The Catalytic-Centre Activity and Kinetic Properties of Bovine Milk Alkaline Phosphatase

By T. E. BARMAN* AND H. GUTFREUND†

National Institute for Research in Dairying, Shinfield, nr. Reading

(Received 25 April 1966)

1. The phosphorylation of milk alkaline phosphatase was studied under various conditions: maximum incorporation occurred at pH 5.0 and 50% incorporation at pH 6.6–7.0. 2. The phosphorylation was shown to be specific and the results suggest that the active centre of the enzyme is involved in the process. 3. Phosphoryl-enzyme is rapidly hydrolysed at alkaline pH. At pH 7.0 the results suggest that a phosphoryl-enzyme could occur as a transient intermediate in the hydrolysis of phosphate esters by the phosphatase. 4. The catalytic-centre activity of the enzyme was found to be 2700sec^{-1} at pH 10.0 and 25° with *p*-nitrophenyl phosphate as substrate.

Alkaline phosphatases (orthophosphoric mono-ester phosphohydrolase, EC 3.1.3.1) from a number of sources have been found to belong to the group of enzymes that have one particularly reactive serine hydroxyl group per molecule (e.g. Schwartz, 1963). In the alkaline phosphatases, the serine residue is readily phosphorylated by incubation with orthophosphate in slightly acid solutions, a property that is not shared by any other protein tested (Engstrom & Agren, 1958; Schwartz & Lipmann, 1961). A number of kinetic investigations of the reactions of the *Escherichia coli* phosphatase have yielded data compatible with the hypothesis that a phosphoryl-enzyme is an intermediate during the enzymically catalysed transfer of phosphate from a phosphate ester substrate to water or some other nucleophilic acceptor (Schwartz, 1963; Aldridge, Barman & Gutfreund, 1964; Wilson & Dayan, 1965). A detailed analysis of the kinetic behaviour of calf-intestinal phosphatase (Fernley & Walker, 1965) led the authors to the conclusion that pH-dependent structural changes of the enzyme are of importance.

The experiments with milk alkaline phosphatase described in the present paper were carried out primarily to investigate the possibility of determining the concentration of active sites of this enzyme and their turnover number in unfractionated biological materials. Further, the kinetic and equilibrium properties of the phosphorylation and dephosphorylation of the phosphatase were investigated to gain information about the catalytic mechanism of this enzyme.

* Present address: Department of Biochemistry, University of California, Berkeley, Calif., U.S.A.

† Present address: Department of Biochemistry, University of Bristol.

MATERIALS AND METHODS

Chemicals. *p*-Nitrophenyl phosphate was obtained from Askell Laboratory Service (London, S.E. 6), Sephadex G-200 from Pharmacia (Uppsala, Sweden), β -glycerophosphate from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and carrier-free [^{32}P]orthophosphate from The Radiochemical Centre, Amersham, Bucks.

Assay methods. Alkaline-phosphatase activity was estimated spectrophotometrically at $406\text{m}\mu$ with *p*-nitrophenyl phosphate as substrate at 25° . At pH 7.5 and above, 50 mM-sodium carbonate-bicarbonate was used as buffer; at pH 7.0 and below 50 mM-sodium acetate-acetic acid was employed. In all cases the reaction mixture (3.0 ml.) contained *p*-nitrophenyl phosphate (5 mM) and magnesium acetate (1 mM). (Mg^{2+} is essential for the activity of the milk enzyme but over the range 1–50 mM its concentration is not critical.) The hydrolysis was observed continuously with a Beckman DB recording spectrophotometer at pH 6.5 and above. At pH values lower than 8.5 the observed extinctions were corrected for the pK_a of *p*-nitrophenol; the data of Biggs (1954) were used for the pH-dependence of extinction at different wavelengths. At pH 6.0 and below the enzyme was assayed as for acid phosphatase by the method of Bingham & Zittle (1963). In the standard assay the reaction mixture was at pH 10.0.

In certain experiments β -glycerophosphate was used as substrate. The final concentrations of the reactants (in 3 ml.) were: β -glycerophosphate (10 mM), magnesium acetate (1 mM) and sodium carbonate-bicarbonate buffer, pH 10.0 (50 mM). At appropriate time-intervals the reaction was stopped by the addition of 0.5 ml. of 2*N*- H_2SO_4 and the concentration of orthophosphate determined by the method of Briggs (1922).

