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Inorganic Pyrophosphate-Glucose Phosphotransferase Activity Associated with Alkaline Phosphatase of *Escherichia coli**

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Wayne B. Anderson‡ and Robert C. Nordlie

From the Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota Medical School, Grand Forks, North Dakota 58201

SUMMARY

Highly purified commercial alkaline phosphatase preparations from *Escherichia coli* have been shown to catalyze the transfer of a phosphoryl group of inorganic pyrophosphate to the hydroxyl group attached to carbon atom 6 of glucose. Phosphotransferase activity also was detected with relatively crude chicken intestinal alkaline phosphatase preparations. The *E. coli* enzyme was further characterized relative to catalytic properties. Adenosine 5′-phosphate, diphosphate, and triphosphate, cytidine 5′-phosphate and diphosphate, guanosine triphosphate, and mannose 6-phosphate functioned as alternate phosphoryl donors. Activity with PP_i as substrate was observed between pH 5 and 9, with a maximum at pH 7.9. K_m (PP_i), 0.83 mM, was independent of glucose concentration. Maximal activity was observed with 1.25 M glucose; higher concentrations of this hexose inhibited both phosphotransferase and phosphohydrolase activities of the enzyme. Maximally, in the presence of 1.25 M glucose, the phosphotransferase activity involved the participation of 9% of reacting inorganic pyrophosphate; the remaining 91% of the reacted phosphoanhydride substrate was hydrolyzed.

The phosphotransferase activity resembled inorganic pyrophosphatase activity of the preparation with respect to effects of pH, pyrophosphate concentration requirement, and thermolability at 30° in the absence of substrates. Glucose 6-phosphatase and AMPase activities of the preparation also were progressively inactivated by mild heating in a manner similar to that of phosphotransferase and inorganic pyrophosphatase activities; however, β-glycerol phosphate hydrolysis was more stable, and *p*-nitrophenyl phosphate hydrolysis activity was considerably more labile to this treatment than were the other four activities studied. A number of alternative explanations for differences in stability of these hydrolytic activities attributed to alkaline phosphatase are presented.

While the high glucose requirement and relatively low ratio of phosphotransferase to phosphohydrolase activity of the enzyme make a biologically significant role for the synthetic activity questionable, it is suggested that the phosphotransferase reaction may provide a tool for further studies relating to the reaction mechanism of alkaline phosphatase.

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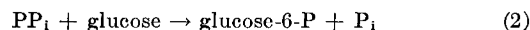
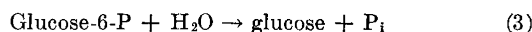
In addition to their hydrolytic activities, alkaline phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.1) are known to catalyze a variety of transphosphorylation reactions (see, for example, References 1 to 3, and review by Atkinson and Morton (4)). Recent studies both in this laboratory (5–7) and elsewhere (8, 9) indicate that a second "specific" phosphatase, liver and kidney microsomal glucose 6-phosphatase (D-glucose-6-P phosphohydrolase EC 3.1.3.9), catalyzes both the hydrolysis of inorganic pyrophosphate (Equation 1) and the transfer of a phosphoryl group from inorganic pyrophosphate (Equation 2) (and a variety of nucleoside di- and triphosphates and mannose 6-phosphate (10)) to glucose. We therefore viewed with particular interest the recent reports that alkaline phosphatases purified in varying degrees from *Escherichia coli* (11, 12), calf intestine (12, 13), yeast (14), and various human tissues (13, 15) exhibit potent inorganic pyrophosphate phosphohydrolase activity. The chromatographically pure enzyme obtained from *E. coli* also catalyzes the hydrolysis of glucose 6-phosphate (Equation 3) and various nucleotides (11). Because (*a*) the

$$PP_i + H_2O \rightarrow 2\ P_i \quad (1)$$



$$PP_i + \text{glucose} \rightarrow \text{glucose-6-P} + P_i \quad (2)$$



$$\text{Glucose-6-P} + H_2O \rightarrow \text{glucose} + P_i \quad (3)$$

hydrolytic activities of both enzymes include a common group of substrates, and (*b*) the reaction mechanisms of glucose 6-phosphatase (6, 16, 17) and alkaline phosphatase (18, 19) are basically similar *i.e.* both appear to involve formation of phosphoryl-enzyme intermediates (6, 16, 17, 19–21) followed by transfer of the phosphoryl group alternatively to water or (in certain documented instances (1–4)) to other phosphoryl acceptors, we have assayed the latter enzyme for possible PP_i-glucose phosphotransferase activity. Evidence for the catalysis of this reaction (Equation 2) by alkaline phosphatase preparations from *E. coli* is presented in this paper, and certain catalytic properties of the activity are described.

