AN INCREASE IN ALKALINE PHOSPHATASE IN AN *IN VITRO* SYSTEM DERIVED FROM *BACILLUS SUBTILIS*[1]

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There have been a number of reports that cell-free preparations from micro-organisms are capable of enzyme synthesis. Increases in the activity of tryptophan synthetase of *Neurospora crassa* (Wainwright, 1959) and in the β-galactosidase of *Escherichia coli* (Nisman and Fukuhara, 1959; Kameyama and Novelli, 1960) were the first to be described; more recently, the formation of tryptophan synthetase of *E. coli* (Yura, et al., 1962) and amylase of *Bacillus subtilis* (Oishi, et al., 1962) by particulate and soluble fractions have been reported. The present communication describes a similar observation with respect to alkaline phosphatase of *B. subtilis* and presents evidence concerning requirements and the effects of inhibitors. The requirement for a supernatant fraction from a genetically competent strain is discussed.

Materials and Methods

Conditions of growth. *B. subtilis* was grown in nutrient broth with shaking until the middle of the logarithmic growth phase. The

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cells were then harvested by centrifugation, washed with distilled water and suspended for derepression in a low phosphate medium (Horiuchi, et al., 1959). Strains BC-6[2] and Al-1[2] were incubated in the latter medium with shaking for 3-4 hours; strain K was derepressed in the same medium, with shaking, for 1/2 to 4 hours, depending on the experiment. The cells were harvested by centrifugation, washed once with distilled water and frozen at -20°C.

Cell-free preparations. The frozen cells were suspended in "standard buffer" (0.01 M Tris buffer, pH 7.4, containing 0.01 M $MgCl_2$, 0.1 M KCl and 0.05 M β-mercaptoethanol) supplemented with 10 % glycerol (vol/vol) and disrupted by passage once through a French pressure cell. Intact cells were removed by centrifugation at 10,000 x g for 20 min. and the supernatant was subjected to 17,500 x g for 30 min. to sediment cell debris. The resulting crude extract was centrifuged at 105,000 x g for 90 min. to yield a reddish-brown sediment ("particles" or "105P") and a clear yellow supernatant fraction ("supernatant" or "105S"). The 105P fraction was used without further manipulation; 105S was dialyzed for 3-6 hours against 100 volumes of standard buffer. All manipulations were carried out in the cold.

Assay. Alkaline phosphatase was measured by the rate of hydrolysis of p-nitrophenyl phosphate (NPP). The enzyme preparation in 0.25 ml was added to 0.75 ml of 0.2 % NPP in 1 M Tris buffer, pH 8.3, containing 0.01 M $MgCl_2$. After incubation at 35°C for the desired time, the reaction was stopped by the addition of 2 ml of 0.4 N NaOH and the optical density at 420 mμ was determined. A unit is defined as that amount of preparation which gave an optical density change of 0.01 per hour.

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Results and Discussion

When the particulate fraction (105P) was incubated with the supernatant fraction (105S) in the presence of the proper supplements, alkaline phosphatase activity was found to increase by 50-200 %. This increase is in the same range as those reported for β-galactosidase (Kameyama and Novelli, 1962), tryptophan synthetase (Yura, et al., 1962) and amylase (Oishi, et al., 1962). The requirements for this increase are shown in Table I. The increase in activity was dependent on an energy generating system, the 4 ribotides, amino acids and was maximal in the presence of a stabilizing agent such as glycerol. The greatest increases in activity were obtained with small amounts of 105S and in the presence of low concentrations of phosphorylated compounds (1.4 μmoles/reaction mixture). Higher concentrations were inhibitory, presumably because

Table I: Requirements for the *in vitro* increase in alkaline phosphatase activity

Component omitted	Increase in units
None	29.0
ATP, CP, CP-kinase	3.8
ATP, GTP, CTP, and UTP	3.05
Amino acids	2.55
Glycerol	13.4
105P	1.1
105S	0.8

Reaction mixtures contained the following in a total volume of 0.5 ml: 50 μmoles of Tris at pH 7.2, 5 μmoles of $MgCl_2$, 50 μmoles of KCl, 0.05 ml of a 50 % solution of glycerol, 0.3 μmole of ATP, 0.25 μmole of creatine phosphate (CP), 50 μg of CP-kinase, 0.05 μmole each of GTP, CTP and UTP, 2.5 μmoles of a mixture of 19 naturally occurring amino acids (minus cysteine), 5 μmoles of β-mercaptoethanol, 105P and 105S from strain BC-6 having 0.5 and 0.12 mg protein, respectively. Incubated for 30 min. at 35°C.

