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The Kinetics of the Reaction of Nitrophenyl Phosphates with Alkaline Phosphatase from *Escherichia coli*

BY D. R. TRENTHAM AND H. GUTFREUND
Department of Biochemistry, University of Bristol

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1. The steady-state rate of hydrolysis of 2,4-dinitrophenyl phosphate catalysed by *Escherichia coli* phosphatase is identical with that of 4-nitrophenyl phosphate over the pH range 5·5–8·5. 2. The increase in the rate of the enzyme-catalysed decomposition of nitrophenyl phosphates in the presence of tris at pH 8·1 and 5·9 is consistent with the hypothesis that tris increases the rate of decomposition of a phosphoryl-enzyme intermediate. At pH 8·1 the rate of decomposition of the phosphoryl-enzyme is approximately twice as fast as the rate of its formation, whereas at pH 5·9 the rate of formation of the phosphoryl-enzyme is considerably faster than its decomposition. 3. Pre-steady-state measurements of the initial transient of the liberation of 2,4-dinitrophenol during the reaction of the enzyme with 2,4-dinitrophenyl phosphate confirmed the above pH-dependence of the ratio of the rates of phosphorylation and dephosphorylation of the enzyme. At optimum pH (above pH 8), when the phosphorylation of the enzyme by the substrate is rate-determining, this step must be controlled by a rearrangement of the enzyme or enzyme–substrate complex.

The evidence for the occurrence of a phosphoryl-enzyme as an intermediate of the reaction between phosphate esters and various alkaline phosphatases has been summarized by Schwartz (1963) and Barman & Gutfreund (1966*b*). The fact that a large number of phosphate esters are hydrolysed at the same rate in the presence of these enzymes, together with other evidence, led Wilson, Dyan & Cyr (1964) to suggest that the hydrolysis of the phosphoryl-enzyme is a common rate-determining step of the overall reaction of alkaline phosphatase from *Escherichia coli*. Aldridge, Barman & Gutfreund (1964) measured the rate of hydrolysis of the phosphoryl-enzyme by a rapid-flow technique and showed that it is approximately twice as fast as the turnover of the enzyme at pH 8 and that this reaction could not be rate-determining under the conditions used by Wilson *et al.* (1964). Fernley & Walker (1966) used the stopped-flow technique to follow fluorescence changes during the steps of the reaction of phosphatase with 4-methylumbelliferyl phosphate. Their elegant experiments confirmed that above pH 7 the hydrolysis of the phosphoryl-enzyme is faster than its formation, and they determined the pH-dependence of the rates of phosphorylation and dephosphorylation below pH 7.

The recent synthesis of 2,4-dinitrophenyl phosphate (Kirby & Varvoglis, 1966) made it possible to investigate the steps of the reaction of phosphatase with two structurally similar substrates with leaving groups of considerably different reactivities. A study of the reaction of alkaline phosphatase with nitrophenyl phosphates offers an opportunity to compare really accurate kinetic data with information obtained from physicochemical studies of these substrates in non-enzymic reactions (Kirby & Varvoglis, 1967).

MATERIALS

E. coli alkaline phosphatase (EC 3.1.3.1) was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). The specific activity at 25° in a solution of 1·00 M-NaCl and 0·050 M-tris adjusted to pH 8·0 with 4 N-HCl with 4-nitrophenyl phosphate as substrate was 14 μmoles/min./mg. of enzyme. Comparing this activity with values obtained by Malamy & Horecker (1964) for the pure crystalline enzyme and corrected to identical conditions, we conclude that approx. 47% of the protein was active enzyme. This value and the molecular weight 86000 (Schlesinger & Barrett, 1965) were used to calculate the molar concentration of active enzyme.

Disodium 4-nitrophenyl phosphate tetrahydrate was obtained from Sigma Chemical Co. The mono-2,6-lutidine salt of 2,4-dinitrophenyl phosphate was given by Dr A. J. Kirby (its synthesis and properties have been described by Kirby & Varvoglis, 1966). Both salts were assayed spectrophotometrically before and after enzyme-catalysed hydrolysis and were more than 98% pure.

