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Evidence for a Phosphoryl–Enzyme Intermediate in Alkaline Phosphatase Catalyzed Reactions*

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ABSTRACT: Kinetic evidence is presented which demonstrates the formation of a catalytic phosphoryl–enzyme intermediate during the hydrolysis of phosphate esters by alkaline phosphatase. Nine phosphate esters were hydrolyzed in the presence of 1 M Tris acting as a phosphate acceptor in competition with water and the ratios of the products were measured. Precisely 1.39 equiv of *O*-phosphoryl Tris was formed for every equivalent of P_i regardless of the particular ester that was hydrolyzed. The phosphoryl–enzyme theory proposes that a phosphoryl–enzyme intermediate is formed as a step in the hydrolysis of phosphate esters followed by reaction of this intermediate with water and with other phosphate acceptors to regenerate enzyme and produce P_i and transphosphorylation products of other acceptors. In this theory, the leaving group of the ester is no longer present when the reaction with Tris and with water takes place and therefore cannot influence the ratio of products. Under these circumstances, the ratio of products must be a constant, independent of the actual ester which is used as a substrate. It is also true that a different ratio of products must be obtained for each ester if these reagents react with an entity which still contains the different leaving groups.

Since a constant ratio of products was obtained, it may be concluded that a phosphoryl–enzyme occurs as an intermediate in the enzymic hydrolysis of phosphate esters.

The most important question concerning the mechanism of alkaline phosphatase is whether a catalytic phosphoryl–enzyme intermediate is formed during the hydrolysis of phosphate esters. Widespread interest in this question was greatly stimulated by the remarkable observations of Engstrom (1961, 1962a,b, 1964), Engstrom and Agren (1958), Schwartz and Lipmann (1963), Schwartz (1961), Pigretti and Milstein (1965), and Milstein (1964), who obtained a phosphoprotein containing a phosphorylated serine side chain by incubating the enzyme (and contaminating proteins) with low concentrations of ^{32}P-labeled P_i in acid pH. There is a tendency to assume that this result, the formation of a phosphoprotein, proves the formation of a transient phosphoryl–enzyme intermediate during enzymic catalysis. However, reflection indicates that this observation in itself does not give us any information on this question. First, we note that the phosphoprotein is thermodynamically very stable; it is 10^5 times more stable than *O*-phosphorylserine (Vladimirova *et al.*, 1961) and *O*-phosphorylethanolamine derivatives (Wilson and Dayan, 1965). Alkaline phosphatase, of course, catalyzes the synthesis as well as the hydrolysis of phosphate esters, the direction being

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dictated by the free-energy change involved. Since, in this case, a serine residue exists which is capable of forming a thermodynamically stable phosphate ester and since a nonspecific catalyst, namely, alkaline phosphatase, along with P_i is present in the solution, the phosphoprotein must necessarily form no matter what the catalytic mechanism of alkaline phosphatase may be.[1] The argument is not altered by the use of glucose phosphate as a phosphate donor because alkaline phosphatase also catalyzes transphosphorylation. Even if we should assume that the phosphoprotein is a derivative of the enzyme rather than of some contaminating protein, the question remains whether the phosphoprotein is the catalytic phosphoryl–enzyme intermediate or, indeed, whether such an intermediate does exist. These questions were recognized by the original investigators.

Some confirmation from other studies is needed and four sources of evidence are available. Using a rapid sampler, Aldridge *et al.* (1964) showed that the phosphoprotein (^{32}P labeled) hydrolyzed rapidly at pH 8.4 with a first-order rate constant of about 115 sec^{-1} which is consistent with hypothesis that the phosphoprotein is the proposed transient phosphoryl–enzyme intermediate. However this rate of hydrolysis is about what would be obtained if the amount of enzyme used in the experiment were to act upon a phosphate ester which could serve as a good substrate. It is therefore possible that the phosphoprotein is not an intermediate but is merely serving as a substrate for alkaline phosphatase. This explanation may seem unattractive but it is a logical alternative to the phosphoryl–enzyme intermediate.