One alkaline-phosphatase unit is defined as that amount of enzyme which will hydrolyse $1\mu\text{mole}$ of *p*-nitrophenyl phosphate/min. at pH 10 and 25° .

Separation of phosphoryl-enzyme from [^{32}P]orthophosphate. In a typical experiment 27 units of alkaline phosphatase (0.88 mg. of protein in 0.1 ml. of 50 mM-magnesium acetate solution) were added to a reaction mixture (0.9 ml.)

containing carrier-free [^{32}P]orthophosphate (2×10^7 counts/min.; experimentally measured, see below), sodium orthophosphate (0.2 mM) and buffer according to the pH of the experiment (see above). The solution was kept at 0° for 1 min., and then 0.2 ml. of 20% (v/v) HClO_4 was added and the resulting precipitate collected by centrifugation. The pellet was washed five times with 6 ml. of 5% (v/v) HClO_4 and dissolved in 90% (v/v) formic acid (0.5 ml.). Water (4.5 ml.) was added and the mixture centrifuged. The phosphoryl-enzyme was precipitated from the supernatant by the addition of 1.0 ml. of 20% HClO_4 and collected by centrifugation followed by washing three times with 6 ml. of 5% HClO_4 . The final pellet was dissolved in 90% formic acid (1.0 ml.) and a portion (0.5 ml.) withdrawn and placed on a planchet. The acid was removed by gentle heating on a hot-plate (80 – 90°) and the activity counted (IDL scintillating counter; Isotope Developments Ltd., Beenhams, Berks.). The efficiency of the counter was 49%.

Determination of molecular weight. Estimates of the molecular weight of milk alkaline phosphatase were obtained by gel filtration on columns of Sephadex G-200. The method used was essentially that of Andrews (1965). Thus 5.4 units of milk alkaline phosphatase (1.7 mg. of protein) were applied to a column (50 cm. $\times$ 2.5 cm.) of Sephadex G-200 that had previously been equilibrated with one of three buffers: (1) pH 5.0; 50 mM-sodium acetate-acetic acid buffer-0.10 M-KCl-30 mM-magnesium acetate; (2) pH 7.5; 50 mM-tris buffer-0.12 M-KCl; or (3) pH 9.5; 50 mM-sodium carbonate-bicarbonate buffer-0.12 M-KCl. The calibration proteins employed were thyroglobulin (mol.wt. 670 000), γ -globulin (mol.wt. 205 000; see Andrews 1965), bovine serum albumin (mol.wt. 67 000), chymotrypsinogen (mol.wt. 25 000) and cytochrome c (mol.wt. 12 500).

Protein. This was determined by the micro-Kjeldahl method.

Treatment of kinetic data. It is generally agreed that orthophosphate is first bound to alkaline phosphatase to form a Michaelis-Menten-type complex before the serine at the catalytic centre is phosphorylated (e.g. see Hummel & Kalnitsky, 1964). Fernley & Walker (1965) concluded from their studies of the hydrolysis of phosphate esters by the calf-intestinal phosphatase that this enzyme occurs in two conformational forms, E_1 and E_2 : only E_1 combines with substrate and only E_2 reacts to release inorganic phosphate. To accommodate the above observations, a mechanism describing the hydrolysis of phosphate esters by alkaline phosphatases must include at least the following steps:

and, consequently, the phosphorylation process may be described by:

Under acid conditions phosphoryl-enzyme (E_1P) is formed and can be isolated by HClO_4 precipitation. E_2P cannot thus be isolated. Now, if it is assumed that, when $[\text{P}]_0 \gg [\text{E}]_0$, $[\text{E}_1] \rightarrow 0$, the steady-state solution for the phosphorylation process can be written as:

$$\frac{[\text{P}]_0}{[\text{E}_1\text{P}]} = [\text{P}]_0 \cdot \frac{1}{[\text{E}]_0} \cdot [K_p(c+1) + 1] + \frac{1}{[\text{E}]_0} \cdot K_p K_i(c+1) \quad (3)$$

where $[\text{P}]_0$ is the total concentration of orthophosphate, $[\text{E}]_0$ is the total concentration of alkaline-phosphatase active sites, $[\text{E}_1\text{P}]$ is the concentration of phosphoryl-enzyme, $K_i = k_{+4}/k_{-4}$, $K_p = k_{+3}/k_{-3}$ and $c = ([\text{P}]_0 - [\text{H}_2\text{PO}_4^-])/[\text{H}_2\text{PO}_4^-]$. At pH 5, $[\text{P}]_0 \rightarrow [\text{H}_2\text{PO}_4^-]$ and eqn. (3) reduces to:

$$\frac{[\text{P}]_0}{[\text{E}_1\text{P}]} = [\text{P}]_0 \cdot \frac{1}{[\text{E}]_0} + \frac{1}{[\text{E}]_0} \cdot \frac{k_{+3}}{k_{-4}} \quad (4)$$

