Relationship between Alkaline Phosphatase and Neomycin Formation in *Streptomyces fradiae*

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Studies on phosphatase activity of *Streptomyces fradiae* 3535 grown in three different media indicate that neomycin formation varies directly with enzyme activity, sodium nitrate–maltose–mineral salts medium giving the highest yields of alkaline phosphatase and neomycin. *S. fradiae* contains more than one alkaline phosphatase and the phosphatase responsible for hydrolysis of neomycin phosphate appears to be substrate specific. The same enzyme apparently hydrolyses both the N–P and P–O–P bonds of neomycin pyrophosphate. The enzyme is stimulated by Ca^{2+} , is inactive at a pH below 7 and is inhibited by EDTA. Enzymic activity increases when mycelia are incubated in mineral salts medium, but decreases when phosphate or glucose is included in the medium, although the latter is more effective. The inhibitory effect of EDTA on neomycin formation by resting mycelia is completely reversed by Ca^{2+} .

Streptomyces fradiae 3535 under certain cultural conditions produces phosphoamido-neomycins (Majumdar & Majumdar, 1969a). These are neomycin B pyrophosphate, neomycin C pyrophosphate and neomycin C dipyrophosphate complex (Majumdar, 1969; Majumdar & Majumdar, 1970). Morton (1955) reported that phosphatase can hydrolyse N–P linkages. Since neomycins are produced under alkaline conditions, alkaline phosphatase may play a role in their formation from phosphoamido-neomycins. Repressible alkaline phosphatases have been reported to occur in a variety of micro-organisms including *Escherichia coli* (Horiuchi, Horiuchi & Mizuno, 1959; Garen & Levinthal, 1960; Torriani, 1960), *Staphylococcus aureus* (Shah & Blobel, 1967), *Bacillus subtilis* (Moses, 1967), *Neurospora crassa* (Nyc, Kadner & Crocken, 1966), *Saccharomyces cerevisiae* (Suomalainen, Linko & Oora, 1960) and *Aspergillus nidulans* (Dorn & Rivera, 1966). But there is no such information on alkaline phosphatase of *Streptomyces* species, although an extracellular alkaline phosphatase from *S. fradiae* (Lechevalier, 1958) active near neutrality has been reported. This paper describes the properties of an alkaline phosphatase in *S. fradiae* 3535, and its role in neomycin formation.

MATERIALS AND METHODS

Media and cultural conditions. The neomycin-producing culture of *S. fradiae* 3535 (Waksman & Lechevalier, 1949) was maintained on a potato–yeast extract–mineral salts–agar slant at 28°C and was subcultured at monthly

intervals. A well-sporulated slant culture (7 days old) was washed with 5ml of sterile water and 0.25 ml of the suspension was used to inoculate an Erlenmeyer flask (100 ml) containing 30 ml of medium. The carbon source and phosphate were sterilized separately and added just before inoculation. The nitrogen source was always included in the basal medium, which was adjusted to pH 7.2 and sterilized. After standing overnight at 28°C, the flasks were placed on a rotary shaker (250 rev./min, eccentricity 0.7 cm) and incubated at 28°C. At intervals during growth mycelia adhering to the surface of the flask were removed and dispersed in the medium. Three different media were selected for study of the relationship between alkaline phosphatase activity and neomycin production: medium 1: maltose, 15 g; sodium nitrate, 5.1 g ($\equiv 0.084\%$ N); K_2HPO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 40 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mg; water, 1 litre, pH 7.5 ± 0.1 (Majumdar & Majumdar, 1967); medium 2: maltose, 15 g; glycine, 4.5 g ($\equiv 0.084\%$ N); mineral salts, as in medium 1; water, 1 litre, pH 7.5 ± 0.1 ; medium 3: glucose, 15 g; glutamic acid, 8.8 g ($\equiv 0.084\%$ N); mineral salts, as in medium 1; water, 1 litre, pH 7.5 ± 0.1 .

Preparation of washed mycelia. Washed mycelia were prepared from cultures in the different media by the method described by Majumdar & Majumdar (1970).

