Covalent (E-P) and noncovalent (E·P) phosphoenzyme intermediates exist on the reaction path of alkaline phosphatase of *Escherichia coli*. Zn(II) and Cd(II) alkaline phosphatase both form E-P and E·P from inorganic phosphate. These intermediates show well separated $^3$P NMR resonances in slow chemical exchange with respect to each other and to unbound phosphate. The $^3$P signals of E·P of all forms of the $^{113}$Cd(II) enzyme are doublets ($J \approx 30$ Hz) due to $^{113}$Cd-O-$^3$P coupling. Heteronuclear decoupling shows the phosphate of E·P to be coordinated to the A site metal of the two metal ions, A and B, $\sim 3.9$ Å apart at each catalytic center. The chemical shifts of E·P vary from $-4$ ppm for the Zn(II) enzyme to 12.6-13.4 ppm for forms of the Cd(II) enzyme and indicate a major influence of the metal ions on the conformation around phosphorus. The phosphoryl group of E·P is not coordinated to either of the two metal ions at the active center as shown by the absence of $^{113}$Cd-O-$^3$P coupling on the $^3$P signals of E·P formed by the $^{113}$Cd(II) enzymes. The chemical shift of E·P is not sensitive to metal ion species or stoichiometry and is 8-9 ppm for all forms of the Zn(II) and Cd(II) enzymes. The E·P = E + P, equilibria are described by analogous pH functions for the Zn(II) and Cd(II) enzymes. At acid pH E·P predominates and is converted to E·P as the pH is raised, following a sigmoid pH profile. For the Zn(II) enzyme the midpoint of the E·P = E·P equilibrium occurs at pH 5, while for the Cd(II), and Cd(II) enzymes the midpoints are pH 8.7 and 10, respectively. The ionization controlling the equilibrium between E·P and E·P may be that of a metal-bound H$_2$O (°OH nucleophile) whose $K_a$ will depend strongly on the hardness of the coordinating metal ion. For the Zn(II), enzyme one of 2 mol of E·P formed by the enzyme at acid pH dissociates readily at pH 7.5-8 where dissociation of E·P ($K_a \approx$ mm) is rate-limiting. Phosphate binds more tightly to the Cd(II) enzyme and 2 mol of phosphate remain bound until about pH 9 where E·P begins to dissociate at mK concentrations. The low $K_a$ for E·P and the alkaline shift in the E·P = E·P pH profile probably account for the slow turnover of the Cd(II) enzyme. Precise chemical shifts of the $^{113}$Cd and $^3$P NMR signals as well as the ratio of E·P/E·P at one active center of the dimer are altered by metal ion binding at the other active center indicating significant subunit-subunit interactions.

Inorganic phosphate is a substrate and inhibitor of the nonspecific hydrolysis of phosphate monoesters by *Escherichia coli* alkaline phosphatase (1, 2). Phosphate turnover can be represented as follows:

$$E + P \rightleftharpoons E \cdot P \rightleftharpoons E \cdot P + H_2O.$$  \hspace{1cm} (1)

The two intermediates, E·P$^-$ and E·P$, represent covalent and noncovalent forms of the enzyme, respectively. Formation of E·P involves phosphorylation of a serine (ser 102) at the active site (3, 4).

Much work has already been done by $^3$P NMR to investigate the system under various conditions of pH and phosphate content as well as at different zinc: enzyme stoichiometries and with metal ions other than zinc (Mn, Co, Cu, and Cd) (5-13). Alkaline phosphatase is well suited to study by $^3$P NMR because it is possible to resolve all three of the phosphorus species present and their resonances are all relatively narrow, $<70$ Hz. Interest in species other than the zinc metalloenzyme stemmed from the unsuitability of zinc for many spectroscopic studies. By using $^{113}$Cd (S = $\frac{1}{2}$) and $^{113}$Cd (S = $\frac{3}{2}$)-substituted alkaline phosphatases it is possible to observe simultaneously both metal and phosphate sites.

While the Cd(II) enzyme forms both E·P and E·P, the activity is $\leq 1\%$ of the Zn(II) enzyme (3). The resonance from E·P has nearly identical $^3$P chemical shifts in both species. The metal ions can be removed from the cadmium phosphoryl enzyme without loss of the phosphoseryl group, which now resonates $\sim 2$ ppm further upfield at 6.2 ppm (9). In contrast the E·P species are very different, resonating near 4 ppm for the zinc enzyme and 13 ppm for the cadmium enzyme (11). It was recently demonstrated that E·P in cadmium-substituted alkaline phosphatase involves direct metal-phosphate coordination so that the large difference in chemical shift may arise from a change in bond order or in the constraints on the metal-O-P bond angle (11). Some data suggest that $^3$P chemical shifts of simple phosphate esters correlate with torsional angles around the phosphorus (14-16). A further difference between the Zn(II) and Cd(II) enzymes, which may relate to the mechanism of phosphorylation-dephosphorylation, is the pH at which the concentrations of E·P and E·P become equal. For both metalloenzymes the E·P form is favored at high pH, yet the zinc enzyme is $\sim 100\%$ in this form at pH 6, while the cadmium enzyme is still $\sim 100\%$ as E·P, not becoming 100% E·P until above pH 9 (7).