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EXPERIMENTAL PROCEDURE

Sources of substrates and other reagents, as well as most analytical procedures, were as previously described (5–7, 10). *p*-Nitrophenyl phosphate was purchased from Sigma. Alkaline phosphatase preparations were obtained from Worthington. Preparations and specific activity values[1] of enzyme purified from *E. coli* were as follows: Worthington preparation BAP-SF, Lot 6167 (22 units per mg of protein); and Worthington preparation BAP-C, "chromatographically purified," Lot 6EA (35.2 units per mg of protein). Worthington chicken intestinal preparation PC (0.9 unit of activity per mg of protein) also was found to possess PP_i-glucose phosphotransferase activity. The bacterial preparation BAP-SF was used in the studies to be described, except where otherwise specifically noted. Glucose-6-P dehydrogenase and phosphoglucomutase were products of the Boehringer and Soehne GmbH, Mannheim. Alkaline phosphatase was diluted with appropriate volumes of 65% (w/v) ammonium sulfate solution, pH 7.5, before use. Glucose-6-P dehydrogenase was diluted with 3.3 M ammonium sulfate solution, pH 8, containing 5 mg of bovine serum albumin per ml. Phosphatase activities were assayed by measuring liberated P_i colorimetrically (24), as described in earlier papers (5–7, 10). Phosphotransferase activity was assayed either (*a*) by the discontinuous spectrophotometric procedure, based on measuring glucose-6-P-dependent reduction of TPN in the presence of an excess of glucose-6-P dehydrogenase, which has been described previously (5, 10, 25), (*b*) by measuring acid-stable ^{32}P-labeled glucose phosphate esters formed by reaction of $^{32}PP_i$ and glucose (10, 25), or (*c*) by continuously monitoring TPN reduction in the presence of an excess of glucose-6-P dehydrogenase in a Beckman model DK-2 recording spectrophotometer. Further specific details pertaining to this last method, which was routinely employed except where otherwise noted, are given in the legends to Figs. 1 and 2. All assays were carried out at 30 ± 0.1°.

RESULTS

Evidence for, and Nature of, Reaction—The dependence of the phosphotransferase reaction on an active alkaline phosphatase preparation, PP_i, and glucose is indicated by the results of the experiments described in Fig. 1, which depicts spectrophotometric tracings of A_{340} *versus* time obtained with a variety of reaction mixtures. No change in A_{340} was noted when glucose was omitted from otherwise complete phosphotransferase reaction mixtures (*Curve D*). The initial slow rate of TPN reduction observed in the presence of glucose (*Curves A, B,* and *C*) prior to the addition of alkaline phosphatase preparation was due to glucose dehydrogenase activity (26) of the glucose-6-P dehydrogenase at the very high concentrations of the hexose used. A marked increase in the rate of TPNH production was noted when active alkaline phosphatase was added to the glucose-6-P dehydrogenase-coupled system containing both glucose and PP_i (*Curve C*). Addition of well boiled alkaline phosphatase preparations to the otherwise complete system (*Curve A*) produced no change in the rate of TPN reduction, thus ruling out the possibility of nonenzymic phosphoryl transfer catalyzed by metal ions (27, 28) or other enzyme impurities. Orthophosphate could not substitute for PP_i, as shown by *Curve B*. This observation rules out the possibility that the observed increase in A_{340} could be accounted for by a sequence of reactions involving (*a*) production of P_i by hydrolysis of PP_i, (*b*) synthesis of glucose-6-P by the reversal of phosphatase-catalyzed glucose 6-phosphatase reaction (equilibrium effect) (1), and (*c*) immediate removal of the formed glucose-6-P by conversion to 6-phosphogluconate with concomitant reduction of TPN. Sharp increases in A_{340} noted in supplementary experiments in which glucose-6-P was added to the described reaction mixtures after the depicted 10-min period indicated that glucose-6-P dehydrogenase was active in all reaction mixtures considered in Fig. 1, and that TPN (which has been reported to be hydrolyzed by alkaline phosphatase (11, 29)) still remained in excess. The absence of analytical complications due to possible alkaline phosphatase-catalyzed TPN-glucose phosphotransferase is shown by the lack of increase in slope of *Curve B* following addition of the phosphatase preparation. The lack of an additional increase in slope of the *right hand segment* of *Curve C* when an excess of phosphoglucomutase was included along with alkaline phosphatase, glucose-

