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On the Localization of Alkaline Phosphatase and Cyclic Phosphodiesterase in *Escherichia coli**

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ABSTRACT: Evidence is presented for the view that alkaline phosphatase, acid phosphatase, and cyclic phosphodiesterase are localized near the surface of *Escherichia coli*, external to the protoplasmic membrane. Phosphate esters which do not penetrate *E. coli* can be hydrolyzed by intact cells, suggesting that these enzymes are outside of the permeability barrier for phosphate esters. However, the per cent of total activity expressed by intact cells (compared with equivalent cell extracts) varies over extremely wide limits, depending upon the substrate and its concentration. It is believed that components of the cell wall are interposed between the enzymes and the external medium. Presumably the different phosphate esters vary in ease of penetration of the wall barrier. A modified procedure for the purification of *E. coli* alkaline phosphatase is also presented.

Alkaline phosphatase is an enzyme formed by *Escherichia coli* when the growth medium lacks P_i; it is P_i repressible (Horiuchi *et al.*, 1959; Torriani, 1960). Malamy and Horecker (1964) discovered that this phosphatase was released into the sucrose medium when *E. coli* was converted into spheroplasts by means of lysozyme and EDTA (Repaske, 1958). Later, it was found that other degradative enzymes were also released on spheroplast formation, including RNase I, 5′-nucleotidase, cyclic phosphodiesterase, and acid hexose phosphatase (Neu and Heppel, 1964a,b, 1965); also DNase I (Cordonnier and Bernardi, 1965); UDPGase and ADPGase (Melo and Glaser, 1966). These same enzymes were also released by a process called osmotic shock in which washed *E. coli* were first exposed to 0.5 M sucrose containing dilute Tris-HCl buffer and EDTA; after this the pellet of cells was rapidly dispersed in "shock medium" consisting of cold water or cold 5×10^{-4} M $MgCl_2$ (Neu and Heppel, 1965; Nossal and Heppel, 1966). The enzymes listed above were selectively released into the shock medium. Many control enzymes were tested and found to remain entirely within the cell during osmotic shock and within the spheroplast during treatment with EDTA and lysozyme.

The selective release suggested that this family of enzymes might be located at or near the surface of the cell. However, examination of broken cell preparations showed almost all of these enzyme activities to be in the supernatant fraction after centrifugation at 100,000*g* for 1 hr, rather than being attached to cell membrane or wall. A consideration of these facts led Malamy and Horecker (1964) to suggest that alkaline phosphatase is localized in the periplasmic space, a region described by Mitchell (1961) between the protoplasmic membrane and the wall layers.

In this paper we present evidence bearing on the localization of alkaline phosphatase, acid hexose phosphatase, and cyclic phosphodiesterase. We also describe a simple procedure for the purification of alkaline phosphatase from shock fluid.

Experimental Section

Materials. Most of the nucleotides and other phosphate esters were Sigma products. Sodium *p*-nitrophenyl phosphate, bis(*p*-nitrophenyl)phosphate, isopropyl β-D-thiogalactopyranoside, and *o*-nitrophenyl β-D-galactopyranoside also were obtained from Sigma Chemical Co., St. Louis, Mo. Uridine 2′,3′-cyclic phosphate, 2′-AMP, and 3′-AMP were from Schwarz BioResearch, Inc., Orangeburg, N. Y. Uniformly labeled [^{14}C]leucine (220 mCi/mmole) was obtained from New England Nu-

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clear Corp., Boston, Mass. *E. coli* tRNA (strain B) was purchased from General Biochemicals, Chagrin Falls, Ohio, and was subjected to an additional phenol extraction. Crystalline lysozyme (muramidase EC 3.2.1.17) was purchased from Worthington Biochemical Corp., Freehold, N. J. Purified leucyl-tRNA synthetase was the generous gift of Dr. Alan Peterkofsky.

Bacterial Strains and Media. With respect to alkaline phosphatase, strain K37, is repressible (obtained from N. Zinder); C90 is constitutive; U7, E15, and HP-58 are deletion mutants (C90 and U7 obtained from A. Garen, E15 and HP-58 from H. H. Winkler). Strain HP-58 also lacks a glucose 6-phosphate transport system.

The high phosphate medium C contained 0.04 M K_2HPO_4, 0.022 M KH_2PO_4, 0.07 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 3×10^{-3} M Na_2SO_4, 1×10^{-3} M $MgCl_2$, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, and 0.5% Bactopeptone. In the low phosphate medium E, the phosphate salts were replaced by 0.12 M Tris adjusted to pH 7.5 with HCl, and it contained 0.12 mM P_i and 0.1 mM organic phosphorus, provided by the Bactopeptone. Supplements of 0.6% glycerol were used, and 0.1 mM isopropyl B-D-thiogalactopyranoside served to induce β-galactosidase.

Growth of Cells and Preparation of Extracts. Cells were grown at 37° on a rotary shaker and harvested in exponential or stationary phases. They were washed with a large volume of cold 0.01 M Tris-HCl (pH 7.3) made 0.03 M with respect to NaCl. (The pH of the buffer solution was actually measured at 23°.) The cells were resuspended in 0.033 M Tris (pH 7.3) to give a reading of 1.0 at 600 mμ in the Beckman spectrophotometer. This corresponds to a concentration of approximately 1 mg of bacterial protein/ml, or 12 mg (wet weight) of cells/ml, or 8×10^8 cells/ml.

Cell extracts were prepared by treating 1 ml of this suspension at 37° in sequence with: (1) 0.01 ml of $CHCl_3$ for 5 min, (2) 50 μg of crystalline muramidase for 5 min, and (3) 0.01 ml of 0.2 M EDTA (pH 7.1) for 10 min.

