Evidence for a Phosphoryl-Enzyme Intermediate in the Catalytic Reaction of Prostatic Acid Phosphatase†

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ABSTRACT: Three independent lines of evidence lead to the conclusion that prostatic acid phosphatase hydrolyzes its substrates by formation of a catalytic covalent phosphoryl-enzyme intermediate. Firstly, a presteady-state burst of *p*-nitrophenol liberation occurs when *p*-nitrophenyl phosphate is mixed with the enzyme. Quantitation of this presteady-state liberation yields a value of two active centers per molecule (102,000 daltons) of the phosphatase. Secondly, transphosphorylation to an alcohol in the enzymic hydrolyses of five phosphoesters occurred, with, in each case, the same ratio of the hydrolysis to the transphosphorylation. This was true at two different ratios, one for ethanol as acceptor and the other for ethanolamine. Thirdly, the finding of Greenberg and Nachmansohn (*J. Biol. Chem. 240*, 1639, 1965) that diisopropyl fluorophosphate inhibits this enzyme was confirmed. This is in an unusual reaction which occurs in a low pH range (~pH 5) of optimum activity, and whose product spontaneously decomposes at pH 7.5. The combined data indicate that a phosphoryl-enzyme is an intermediate directly upon the catalytic pathway. Important mechanistic differences must exist, however, between this acid phosphatase and the well-studied alkaline phosphatases.

Some recent discussions of mechanisms of enzymic phosphate transfer have focussed on the acid phosphatases, which in interesting and important respects differ in their catalysis from those alkaline phosphatases that have been well-studied (Feldman and Butler, 1969; Boer and Steyn-Parvé, 1970; Igarashi *et al.*, 1970; Ostrowski and Barnard, 1971). Work in a number of laboratories has established that the alkaline phosphatases can form an *O*-phosphorylserine-containing derivative (for a review, see Reid and Wilson, 1971). To analyze the mechanism of action of an acid phosphatase, it is first of all essential to determine if such a covalent intermediate is involved. Secondly, if evidence for such a derivative can be obtained, it is necessary to relate it to the catalytic reaction sequence. It has on several occasions been pointed out, for the case of alkaline phosphatase, that the possibility must be tested that the phosphoryl-enzyme that can be isolated might be formed in a side pathway and not be of catalytic importance (Reid *et al.*, 1969; Halford, 1972).

One method for recognizing a catalytically essential covalent intermediate has been described by Barrett *et al.* (1969). If the enzyme can utilize an alternative phosphate acceptor present in addition to the water, then the ratio of the extent of the transfer reaction to that of the hydrolysis reaction would be independent of the substrate employed, if a phosphoryl-enzyme, E–P, is always the catalytic intermediate. The reaction rate of E–P with, say, a given alcohol will be independent of the nature of the original substrate (and also independent of whether the formation of E–P from that substrate was relatively fast or slow), and the same will be true of the (different) reaction rate of E–P with water. Hence, a constant ratio of the two reactions should be obtained with all substrates, for this mechanism. Barrett *et al.* (1969) concluded that *Escherichia coli* alkaline phosphatase hydrolyzes substrate through a phosphoryl-enzyme intermediate, since nine substrates gave the same partitioning of their phosphate groups between water and ethanolamine. We have now used this method, with positive results, with human prostatic acid phosphatase. We have also used two acceptors—one much more efficient than the other—since a different, although substrate-independent, ratio should be obtained for the two series if this intermediate is always involved.

In addition, we report an all-or-none titration of the active centers of this acid phosphatase, based upon formation of such a phosphoryl-enzyme. We also reinvestigate the inhibition by Dip-F[1] (Greenberg and Nachmansohn, 1965) in this connection.

Materials and Methods

Materials. Acid phosphatase I from hypertrophic human prostate glands was isolated and purified as described by Ostrowski and Barnard (1971). It was completely pure by the chromatographic and gel electrophoretic criteria reported there, but had three components in isoelectric focussing (Ostrowski *et al.*, 1970). These appear to differ only in minor degrees of sialic acid content (Ostrowski *et al.*, 1970). The specific enzymic activity at pH 5.0 was 243 units (μmol/min of *p*-nitrophenyl phosphate in 0.02 M solution, hydrolyzed per min at 25°).[2]

Dip-F, phenyl phosphate, sodium β-glycerophosphate, galactose 6-phosphate, uridine, and *p*-nitrophenyl phosphate (type 104) were the purest grades available from Sigma. Phenol (redistilled) was from Baker Chem. Co. Adenosine was

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[1] Abbreviation used is: Dip-F, diisopropyl fluorophosphate.