Initial activity for reaction mixtures containing 105P and 105S = 23.4; initial activity in the absence of 105S = 23.0; initial activity in the absence of 105P = 1.2.

of the effect of inorganic phosphate on NPP hydrolysis (Garen and Levinthal, 1960). The supernatant fraction contained an ATP-ase and the activity of 105P was inhibited approximately 50 % by 2.5 μmoles of inorganic phosphate/reaction mixture.

Enzyme activity was located chiefly in the particulate fraction but both fractions were required (Table I). The requirement for 105S was specific (Table II). 105S derived either from strain BC-6 (a partially repressed mutant) or strain K (wild type) when added to reaction mixtures containing 105P from strain BC-6 gave rise to an increase in alkaline phosphatase activity. However, 105S from A1-1 (a negative mutant) was not effective. It should be noted that 105S from strain K derepressed for 4 hours was more effective than that obtained after shorter periods of derepression. Supernatant obtained from cells prior to derepression, when the culture had 1/50th the activity found after 4 hours of derepression, stimulated a smaller increase in alkaline phosphatase.

Table II: Specificity of requirement for supernatant fraction

105S from:	Hours of derepression:	Increase in units
None	--	4.1
strain BC-6	4.0	23.5
strain A1-1	4.0	3.3
strain K	0	8.0
"	0.75	11.2
"	2.0	14.9
"	4.0	20.4

Conditions as in Table I using 105P from strain BC-6. This contained 0.7 mg protein whereas the 105S preparations contained 0.3-0.4 mg protein. All 105S preparations were dialyzed for 12 hours against 3 changes of buffer.

Initial activity of the first 3 reaction mixtures = 17.5 units; initial activity of the last 4 reaction mixtures = 18.0 units.

These results suggest that the increase in alkaline phosphatase activity depends on the genotype and degree of repression and

that the controlling factor(s) is present in the supernatant fraction. Preliminary experiments with 105P derived from the negative strain confirm this suggestion. Similar results concerning the requirement for a supernatant fraction from a genetically competent strain were obtained with β-galactosidase and tryptophan synthetase and the responsible factor was shown to be DNA (Nisman and Fukuhara, 1960; Eisenstadt, et al., 1962) and messenger RNA (Wainwright and McFarlane, 1962), respectively.

The increase in alkaline phosphatase activity could be partially inhibited by the addition of compounds known to interfere with protein synthesis or gene expression (Table III). The most reproducible inhibition was obtained with RNA-ase (60-90 % inhibition); results with chloramphenicol, puromycin, actinomycin and DNA-ase (30-80 % inhibition) were more variable, depending on the preparation.

Table III: Effect of inhibitors on the increase in alkaline phosphatase

Amount per reaction mixture	Increase in units
None	24.5
100 µg chloramphenicol	11.8
100 µg puromycin	9.0
100 µg DNA-ase	3.6
10 µg actinomycin D	4.5
10 µg RNA-ase	2.0

Conditions as in Table I. 105P and 105S preparations contained 0.61 and 0.12 mg protein, respectively. Initial activity = 39.5 units.

These results are consistent with the interpretation that the observed increase in alkaline phosphatase was due to protein synthesis, either de novo or by completion of peptide chains whose synthesis was initiated in vivo. Supporting this interpretation are the findings that the B. subtilis preparations are capable of incorpo-

rating labeled amino acids into protein and have an RNA polymerase (details of these results will be presented elsewhere). However, it is also possible that the increase in activity results from activation or release of preformed enzyme. The latter has been reported for the "formation" of tryptophan synthetase by certain particulate fractions of E. coli (Yura, et al., 1962). The in vitro release of preformed proteins by cell-free systems may have requirements similar to those for de novo synthesis (Morris, 1963).

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