Similarly the formation of a rapid burst of substituted phenol (2,4-dinitrophenol and umbelliferone) when the phosphate ester and high concentrations of enzyme are rapidly mixed together at pH $\leq$ 6 may be taken as support for the hypothesis of a phosphoryl–enzyme intermediate (Fernley and Walker, 1966; Fife, 1967; Williams, 1966; Ko and Kézdy, 1967), or may alternatively be explained as arising from the formation of a phosphoprotein which is not an intermediate. Evidence has also come from experiments using a kinetic method for evaluating the free energy of hydrolysis of a postulated phosphory–enzyme intermediate which showed that the intermediate, assuming it exists, is, similar to the phosphoprotein, thermodynamically stable relative to free enzyme and P_i. But this work was at pH 8.0 where a phosphoprotein does not form (Wilson and Dayan, 1965).

The fact that almost all phosphate esters are hydrolyzed at about the same rate and none at a higher rate is rather puzzling (Garen and Levinthal, 1960). One would think that the nature of the leaving group must surely influence the rate of hydrolysis. However, if a phosphoryl–enzyme is involved, one has only to assume that the dephosphorylation of the phosphoryl–enzyme is rate controlling. Then even though the various phosphate esters may phosphorylate the enzyme at widely varying rates the over-all rates of the reactions would be identical.

We have two questions which are interwoven in the above studies: (1) Is a phosphoryl–enzyme formed as a catalytic intermediate? (2) If a phosphoryl–enzyme is formed, is it the same as the phosphoprotein? These questions are so fundamental to the mechanism of alkaline phosphatase that they must be thoroughly investigated and it is especially important to have kinetic evidence bearing on these questions even though the observations briefly described above are consistent with the theory of the phosphoryl–enzyme intermediate and the alternative explanation which is logically possible may seem emotionally unattractive. In this connection it may be remarked that the idea that the phosphoryl–enzyme intermediate would be thermodynamically very stable was not suggested by anyone, and would surely have been widely regarded as unattractive.

We have therefore undertaken further kinetic experiments of a type which can unequivocally show whether or not a transient phosphoryl–enzyme intermediate occurs. The present paper does not deal with the question of the identity of the phosphoprotein and the hypothetical phosphoryl–enzyme intermediate, but only with the question whether a phosphoryl–enzyme intermediate is formed during enzymic hydrolysis. The method used here has been used previously (Epand and Wilson, 1963).

The method is illustrated in reaction I showing

$$\begin{array}{l} E + ROP(OH)_2 \rightleftarrows \\ \qquad\qquad E \cdot ROP(OH)_2 \\ \text{Tris} \qquad\qquad\quad H_2O \\ \text{Tris-P} + \text{ROH} \quad E + P_i + \text{ROH} \end{array} \qquad \begin{array}{c} ? \\ \left[\begin{array}{l} \text{E–P} + \text{ROH} \\ \text{Tris} \qquad H_2O \\ \text{Tris-P} \qquad E + P_i \end{array}\right] \end{array} \quad (I)$$

the hydrolysis of a phosphate ester in the presence of Tris which can serve as a phosphate acceptor, so that *O*-phosphoryl Tris is a product as well as P_i. It has been shown that in the presence of alcohols such as Tris and ethanolamine the rate of utilization of *p*-nitrophenol is increased, that the formation of *p*-nitrophenol exceeds phosphate, and that the difference is due to the formation of the *O*-phosphorylamino alcohol (Dayan *et al.*, 1964). The question is: Do the reactions with water and with Tris emanate from the Michaelis complex or from a phosphoryl–enzyme intermediate (E–P)? If the reactions stem from a phosphoryl–enzyme, the ratio of products Tris-P and P_i must be independent of the leaving group RO, but if the reactions stem from the reversible complex containing the leaving group, the ratio of products must depend upon the structure of R.

The method then is to partially hydrolyze a number of esters with different leaving groups in the presence of 1 M Tris, measure the amounts of the two products and see whether their ratio is independent of the ester used as substrate.

[1] Bovine serum albumin added as a carrier protein is also phosphorylated but only to a slight extent because the phosphate esters that are formed are not, in this case, unusually stable.

