# Unusual Alkaline Phosphatase Levels in Streptomycin-Dependent Strains of *E. coli**

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Streptomycin-dependent strains of *Escherichia coli* contain a high level of alkaline phosphatase even when grown in a medium which represses the elaboration of this enzyme in wild strains. The presence of alkaline phosphatase in streptomycin-dependent bacteria might be the result of an altered permeability of these cell for inorganic phosphorus. The enzyme might also remove the sensitive site, within the bacteria, with which the antibiotic combines, *i.e.*, the phosphate end of double-stranded deoxyribonucleic acid (DNA).

Several sites of action have been proposed for streptomycin. It has been suggested that the ribosomes are most directly affected by the drug (Spotts and Stanier, 1961; Speyer *et al.*, 1962; Flaks *et al.*, 1962a). Anand and his colleagues (Anand and Davis, 1960; Anand *et al.*, 1960) and Landman and Burchard (1962) indicate, however, that the cell membrane may be the most susceptible site of attack of this antibiotic, although Plotz and Davis (1962) suggest that this is not the only structure that is affected. Recent chemical and biological studies indicate that the nucleic acids may be the target of streptomycin (Rosenkranz and Cohen, 1963). More specifically, streptomycin seems to combine with the terminal phosphate group of double-stranded deoxyribonucleic acids, and it was found, in an *in vitro* experiment, when the terminal phosphate groups of the polydeoxyribonucleotide were removed enzymatically by alkaline phosphatase, that the macromolecule could no longer be complexed efficiently by the antibiotic. The present study was undertaken in order to investigate whether the resistance to and dependence of several *E. coli* strains on streptomycin were perhaps related to the unavailability of such terminal DNA phosphoryl groups. Accordingly, the alkaline phosphatase contents of sensitive, resistant, and dependent mutants were determined.

## EXPERIMENTAL

Bacterial strains were received from a number of different sources, and the author is especially grateful to Dr. J. G. Flaks, University of Pennsylvania, Drs. D. S. Feingold and B. D. Davis, Harvard Medical School, and Dr. A. Torriani, Massachusetts Institute of Technology, for generous gifts of streptomycin-sensitive, resistant, and dependent strains of *E. coli*. Each strain was tested for its ability to grow in the presence and absence of the antibiotic, and it was found that several strains of supposedly streptomycin-dependent bacteria no longer possessed this requirement. This change was probably due to a modifier mutation (Matney *et al.*, 1960), or possibly it was a function of the media that were used; in any event, the data obtained with such strains were not included in the present study. Another strain (ATCC 11370), designated as a resistant mutant, was in fact streptomycin-dependent. The various strains and their sources are listed below:

*Sensitive Bacteria.*—The strains used were *E. coli* $B_f$, $B_d$, and $B_c$, obtained from Drs. Flaks, Davis, and S. S. Cohen, respectively; *E. coli* ML35, obtained from Drs. Feingold and Davis (this strain, which they obtained from Dr. J. Monod, is cryptic and constitutive for β-galactosidase); *E. coli* K 10 (Echols *et al.*, 1961), obtained from Dr. Torriani; *E. coli* C 600 (Appleyard *et al.*, 1956); *E. coli* W.

*Resistant Bacteria.*—*E. coli* $B/Sr_3$, $B/Sr_5$, $B/Sr_{11}$, derived from *E. coli* $B_f$, were obtained from Dr. Flaks. They were grown either in the absence or presence of 100 μg of streptomycin sulfate per ml. *E. coli* $B_d/Sr$ and *E. coli* $ML35/Sr_2$, derived from *E. coli* $B_d$ and *E. coli* ML 35 by Drs. Feingold and Davis, were grown in the presence of antibiotic concentrations of 25 and 250 μg/ml respectively.

*Dependent Strains.*—*E. coli* B Sd9 (derived by Dr. Flaks from *E. coli* $B_f$), and *E. coli* ATCC 11370 (see above), were grown in 100 μg of streptomycin per ml.

*Isolation of Streptomycin-Resistant and Streptomycin-Dependent Mutants.*—The replica plating technique of Lederberg and Lederberg (1952) was used to obtain mutants of *E. coli* $B_c$, *E. coli* C 600, and *E. coli* K 10. Sensitive cells ($1 \times 10^9$ per plate) were spread on nutrient agar containing 10 μg of streptomycin per ml. (The media used for *E. coli* C 600 were supplemented with thiamine hydrochloride, 10 mg per liter.) After incubation at 37° for 5 days these master plates were replicated on nutrient agar free of the antibiotic. After overnight incubation, these plates were replicated once more to insure a complete absence of streptomycin. Colonies obtained in this manner were prototrophic with respect to the antibiotic and were classified as streptomycin-resistant bacteria.