The pH dependence of the three-stage equilibrium, E + P $\rightleftharpoons$ E·P $\rightleftharpoons$ E·P, and the chemical reactivity of the intermediates is central to the mechanism of action of alkaline phosphatase. This paper presents a detailed study of this equilibrium by $^3$P NMR following these phosphointermediates as formed by enzymes containing 2-6 Zn(II) or Cd(II) ions and in the presence or absence of Mg(II).
MATERIALS AND METHODS

Enzyme Preparation—Alkaline phosphatase was isolated from CW3747 E. coli by osmotic shock and purified by heat treatment, ammonium-sulfate precipitation, and gel chromatography using Sephadex G-100 superfine (3). Enzyme concentrations were determined spectrophotometrically at 278 nm using E\textsubscript{1%26;cm} = 0.72 (17) and a molecular weight for the dimer of 94,000 based on the complete sequence (4). Enzyme activity was measured by the release of p-nitrophenol from p-nitrophenyl phosphate (Sigma) in 1 M Tris-HCl at pH 8 and 22 \degree C (18). The native enzyme had a specific activity of 2300 ± 500 of substrate hydrolyzed/h/mg of protein. All buffer solutions were prepared metal-free (19) and contained 0.01 M Tris-HCl, 0.01 M acetate, and 0.1 M NaCl.

Apoprotein phosphatase was prepared by dialysis of enzyme (−10 ng/mg) against 2 liters of 2 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} as described in the previous paper (20).

\textit{Me(II)} alkaline phosphatases were prepared by addition of stoichiometric quantities of stock solutions of Zn\textsubscript{II} or MgSO\textsubscript{4} (spectro-grade from Johnson Matthey Chemicals Ltd., London), \textit{111}\textsuperscript{Cd}(CH\textsubscript{3}COO), \textit{111}\textsuperscript{Cd}(CH\textsubscript{3}COO)\textsubscript{2} or \textit{111}\textsuperscript{Cd}(CH\textsubscript{3}COO)\textsubscript{3} (prepared from 96% isotopically enriched oxides, Oak Ridge National Laboratory) to concentrated AP\textsuperscript{m} samples. Metal ion stoichiometries were checked by atomic absorption spectroscopy (21).

\textit{P} NMR Studies—\textit{P} NMR spectra were recorded at 89.08 MHz on a Bruker CXP-200 spectrometer equipped with a broadband tunable probe. Spectra were recorded at 293 K with a sweep width of 4000 Hz. Proton decoupling (2W) was employed. Pulse widths of −30° were used with a cycle time of 1.1 s unless otherwise stated. Chemical shifts are reported relative to 85% H\textsubscript{3}PO\textsubscript{4}. Samples of 1.8 cm\textsuperscript{3} were contained in 10-mm NMR tubes fitted with a coaxial capillary containing D\textsubscript{2}O for the external field-frequency lock and methyl phosphonate as an external reference. A line broadening of 10 Hz was employed. The experiment in which the phosphorus resonance of the E-P complex of \textsuperscript{111}\textsuperscript{Cd}(II) was decoupled from \textsuperscript{111}\textsuperscript{Cd} was performed using the same probe in modified form. No field-frequency lock was used. Instead the deuterium channel was tuned to the frequency of cadmium 111 at this field strength (42.4 MHz) and used to irradiate \textsuperscript{111}\textsuperscript{Cd} while observing the \textit{P} spectrum.

RESULTS

Zn\textsubscript{II}-containing Alkaline Phosphatase Species—At zinc-enzyme stoichiometries of less than 4:1 the \textit{P} NMR spectrum resulting from phosphorylation is usually heterogeneous (5). This arises from the high affinities of both A and B as well as other sites for zinc (21, 22), so that binding is not sufficiently selective to give homogeneous Zn\textsubscript{II}(II)-AP species when added at the millimolar concentrations used for NMR experiments. In the absence of high Mg\textsubscript{II}, sufficient zinc must be added to occupy both pairs of A and B\textsuperscript{m} sites fully before a homogeneous \textit{31P} NMR spectrum can be observed. Spectra for this species, Zn\textsubscript{II}(II)-AP, are shown in Fig. 1 for the pH range 5.0–6.0. At pH 5.0 two resonances are seen, one at 8.60 ppm corresponding to E-P and a second at 4.23 ppm arising from E-P. The percentage of phosphate bound as E-P decreases, while that bound as E-P increases as the pH is raised. The midpoint [E-P] = [E-P] of the transition is −pH 5. There is no variation in chemical shift for either E-P or E-P resonances over this pH range. At pH values above 6 no E-P is seen, while above pH 7 a growing resonance from dissociated P, is visible.

