The Biosynthesis of Alkaline Phosphatase with a Particulate Fraction of *Escherichia coli*

THE MECHANISM OF INHIBITION BY INORGANIC PHOSPHATE

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1. By digitonin lysis of penicillin spheroplasts of *Escherichia coli* a particulate fraction P_1 was previously obtained that supported the sustained synthesis of alkaline phosphatase when supplied with amino acids, nucleotide triphosphates and other cofactors. This P_1 fraction, when subjected to mild ultrasonic treatment in the presence of sucrose and Mg^{2+} , yielded the $P_1(S)$ fraction, consisting of integrated particulate subcellular particles containing DNA and RNA. 2. The $P_1(S)$ fraction from *E. coli* K10 wild type ($R^+R^+P^+$) grown under repressed conditions supported the immediate synthesis of alkaline phosphatase *in vitro*. The synthesis occurred in phases. The first was followed by a lag, and then there was a linear rapid phase that continued for at least 3 hr. Actinomycin D inhibited the appearance of the second phase. It was concluded that the particles are programmed to synthesize enzyme even when prepared from repressed cells, and therefore that synthesis of the specific messenger RNA for alkaline phosphatase *in vivo* was not inhibited when the bacteria were grown in an excess of inorganic phosphate. 3. Phosphate inhibited synthesis of enzyme to the same extent with the $P_1(S)$ fractions of two constitutive strains as with the $P_1(S)$ fraction of the wild-type strain. 4. Inorganic phosphate inhibited amino acid incorporation with the $P_1(S)$ fraction and also inhibited enzyme synthesis *in vitro*. The effect on amino acid incorporation could be partially overcome by adding Mn^{2+} to the incubation mixtures. However, Mn^{2+} inhibited the synthesis of alkaline phosphatase. Also, inhibition of the incorporation of $[^{32}P]CTP$ into RNA was overcome by Mn^{2+} . The effect of phosphate on amino acid uptake was most probably due to a phosphorolysis of RNA by polynucleotide phosphorylase, also present in the $P_1(S)$ fraction. This phosphorolysis may be responsible for the instability of messenger RNA *in vitro* and *in vivo*. 5. Phosphate also specifically inhibited the formation of alkaline phosphatase, since it did not affect markedly the induced formation of β -galactosidase by the same $P_1(S)$ fraction. The specific effect is attributed to the prevention of formation of the enzymically active dimer from precursors, a Zn^{2+} -dependent reaction. It is suggested that the repression of the synthesis of alkaline phosphatase *in vivo* in the wild-type strain was the sum of these two effects.

The discovery that digitonin lysis of penicillin spheroplasts of *Escherichia coli* yielded a subcellular particulate preparation that would synthesize β -galactosidase (Nisman & Fukuhara, 1959) and alkaline phosphatase (Nisman, Fukuhara, Demailly & Genin, 1962; Bishop, Roche & Nisman, 1964) when appropriately supplemented with amino acids, ribonucleoside triphosphates and other cofactors has prompted an investigation of the properties of this fraction. This fraction, called P_1 , contained DNA and RNA integrated into a particulate lipoprotein structure. The biosynthetic properties of the P_1 fraction have been

reviewed by Nisman, Pelmont, Bishop, Demailly & Yapo (1963a) and Nisman & Pelmont (1964).

Further fractionation of the P_1 preparation by mild ultrasonic treatment and differential centrifugation in sucrose-containing media has yielded a particulate fraction, $P_1(S)$, that still contains RNA, DNA and protein in essentially the same proportions as in the P_1 fraction. As demonstrated in the present paper, these particles have all the activities necessary for the synthesis of enzyme proteins but at a decreased efficiency as compared with the P_1 fraction.

The availability of such a preparation has

allowed a detailed investigation of the mechanism of biosynthesis of the repressible enzyme alkaline phosphatase (Horiuchi, Horiuchi & Mizuno, 1959; Torriani, 1960). The particles supported the synthesis of the enzyme and this synthesis was inhibited *in vitro* by inorganic phosphate, a repressor *in vivo*. Inorganic phosphate had two inhibition effects *in vitro*. The first was a general inhibition of protein synthesis that was reversible by Mn^{2+} . The second was a specific effect on the synthesis of alkaline phosphatase, the inhibition of the Zn^{2+} -catalysed formation of the enzymically active dimer. A preliminary account of this work has been published (Manson, Pelmont & Nisman, 1964).

MATERIALS AND METHODS

Method of preparation of the particulate fraction $P_1(S)$. The method of preparation of the P_1 fraction from 500 ml. of an overnight culture of the various strains used in the present study was as reported by Bishop *et al.* (1964). The P_1 fraction was washed by centrifugation in a Servall refrigerated centrifuge (model RC-2, rotor SS-34) for 20 min. at 25000g three times in medium 1 (0.5 M-sucrose–15 mM- $MgCl_2$ –10 mM-tris buffer, pH 7.3) by suspending gently with wide-bore pipettes to minimize shear forces. The final suspension in 40 ml. of medium 1 was passed vigorously 60 times through a 100 ml. syringe without a needle. The resulting viscous suspension in 5 ml. amounts in capped plastic centrifuge tubes was placed inside the stainless-steel cup of the Raytheon (250 w; 10 kcy./sec.) ultrasonic oscillator, containing 20 ml. of cold water, and subjected to full-power ultrasonic treatment for 3 min. The suspension was then centrifuged for 20 min. at 25000g. The particles were resuspended in medium 1 and centrifuged again at the same speed. The final pellet was suspended in 15–20 ml. of medium 7 [1.2 M-sucrose–15 mM- $MgCl_2$ –10 mM-tris buffer, pH 7.3, containing penicillin (500 units/ml.) and bentonite suspension (0.2 ml./100 ml.; see below)] and centrifuged for 10 min. at 10000g. The final supernatant fluid, containing a suspension of particles, is called the $P_1(S)$ fraction. The RNA/protein and DNA/protein ratios of the P_1 fraction for 40 different preparations were 0.32 and 0.12 respectively (Bishop *et al.* 1964), whereas the same ratios for the $P_1(S)$ fraction for 11 different preparations were 0.27 and 0.09.