[2] In previous papers (*e.g.*, Ostrowski, 1968; Ostrowski and Barnard, 1971) a value of maximum specific activity of about 1500–1700 units/mg has been reported for the same conditions. This has now been found to be in error, due to use of an incorrect standard for the units used. The present value of 243 International enzyme units/mg was for completely pure enzyme, which gave 1750 units/mg in the previous notation.

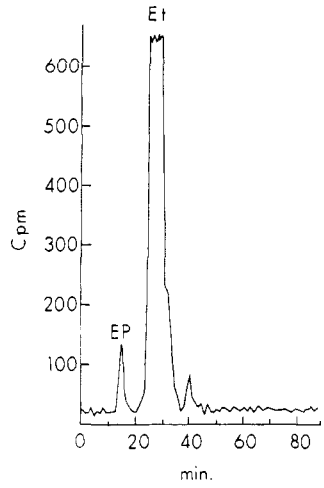

FIGURE 1: Separation of [^{14}C]ethyl phosphate (EP) from excess of [^{14}C]ethanol (Et) on the amino acid analyzer column, at pH 2.2, 35°. The radioactivity in the effluent, as read continuously in the flow-cell system, is plotted.

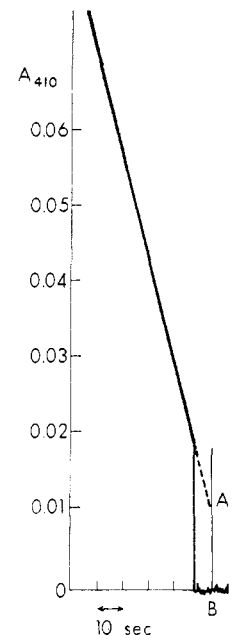

FIGURE 2: An example of the prostatic acid phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate at 25°. The reaction mixture consisted of 50 μg of pure enzyme and 285 μg of *p*-nitrophenyl phosphate in a total volume of 2.5 ml of 0.2 M glycine–NaOH buffer (pH 8.1). The reaction was started by the rapid addition (see Methods) of 50 μl of enzyme solution in water at 25°. The heavy line (starting at B and moving from right to left) is the experimental tracing, and the broken line is the extrapolation made. A-B represents, therefore, the absorbance value of *p*-nitrophenol released in the "instantaneous burst." (The slope illustrated is the greatest employed, others being considerably below this by virtue of higher pH or lower temperature.)

from Boehringer. 3′-AMP and 3′-UMP were from Sigma and were pure by paper chromatographic analysis. 1-[^{14}C]Ethanol (21 Ci/mol) was from Amersham Searle. Ethanolamine and other unspecified materials were as used by Ostrowski and Barnard (1971).

Measurement of Phosphate Transfer. With ethanolamine as acceptor, the experiments with each substrate were conducted as described previously for the *p*-nitrophenyl phosphate reaction, with analysis of the *O*-phosphorylethanolamine product on the amino acid analyzer (Ostrowski and Barnard, 1971). With ethanol as acceptor, [^{14}C]ethanol (1.56 M, 4.05 Ci/mol) was used similarly instead of ethanolamine, and the [^{14}C]ethyl phosphate was determined by ion-exchange chromatographic separation (Figure 1), measuring the radioactivity peak in a scintillation flow-cell system as described previously (Goren *et al.*, 1968). In each case, in the samples withdrawn at intervals the reaction was arrested by dilution with four times their volume of 0.2 M citrate buffer (pH 2.2) and freezing.

Other aliquots were taken from each sample without freezing, and the content of free orthophosphate therein was determined by the method of Chen *et al.* (1956).