The complete transition is illustrated in Fig. 2 with a sample of Zn\textsubscript{II}(II)-AP at pH 5 containing 1 equivalent of phosphate and 2 equivalents of magnesium. The resulting \textit{31P} NMR spectrum shows all the phosphate to be bound, with resonances from both E-P and E-P species. Whereas the E-P resonance appears to be homogeneous and has a chemical shift the same as for the Zn\textsubscript{II}(II)-AP species (8.60 ppm), the E-P peak is clearly composed of two components, one at 4.12 ppm (the same as Zn\textsubscript{II}(II)-AP) and a new one at 3.49 ppm. The binding of Mg\textsubscript{II} is expected to be p-phosphate dependent, hence the inhomogeneity may result from partial occupancy of the C site at this low pH, although the identification of the site involved is not certain. Addition of more Mg\textsubscript{II} increases the amplitude of the 3.49 ppm resonance at the expense of the 4.12 ppm resonance. If the pH is then raised to 6.5, not only does the E-P resonance disappear, but the E-P resonance becomes homogeneous with a chemical shift of 3.50 ppm. This is probably due to the higher affinity of magnesium for an additional pair of sites rendering all of the alkaline phosphatase Zn\textsubscript{II}(II)-Mg\textsubscript{II}(II)-AP. The distribution between Mg\textsubscript{II} binding at separate sites or competition between Zn\textsubscript{II} and Mg\textsubscript{II} for similar types of site cannot be made from \textit{31P} data alone (21). The E-P resonance remains homogeneous to higher pH, but some phosphate dissociates to give a free P, resonance.

Homogeneous Cd\textsubscript{II}(II)-containing Alkaline Phosphatases—The effects on the \textit{31P} NMR spectrum of phosphorylated \textit{111}\textsuperscript{Cd}(II)AP (1 equivalent of phosphate added) as the pH is raised are shown in Fig. 3. At low pH, where the species is first formed, only a single resonance is seen, arising from the phosphoserine residue of the E-P complex. Its chemical shift is 8.45 ppm and it is a singlet in the proton-decoupled spectrum, though any coupling less than 20 Hz would go undetected. The line width of 43 Hz. By pH 8 the percentage of bound phosphate existing as E-P is sufficiently high (~10%) to make its resonance clearly discernible at 13.02 ppm. The \textit{31P} resonance of E-P is a doublet with a coupling of ~33 Hz to a single \textit{111}\textsuperscript{Cd}(II) nucleus. The intrinsic line width of the resonance is ~40 Hz. Both the E-P and E-P resonances have comparatively narrow line widths, which may reflect some rotational freedom about bonds to the phosphorus. In marked contrast to the Zn\textsubscript{II}(II) enzyme, phosphate does not dissociate from the Cd\textsubscript{II} enzyme until above pH 9.0 when a resonance from free P begins to appear (Fig. 3).

A second equivalent of phosphate added to the enzyme (not shown) is bound as E-P and E-P without altering their relative proportions. The chemical shifts of the two resonances also appear to be unaffected by the presence of the second equivalent of bound phosphate (Fig. 4). While both E-P and E-P resonances of the diphosphoenzyme show a slight pH dependence of their chemical shifts, each remains homogeneous. The E-P resonance moves downfield by 0.4 ppm between pH 7.3 and 8.3 and then returns upfield by 0.1 ppm between pH 8.3 and 10. In contrast, the E-P resonance titrates continuously downfield a total of 0.35 ppm over the range pH 7.3 to 10 (Fig. 4).
$^{31}$P NMR of Alkaline Phosphatase

**Fig. 1 (left).** 80.9 MHz $^{31}$P NMR spectra of Zn(II)$_2$ alkaline phosphatase containing 2 equivalents of phosphate as a function of pH. The enzyme concentration was 1.72 mM. Each spectrum represents the average of 9000 transients.

**Fig. 2 (right).** $^{31}$P NMR spectra (80.9 MHz) of Zn(II)$_2$,Mg(II)$_3$AP, containing 1 equivalent of phosphate, as a function of pH. The enzyme concentration was 2.13 mM. Spectra are the average of 14,000 transients. The most downfield resonance is external methyl phosphonate.

$^{113}$Cd(II)$_2$AP forms E-P and E-P intermediates whose $^{31}$P chemical shifts are generally similar to those of the same intermediates formed by $^{113}$Cd(II)$_2$AP. The Cd(II)$_2$AP, however, will bind only 1 equivalent of phosphate, hence a prominent P resonance from the second equivalent of phosphate is apparent throughout the pH titration. As shown in the previous paper (20), phosphorylation of Cd(II)$_2$AP results in migration of half the Cd(II) from A to B sites, hence the phosphoenzymes of both Cd(II)$_2$ and Cd(II)$_3$ species represent active sites with both A and B sites filled. The chemical shift of E-P formed by the Cd(II)$_2$ enzyme is at significantly higher field than for Cd(II)$_2$AP, 8.02 ppm versus 8.56 ppm when compared at pH 7.58 (Fig. 4). The corresponding E-P resonance also shows the spin-spin coupling of ~30 Hz to a single
**31P NMR of Alkaline Phosphatase**

**Fig. 3.** 31P NMR spectra (80.9 MHz) of 113Cd(II)₆AP, containing 1 equivalent of phosphate, as a function of pH. The enzyme concentration was 2.0 mM. Spectra are the average of 7000 transients. The most downfield resonance is external methyl phosphonate.