Ultrasonic disintegration of cells was accomplished by means of a Branson Sonifier, Model LS75. Cells suspended in 0.033 M Tris (pH 7.3) at 0° were disintegrated by exposure to bursts of ultrasonic energy of 30-sec duration. Three 30-sec treatments usually were sufficient to clarify the suspension.

Enzyme Assays. ALKALINE PHOSPHATASE. (a) The reaction mixture (0.2 ml) contained 0.5 M Tris buffer (pH 8.3), 0.01 M $MgCl_2$, substrate, and enzyme (cell suspension, cell extract, or purified enzyme). After 20 min at 37° the release of P_i was measured. When the reaction mixture was modified to contain only 0.05 M Tris-HCl buffer, essentially similar results were obtained. (b) Hydrolysis of *p*-nitrophenyl phosphate was measured by following the change in absorbancy at 410 mμ in a Beckman Model DU spectrophotometer at 23–24° (Garen and Levinthal, 1960). The reaction mixture consisted of 0.5 M Tris buffer (pH 8.3), 0.01 M $MgCl_2$, *p*-nitrophenyl phosphate, enzyme, and water to 1 ml. For routine assays the substrate concentration was 5×10^{-3} M. One unit of activity represents an increase in absorbancy of 1.0/min.

CYCLIC PHOSPHODIESTERASE. (a) For hydrolysis of U cyclic P the reaction mixture (0.1 ml) contained 50 mM sodium acetate buffer (pH 6), 5 mM $MgCl_2$, 1 mM $CoCl_2$, 1.2 mM U cyclic P, excess purified *E. coli* alkaline phosphatase, and cyclic phosphodiesterase. After 20 min at 37° the release of P_i was measured. The hydrolysis of 3′-AMP was measured in the same way except that phosphatase was omitted. (b) In an alternative assay the reaction mixture (0.1 ml) contained 50 mM sodium acetate buffer (pH 6), 5 mM $MgCl_2$, 1 mM $CoCl_2$, 0.1 mg of Bis-PNPP,[1] and enzyme. After 20 min at 37° the reaction was stopped with 1.0 ml of 0.1 N NaOH and the absorbance at 410 mμ was measured. One unit of activity represents a change in absorbance at 410 mμ of 2.0/20 min.

For all assays in which P_i was measured, the method of Chen *et al.* (1956) as modified by Ames and Dubin (1960) was employed, except for substrates with pyrophosphate linkages, when the procedure of Fiske and Subbarow (1925) was used. For both enzymes one unit of activity represents the release of 1 μmole of P_i/hr, and corresponds to approximately 0.5 unit of assay b.

Protein was determined according to Lowry *et al.* (1951).

Disc Electrophoresis was performed in polyacrylamide gel at 4° in Tris-glycine buffer (pH 9.5) for 2 hr, using the general procedure of Reisfeld *et al.* (1962).

Purification of Alkaline Phosphatase from E. coli. E. coli C-90 cells were grown to stationary phase in 10 l. of medium E (see above) supplemented with 0.6% glycerol. Even with this constitutive strain the formation of alkaline phosphatase was increased when cells were grown in a low phosphate medium. The cells were harvested in a refrigerated continuous-flow centrifuge (Servall RC-2) at 30,000*g* and a flow rate of 200 ml/min. Cells were washed by resuspending them in 600 ml of cold 0.01 M Tris–0.03 M NaCl (pH 7.3) followed by centrifugation. (All pH measurements were made at 23°, even if the solutions were then chilled.) The washing process was repeated twice; the yield of washed cells was 18.4 g (wet weight). The cells were resuspended at room temperature in 0.033 M Tris (pH 7.2) at a concentration of 1 g (wet weight)/10 ml of suspension; the total volume used was 182.5 ml. An equal volume of 40% sucrose was mixed with the cell suspension and 0.2 M EDTA (pH 7.6) was added to give a final concentration of 5×10^{-3} M. This suspension was shaken in a 2-l. flask for 10 min at room temperature on a rotary shaker and then centrifuged for 12 min at 30,000*g* in the cold. The slightly opalescent sucrose–Tris–EDTA supernatant was decanted and the cells were resuspended in cold water with stirring; the suspension was shaken at ice temperature for 10 min. The suspension was centrifuged for 15 min at 30,000*g* and 365 ml of supernatant, designated cold water wash, was collected. The cold water wash was adjusted to a concentration of 0.01 M Tris (pH 7.3)–

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: PNPP, *p*-nitrophenyl phosphate; Bis-PNPP, bis(nitrophenyl)phosphate; U cyclic P, uridine 2′,3′-cyclic phosphate; TCA, trichloracetic acid. Nucleoside monophosphates are 2′, 3′, or 5′ substituted or an isomeric mixture (2′,-3′).