Active-Site Titration. A modification of the method of Fife (1967) was used. *p*-Nitrophenyl phosphate (free of *p*-nitrophenol, as checked by spectrophotometry) was used in solution in 0.2 M glycine–NaOH buffer (except where noted) at the stated pH. To 2.5 ml of the solution in a cuvette thermostatted at 25°, 50 or 100 μl of the enzyme solution (1.0 mg/ml in water, predialyzed) was added at zero time by plunging it rapidly into a rapidly vibroagitated cuvette. The mixing phase, determined by similar injection of colored solutions, was reproducibly complete in 2 sec, most of it occurring in the first second. The cuvette was placed in the Cary 15 spectrophotometer (0–0.1 slide-wire), and recording at 410 nm was initiated at a timed interval of about 7 sec after entry of the enzyme. Extrapolation to zero time was made to give the "instantaneous burst" (Figure 2). The amount of *p*-nitrophenol released thus was determined by the extinction coefficient (ϵ_{410}) of pure *p*-nitrophenol in the same buffers. Thus, ϵ_{410} measured in a calibration experiment at pH 9.1 was 1.80×10^4, the same as the reported value in alkali (Trentham and Gutfreund, 1968). The pH values of the reaction mixtures were measured at the end of the reaction (and were unchanged from those of the media used).

Reaction with Dip-F. The Dip-F stock solution was 1.0 M in anhydrous propane-1,2-diol (Lazarus *et al.*, 1966), stored at −20°, and was diluted, just before use, in water to 0.1 M. This solution was treated with about one-tenth of its volume of beads of Bio-Rad AG501-X8 mixed-bed resin in water, to remove any traces of fluoride, and centrifuged. The final Dip-F solution was at once prepared from this by dilution in pH 5.0 (50 mM acetate) or pH 7.5 (50 mM sodium Veronal) buffer and used immediately. The final concentration of Dip-F present was checked by assay on α-chymotrypsin. The protease was treated with (nominal) 10^{-5} M deionized Dip-F at 25° and the rate of loss of activity on benzoyl-L-tyrosine ethyl ester was determined by spectrophotometric assay conducted at 256 nm (Zendzian and Barnard, 1967). A standard Dip-F solution prepared directly from pure Dip-F without deionization was used in a parallel assay for calibration.

Assay of acid phosphatase, and other methods not specified, were as used by Ostrowski and Barnard (1971).

Results

Active-Site Titration. Pure prostatic acid phosphatase was allowed to hydrolyze *p*-nitrophenyl phosphate in conditions where an initial "instantaneous burst" of liberated *p*-nitrophenol (*i.e.*, liberation in the presteady-state phase) can be measured (Figure 2).[3] This corresponded, within experimental

[3] At the completion of this work, a report has appeared by Hickey and van Etten (1972) which shows that wheat germ acid phosphatase gives a similar burst with *p*-nitrophenyl phosphate, at a level of about one active site per molecule of mol wt 59,000. The same substrate labeled with ^{32}P introduced bound radioisotope into the enzyme, from which 3-[^{32}P]phosphohistidine was isolated. It is mentioned that similar results were obtained with human prostate acid phosphatase.

TABLE I: Active Sites per Molecule of Phosphatase.[a]

pH	Substrate (mM)	Active Sites/Molecule
8.0	1.00	1.97[b,c]
8.1	0.50	2.40
8.2	0.50	1.93
8.5	0.50	1.61
8.5	2.10	1.89
9.1	1.00	1.93
9.1	1.00	1.90[c]
9.1	2.00	1.68
8.1–9.1	0.5–2.0	1.95[d] (SD = 0.27)

[a] Measured by the apparently instantaneous liberation (presteady-state "burst") of *p*-nitrophenol from *p*-nitrophenyl phosphate; in glycine buffer at 25°, except where noted. [b] This reaction, only, was conducted in 0.05 M Tris-HCl buffer. [c] At 10° (mean of two experiments). [d] Mean of 15 individual experiments, at 25° only; SD = standard deviation of the mean.

error (Table I), to two active sites per molecule, using the previously determined molecular weight (Derechin *et al.*, 1971) of this enyzme, 102,000. This value was maximal at a substrate concentration of 0.5 mM (up to 2 mM). Reduction of the rate of product release considerably below that shown in Figure 2, by change of temperature from 25 to 10°, did not alter the value obtained (Table I). This value was constant with pH, in the range 8.0–9.2.

