $\Delta F^\circ \leqq -6160$ cal/mole. At first sight such a figure seems unreasonably large, but a closer analysis of the energy relationships in the reaction sequence shows that it is not.

Thermodynamics. COMPARISON WITH SPECIFIC PHOSPHATASES. The information thus far developed can be used to calculate the equilibrium constants, or at least one of their limiting values, for the other three steps of the proposed mechanism (15). For the first two steps, the constants differ for the two substrates; the last two steps are of course identical for all substrates. From the relationships used to develop inequality 17, it is seen that the constants for the first step are again the reciprocals of the respective values for K_{ia}. When k_5/k_6 is large the equilibrium constant for the last step, k_7/k_8, = K_{iq} very nearly. Upper limits for k_3/k_4 (second step) are set by the values of K_{ip}. For glycerophosphate as substrate the value for k_1/k_2 (G-P), the lower limiting value for k_5/k_6, the value for K_{iq}, and the value for K_{eq} (G-P) may be used in eq 16 to give a revised upper limit for k_3/k_4. The k_3/k_4 for *p*-nitrophenyl phosphate cannot be further refined in the absence of a definite value for K_{eq} (NP-P).

Conversion of the computed equilibrium constants to ΔF° values yields the diagram of Figure 8. This diagram shows the free energy level of the system at each stage of the reaction (if all components at that stage were at unit activity) referred to a mixture of enzyme and products all at unit activity. A range is shown in the first two stages for *p*-nitrophenyl phosphate as substrate, and for the third stage (first enzyme–phosphate complex) the values range upward from the limit set by the limiting values of the k_3/k_4.

The diagram reveals in a striking way the strong binding of phosphate to enzyme in the last complex (EQ′), which is at an energy level 4900 cal/mole below products plus free enzyme. (Note that the value is the same, *viz.*, $-RT \ln K_{eq}$, whether mechanism 15 or mechanism 2 is assumed.) Further, it is seen that if there are two enzyme–phosphate complexes, this 4900 cal/mole is a part of the energy difference between them, so that the lower limit for the energy level of the first complex is brought close enough to that of the final products to make the postulation of this complex quite reasonable. It may be concluded, then, that the four-step mechanism (15) is fully compatible with all data and calculations, and it is the simplest mechanism which is thus compatible.

In view of the fact that the product inhibition patterns of glucose 6-phosphatase and phosphoserine phosphatase (Byrne, 1961) are essentially the same as that of potato phosphatase, it was of interest to further compare these enzymes. When the kinetic data of Hass and Byrne (1960) for glucose 6-phosphatase were analyzed by the methods applied to our data in the preceding section, a complete parallelism was found. For glucose 6-phosphatase, also, it is necessary to postulate a mechanism with isomerization of the enzyme phosphate complex EQ to EQ′ (mechanism 15). Accordingly, it seems likely that a large number of phosphatases, both specific and nonspecific, will be found to share this common mechanism.

If two enzyme–phosphate complexes are formed during the course of some, perhaps many, phosphatase reactions, the nature of the bonds between the enzyme and phosphate in these complexes becomes of particular interest. Some indication comes from the energy-level calculations performed here, which show that the first complex of both potato phosphatase and glucose 6-phosphatase is in the range suggestive of covalent

bonding. The value is low enough to be compatible with an ester bond to a hydroxyl of serine, but since only the lower limit can be estimated higher energy structures such as acyl or imidazole phosphate must be considered. For the second complex, ionic bonding seems likely. An argument favoring the participation of one covalently bonded complex in phosphatase reactions is the ability of these enzymes, including glucose 6-phosphatase, to catalyze transphosphorylation (Morton, 1955; Byrne, 1961). Another is the phosphorylation by inorganic phosphate of serine hydroxyls, presumably at the active site, in two nonspecific alkaline phosphatases (Engstrom, 1961; Schwartz and Lipmann, 1961). None of these arguments are completely compelling, however. More work on phosphatases will be required to elucidate the nature of the complexes formed during the course of their action.

References

Alvarez, E. F. (1962), *Biochim. Biophys. Acta 59*, 663.

Andreu, M., Alvarez, E. F., and Lora-Tamayo, M. (1960), *Anales Real Soc. Espan. Fis. Quim. (Madrid), Ser. B 56*, 67.

Bessey, O. A., Lowry, O. H., and Brock, M. J. (1946), *J. Biol. Chem. 164*, 321.

Byrne, W. L. (1961), *in* The Enzymes, Boyer, P. D., Lardy, H. A., and Myrbäck, K., Ed., Vol. 5, 2d ed, New York, Academic, p 73.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67*, 104.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67*, 173.

Courtois, J. E., and Lino, A. (1961), *Fortschr. Chem. Org. Naturstoffe 19*, 316.

Engstrom, L. (1961), *Biochim. Biophys. Acta 52*, 49.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Hass, L. F., and Byrne, W. L. (1960), *J. Am. Chem. Soc. 82*, 947.

Helferich, B., and Bruck, G. v. (1953), *Z. Physiol. Chem. 295*, 114.

Helferich, B., and Stetter, H. (1948), *Ann. 560*, 191.

Jørgensen, O. B. (1959), *Acta Chem. Scand. 13*, 900.

Lambert, M., and Neish, A. C. (1950), *Can. J. Res. B28*, 83.

Layne, E. (1957), *Methods Enzymol. 3*, 450.

Lora-Tamayo, M., and Alvarez, E. F. (1959), *Fette, Seifen, Anstrichmittel 61*, 1079.

Lora-Tamayo, M., Alvarez, E. F., and Andreu, M. (1962), *Bull. Soc. Chim. Biol. 44*, 501.

Lowry, O. H., and Lopez, J. A. (1946), *J. Biol. Chem. 162*, 421.

Meyerhof, O., and Green, H. (1950), *J. Biol. Chem. 183*, 377.

Morton, R. K. (1955), *Discussions Faraday Soc. 20*, 149.

Naganna, B., Raman, A., Venugopal, B., and Sripathi, C. E. (1955), *Biochem. J. 60*, 215.

Schmidt, G., and Laskowski, M., Sr. (1961), *in* The Enzymes, Boyer, P. D., Lardy, H. A., and Myrbäck, K., Ed., Vol. 5, 2d ed, New York, Academic, p 3.

Schwartz, J. H., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1996.