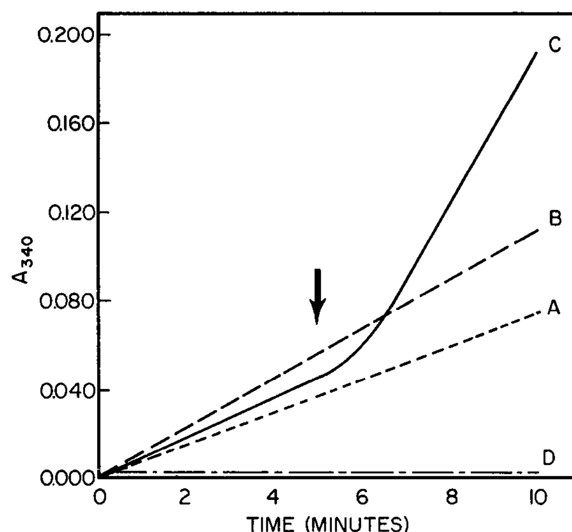

FIG. 1. Dependence of the phosphotransferase reaction on PP_i, glucose, and active alkaline phosphatase preparation. With the exception of the experiment depicted by *Curve D*, in which glucose was omitted, reaction mixtures, pH 7.5, initially contained 150 μmoles of Tris-chloride buffer, 2 μmoles of TPN, 1000 μmoles of glucose, 0.35 unit of glucose-6-P dehydrogenase, and 30 μmoles of PP_i (*Curves A, C,* and *D*) or 30 μmoles of P_i (*Curve B*) in a total volume of 3.0 ml. Absorbance at 340 mμ, due to TPN reduction, initially was monitored for a period of 5 min in each instance. Alkaline phosphatase protein, 60 μg, either active (*Curves B, C,* and *D*) or previously heated at 100° for 15 min (*Curve A*), then was added (*vertical arrow*) to appropriate cuvettes, and A_{340} was monitored for an additional 5 min. The increase in A_{340} observed during the first 5-min period, in the absence of alkaline phosphatase preparation, was due to TPN reduction accompanying the glucose dehydrogenase activity of glucose-6-P dehydrogenase with the high concentrations of glucose employed (26). The results described by *Curve C* also were obtained when an excess (1.3 units) of phosphoglucomutase was included in assay mixtures. (Addition of glucose-1-P to the phosphoglucomutase-containing system after the depicted 10-min period produced marked immediate elevation in A_{340}, indicating that the mutase was active under the experimental conditions used.)

[1] One unit of activity is that amount of enzyme catalyzing the hydrolysis, at 25°, of 1 μmole per min of *p*-nitrophenyl phosphate under the conditions described by Garen and Levinthal (22) (*E. coli* enzyme), or of *o*-carboxyphenyl phosphate under the conditions described by Hofstee (23) (chicken intestinal enzyme).

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6-P dehydrogenase, TPN, and substrates in the reaction system rules out the possibility that glucose-1-P was formed directly as a product of transphosphorylation from PP_i.

The rate of glucose-6-P formation was a linear function of alkaline phosphatase concentration in the range, 15 to 75 μg of enzyme protein, as indicated by the experiment described in Fig. 2*a*.

As an independent check on the validity of the coupled enzymic assay used, glucose phosphorylation also was assayed (*a*) by the discontinuous spectrophotometric procedure previously employed in our laboratory for study of phosphotransferase activity of microsomal glucose 6-phosphatase (5, 10, 25), and (*b*) with the use of $^{32}PP_i$ as substrate, and isolating and measuring glucose-^{32}P formed; a technique also previously used with the microsomal enzyme (10, 25). Phosphorylation of the hexose was detected by both methods[2] (Table I). Further, amounts of glucose phosphate detected by both methods were in close agreement. This observation, along with the lack of stimulation of TPNH formation when phosphoglucomutase was included in reaction mixtures along with glucose-6-P dehydrogenase (Fig. 1), indicates that the reaction involves exclusively phosphorylation of the hydroxyl group attached to carbon atom 6 of glucose.