Thus a plot of $[\text{P}]_0/[\text{E}_1\text{P}]$ against $[\text{P}]_0$ should give a straight line from which $[\text{E}]_0$ can be obtained.

The rate of decomposition of phosphoryl-enzyme can be described as either:

$$\ln \left(\frac{[\text{E}_1\text{P}]_0 - [\text{E}_1\text{P}]_t}{[\text{E}_1\text{P}]_0 - [\text{E}_1\text{P}]_e} \right) = (k_{+3} + k_{-3})t \quad (5)$$

if $k_{+4} \gg k_{+3}$ or:

$$\ln \left(\frac{[\text{E}_1\text{P}]_0^2 - [\text{E}_1\text{P}][\text{E}_1\text{P}]_t}{([\text{E}_1\text{P}]_0 - [\text{E}_1\text{P}]_e)[\text{E}_1\text{P}]_0} \right) = k_{+4} \cdot \frac{[\text{E}_1\text{P}]_0 + [\text{E}_1\text{P}]_t}{[\text{E}_1\text{P}]_0 - [\text{E}_1\text{P}]_e} \cdot t \quad (6)$$

if $k_{+4} \ll k_{+3}$ (Frost & Pearson, 1962), where $[\text{E}_1\text{P}]_0$ is the initial concentration of phosphoryl-enzyme, $[\text{E}_1\text{P}]_e$ is the equilibrium concentration of phosphoryl-enzyme and $[\text{E}_1\text{P}]_t$ is the concentration of phosphoryl-enzyme at time t .

RESULTS

Purification of milk alkaline phosphatase. The enzyme was purified by a modification of the method of Lyster & Aschaffenburg (1962). Raw buttermilk (24 l.) was treated in 31 batches at pH 6.6 and at room temperature with 1.2 l. of n -butanol and then adjusted to pH 5.0 with 0.25 N-acetic acid. The serum was filtered through Hyflo Super-Cel and, after adjustment to pH 8.5 with 0.2 N-sodium hydroxide, left at 4 – 5° overnight.

The supernatant was collected by decantation, adjusted to pH 6.3 and ice-cold acetone added to 50% (v/v). The mixture was left overnight at room temperature and the resulting precipitate collected and extracted with 50 mM-magnesium acetate (3×200 ml.). After centrifugation, the combined extracts were fractionated with ice-cold acetone, the material precipitated at $33\frac{1}{3}$ –50% (v/v) acetone was dissolved in 50 mM-magnesium acetate (15 ml.) and excess of acetone was removed by freeze-drying. The residue was extracted with 15 ml. of water and centrifuged at 105 000 g for 60 min. to yield a red solution containing 8.8 mg. of protein/ml. The specific activity of the preparation was 31 units/mg. of protein at pH 10.0 and 25° .

Fig. 1. pH profile of the $[^{32}\text{P}]$ orthophosphate fixation (○) and of the *p*-nitrophenyl-phosphatase activity (●) of milk alkaline phosphatase. It was assumed that 100% of the attainable incorporation of orthophosphate and 100% *p*-nitrophenyl-phosphatase activity occurred at pH 5.0 and 10.0 respectively. For experimental details see the Materials and Methods section.

pH optima of phosphorylation and of *p*-nitrophenyl-phosphatase activity. Maximum phosphorylation occurred at about pH 5.0 and on the alkaline side of this optimum 50% phosphorylation occurred at pH 6.7 (Fig. 1). The pH profile of V_{max} (*p*-nitrophenyl-phosphatase activity) rises to a maximum at pH 10, after which it rapidly declines.

