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THE PROPERTIES OF THE ALKALINE PHOSPHATASE OF STREPTOMYCIN-DEPENDENT STRAINS OF *ESCHERICHIA COLI*

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SUMMARY

Enzymic and immunochemical studies indicate that the alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) extracted from streptomycin-dependent strains of *Escherichia coli* is similar to the enzyme isolated from induced cultures of *E. coli*.

INTRODUCTION

Although wild strains of *E. coli* are capable of synthesizing an alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) this synthesis is repressed when the bacteria are grown in media containing increased concentrations of inorganic phosphates[1,2]. Under similar conditions streptomycin-dependent[3] and some streptomycin-resistant[4] mutants of *E. coli* characteristically produce large amounts of an alkaline phosphatase; in this respect they resemble alkaline phosphatase-constitutive mutants[5]. In view of the possible role of this enzyme in the resistance to and dependence upon streptomycin of *E. coli* mutants (see also ref. 6), it was of interest to investigate its properties as a constitutive enzyme of streptomycin-dependent strains in comparison with the properties of the enzyme elaborated by wild bacteria under de-repressed conditions (*i.e.*, grown in the absence of an excess of inorganic phosphates).

EXPERIMENTAL

Bacteria: The bacterial strains used in this study are listed in Table I. The authors are especially grateful to Dr. A. TORRIANI, Massachusetts Institute of Technology, and Dr. J. G. FLAKS, University of Pennsylvania, for several of these strains.

Media: Medium VLP is the Tris medium of TORRIANI[2], adjusted to 0.2 mM KH_2PO_4; medium A is the minimal medium of DAVIS AND MINGIOLI[7] containing 0.5% glucose; medium F consisted of 3 g tryptone, 3 g yeast extract, 11 g K_2HPO_4 and 8.5 g KH_2PO_4 per l of de-ionized water. Glucose was sterilized separately and added to a final concentration of 10 g/l. The media were supplemented with streptomycin for the growth of the streptomycin-dependent strains.

Growth of bacteria: 20 ml of overnight cultures of the bacteria were diluted to

Abbreviation: TM-buffer, 0.2 M Tris buffer (pH 8.0) in 0.01 M $MgCl_2$.

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TABLE I

STRAINS OF *E. coli* USED IN THIS STUDY

Strain	*Description*	*Source*
B	Wild	This laboratory
C 600	K 12, thiamine, leucine and methionine requiring, F^-	Dr. R. K. APPLEY *et al.*[a]
K 10	Hfr strain	Dr. A. TORRIANI[b]
C 4	Alkaline phosphatase constitutive, derived from K 10	Dr. A. TORRIANI[b]
B-Sd 9	Streptomycin-dependent strain isolated from *E. coli* B	Dr. J. G. FLAKS[c]
B-Sd 101, B-Sd 102, B-Sd 103	Streptomycin-dependent strains isolated from *E. coli* B	This laboratory[d]
C 600-Sd 101, C 600-Sd 102	Streptomycin-dependent strains isolated from *E. coli* C 000	This laboratory[d]

[a] R. K. APPLEYARD, J. F. MCGREGOR AND K. M. BAIRD, *Virology*, 2 (1956) 565.
[b] A. TORRIANI AND F. ROTHMAN, *J. Bacteriol.*, 81 (1961) 835; H. ECHOLS, A. GAREN, S. GAREN AND A. TORRIANI, *J. Mol. Biol.*, 3 (1961) 425.
[c] J. G. FLAKS, E. C. COX, M. L. WITTING AND J. R. WHITE, *Biochem. Biophys. Res. Commun.*, 7 (1962) 390.
[d] H. S. ROSENKRANZ, *Biochem.*, 2 (1963) 122.

200 ml with fresh media and aerated at 37° until a bacterial concentration of $2 \cdot 10^8$ cells/ml was reached. The cells were then chilled and harvested by centrifugation.

Viable counts: Samples were diluted in cold minimal medium (medium A) and spread on nutrient agar plates (23 g Difco nutrient agar, 5 g NaCl per l.). The plates were incubated for 16 h at 37°.