A.R.-grade salts and amines were used without further purification. Water was glass-distilled. 2,4-Dinitrophenol and 4-nitrophenol were recrystallized.

METHODS AND RESULTS

The rates of hydrolysis of nitrophenyl phosphate esters were calculated from the extinction change associated with the release of 4-nitrophenol and 2,4-dinitrophenol from their respective phosphates.

For the steady-state reactions, the rates of hydrolysis were followed spectroscopically in a Beckman DB recording spectrophotometer with a thermostat cell compartment and 1cm. glass or silica cuvettes. The cell compartments were maintained at $25 \pm 1°$. The steady-state reactions were initiated by adding the enzyme to the assay medium. The enzyme suspension was used directly after appropriate dilution without removing ammonium sulphate except where the contrary is stated.

The pH values of the reaction mixtures were measured at room temperature at the end of the reactions. The rate of uncatalysed hydrolysis of the substrates was always at least 100-fold less than that of the enzyme-catalysed hydrolysis. In general, spectrophotometric records of 4-nitrophenyl phosphate hydrolysis were made at 400mμ and of 2,4-dinitrophenyl phosphate hydrolysis at 360mμ. 4-Nitrophenolate anion had $\epsilon_{max.}$ $1{\cdot}83 \times 10^4$ at 400mμ (Biggs, 1954) and 2,4-dinitrophenolate anion had $\epsilon_{max.}$ $1{\cdot}45 \times 10^4$ at 360mμ (Gutfreund, 1965). The spectra in the range 320–500mμ of known concentrations of the phenols were recorded at pH 1·1 and 10·0. The extinction values of the phenols and their phosphates were measured at the same pH and salt and buffer concentrations as used in the assays. Hence the pK of the phenols and the molar extinction coefficients of the materials giving rise to the observed extinction change could be calculated.

The relative rates of hydrolysis of 4-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate catalysed by alkaline phosphatase were studied in the pH range 5·5–8·5 at 25°. Within experimental error the rates of the enzyme-catalysed hydrolysis of the two phosphates are identical at any pH value in this range (Fig. 1). The rate of hydrolysis of both phosphates was inversely proportional to H^+ ion concentration in the range pH 5·5–6·8. 4-Nitrophenol had pK 6·84 and 2,4-dinitrophenol had pK 3·76 when measured under the experimental conditions.

The rate of the enzyme-catalysed hydrolysis of each of the two phosphates was measured with both present in the same solution by following the rate of extinction change at 345, 360 and 400mμ at 25°. At 345mμ there was no change in extinction due to the hydrolysis of 4-nitrophenyl phosphate. The

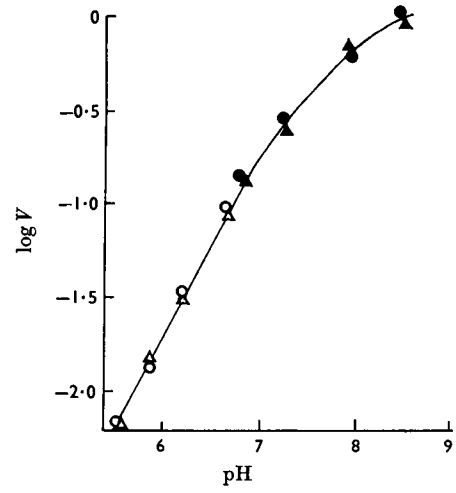

Fig. 1. Dependence of rate of the enzyme-catalysed hydrolyses of 2,4-dinitrophenyl phosphate and 4-nitrophenyl phosphate on pH at 25°. V is the relative rate of hydrolysis of substrate at arbitrary unit concentration of enzyme. The assay media contained substrate (200μM), alkaline phosphatase (0·50–33·3μg./ml.), NaCl (1·00M) and either EDTA (0·50M) adjusted to the required pH with N-NaOH or tris (0·050M) adjusted to the required pH with 4N-HCl. ○, 2,4-Dinitrophenyl phosphate, EDTA buffer; △, 4-nitrophenyl phosphate, EDTA buffer; ●, 2,4-dinitrophenyl phosphate, tris buffer; ▲, 4-nitrophenyl phosphate, tris buffer. The line was drawn by eye above pH 6·8 and by regression as a straight line in the pH range 5·5–6·8.