Transfer of Phosphate to Ethanol. The acid phosphatase reaction was conducted in the presence of 1.56 M [^{14}C]ethanol, and the [^{14}C]ethyl phosphate formed was separated by a chromatographic method with a flow-cell counting system (Figure 1). The radioactivity in the [^{14}C]ethyl phosphate peak increased with time, but was always fully resolved from the large [^{14}C]ethanol peak. When the production of ethyl phosphate was followed thus as a function of time, a plateau level was reached in the case of *p*-nitrophenyl phosphate (Figure 3A), and similarly (in decreasing order of the initial rate) with the substrates phenyl phosphate, 3′-UMP and 3′-AMP; there was no change in this level after 3 hr upon a further doubling of the reaction period (in each of duplicate experiments, with constant enzyme and initial substrate concentrations throughout) for these four cases. This level corresponded to the complete destruction of the substrate, as shown (in the case of *p*-nitrophenol phosphate) by the simultaneous course of *p*-nitrophenol liberation (Figure 3A, upper curve). With β-glycerophosphate as substrate, the overall reaction was much slower (lowest curve, Figure 3A), but the same final value was reached.

The hydrolysis of the product, ethyl phosphate (50 mM), by the concentration of enzyme used in the experiments of Figure 3 was tested separately in otherwise similar conditions. Phosphate liberation was below the limit of detection after 4-hr reaction, and was only 5% after 14 hr. Hence, any effect of product hydrolysis in these experiments can be neglected.

The course of orthophosphate liberation was simultaneously determined in these reactions (Figure 3B), and rose to a plateau in each case. A constant final ratio was reached for transphosphorylation/hydrolysis, *i.e.*, ethyl phosphate/orthophosphate produced; this ratio did not change significantly between 6- and 14-hr reaction period. The ethyl phosphate was determined here by subtraction of the amount of orthophosphate produced from the amount of the total organic phosphate initially present. This ratio was about 0.29 in every case (Table II). This constant ratio was confirmed by the observations made on the production of [^{14}C]ethyl phosphate (Figure 3A), which gave the same value (second column of figures in Table II).

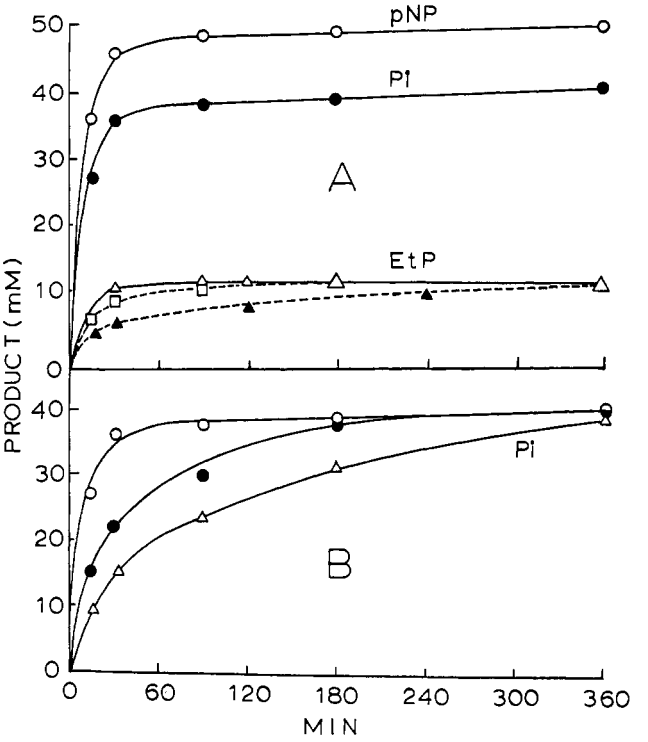

FIGURE 3: Product formation during reaction of prostatic acid phosphatase with substrates in the presence of ethanol as phosphate acceptor. The reaction mixture in 0.09 M acetate buffer (pH 4.9), contained 1.56 M [^{14}C]ethanol (4.05 Ci/mol), 0.049 M initial substrate, and 21.5 μg/ml of enzyme, at room temperature. At intervals 50-μl aliquots were withdrawn, diluted four times with 0.2 M citrate buffer (pH 2.2), and radioactivity was determined as described in Materials and Methods. (A) [^{14}C]Ethyl phosphate (EtP) formation is shown for the reaction with *p*-nitrophenyl phosphate (△), 3′-UMP (□), and β-glycerophosphate (▲). Similar curves were obtained with phenyl phosphate and 3′-AMP, these lying between the *p*-nitrophenyl phosphate and 3′-UMP curves; the larger triangles at 180 and 360 min represent the envelope of the values at those times for all of the five substrates (excepting β-glycerophosphate at 180 min). In the case of *p*-nitrophenyl phosphate, the concurrent formation of nitrophenol (pNP) (measured as in the standard assay procedure for the enzyme) and of orthophosphate (P_i) were also followed on parallel aliquots. (B) Orthophosphate release from *p*-nitrophenyl phosphate (○), 3′-UMP (●), or β-glycerophosphate (△). There was no change after the 360 min values (see text).