113Cd nucleus, but the chemical shift is 0.35–0.45 ppm to lower field than that for E·P of Cd(II)₆AP (Fig. 2). The chemical shifts of all E·P and E·P species are summarized in Table I. **E·P = E·P Equilibrium**—Assuming the T₁ (spin-lattice relaxation time) values of the E·P, E·P and P resonances do not change significantly as a function of pH, it is possible to obtain estimates of the percentage of each species present at each pH from the resonance areas and these percentages are plotted in Fig. 5 for Zn(II)₆, Zn(II)₆Mg(II)₂, Cd(II)₆, and Cd(II)₆AP. Of the three homogeneous species plotted, only the Zn(II)₆ and Cd(II)₆ enzymes exhibit the complete shift.
from E-P to E-P and finally dissociation of P, over the pH range examined, 5 to 10. They have several features in common. At low pH (below pH 5 for Zn(II),AP and Zn(II),Mg(II),AP and below pH 8 for Cd(II),AP; E-P predominates. As the pH is raised there is a shift in the equilibrium position towards E-P, which occurs at the same rate for all three systems, so that at 1.5 pH units above the midpoint of the transition E-P is no longer of measurable intensity. At still higher pH inorganic phosphate, which was previously bound, starts to dissociate. This is evident above pH 7 for Zn(II),AP and Zn(II),Mg(II),AP and above pH 9.5 for Cd(II),AP (Fig. 5). It should also be noted that the profiles for Zn(II),AP and Zn(II),Mg(II),AP are superimposable, despite the differences in chemical shift noted above for the 31P resonances of their E-P and E-P intermediates. The similarity between Zn(II),AP and Cd(II),AP highlights the unimportance of the third pair of sites in influencing the mechanism and is the justification for drawing comparisons between a Me(II), species (Zn(II),AP) and a Me(II), species (Cd(II),AP). Indeed, with the Zn(II),AP enzyme, the same chemical shifts and titration behavior are observed as for the Zn(II),AP system. The need for using a Me(II), species for cadmium arises from the instability of Cd(II),AP with respect to disproportionation upon phosphorylation (20). In contrast to the Zn(II),AP species, if only two Zn(II) ions are added along with 10 mM Mg(II) followed by dialysis, a homogeneous Zn(II),Mg(II),AP species is produced whose phosphoenzymes show quite different chemical shifts and titration behavior for those for the two Zn(II) species shown in Fig. 5. This probably represents occupancy of the B sites by Mg(II).

**Stoichiometry of Phosphate Binding**—From the pH titrations of the homogeneous phosphoenzyme derivatives, it is apparent that phosphate binds more weakly at higher pH such that both phosphate binding sites may not be fully saturated at stoichiometric concentrations of inhibitor:enzyme. To quantitate this, phosphate was titrated into Cd(II),AP and Zn(II),AP at pH 6 and pH 9 or 8 and the fraction bound determined from the areas of the resultant 31P resonances. The data expressed as phosphate added versus phosphate bound are shown in Fig. 6. For the cadmium derivative with all metal sites occupied the affinity constant characterizing both sites remains >10^6 M^-1 so that phosphate in excess of 2 equivalents per dimer is required before a resonance due to free phosphate is seen. As already indicated above (Fig. 5), the affinity of the zinc enzyme for phosphate is lowered substantially by raising the pH.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Chemical shifts</th>
<th>Chemical shifts</th>
</tr>
</thead>
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<tr>
<td>ApoAP</td>
<td>4-8</td>
<td>6.2 ppm</td>
<td>3.2 ppm</td>
</tr>
<tr>
<td>Native AP (Zn(II),Mg(II))</td>
<td>7.1</td>
<td>8.63 ppm</td>
<td>4.26 ppm</td>
</tr>
<tr>
<td>Zn(II),AP</td>
<td>6.2</td>
<td>8.60 ppm</td>
<td>3.47 ppm</td>
</tr>
<tr>
<td>Zn(II),Mg(II),AP</td>
<td>6.0</td>
<td>8.60 ppm</td>
<td>3.47 ppm</td>
</tr>
<tr>
<td>Cd(II),AP A and B</td>
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<td>7.9 ppm</td>
<td>4.36 ppm</td>
</tr>
<tr>
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<td>12.79 ppm</td>
</tr>
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<tr>
<td>Cd(II),Mg(II),AP</td>
<td>9.0</td>
<td>8.99 ppm</td>
<td>12.89 ppm</td>
</tr>
</tbody>
</table>

* From Ref. 9.

† Applies to phosphoenzyme prior to migration of Cd(II) from A to B site. 113Cd signal is split into A and A'.

‡ From Ref. 36.