The master plates were also replicated on streptomycin-containing nutrient agar. The colonies that grew only in the presence of the antibiotic (10 μg per ml) were designated streptomycin-dependent.

Discrete colonies of resistant and dependent cells were selected, subjected to two cycles of single colony isolations, and tested for their ability to grow in the presence and absence of streptomycin.

*Selection of Streptomycin-Dependent Mutants by Spraying with p-Nitrophenylphosphate.*—The resolution of the replica plating technique being quite small (Lederberg and Lederberg, 1952; Cavalli-Sforza and Lederberg, 1956), another method, based on the alkaline phosphatase content of streptomycin-dependent bacteria, was also used to differentiate between streptomycin-resistant and dependent mutants. Levinthal (1959) showed that alkaline phosphatase-producing bacteria could be detected by spraying the agar on which they were growing with *p*-nitrophenylphosphate. Those cells which contained the enzyme converted the substrate to the yellow-colored *p*-nitrophenol.

*E. coli* W cells were spread on nutrient agar ($1 \times 10^9$ bacteria per plate) containing 20 μg of streptomycin per ml. After 7 days at 37°, the plates were sprayed with sodium-*p*-nitrophenylphosphate (0.01 g per ml of 0.2 M Tris buffer, *p*H 8.0, in 0.01 M $MgCl_2$).

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Forty-five per cent of the bacterial colonies (562/1236) were found to contain alkaline phosphatase. Eight yellow colonies were selected, purified by two cycles of single colony isolations, and inoculated into medium F (see below) containing 20 $\mu$g of streptomycin per ml. Portions of exponentially growing bacteria ($2 \times 10^8$ cells per ml) were washed twice with 1 M NaCl to remove bound antibiotic (Engelberg and Artman, 1962) and inoculated into medium F not supplemented with streptomycin in order to test for streptomycin independence. The eight colonies so tested were found to require streptomycin for growth. All of the unstained colonies similarly examined (sixteen) were prototrophic in this respect and hence could be classified as streptomycin-resistant.

The reliability of the method was tested by replica plating (see above). None of the colonies which grew on streptomycin-free agar after two transfers became colored when sprayed with *p*-nitrophenylphosphate. The yellow reaction was given only by colonies which required the antibiotic for growth.

*Media.*—*Medium F* consisted of 3 g tryptone, 3 g yeast extract, 11 g $K_2HPO_4$, and 8.5 g of $KH_2PO_4$ per liter of de-ionized water. Glucose was sterilized separately and added to a final concentration of 10 g per liter. The author is grateful to Dr. Flaks for suggesting this medium. *Medium VLP* was the Tris–low phosphorus medium of Torriani (1960) adjusted to $2 \times 10^{-4}$ M $KH_2PO_4$. *Medium LP* and *medium HP* were the same as VLP, but adjusted to $2 \times 10^{-3}$ and $1.6 \times 10^{-2}$ M $KH_2PO_4$ respectively. Streptomycin was added as needed (see above).

*Growth of Bacteria.*—Routinely, 10 ml of an overnight culture was inoculated into 100 ml of complete medium and incubated at 37° with aeration. The bacteria were harvested in the middle of the exponential growth phase ($2 \times 10^8$ cells per ml). The cells were washed twice with 0.2 M Tris buffer (*p*H 8.0) in 0.01 M $MgCl_2$, ground with alumina, and extracted with 4 ml of the Tris-$MgCl_2$ buffer. The cell debris and alumina were removed by centrifugation and discarded. The protein content and alkaline phosphatase activity of the supernatant fluid were determined.

*Viable Counts.*—Samples were taken at appropriate intervals, diluted in cold mineral medium (Cohen and Arbogast, 1950), and spread on nutrient agar (23 g Difco-Nutrient Agar, 5 g NaCl per liter). The plates were incubated for 16 hours at 37°.

*Chemicals.*—Sodium *p* - nitrophenylphosphate was purchased from the Sigma Chemical Co. and streptomycin sulfate (streptomycin) from E. R. Squibb & Sons.

*Analyses.*—Protein contents of the extracts were determined in duplicate by the procedure of Lowry *et al.* (1951), with a bovine serum albumin standard.