Transfer of Phosphate to Ethanolamine. The reaction was also conducted in the presence of 1.60 M ethanolamine, and the *O*-phosphorylethanolamine formed was measured on the amino acid analyzer (Ostrowski and Barnard, 1971). With each of four substrates, a plateau of *O*-phosphorylethanolamine was reached, as illustrated in Figure 4 for *p*-nitrophenyl phosphate and 3′-AMP. The final ratio of transphosphorylation/hydrolysis was again essentially constant in each of four cases (Table II). This ratio was, however, about sixfold lower with ethanolamine as acceptor compared to ethanol. For these cases, the differences between substrates were no larger than the differences found in duplicate experiments on the same substrate, as was true also for all of the values for the reaction with ethanol. In the case of galactose 6-phosphate (Table II),

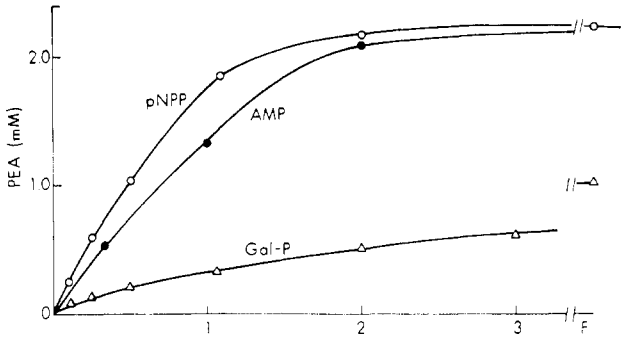

FIGURE 4: Reaction of acid phosphatase with *p*-nitrophenyl phosphate (○), 3′AMP (●) and galactose 6-phosphate (△) in the presence of 1.60 M ethanolamine as phosphate acceptor. The concentration of *O*-phosphorylethanolamine in the reaction mixture is plotted against time in hr. Experimental conditions as detailed by Ostrowski and Barnard (1971). F marks the final value reached: this was at both 6 and 12 hr with *p*-nitrophenyl phosphate and 3′-AMP (value, not shown, coincident with the open circle), and at 12 hr for the maximal value, prior to decline, with galactose 6-phosphate.

the initial rate was very much slower (Figure 4) and no plateau was obtained before the hydrolysis of *O*-phosphorylethanolamine became significant. The rate of the *O*-phosphorylethanolamine hydrolysis reaction has previously been determined for these conditions (Ostrowski and Barnard, 1971), and is sufficient to prevent the maximal amount of *O*-phosphorylethanolamine present reaching more than about half the theoretical value, when treated as a case of consecutive reactions. Galactose 6-phosphate was too poor a substrate, therefore, to provide a measure of the ratio of products at the maximum of the transfer reaction.

Reaction with Diisopropyl Fluorophosphate. Greenberg and Nachmansohn (1965) reported, using an impure preparation of human prostatic acid phosphatase, that the organophosphate *Dip-F* inhibits the enzyme. Their attempt to isolate

TABLE II: Ratio of Transphosphorylation/Hydrolysis Reactions with Two Acceptor Alcohols.

Substrate	Ratio		
	Ethanol[a]		Ethanolamine[b]
p-Nitrophenyl phosphate	0.30	0.28	0.044
Phenyl phosphate	0.26	0.27	0.044
3′-UMP	0.28	0.28	
3′-AMP	0.30	0.29	0.046
β-Glycerophosphate	0.29	0.27	0.041
Galactose 6-phosphate			0.020[c]

[a] The ratio of the production of ethyl phosphate to that of orthophosphate, reached at the end of 6 hr reaction in the conditions noted for Figure 3. The first column of figures is obtained from the final orthophosphate concentrations, and the second column is obtained independently from the final [^{14}C]ethyl phosphate concentrations (in both cases using the phosphate content of the initial substrate mixture, in determining the ratio). The mean values from duplicate experiments (one shown in Figure 3 for 3 of the substrates) are listed in each case. [b] The same ratio obtained from maximal values of the [^{14}C]ethanolamine phosphate formation, in experiments similar to those of Figure 4. [c] End of this reaction not reached (see text).