assay medium (final volume 3ml.) contained 4-nitrophenyl phosphate (70μM), 2,4-dinitrophenyl phosphate (70μM), sodium chloride (1·00M) and tris (0·050M) adjusted to pH 8·08 with 4N-hydrochloric acid. The reaction was initiated by the addition of 1·0μg. of enzyme. The molar extinction coefficients at the different wavelengths of the two products arising from hydrolysis were calculated. Hence the relative proportion of extinction change due to the two phosphate hydrolyses could be determined. The relative rate of hydrolysis of the two phosphates was $1{\cdot}0 \pm 0{\cdot}15$ (maximum error).

The experiment was repeated at lower pH values to pH 6. However, the results were less accurate since the molar extinction coefficient of 4-nitrophenol becomes an order of magnitude less than that of 2,4-dinitrophenol at pH 6. In all experiments the relative hydrolysis rates were close to 1.

Wilson *et al.* (1964) reported that the presence of tris during the hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase at pH 8 increased the rate of 4-nitrophenol liberation with concomitant phosphorylation of tris. They concluded that this rate enhancement by a phosphoryl acceptor more effective than water indicates that

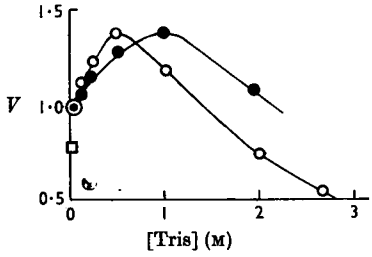

Fig. 2. Dependence of the relative rate of the enzyme-catalysed hydrolyses of 2,4-dinitrophenyl phosphate and 4-nitrophenyl phosphate on tris concentration at 25° at pH 8·11. The assay media contained tris adjusted to pH 8·11 with 4 N-HCl; in addition: ●, 4-nitrophenyl phosphate (100 μM), alkaline phosphatase (0·67 μg./ml.) and NaCl to yield final ionic strength 1·0; ○, 2,4-dinitrophenyl phosphate (100 μM), alkaline phosphatase (1·0 μg./ml.) and NaCl to yield final ionic strength 2·0; □, as ○, but final ionic strength 0·075.

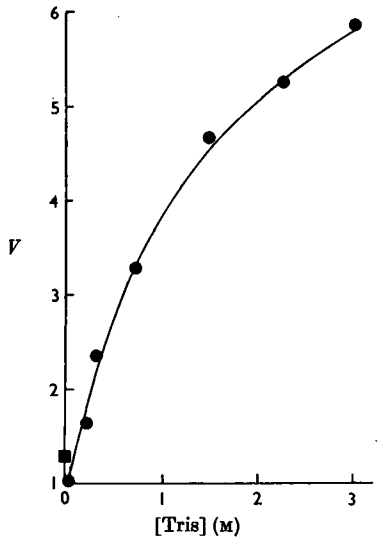

Fig. 3. Dependence of the relative rate of the enzyme-catalysed hydrolysis of 2,4-dinitrophenyl phosphate on tris concentration at 25° at pH 5·90. The assay media contained: ●, 2,4-dinitrophenyl phosphate (60 μM), alkaline phosphatase (27 μg./ml.), tris hydrochloride, sodium citrate (0·33 M) adjusted to pH 5·90 with 4 N-HCl and NaCl to yield ionic strength 4·0; ■, as ●, but ionic strength 1·4.

the dephosphorylation of the enzyme is rate-determining for the overall reaction. This conclusion had to be qualified when Aldridge *et al.* (1964) showed that dephosphorylation is somewhat faster than phosphorylation of the enzyme at pH 8. The 1·4-fold enhancement at optimum tris concentration at pH 8·11, which was confirmed by us (see Fig. 2), is consistent with the observations of