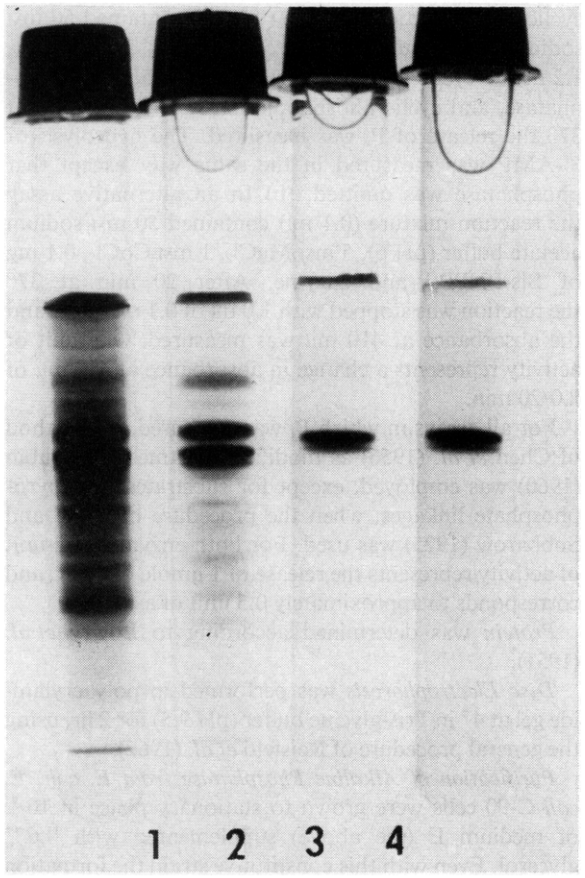

FIGURE 1: Polyacrylamide disc gel electrophoresis of *E. coli* C90 sonic extract (1), cold water wash (2), heated cold water wash (3), and ammonium sulfate precipitate (4). See Table I for a summary of the enzyme activity of these fractions. Both bands in 3 and 4 show alkaline phosphatase activity.

0.01 M $MgCl_2$; 360 ml of this solution was heated to 80° in a boiling-water bath, held at 80° for 15 min, and then cooled to 4°. The flocculent precipitate that formed was removed by means of a Buchner filter funnel in the cold.

Protein was concentrated from this solution by slow addition of Mann's Enzyme Grade crystalline ammonium sulfate to 85% saturation. The precipitate was redissolved in 15 ml of 0.01 M Tris–0.01 M $MgCl_2$ (pH 7.3). To this solution was added 4.36 g of ammonium sulfate, with adjustment to pH 8; the solution was centrifuged to remove air bubbles and slight turbidity. The supernatant solution was immersed in a large reservoir of ice water and adjusted to a faint turbidity with cold, saturated ammonium sulfate. The insulated ice bath was permitted to warm slowly to room temperature over a period of several days. The turbidity of the protein solution increased and microscopic examination showed the formation of crystals similar to those observed by Malamy and Horecker (1964).

Table I summarizes this purification of alkaline phosphatase. The purification also was followed by means of analytical polyacrylamide disc gel electrophoresis with the results shown in Figure 1.

Test of Ribonuclease Activity against tRNA. We were interested in whether or not the purified alkaline phosphatase contained ribonuclease activity against tRNA.

TABLE I: Preparation and Purification of *E. coli* Alkaline Phosphatase.[a]

Fraction	Alkaline Phosphatase Act.		
	Units/g of Cells	Units/mg of Protein	Total Units
Sonic extract	6,000	104	110,400
Sucrose–Tris–EDTA supernatant	180		3,300
Cold water wash	3,200	294	59,000
85% ammonium sulfate precipitate	3,000	815[b]	55,300

[a] Alkaline phosphatase activity was assayed spectrophotometrically using *p*-nitrophenyl phosphate as substrate. [b] The specific activity of the crystalline enzyme and of material purified by DEAE-cellulose chromatography was similar to this value.

Leucine acceptor activity was used as a sensitive measure of tRNA degradation.

A. ASSAY OF tRNA FOR LEUCINE ACCEPTOR ACTIVITY. The standard reaction mixture contained 0.1 M potassium cacodylate (pH 7.0), 1×10^{-3} M ATP, 2×10^{-3} M $MgCl_2$, 1×10^{-3} M β-mercaptoethanol (freshly diluted), 0.2 μCi of [^{14}C]leucine, 1 A_{260} unit of *E. coli* tRNA, and an excess (2 μl) of purified *E. coli* leucyl-tRNA synthetase in a final volume of 0.2 ml. The mixture was incubated at 37° for 15 min, and the reaction was terminated by addition of 1 ml of cold 5% TCA. The TCA-insoluble precipitate was transferred to Millipore filters (0.4 μ) with two 1-ml cold TCA washes, and the precipitate on the filter was washed three times with 10-ml portions of cold 5% TCA. The Millipore filters were then attached to planchets by means of paper cement and air dried; radioactivity was determined quantitatively in a Nuclear-Chicago thin-window gas-flow proportional counter. The labeling of tRNA by [^{14}C]leucine was linear with respect to tRNA concentrations up to 4 A_{260} units/0.2-ml reaction mixture, the highest concentration of tRNA used in these experiments. Leucine fixation was proportional to the amount of leucyl-tRNA synthetase added to the reaction mixture. At a tRNA concentration of 4 A_{260} units/0.2 ml the amount of undiluted leucyl-tRNA synthetase required for maximum fixation of [^{14}C]leucine was 0.3 μl. It is evident that a considerable excess of enzyme was present in the standard assay of tRNA for acceptor activity. The synthetase was purified by Dr. Alan Peterkovsky, using an unpublished procedure developed by him.