Alkaline phosphatase activities were assayed by an adaptation of the methods of Torriani (1960) and Garen and Levinthal (1960): 0.1 or 0.2 ml of cell extracts was added to 2 ml of a solution containing 0.2 mg of *p*-nitrophenylphosphate per ml of Tris-$MgCl_2$ buffer, pre-equilibrated at 37°. The increase in optical density (410 m$\mu$) of the reaction mixture was followed in a Beckman DU spectrophotometer thermostated at 37°. An increase in optical density of 0.001 per minute was defined as one unit of enzyme activity. All assays were performed in triplicate. The specific enzymatic activities of extracts could be reproduced within $\pm$ 15% in separate experiments.

*Dialyses.*—The cell extracts were dialyzed against five changes of 500 ml of Tris-$MgCl_2$ buffer; the protein contents and enzymatic activities were determined.

TABLE I

ALKALINE PHOSPHATASE LEVELS OF STREPTOMYCIN-SENSITIVE, RESISTANT, AND DEPENDENT STRAINS OF *E. coli* OBTAINED FROM OUTSIDE SOURCES[a]

| Classification | Strain Designation | Specific Enzymatic Activity (units/mg protein) |
|---|---|---|
| Sensitive | $B_f$ | 2.0 |
| Resistant | $B/Sr_3$ | 3.0 |
| Resistant | $B/Sr_5$ | 3.4 |
| Resistant | $B/Sr_{11}$ | 5.0 |
| Resistant | $B/Sr_{11}$ minus streptomycin[b] | 7.3 |
| Dependent | B-Sd 9 | 1487 |
| Sensitive | ML 35 | 0 |
| Resistant | ML $35/Sr_2$ | 2.2 |
| Sensitive | $B_d$ | 1.7 |
| Resistant | $B_d/Sr$ | 2.2 |
| Dependent | ATCC 11370 | 129 |

[a] Except as otherwise indicated, the resistant strains were grown in the presence of the streptomycin levels given in the text. All the data were obtained with strains grown in medium F. [b] Grown in medium F free of streptomycin.

TABLE II

ALKALINE PHOSPHATASE LEVELS OF STREPTOMYCIN-RESISTANT AND DEPENDENT STRAINS OF *E. coli* ISOLATED IN THIS LABORATORY BY THE REPLICA PLATING TECHNIQUE[a]

| Classification | Strain Designation | Specific Enzymatic Activity (units/mg protein) |
|---|---|---|
| Sensitive | $B_e$ | 0 |
| Resistant | $B/Sr_{101}$ | 1.8 |
| Resistant | $B/Sr_{102}$ | 0 |
| Dependent | B-Sd 101 | 79 |
| Dependent | B-Sd 102 | 481 |
| Dependent | B-Sd 103 | 234 |
| Sensitive | C600 | 0 |
| Resistant | $C600/Sr_{101}$ | 0 |
| Dependent | C600 Sd 101 | 173 |
| Dependent | C600 Sd 102 | 284 |
| Sensitive | K 10 | 0 |
| Resistant | $K\ 10/Sr_{101}$ | 0 |
| Resistant | $K\ 10/Sr_{102}$ | 0 |
| Resistant | $K\ 10/Sr_{103}$ | 1.1 |
| Dependent | K 10 Sd 101 | 109 |
| Dependent | K 10 Sd 102 | 536 |
| Dependent | K 10 Sd 103 | 394 |

[a] All mutants were grown in medium F containing 10 $\mu$g of streptomycin per ml.

## RESULTS

The alkaline phosphatase contents of a number of streptomycin-sensitive strains of *E. coli* and the streptomycin-resistant and dependent mutants derived therefrom are shown in Tables I and II. These data are for strains obtained from outside sources (Table I) as well as for mutants isolated in this laboratory by replica plating (Table II). Under conditions which repressed the elaboration of alkaline phosphatase (Horiuchi, 1959; Torriani, 1960) in the wild strain (*i.e.*, medium with a high phosphorus content), the streptomycin-dependent mutants all showed a high level of this enzyme. In the resistant mutants the

enzyme was still repressed. The extracts of wild and streptomycin-resistant *E. coli* B ($B_f$ and $B/Sr_{11}$) did not contain an inhibitor of alkaline phosphatase activity. This was shown by making 1:1 mixtures of these extracts with an extract of *E. coli* B Sd9 and demonstrating that the mixture had the expected activity. None of these extracts ($B_f$, $B/Sr_{11}$, or B Sd9) showed any change in their specific activities upon dialyses. This therefore demonstrated that a dialyzable inhibitor (or activator) was not involved.