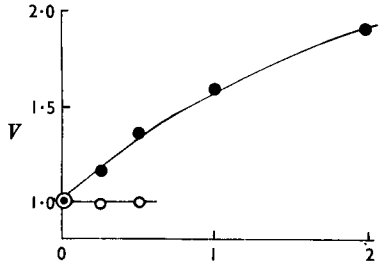

Fig. 4. Dependence of the relative rate of the enzyme-catalysed hydrolysis of 2,4-dinitrophenyl phosphate on hydroxylamine concentration at 25°. The assay media contained: ●, 2,4-dinitrophenyl phosphate (65 μM), alkaline phosphatase (27 μg./ml.), sodium citrate (0·10 M) adjusted to pH 5·83 with 4 N-HCl and NaCl to yield final ionic strength 1·3; ○, 2,4-dinitrophenyl phosphate (65 μM), alkaline phosphatase (1·33 μg./ml.), tris (0·050 M) adjusted to pH 7·96 with 4 N-HCl and NaCl to yield final ionic strength 1·0.

Aldridge *et al.* (1964) as discussed below. At lower pH, when the rate of hydrolysis is lower, the rate-determining step (the dephosphorylation of the enzyme) must be slower. It is therefore to be expected that tris will enhance the reaction by a larger factor at lower pH. Fig. 3 shows that, at pH 5·90, up to sixfold enhancement of the rate of liberation of 2,4-dinitrophenol was observed in the presence of tris.

The effect of the presence of other nitrogen bases was also investigated. Hydroxylamine (pK 6·0) did not increase the rate of hydrolysis of 2,4-dinitrophenyl phosphate at pH 7·96 and increased it only twofold at pH 5·83 (Fig. 4).

Assays were carried out in the presence of ammonia (pK 9·2) (0·5 M) at pH 8·1, 2,6-lutidine (pK 6·8) (0·6 M) at pH 5·9 and 8·1, and a mixture of 2,4- and 2,5-lutidine (total base concn. 0·6 M) at pH 5·9, to study the effect of these bases on the rate of the enzyme-catalysed hydrolysis of 2,4-dinitrophenyl phosphate. In every case the substrate was hydrolysed at a rate in the range 0·6–0·8 that of the rate of hydrolysis of control assays carried out at the same ionic strength. The lutidines were studied to see whether a difference could be observed between the effects of 2,6-lutidine and 2,4- or 2,5-lutidine, since 2,6-lutidine has a similar proton-accepting ability to 2,4- or 2,5-lutidine but is a weaker nucleophile.

The stopped-flow technique was used to study the kinetics of the enzyme-catalysed hydrolysis of 2,4-dinitrophenyl phosphate near pH 6, when a two-step reaction should be observed at high enzyme concentration if the cleavage of the phosphoryl-enzyme complex is rate-determining. The apparatus and

techniques for the stopped-flow equipment were those of Barman & Gutfreund (1966*a*). For these experiments the enzyme was first dialysed against sodium chloride (1·0M) and sodium citrate (0·10M) adjusted to pH 5·9 with 4N-hydrochloric acid.

An example of the change of extinction with time on mixing the solution is shown in Fig. 5. The initial rapid liberation of 2,4-dinitrophenol with a first-order rate constant of 30sec.$^{-1}$ (± 3 max. error) corresponds to approx. 1 mole of 2,4-dinitrophenol/mole of active enzyme. This is followed by the steady-state rate of 2,4-dinitrophenol liberation. Correlation of the observed spectral change with 2,4-dinitrophenol release was obtained by comparing the turnover rate in the stopped-flow experiment with that in a steady-state assay carried out under identical conditions except that the enzyme solution had been diluted 50-fold.

The effects of tris on the pre-steady-state rate (initial transient) and on the steady-state rate at pH 5·9 were also studied in stopped-flow experiments. The control experiments were carried out with solutions described in the legend to Fig. 5 and in parallel experiments tris hydrochloride (2·0M) was substituted for sodium chloride in the syringe containing enzyme. This resulted in tris being 1·0M in the reaction mixture. The initial rapid rate of the reaction was 0·7 of that of the control, whereas the steady-state rate was 2·1 times that of the control.