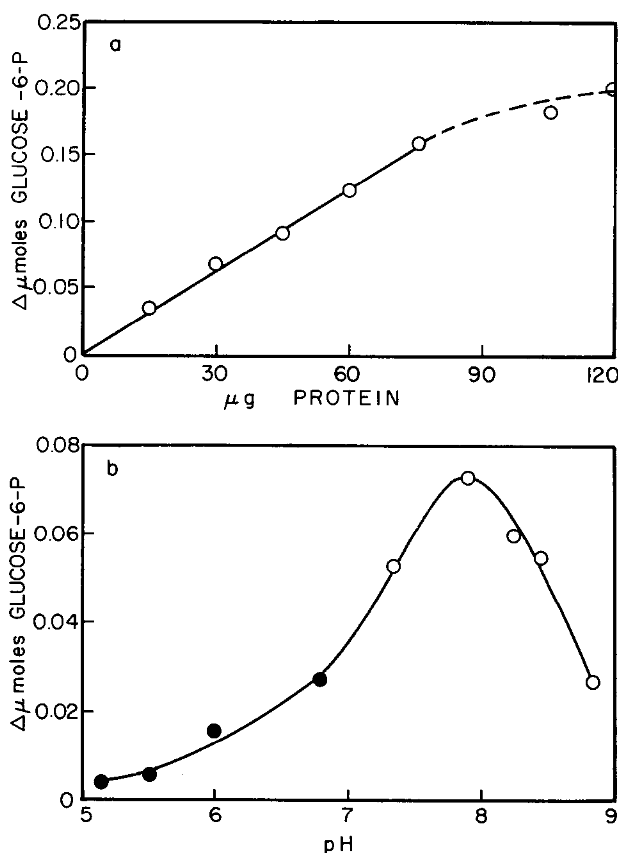

FIG. 2. *a*, effects of alkaline phosphatase concentration on PP_i-glucose phosphotransferase activity. Assay mixtures were identical with those used in the experiment described in *Curve C* in Fig. 1, except that alkaline phosphatase protein concentration was varied in the indicated range. PP_i-glucose phosphotransferase activity was calculated as the difference in slopes of linear portions of plots of A_{340} against time obtained after and before addition of alkaline phosphatase preparations to reaction mixtures (for example, see *Curve C* in Fig. 1). A reference molar absorbance index of 6.22×10^3 M^{-1} cm^{-1} (see References 30 and 31) was used to convert A_{340} readings to molar concentration values. The glucose-6-P dehydrogenase preparation employed catalyzed the reduction of 1 μmole of TPN per μmole of glucose-6-P present. Activity is expressed in terms of glucose-6-P formed per min × 10. *b*, effects of pH on PP_i-glucose phosphotransferase activity. Assay mixtures contained in 3.0 ml, 15 μmoles of sodium PP_i, 1000 μmoles of glucose, 2 μmoles of TPN, 150 μmoles of Tris-chloride (○) or sodium cacodylate (●) buffer, 60 μg of alkaline phosphatase protein, and 0.35 unit of glucose-6-P dehydrogenase. pH was measured with a Beckman expanded scale meter in a duplicate set of reaction mixtures prepared concurrently with those utilized for enzymic activity assays. Activity, which was linear with incubation time in all instances, is expressed in terms of glucose-6-P produced per 5 min.

TABLE I

Comparison of spectrophotometric and radioactive isotopic assays of reaction products

Assay mixtures, at pH 7.5, contained in 1.5 ml, 60 μmoles of Tris-chloride buffer, 1500 μmoles of glucose, 15 μmoles of sodium $^{32}PP_i$ (5×10^5 cpm), and 60 μg of alkaline phosphatase protein. Incubations were carried out at 30° for the periods indicated. Reactions were terminated by the addition of 0.5-ml aliquots of 12% (w/v) perchloric acid. After neutralization with predetermined aliquots of 5 N potassium hydroxide solution and removal of potassium perchlorate by centrifugation, aliquots of the resulting solution were assayed for glucose-6-P spectrophotometrically by coupling with glucose-6-P dehydrogenase, as previously described (5, 10). Following spectrophotometric assay, aliquots of solution were removed from cuvettes and subjected to differential acid hydrolysis with 1 M HCl, extraction of $^{32}P_i$ produced from the hydrolysis of $^{32}PP_i$, and counting of ^{32}P-labeled glucose phosphate ester remaining, also as previously described (10, 25). Values recorded are averages of duplicate determinations.

Incubation time	Observed glucose phosphate produced	
	Spectrophotometric assay	Radioactive isotopic assay
min	*μmole/1.5 ml*	
5	0.31	0.33
10	0.51	0.46

Catalytic Properties—The phosphotransferase was active over a wide range of pH values (Fig. 2*b*). Activity was observed over the entire range studied, pH 5 to 8.9, with a maximum at pH 7.9. A very similar plot, with a maximum at pH 8.2, was obtained for PP_i phosphohydrolase activity of this same preparation (see Fig. 5*b*).

Both P_i liberation from PP_i and glucose-6-P formation were measured in the presence of various concentrations of glucose in the experiment described in Fig. 3. Phosphotransferase activity increased progressively with elevation of glucose concentrations up to 1.25 M, and then dropped slightly. PP_i disappearance, calculated as one-half the sum of glucose-6-P + P_i produced, remained essentially constant up to 1.25 M glucose, and then also decreased. Maximally (with 1.25 M glucose present) phosphotransferase activity involved the participation of 8.7% of the reacted PP_i; the remainder of the reacting phosphate substrate was hydrolyzed. Half-maximal phosphotransferase

[2] Glucose-1-^{32}P formation, if it occurred, would not have been detected by the isotopic method, since this compound also is decomposed by the selective acid hydrolysis technique employed for breakdown of $^{32}PP_i$ remaining after incubation. Other glucose phosphate esters are stable to this treatment.