Cell extracts: The harvested cells were washed three times with 0.2 M Tris buffer (pH 8.0) in 0.01 M $MgCl_2$ (TM-buffer), ground with alumina and extracted with 4 ml of TM buffer. The cell debris and alumina were removed by centrifugation and discarded.

Solubilization of cell walls by lysozyme: In essence the procedure of MALAMY AND HORECKER[8] was followed. The harvested cells were washed with chilled 1 M NaCl to remove bound streptomycin of the antibiotic-dependent cultures[9]. This was followed by washing with 0.5 M sucrose in 0.1 M Tris buffer (pH 8.0). The cells were resuspended in 40 ml of sucrose–Tris buffer to which were added 0.2 mg EDTA-Na_2 and 50 μg of lysozyme (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17) per ml. Incubation at 37° was allowed to proceed for 1.5 h. The formation of protoplasts was verified by microscopic examination. The protoplasts so formed were collected by centrifugation, washed with 40 ml of sucrose–Tris buffer and finally lysed by the addition of 2 ml of TM-buffer. The supernatant solutions containing the solubilized cell walls were assayed for their enzymic content.

Enzymes: Chromatographically pure *E. coli* alkaline phosphatase was purchased from Worthington Biochemicals Corp. and lysozyme was obtained from California Corporation for Biochemical Research.

Chemicals: Sodium *p*-nitrophenylphosphate and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co.; 8-hydroxyquinoline from Fisher Scientific Co.,

and sodium β-glycerophosphate·5 H_2O and AMP from California Corporation for Biochemical Research.

Analyses: Phosphorus was determined by the procedure of KING[10] and protein concentrations by the method of LOWRY *et al.*[11], using a bovine serum albumin standard.

Alkaline phosphatase assay: Except as otherwise indicated, either 0.1 ml of cell extract (2.54 mg protein per ml of 0.1 M Tris buffer (pH 7.9) in 5 mM $MgCl_2$) or 0.1 ml of solubilized cell-wall material were added to 2.0 ml of 1.33 mM sodium *p*-nitrophenylphosphate in 1 M Tris buffer (pH 7.9) pre-equilibrated at 37°. The increase in absorbancy at 410 mμ was determined at 15-sec intervals in a Beckman DU spectrophotometer at 37° under constant thermostatic control. An increase in absorbancy of 0.001 per min was defined as 1 unit of enzymic activity.

Determination of pH optimum: The standard enzyme assay was modified in such a way that the *p*-nitrophenylphosphate was diluted in 1 M Tris buffer solutions of various pH values. After equilibration at 37°, 440 units of enzyme obtained from an extract of *E. coli* B-Sd 9 were added in 0.1 ml of 0.1 M Tris buffer (pH 7.9) in 5 mM $MgCl_2$.

Phosphate inhibition: The procedure was similar to the one adapted for determination of the pH optimum, except that the substrate (1.33 mM) was dissolved in 1 M Tris buffer (pH 7.9) solutions containing various concentrations of KH_2PO_4. The data were plotted in the manner described by DIXON AND WEBB[12].

Determination of Michaelis constant: Various concentrations of the substrate were dissolved in 1 M Tris buffer (pH 7.9). The enzymic activities of an extract of *E. coli* B-Sd 9 were assayed in the usual manner at 37°. The K_m value was determined from a LINEWEAVER–BURK double-reciprocal plot[13].

Activation energy: The substrate (1.33 mM *p*-nitrophenylphosphate in 1 M Tris buffer, pH 7.9) was pre-equilibrated at the ambient temperature prior to the addition of 515 units of enzymic activity obtained from an extract of *E. coli* B-Sd 9. The temperature inside the spectrophotometer quartz curvettes could be maintained to within ± 0.2° by means of a Haake thermostat connected to a series of four thermospacers. The authors are very grateful to Dr. A. BENDICH, Sloan-Kettering Institute, for advise on the assembling of this apparatus, as well as for the gift of a specially constructed heating-chamber attachment that permitted recording of the ambient temperature.

Effect of inhibitors: 440 units of enzyme (from *E. coli* B-Sd 9) were added to 2-ml portions of inhibitors in 1 M Tris buffer (pH 7.9). The solutions were equilibrated at 37° for 1 h. At zero time, the solutions were adjusted to 1.33 mM *p*-nitrophenylphosphate and the residual enzymic activities determined.