B. TREATMENT OF tRNA WITH PURIFIED ALKALINE PHOSPHATASE; EFFECT ON LEUCYL-tRNA SYNTHESIS. In order to provide evidence for the absence of ribonuclease activity in the purified alkaline phosphatase preparations, *E. coli* tRNA was pretreated with alkaline phos-

TABLE II: Alkaline Phosphatase Activity of *E. coli* Suspensions Expressed as Per Cent of Activity of Equivalent Cell Extracts.[a]

Substrate	Act. of Suspension (% of extract)	Substrate	Act. of Suspension (% of extract)
5′-UMP	76	Phenolphthalein P	10
2′,3′-UMP	45	Sodium pyrophosphate[b]	19
5′-CMP	89	α-Glycerophosphate	93
2′,3′-CMP	76	β-Glycerophosphate	92
5′-GMP	65	D-Glucose-6-P	92
2′,3′-GMP	25	D-Galactose-6-P	90
5′-AMP	21	D-Mannose-6-P	80
3′-AMP	7	PNPP[b]	45
2′-AMP	8	Ethanolamine P	96
ATP	36	Serine P[b]	99

[a] Strain C90, constitutive for alkaline phosphatase, was used. Cells were grown to stationary phase and were treated as described in Methods. The activity of a suspension of washed cells is given as a per cent of the activity of an equivalent sonic extract. Assays were at 37° in Tris-HCl buffer (pH 8.3). Under these conditions no other phosphatase shows appreciable activity in *E. coli*. The concentration of substrate was 2.5×10^{-3} M except as noted. [b] Concentration of substrate was 4×10^{-3} M.

phatase and then examined for leucine acceptor activity as previously described.

Each milliliter of the reaction mixtures contained 1 M Tris (pH 8.1) *E. coli* tRNA (10 mg) and *E. coli* alkaline phosphatase (20 units; dialyzed against 0.01 M Tris). Reaction mixtures were incubated at 45° and samples were taken at various time intervals; 0.1-ml samples were added to 0.3 ml of cold uranyl acetate–perchloric acid (Harkness and Hilmoe, 1962). The supernatant fraction obtained after centrifugation was examined for acid-soluble $A_{260\,m\mu}$ material and for inorganic phosphate released from tRNA. In addition, 0.1-ml samples were taken at similar time intervals and added to 0.4 ml of cold 80% ethanol–0.3 M NaCl. The precipitate was collected by centrifugation and redissolved in 0.25 ml of 0.01 M Tris buffer; an aliquot of this solution containing 1 A_{260} unit was used as a source of tRNA in the amino acid acceptor assay described above.

The results, summarized in Figure 2, show that phosphate was rapidly released from the 5′-terminal position of tRNA by the action of alkaline phosphatase without effect on leucine acceptor activity. This observation is in agreement with previous work (Harkness and Hilmoe, 1962) in which alkaline phosphatase prepared by another method was used. Also, there was no detectable release of acid-soluble A_{260} material from tRNA treated with alkaline phosphatase. This is to say, alkaline phosphatase purified as described above is essentially free of ribonuclease activity. Similar results were obtained when the incubation was carried out at 60°.

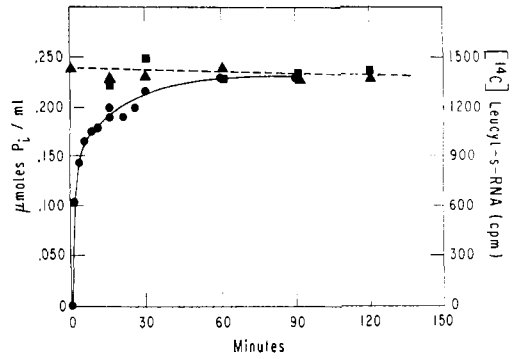

FIGURE 2: Effect of purified *E. coli* C90 alkaline phosphatase on *E. coli* tRNA. Phosphate was released from the 5′-terminal position of tRNA but there was no effect on leucine acceptor activity of phosphatase-treated tRNA. Experimental details in text. (●) P_i, (■) tRNA with phosphatase, and (▲) tRNA without phosphatase.

Results

Expression of Alkaline Phosphatase Activity by Intact Cells. We define "expression" as the per cent of total cellular enzyme that can be measured with intact cells in the assay. It was observed some time ago that the hydrolysis of PNPP can be measured with intact *E. coli* possessing alkaline phosphatase activity (Torriani, 1960). This may be taken as evidence for a surface localization of the enzyme since the only phosphate esters for which a transport system is known are certain hexose phosphates (Fraenkel *et al.*, 1964) and α-glycerophosphate (Hayashi *et al.*, 1964). We have confirmed the fact that PNPP is hydrolyzed by intact cells and we have found that many other phosphate esters are also split under similar conditions (Table II). However, the compounds showed wide variation in the rate of cleavage by cells, on the one hand, and by equivalent sonic extracts, on the other. That is to say, a variable fraction of the enzyme activity appeared to be masked in intact cells.

It should be mentioned that under the conditions of the assay essentially only alkaline phosphatase is measured; other phosphatases are inactive. In alkaline phosphatase negative cells there is no splitting of certain esters such as 2′-AMP and phosphoethanolamine, even at neutral pH.

Effect of Substrate Concentration. An examination of the rate of hydrolysis of PNPP as a function of substrate concentration showed that at higher concentrations the rate of cleavage by cell suspensions approached the maximum velocity attained by cell extracts (Figure 3). Similar results were obtained with other compounds, including α-naphthyl phosphate, β-naphthyl phosphate, and ATP. In general, the per cent expression of activity

TABLE III: Effect of Substrate Concentration on the Expression of Alkaline Phosphatase Activity by *E. coli* Cells.[a]

Substrate	Growth Phase	Per Cent Activity			
		1×10^{-2} M	5×10^{-3} M	1×10^{-3} M	5×10^{-4} M
Isoamyl phosphate	S	88	85	77	69
α-Glycerophosphate	S	76	73	61	63
β-Glycerophosphate	S	76	72	74	66
3-Phosphoglyceric acid	S	82	82	92	
Choline phosphate	S	93	94	87	
D-Fructose 1,6-diphosphate	S	67	73	75	75
D-Fructose 6-phosphate	S	77	73	68	66
D-Ribose 5′-phosphate	S	70	75	63	60
α-Naphthyl phosphate	S	80	64	30	20
β-Naphthyl phosphate	S	79	66	43	30
ATP	S	70	62	38	29
5′-AMP	S	48	32	16	11
PNPP	S	93	77	33	27
PNPP	E	88	72		
2′-AMP	S	22	14	9	7
2′-AMP	E	26	14	12	10
NADP	S		10	8	8
NADP	E		12	15	15