**Fig. 5. Relative equilibrium concentrations of phosphorus species for zinc and cadmium-containing alkaline phosphatases as a function of pH.**

- **C**: E-P:
- **E-P**: P, A: 1.98 mM Zn(II),AP plus 1 equivalent P;
- **E-P**: P: B: 2.13 mM Zn(II),Mg(II),AP plus 1 equivalent P;
- **E-P**: P: C: 2.00 mM Cd(II),AP plus 2 equivalents P;
- **E-P**: P: D: 1.83 mM Cd(II),AP plus 2 equivalents P.

Phosphate was added at pH 6.5 and equilibrium attained before the pH was adjusted. Only 1 equivalent of phosphate remained bound throughout the titration and therefore the percentages of E-P and E-P are normalized to this concentration rather than to 2 equivalents. Solid lines are for visual aid. Broken lines represent E-P concentrations determined previously from 31P measurements (7).
NMR spectra of E-P formed by all species of \(^{113}\text{Cd(II)}\) alkaline phosphatase, the noncovalent phosphate complex shows a \(~30\) Hz coupling expected for a typical \(^{113}\text{Cd}-\text{O}^{31}\text{P}\) bond (Figs. 3 and 7 plus \(^{31}\text{P}\) spectra in the preceding paper (20)). Thus, the phosphate oxygen of E-P is coordinated to one, but not both of the two \(\text{Cd(II)}\) ions, A and B, at each active center. In order to determine which \(\text{Cd(II)}\) ion is coordinated, we prepared a \(^{111}\text{Cd(II)}\)AP at pH 9.1 in which approximately equal concentrations of E-P and E-P were present (Fig. 3). The \(^{113}\text{Cd}\) NMR spectrum of this species shows two resonances for site A separated by \(~14\) ppm, the upfield one corresponding to E-P, the downfield to the E-P species. Likewise, two resonances occur for site B separated by \(6\) ppm, the upfield corresponding to E-P and the downfield to E-P (see Fig. 8 in the preceding paper for the corresponding \(^{113}\text{Cd}\) spectrum (20)). Using the heteronuclear decoupling arrangement described under "Materials and Methods," the above set of \(^{111}\text{Cd}\) signals were irradiated while observing the \(^{31}\text{P}\) spectrum. Irradiation of the B site \(^{113}\text{Cd}\) signal corresponding to E-P causes no loss of coupling. Irradiation of the A site signal corresponding to E-P causes partial collapse of the coupling on the \(^{31}\text{P}\) signal of E-P. The heteronuclear decoupling employing the deuterium lock coil to feed in the \(^{113}\text{Cd}\) frequency is a continuous wave experiment which involves a very narrow \(^{113}\text{Cd}\) frequency band. In contrast the \(^{111}\text{Cd}\) enzyme resonances are much broader, hence, complete collapse of the coupling is not achieved.

In contrast to the E-P resonance, if the downfield A site \(^{111}\text{Cd}\) signal, corresponding to the E-P species, is irradiated no alteration of the coupling on the \(^{31}\text{P}\) signal of E-P is observed. Moreover, no alteration in line width of the \(^{31}\text{P}\) signal of E-P is observed either, confirming the impression that the phosphate of E-P is not coupled to either cadmium ion. Hence, the phosphate in E-P appears to be coordinated to the A site metal ion. Careful analysis of the line shape of the \(^{31}\text{P}\) reso-
nances for both E-P and E-P during the initial stages of phosphorylation of $^{115}$Cd(II)AP, when both metal ions remain in A sites, tends to support the same conclusion (see Fig. 3 in the preceding paper (20)). $^{31}$P spectra over the first 2 h of phosphorylation were summed and show the resonance from E-P to be much broader than that for E-P and coupling appears to be present, although good resolution is not possible because of the transient appearance of E-P at pH 6.5.

**Inter-site Metal Rearrangement**—Prior to the above described decoupling experiment, an attempt was made to assign the metal responsible for phosphate coordination by ordered addition of spin = $\frac{1}{2}$ and spin = 0 cadmium isotopes. To a sample of apoenzyme at pH 9.0 were added sequentially 2 equivalents each of cadmium 112 and cadmium 113 followed by 3 equivalents of inorganic phosphate. Data collection was started 30 min after addition of phosphate. The $^{31}$P spectrum representing the first 2000 scans (40 min) is shown in Fig. 7A. In addition to the heterogeneity of the E-P resonance, which is composed of peaks at the positions of Cd(II)${}_n$ and Cd(II)${}_h$ E-P species, the E-P resonance exhibits spin-spin coupling. The next 2000 scans show the disappearance of any distinguishable doublet without a change in chemical shift or in the proportions of the three phosphorus species present (Fig. 7B). Hence, on initial formation of the Cd(II) enzyme at pH 9, there exists the possibility for facile exchange between A and B site Cd(II) ions. This exchange is facilitated by phosphate binding. Since the A site metal ion is coupled to E-P, it must be the B site that is preferentially occupied by the first pair of Cd(II) ions at pH 9.0, since coupling is initially present only when the second pair is $^{115}$Cd(II) (Fig. 7A). Distribution of two $^{115}$Cd(II) ions between sites A and B of the phosphorylated Cd(II)AP at pH 9.0 can be demonstrated by addition of two $^{117}$Cd(II) plus P, in which case the phosphorus of E-P is coupled to the $^{115}$Cd(II) (Fig. 7C). If two $^{112}$Cd(II) ions are then added, the resonance of E-P becomes a pseudotriplet showing that half the A sites contain $^{115}$Cd, while half contain $^{117}$Cd (Fig. 7D).