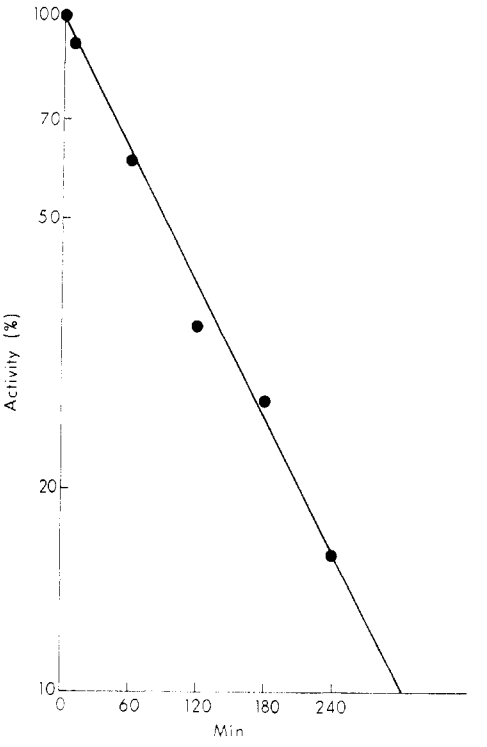

FIGURE 5: Dip-F inhibition of prostatic acid phosphatase at pH 5.0, 25°, plotted semilogarithmically. The reaction mixture consisted of 100 μl of 50 mM acetate buffer containing 0.2 mg of bovine serum albumin/ml and 6 mM resin-treated Dip-F (*cf.* Materials and Methods), plus 10 μl of phosphatase solution (2.9 μg). A control was prepared in the same way, without Dip-F; it showed virtually no activity loss throughout the experiment. At intervals 2-μl portions of the reaction mixture were withdrawn and the enzymic activity determined in standard conditions. At much longer reaction times than those shown, the Dip-F reaction completely abolished the enzymic activity: the line (fitted by least squares) takes into account, also, later values at up to 12 hr reaction, in the range 0–5% residual activity.

[^{32}P]phosphorylated serine from the product gave only an extremely low yield. In view of the potential significance of this reaction for the mechanism of phosphorylation by substrate, it was reinvestigated with the pure enzyme.

Reaction with Dip-F (1 mM) inhibited the enzyme at pH 5 (Figure 5). The reaction is very much slower than that with active-center serine enzymes such as chymotrypsin, but it proceeds to completion, and is pseudo-first-order in behavior. In the more usual conditions for Dip-F inhibition of such enzymes, *e.g.*, at pH 7, the reaction was much slower; after 30 min at pH 7 in similar conditions, the activity loss was only 12% and did not increase at a first-order rate. Upon exposure of the inhibited enzyme (prepared at pH 5) to pH 7.5 (25°), the inhibition was lost spontaneously, to give complete recovery (Figure 6).

This phosphatase is known to be very sensitive to fluoride ion (Reiner *et al.*, 1955), and it was necessary, in order to obtain reliable results in the Dip-F experiments, to remove the low levels of fluoride present in that reagent arising from prior hydrolysis. This was accomplished by treatment with a mixed-bed resin, after which any zero-time inhibition by the Dip-F-containing medium disappeared. (Parallel reactions, in which the deionized Dip-F solution was preincubated without enzyme, showed that none of the original inhibition by Dip-F was attributable to the amount of fluoride released by spontaneous Dip-F hydrolysis, this being quite insignificant in the time periods involved.)