Specificity of the enzyme: Solutions of organic phosphates, 0.01 M in 1 M Tris buffer (pH 7.9), were exposed to 515 units of enzyme (*E. coli* B-Sd 9) for 8 min at 37°. The reaction was stopped by the addition of perchloric acid. The amount of inorganic phosphorus released was compared with a control consisting of unhydrolyzed substrate. Relative reaction velocities were computed using the hydrolysis of *p*-nitrophenylphosphate as unity.

Preparation of antisera: Rabbits were immunized with chromatographically pure alkaline phosphatase isolated from de-repressed wild *E. coli*. The method and schedule of immunization have been described previously[14].

TABLE II

LOCALIZATION OF ALKALINE PHOSPHATASE

The enzymic activity is expressed as the units of enzymic activity per fraction. Bacteria were suspended in 0.5 M sucrose and 0.1 M Tris buffer (pH 7.9). After the addition of EDTA and lysozyme the cells were incubated at 37° for 1.5 h. The solubilized enzyme was removed by centrifugation and the protoplasts were washed with 40 ml of sucrose–Tris buffer ("Protoplast wash"). Finally the "protoplast-bound" activity was determined by lysing the protoplasts with 2 ml of 0.2 M Tris buffer (pH 8.0) in 0.01 M $MgCl_2$ and removing the debris by low-speed centrifugation. The E. coli B was grown in the Tris–low phosphorus medium (VLP) and the streptomycin-dependent mutants were grown in medium F.

	E. coli B de-repressed	*E. coli B streptomycin-dependent (B-Sd 9)*	*E. coli B streptomycin-dependent (B-Sd 102)*
Solubilized by lysozyme	1670	25 200	44 199
Protoplast wash	822	424	3 332
Protoplast-bound	785	498	14 310

Comparison of immunochemical properties: An immunochemical comparison of the alkaline phosphatase present in the different cell extracts derived from various streptomycin-dependent strains of *E. coli* and the alkaline phosphatase of the de-repressed *E. coli*, used as the antigen, was made by the OUCHTERLONY agar-gel diffusion technique[15]. The medium consisted of 0.6% agar, 0.85% NaCl, 0.067 M barbital, adjusted to pH 7.5. Diffusion was allowed to proceed at 4° in a moist chamber.

RESULTS AND DISCUSSION

Localization of enzyme

MALAMY AND HORECKER[8] showed that the alkaline phosphatase of induced *E. coli* was solubilized after treatment of the bacteria with lysozyme, thus indicating that the enzyme is located between the cell wall and the cell membrane. The data

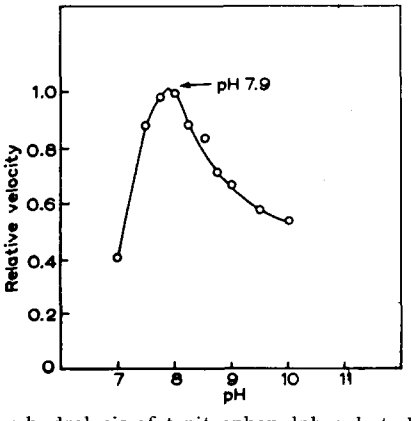

Fig. 1. pH dependence of the hydrolysis of *p*-nitrophenylphosphate by an enzyme present in an extract of a streptomycin-dependent strain of *E. coli* (B-Sd 9).

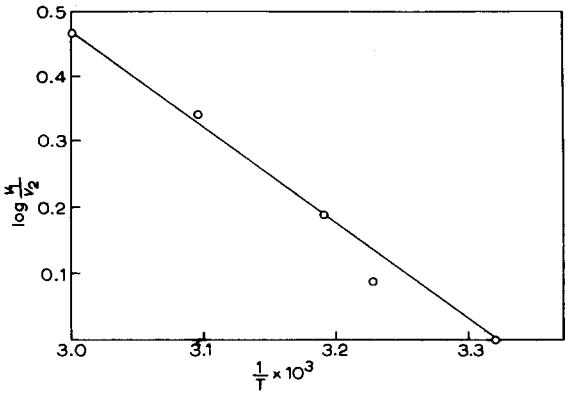

Fig. 2. Effect of temperature on the rate of hydrolysis of *p*-nitrophenylphosphate by the alkaline phosphatase present in an extract of *E. coli* B-Sd 9. Such an Arrhenius plot permits the determination of the activation energy (6640 cal/mole).

of Table II indicate that a similar location can be ascribed to a major portion of the constitutive alkaline phosphatase of streptomycin-dependent *E. coli* strains.