Determination of neomycin potency and growth. The culture filtrates and neomycin B base, which was used as standard, were diluted with 0.1 M-potassium phosphate buffer, pH 8, and the antibiotic activity was determined by a conventional cup-plate method with *Bacillus subtilis* (strain B₃) as the test organism. The results are expressed in terms of μg of neomycin B/ml of culture filtrate. Growth was determined as the dry weight of cells (Majumdar & Majumdar, 1967).

Preparation of cell-free extract. Mycelia from different

defined media cultures were separated by filtration through a sintered-glass filter and then washed with 0.5% KCl and water (as described in 'preparation of washed mycelium'). Mycelium (1g wet wt.) was made to 10ml volume with 0.1M-glycine-NaOH buffer, pH9.0, and homogenized in an all-glass homogenizer. The mycelial and glass debris (from mechanical disintegration of the homogenizer) was removed by centrifugation and discarded. The cloudy supernatant was kept overnight at -20°C , when a precipitate formed. The material was then shaken for 5min with 500mg of Dowex 1 (X8; Cl^- form; 200-400 mesh) to remove phosphate, and centrifuged to give a clear yellowish supernatant. This was used as the enzyme source.

Substrates in alkaline phosphatase assay. Neomycin B pyrophosphate, neomycin C pyrophosphate and neomycin C dipyrophosphate complex were prepared by the method of Majumdar & Majumdar (1970). Glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate, fructose 1,6-diphosphate, *p*-nitrophenyl phosphate and sodium pyrophosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Neomycin B pyrophosphate, neomycin C pyrophosphate and neomycin C dipyrophosphate complex were dissolved first in the minimum quantity of 0.5M- NH_3 , and then the solutions were brought to pH9.0 with 0.1M-glycine buffer.

Assay of alkaline phosphatase. As neomycin phosphates are pyrophosphate linked, enzyme activity was determined by measurement of P_i liberated as well as by microbiological assay of neomycin, indicating the hydrolysis of the P-O-P bond in the former case and that of the N-P bond in the latter.

When *p*-nitrophenyl phosphate was used as substrate, enzymic activity was estimated on the basis of the spectrophotometric measurement at 410nm of the *p*-nitrophenol released with the method of Bessey, Lowry & Brock

(1946). With other substrates, P_i was determined by King's (1932) method.

All measurements of alkaline phosphatase activity by P_i release have been reported as μmol of P_i released; no allowance has been made for the different molar amounts of P_i that would be released from different substrates.

For microbiological assay of neomycin, enzyme was inactivated at the end of incubation by treatment at 30°C with 0.1ml of 3M-NaOH for 20min and the mixture diluted appropriately with 0.1M-potassium phosphate buffer pH8, and subsequently mixed with 0.1ml of 3M-HCl. The assay of neomycin was made by a conventional cup-plate method, with *Bacillus subtilis* (strain B₃) as the test organism. In a blank experiment, substrate was added after inactivation of enzyme with NaOH.

Identification of neomycin phosphate. Supernatants (20ml) from defined media after growth of *S. fradiae* were passed through a small column (1ml; diam. 8mm) of Amberlite IRC50 (NH_4^+ form; 200-400 mesh), which was washed with 5ml of water and eluted with 2ml of 1M- NH_3 . Samples (containing 5-25 μg of compound) of the eluate were spotted on Whatman no. 1 paper together with a spot of a mixture of neomycins B and C. The paper was rolled and soaked in 10% (v/v) formic acid in butan-1-ol for 12h at 37°C to hydrolyse N-P bonds, and finally air-dried to remove formic acid. Samples of the untreated eluate (from the column) were spotted in two other positions on the treated paper, which was subsequently developed with the solvent system butan-2-one-2-methylpropan-2-ol-methanol-6.5M- NH_3 (16:3:1:6, by vol.) (Majumdar & Majumdar, 1969b). The paper was divided into parallel strips, and spots were detected on the strips with ninhydrin and ammonium molybdate reagents. Before hydrolysis of the eluate a ninhydrin- and organic phosphate-positive spot was detected at the base line, but after dephosphorylation with formic acid, two

Table 1. *Alkaline phosphatase activity of S. fradiae and neomycin production*

S. fradiae was grown in three different defined media (see the Materials and Methods section) and cell-free extracts were prepared. P_i was determined in a mixture containing crude cell-free extract, 0.2ml; substrate (mixture of 45% neomycin B pyrophosphate, 30% neomycin C pyrophosphate and 25% neomycin C dipyrophosphate complex), 250 μg ; 160 μmol of glycine-NaOH buffer, pH9.0, in a total volume of 1.5 ml after incubation for 3h (arbitrarily chosen) at 37°C . Specific activity and total activity were expressed respectively as μmol of P_i liberated/mg dry cell wt. in 3h and μmol of P_i liberated/culture flask in 3h.