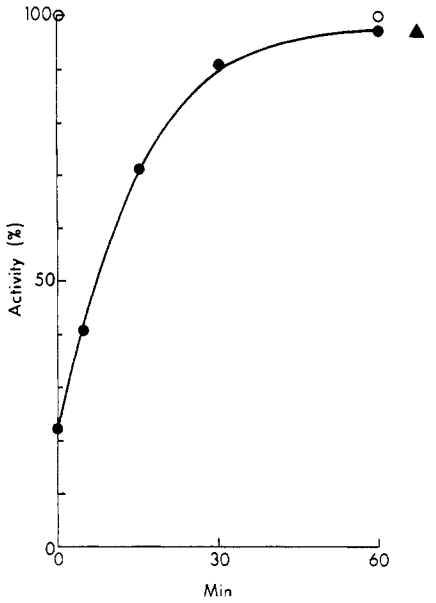

FIGURE 6: Reversal of Dip-F inhibition of prostatic acid phosphatase. Enzyme was preincubated with 6 mM Dip-F for 4 hr at pH 5.0, in the conditions noted for Figure 5. The pH was then raised to 7.5 by adding to 50 μl of the reaction mixture 20 μl of 50 mM Veronal buffer (pH 8.7). Enzymic activity was determined, 1 min after this addition (zero time on this scale) and after intervals during incubation at 25°; the values were corrected for dilution and plotted (on a linear scale) as a percentage of the original activity. (●) Dip-F-treated sample; (○) control, treated similarly throughout but with absence of Dip-F. Each point represents the mean of values (in close agreement) obtained in duplicate experiments. In addition, another sample, that had been initially preincubated with Dip-F at pH 5.0 until no significant activity remained, was exposed similarly to pH 7.5 and measured after 2 hr (▲).

Discussion

These results provide very strong evidence for the existence of a catalytic phosphoryl-enzyme intermediate with this enzyme, although this has not yet been isolated and characterized chemically.[3] The constant ratios of alcoholysis to hydrolysis establish in such a case that a common intermediate occurs. It is difficult to see how any other explanation could account for this uniformity of the relative rates throughout a series of chemically very different substrates, and a parallel behavior with another acceptor of quite different efficiency. This evidence, taken together with the stoichiometric behavior in the labeling burst experiment, leads us to the inference that a phosphoryl-enzyme, E–P, is indeed a catalytic intermediate.

Formation of a noncovalent intermediate, $E \cdot P_i$, could theoretically also lead to such a burst (Reid and Wilson, 1971), but the combined evidence obtained weighs strongly against this alternative. The reaction with Dip-F, and its slow rate of loss when the pH is changed, suggests that a phosphorylation of the active center can indeed occur. Direct reaction of $E \cdot P_i$ with the alcohol is unlikely mechanistically, and was shown to be absent (in the case of ethanolamine as acceptor) by Ostrowski and Barnard (1971) since no significant rate of ester formation was found when orthophosphate replaces the organic phosphate in the transfer reaction mixture, and since no exchange with added [^{32}P]orthophosphate occurred in the phosphate participating in the normal transfer reaction. There is, however, no evidence to indicate a phosphoserine structure as found in alkaline phosphatase phosphorylated by substrate or orthophosphate. In contrast to the latter, the product here cannot be isolated after quenching at acid pH (Ostrowski and Barnard, 1971). The rapid loss at pH 7.5 of the inhibition previously introduced by Dip-F (Figure 6) is not found with serine residues in other enzymes phosphorylated by this reagent. Greenberg and Nachmansohn (1965) in their study with [^{32}P]Dip-F found that the radioactivity was totally removed from the inhibited enzyme in a gel filtration (in 0.1 M NaCl solution at 4°), again suggesting great lability.

All of the data considered would be compatible with a phosphorylation at a histidine side chain, as has been found to occur with acid phosphatase from rat liver reacted with [^{32}P]-orthophosphate (Igarashi *et al.*, 1970). Results equivalent to the latter have also been found with insoluble microsomal acid phosphatase reacted with glucose 6-[^{32}P]phosphate (Feldman and Butler, 1969). The phosphorylation at a serine in alkaline phosphatase, contrasted with the reaction of a different type of site in prostatic acid phosphatase, could provide the basis for a difference in the positions of the equilibria between the phosphorylated enzyme, E–P, and a noncovalent enzyme–phosphate complex (Reid *et al.*, 1969) in the two cases: such a distinction was suggested (Ostrowski and Barnard, 1971) when a large difference was observed between the two enzymes in the occurrence of [^{32}P]phosphate exchange in their transferase reactions.

The stoichiometry of the "burst" reaction shows that there are (as a minimum value, since higher substrate concentrations were not used) two active centers per molecule of 102,000 molecular weight. Physical measurements have indicated that the molecule has two subunits (Derechin *et al.*, 1971). Hence, we suggest that each carries an active center, and that both active centers are readily phosphorylated. Isolation of a phosphorylated peptide at alkaline pH should, therefore, identify an important element of the catalytic site of this enzyme.