Enzymic properties of the alkaline phosphatase of streptomycin-dependent E. coli

The enzyme present in extracts obtained from *E. coli* B-Sd 9 cells was selected for a more detailed study of its enzymic properties. The pH-dependence of the reaction is shown in Fig. 1; maximum activity is obtained at pH 7.9. An Arrhenius plot of the temperature-dependence of the enzymic velocity is given in Fig. 2, from which the activation energy may be estimated. The double-reciprocal plot shown in Fig. 3 permits the calculation of the Michaelis constant of the reaction, using sodium *p*-nitrophenylphosphate as the substrate. The inhibition of hydrolysis of the sub-

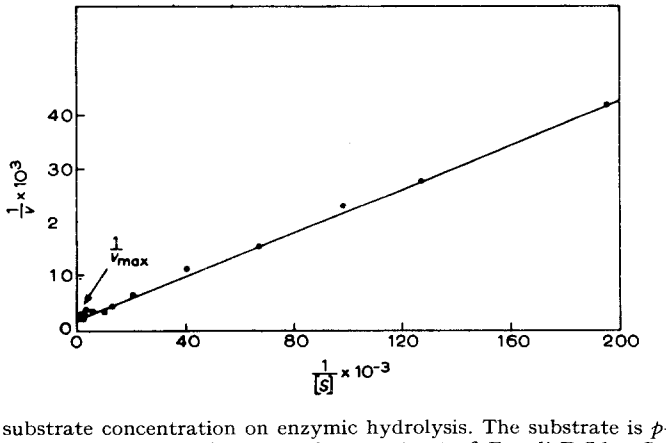

Fig. 3. Effect of substrate concentration on enzymic hydrolysis. The substrate is *p*-nitrophenylphosphate and the enzymic activity is present in an extract of *E. coli* B-Sd 9. Such a double-reciprocal plot allows the calculation of the Michaelis constant ($K_m = 1.3 \cdot 10^{-4}$).

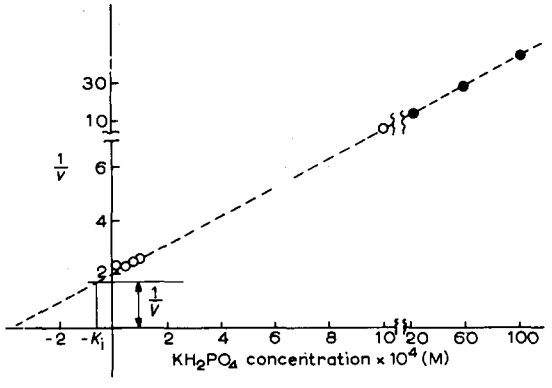

Fig. 4. Inhibition by KH_2PO_4 of the enzymic hydrolysis of *p*-nitrophenylphosphate by an extract of *E. coli* B-Sd 9 ($K_i = 4.5 \cdot 10^{-5}$).

strate (*p*-nitrophenylphosphate) by KH_2PO_4, shown in Fig. 4, together with the value of V_{max} obtained from the data of Fig. 3, allow the estimation of K_i, using the procedure outlined by DIXON AND WEBB[12].

The properties of the enzyme present in the streptomycin-dependent strain were compared with the properties reported by other investigators for the alkaline phosphatase present in de-repressed wild strains (see Table III). The main difference between the enzymes isolated from wild and streptomycin-dependent strains is in the values for K_m and K_i, but these may simply depend on variations in the experimental conditions used in different laboratories. For example, GAREN AND LEVINTHAL[16] determined their values at 25°, whereas 37° was used in this study. The ratios K_i/K_m are identical for both preparations, however, and the data in Table III indicate that the two enzymes have many properties in common. These similarities are emphasized by the results of inhibition studies.