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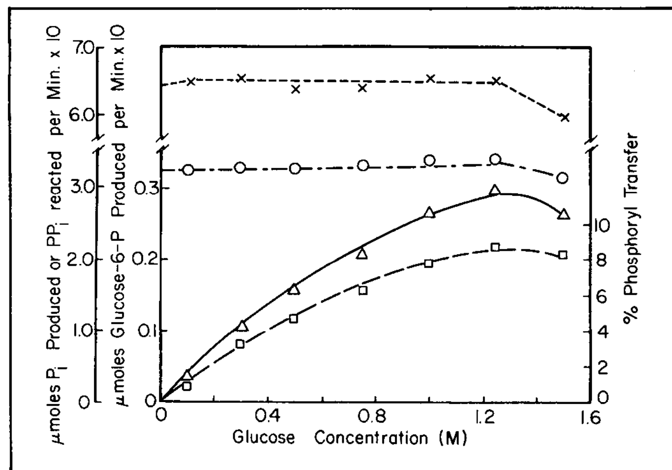

FIG. 3. Effects of glucose concentration on rates of PP_i-glucose phosphotransferase and pyrophosphate phosphohydrolase reactions. Assay mixtures, pH 7.5, contained in 3.0 ml, 90 μmoles of Tris-chloride, 3 μmoles of TPN, 30 μmoles of PP_i, 0.50 unit of glucose-6-P dehydrogenase, and 60 μg of alkaline phosphatase protein. PP_i-glucose phosphotransferase activity (△) was determined as described in the legends to Figs. 1 and 2*a*. After 10 min of incubation, with continuous monitoring of A_{340}, reactions were terminated by the addition of 1.0 ml of 20% (w/v) trichloracetic acid solution, and P_i (×) was assayed colorimetrically (24). Total amount of PP_i (in micromoles) reacted (○) was calculated as (Δ *μmoles* P_i + Δ *μmoles glucose-6-P*)/*2*. Percentage phosphoryl transfer (□) = (Δ *μmoles glucose-6-P*/Δ *μmoles* PP_i) × *100*.

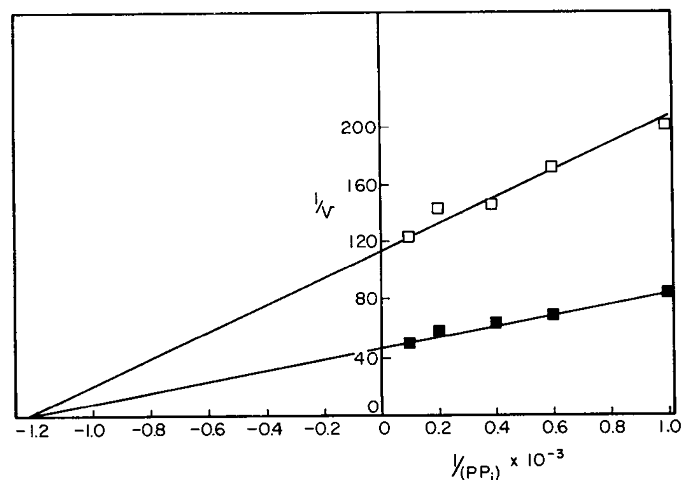

FIG. 4. Effects of PP_i concentration on PP_i-glucose phosphotransferase activity. Assay mixtures, pH 7.5, contained in 3.0 ml, 150 μmoles of Tris-chloride, 2 μmoles of TPN, 0.75 M (■) or 0.17 M (□) glucose, indicated varied amounts of PP_i in the range 1 to 10 mM, 0.35 unit of glucose-6-P dehydrogenase, and 60 μg of alkaline phosphatase protein. Activities (*v*) were measured as described in the legends to Figs. 1 and 2*a*, and are expressed in terms of glucose-6-P formed per min. K_m (PP_i) values, calculated as negative reciprocals of *x* axis intercepts of conventional double reciprocal plots (32) of data, were 0.83 mM in both instances.

reaction velocity was observed with approximately 0.45 M glucose.

The Michaelis constant for PP_i in the phosphotransferase reaction was independent of glucose concentration (see Fig. 4). The observed value for this kinetic parameter, 0.83 mM, agreed well with the K_m (PP_i) value determined for the phosphohydrolase reaction with this same preparation (Fig. 5*a*) and also compared favorably with the value of 1.4 mM obtained earlier by Heppel, Harkness, and Hilmoe (11) for inorganic pyrophosphatase activity of *E. coli* alkaline phosphatase at relatively high substrate concentrations.