Medium	Time of harvest (days)	Average wt. of mycelium (mg dry wt./culture flask)	Alkaline phosphatase activity		Neomycin production in culture filtrates (μmol /culture flask)
			Specific activity	Total activity	
NaNO_3 -maltose-salts	3	60	0.115	6.9	2.50
	5	59.3	0.301	17.85	9.99
	7	39.9	0.386	15.40	14.78
Glycine-maltose-salts	3	32.1	0.023	0.72	0.08
	5	147.8	0.039	5.25	2.45
	7	110.0	0.050	5.58	3.92
Glutamic acid-glucose-salts	3	78.8	0.020	1.60	0.73
	5	148	0.014	2.02	0*

* pH was 6.4

spots were obtained having R_f values the same as those of neomycins B and C.

RESULTS AND DISCUSSION

Alkaline phosphatase activity of *S. fradiae*. Three media were selected for study of the relationship between phosphatase activity of *S. fradiae* and neomycin production. Of the synthetic media studied, medium 1 produced the highest amount of

neomycin, medium 2 yielded neomycin phosphates along with neomycin, and medium 3 gave almost no neomycin after 5 days of incubation. Majumdar & Majumdar (1967) reported that chemically defined media support little or no neomycin production during the early growth period; neomycin is produced in quantity only by mature cultures. Consequently, changes in phosphatase activity of cells were studied at different periods of growth. Table 1 shows the variation in the phosphatase activity of *S. fradiae* and in neomycin production during growth in the three media. Medium 1 yielded the highest enzyme activity and medium 3 the lowest, neomycin formation varying directly with enzyme activity.

In subsequent experiments, cell-free extracts were prepared from 6-day-old mycelia grown in medium 1. The extracts liberated orthophosphate from various phosphate esters including neomycin pyrophosphates and neomycin dipyrophosphate complex (Table 2). Among the substrates tested, the extract showed the highest activity with neomycin B pyrophosphate and the lowest with fructose 6-phosphate. Since the cell-free preparation showed such a broad range of activity with different classes of phosphates, it seemed that more than one phosphatase might be present in the extract. To test whether the activity was due to one enzyme or several, the effects of certain environmental factors on the release of phosphate from four substrates, neomycin B pyrophosphate, sodium pyrophosphate, fructose 1,6-diphosphate, *p*-nitrophenyl phosphate, were studied by two methods

Table 2. Rate of orthophosphate release from different substrates by cell-free extract of *S. fradiae*

P_i was determined in a reaction mixture containing crude cell-free extract, 0.1 ml; substrate, 0.2 μ mol; 160 μ mol of glycine-NaOH buffer, pH 9.0, in a final volume of 1.5 ml, after incubation for 3 h at 37°C. The maximal P_i release (0.019 μ mol under the conditions of the assay with neomycin B pyrophosphate) has been taken as 100% activity.

Substrate	Relative rate of hydrolysis (%)
Neomycin C dipyrophosphate complex	76
Neomycin C pyrophosphate	54
Neomycin B pyrophosphate	100
Glucose 6-phosphate	52
Fructose 6-phosphate	21
Sodium pyrophosphate	25
Glucose 1-phosphate	39
<i>p</i> -Nitrophenyl phosphate	62
Fructose 1,6-diphosphate	71

Table 3. Effect of inhibitors on the activity of the alkaline phosphatase of *S. fradiae*

Inhibitor (indicated amount) was included in the reaction mixture described in Table 2. After incubation for 3 h at 37°C, P_i (method I) and neomycin (method II) were determined.