TABLE III

PROPERTIES OF THE ALKALINE PHOSPHATASE ACTIVITIES PRESENT IN A STREPTOMYCIN-DEPENDENT STRAIN AND A WILD DE-REPRESSED STRAIN OF *E. coli*

Property	*E. coli B-Sd 9**	*E. coli wild*** *de-repressed*
pH optimum	pH 7.9	pH 8.0
Activation energy	6640 cal/mole	6880 cal/mole
Effect of exposure to 82°	None	None
K_m (p-nitrophenylphosphate)	$1.3 \cdot 10^{-4}$	$1.2 \cdot 10^{-5}$
K_i (KH_2PO_4)	$4.5 \cdot 10^{-5}$	$3.8 \cdot 10^{-6}$
K_i/K_m	0.34	0.32
Localization	External	External**

* A streptomycin-dependent mutant of *E. coli* B.

** See M. MALAMY AND B. L. HORECKER, *Biochem. Biophys. Res. Commun.*, 5 (1961) 104.

*** The production of alkaline phosphatase was induced by growing the bacteria in a low-phosphorus medium. Data taken from A. GAREN AND C. LEVINTHAL, *Biochim. Biophys. Acta*, 38 (1960) 470.

Effect of inhibitors on the alkaline phosphatase of streptomycin-dependent bacteria

PLOCKE *et al.*[17] have reported the alkaline phosphatase isolated from de-repressed cells as well as from bacteria constitutive for alkaline phosphatase to be a zinc metallo-enzyme. These authors found that zinc-binding agents inhibited the expression of the enzymic activities of their preparations. HEPPEL *et al.*[18] investigated the effectiveness of a number of other agents in inhibiting the reaction catalysed by alkaline phosphatase. In Table IV the effects of four inhibitors on the enzyme

TABLE IV

EFFECT OF INHIBITORS ON ALKALINE PHOSPHATASE ACTIVITIES

The activities were measured by their effects on the rate of hydrolysis of *p*-nitrophenylphosphate

	E. coli B-Sd 9	*E. coli wild de-repressed*
NaF (0.1 M)	−1.5%	+15%*
p-Hydroxymercuribenzoate (0.015 M)	−6.5%	+20%*
8-Hydroxyquinoline for 50% inhibition	$7 \cdot 10^{-3}$ M	$3 \cdot 10^{-5}$ M**
Na_2S for 50% inhibition	$7 \cdot 10^{-2}$ M	$6 \cdot 10^{-4}$ M**

* L. A. HEPPEL, D. R. HARKNESS AND R. J. HILMOE, *J. Biol. Chem.*, 237 (1962) 841.
** D. J. PLOCKE, C. LEVINTHAL AND B. L. VALLEE, *Biochem.*, 1 (1962) 373.

activity isolated from *E. coli* B-Sd 9 and induced wild *E. coli* are compared. Neither NaF nor *p*-hydroxymercuribenzoate appreciably affected either of the preparations. On the other hand, metal-binding agents which were found by PLOCKE *et al.*[17] to be very efficient in inhibiting the activity of alkaline phosphatase isolated from wild and constitutive bacteria were not as effective in decreasing the activity of the enzyme present in extracts of streptomycin-dependent cells. It should be noted, however, that PLOCKE *et al.*[17] were dealing with a purified enzyme preparation,

TABLE V

SUBSTRATE SPECIFICITIES OF THE ALKALINE PHOSPHATASES PRESENT IN INDUCED WILD *E. coli* AND ITS STREPTOMYCIN-DEPENDENT MUTANT

	Relative velocities of enzymic hydrolysis	
	*E. coli B-Sd 9**	*E. coli wild de-repressed***
p-Nitrophenylphosphate	1.00	1.00
β-Glycerophosphate	0.9	0.9
Sodium pyrophosphate	0.8	0.9
AMP	0.8	1.00

* Determined in this laboratory.
** Data taken from A. GAREN AND C. LEVINTHAL, *Biochim. Biophys. Acta*, 38 (1960) 470.

whereas a crude extract containing Mg^{2+} (added in the course of the extraction) as well as the other trace elements originally present in the cells, was used in this study. Nevertheless, the data shown in Table IV indicate an inhibitory effect of the metal-binding agents on the enzymic activity present in extracts of *E. coli* B-Sd 9.