[a] Cells of strain C90 were harvested in midexponential (E) or stationary (S) phase of growth and treated as described in Methods. The per cent expression of activity is the activity of a suspension of cells given as per cent of the activity of an equivalent sonic extract. Incubations were at 37° in Tris-HCl buffer (pH 8.3) using four different concentrations of substrate indicated at the top of each vertical column.

by cells increased as the concentration of substrate was increased, but, in a number of cases, relatively large concentrations (0.01–0.015 M) were required to approach saturation with intact cells (Table III, Figure 3). With purified alkaline phosphatase a biphasic Lineweaver–Burk plot was obtained from which two values of K_m were calculated, 1.4×10^{-5} and 3×10^{-3} for PNPP, and 3.3×10^{-5} and 1.1×10^{-3} for 5′-AMP (Heppel *et al.*, 1962). It is apparent that the rates observed with intact cells were increasing with increasing concentration of substrate in a range where the purified enzyme and cell extracts were saturated. Furthermore, with certain substrates, such as 2′-AMP, 3′-AMP, and NADP

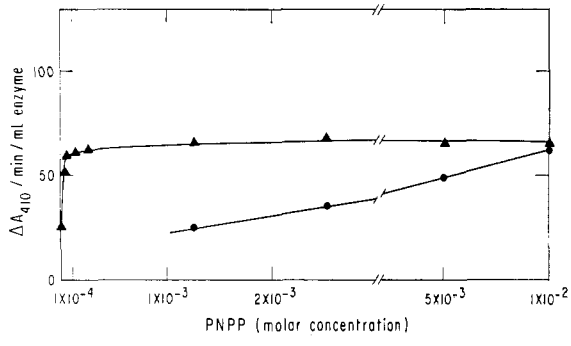

FIGURE 3: Alkaline phosphatase activity of *E. coli* C90 cell extract and cell suspension as a function of *p*-nitrophenyl phosphate concentration. Experimental details in text. (▲) Cell extract and (●) cell suspension.

low rates were observed even with 1×10^{-2} M substrate (Table III, Figure 4A). The relatively nonpolar substrate, isoamyl phosphate, was hydrolyzed nearly as well by whole cells as by sonic extracts over a wide range of concentration (Figure 4B).

Effect of Other Conditions. The per cent expression of alkaline phosphatase activity by intact cells was the same whether or not the cell suspension was shaken during incubation with substrate. The concentration of Tris-HCl buffer was varied from 0.05 to 0.5 M with similar results. No effect of temperature was observed, for the per cent expression with PNPP was the same at 23 and 37°. In general, the results obtained with cells in stationary phase and in exponential phase were comparable (Table III). Similar effects were observed with an eightfold variation in the content of alkaline phosphatase. Also, it did not seem to matter whether the enzyme was constitutive as in strain C90 or induced as in strains K12, K37, or W.

These data indicate that a barrier exists between the substrate containing medium and the enzyme in the cell, presumably a cell wall barrier. Treatment with EDTA (Leive, 1965a,b) affects permeability to actinomycin D and causes the removal of part of the lipopolysaccharide layer. The per cent of expression of alkaline phosphatase by cells treated in this way was unchanged.

It is not apparent why the phosphate esters differ so greatly in ease of hydrolysis by whole cells. The results cannot be correlated with the presence or absence of polar groups. Actually, striking differences were ob-

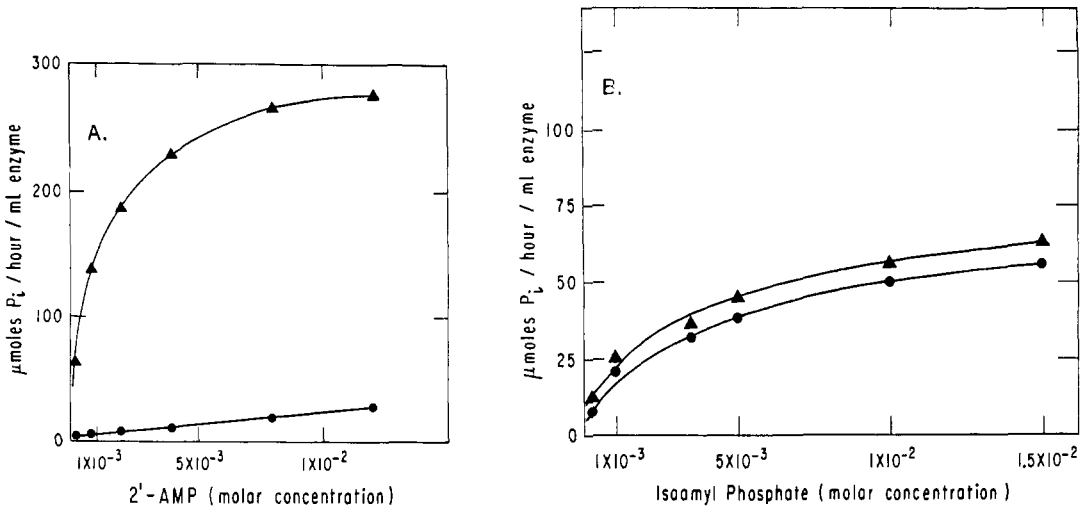

FIGURE 4: Alkaline phosphatase activity. (A) Of *E. coli* C90 cell extract and cell suspension as a function of 2'-AMP concentration. Experimental details in text. (B) Of *E. coli* C90 cell extract and cell suspension as a function of isoamyl phosphate concentration. (▲) Cell extract and (●) Cell suspension.

served among isomeric nucleotides (Table II); thus, the per cent expression was 65% for 5'-GMP and only 25% for 2',3'-GMP.