Effect of denaturation on the incorporation of $[^{32}\text{P}]$ orthophosphate. The enzyme was found to be slowly denatured when kept at pH 4.6 and 40° and the effect of this treatment on its ability to incorporate $[^{32}\text{P}]$ orthophosphate was investigated. Thus samples (0.7 ml.), each containing 27 phosphatase units (0.87 mg. of protein) in 66 mM-sodium acetate-acetic acid buffer, pH 4.6, were kept at 40° and removed at appropriate time-intervals (1 min. to 1 hr.) and placed on ice. Portions (0.02 ml.) were assayed for alkaline-phosphatase activity by the standard method except that the increase in extinction was observed at 450 m μ rather than at 400 m μ . Little reactivation of phosphatase activity in denatured samples occurred over a period of 10 min. but over longer intervals (30–40 min.) the activity exhibited a definite increase. After 1 hr. at pH 9.6 the enzyme left in the remaining 0.68 ml. was immediately phosphorylated by the addition of $[^{32}\text{P}]$ orthophosphate and magnesium acetate. The final concentrations (in 1 ml.) were: sodium orthophosphate (1 mM, 8.5×10^6 counts/min.), magnesium acetate (35 mM) and sodium acetate buffer,

Fig. 2. Inactivation of alkaline phosphatase at pH 4.6. The enzyme was incubated at pH 4.6 and 40°, and samples were removed at appropriate time-intervals and assayed for *p*-nitrophenyl-phosphatase activity and their ability to incorporate $[^{32}\text{P}]$ orthophosphate. Both phosphate fixation and *p*-nitrophenyl-phosphatase activity are plotted as percentages of the initial enzyme properties of the preparation. For experimental details see the Results section.

pH 5.0 (50 mM). The mixture was kept at 0° for 1 min. and then quenched by the addition of 20% perchloric acid (0.2 ml.), and the precipitated protein was separated from excess of $[^{32}\text{P}]$ orthophosphate as described in the Material and Methods section. Fig. 2 indicates that the enzyme loses its ability to incorporate phosphate when it is heat-denatured and that this loss closely parallels the decrease in *p*-nitrophenyl-phosphatase activity.

Molecular weight. The enzyme behaved on a column of Sephadex G-200 as a protein of mol.wt. 190 000 ($\pm 10 000$) at pH 5.0, 7.5 or 9.5.

Catalytic-centre activity of the enzyme. According to eqn. (4) (see the Materials and Methods section) the concentration of alkaline-phosphatase active sites in a given preparation can be obtained by titration with $[^{32}\text{P}]$ orthophosphate. Thus reaction mixtures (1.0 ml.) containing enzyme (27.6 units), magnesium acetate (35 mM), sodium acetate-acetic acid buffer, pH 5.0 (50 mM), and carrier-free orthophosphate (1.3×10^7 counts/min.) were incubated for 1 min. at 0° with various amounts of orthophosphate (1 μM –1 mM). $[^{32}\text{P}]$ Phosphoryl-enzyme

Fig. 3. Titration of alkaline phosphatase with [^{32}P]orthophosphate at pH 5.0. The data were plotted as described by eqn. (4). For experimental details see the Results section.

Fig. 4. Titration of alkaline phosphatase with [^{32}P]orthophosphate at pH 7.0. The data were plotted as described by eqn. (3). For experimental details see the Results section.

was precipitated and purified as described in the Materials and Methods section. The results are shown in Fig. 3, from which it was calculated that the concentration of active sites used was $0.17 \mu\text{M}$, and thus the catalytic-centre activity of the enzyme is 2700 sec^{-1} with *p*-nitrophenyl phosphate or 2240 sec^{-1} with β -glycerophosphate as substrate. If it is assumed that the enzyme has one catalytic site/mol. of mol.wt. 190000 it can be concluded that the specific activity of the pure enzyme is 854 units/mg. of protein (also see the Discussion section). The purity of the preparation used was therefore 3.6% and that of the purest to date (Lyser & Aschaffenburg, 1962) 62%, and, further, the concentration of alkaline phosphatase in whole milk is $0.24 \text{ m}\mu\text{M}$.

Incorporation of orthophosphate at pH 7.0. Eqn. (3) shows that K_i and K_p can be obtained from phosphorylation experiments at pH 7.0. Consequently, enzyme (27.6 units or $0.17 \mu\text{M}$) was incubated with magnesium acetate (35 mM), sodium acetate-acetic acid buffer (50 mM), carrier-free orthophosphate (2.1×10^7 counts/min.) and various amounts of orthophosphate at pH 7.0. Fig. 4 shows that a straight line is obtained from which $K_i = 1.94 \times 10^{-4} \text{ M}$ and $K_p = 0.5$.