Phosphoryl Donor Specificity—A variety of phosphate compounds, most of which previously (11, 22) have been shown to be hydrolyzed by alkaline phosphatase of *E. coli*, were tested as potential phosphoryl donors at concentrations of 10 mM. They were substituted for PP_i in reaction mixtures identical with that described for the experiment depicted in *Curve C* in Fig. 1. Observed relative activities under these conditions were as follows: PP_i, 1.0; ATP, 0.69; ADP, 0.90; AMP, 0.82; CDP, 0.75; CMP, 0.82; GMP, 0.69; and mannose-6-P, 0.68. Morton (2) previously noted AMP-glucose phosphotransferase activity with alkaline phosphatase prepared from milk.

Relative Stabilities of PP_i*-Glucose Phosphotransferase and Various Phosphohydrolase Activities of Alkaline Phosphatase*—

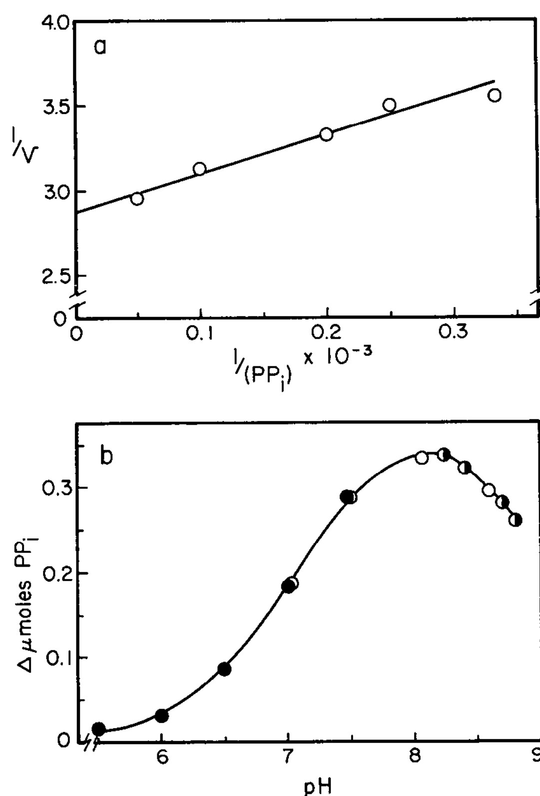

FIG. 5. Effects of PP_i concentrations (*a*) and pH (*b*) on PP_i phosphohydrolase activity of alkaline phosphatase. In the experiment described in *a*, assay mixtures, pH 8.2, contained in 1.5 ml, 60 μmoles of Tris-chloride buffer, 5 μg of alkaline phosphatase protein, and indicated, varied concentrations of sodium PP_i. A K_m value of 0.80 mM for PP_i was calculated from the conventional double reciprocal plot (32) of data in *a*. In the experiment depicted in *b*, assay mixtures contained in 1.5 ml, 60 μmoles of sodium cacodylate (●), Tris-chloride (○), or NaOH-glycine (◑) buffer, 30 μmoles of sodium PP_i, and 5 μg of alkaline phosphatase protein. Reaction mixtures were prepared in duplicate; enzymic activity was assayed with one series while pH was measured in the other. In experiments described in both *a* and *b*, reactions were carried out for 5 min at 30°. P_i production was measured colorimetrically (24). Activity is expressed in terms of micromoles of PP_i hydrolyzed, which was calculated as ½ Δ *μmoles* P_i.

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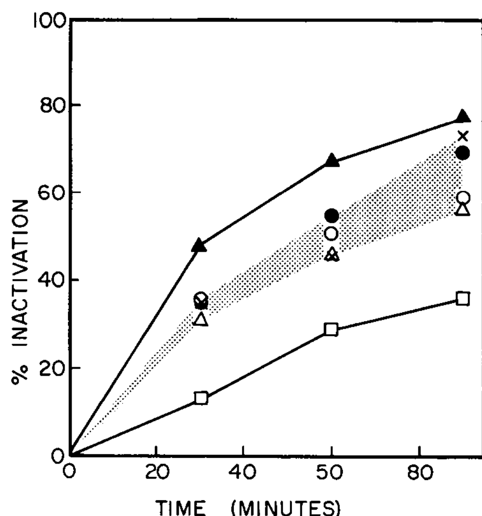

FIG. 6. Comparative study of the stability at 30° in the absence of substrates of various activities of alkaline phosphatase. A commercial enzyme preparation (Worthington Preparation BAP-C) was diluted with 4 volumes of 65% (w/v) ammonium sulfate solution, pH 7.5, containing 5 mg of bovine serum albumin per ml, and allowed to stand in a water bath at 30° for the indicated time intervals. At designated times, aliquots were removed and placed in tubes in an ice bath. Activities were quickly assayed. PP_i-glucose phosphotransferase (×) assay mixtures and methods are as described in the legend to Fig. 2 (*a*), except that 60 μg of alkaline phosphatase protein routinely was assayed. Hydrolysis assay mixtures, pH 8.2, contained in 1.5 ml, 60 μmoles of Tris-chloride buffer, 30 μmoles of phosphate substrate (either PP_i (●), *p*-nitrophenyl phosphate (▲), β-glycerol phosphate (□), AMP (○), or glucose-6-P (△)), and 5 μg of alkaline phosphatase protein. P_i liberated was measured colorimetrically (24); tubes to which trichloracetic acid was added before enzyme served as controls. All incubations were carried out at 30° for 5 min. *Percentage inactivation* values are based on activities determined with the enzyme preparation just prior to preincubation at 30°.