Substrate specificity of the alkaline phosphatase of E. coli B-Sd 9

The specificity of the enzyme present in the extract of the streptomycin-dependent strain B-Sd 9 was compared with that of the enzyme isolated from induced wild strains and studied by GAREN AND LEVINTHAL[16] and HEPPEL *et al.*[18]. The few substrates that were studied were hydrolyzed at identical rates (see Table V).

Immunochemical comparison

The identity of the enzyme present in extracts of streptomycin-dependent strains with alkaline phosphatase isolated from both de-repressed wild strains and constitutive strains was demonstrated by double diffusion in agar, as shown in Fig. 5–7. It should be noted that no bands are seen when extracts of repressed cultures are used, this indicates that the antiserum contains antibodies directed specifically against the enzyme.

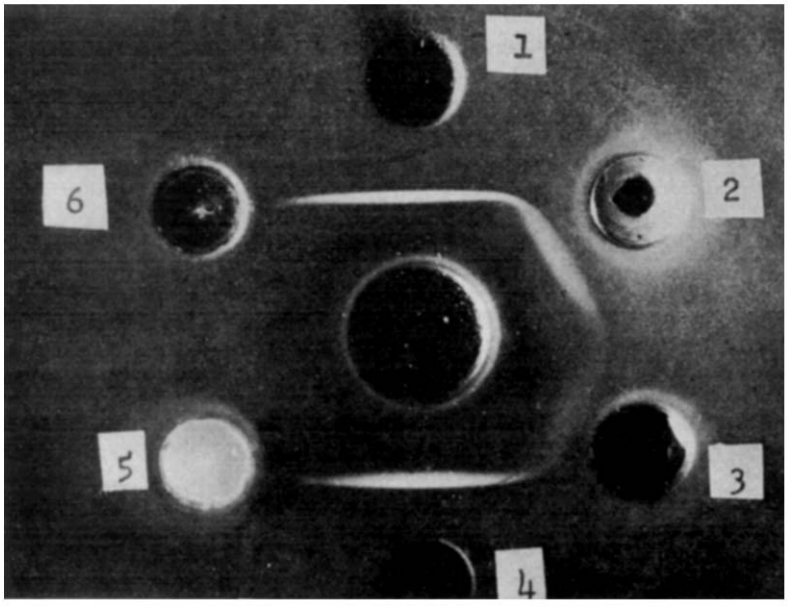

Fig. 5. Double agar-gel diffusion: The central well contains rabbit antibody prepared against purified alkaline phosphatase isolated from de-repressed wild *E. coli*. Wells Nos. 1 and 4 contain the antigen (*i.e.*, purified alkaline phosphatase). Wells Nos. 2, 3, 5 and 6 contain, respectively, extracts from *E. coli* B-Sd 9 (streptomycin-dependent), C 4 (alkaline phosphatase constitutive), K 10 (wild, repressed), and B (wild, repressed).