Bentonite suspension. Bentonite (50 g.) was suspended in 1 l. of distilled water with 5 g. of EDTA (sodium salt). The mixture was agitated vigorously with a magnetic stirrer at approx. 75° for 3 hr. The suspension was centrifuged for 10 min. at 3000g and the pellet discarded. The supernatant fluid was then centrifuged for 20 min. at 25000g. The drained sediment was suspended in 200 ml. of 0.01 M-tris buffer, pH 7.3, containing $MgCl_2$ (1 mM). This suspension was autoclaved for 15 min. and stored in the cold.

Incubation conditions for the synthesis of alkaline phosphatase by the $P_1(S)$ fraction. The following substances were added per ml. of incubation mixture for the synthesis of enzyme [the concentrations, with minor exceptions, being the same as those reported by Bishop *et al.* (1964) to be effective with the P_1 fraction]: tris buffer, pH 7.3, 100 μ moles; amino acid mixture, 0.05 ml. (the composition

of the amino acid mixture, containing all 20 naturally occurring α -amino acids, was twice that used by Bishop *et al.* 1964); sucrose, 300 μ moles; $CaCl_2$, 6 μ moles; ATP, UTP, GTP and CTP, 0.05 μ mole each; NADP⁺, 3 m μ moles; KCl, 100 μ moles; $MgCl_2$, 8 μ moles; glucose, 3.0 μ moles; phosphoenolpyruvate, 1 μ mole; pyruvate kinase, 0.05 m μ g.; penicillin G, 5000 units; $P_1(S)$ fraction, 25–100 μ g. of protein. The incubation mixture was equilibrated at 0° for 15 min., zero-time samples were removed and the tubes were incubated for 4 hr. at 37°. Duplicate samples (0.2 ml.) of the incubation mixture were removed for enzyme assay at various times, chloramphenicol was added (final concn. 1 mg./ml.) and the samples were refrigerated until assay was performed. There was no change in enzyme assay after storage for several days at 0°; therefore assays were usually performed the next day. All the experiments reported in the present paper were carried out on the same day that the $P_1(S)$ preparation was made. The $P_1(S)$ fraction made from strain K10 retained 70% of its activity when stored for 24 hr. at 0°.

To exclude the possibility that the observed enzyme synthesis was due to contaminating bacteria, one experiment after 5 hr. of incubation was sampled and plated for viable cells. A cell count of 10⁴ viable cells/ml. was found. This number of cells would account for 0.1% of the amount of enzyme that was found to have been synthesized. Each $P_1(S)$ preparation was examined with phase-contrast optics under oil immersion with a Leitz microscope. No whole bacteria were seen, nor were any whole spheroplasts evident.

Alkaline-phosphatase assay. The assay mixture contained 0.2 ml. of the sample, equilibrated at 32°, 0.2 ml. of 1.0 M-tris buffer, pH 8.0, containing β -mercaptoethanol (1 mM) and 0.2 ml. of a solution of *p*-nitrophenyl disodium phosphate (10 mg./ml.) dissolved in 1.0 M-tris buffer, pH 8.0, containing β -mercaptoethanol (1 mM) and $MgCl_2$ (10 mM). Incubation was continued until a sufficient amount of colour (extinction 0.2–1.0) had developed. The reaction was stopped by the addition of 0.6 ml. of 0.5 M-phosphate buffer, pH 7.5, containing EDTA (10 mM). Under these conditions the colour formed was stable at room temperature for 2 hr. The amount of colour was determined

Table 1. *Effect of orthophosphate on assay of alkaline phosphatase*

The conditions of assay were those described in the text. The enzyme used was a purified preparation of alkaline phosphatase from *E. coli* strain C4. Three separate solutions of *p*-nitrophenyl phosphate, made in the usual buffer (see the text), were used.

Concn. of substrate ... Addition to assay tube	Observed alkaline-phosphatase activity (units)		
	0.6 mg./ml.	6.5 mg./ml.	10 mg./ml.
None (control)	625	656	675
Phosphate (0.2 μ mole)	475	698	685
Phosphate (0.4 μ mole)	405	672	673
Phosphate (0.6 μ mole)	383	660	669
Phosphate (0.8 μ mole)	352	590	642
Phosphate (1.0 μ mole)	345	520	586

with a Beckman spectrophotometer at 410μ , a reagent blank being used. Since orthophosphate is a competitive inhibitor for the enzyme (Garen & Levinthal, 1960), it was necessary to use the *p*-nitrophenyl phosphate reagent at 10mg./ml. to ensure that the phosphate that was liberated or added during the original incubation conditions did not interfere with the assay (Table 1). This amount of *p*-nitrophenyl phosphate was empirically found to give a correct assay with a known amount of enzyme in the presence of as much as 0.8μ mole of inorganic phosphate in the assay mixture (or 4μ moles of inorganic phosphate/ml. of original incubation mixture). One unit of enzyme activity is defined as that amount of enzyme that liberates 1μ mole of *p*-nitrophenol/hr., the extinction coefficient for nitrophenol being taken to be 1.62×10^4 (Plocke, Levinthal & Vallee, 1962). Enzyme activity in the *E. coli* preparations used was linear with respect to time and enzyme concentration under the conditions described above.

Bacterial strains. The following strains of *E. coli* were kindly supplied by Dr A. Garen and Dr H. Echols: wild-type ($R_1^+R_2^+P^+$), Hfr strain K10 of *E. coli* K12 and two mutant strains C2 ($R_1^-R_2^+P^+$) and C4 ($R_1^-R_2^-P^+$). The two mutant strains are constitutive, i.e., the enzyme is not repressible by inorganic phosphate.

Determination of protein, RNA and DNA, and incorporation of radioactive amino acids into proteins. The methods used were those described by Nisman *et al.* (1962).

Inorganic phosphate. This was measured by the method of Sumner (1944).