K_m for *p*-nitrophenyl phosphate and K_i for orthophosphate at pH 10.0 and 7.0. At pH 10.0 the constants were readily obtained by conventional means: the K_m for *p*-nitrophenyl phosphate was $4.7 \times 10^{-4} \text{ M}$ and the K_i for orthophosphate $3 \times 10^{-4} \text{ M}$ (with *p*-nitrophenyl phosphate as substrate). However, the K_m for *p*-nitrophenyl phosphate at pH 7.0 was so small that it was difficult to measure accurately (approx. $2 \times 10^{-6} \text{ M}$). The K_i for orthophosphate was approx. $1 \times 10^{-4} \text{ M}$. Both values were obtained from experiments where at different

orthophosphate concentrations the *p*-nitrophenyl phosphate concentration was varied, thus giving rise to a series of apparent Michaelis constants. A plot of $K_m(\text{app.})$ against orthophosphate concentration yielded the required constants. At both pH values orthophosphate competitively inhibited the hydrolysis of *p*-nitrophenyl phosphate.

Alkaline hydrolysis of the phosphoryl-enzyme. The technique used was essentially that described by Aldridge *et al.* (1964). Phosphoryl-enzyme was prepared by incubating at room temperature ($20-23^\circ$) alkaline phosphatase ($1.7 \mu\text{M}$, 276 units), carrier-free [^{32}P]orthophosphate (8×10^7 counts/min.), orthophosphate (0.2 mM), magnesium acetate (60 mM) and sodium acetate-acetic acid buffer, pH 5.0 (0.4 M). The rate of dephosphorylation of the phosphoryl-enzyme was studied by mixing the above solution in the rapid-flow quenching apparatus of Barman & Gutfreund (1964) in different experiments with the same volume of (1) 50 mM -sodium acetate-acetic acid buffer, pH 5.0, (2) 90 mM -sodium hydroxide or (3) 0.2 M -sodium carbonate buffer, pH 10.0. The buffers contained 0.4 mg. of serum albumin/ml. The final pH values of the reaction mixtures ($3.4-3.8 \text{ ml.}$) were 5.0, 7.0 and 9.6 respectively. These were quenched after the indicated reaction times (see Fig. 5) in 3 ml. of 20% perchloric acid and the precipitated protein was separated from excess of [^{32}P]orthophosphate and counted as described in the Materials and Methods section.

At pH 9.6 the dephosphorylation was extremely rapid and little phosphoryl-enzyme could be detected after a reaction time of 3 msec. Figs. 5 and 6 show the results obtained at pH 7.0. As noted above, these results can be interpreted in two ways: (1) $k_{+4} \gg k_{+3}$: eqn. (5) yields $k_{+3} + k_{-3} = 164 \text{ sec}^{-1}$ and, since $K_p = 0.5$, $k_{+3} = 54 \text{ sec}^{-1}$ (Fig.

Fig. 5. Decomposition of phosphoryl-enzyme at pH 7.0 in the rapid-flow quenching apparatus. For experimental details see the Results section.

Fig. 6. Decomposition of phosphoryl-enzyme at pH 7.0. The data obtained in Fig. 5 were plotted according to (a) eqn. (5), where $k_{+4} \gg k_{+3}$, or (b) eqn. (6), where $k_{+4} \ll k_{+3}$.

Table 1. Kinetic constants of partially purified milk alkaline phosphatase at pH 7.0

Experimental details are given in the Results Section.

Constant	Value	Method
K_m	$\sim 2 \times 10^{-7} M$	<i>p</i> -Nitrophenyl phosphate as substrate
K_i	$\sim 1 \times 10^{-4} M$	Inhibitor studies with <i>p</i> -nitrophenyl phosphate as substrate
K	$1.94 \times 10^{-4} M$	Phosphorylation experiments
K_p	0.5	Phosphorylation experiments
$k_{obs.}$	15 sec. ⁻¹	<i>p</i> -Nitrophenyl-phosphatase activity
k_{+3}	54 sec. ⁻¹ ($k_{+4} \gg k_{+3}$)	Dephosphorylation experiments
k_{+4}	119 sec. ⁻¹ ($k_{+4} \ll k_{+3}$)	Dephosphorylation experiments

6a). (2) $k_{+4} \ll k_{+3}$: in this case, eqn. (6) must be used and, from Fig. 6(b), $k_{+4} = 119 \text{ sec.}^{-1}$. Since at pH 7.0 $k_{obs.} = 15 \text{ sec.}^{-1}$, the dephosphorylation of phosphoryl-enzyme cannot be the rate-limiting step in the hydrolysis of phosphoryl esters by milk alkaline phosphatase.