Inhibitor	Substrate ... Amount (μ mol)	Relative inhibition (%)		
		<i>p</i> -Nitrophenyl phosphate	Neomycin B pyrophosphate	
			Method I	Method II
EDTA	0.04	17.5	62	58
	0.2	100	100	100
	0.8	100	100	100
8-Hydroxyquinoline	0.04	29.5	0	0
	0.2	58	0	0
	0.8	58	35	27
Ammonium molybdate	0.04	0	—	0
	0.2	0	—	24
	0.8	0	—	45
K_2HPO_4	0.4	23.5	—	26
	0.8	32.5	—	50.5
	4.0	67.6	—	71.6
	8.0	82.3	—	83.4
	40.0	100	—	100

based on hydrolysis of R-O-P, P-O-P and N-P bonds (see assay of alkaline phosphatase in the Materials and Methods section).

Table 3 shows that *S. fradiae* alkaline phosphatase activity was inhibited by phosphate. Molybdate inhibited only the hydrolysis of neomycin B pyrophosphate, but borate and NaF were ineffective (not shown in the Table). A similar observation was also reported on the pyrophosphatase activity of *Desulfovibrio desulfuricans* (Akagi & Campbell, 1963). Metal-chelating agents such as EDTA and 8-hydroxyquinoline were inhibitory to phosphatase action, EDTA causing complete inactivation at a lower equimolar concentration. EDTA also inhibited hydrolysis of other substrates such as PP_i and fructose 1,6-diphosphate. It appeared that alkaline phosphatase of *S. fradiae* requires the presence of metals as activators. EDTA inhibits the Mg²⁺-requiring alkaline pyrophosphatase of *Saccharomyces mellis* (Weimberg & Orton, 1963) but alkaline phosphatase of *Neurospora crassa* (Nyc *et al.* 1966) is stimulated by EDTA.

Effects of metals on EDTA inhibition. Table 4 indicates that when *p*-nitrophenyl phosphate was used as substrate, inhibition of the enzyme by EDTA was reversed fully by Mn²⁺. At equimolar concentration, Ca²⁺ and Mg²⁺ were 81% and 56.5% as effective but Zn²⁺ and Cu²⁺ did not exert any effect. But when neomycin B pyrophosphate was used, only Ca²⁺ reversed EDTA inactivation. With neomycin C pyrophosphate and neomycin C dipyro-

phosphate, a similar behaviour was noted (not shown in the table). It is notable that Fe²⁺ or Zn²⁺ was highly effective in reversing EDTA inhibition of fructose 1,6-diphosphate hydrolysis. Either Ca²⁺ or Mg²⁺ was required for hydrolysis of PP_i when the enzyme was inhibited by EDTA, Ca²⁺ giving greater stimulation. These variable metal requirements for hydrolysis of different substrates might be due to the presence of more than one phosphatase. It has been reported that repressible alkaline phosphatase of *E. coli* (Garen & Levinthal, 1960; Torriani, 1960), alkaline pyrophosphatase of *S. mellis* (Weimberg & Orton, 1963) are stimulated by Mg²⁺, alkaline phosphatase of yeast (Stadtman, 1959) is Fe²⁺-dependent and neutral pyrophosphatase in spore extracts of *B. megaterium* (Levinson Sloan & Hyatt, 1958) is activated by Mn²⁺. Repressible alkaline phosphatase of *N. crassa* is stimulated by EDTA but unaffected by a number of metal ions tested in the absence of EDTA. The alkaline phosphatase requiring Ca²⁺ for hydrolysis of neomycin phosphate seems to be distinct from other alkaline phosphatases of *S. fradiae*, which are activated by more than one metal ion.

Stability of the enzyme. The crude enzyme could be stored at -20°C for several weeks without loss of activity and retained 95-97% of its activity when heated for 10 min at 55°C. The repressible alkaline phosphatase (crude) of *E. coli* (Garen & Levinthal, 1960) could withstand a temperature of 85°C for at least 30 min without loss of enzyme activity.

Table 4. *Effect of metal ions on EDTA inhibition of alkaline phosphatase activity of S. fradiae*

EDTA (0.8 μmol) and metal ion (indicated amount) were included in the reaction mixture (see Table 2). Other conditions were similar to those in Table 3. Results are expressed relative to the maximal stimulation observed with each substrate taken as 100%. Separate 100% values have been chosen for each substrate, and these correspond to the release under the stated assay conditions and in the presence of the appropriate metal ion, of 0.012, 0.009, 0.004 and 0.025 μmol of P_i from *p*-nitrophenyl phosphate, fructose 1,6-diphosphate, PP_i and neomycin B pyrophosphate respectively.