RESULTS

Synthesis of alkaline phosphatase by the $P_1(S)$ fraction. The supplements that were found necessary for the $P_1(S)$ fraction isolated from wild-type *E. coli* for optimum synthesis of alkaline phosphatase *in vitro* are shown in Table 2. These requirements were essentially the same as those reported by Bishop *et al.* (1964) for the synthesis of this enzyme *in vitro* with the P_1 fraction. Sucrose was essential for maintaining the structure of the

particles that carry out the synthesis. A requirement for a polyhydroxy non-ionic substance for the synthesis of alkaline phosphatase with a particulate fraction of *Bacillus subtilis* has been reported by Whitely & Oishi (1963). In the latter case, enzyme formation was observed in the presence of approx. 540μ moles of glycerol/ml.

An amino acid mixture must be supplied for optimum synthesis, as must also $NADP^+$, glucose, Ca^{2+} and Mg^{2+} . The omission of phosphoenolpyruvate and pyruvate kinase did not affect the amount of enzyme synthesized with these preparations. This test was carried out in the presence of glucose and $NADP^+$. Since it has been demonstrated that the P_1 fraction can carry out oxidative phosphorylation (Nisman, Zubrzycki, Bishop & Pelmont, 1963c), it is likely that the energy requirements of the system were met in this way. In addition, phosphoenolpyruvate is a source of orthophosphate, as are all phosphate esters as a result of phosphatase action, and, as demonstrated below, orthophosphate inhibited enzyme formation. These effects together may explain the inability to demonstrate a marked effect on enzyme synthesis by the omission of phosphoenolpyruvate.

Omission of the nucleoside triphosphates led to only a slight decrease in enzyme synthesis. Bishop *et al.* (1964) did demonstrate a significant decrease in enzyme formation by omitting the triphosphates with the P_1 fraction. However, during the preparation of $P_1(S)$ from the P_1 fraction, the synthesizing activity of the preparation decreases four- to five-fold, and the maximum amount of enzyme synthesized/mg. of protein with the $P_1(S)$ fraction was slightly less than the amount of enzyme that these authors observed with the P_1 fraction without added nucleotides.

Table 2. Requirements for the synthesis of alkaline phosphatase *in vitro* with the $P_1(S)$ fraction of wild-type *Escherichia coli* K10 ($R_1^+R_2^+P^+$)

The $P_1(S)$ fraction was incubated with complete incubation mixture (see the Materials and Methods section) or with omissions as indicated. Values in the Table are means of duplicate determinations. The standard deviation for zero-time values was ± 24 units/mg. of protein. The increase in alkaline-phosphatase activity in the complete system was arbitrarily taken as 100.

	Alkaline-phosphatase activity (units/mg. of protein)			Relative increase in alkaline-phosphatase activity
	Initial	Final	Increase	
Omission from complete system				
None (control)	760	1260	500	(100)
Pyruvate kinase	782	1288	506	101
Amino acids	773	1014	241	48
Ribonucleoside triphosphates	751	1210	459	92
$NADP^+$	750	1120	370	74
Glucose	749	990	241	48
Sucrose	759	924	165	33
Ca^{2+}	768	1038	270	54
Mg^{2+}	750	1155	405	81

To see if the concentration of supplements was optimum, the formation of alkaline phosphatase was observed under conditions where the concentration of supplements was kept constant and the concentration of the $P_1(S)$ fraction was varied (Table 3). The efficiency of synthesis/mg. of $P_1(S)$ fraction decreased only slightly as the concentration of $P_1(S)$ fraction/ml. of incubation mixture increased. The effect on amino acid incorporation was not as marked. Since the enzyme whose synthesis was being studied was alkaline phosphatase and there were large amounts of phosphate esters in the incubation mixtures, the concentration of inorganic phosphate was assayed

during the incubation. It was found, not unexpectedly, that the inorganic phosphate concentration was proportional to the amount of $P_1(S)$ fraction used. The omission of Ca^{2+} doubled the amount of phosphate released. The omission of glucose resulted in a small but significant rise in the concentration of phosphate. The omission of $NADP^+$ had no effect on the phosphate concentration but did affect enzyme synthesis. Also Ca^{2+} in these concentrations did not affect alkaline-phosphatase activity. The orthophosphate concentration was, in all cases, less than $0.01 \mu\text{mole/ml.}$ at zero time and increased linearly with time during the incubation.

Table 3. *Effect of concentration of the $P_1(S)$ fraction on the alkaline phosphatase synthesized and orthophosphate released by the $P_1(S)$ fraction of Escherichia coli K 10*

The experimental conditions and composition of complete incubation systems were as given in the Materials and Methods section. The initial alkaline-phosphatase activity of the $P_1(S)$ fraction was 275 ± 8 units/mg. of protein. $L-[Me-^{14}C]$ Methionine was added to the incubation mixture to give a final concentration of $0.8 \mu\text{C/ml.}$ The specific radioactivity of zero-time controls was less than 100 counts/min./mg. of protein.

Omission(s) from complete system	Concn. of $P_1(S)$ fraction (mg. of protein/ml.)	Alkaline phosphatase formed (units/mg. of protein)	Amino acid incorporation (counts/min./mg. of protein)	Phosphate found ($\mu\text{mole/ml.}$)
None (control)	0.036	583	24 420	0.07
None (control)	0.072	525	25 200	0.11
None (control)	0.108	454	22 600	0.17
Glucose	0.072	443	23 100	0.13
$NADP^+$	0.072	388	21 100	0.11
$NADP^+$ + glucose	0.072	410	24 000	0.13
Ca^{2+}	0.072	392	21 300	0.22

Table 4. *Effect of various inhibitors on the synthesis of alkaline phosphatase with the $P_1(S)$ fraction of Escherichia coli K 10 in vitro*

The composition of complete incubated system and the experimental conditions were as given in the text. Inhibitors were added before the addition of the $P_1(S)$ fraction to the incubation mixtures. All tubes were equilibrated for 45 min. at 0° , then incubated for 4 hr. at 37° . The initial alkaline-phosphatase activity of the $P_1(S)$ fraction was 425 ± 20 units/mg. of protein. The initial concentration of the $P_1(S)$ fraction was $43 \mu\text{g.}$ of protein/ml. of incubation mixture. $L-[Me-^{14}C]$ Methionine was added to give a final concentration of $0.8 \mu\text{C/ml.}$ The specific radioactivity of the zero-time control incubation was 260 counts/min./mg. of protein.