**Discussion**

The reactivities of the phosphoenzyme intermediates, E-P and E-P, the hydrolysis of the former and dissociation of the latter, are the key elements in the alkaline phosphatase mechanism. Studies of the exchange of $^{18}$O from H$_2$O into inorganic phosphate catalyzed by alkaline phosphatase (23, 24) and the demonstration using $^{18}$O, $^{17}$O, $^{16}$O chiral phosphate esters that enzymatic hydrolysis proceeds by retention of configuration around phosphate (25) strongly suggest that E-P is an essential intermediate even at alkaline pH where equilibrium concentrations of this intermediate are small. With the proper preparative sequence homogeneous species can be obtained and the $^{31}$P NMR of their E-P and E-P intermediates provide much information about the chemical state of these intermediates. One of the advantages of $^{31}$P NMR is that it permits simultaneous observation of both E-P = E-P and E-P = E + P, equilibria without perturbing the system. The data obtained previously on formation of E-P by labeling with H$^{32}$PO$_4^-$ (7) suffer from the limitation of being able to measure directly only covalently bound phosphate and from the perturbing nature of the method. While $^{31}$P-labeling suggested the alkaline stability of E-P formed by the Cd(II) enzyme (7), precise data were not available and the dramatic influence of the metal ion stoichiometry on the pH dependence of the E-P equilibrium was not detected.

**Factors Affecting the Chemical Shift of E-P and E-P—**

Phosphoserine ionizes from the monoaionic to the diaionic state with a pK$_s$ of 5.5 and with a concomitant change in chemical shift from −0.3 ppm to 3.7 ppm (26). Inorganic phosphate behaves similarly and exhibits a downfield shift of 2.5 ppm (from 0.2 to 2.7 ppm) for the same ionization. In contrast, the $^{31}$P resonance of the E-P forms of alkaline phosphatase is in the range 8.0–8.9 ppm for the metalloenzymes (both Zn(II) and Cd(II)) and occurs at 6.2 ppm in apophosphoryl alkaline phosphatase (12). Even more striking are the chemical shifts for the noncovalent E-P complexes which are between 12.8 and 13.4 ppm when Cd(II) occupies the corresponding sites (Fig. 4). In addition, unless the enzyme is denatured, there is no radical pH dependence of the chemical shift of the E-P resonance to indicate protonation at low pH (Fig. 2), so that it presumably exists solely as the diatomic as low as pH 4 in both Zn(II) and Cd(II) enzymes. E-P is not formed as an equilibrium species below pH 7 except for the Zn(II)-containing enzyme. Over the observable pH range for this species (>pH 4.8) the chemical shift is also independent of pH suggesting that it is also the diatomic.

Compared with free phosphoserine there is a downfield shift of 2.5 ppm for the phosphoserine group at the active center in the absence of metal ion (apophosphoryl enzyme) accompanied by a fall in the pK$_s$, by at least 2.5 pH units (9). Binding of metal ions induces an additional downfield shift of 1.5–2.4 ppm (Table I). The lowering of the pK$_s$, presumably results from the existence of positive charges close to the phosphate moiety which stabilize the diatomic form. E-P is coordinated to one A site metal ion, a divalent metal ion is clearly one of these groups. The presence of proximal metal ions could stabilize both E-P and E-P and lower the pK$_s$ of both intermediates. On the other hand, the pK$_s$ of E-P in the apophosphoryl enzyme is also lowered implying the involvement of protein side chain(s) as well. This may be the arginine side chain, which has been implicated in the mechanism from chemical modification and protection studies (27, 28).

The chemical shift of the E-P complex is thus much more sensitive to the change of metal ion species than is E-P, the former differing by −9 ppm from Zn(II) to Cd(II), while the latter differs by at most 1 ppm between metal ion species. The major source of the difference appears to be the nature of the metal at A site, since E-P in Cd(II)AP at pH 6.5, prior to occupation of the B sites by cadmium migration, has a chemical shift of 12.79 ppm, as described in the previous paper (20). It is unlikely that the difference between Zn(II) and Cd(II) arises from a difference in mechanism, since both metalloenzymes appear to proceed via the same intermediates. Along with the absence of coupling of E-P to $^{115}$Cd(II), this suggests that the phosphoseriny residue is less closely connected to the metal ion than the phosphate group of the noncovalent complex. Perhaps the phosphate leaves the coordination sphere on formation of the ester. In contrast, the larger ionic radius of Cd(II) compared with Zn(II) (0.97 Å versus 0.74 Å) must have a significant effect on the geometry of the E-P complex. It is unclear how this alters the properties of the phosphate group, but it may arise from a distortion of the group which