In order to determine whether the alkaline phosphatase was part of a defense system to overcome the lethal effect of the antibiotic, a growing culture of *E. coli* $B_f$ ($1 \times 10^8$ cells per ml of medium F) was exposed to streptomycin (60 $\mu$g/ml) for 3 or 4 hours so that more than 99.9% of the cells were killed. There was a 4-fold increase in the level of alkaline phosphatase of the extracts (Table III). Even though the cells were dying, they had not disintegrated to a large extent as judged by the protein contents of the extracts (Table III). Still, the specific enzymatic activity of the extract prepared from the streptomycin-treated bacteria may be deceptively low, as the enzyme might have been solubilized as a result of the action of the antibiotic on the external cell structure.

TABLE III

EFFECT OF EXPOSURE TO STREPTOMYCIN ON ALKALINE PHOSPHATASE LEVEL OF GROWING CULTURES OF *E. coli* B[a]

| Bacterium | Streptomycin Concentration | mg Protein/ml Extract | Specific Activity (units/mg protein) |
|---|---|---|---|
| *E. coli* B | 0 | 3.8 | 1.5 |
| *E. coli* B | 60 $\mu$g/ml | 3.4 | 6.0 |

[a] A growing culture of *E. coli* $B_f$ ($1 \times 10^8$ cells per ml of medium F) was exposed to streptomycin for 4 hours, and there was a 99.9% decrease in viability of the culture as a result of this treatment.

The exposure of a streptomycin-dependent strain (B Sd9) to elevated levels of streptomycin (250 $\mu$g per ml of medium F) resulted in a 2-fold decrease of enzymatic activity (Table IV). This is consistent with the results of Flaks *et al.* (1962b), who found that an *in vitro* protein-synthesizing system prepared from this bacterium was inhibited by high streptomycin concentrations.

The effect of streptomycin starvation on the alkaline phosphatase level of a dependent strain (B Sd9) was also studied. The bacteria were brought to the exponential stage ($2 \times 10^8$ cells/ml) in medium F supplemented with streptomycin (100 $\mu$g/ml). The cells were harvested, washed twice with physiological saline to remove the bound streptomycin (Engelberg and Artman, 1962), and resuspended in medium F devoid of the antibiotic. Incubation was allowed to proceed 5 more hours before the cells were again harvested. The data in Table IV show no significant decrease (11.3%) in the enzyme level.

TABLE IV

EFFECT ON ALKALINE PHOSPHATASE LEVEL OF EXPOSURE OF DEPENDENT STRAIN OF *E. coli* TO VARIOUS LEVELS OF STREPTOMYCIN

| Bacterium | Streptomycin Concentration ($\mu$g/ml) | Condition for Growth | Specific Activity (units/mg protein) |
|---|---|---|---|
| *E. coli* B Sd 9[a] | 0 | Starvation | 978 |
| *E. coli* B Sd 9 | 100 | Optimal | 1104 |
| *E. coli* B Sd 9 | 250 | Elevated | 617 |

[a] A growing culture of *E. coli* B Sd9 ($2 \times 10^8$ cells/ml) in medium F containing 100 $\mu$g of streptomycin per ml was harvested, washed twice with chilled physiological saline, and then resuspended in medium F free of streptomycin. It was incubated for 5 hours at 37°.

The effect of the growth medium on the alkaline phosphatase of a dependent strain (B Sd9) is illustrated in Table V. It is noteworthy that production of the phosphomonoesterase in streptomycin - dependent bacteria was diminished when the bacteria were grown in the low-phosphorus medium, which permits the synthesis of the enzyme in the wild strain (Horiuchi, 1959; Torriani, 1960).

TABLE V

EFFECT OF GROWTH MEDIUM ON ALKALINE PHOSPHATASE LEVEL OF WILD AND STREPTOMYCIN-DEPENDENT STRAINS OF *E. coli*[a]

| Medium[b] | Specific Activity (units/mg protein) *E. coli* B | *E. coli* B Sd |
|---|---|---|
| VLP | 678 | 714 |
| LP | 0 | 693 |
| HP | 0 | 1395 |
| F | 0 | 1104 |

[a] Strain *E. coli* B Sd9 was grown in the appropriate medium in the presence of 100 $\mu$g of streptomycin per ml. [b] The media are described in the text.