Effect of Presence or Absence of Transport Systems on Expression of Enzyme Activity by Intact Cells. The per cent expression of alkaline phosphatase activity by intact *E. coli* was the same for α-glycerophosphate and β-glycerophosphate (Table III) even though a transport system exists only for the former (Hayashi *et al.*, 1964). Similarly, the rates of hydrolysis of glucose 6-phosphate and galactose 6-phosphate by whole cells and sonic extracts were nearly the same (Table II) although a transport system cannot be induced for galactose 6-phosphate (Winkler, 1966).

The per cent expression of acid hexose phosphatase was measured with glucose 6-phosphate in cells of strain E15 and strain HP-58. Both carry a deletion for alkaline phosphatase. A transport system for glucose 6-phosphate was induced in E15 by growth in the presence of this phosphate ester, but HP-58 is a mutant that is unable to transport hexose 6-phosphates under any conditions (Winkler, 1966). When the rates of hydrolysis of glucose 6-phosphate by intact cells and equivalent sonic extracts are compared (Table IV), it is evident that acid hexose phosphatase is expressed as well by cells of H58 as by those of strain E15. Thus, the ester is hydrolyzed efficiently by intact cells, even when no uptake mechanism exists.

Expression of Cyclic Phosphodiesterase Activity by Intact Cells. Cyclic phosphodiesterase catalyzes the hydrolysis of bis(*p*-nitrophenyl)phosphate. It also catalyzes the following, two-stage reaction (Anraku, 1964)

$$\text{U cyclic P} \longrightarrow \text{3'-UMP} \qquad \text{(a)}$$

$$\text{3'-UMP} \longrightarrow \text{uridine} + P_i \qquad \text{(b)}$$

Step b is rate limiting, being only one-fourth as rapid as step a in the enzyme-catalyzed reaction. Figure 5A shows the rate of release of P_i from U cyclic P catalyzed by cell suspensions and extracts of strain K37 that had been grown in the high P_i medium to suppress alkaline phosphatase. Activity measured in this way is almost completely expressed at concentrations of U cyclic P in excess of 1×10^{-3} M. If the reaction mixture contained an excess of purified alkaline phosphatase the rate of P_i formation rose fourfold with the sonic extract because step b was no longer rate limiting (Figure 5B). Now, however, the per cent expression by intact cells was much less, showing that 3'-UMP was formed at a site where it was not readily available to the excess of purified alkaline phosphatase in the medium. An experiment similar to that of Figure 5B was then performed using strain C90 (constitutive alkaline phosphatase) and the results are shown in Figure 5C. The per cent of expression was considerably greater in Figure 5C than in Figure 5B, even though the substrate under study was the same, namely U cyclic P. We assume that formation of P_i from the intermediate, 3'-

TABLE IV: Acid Hexose Phosphatase Activity of *E. coli* Strains Possessing or Lacking a Glucose 6-Phosphate Transport System.[a]

Strain	Glucose-6-P Transport	Act. against Glucose-6-P Intact Cells: Sonic Extract % Act.
E15	+	84
HP-58	−	90

[a] Cells were grown to midexponential phase under conditions described by Winkler (1966), washed, and suspended as described in Methods. The assay procedure for acid hexose phosphatase is also described in Methods.

UMP, was more effectively assisted by cell-bound alkaline phosphatase than by phosphatase present in the medium. We conclude that U cyclic P readily penetrates a barrier to reach a site where it is hydrolyzed by cyclic phosphodiesterase to form 3′-UMP and, at a much slower rate, uridine plus P_i. The intermediate, 3′-UMP, does not readily diffuse into the medium, else it would be rapidly split by the excess of purified alkaline phosphatase present in the medium in the case shown in Figure 5B. The hydrolysis of Bis-PNPP by intact cells and by an equivalent extract is shown in Figure 5D. The per cent expression rises from 50% at 1×10^{-3} M to 90% at 8×10^{-3} M.

Similar experiments were carried out with 3′-AMP as a substrate to measure cyclic phosphodiesterase activity of intact cells and equivalent sonic extracts. Strain K37 was grown in medium C to repress alkaline phosphatase, an enzyme that would interfere with the assay. The per cent expression of cyclic phosphodiesterase with 3′-AMP was 30% at 1×10^{-2} M, 14% at 5×10^{-3} M, 12% at 1×10^{-3} M, and 10% at 5×10^{-4} M. These results are not greatly different from those obtained when alkaline phosphatase activity was measured with this substrate (Table II).[2]

Discussion

The purpose of this investigation was to provide additional evidence for localization near the cell surface of a group of degradative enzymes which are selectively released by osmotic shock or during the conversion of *E. coli* into spheroplasts. A total of nine such enzymes has been described (Heppel, 1967); they include alkaline phosphatase, cyclic phosphodiesterase, 5′-nucleotidase, and acid hexose phosphatase. Quite recently, asparaginase II has been added to the list (Schwartz, 1967). These enzymes are not attached to any particulate fraction in broken cell preparations, so evidence for their localization must necessarily be indirect. We agree with previous workers (Mitchell, 1961; Malamy and Horecker, 1964) that these enzymes probably exist external to the cytoplasmic membrane and within the cell wall. This would account for their selective release by the procedures just mentioned, which affect the integrity of the cell wall. Such a location would also be consistent with selective release of alkaline phosphatase into the medium during growth of an *E. coli* mutant which was osmotically sensitive and had a defective wall (Mangiarotti *et al.*, 1966). Histochemical data also suggest a localization between membrane and wall (Done *et al.*, 1965; Spicer *et al.*, 1966).