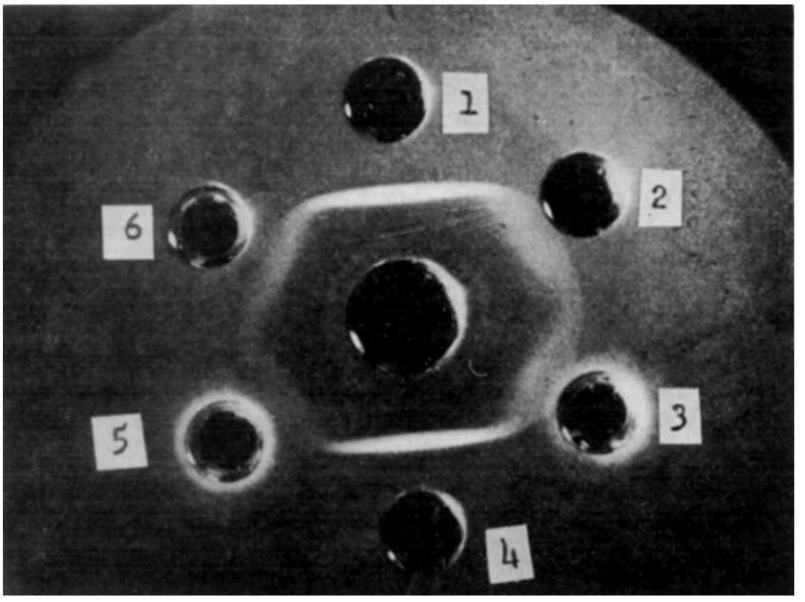

Fig. 6. Double agar-gel diffusion: The central well contains rabbit antibody to purified alkaline phosphatase isolated from de-repressed *E. coli*. Wells Nos. 1 and 4 contain the antigen. Wells Nos. 2, 3, 5 and 6 contain, respectively, extracts from *E. coli* B-Sd 9 (streptomycin-dependent), B-alkaline phosphatase de-repressed, B-Sd 102, and B-Sd 101.

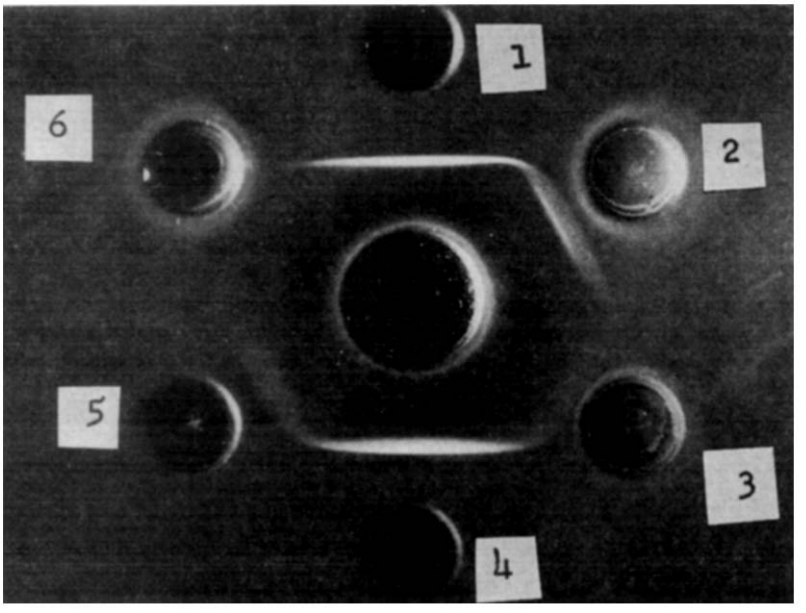

Fig. 7. Double agar-gel diffusion: The central well contains rabbit antibody to purified alkaline phosphatase isolated from de-repressed *E. coli*. Wells Nos. 1 and 4 contain the antigen. Wells Nos. 2, 3, 5 and 6 contain, respectively, extracts from *E. coli* C 600-Sd 101 (streptomycin-dependent), C 600-Sd 102 (streptomycin-dependent), *E. coli* B-Sd 103 (streptomycin-dependent), and *E. coli* C 600 (repressed).

CONCLUSIONS

The data indicate that the alkaline phosphatase elaborated by streptomycin-dependent strains of *E. coli* is identical with the enzyme present in *E. coli* cells induced to produce this enzyme by the removal of inorganic phosphates from the medium. The exact relationship of alkaline phosphatase to streptomycin-dependence is still not understood; it may very well be a secondary effect resulting from changes in the cell wall. It is known that streptomycin-dependent cells have defective external cell structures[19–21] which require the antibiotic for maintenance of cellular integrity and it is not inconceivable that this results in an inhibition of the normal transport of inorganic phosphates into the cell. The affected cell, in turn, compensates by elaborating an enzyme, *i.e.*, alkaline phosphatase, which can use organic phosphates as an alternative source of phosphorus. Genetic studies are being carried out to determine whether the constitutive alkaline phosphatase in streptomycin-dependent strains is (a) the result of a mutation linked to the locus for streptomycin-dependence, (b) a separate mutation that can be mapped on the bacterial chromosome, or (c) a de-repression of the enzyme due to an altered effective concentration of inorganic phosphates.

ACKNOWLEDGEMENTS

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