The sedimentation coefficient of alkaline phosphatase was determined by the activity sedimentation technique (Cohen & Hahn, 1965) with a Spinco model E analytical ultracentrifuge equipped with an RTIC control unit at 59780rev./min. near 20°. A 406mμ interference filter was used. The valve-type artificial-boundary cell contained 4-nitrophenyl phosphate (100μM), sodium chloride (1·0M) and tris (0·050M) adjusted to pH 7·8 with 4N-hydrochloric acid. The enzyme dissolved in the same solution at a concentration of 10μg./ml. was layered on as the centrifuge speeded up. $S_{20,w}$ was 6·2s. This result is consistent with that of earlier work when the sedimentation coefficient of highly purified enzyme was measured as 6·1s and the molecular weight of 86000 was estimated (Schlesinger & Barrett, 1965).

The ratio of the specific activities of the enzyme was 1·08 when measured at enzyme concentrations of 10·0μg./ml. and 1·00μg./ml. in the assay medium of the sedimentation study.

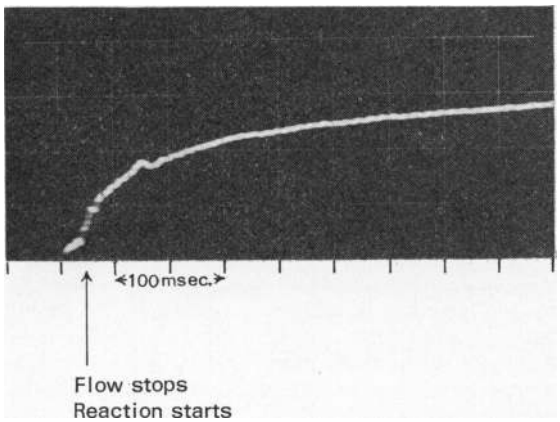

Fig. 5. Record of a stopped-flow observation of the liberation of 2,4-dinitrophenol at 365mμ during the reaction of 2,4-dinitrophenyl phosphate (70μM) with alkaline phosphatase (1·5mg./ml.) in NaCl (1·8M) and sodium citrate (0·10M) adjusted to pH 5·90 with 4N-HCl. The ordinate is percentage transmission in arbitrary units.

DISCUSSION

In recent years a general mechanism has been proposed for many hydrolytic and acyl- and phosphoryl-transfer reactions catalysed by a wide range of enzymes:

$$E + AB \underset{}{\overset{k_1}{\rightleftharpoons}} EAB \xrightarrow{k_2} EA + B \qquad (1)$$

$$EA + C \xrightarrow{k_3} E + AC \qquad (2)$$

where E is the free enzyme, EAB the Michaelis complex, EA the acyl- or phosphoryl-enzyme and C either water or some other acyl or phosphoryl acceptor (Hartley & Kilby, 1954; Bernhard & Gutfreund, 1960; Bender & Kézdy, 1965). Analyses of steady-state kinetic data and of transients of reactions of a number of these enzymes with special reagents and pseudo-substrates have been interpreted in terms of this simple scheme. However, Bernhard & Gutfreund (1965) and Barman & Gutfreund (1966*a*) have pointed out that, with the proteolytic enzymes chymotrypsin and trypsin, the reactions with specific synthetic substrates cannot be analysed simply in terms of two kinetic steps (acylation and hydrolysis of the acyl-enzyme) preceded by a second-order enzyme–substrate combination. The formation of the acyl-enzyme from the enzyme–substrate complex includes at least one additional kinetically significant step, which makes the direct correlation of k_2 with the reactivity of leaving group B (eqn. 1) less reliable than proposed by Gutfreund & Hammond (1959) and Zerner, Bond & Bender (1964).