The present evidence for localization of alkaline phosphatase, cyclic phosphodiesterase, and acid hexose phosphatase near the bacterial cell surface is based on the generally accepted premise that phosphorylated compounds do not freely pass through cell membranes, with the exception of L-α-glycerophosphate (Lin *et al.*, 1962; Hayashi *et al.*, 1964) and certain hexose phosphates (Fraenkel *et al.*, 1964; Hagihira *et al.*, 1963; Winkler, 1966). In bacteria this premise has been substantiated for nucleotides, especially by the work of Lichtenstein *et al.* (1960), who found that exogenously supplied nucleotides, such as deoxycytidine 5′-phosphate, did not penetrate as such into *E. coli*. Their d-CMP contained [^{14}C]cytosine and ^{32}P. The [^{14}C]cytosine moiety was utilized as an intact cytidine residue in the formation of bacterial thymidylate whereas the phosphate moiety of the doubly labeled nucleotide was slowly liberated as inorganic phosphate *and excluded from the bacterial cell*. The growth medium contained large amounts of inorganic phosphate. The authors suggested that deoxycytidine 5′-phosphate is dephosphorylated to deoxycytidine at the bacterial surface, whereupon the labeled phosphate moiety mixes with the excess of phosphate in the medium.

In interpreting our own experiments, we conclude that the hydrolysis of nonpenetrating nucleotides by whole cells under conditions where alkaline phosphatase or cyclic phosphodiesterase are active implies that these enzymes are located external to the cytoplasmic membrane, where they can be reached by substrates from the medium. Similar conclusions were reached with respect to 5′-nucleotidase (Neu and Heppel, 1964b). However, in the present investigation we find that the rate of hydrolysis by whole cells is less than for an equivalent cell extract; the fractional activity displayed by cell suspensions is termed the per cent expression. The per cent expression varies widely with the nature of the substrate and in general increases with the concentration of phosphate ester in the incubation medium over a range far in excess of that needed to saturate the isolated enzyme. We presume that the per cent expression reflects a barrier presented by the cell wall. Phosphate esters vary widely in their ability to penetrate this barrier, but it is not clear just what factors (size, shape, charge distribution, polar character of the molecule, etc.) are critical for entry through the cell wall. In a recent study, it was suggested that certain small polypeptides also are restrained by a cell wall barrier in *E. coli* rather than by the cytoplasmic membrane (Losick and Gilvarg, 1966).

Torriani and Levinthal (1961) reported that the apparent K_m for hydrolysis of PNPP was greater for intact *E. coli* than for cell extracts, a result which we here confirm and extend to include many other substrates and other enzymes. They suggest a passive barrier, or an internal inhibitor in the local environment of the enzyme. Since we observe such large differences among the phosphate esters, it seems more reasonable to postulate a barrier that affects various substrates differently.

Caution must be used in interpreting activity measurements with the whole cells, and we must be sure that the cells have not developed a partially altered cell permeability because of their treatment. Thus, if the bacteria became abnormally permeable to nucleotides, hydrolysis could be observed with whole cells regardless of whether the hydrolases were external to the cell mem

[2] It is possible to measure alkaline phosphatase activity against 3′-AMP using whole cells and extracts because cyclic phosphodiesterase activity is relatively insignificant at pH 8.3. However, cyclic phosphodiesterase activity against 3′-AMP cannot be measured in the presence of substantial amounts of alkaline phosphatase and the latter enzyme must, therefore, be repressed by growth of cells in a high phosphate medium.