TABLE I: Product Ratios and Spectral Data for Phenols and Phosphate Esters. All Measurements at 26° in a Solution 1 M in both Tris and NaCl, pH 8.[a]

Phosphate Ester	Phenol		Ester		λ (Difference Spectrum)	Δε	(Phenol)/PO_4 Ratio
	λ_{max}, mμ	ε	λ_{max}, mμ	ε			
Phenyl	270	1,679	267	674	270	955	2.42 ± 0.02
p-Cresyl	277	1,734	274	902	284	1,165	2.41 ± 0.01
p-Chlorophenyl	280	1,396	276	766	288	1,084	2.38 ± 0.01
p-*t*-Butylphenyl	275	1,600	271.5	802	282	1,268	2.37 ± 0.025
p-Nitrophenyl	400	17,390	308	10,300	400	17,110	2.37 ± 0.01
			326	8,408			
O-Methoxy-*p*-methylphenyl	280	2,712	276	2,286	288	1,327	2.34 ± 0.01
α-Naphthyl	294.5	4,460	286	5,368	322.5	2,180	2.40 ± 0.06
	322	2,720					
β-Naphthyl	274	4,427	274	4,544	330	1,321	2.40 ± 0.04
	328	1,704					
Pyruvic acid	See text		240	1,410	240	See text	2.40
							Av 2.39 ± 0.02

[a] The ratio of products obtained during the partial hydrolysis of phosphate esters in the presence of Tris. The concentration of phenol produced during the reaction is equal to the sum of the concentrations of Tris phosphate and P_i that are also produced as products. The ratio 2.39 indicates that 1.39 equiv of Tris phosphate are formed for every equivalent of P_i.

The concentration of *O*-phosphoryl Tris is not easily measured, but inorganic phosphate and ROH, if ROH is a phenol, can be easily measured, the latter by its optical absorption. We have, therefore, measured ROH and P_i and reported the ratio ROH:P_i for nine different esters.

There is a potential experimental difficulty in the method which, however, turned out to be unimportant. If *O*-phosphoryl Tris were a good substrate relative to the phosphate ester being hydrolyzed, it would, as it is formed as a product, serve as a substrate. The effect would be to lower the ratio of products and in the extreme case the ratio would approach 1. For the method to work, *O*-phosphoryl Tris must be a relatively poor substrate or at least not a better substrate than the test substrates.

Materials

The phosphate esters used as substrates in this work are listed in Table I along with spectral data obtained on a Zeiss PMQ II spectrophotometer. After preliminary purification, all phenols, except *O*-methoxy-*p*-methylphenol, were further purified by distillation and condensation as a solid in a sublimation apparatus. *O*-Methoxy-*p*-methylphenol acid was purified by fractional distillation. Since all these substances deteriorate upon standing, molar extinction coefficients, ε, were measured within a day after purification. All esters, except phosphoenolpyruvate, purchased as the triple salt of cyclohexylamine, were recrystallized in the acid form from mixtures of chloroform with acetone or methanol, or from mixtures of ether and benzene. Precipitation was facilitated by storing the solutions at 0–5°, and occasionally by adding a little hexane. Traces of solvent were removed by heating the esters in an evacuated drying pistol. Four esters (*p*-cresyl, *p*-chlorophenyl, *p*-*t*-butylphenyl, and *O*-methoxy-*p*-methylphenyl) were prepared by the general method of Freeman and Colver (1938) and confirmed as monoesters by C and H analysis. Esters purchased as hydrated sodium salts proved to be of uncertain composition, hence were converted into the acid form by suspending in a mixture of ether and chloroform, passing in HCl gas, and recrystallizing the material recovered by filtration. All esters and phenols were obtained as colorless crystalline products. With two exceptions, melting points agreed with those in the literature; our purified β-naphthyl phosphate melted at 177–179° on a Fisher-Johns apparatus, reported as 167° (Kung, 1894); *p*-nitrophenyl phosphate, recrystallized in the acid form from methanol–chloroform or from ether–benzene, melted at 156–157°, lit. (Rapp, 1884) mp 122°. This melting point, originally reported by Rapp, was obtained on a yellow solid prepared by nitration of phenyl phosphate and recrystallized from hot water. It was stated to be insoluble in cold water, but similar to other phosphate esters, our product was appreciably soluble in cold water, alcohol, and ether, slightly soluble in chloroform.

Alkaline phosphatase was purchased from Worthington Chemical Corp.