With alkaline phosphatase this lack of correspondence between the rate of formation of the phosphoryl-enzyme and the leaving group of the substrate is even more marked. It has been shown (Aldridge *et al.* 1964; Barman & Gutfreund, 1966*b*; Fernley & Walker, 1966) that at optimum pH the

rate of phosphorylation of the enzyme is largely rate-determining for the rate of hydrolysis of a number of phosphate esters. This fact only becomes significant in relation to the above problem when one can compare accurate data for a well-defined pair of substrates. Kinetic data obtained by phosphate analyses are of doubtful significance because of the insensitivity of this method and the marked inhibition of ester hydrolysis by orthophosphate (Barman & Gutfreund, 1966*b*). The sensitivity and accuracy of kinetic data obtained from studies of the alkaline-phosphatase-catalysed hydrolysis of 4-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate allow one to emphasize with confidence that these two substrates, whose leaving groups have pK values 3·1 units apart, are hydrolysed at identical rates over the pH range 5·5–8·5. This in turn clearly proves that the overall rates of phosphorylation of the enzyme by these two substrates are identical, at least above pH 7, when this rate must contribute significantly to the steady-state rate. This result is striking because Kirby & Varvoglis (1967) showed that the rate of hydrolysis of the phosphate monoester dianion is very sensitive to the pK_a values of the leaving group. This emphasis on the choice of a pair of substrates that can be used for accurate kinetic studies is made because of the critical relation between the turnover, k_0, the rate of phosphorylation, k_2, and the rate of dephosphorylation of the enzyme, k_3, under conditions of substrate saturation:

$$k_0 = \frac{k_2 k_3}{k_2 + k_3}$$

Correction to identical conditions (0·1 M-tris, pH 8, 25°) of the data of Malamy & Horecker (1964) for the pure crystalline enzyme with 4-nitrophenyl phosphate as substrate gives $k_0 = 45\,\text{sec.}^{-1}$ This, together with $k_3 = 120\,\text{sec.}^{-1}$ obtained by Aldridge *et al.* (1964), enables one to calculate $k_2 = 72\,\text{sec.}^{-1}$ for the first-order rate of conversion of the initial enzyme–substrate complex into the phosphoryl-enzyme. Under these conditions changes in k_2 from 36 to 72 to $144\,\text{sec.}^{-1}$ would result in changes of k_0 from 28 to 45 to $65\,\text{sec.}^{-1}$ at constant k_3.

The fact that at equal concentrations the two nitrophenyl phosphates in the same solution are hydrolysed at the same rate by *E. coli* alkaline phosphatase leads to an important conclusion if it is assumed that each substrate had a similar binding constant. The results of Kirby & Varvoglis (1967) indicate that, if the two substrates react as dianions, they would lose their phosphate groups at different rates. If this is so, then the conclusion is that there must be a step in the reaction mechanism that is not related to the release of the phosphate group that is rate-determining and occurs between the formation of the enzyme–substrate complex and the formation of the phenol. A conformation change of the enzyme induced by the substrate could be such a step whose rate constant would be k_2.

Extension of the studies of Wilson *et al.* (1964) of the effect of increasing concentrations of tris at constant ionic strength on k_0 gives support to the conclusion that k_2/k_3 is very much larger than 1 at pH 5·9 and smaller than 1 at pH 8·1. If one accepts the conclusion of Wilson *et al.* (1964) that tris increases the rate of dephosphorylation of the enzyme (k_3) and our conclusion that an increase in k_3 should lead to conditions $k_2 = k_0$, one can make the following calculation. The maximum increase in k_0 when tris is present at pH 8·1 is 1·4-fold, which gives $k_2 = k_0 = 63\,\text{sec.}^{-1}$, compared with $k_2 = 72\,\text{sec.}^{-1}$ calculated above. This is in very reasonable agreement, especially when one considers the fact that higher concentrations of tris inhibit the steady-state rate at pH 8·1. The observation of the initial transient discussed below also indicates a decrease of k_2 at high tris concentrations at pH 5·9. At this pH, however, k_3 is rate-determining by a large factor and tris can lead to a very much greater enhancement of k_0 by increasing k_3 (Fig. 3).