Addition to complete system	Alkaline phosphatase formed (units/mg. of protein)	Relative increase in alkaline phosphatase	Amino acid incorporation (counts/min./mg. of protein)	Relative increase in amino acid incorporation
None (control)	635	(100)	52 600	(100)
Ribonuclease ($25 \mu\text{g./ml.}$)	387	61	37 350	71
Ribonuclease ($50 \mu\text{g./ml.}$)	355	56	32 100	61
Deoxyribonuclease ($50 \mu\text{g./ml.}$)	317	50	26 300	50
Deoxyribonuclease ($75 \mu\text{g./ml.}$)	324	51	27 350	52
Actinomycin D ($20 \mu\text{g./ml.}$)	286	45	17 900	34
Actinomycin D ($40 \mu\text{g./ml.}$)	178	28	15 590	30
Chloramphenicol ($250 \mu\text{g./ml.}$)	0	0	502	1
Phosphate ($1 \mu\text{mole/ml.}$)	267	42	29 450	56
Phosphate ($2 \mu\text{moles/ml.}$)	121	19	21 600	41

Effect of inhibitors on the synthesis of alkaline phosphatase by the $P_1(S)$ fraction. Table 4 shows the effects of various inhibitors on enzyme synthesis and amino acid incorporation into protein catalysed by the $P_1(S)$ fraction. The sensitivity to ribonuclease was not great, and this was most probably due to the presence in the preparations of significant amounts of bentonite. The sensitivity to deoxyribonuclease was partial and was similar to that observed with the P_1 fraction by Bishop *et al.* (1964). Actinomycin D, a known inhibitor of the DNA-primed RNA polymerase, also inhibited synthesis but again only partially. Chloramphenicol abolished all amino acid incorporation and enzyme synthesis. In general, there was a correspondence between the effect of an inhibitor on enzyme synthesis and amino acid incorporation into protein except for that seen with inorganic phosphate. In this case, the inhibition of enzyme formation was much more severe than the effect on amino acid uptake. This result was always observed

when the comparison was made. This phenomenon is discussed below.

Kinetics of the synthesis of alkaline phosphatase with the $P_1(S)$ fraction. The kinetics of the synthesis of alkaline phosphatase and the effects of several inhibitors on the kinetics are shown in Fig. 1. Two phases in the formation of enzyme by the $P_1(S)$ fraction were always observed. There was an immediate formation of enzyme that slowed down between 60 and 150 min. Then there was a rapid rise in the rate of enzyme appearance and the kinetics observed were linear for at least another 3 hr. Actinomycin D inhibited the appearance of the second phase of enzyme formation. Inorganic phosphate inhibited both the first and second phases of enzyme appearance. Similar kinetics were seen with seven different $P_1(S)$ preparations of strain K 10 as well as with the $P_1(S)$ fractions of the two constitutive strains C2 and C4.

In contrast with the observed kinetics of enzyme synthesis, the incorporation of labelled amino acid protein was linear with time (Fig. 2). The inhibitory

Fig. 1. Kinetics of the synthesis of alkaline phosphatase with the $P_1(S)$ fraction of *E. coli* K10. The composition of the incubation mixtures and the experimental conditions were as given in the Materials and Methods section. Actinomycin D ($20 \mu\text{g./ml.}$), orthophosphate ($2 \mu\text{moles/ml.}$) and chloramphenicol ($250 \mu\text{g./ml.}$) were added to the incubation mixture (at 0°) 45 min. before incubation at 37° was begun. The $P_1(S)$ fraction was the same as that used in Table 4. ○, Control; △, system with actinomycin D; □, system with inorganic phosphate; ●, system with chloramphenicol.

Fig. 2. Kinetics of amino acid incorporation into protein with the $P_1(S)$ fraction of *E. coli* K10. The experimental conditions for the incubation systems were the same as those listed for Table 4. Samples of the incubation system (0.5 ml.) were removed and precipitated with trichloroacetic acid. The trichloroacetic acid precipitate was washed, plated and counted by the methods described by Nisman *et al.* (1962). ○, Control; △, system with added phosphate ($1 \mu\text{mole/ml.}$); □, system with added phosphate ($2 \mu\text{moles/ml.}$).

Table 5. *Effect of orthophosphate on the synthesis of alkaline phosphatase by the P₁(S) fraction of Escherichia coli K 10 (wild type), C 2 (constitutive, R₁-R₂+P+) and C 4 (constitutive, R₁+R₂-P+)*

The P₁(S) fraction of each strain was incubated with the complete incubation mixture (see the Materials and Methods section) for 4 hr. at 37°. The amount of P₁(S) fraction used in each experiment was: strain K 10, 36 µg./ml.; strain C 2, 71 µg./ml.; strain C 4, 29 µg. of protein/ml. of incubation mixture.

	Strain K 10		Strain C 2		Strain C 4	
	Alkaline-phosphatase activity (units/mg. of protein)	Relative increase in alkaline-phosphatase activity	Alkaline-phosphatase activity (units/mg. of protein)	Relative increase in alkaline-phosphatase activity	Alkaline-phosphatase activity (units/mg. of protein)	Relative increase in alkaline-phosphatase activity
Control (zero time)	422 ± 18		905 ± 32		5620 ± 125	
Control (incubated)	1078 ± 28		1145 ± 35		6274 ± 118	
Increase in control	656	(100)	240	(100)	654	(100)
Control + phosphate (1 µmole/ml.)	328	51	96	40	209	32
Control + phosphate (2 µmoles/ml.)	138	21	38	16	124	19

effect of inorganic phosphate on amino acid incorporation is also shown. The establishment of the inhibition by inorganic phosphate must have been rapid, since it was already evident at the end of the first hour of incubation. Linear kinetics of amino acid incorporation into protein were also observed in the presence of added inorganic phosphate.