Metal ion	Amount (μmol)	Substrate ...	Relative stimulation (%)				
			<i>p</i> -Nitrophenyl phosphate	Fructose 1,6-diphosphate	PP _i	Neomycin B pyrophosphate	
						Method I	Method II
Ca ²⁺	0.4		51.5	—	—	100	100
	2.0		81	52	100	100	95
Mg ²⁺	0.4		32.4	—	—	0	0
	2.0		56.5	0	83	0	0
Mn ²⁺	0.4		62	—	0	0	0
	2.0		100	49	0	0	0
Zn ²⁺	0.4		—	—	0	0	0
	2.0		0	100	0	0	0
Fe ²⁺	0.4		—	—	—	0	0
	2.0		—	100	—	0	0
Cu ²⁺	0.4		0	0	0	0	0
	2.0		—	0	0	0	0

Fig. 1. Effect of pH on alkaline phosphatase activity of *S. fradiae*. The reaction mixture contained: 0.1 ml of crude enzyme extract; 0.2 μ mol of substrate (O, neomycin B pyrophosphate; ●, *p*-nitrophenyl phosphate; Δ, fructose 1,6-diphosphate); 160 μ mol of buffer, barbital-HCl for pH 6.8–8.6 (—) and glycine-NaOH for pH 8.6–10.6 (----) in a final volume of 1.5 ml. After incubation for 3 h at 37°C, phosphate and neomycin (from neomycin B pyrophosphate) were measured. The maximal P_i release (with neomycin B pyrophosphate at pH 9.2) has been taken as 100% activity.

Effect of pH on enzyme activity. The effect of pH was studied in the range pH 6.8–10.6 by using barbital-HCl and glycine-NaOH buffers. Fig. 1 shows that the optimum pH for enzyme activity with *p*-nitrophenyl phosphate as substrate is 8.6 whereas that with neomycin phosphate is 9.2, the shape of the curve also being different. In both cases, enzyme was almost inactive below pH 7.0. It was reported by Sebek (1955) and Majumdar & Majumdar (1967) that little or almost no neomycin was produced at a pH below 7. The inactivation of alkaline phosphatase at acid pH may be responsible for the very low synthesis of neomycin at pH values below neutrality. In the alkaline pH range, the phosphatase(s) were more active in glycine buffer than in barbital buffer. There are two pH optima for enzyme activity with fructose 1,6-diphosphate, one at 8.0 and another above 10.0. The existence of two pH optima may imply the presence of two different alkaline phosphatases active on the same substrate. However, Dorn (1968) has reported that the homogeneous alkaline phosphatase of *A. nidulans* has two pH optima.

Effect of substrate concentration. Paper-chromatographic study by the method of Majumdar & Majumdar (1969b) showed that alkaline phosphatase of *S. fradiae* could break the N-P bond of neomycin B pyrophosphate, neomycin C pyrophosphate and neomycin C dipyrophosphate, thereby releasing neomycin B, neomycin C and neomycin

Fig. 2. Effect of substrate concentration on alkaline phosphatase activity of *S. fradiae*. The reaction mixture contained: 0.1 ml of crude enzyme extract; the indicated amount of substrate (....., neomycin B pyrophosphate; ----, neomycin C dipyrophosphate complex; and —, neomycin C pyrophosphate); 160 μ mol of glycine-NaOH buffer (pH 9.2); 1 μ mol of $CaCl_2$ in a final vol. of 1.5 ml. Neomycin was determined after incubation for 3 h at 37°C.

Table 5. Effect of EDTA on neomycin formation by washed mycelium of *S. fradiae* in sodium sulphate medium

The washed mycelium was a 60 h-old mycelium suspension (6.0 mg dry wt./ml). The medium (10 ml/50 ml Erlenmeyer flask) was 0.5% Na_2SO_4 (pH 8) and the suspension was incubated on a rotary shaker at 250 rev./min at 28°C.