Why tris accelerates the decomposition of the enzyme–phosphate complex can only be conjectured. Ethanolamine has also been shown by Wilson *et al.* (1964) to accelerate cleavage of the enzyme–phosphate complex. Moreover, even when the cleavage of the enzyme–phosphate complex is not rate-determining the *O*-ethanolamine phosphate/free phosphate ratio is greater than the ethanolamine/water molar ratio. It has been shown that hydroxyl compounds other than water are phosphate acceptors to substrates cleaved by phosphatases (Wilson *et al.* 1964; Anderson & Nordlie 1967). Kirby & Jencks (1965) noted that the rates of reaction of monocations of diamines, including that of the conjugate acid of triethylenediamine, with the dianions of 4-nitrophenyl phosphate are abnormally rapid. They suggested that the enhanced reactivity of these monocations results from electrostatic interaction between the protonated nitrogen atom and the two negative charges of the phosphate ester. However, they could detect no catalysis by tris of 4-nitrophenyl phosphate hydrolysis. In view of these observations and those of Wilson *et al.* (1964), together with our own on the ineffectiveness of amines other than hydroxylamine to act as catalysts, it is probable that a hydroxyl group of tris is acting as the phosphate acceptor. One reason for the relative increase of reactivity of tris over other hydroxyl derivatives may be as a result of the protonated amine group being involved in an electrostatic interaction.

Ammonia and 2,6-lutidine have relatively small effects on the rate of catalytic hydrolysis of phosphate esters. This allows one to neglect their

presence in small amounts in many of the assays described.

Various factors may contribute to the decrease in the rate of catalytic hydrolysis of the phosphate esters at high amine concentration and constant ionic strength (Figs. 2 and 4). For example, this decrease may be due to solvent effects [at pH 5·9 a 2·0 M solution of hydroxylamine contains about 3% (w/v) of free hydroxylamine]. The specific action of cations in affecting the rate of aryl phosphate dianion hydrolysis has been noted by Bunton, Fendler & Fendler (1967), and also in the special case of monoprotonated diamines referred to above. It is likely therefore that cations will show specific effects in relation to the rate of breakdown of the enzyme–phosphate complex.

Stopped-flow spectrophotometric observations of the reaction of 2,4-dinitrophenyl phosphate with phosphatase at high enzyme concentration at pH 5·9 can be interpreted in terms of a rapid phosphorylation step with concomitant liberation of 1 mole of 2,4-dinitrophenol/mole of enzyme, and subsequent slow steady-state liberation of 2,4-dinitrophenol corresponding to the steady-state rate in agreement with the findings of Aldridge *et al.* (1964) and Fernley & Walker (1966). Although the addition of tris at pH 5·9 accelerates the steady-state rate 2·1-fold in the transient experiment, the corresponding factor in the steady-state experiment at pH 5·9 is 3·8 (Fig. 3). Hence one must accept the amount of 2,4-dinitrophenol released/mole of enzyme with some reservation. Nevertheless it is noteworthy that both the rapid turnover of [^{32}P]-phosphate (Aldridge *et al.* 1964) and the rapid liberation of 2,4-dinitrophenol correspond to 1 mole/86 000 g. of active enzyme. The role of two sub-units with a serine hydroxyl group in identical surroundings (Pigretti & Milstein, 1965) in enzymes of molecular weight 86 000 remains to be elucidated. The fact that tris decelerates the burst rate at pH 5·9 relative to the rate in sodium chloride may be an example of specific salt effects referred to above.

Since the sedimentation coefficient of the enzyme at concentrations of 10 μg./ml. and 4 mg./ml. is the same as deduced from activity sedimentation studies and velocity sedimentation studies (Schlesinger & Barrett, 1965), and the turnover rate at pH 7·8 of the enzyme with 4-nitrophenyl phosphate as substrate is linear with enzyme concentration in the range of concentrations of the steady-state experiments up to 10 μg./ml., there seems no reason to doubt that in all our experiments the enzyme was in the associated form of molecular weight 86 000.

In summary, the most challenging problems in the elucidation of the mechanism of action of this enzyme remain the explanation of the step controlling the rate of phosphorylation of the enzyme by the substrate, and the explanation of the phenomenon of an enzyme having two sub-units and apparently only one active site at any instant. Work is being directed at looking for a conformation change in the enzyme when the substrate is bound, and seeing if the rate-determining step, which is known to be independent of substrate in the pH range 5·5–8·5, is involved with this proposed change. The conformation change could be induced by the phosphate group itself.

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