Mechanism of the inhibition by phosphate of the synthesis of alkaline phosphatase. (a) Effect of phosphate on the synthesis of alkaline phosphatase by the P₁(S) fractions of constitutive strains. At this stage of the investigation it seemed apparent that the isolation procedure had yielded an organized particle, the P₁(S), able to carry out the sustained synthesis *in vitro* of alkaline phosphatase. This enzyme was repressible in wild-type *E. coli* (Horiuchi *et al.* 1959; Torriani, 1960), i.e., very small amounts of enzyme were formed when the cells were grown in the presence of high concentrations of inorganic phosphate in the growth medium. Echols, Garen, Garen & Torriani (1961) have isolated a large number of mutant strains of *E. coli* K 10 in which the enzyme was found to be constitutive, i.e., even when the cells were grown in high concentrations of inorganic phosphate the cells formed substantial amounts of the enzyme. We therefore examined the sensitivity of the P₁(S) fractions of two mutant strains to orthophosphate (Table 5). Under identical conditions and regardless of the initial content of enzyme in the P₁(S) fractions, orthophosphate inhibited synthesis of the enzyme *in vitro* to the same extent with the P₁(S) fractions of each of the three strains used.

(b) Effect of phosphate on the synthesis of β -galactosidase by the P₁(S) fraction. From parallel studies carried on in this Laboratory, it was known that the P₁(S) fraction of *E. coli* K 10 would synthesize β -galactosidase when induced *in vitro* with an appropriate inducer such as isopropyl β -D-thiogalactoside. If the inhibition by phosphate of the synthesis of alkaline phosphatase that was seen in the previous experiments was specific, then there should be no comparable inhibition of the synthesis of β -galactosidase by the same particles under the same conditions. An experiment was therefore undertaken to induce the formation of β -galactosidase by using identical incubation conditions but omitting glucose (Table 6). A small inhibition of β -galactosidase was seen (20%), but this inhibition was considerably less severe than that seen with the synthesis of alkaline phosphatase in the presence of 2 µmoles of inorganic phosphate/ml. (Table 5). The conditions for β -galactosidase synthesis were not optimum, since Mn²⁺, a potent stimulator of β -galactosidase synthesis, could not be used in these studies as it inhibited the synthesis of alkaline phosphatase (Table 8). In the presence of Mn²⁺, phosphate had

Table 6. *Effect of orthophosphate on the synthesis of β -galactosidase by the $P_1(S)$ fraction of Escherichia coli K 10*

Incubation was for $3\frac{1}{2}$ hr. at 37° . The final concentration of isopropyl β -D-thiogalactoside (IPTG) was 0.5 mM. All other supplements were the same as indicated in the Materials and Methods section, except that glucose was omitted. The standard assay for β -galactosidase was that used by Nisman *et al.* (1962). The same $P_1(S)$ fraction was used for this experiment as that used in Table 3.

Addition(s) to complete system	β -Galactosidase activity	
	(units/mg. of protein)	(%)
None (control)	3	1
IPTG	310	(100)
IPTG + phosphate (1 μ mole/ml.)	250	81
IPTG + phosphate (2 μ moles/ml.)	250	81

Table 7. *Effect of orthophosphate on the incorporation of cytidine [α - 32 P]triphosphate into ribonucleic acid with $P_1(S)$ fraction of Escherichia coli K 10*

The incubation mixture contained (per ml.): tris buffer, pH 7.3, 100 μ moles; amino acids, 0.1 ml.; CTP, 0.3 μ mole; UTP, 0.6 μ mole; GTP, 0.6 μ mole; ATP 0.7 μ mole; Mg^{2+} , 6 μ moles; KCl, 133 μ moles; [α - 32 P]CTP, 92500 counts/min. Samples were taken at zero time and after incubation at 37° , and precipitated with an equal volume of 10% (w/v) trichloroacetic acid filtered on a Millipore filter. The filter was washed several times with cold 10% trichloroacetic acid, dried and counted in a Geiger counter. The radioactivity of the zero-time control was 60 counts/min./mg. of protein.

Addition(s) to complete system	Radioactivity (counts/min./mg. of protein)	
	At 15 min.	At 30 min.
None (control)	1920	2210
Phosphate (1 μ mole/ml.)	686	785
Mn^{2+} (3 μ moles/ml.)	8240	11650
Mn^{2+} (3 μ moles/ml.) + phosphate (1 μ mole/ml.)	9160	13330

Table 8. *Effect of Mn^{2+} , Zn^{2+} and orthophosphate on the synthesis of alkaline phosphatase by the $P_1(S)$ fraction of Escherichia coli*

The initial activity of the $P_1(S)$ fraction was 760 ± 22 units/mg. of protein. DL-[14 C]Lysine was added to the amino acid mixture to give a final concentration of 1.6 μ Ci/ml. The radioactivity of the zero-time control was less than 40 counts/min./mg. of protein. The composition of the complete incubation system and experimental conditions are given in the Materials and Methods section.

Addition(s) to complete system	Alkaline phosphatase formed (units/mg. of protein)	Amino acid incorporation (counts/min./mg. of protein)
None (control)	500	2780
Mn ²⁺ (3 μmoles/ml.)	18	3820
Phosphate (2 μmoles/ml.)	199	2490
Mn ²⁺ (3 μmoles/ml.) + phosphate (2 μmoles/ml.)	—23	3490
Zn ²⁺ (0.1 μmole/ml.)	195	—
Zn ²⁺ (1.0 μmole/ml.)	93	1826

no effect on β -galactosidase synthesis at all (J. Pelmont, unpublished work).

(c) Effect of phosphate on DNA-primed RNA polymerase of the $P_1(S)$ fraction. It has been reported by Pelmont, Nisman, Demailly & Yapo (1963a) and Pelmont, Yapo, Demailly & Nisman (1963b) that the DNA-primed RNA polymerase is present in the P_1 and the $P_1(S)$ particles and that the 32 P of [α - 32 P]CTP is largely incorporated into an RNA fraction in the P_1 preparation that has the characteristics of messenger RNA (Bishop *et al.* 1964). Similar experiments have been performed with the $P_1(S)$ fraction and essentially the same results have been obtained (J. Pelmont, unpublished work).