Streptomycin-resistant and dependent cells arising in a homogeneous population of sensitive bacteria can be selected by exposure of the bacteria to the antibiotic. One can use the high alkaline phosphatase level of streptomycin-dependent cells to distinguish between them and the resistant cells by spraying the streptomycin-containing nutrient agar plate on which they are growing with *p*-nitrophenylphosphate. The details of the procedure are given in the Experimental section of this report. The alkaline phosphatase levels of a number of streptomycin-dependent mutants isolated in this manner are given in Table VI. The variations in the specific enzymatic activities among the different mutants probably reflect the fact that the streptomycin concentration of the medium employed (20 $\mu$g/ml) may not have been at the appropriate level for optimal growth of all the mutants. This in turn influences the enzymatic content of the cells (Table IV).

## DISCUSSION

It has been suggested that exposure of sensitive strains of *E. coli* to streptomycin results in a lesion of the cell membrane, which is followed by penetration of the drug into the interior (Anand *et al.*, 1960). Resistant strains seem to possess a modified membrane which is not damaged by absorbed streptomycin and there is thus no concomitant passage of the antibiotic beyond the membrane (Anand *et al.*, 1960). When the membranes of such resistant cells are removed, the altered bacteria (protoplasts) become sensitive to the action of the drug (Lederberg and St. Clair, 1958). Streptomycin appears to be required for the maintenance of the integrity of the cell membrane in dependent strains (Landman and Burchard, 1962), and there may also be some penetration of the agent into the cell (Szybalski and Mashima, 1959; see also Spotts and Stanier, 1961; and Engelberg and Artman, 1962). Alkaline phosphatase is elaborated by wild strains of *E. coli*

TABLE VI

ALKALINE PHOSPHATASE LEVELS OF A NUMBER OF STREPTOMYCIN-RESISTANT AND DEPENDENT STRAINS OF *E. coli* W SELECTED BY SPRAYING WITH *p*-NITROPHENYL-PHOSPHATE[a]

| Classification | Strain Designation | Specific Enzymatic Activity (units/mg protein) |
|---|---|---|
| Sensitive | W | 0 |
| Resistant | $Sr_{103}$, $Sr_{104}$, $Sr_{107}$, | 0 |
| Resistant | $Sr_{109}$, $Sr_{110}$, $Sr_{111}$, | 0 |
| Resistant | $Sr_{112}$, $Sr_{114}$, $Sr_{115}$ | 0 |
| Resistant | $Sr_{101}$, $Sr_{105}$, $Sr_{108}$ | 0.8–1.5 |
| Resistant | $Sr_{106}$, $Sr_{116}$ | 1.5–2.5 |
| Resistant | $Sr_{113}$ | 3.6 |
| Resistant | $Sr_{102}$ | 7.8 |
| Dependent | Sd 101 | 48.6 |
| Dependent | Sd 102 | 283 |
| Dependent | Sd 103 | 582 |
| Dependent | Sd 104 | 514 |
| Dependent | Sd 105 | 354 |
| Dependent | Sd 106 | 220 |
| Dependent | Sd 107 | 383 |
| Dependent | Sd 108 | 197 |

[a] All mutants were grown in medium F adjusted to 20 μg streptomycin per ml.

during growth in a medium of restricted phosphorus content. The enzyme is thought to be associated with the external cell structure (Malamy and Horecker, 1961, 1962).

The findings reported in the present study can possibly be explained by either or both of the following:

1. In dependent strains, streptomycin combines with the cell membrane in such a manner that an alteration of the permeability takes place which may cause an inhibition of the uptake and transport of inorganic phosphorus. This is equivalent to exposing the cells to a low phosphorus environment, thus leading to a derepression of the synthesis of alkaline phosphatase.

2. When streptomycin penetrates vulnerable bacterial cells, its ability to form complexes with the nucleic acids may result in a lethal effect. Streptomycin-dependent strains possibly have evolved a mechanism which renders their nucleic acids immune to the action of the antibiotic. This protective effect might be achieved by the enzymatic removal of the sensitive site (*i.e.*, the phosphate end of the DNA). Sensitive strains of *E. coli*, when exposed to the drug, do not produce alkaline phosphatase in sufficient quantities (Table III) and may thus lack an essential defensive element.

Preliminary results obtained in a study dealing with the lethal action of streptomycin on *E. coli* constitutive for alkaline phosphatase do not permit a clear-cut distinction between the two possible causes for the elaboration of the enzyme by the antibiotic-requiring mutants. Further investigations on this subject are therefore under way. Experiments are also being carried out to determine the relationship of the enzyme in streptomycin-dependent cells to the alkaline phosphatase elaborated by sensitive bacteria under de-repressed conditions.

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