Methods

All measurements were carried out at 26° in a buffer solution, 1 M in both Tris and NaCl, and adjusted to pH 8 with concentrated HCl. To measure release of phenol, a solution containing ester but no enzyme was used as a blank in one cuvet. Ester solution with a

measured amount of enzyme was added to the remaining cuvets. Beginning 10 min after addition of enzyme, ten optical density readings were taken over a period of 2.5–3 hr at a wavelength which gave the best difference, $\Delta\epsilon$, between the molar extinction coefficients of the phenol and ester. Immediately after reading a particular cuvet, it was removed and the contents were mixed with 3 ml of 2 N HCl to quench the reaction and to bring the solution to the proper acidity for phosphate determination by the method of Itaya and Ui (Itaya and Ui, 1966). The molar concentration of free phenol at any time was given by the relation: (phenol) = optical density/$\Delta\epsilon$. Normally, with an ester concentration of 5×10^{-4} M and 0.06 μg of enzyme/ml, 70–90% hydrolysis was achieved in 2.5–3 hr. Both naphthyl phosphates were run at $1\text{–}2 \times 10^{-4}$ because of their low solubility in acid solution and consequent interference with the phosphate determination. *p*-nitrophenyl phosphate was run at a similar concentration because of the high extinction coefficient of the free phenol. The enzyme concentration was adjusted proportionately.

Phosphoenolpyruvate was run to provide an entirely different type of substrate for testing the hypothesis. Neither phosphoenolpyruvate nor pyruvate, in Tris buffer at pH .8, gave any absorption maximum between 225 and 400 mμ. At this concentration of Tris, wavelengths below 225 mμ cannot be used. However, phosphoenolpyruvate gives a stable extinction coefficient at 240 mμ where it is usually measured, but the extinction coefficient of pyruvate is not stable. To obtain usable values for pyruvate, a freshly distilled sample was crystallized at 10°, a weighed sample of the crystals was added to a cuvet containing 3 ml of Tris buffer, optical density readings were taken from 2–35 min, and extinction coefficients were calculated for each 3-sec interval. During this time, the extinction coefficient decreased from 375 to 318, or about 15%. A solution of phosphoenolpyruvate, containing ten times the usual enzyme concentration, was then run for 35 sec, and optical density readings were taken every 3 sec. Pyruvate was calculated from: (pyruvate) = optical density/ϵ-ester − ϵ-pyruvate. At any particular time ϵ-pyruvate was taken as the cumulated average; *i.e.*, ϵ at 9 sec was taken as the average of the 3-, 6-, and 9-sec values. This procedure overestimates ϵ, especially in the latter part of the experiment, since hydrolysis of the phosphate esters decreases as phosphate accumulates. However, the 35-sec reading, which would be most affected, gave a (pyruvate/phosphate) ratio of 2.40. The largest possible error would be made by using the value obtained for ϵ for pyruvate at either 3 or 35 sec. Calculations based on these values gave, respectively, ratios of 2.45 and 2.35, or a difference of 2% from 2.40. Hence, the error incurred using the average must have been quite small.

The phosphate method of Itaya and Ui proved to be the most sensitive of those tested, and permitted use of dilute ester solutions. The green color produced by this reagent with phosphate is affected by acidity, time and temperature, hence the acidity was carefully controlled and the solutions were allowed to sit for 2–3 hr at 26° after mixing, at which time the optical density was near maximum. Even so, the solutions do not follow Beer's law exactly. Therefore, a set of standards was prepared for each experiment, an increment was calculated as optical density units/μmoles of P_i for each concentration, and appropriate interpolations were made for the experimental solutions. It is also advisable to add the color reagent to all tubes serially, and subsequently read them in the same order. With these precautions, the method proved capable of giving satisfactorily precise results.

Results

Table I gives spectral data for the phenols and esters and the (phenol/phosphate) ratios. Ratios were determined by plotting micromoles of phenol as ordinate against micromoles of phosphate on large graph paper, drawing by eye the best straight line through the points, and calculating the slope of the line. Figure 1 shows a typical plot on smaller scale paper obtained with *p*-chlorophenyl phosphate, and Figure 2 shows the time course of the reaction. Referring to Figure 1, at some point beyond 80% hydrolysis, the ratio invariably decreased, presumably because the enzyme begins to hydrolyze *O*-phosphoryl Tris as the latter increases in concentration and the original substrate diminishes. Hence, the last point is ignored in determining the phenol/phosphate ratio.