The effect of inorganic phosphate on the incorporation of [32 P]CTP into RNA as catalysed by the $P_1(S)$ fraction was examined (Table 7). This incorporation was severely inhibited by inorganic phosphate. The addition of Mn^{2+} to the inhibition mixture stimulated the incorporation of the CTP into RNA and completely abolished the inhibition by inorganic phosphate. It was known from the studies of Chamberlain & Berg (1962) that Mn^{2+} is an activator of the RNA polymerase and that, with the P_1 fraction, Mn^{2+} stimulated both the rate of induced β -galactosidase formation and amino acid incorporation into protein (Nisman & Hirsch, 1958; Nisman *et al.* 1962). However, as pointed out above, Mn^{2+} cannot be added to incubation mixtures for alkaline phosphatase since it severely inhibited enzyme formation (Table 8).

(d) Effect of phosphate on Zn^{2+} -catalysed dimer formation. In addition to the general inhibitory effect on protein synthesis induced by orthophosphate, there was a specific inhibition of the formation of alkaline phosphatase (Tables 3, 4 and 5). Plocke *et al.* (1962) reported that alkaline phosphatase of *E. coli* is a zinc-containing enzyme, with 2.1 moles of zinc/mole of enzyme. Schlessinger & Levinthal (1963) have demonstrated that it is

possible to dissociate the active dimer into inactive monomers and that a subsequent incubation with Zn^{2+} would re-form the active enzyme; Mn^{2+} would not reactivate the enzyme. Zinc phosphate is an insoluble substance (solubility product 9.1×10^{-33}) that might be expected to form at pH 7.3–8.0, the pH range in which dimerization was studied and synthesis was observed. There was the possibility that orthophosphate might bind the available Zn^{2+} in the $\text{P}_1(\text{S})$ fraction and thus inhibit the formation of the active enzyme. Attempts to stimulate the $\text{P}_1(\text{S})$ fraction with Zn^{2+} failed, since Zn^{2+} was found to inhibit enzyme synthesis completely and inhibit severely amino acid incorporation into proteins (Table 8).

A study of the effect of Zn^{2+} and orthophosphate on the formation of the active dimer from acid-formed monomer was undertaken. It has been reported that acid treatment converts the enzyme with S_{20} 6.77 s into a homogeneous substance with S_{20} 2.6 s (Malamy, 1963). Acid treatment of a

semi-purified alkaline-phosphatase preparation obtained from a C4 strain did result in a loss of activity that could be recovered by incubation in the presence of Zn^{2+} (Table 9). Incubation of the acid-treated material in the absence of added Zn^{2+} restored some activity. Incubation with orthophosphate alone restored some activity. Incubation with added Zn^{2+} restored all the initial activity, and this restoration was inhibited by an equimolar amount of orthophosphate. The order of addition of supplements to the acid-treated preparation had no effect on the outcome.

The fact that enzymically inactive monomer can be formed *in vitro* from the isolated enzyme is not by itself direct proof that the monomer is a precursor of the active enzyme in the biosynthetic chain. Experiments were therefore attempted to demonstrate directly that in the presence of orthophosphate some material was formed that could be converted into active enzyme in a subsequent Zn^{2+} -dependent reaction under conditions

Table 9. *Effect of orthophosphate on the Zn^{2+} -catalysed activation of alkaline phosphatase*

A semi-purified preparation of alkaline phosphatase from *E. coli* C4 was treated for 30 min. at pH 4 in acetate buffer (Schlessinger & Levinthal, 1963). Portions were subsequently incubated for 1 hr. at 37° in 1 M-tris buffer, pH 8.0, with the listed additions. Samples were cooled to 25° and assayed for enzyme activity. The Zn^{2+} was supplied as zinc acetate.

	Alkaline-phosphatase activity (units)	Relative alkaline-phosphatase activity
Before acid treatment	330 ± 6	(100)
After acid treatment	96	29
Incubated without Zn^{2+}	127	38
Incubated with added Zn^{2+} (1.0 $\mu\text{mole/ml.}$)	326	99
Incubated with phosphate (1.0 $\mu\text{mole/ml.}$)	207	63
Incubated with phosphate (1.0 $\mu\text{mole/ml.}$) and Zn^{2+} (1.0 $\mu\text{mole/ml.}$)	225	68

Table 10. *Effect of a post-incubation with Zn^{2+} on the alkaline phosphatase synthesized in vitro by the P_1 fraction of Escherichia coli K 10*

The experimental conditions and composition of incubation mixtures with listed additions were as given in the text. All assays were performed in duplicate, and zero-time values are averages of four replicates. Incubation was at the indicated temperatures. At 2 and 4 hr., replicate samples were treated with chloramphenicol (final concn. 1 mg./ml.) and stored in ice overnight. Post-incubation was in 0.5 M-tris buffer, pH 8.0, containing β -mercaptoethanol (1 mM) with or without zinc acetate (1.5 mM) for 90 min. at 37°. Assays for enzyme activity were then performed as usual. The zinc/phosphate ratio in the post-incubation mixture was 3:2. Values in parentheses are the additional units of enzyme activity revealed by post-incubation with Zn^{2+} as compared with that obtained without Zn^{2+} .