Acknowledgments

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Biosynthesis of Chondroitin Sulfate. Microsomal Acceptors of Sulfate, Glucuronic Acid, and *N*-Acetylgalactosamine†

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ABSTRACT: A microsomal preparation from chick embryo epiphyseal cartilage has previously been shown to accept [^{35}S]sulfate, [^{14}C]glucuronic acid, and *N*-[^{3}H]acetylgalactosamine into endogenous glycosaminoglycan. The endogenous glycosaminoglycan has now been characterized further. Incubations of microsomal preparations at pH 6.5 with 3′-phosphoadenosine 5′-phosphosulfate resulted in incorporation of sulfate into endogenous chondroitin 6-sulfate (60–70%) and chondroitin 4-sulfate (30–40%) while incubations at pH 7.8 resulted in incorporation into chondroitin 6-sulfate exclusively. Incorporation appeared to be into occasional nonsulfated galactosamine units in predominantly sulfated molecules with no "oversulfation" to form 4,6-disulfated hexosamine units. Endogenous polysaccharide molecules of low sulfate content were not found. Gel filtration demonstrated that the endogenous sulfate-accepting chondroitin sulfate ranged in size from mol wt ~2000 to ~40,000 relative to chondroitin sulfate standards. Of the total, approximately 90% was of mol wt 16,000 or larger. Incubation of microsomal preparations with UDP-[^{14}C]glucuronic acid or UDP-*N*-[^{3}H]-acetylgalactosamine also resulted in incorporation of the appropriate sugar into similar endogenous chondroitin sulfate. Essentially all the acceptor glycosaminoglycan was linked to protein by alkali labile bonds.

Considerable information is now available on the intermediates involved in the synthesis of the heteropolysaccharide (glycosaminoglycan) portion of the proteoglycan, chondroitin sulfate. Uridine sugar nucleotides have been shown to be the precursors for the sugars of the chondroitin chains (Silbert, 1964; Perlman *et al.*, 1964) and significant sulfation of newly synthesized chondroitin (Silbert and DeLuca, 1969) has been demonstrated with 3′-phosphoadenosine 5′-phosphosulfate as sulfate donor. It is also well established that at least some of the chondroitin sulfate heteropolysaccharide found in extracellular matrix is linked to serine units of protein by an alkali-labile xylosyl-serine bond which is part of a glucuronosyl-galactosyl-galactosyl-xylosyl-serine protein–polysaccharide "bridge" (Rodén and Smith, 1966; Lindahl and Rodén, 1966; Helting and Rodén, 1968). Uridine sugar nucleotides have been shown to be intermediates in the synthesis of this "bridge" (Helting and Rodén, 1969a,b; Brandt *et al.*, 1969; Baker *et al.*, 1972). Alternatively, it has been suggested that some of the polysaccharide chains may be attached to protein by an as yet unidentified bond which is alkali stable (Katsura and Davidson, 1966; Lyons and Singer, 1971). This could represent a different type of separately formed glycosaminoglycan or could be an intermediate in the formation of the glycosaminoglycans attached by the alkali labile bond.

Studies on the structure of proteoglycans have mainly involved the examination of end products of synthesis, such as are found in connective tissue matrix. The structure of intracellular proteolycans at the time of synthesis has not been investigated to any great extent, for the most part because presently available sources do not contain enough intracellular polysaccharide for chemical characterizations. Incorporation of radioactive sugars or [^{35}S]sulfate into this material provides an alternative method of characterization by looking at intracellular glycosaminoglycans as "acceptors." ("Acceptor" will hereafter be the term used to describe material into which sulfate or a single sugar can be incorporated.)

Addition of sulfate to chondroitin or chondroitin sulfate has been reported from many laboratories where soluble (105,000*g* supernatant) enzymes were utilized with either exogenous or extracellular endogenous acceptors (D'Abramo and Lipmann, 1957; Adams, 1960; Suzuki and Strominger, 1960a; Perlman *et al.*, 1964; Meezan and Davidson, 1967; Robinson, 1969). The addition of sulfate has been low relative to the large amount of acceptor present. In contrast, when microsomal preparations have been utilized to demonstrate sulfate incorporation into intracellular endogenous particulate material, a much greater efficiency in sulfation has been observed (DeLuca and Silbert, 1968). Products were found to be protein-linked macromolecules that were identified as chondroitin sulfate.

Certain questions, however, remained unanswered by this

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