Individual points differ from the line in the phenol-phosphate plot by about 4% on the average which for nine points corresponds to an error of about 1.3% in the ratio derived from the line. This is about the error which we actually obtain from duplicate experiments

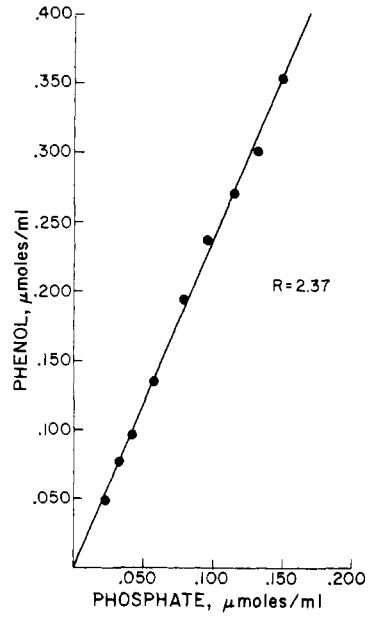

FIGURE 1: The concentration of *p*-chlorophenol is plotted *vs.* the concentration of P_i at the same time during the course of the hydrolysis of the phosphate ester in the presence of 1 M Tris–1 M NaCl (pH 8.0). The ratio of products, *R*, is 2.37 indicating that 1.37 equiv of *O*-phosphoryl Tris is formed for every equivalent of P_i.

and is the average deviation of the ratio obtained with the different compounds from the average value for all the compounds. Thus, the differences in the ratios that we have obtained from compound to compound are within the small experimental error. Two runs were made with each ester.

During the first 10–15 min of hydrolysis, release of phenol was essentially linear. The hydrolysis rate of the aromatic esters fell in the range 33–42 μmoles/(min mg) of enzyme preparation and phosphoenolpyruvate gave a rate of 18.

Thus, all of the substrates, except phosphoenolpyruvate which was hydrolyzed at half the rate of the others, were hydrolyzed at about the same rate. No attempt was made to obtain precise comparisons and some runs were made using relatively old stock enzyme solutions (up to 3 weeks) which were *not* assayed each day against a standard substrate, but the low rate with phosphoenolypruvate was confirmed by comparison with *p*-nitrophenyl phosphate. The lower rates in the group were obtained with older solutions, which may have declined in activity. The results suggest that these substrates are probably hydrolyzed at nearly the same rate. No measurements of K_m were made, but since our concentrations were very high compared with K_m for α-naphthyl phosphate and *p*-nitrophenyl phosphate, it seems likely that most, if not all, of our measurements were made at close to maximum velocity.

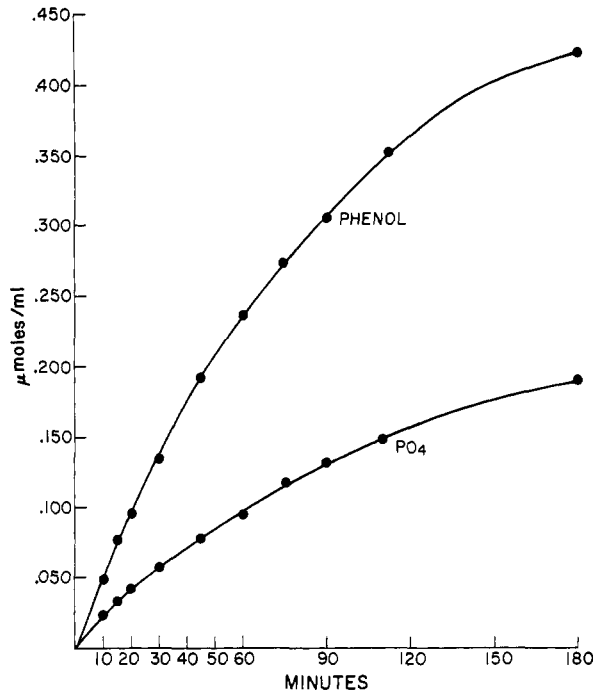

FIGURE 2: Time course for the hydrolysis of *p*-chlorophenyl phosphate; 80% of the ester has been hydrolyzed at 145 min and the rate of hydrolysis at this time is one-fifth the initial rate. The concentration of *O*-phosphoryl Tris is given by the difference in the two curves.

Discussion

The purpose of this study was to ascertain whether the ratio of products, phenol to P_i, in the presence of Tris as a phosphate acceptor competing with water, is independent of the nature of the phosphate ester being catalytically hydrolyzed by alkaline phosphatase. The ratios presented in Table I show that the ratio is independent of the leaving group.

It is self-evident that an invariant ratio of products is a necessary condition for the correctness of the phosphoryl–enzyme theory. We may enquire to what extent it may be a sufficient condition. The answer is that it is to a very high probability a sufficient condition. The two reagents, Tris and water, used in this study are obviously very different in structure and they react as phosphate acceptors at very different rates, 1 M Tris being more than equivalent to 55 M water. The mechanism may also be somewhat different because the effectiveness of Tris and similar compounds seems to depend upon an amino group no further than three carbon atoms removed from the nucleophilic oxygen (Dayan *et al.*, 1964). A series of reactants such as our phosphate esters simply do not give a constant ratio of products when they react with two even rather closely related reagents, let alone such different reagents as water and Tris, unless a common intermediate is involved. The common intermediate here must, of course, be the phosphoryl–enzyme.