		Alkaline-phosphatase activity (units/ml. of incubation mixture)					
Addition to complete system	Temp. of incubation	At zero time		At 2 hr.		At 4 hr.	
		Zn^{2+} absent	Zn^{2+} present	Zn^{2+} absent	Zn^{2+} present	Zn^{2+} absent	Zn^{2+} present
None (control)	30°	78 ± 5	82 ± 3	101	103 (+2)	143	152 (+9)
Phosphate (2 $\mu\text{moles/ml.}$)	30	75 ± 4	78 ± 2	49	74 (+25)	64	94 (+30)
None (control)	37	76 ± 6	77 ± 4	128	123 (–5)	149	152 (+3)
Phosphate (2 $\mu\text{moles/ml.}$)	37	69 ± 4	75 ± 1	63	83 (+20)	78	91 (+13)

Table 11. *Effect of post-incubation with Zn^{2+} on the alkaline phosphatase synthesized in vitro by the $P_1(S)$ fraction of Escherichia coli K10*

The compositions of the incubation systems were as given in the text. Incubation was for 4 hr. at 37°. Post-incubation was in 0.5M-tris buffer, pH 8.0, containing β -mercaptoethanol (1 mM) with or without zinc acetate (1 mM) for 90 min. at 37°. Assays for enzyme activity were performed as usual. Duplicate incubation mixtures at two concentrations of the $P_1(S)$ fraction (69.5 and 34.8 μ g./ml.) were carried out with replicate samples taken of each. Each determined value in the Table is the mean of eight replicate assays. The standard deviation for each determination was ± 17 units/mg. of protein.

	Alkaline phosphatase synthesized (units/mg. of protein)		
	Initial	Final	Increase
No phosphate added			
Not post-incubated (control)	583	781	198
Post-incubated	578	781	203
Post-incubated with Zn^{2+}	608	801	193
Phosphate (0.5 μ mole/ml.) added			
Not post-incubated (control)	580	684	104
Post-incubated	573	677	104
Post-incubated with Zn^{2+}	591	729	138
Phosphate (1.0 μ mole added)			
Not post-incubated (control)	602	706	104
Post-incubated	585	679	94
Post-incubated with Zn^{2+}	591	732	141

where protein synthesis was inhibited. The P_1 fraction (Table 10) and the $P_1(S)$ fraction of *E. coli* K10 (Table 11) were incubated under the usual conditions and also in the presence of added orthophosphate. Replicate samples were taken, and chloramphenicol was added (final concn. 1 mg./ml.). The samples were refrigerated overnight. Samples were then incubated at pH 8.0 with and without added Zn^{2+} for 90 min. at 37° (Schlessinger & Levinthal, 1963). All the samples, including those that were not post-incubated, were assayed together under the usual assay conditions. In the control incubations, with no added orthophosphate, the enzyme activity found was not increased significantly by a post-incubation with Zn^{2+} . However, in the presence of orthophosphate, the activity found after a post-incubation with Zn^{2+} was significantly higher than that found either with no post-incubation or a post-incubation without added Zn^{2+} . This phenomenon was observed in seven out of the nine experiments performed. In the two experiments where no post-incubation effect was seen, the amount of the $P_1(S)$ fraction used was extremely small and a statistical significance could not be attributed to the observed results.

DISCUSSION

The subcellular particulate fraction, P_1 , isolated by digitonin lysis of penicillin spheroplasts of *E. coli* appears to be a complex structure containing DNA, RNA, lipoproteins and many enzyme activities (Nisman *et al.* 1963a; Nisman & Pelmont, 1964). These particles have been demonstrated to

support the synthesis of alkaline phosphatase, and in addition they will support the induced synthesis of β -galactosidase but only in the presence of an inducer, such as isopropyl β -D-thiogalactoside or methyl β -D-thiogalactoside. The DNA of the P_1 fraction is integrated into the particles in such a way that relatively large amounts of deoxy-ribonuclease and actinomycin D are required to inhibit enzyme synthesis. The particles also contain RNA attached in such a way that it does not exhibit the usual sensitivity to ribonuclease. A purified lipoprotein fraction has been isolated from the P_1 fraction and found to be an integrated component of the particle (Nisman *et al.* 1964). The RNA polymerase that can synthesize messenger RNA by using DNA as a template has been demonstrated to be in the P_1 and the $P_1(S)$ fractions (Pelmont *et al.* 1963a,b). Some of the amino acid-activating enzymes have been demonstrated to be present in the P_1 fraction (Nisman & Fukuhara, 1959). A comparison has been made of all these activities of the P_1 fraction with the $P_1(S)$ fraction used in the present study, and similar results have been obtained (Pelmont *et al.* 1963a; Nisman *et al.* 1964). The major difference seems to be that the $P_1(S)$ particle is not as efficient in supporting cell-free enzyme synthesis as the P_1 particle. For example, Bishop *et al.* (1964) observed that in 4 hr. the alkaline-phosphatase activity observed was more than 4000 units/mg. of protein with the P_1 fraction of *E. coli* K10, whereas that of the $P_1(S)$ fraction rarely reached more than 1500 units/mg. of protein in the same time. Whether this decreased efficiency is a function only of the

lower nucleic acid content of the $P_1(S)$ fraction or also a deficiency of other enzyme activities (Nisman *et al.* 1963b) is not clear at the present time. In summary, it seems as if the P_1 and the $P_1(S)$ particles contain all the activities necessary for the formation of proteins under the regulation of the structural gene.

A study of the kinetics of the synthesis of alkaline phosphatase with the $P_1(S)$ fraction showed that there was an initial synthesis of enzyme that was sensitive to chloramphenicol. This first phase of enzyme synthesis was not affected markedly by deoxyribonuclease or by actinomycin D, and it took place without an initial lag. Actinomycin D is believed to inhibit the transcription of genetic information by the DNA-primed RNA polymerase (Reich, Goldberg & Rabinowitz, 1962). Since enzyme synthesis took place in the cell-free extract in spite of the presence of large amounts of this inhibitor, the programming for the synthesis of alkaline phosphatase must have already occurred *in vivo*, in spite of the fact that the cells were grown in the presence of large amounts of inorganic phosphate.

The second phase of enzyme synthesis, which was seen after the lag, was sensitive to actinomycin D. This suggested that this phase was dependent on the formation of new messenger RNA and that this could take place if the integrity of the $P_1(S)$ particles was maintained. The results presented in Fig. 1 are those obtained with the $P_1(S)$ fraction of *E. coli* K10; however, similar results were obtained with the $P_1(S)$ fractions of C2 and C4, the mutants in which the enzyme is constitutive.