1 One possible explanation for the large difference in chemical shifts of the cadmium and zinc E-P complexes is coordination of phosphate to cadmium but not to zinc. However, a phosphorylated $^{115}$Cd(II)AP enzyme has been observed that did not show the normal $^{115}$Cd–P coupling. Although an E-P resonance was seen at low field (~12 ppm) it is exhibited by $^{115}$Cd–P–P coupling indicating an absence of direct coordination. Despite a chemical shift only 1 ppm upfield from normal cadmium E-P such uncoupled low field E-P complexes have also been seen in $^{115}$Cd(II)Zn(II)AP hybrids in which cadmium occupies A sites and zinc B sites. It is, therefore, unlikely that the presence of direct phosphate–metal coordination for the cadmium but not for the zinc enzymes is an adequate explanation for the large difference in chemical shifts of the E-P species of the two systems (P. Gettins and J. E. Coleman, unpublished results).
is bound more tightly by the Cd(II) enzyme (see below).

The chemical shifts of both E·P and E·P for any individual enzyme species are subject to small, but significant changes in chemical shift as a function of pH (Fig. 4). These small shifts do not appear to be related to single ionizations and probably do not involve the metal ions or phosphate directly, but may reflect pH-dependent changes in adjacent enzyme conformation.

The E·P = E·P Equilibrium—The pH where [E·P] = [E·P] varies from pH 5 to 10 depending on both the metal ion species and the metal protein stoichiometry. The sigmoidal shape of the E·P → E·P transition suggests that an ionization in the active center is responsible. The most remarkable change in this function is the shift from a midpoint of pH 5 to a midpoint of pH 8.7 on changing from the Zn(II) to the Cd(II) enzyme (Fig. 5). This implies an alkaline shift of 3.7 pH units in the pKₐ of the group controlling the E·P = E·P equilibrium. An attractive candidate for the ionizing group influencing the E·P = E·P equilibrium is a metal-coordinated water, whose pKₐ is lowered from 15.7 to 7.5-8 by the mixed enzyme-Zn(II) complex. The actual value of this pKₐ need not correspond to the midpoint of the E·P → E·P equilibrium, but may be several units higher depending on the absolute values of k₁ and k₋₂ (Equation 1). Thus, it may correspond to the apparent pKₐ for enzymatic activity. Substitution of Cd(II) for Zn(II) could thus be interpreted to result in a far less effective lowering of the pKₐ of the coordinated solvent. A similar effect has been observed for carbonic anhydrase, where there is evidence to support the existence of a Zn(II)-OH-containing enzyme species as the active form (29, 30). There is also a shift of the pH rate profile by 2 to 3 units toward alkaline pH upon substitution of Cd(II) for Zn(II) in this enzyme (31).

Dissociation of E·P—At H⁺ concentrations where significant equilibrium concentrations of E·P are present, both Zn(II) and Cd(II)·AP bind phosphate sufficiently tightly to form 2 mol of phosphoenzyme (E·P + E·P) at millimolar concentrations of enzyme and phosphate (Figs. 5 and 6). This is, of course, the pH region where dephosphorylation of E·P is expected to be rate-limiting as has been shown by many previous investigations of both Zn(II) and Cd(II) enzymes (7, 12, 32–35). In contrast, when alkaline pH values are reached, where E·P becomes the exclusive equilibrium species, phosphate binding affinity decreases dramatically and for the Zn(II) enzyme Kₐ for at least one phosphate must be >mm (Fig. 5). The binding curve is nonlinear, not even hyperbolic (shown by the dashed curve), suggesting that not only has Kₐ for phosphate increased, but two different binding constants may apply. One site has a Kₐ ≪ mm and the second has a Kₐ ~ mm. For the Zn(II) enzyme the phosphate dissociation follows a sigmoid curve very close to that describing the appearance of enzyme activity (35). Since dissociation of E·P is the rate-limiting step at alkaline pH as shown by previous NMR studies (36), this increase in the dissociation rate of phosphate would appear to have functional significance. The appearance of a negative charge in the active center, e.g. a metal-coordinated hydroxyl, could be responsible for reducing the phosphate binding constant.

The same formulation could apply to the Cd(II) enzyme except that the corresponding pH range is shifted ~2 pH units more alkaline (Fig. 4) and the affinity for phosphate is sufficiently high that 2 equivalents of phosphate remain bound up to pH 9 (Fig. 6). On this basis one of the reasons for the low activity of the Cd(II) enzyme is its much higher affinity for phosphate in the normal pH range. Activity might appear above pH 9.0 in the region where phosphate begins to dissociate. Another effect of Cd(II) substitution, however, may be primarily responsible for slow turnover and that is the slow dephosphorylation rate associated with the distortion of E·P produced by Cd(II) substitution. As shown in the previous paper, dephosphorylation of the seryl hydroxyl from E·P is very slow (hours to days) at both pH 6.5 and 9.0 (20). Thus, the dephosphorylation rate for HOP remains slower than P, dissociation, although phosphorylation of the Cd(II) enzyme by ROP might be faster and allow significant turnover in the region of phosphate dissociation, >pH 9.