The kinetic constants obtained at pH 7.0 are summarized in Table 1.

DISCUSSION

Our results are interpreted within the kinetic scheme given above. Thus the enzyme has a catalytic-centre activity of 2700 sec.^{-1} with *p*-nitrophenyl phosphate as substrate. This must be a maximum value; it assumes that the phosphorylation is complete under the conditions used. Only detailed chemical studies, involving the determination of amino acid sequences of ^{32}P -labelled peptides, will be able to establish whether extrapolation to infinite phosphate concentration at pH 5 (see Fig. 3) results in a real 100% phosphorylation of active sites. The value for the specific activity of the pure enzyme is less certain. Pigretti & Milstein (1965) concluded that both chains of the native *E. coli* phosphatase (Rothman & Byrne, 1963; Schlesinger, 1964) can incorporate inorganic phosphate and, consequently, if the milk enzyme is composed of four sub-units (each of mol.wt. 47 500) that can all be phosphorylated, then the specific activity of this enzyme could be 3440 units/mg. If this were the case, each sub-unit must be phosphorylated independently of the others since no co-operative effect was observed in our experiments (Fig. 3). It is noteworthy that the molecular activity of the bovine liver enzyme is about

2650 sec.⁻¹ and that this enzyme also behaves on a Sephadex G-200 column as a protein of mol.wt. 190 000 (Engstrom, 1964).

The assumption that the phosphorylation occurs at the catalytic centre of the phosphatase is supported by the following (see also Schwartz, 1963):

(1) Orthophosphate inhibited the enzyme competitively at pH 7.0 where the enzyme at once hydrolyses *p*-nitrophenyl phosphate and incorporates orthophosphate.

(2) No phosphorylation by [³²P]orthophosphate occurred in the presence of substrates at pH 5.0. The lack of incorporation could not be accounted for by isotope dilution.

(3) In denaturation experiments (Fig. 2) loss of *p*-nitrophenyl-phosphatase activity closely paralleled the concomitant loss of ability to incorporate orthophosphate.

(4) Phosphoryl-enzyme was rapidly hydrolysed by alkali.

The mechanism summarized by eqn. (3) assumes that the orthophosphate species active in the phosphorylation process is H_2PO_4^- . Thus, in the presence of constant orthophosphate and enzyme concentrations, the pH profile of the formation of phosphoryl-enzyme should be in the form of a titration curve. Such a relationship is indeed obtained (Fig. 1), 50% of the attainable phosphorylation occurring at pH 6.6–6.7, a value close to the $\text{p}K_2$ of orthophosphate. There is, however, some doubt about the pH at which 50% of the theoretically possible phosphorylation occurs, since 100% incorporation may not be attainable. The decrease in the amount of phosphoryl-enzyme formed on the acid side of the pH optimum is probably due to non-specific inactivation of the enzyme (Pigretti & Milstein, 1965).

Ray & Koshland (1963) introduced an elegant concept for determining the total concentration of catalytic sites in a mixture of fully or partially active enzyme molecules that they call an 'all-or-none' assay. This method can only be used where stable and determinable enzyme-substrate or enzyme-inhibitor complexes are formed. The alkaline phosphatases fall within this category (Ray & Koshland, 1963; Pigretti & Milstein, 1965). Our experiments, for example, show that the milk enzyme can be phosphorylated specifically in crude preparations and that the phosphorylation almost certainly involves the active centre of the enzyme. An enzyme-inhibitor complex is formed, namely phosphoryl-enzyme, and this can be isolated and its concentration estimated. The phosphorylation process is rapid and is complete in less than 15 sec. with 0.1 mM-phosphate at pH 5 or 7. In the presence of a large excess of orthophosphate, alkaline-phosphatase active sites can readily be determined (Fig. 2). However, it has not been

demonstrated that altered enzyme molecules can be labelled specifically and, furthermore, additional labelling of the *E. coli* enzyme is known to occur on prolonged exposure to orthophosphate (Aldridge *et al.* 1964). It should be possible, however, to differentiate between specific and non-specific labelling by dephosphorylation experiments.