The preceding experiments provide evidence for the existence of PP_i-glucose phosphotransferase activity in highly purified alkaline phosphatase preparations, and describe some catalytic properties of this system. However, they do not in themselves constitute direct proof that the phosphotransferase activity is indeed due to the alkaline phosphatase enzyme itself. To gain additional insight regarding the possible identity of the enzyme catalyzing the observed phosphotransferase and phosphohydrolase reactions, we maintained a diluted "chromatographically purified" alkaline phosphatase preparation at 30° in the absence of substrates and removed samples at intervals for analysis of a variety of activities. Results of these experiments are presented in Fig. 6. Reasonably similar progressive losses of PP_i-glucose phosphotransferase, inorganic pyrophosphatase, AMPase, and glucose 6-phosphatase activities were observed after 30, 60, and 90 min of preincubation. However, β-glycerol phosphate hydrolysis activity was considerably more stable to this treatment during the first 30-min preincubation than were the former activities, while *p*-nitrophenylphosphate hydrolysis activity was lost more rapidly than any of the other activities during this initial period. After the first 30 min, all activities were progressively inactivated in a parallel fashion. The significance of these findings will be considered further under "Discussion," below.

DISCUSSION

The presence in highly purified alkaline phosphatase preparations of an enzymic activity involving the transfer of a phosphoryl group from PP_i to the hydroxyl group on carbon atom 6 of glucose has been substantiated by the experiments described. In addition, catalysis of glucose-6-P formation by phosphoryl transfer from a variety of nucleotides and mannose-6-P to glucose by this enzyme preparation also has been established. Although the possibility that these reactions are catalyzed by a contaminating protein in the alkaline phosphatase preparation cannot unequivocally be ruled out on the basis of the data presented, it is suggested that these activities reasonably may be added to the growing list (1-4) of phosphotransferase reactions catalyzed by this nonspecific phosphatase. This conclusion appears to be justified on the following bases: (*a*) potent phosphotransferase activity was observed with highly purified, chromatographically prepared alkaline phosphatase preparations of very high specific activity;[3] (*b*) pH activity profiles and optima for the phosphotransferase and inorganic pyrophosphatase activities were very similar; (*c*) K_m (PP_i) values for the phosphotransferase agree quite well with the corresponding values determined with, and previously reported for (11), inorganic pyrophosphatase activity of *E. coli* alkaline phosphatase; and (*d*) the thermal stability of the phosphotransferase activity was similar to that of three hydrolytic activities which are considered characteristic of this phosphatase (11, 22, 33). The PP_i-glucose phosphotransferase also is similar to glucose-involving phosphotransferase activity of milk alkaline phosphatase (2) with respect to glucose concentration requirement, inhibition of both phosphotransferase and phosphohydrolase activities by hexose in concentrations greater than 1.25 M (bacterial enzyme) or 1.50 M (milk enzyme), and the involvement of AMP as an alternate phosphoryl donor. A somewhat higher maximal participation of phosphate in phosphotransferase activity with glucose (approximately 16%) with creatine phosphate or AMP as phosphoryl donor, was observed with the milk enzyme, however (2).

A reaction mechanism describing glucose 6-phosphatase, inorganic pyrophosphatase, and PP_i-glucose phosphotransferase activities of microsomal glucose 6-phosphatase previously has been proposed by Arion and Nordlie (6). This mechanism, which involves successively (*a*) formation of a binary enzyme-phosphoryl-substrate complex, (*b*) a dissociation leaving a phosphoryl-enzyme intermediate, and (*c*) transfer of the phosphoryl group from enzyme either to water (hydrolysis) or to glucose or other acceptor (phosphotransferase), may apply also to the alkaline phosphatase system. Such a contention is supported by the observations that PP_i-glucose phosphotransferase and the hydrolysis of PP_i and glucose-6-P all appear to be catalyzed by the alkaline phosphatase, and isolation of a ^{32}P-labeled phosphoryl-enzyme complex formed from glucose-6-^{32}P and *E. coli*