EDTA (μ g/ml)	Neomycin (μ g/ml)	
	24 h	48 h
0	45	120
10	34	85
20	20.9	62.5
40	17.8	44.5
80	12.6	20.0
400	9.2	14.0

C respectively. Fig. 2 shows the effect of the concentration of these substrates on enzyme activity, measured from the amount of neomycin liberated (microbiological method). Fig. 2 shows that higher substrate concentrations had no adverse effect on enzyme activity.

Effect of EDTA on neomycin formation in washed cell suspensions of *S. fradiae*. As EDTA completely inhibited the hydrolysis of neomycin phosphates by alkaline phosphatase, the effect of EDTA on neomycin biosynthesis by *S. fradiae* was investigated. The experiment was carried out with a washed cell suspension in 0.5% sodium sulphate

Table 6. *Effect of metal ions on EDTA inhibition of neomycin formation by washed mycelium of S. fradiae in sodium sulphate medium*

The washed mycelium was a 60 h-old mycelium suspension (5.6 mg dry wt./ml). The culture conditions were similar to those in Table 5. After addition of EDTA, the flasks were incubated for 3 h; then the indicated amounts of metal were added.

Metal ion	Amount ($\mu\text{g/ml}$)	EDTA (40 $\mu\text{g/ml}$)	Neomycin ($\mu\text{g/ml}$)	
			24 h	48 h
None	—	0	32.3	83
None	—	+	12.4	26.2
Ca^{2+}	40	+	45.2	136
	80	+	60.3	158
	160	+	51.5	143
Mg^{2+}	40	+	11.6	24.8
	80	+	10.4	23.6
	160	+	10.2	22
Mn^{2+}	40	+	18.8	34.6
	80	+	22.5	41.2
	160	+	16.6	32.8
Fe^{2+}	40	+	14.3	28.2
	80	+	18.7	34.4
	160	+	16.8	30.6
Zn^{2+}	40	+	14.4	29.2
	80	+	17.2	33.3
	160	+	15.3	31.4

(pH 8.0), because the effect of growth of the microbe on antibiotic formation can be eliminated under this condition. Table 5 shows that EDTA inhibited neomycin formation by resting cells. This inhibitory effect on antibiotic formation was reversed completely by Ca^{2+} and slightly by Mn^{2+} , Fe^{2+} and Zn^{2+} (Table 6). There might be partial release of Ca^{2+} from its chelation complex by Mn^{2+} , Fe^{2+} and Zn^{2+} , which have a greater affinity for EDTA (Welcher, 1957). A paper-chromatographic study showed that accumulation of neomycin phosphate occurred in the presence of EDTA, and was highest at an EDTA concentration of 80 $\mu\text{g/ml}$. Of the various metal ions tested, only when the incubation medium was supplemented with Ca^{2+} (80 $\mu\text{g/ml}$ for 40 μg of EDTA/ml; Table 6) was the accumulation of neomycin phosphate prevented. Growing cultures of *S. fradiae* in sodium nitrate-maltose-salts medium or the mycelium collected from this medium and kept under resting conditions in 0.5% sodium sulphate did not produce neomycin phosphate. The fact that the inhibitory effect of EDTA on the action of the alkaline phosphatase active on neomycin phosphates and its effect on the formation of neomycin in resting-cell suspensions can both be reversed only by Ca^{2+} suggests that the production of neomycin phosphate in the presence of EDTA is due to inhibition of phosphatase activity. Under normal conditions, alkaline phosphatase hydrolyses neomycin phosphate completely to produce neo-

mycin. It is possible that the formation of neomycin phosphate in glycine-maltose-salts medium (medium 2) is due to a relatively lower alkaline phosphatase activity (Table 1).