Subunit-Subunit Interactions—The dimeric nature of alkaline phosphatase provides the opportunity for cooperativity of substrate binding. There have been both claims (1, 3, 37) and rebuttals (38, 39) that phosphate binding is negatively cooperative. We will now summarize results from this and the preceding paper that are relevant both to negative cooperativity of phosphate binding and more generally to subunit-subunit interactions.

Cd(II)·AP at alkaline pH has both A and B sites of one monomer occupied by cadmium, while both sites of the other monomer are vacant. Yet °Cd(II)·AP, with A and B sites of both monomers filled, does not simply show the same °Cd NMR spectrum as twice the intensity, but a different character to the resonances. Not only are the precise chemical shifts different, but some type of chemical exchange modulation is present (20). This is further emphasized by the change in the pH where [E·P] = [E·P] between the Cd(II), and Cd(II), phosphoenzymes, from pH 10.2 in the former to pH 8.7 in the latter (Fig. 5). If changes in structure at the active center of one monomer do not influence the other, these changes are very difficult to explain.

Further support for the dissimilarity of Cd(II)-containing subunits in Cd(II)·AP versus Cd(II)·AP comes from the °P chemical shift data on the phosphorylated species. The equilibrium E·P species of Cd(II)·AP formed at pH 6.5 has a °P chemical shift of 7.96 ppm compared with 8.42 ppm for E·P of the Cd(II)·AP at the same pH value. Similarly, the °Cd chemical shifts reflect the same difference, A and B site cadmium in phosphorylated °Cd(II)·AP have resonances at 142 and 56 ppm compared with 155 and 70 ppm in °Cd(II)·AP (20).

The most compelling evidence for negative cooperativity was provided by the sequential spectra during the phosphorylation of °Cd(II)·AP at pH 6.5 (20). Prior to addition of phosphate, the subunits are identical with cadmium in both A sites. Phosphate binds, however, at only one active center. The subsequent migration of cadmium from the A site of the unphosphorylated monomer to the B site of the phosphorylated subunit is, thus, an effect and not a cause of the phosphate-induced asymmetry (see "Discussion" in preceding paper (20)).

Mechanistic Implications—The analogous pH profiles for the formation of E·P and E·P by the Zn(II) and Cd(II) enzymes (Figs. 1, 2, and 5) suggest that the latter is a reasonable model for the native enzyme. The great differences in catalytic activity at pH 8, dependent upon whether the enzyme contains Zn(II) or Cd(II), arise from two causes. First, the binding of inorganic phosphate is much weaker to Zn(II)·AP than to Cd(II)·AP. Second, it can be seen from the pH profiles of the E·P = E·P interconversion (Fig. 5) that the equilibrium favors E·P for the cadmium enzyme, but E·P almost exclusively for the zinc enzyme in the physiological pH range. The slowness of the dephosphorylation of the Cd(II) enzyme in the alkaline pH region can be compared with the slow dephosphorylation of the Zn(II) enzyme at pH 5 and presumably results directly from the different pKₐ values of the nucleophile (metal coordinated OH⁻) responsible for hydrolysis, which is related to the greater polarizing power
of the smaller zinc ion.

Formation of E-P by the Cd(II) enzymes appears to involve coordination to the A site Cd(II) ion. Both formation of E-P and hydrolysis of E-P are metal ion dependent (3) and can proceed only with only A site occupied (20). Coordination of the phosphate to A site would explain the metal ion dependence of phosphate binding. Such coordination must also place the phosphorus in a suitable position for nucleophilic attack by the hydroxyl oxygen of seryl 102. Formation of the seryl ester appears to move the phosphate out of the coordination sphere, since no coupling is observed on the 31P signal from E-P and the chemical shift of E-P is relatively insensitive to metal ion species, in contrast to E-P (Fig. 4).

The precise function of the B-site metal ion is less clear. Its presence clearly accelerates phosphorylation, as shown in the first paper of this series (21). This could result from enhanced nucleophilic attack made possible by the additional neutralization by the B site metal ion of the negative charge at the active site arising from the phosphate dianion. The possible coordinated hydroxyl ion postulated to participate in the hydrolysis of the phosphoserine ester, as outlined above, could be associated with either A or B site metal ions. Since phosphorylation of any Cd(II) enzyme species always induces occupancy of A and B sites at equilibrium, we do not know whether dephosphorylation can proceed in the presence of A site metal alone. Since phosphate has apparently moved out of the inner coordination sphere of the A site metal ion accompanying phosphorylation, there would appear to be an open coordination site on the A site metal ion available for a coordinated solvent molecule, which could create the nucleophile for the final step of the mechanism.

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