Having available a cell-free system that would synthesize a repressible enzyme, and having demonstrated that synthesis could be inhibited *in vitro* by orthophosphate, which was an effective repressor *in vivo*, we decided to study further the mechanism of this inhibition. Inorganic phosphate was shown in the present study to have two distinct effects. The first was an inhibition of amino acid incorporation into protein, of CTP incorporation into RNA, and a slight inhibition of β -galactosidase synthesis. Not only were these inhibitions diminished or abolished by the addition of Mn^{2+} to the incubation systems, but each activity was stimulated above the control values in the presence of Mn^{2+} . It has been reported by Sekiguchi & Cohen (1963) and Andoh, Natori & Mizuno (1963) that polynucleotide phosphorylase can break down messenger RNA in cell-free extracts. Inorganic phosphate is a substrate for this enzyme and would activate it if it were present. Littauer & Kornberg (1957) observed that polynucleotide phosphorylase is a particle-bound enzyme in *E. coli*, since even after 10 min. of ultrasonic treatment none of the enzyme was found in soluble

form. They also observed that 0.1 mM-Mn^{2+} inhibited the enzyme by 85%. The enzyme has also been found in the $P_1(S)$ fraction (Pelmont *et al.* 1963a). It is therefore a reasonable possibility that one of the effects of orthophosphate on the $P_1(S)$ fraction is to activate polynucleotide phosphorylase, and that this activation results in a breakdown of messenger RNA and perhaps other RNA, which then results in an inhibition of protein synthesis.

It is likely that this kind of inhibition by inorganic phosphate is more general than has been imagined. It is well known that alkaline phosphatase in *E. coli* and *B. subtilis* (Whitely & Oishi, 1963) is repressed by extracellular phosphate. Freese & Oosterwyk (1963) reported that whole cells of *B. subtilis* make more L-alanine dehydrogenase, an inducible enzyme, in the presence of very low concentrations of inorganic phosphate. They also show that the inhibition of induction of alanine dehydrogenase by inorganic phosphate parallels the repression of alkaline phosphatase with the same strain of bacteria. This phenomenon should be investigated with other repressible and inducible systems, since the phenomenon of inhibition by phosphate of protein synthesis by the activation of polynucleotide phosphorylase may be widespread.

There is a second effect of inorganic phosphate on the biosynthesis of alkaline phosphatase. In Table 4 it was demonstrated that the synthesis of alkaline phosphatase was more severely depressed than was amino acid incorporation into protein. It has been established that alkaline phosphatase is a zinc-containing enzyme (Plocke *et al.* 1962), that the enzyme consists of two identical monomers (Rothman & Byrne, 1963) that by themselves are enzymically inactive, and that dimerization can occur by incubation of the monomers with Zn^{2+} (Schlessinger & Levinthal, 1963). It has been demonstrated in the present study that the acid-treated enzyme can be converted into fully active material by incubation with Zn^{2+} and that this reactivation can be partially prevented by an equimolar amount of inorganic phosphate. Finally, it was demonstrated that, during the biosynthesis of alkaline phosphatase with the P_1 and the $P_1(S)$ fractions in the presence of added inorganic phosphate, some precursors were formed that could be converted into active enzyme by post-incubation with Zn^{2+} in the presence of large amounts of chloramphenicol. The presence of chloramphenicol ensured that during this post-incubation no new protein was synthesized. No such precursor of enzyme was found when orthophosphate was not added. We therefore conclude that the specific inhibition induced by inorganic phosphate on the synthesis of alkaline phosphatase *in vitro* is

the inhibition of the formation of the enzymically active zinc-containing dimer. Final proof of this conclusion will require a direct demonstration that monomer accumulated on the particles in the presence of orthophosphate, a difficult experiment to perform since only small amounts of such a precursor have been observed.

The mechanism of repression of the synthesis of alkaline phosphatase suggested in the present study does not fit the theory of alkaline-phosphatase regulation postulated by Echols *et al.* (1961). The fact that some enzyme is synthesized immediately by the $P_1(S)$ fraction of wild-type *E. coli* in the absence of added orthophosphate, even though the cells used to make the $P_1(S)$ fraction were grown under repressed conditions, suggests that some late step in enzyme assembly is a likely site of inhibition. Since orthophosphate can directly interfere with dimer formation it is most likely that repression *in vivo* takes place at this step. Synthesis *in vitro* slows up only as orthophosphate accumulates, and it is resumed after a lag. Since the resumption of enzyme synthesis is sensitive to actinomycin D and deoxyribonuclease, it is most likely dependent on the formation of new messenger RNA catalysed by the DNA-dependent RNA polymerase present in the particles (Pelmont *et al.* 1963a,b). This synthesis must take place in the presence of a low concentration of orthophosphate, and the lag suggests that it takes some time for it to occur and a new dynamic state established. If these suggestions are correct, there is no need to invoke a genetically determined repressor for alkaline phosphatase. A number of strains of *E. coli* have been isolated in which the enzyme was found to be constitutive (Echols *et al.* 1961). These strains were isolated by growing the culture on a phosphate ester as a sole carbon source. The only strains that could grow under these circumstances were those that were capable of making the enzyme in the presence of large amounts of orthophosphate. The constitutive mutants have been genetically mapped and have been found to fall into two groups: those characterized by a mutation at the R_1 locus and these characterized by a mutation at the R_2 locus. Both the R_1 and R_2 loci were outside the *P* cistron (i.e. the structural gene for alkaline phosphatase). No further characterisation of these mutants has as yet been reported. Since the biosynthesis of the enzyme by the $P_1(S)$ fraction of two of these strains, the R_1^- and the R_2^- , showed the same sensitivity to inorganic phosphate *in vitro* as the $P_1(S)$ fraction of the wild-type strain, one must look for an explanation for constitutivity elsewhere than in the regulation of biosynthesis of the enzyme-forming system directly. Whether alkaline-phosphatase regulation as suggested in the present study is an

exception to the general case will be determined by further investigations of other enzyme-forming systems *in vitro*.

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