To elaborate on this question of the ratio of products obtained when a series of reactants react with two reagents, we may consider the restricted series of *meta*- and *para*-substituted phenyl derivatives. Such series show considerable regularity; they obey the Hammett linear σ–ρ relationship in their reactions. To obtain a constant ratio of products with two reagents, the two reactions series would have to have the same value of ρ. The difference in σ for *p*-CH_3 and *p*-NO_2 is about 1.5, so that if ρ were to differ by as little as 0.2, there would be a range of a factor of 2 in the ratio of products. There is a case listed by Hammett (1940) in which the ρ values differ by only 0.06 which corresponds to a range of only 23% in the ratio of rates. This is the reaction of substituted benzoyl chlorides with methanol and ethanol (Norris *et al.*, 1935; Norris and Young, 1935). These two reagents are clearly more similar than water and Tris. However, even though the ρ values (which involves averaging) are nearly the same, the original data show a range in the ratio of rates from 3.6 for *m*-NO_2 to 6.00 for the unsubstituted compound to 7.9 for *m*-CH_3. Thus, there is actually a range of a factor of 2.2 in the ratio of products. More important, the ratio of the rates for the reaction of *meta*- and *para*-substituted benzoyl chlorides with ethanol and water have a range of a factor of 10 (Norris and Young, 1935). We present this case, which, on initial inspection, seems to indicate only a 23% range in ratio of rates, only to illustrate how unlikely it is that two quite different reagents such as Tris and water could give a constant ratio of rates even in a highly restricted series without the advent of a common intermediate. In this connection, we note that our ratio of rates is constant within an experimental error of 1–2%.

We must also note that our own series of compounds is of a type that does not show the regularity of a linear

σ–ρ relationship in its reactions since our group contains an *ortho*-substituted derivative, two naphthyl derivatives and a compound without a ring, as well as the *para*-substituted phenyl phosphates. If we had restricted our series to say *m*-iodo, *m*-bromo, and *m*-chlorophenyl phosphates, we would, of course, have no argument since iodine, bromine, and chlorine have almost the same σ values.

To summarize, if the members of the series are reasonably different and if the two reagents are also reasonably different, the possibility of obtaining equal ratios of products to the precision that we have obtained is vanishingly small unless a common intermediate is involved. Our compounds easily satisfy the difference requirements and our conclusion that a common intermediate is involved is, we believe, quite sound.

The constant ratio of products does not tell us what the common intermediate is. One might question whether a different intermediate, rather than the phosphoryl–enzyme, is involved. One other possibility does come to mind. It has been proposed that a hypothetical, rapidly hydrating, metaphosphate anion, PO_3^-, may be involved in the hydrolysis (nonenzymic) of the monoanion of phosphate esters (Butcher and Westheimer, 1955). Our results do not, by themselves, distinguish between these possibilities. However, other observations, such as the increased rate of reaction of phosphate ester in the presence of Tris, the burst phenomenon, and the "nonspecificity of the enzyme" can be accommodated by the phosphoryl–enzyme theory, but cannot be explained by the metaphosphate theory. The metaphosphate ion is conceived as an unstable highly reactive species which reacts rapidly with water and other acceptors (Butcher and Westheimer, 1955). Therefore its formation is rate controlling. Since it is formed from different esters it must be formed at different rates and we cannot account for the same high rate of hydrolysis of a large number of different esters. On the other hand, this is easily accounted for, if the mechanism involves a phosphoryl–enzyme, by assuming that dephosphorylation is rate controlling. Similarly the addition of a second acceptor cannot speed up a process involving "metaphosphate ion" because its formation is rate controlling but can speed up a process involving a phosphoryl–enzyme because diphosphorylation can be a rate-controlling step. Even if the formation of metaphosphate is assumed to be rapid rather than slow, metaphosphate would then be a transient *product* the enzyme would be free and enzymic action would not be limited by its persistence. Thus in either case we would not be able to explain the enhanced utilization of substrate in the presence of phosphate acceptors, the nonspecificity of the enzyme, nor the "burst."

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