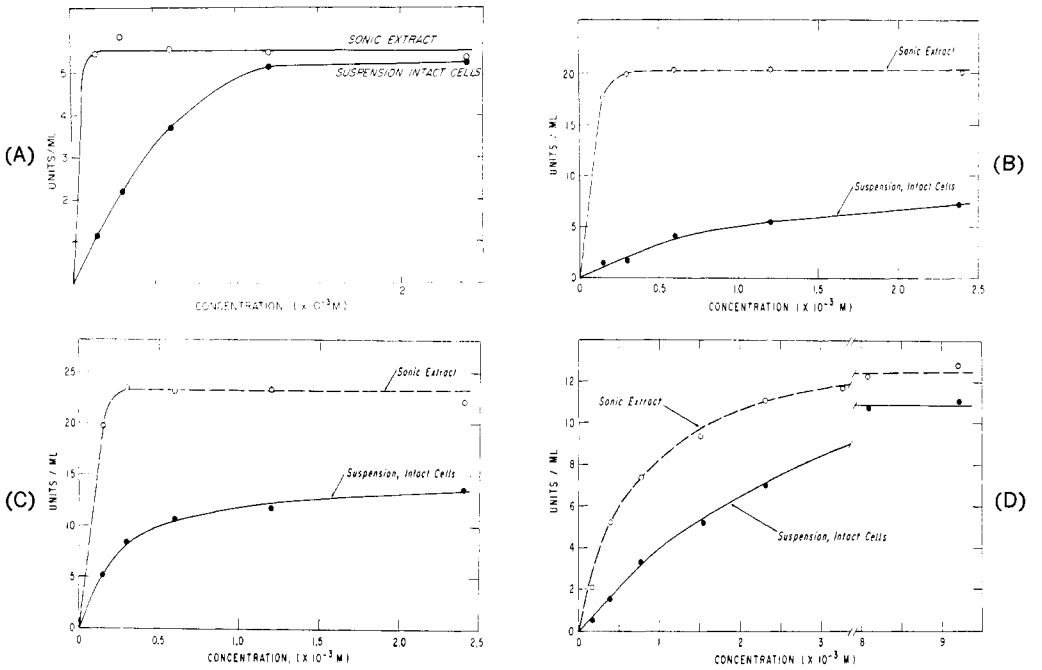

FIGURE 5: Expression of cyclic phosphodiesterase activity by intact *E. coli* compared with sonic extracts. *E. coli*, strain K37 or C90, was grown to stationary phase in medium C or E, respectively, and suspensions were prepared as described in Methods. Assays for the hydrolysis of Bis-PNPP and U-cyclic-P are described in Methods. Incubation volumes and time of incubation were arranged so that only 7% or less of the substrate was hydrolyzed, even at the lowest concentration used. (A) Strain K37, grown in medium C (high phosphate, suppresses alkaline phosphatase). Hydrolysis of U cyclic P was measured by rate of P_i formation by cell extracts and intact cells. Assay a (see Methods) was used but *no* purified alkaline phosphatase was added to the assay medium. Complete hydrolysis of U cyclic P was entirely dependent upon cyclic phosphodiesterase in cells and extracts, and monoesterase component of cyclic phosphodiesterase limits rate of P_i formation. (B) Same, except that excess purified alkaline phosphatase was added to the assay medium. Thus, alkaline phosphatase is present in the medium but *not* in the suspended cells. (C) Strain C90 (constitutive alkaline phosphatase mutant) was grown in medium E; high levels of alkaline phosphatase were contained in the cells. Assay a was used (Methods). In this case, excess alkaline phosphatase was present both in the assay medium and within the suspended cells. (D) Strain K37 was grown in medium C. Cyclic phosphodiesterase activity was determined by measuring the rate of hydrolysis of Bis-PNPP (assay b, Methods).

brane or not. We have observed the same per cent expression of alkaline phosphatase and 5′-nucleotidase activity when cells were washed with dilute Tris-HCl, or with NaCl, or when cells were tested in growth medium, with no washing step. In this paper we report that, with 3′-AMP as substrate, the per cent expression of activity of whole cells was the same whether we measured alkaline phosphatase or cyclic phosphodiesterase activities. The conditions of incubation were very different for the two assays. Finally, we tested cells that had been washed with Tris buffer for permeability to ^{14}C-labeled UTP. For this purpose we measured incorporation of radioactivity into an acid-insoluble fraction, using a mutant that was unable to utilize breakdown products such as [^{14}C]uridine or [^{14}C]uracil. No significant uptake of radioactivity was observed, from which we conclude that UTP did not penetrate as such. The same intact cells were able to hydrolyze UTP when alkaline phosphatase was induced, suggesting that the enzyme was located outside the permeability barrier for this compound.

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On the Possible Involvement of an Anhydride Intermediate in Papain-Catalyzed Hydrolyses*

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ABSTRACT: The papain-catalyzed hydrolysis of *p*-nitrophenyl benzyloxycarbonylglycinate was carried out in $H_2{}^{18}O$, and the papain was isolated from the reaction and allowed to react with more of the substrate in $H_2{}^{16}O$. Benzyloxycarbonylglycine isolated from the reaction in $H_2{}^{18}O$ was labeled with ^{18}O to the same extent as the ^{18}O content of the solvent; however, benzyloxycarbonylglycine isolated from the second hydrolysis in $H_2{}^{16}O$ was not enriched in ^{18}O. In a model system, *o*-mercaptobenzoic acid was used to catalyze the hydrolysis of *p*-nitrophenyl benzoate in $H_2{}^{18}O$. While the benzoic acid isolated from the reaction mixture was enriched in ^{18}O to nearly the extent of the ^{18}O content of the solvent, there was no ^{18}O enrichment of *o*-mercaptobenzoic acid. These data suggest that neither of the catalyzed reactions involve an anhydride intermediate. The essential carboxyl group in papain and the carboxyl group of *o*-mercaptobenzoic acid probably function as a general base in the catalyses.

On the basis of the pH–activity profiles, heats of ionization, and chemical inhibition studies, it has been suggested that the activity of papain is dependent upon a carboxyl group and a sulfhydryl group in the active center (Stockell and Smith, 1957; Smith *et al.*, 1958). These studies have been confirmed and extended (Sanner and Pihl, 1963; Sluyterman, 1964; Whitaker and Bender, 1965; Bender and Brubacher, 1966). Recently, it was indicated that these groups may be cysteine residue 25 (the only cysteine residue in papain) and aspartic acid residue 163 (Light *et al.*, 1964).

The function of the sulfhydryl group as acceptor of the acyl portion of the substrate molecule has been indicated by isolation and spectrophotometric observation of the acyl-enzyme intermediate (Lowe and Williams, 1964; Bender and Brubacher, 1964; Brubacher and Bender, 1966). The function of the carboxyl group in the mechanism of action of papain and related enzymes is not clear. Bender and collaborators (Bender, 1957; Bender *et al.*, 1958; Schonbaum and Bender, 1960) have studied some intramolecular catalyzed reactions which were postulated to simulate the action of the sulfhydryl proteolytic enzymes. In these models, it was proposed that the carboxylate group participates as a nucleophile to accept the acyl moiety from the sulfhydryl group so as to form an anhydride intermediate. On the basis of these models it has been suggested that such an

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