[3] The purity of alkaline phosphatase preparations used in the present studies compares favorably with that used by Heppel, Harkness, and Hilmoe (11) in their studies of hydrolysis of PP_i and a variety of other phosphoanhydrides and phosphate esters by the *E. coli* enzyme. Their best preparations, supplied by Garen and Levinthal (22) and Worthington, catalyzed the hydrolysis, respectively, of 1230 and 1130 μmoles of AMP per hour per mg of protein at 37° (11). As has previously been shown (11), the relative velocities of the *E. coli* alkaline phosphatase-catalyzed hydrolysis of saturating levels of *p*-nitrophenyl phosphate and AMP are identical. Thus, the alkaline phosphatase preparations employed in the present studies had specific activities calculated on the basis of the definition of Heppel *et al.* (11), of 1320 (Preparation BAP-SF) and 2162 (Preparation BAP-C), based on assays at 25°. Presumably these values would be more than doubled if reactions were carried out at 37°.

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alkaline phosphatase recently has been accomplished (20, 21). Such a mechanism also is generally identical with that proposed by Morton (18) to describe phosphotransferase and phosphohydrolase activities of milk and intestinal alkaline phosphatase.

Although the general reaction mechanisms for microsomal glucose 6-phosphatase and alkaline phosphatase may be similar, the two enzymes differ in that the latter has a considerably higher requirement for glucose than does the former (K_m for glucose = 90 mM for the liver microsomal glucose 6-phosphatase (6)). In addition, maximal phosphotransferase reaction velocity of the liver enzyme equals that for the hydrolysis reaction determined in the absence of glucose (6), while PP_i-glucose phosphotransferase activity of the alkaline phosphatase was in all instances less than 10% as rapid as the rate of PP_i hydrolysis by this enzyme.

The differences in thermolability of a number of the phosphohydrolase activities studied in the experiment described in Fig. 6 suggest a variety of interesting possibilities. With the exception of the phosphotransferase activity, all the reactions studied have been attributed to alkaline phosphatase by at least some groups of workers[4] (11, 22, 33). The marked differences in thermostability of β-glycerol phosphate and p-nitrophenylphosphate phosphohydrolase activities, compared with the other four activities studied, may be rationalized in a number of ways, including the following: (a) the commercial preparations of alkaline phosphatase employed possibly may have been contaminated with other phosphatases, although this possibility appears remote in view of the high specific activity and "chromatographically purified" nature of the preparation. (b) Isozymes of alkaline phosphatase, with slightly varying stability and specificity, may be present. (Both possibilities, (a) and (b), are consistent with the findings of Heppel, Harkness, and Hilmoe (11) who observed two K_m values, varying by two orders of magnitude, for each of a number of substrates of the hydrolytic activity of this enzyme. We have observed K_m (PP_i) values of approximately 10^{-5} M rather than 10^{-3} M with certain lots of commercial alkaline phosphatase.) (c) That portion of the active enzymic site initially occupied, prior to phosphoryl-enzyme formation, by that part of the substrate molecule other than the transferable phosphoryl group, may differ for various substrates and may respond differentially to thermally induced conformational alterations accompanying progressive inactivation of the enzyme at 30° in the absence of substrates. These interesting alternative possibilities presently are being investigated in this laboratory.

Although the high glucose requirement of this system makes a biological role for the synthetic activity of alkaline phosphatase questionable (unless, perhaps, this hexose requirement is considerably lower for the enzyme in the living bacterial cell than for the purified enzyme *in vitro*, in the presence of approximately 55 M water), the PP_i-glucose phosphotransferase activity may be of practical value in providing a convenient additional tool for the investigation of the reaction mechanism of alkaline phosphatase. Such studies, suggested generally by Hummel and Kalnitsky (36), are under way in this laboratory.

Acknowledgment—The authors are indebted to Dr. Ernst Noltmann for helpful discussions.

[4] Note, however, that PP_i hydrolysis could not be demonstrated by Morton (34) with milk or calf intestinal alkaline phosphatase preparations, or by Torriani (35) and Garen and Levinthal (22) with alkaline phosphatase prepared from *E. coli*. (Heppel *et al.* (11), however, obtained hydrolysis of PP_i with enzyme preparations provided by Garen and Levinthal, and have concluded that the latter workers may have had an inhibitor present in their assay system which specifically blocked hydrolysis of this phosphoanhydride.) It also has been pointed out that "... the rate of hydrolysis of a particular ester such as p-nitrophenyl phosphate may not be a true measure of nonspecific alkaline phosphomonoesterase activity of a given biological material ... (since), for example, enzymes have been found in yeast and certain anaerobic bacteria that readily decompose this substrate but have little or no activity on phosphomononucleotides, sugar phosphates, and other commonly occurring phosphate esters that are actively decomposed by the nonspecific phosphatase" (33).

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Wayne B. Anderson and Robert C. Nordlie

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