Until recently it was generally agreed that a phosphoryl-enzyme complex was an obligatory intermediate in the hydrolysis of phosphoryl esters and that its decomposition into free enzyme and orthophosphate was the rate-limiting step in the sequence of events leading to products (e.g. see Morton, 1953; Schwartz, 1963; Hummel & Kalnitsky, 1964). This conveniently explained the observation that these enzymes catalyse the hydrolysis of a large number of phosphoryl esters with widely different leaving groups with equal facility. However, the work of Aldridge *et al.* (1964) showed that a step preceding the dephosphorylation reaction must be rate-limiting, and Fernley & Walker (1965) concluded that conformational changes of the enzyme protein are important in determining the rate of hydrolysis of phosphoryl esters. Now, since a conformational change appears to be involved in the hydrolysis of phosphoryl-enzyme and since our experiments and those of Aldridge *et al.* (1964) suggest that this step is rapid, it appears reasonable that the step characterized by k_{+5} is the rate-limiting step in the hydrolysis of phosphoryl esters. However, it is conceivable that the reaction mechanism does not involve a phosphoryl-enzyme at all and that, instead, an intermediate breaks down with the simultaneous liberation of products. A rate-limiting conformational change could precede this reaction. Thus the formation of phosphoryl-enzyme could be due to a side reaction arising from an intermediate by the quenching. A similar note of caution was given by Barman & Gutfreund (1966) in their discussion of trypsin- and chymotrypsin-catalysed ester hydrolysis. However, our experiments are consistent with a phosphoryl-enzyme intermediate, though at best it can only have a transient existence.

These studies were supported by grants from the U.S. Department of Agriculture, PL480, and by the U.S. Public Health Service.

REFERENCES

- Aldridge, W. N., Barman, T. E. & Gutfreund, H. (1964). *Biochem. J.* **92**, 23c.
- Andrews, P. (1965). *Biochem. J.* **96**, 595.
- Barman, T. E. & Gutfreund, H. (1964). In *International Colloquium on Rapid Mixing and Sampling Techniques in Biochemistry*, p. 339. Ed. by Chance, B., Eisenhardt, R. H., Gibson, Q. H. & Lonberg-Holm, K. K. New York: Academic Press Inc.

- Barman, T. E. & Gutfreund, H. (1966). *Biochem. J.* **101**, 411.
- Biggs, A. I. (1954). *Trans. Faraday Soc.* **50**, 800.
- Bingham, E. W. & Zittle, C. A. (1963). *Arch. Biochem. Biophys.* **101**, 471.
- Briggs, A. P. (1922). *J. biol. Chem.* **53**, 13.
- Engstrom, L. (1964). *Biochim. biophys. Acta*, **92**, 71.
- Engstrom, L. & Agren, G. (1958). *Acta chem. scand.* **12**, 357.
- Fernley, H. N. & Walker, P. G. (1965). *Biochem. J.* **97**, 95.
- Frost, A. A. & Pearson, R. G. (1962). *Kinetics and Mechanism*, p. 186. New York: John Wiley and Sons Inc.
- Hummel, J. P. & Kalnitsky, G. (1964). *Annu. Rev. Biochem.* **33**, 15.
- Lyster, R. L. J. & Aschaffenburg, R. (1962). *J. Dairy Res.* **29**, 21.
- Morton, R. K. (1953). *Biochem. J.* **55**, 786.
- Pigretti, M. M. & Milstein, C. (1965). *Biochem. J.* **94**, 106.
- Ray, W. J. & Koshland, D. E. (1963). *J. Amer. chem. Soc.* **85**, 1977.
- Rothman, F. & Byrne, R. (1963). *J. molec. Biol.* **6**, 330.
- Schlesinger, M. J. (1964). In *Brookhaven nat. Lab. Symp. Subunit Structure of Proteins: Biochemical and Genetic Aspects*, p. 66.
- Schwartz, J. H. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 871.
- Schwartz, J. H. & Lipmann, F. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1996.
- Wilson, I. B. & Dayan, J. (1965). *Biochemistry*, **4**, 645.