Effect of phosphate and glucose on alkaline phosphatase formation by washed mycelium of S. fradiae. It has been reported that phosphate represses the formation of phosphatase in *E. coli* (Horiuchi *et al.* 1959; Torriani, 1960), *S. cerevisiae* (Schmidt & Laskowski, 1961; Heredia, Yen & Sols, 1963), *N. crassa* (Nyc *et al.* 1966) and *A. nidulans* (Dorn & Rivera, 1966). To study the effect of phosphate on phosphatase synthesis in *S. fradiae*, an experiment was conducted with a washed cell suspension so as to decrease the effect of inducer that might form in a growing culture or be already present in the medium. The maximum rate of synthesis of an inducible enzyme is obtained when the inducer is added to the growth medium or when the repressor is removed. The effect of glucose, a powerful repressor of the formation of many enzymes (Magasanik, 1957), was also examined. Table 7 shows that, when neomycin B pyrophosphate was used as the substrate, the activity of alkaline phosphatase was lower when phosphate or glucose had been present in the medium, the extract from cells incubated in glucose medium showing much lower activity. With *p*-nitrophenyl phosphate and fructose 1,6-diphosphate as the substrates, phosphate caused the greater decrease in phosphatase

Table 7. *Effect of phosphate and glucose on synthesis of alkaline phosphatase by washed mycelium of S. fradiae*

The washed mycelium was a 60 h-old mycelium suspension (5.9 mg dry wt./ml). The mineral salts medium (10 ml/50 ml Erlenmeyer flask), containing (per litre) K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 5 mg; pH 7.5 ± 0.1 , was inoculated with 59 mg dry wt. of washed mycelium and incubated on a rotary shaker at 250 rev./min at 28°C. The mycelia were harvested and cell-free extracts were prepared. Alkaline phosphatase activity of the extracts was estimated by determination of P_i (method I) and neomycin from neomycin B pyrophosphate (method II) in a mixture containing crude cell-free extract, 0.1 ml; indicated substrate, 0.2 μ mol; indicated metal ion, 1 μ mol; 160 μ mol of glycine-NaOH buffer (at the indicated pH) in a final volume of 1.5 ml after incubation for 3 h at 37°C. Activity was expressed as μ mol of P_i or neomycin liberated/mg dry wt. of mycelium in 3 h.

System	Substrate ...	Alkaline phosphatase activity				
		<i>p</i> -Nitrophenyl phosphate + Mn^{++} (pH 8.6)	Fructose 1,6-diphosphate + Zn^{++} (pH 10)	PP_i + Ca^{2+} (pH 9.0)	Neomycin B pyrophosphate + Ca^{2+} (pH 9.0)	
					Method I	Method II
Mycelium, 0 h		0.218	2.18	0.073	0.104	0.051
Mycelium incubated for 48 h in mineral salts medium		0.221	0.656	0.067	0.445	0.216
Mycelium incubated for 48 h in mineral salts medium + 0.45% K_2HPO_4		0.072	0.059	0.061	0.206	0.096
Mycelium incubated for 48 h in mineral salts medium + glucose*		0.133	0.812	0.063	0.040	0.018

* 0.5% glucose added at 0 h and 0.25% at 24 h.

activity. The decrease of phosphatase activity observed in a number of cases suggests that destruction of phosphatase has occurred during the incubation, though the amounts of activity found presumably represent a balance between the synthesis and the destruction of the enzyme. The present experiments do not allow one to decide which of these processes it is that is affected by the presence of phosphate-glucose. However, analogy with the effects of these compounds in other organisms suggests that they may be acting as repressors of enzyme synthesis. PP_i -hydrolysing properties of the extract seem to be constitutive, because they were affected by neither glucose nor phosphate. The ratio of the phosphatase activities on four different substrates with extracts from cells incubated under different conditions is different, but the ratio of the activity determined on the basis of neomycin estimation and P_i estimation with neomycin phosphate as substrate under different conditions is the same. These findings indicate that the same alkaline phosphatase is hydrolysing the N-P and the P-O-P bond of neomycin phosphate, although the cell-free extract contains more than one alkaline phosphatase. On incubation of washed mycelium in nutrient-free mineral medium (truly non-growing condition) for 48h, activity of other alkaline phosphatases either remained the same or decreased but the activity on neomycin phosphates increased by more than 4 times (Table 7). These distinct characteristics of the alkaline phosphatase responsible for the hydrolysis of neomycin phosphate, with respect to metal requirement, pH optimum and mode of formation, differentiate it from other alkaline phosphatases of *S. fradiae*. It also appears from these findings that the neomycin phosphates are hydrolysed only by the enzyme specific for neomycin phosphates and not by the other alkaline phosphatase(s).

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