# STUDIES ON THE NUTRITION AND PHYSIOLOGY OF *PASTEURELLA PESTIS*

## V. Inhibition of Growth by D-Serine and Its Reversal by Various Compounds

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The inhibitory effects of D-serine on growth have been shown in a variety of bacterial systems: *Bacillus anthracis* (Gladstone, 1939), *Streptococcus lactis* R (Snell and Guirard, 1943), *Escherichia coli* (Davis and Maas, 1949; Maas and Davis, 1950), *Mycobacterium tuberculosis* (Dubos, 1949), *Bacillus subtilis* (Teas, 1950), *Lactobacillus leichmannii* (Teeri, 1954), and *Micrococcus pyogenes* (Castellani *et al.*, 1955). Other physiological effects of D-serine such as depression of toxin production by *Clostridium tetani* (Mueller and Miller, 1949) and suppression of spore germination in *B. subtilis* (Woese *et al.*, 1958) also have been noted. Among animals, a nephrotoxic effect was observed in rats given D-serine (Fishman and Artom, 1944; Wachstein, 1947).

Previous work reported from our laboratory had disclosed that the growth of *Pasteurella pestis* was highly sensitive to D-serine (Higuchi and Carlin, 1958). Further studies on the mode of D-serine inhibition have indicated that certain aspects of glycine metabolism are involved. Details of these investigations are reported below.

### MATERIALS AND METHODS

*Organism.* The Alexander strain of *P. pestis* employed in the present work was originally isolated in 1949 from a human case of bubonic plague in New Mexico (Link, 1950).

*Medium and cultural conditions.* The basal medium (table 1) with D-xylose and vitamins omitted was adjusted to pH 7.5, and sterilized by autoclaving for 15 min at 121 C. D-Xylose and the vitamin mixture were autoclaved separately and added aseptically to the medium prior to inoculation. D-Serine and test compounds were autoclaved in the medium unless indicated otherwise. Twenty-five ml of medium contained in a 500-ml Erlenmeyer flask were inoculated with 0.5 ml of a washed suspension of cells from a 24-hr culture grown in a casein hydrolyzate medium (Higuchi and Carlin, 1957) to give an initial count of approximately $5 \times 10^8$ cells per ml. Cultures were incubated at 27 C ($\pm 1$) for 30 hr on a reciprocating shaker operating through a 3-inch stroke at 100 cycles per minute.

*Measurement of growth.* After 30 hr incubation, growth was measured nephelometrically in a Coleman model 9 Nepho-colorimeter after a 1:10 dilution of the cultures. An arbitrary turbidity standard was employed for reference. A reading of 50 turbidity units with a 1:10 dilution of a culture in a standardized 18-mm test tube corresponded to an optical density of approximately 0.5 determined with a colorimeter at 650 m$\mu$ wave length. These values corresponded to approximately $10^{10}$ cells per ml in the culture medium.

### RESULTS

*Reversal of inhibition by amino acids.* The inhibitory effects of D-serine on the growth of *P. pestis* are shown by the data plotted in figure 1. Almost complete inhibition was obtained at approximately 1 mM concentration of D-serine, whereas, no inhibitory effects were obtained with L-serine, even at concentrations as high as 25 mM. Several amino acids were capable of reversing the inhibitory effects of D-serine as shown in figure 2. Glycine was particularly effective. The data indicate that on a molar basis glycine was approximately 4 times as effective as L-alanine and 10 times as effective as L-serine. D-Alanine was even less active than L-serine. When varying ratios of D-serine and glycine were employed in the medium, a competitive type of reversal was observed. Approximately three moles of glycine were required to obtain 50 per cent reversal of

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TABLE 1

*Composition of the basal medium*

| Component | Conc | Component | Conc |
|---|---|---|---|
| | *mM/L* | | *mM/L* |
| L-Glutamic acid | 82.0 | $K_2HPO_4$ | 25.0 |
| DL-Phenylalanine | 5.0 | $NH_4$-acetate | 10.0 |
| DL-Methionine | 3.2 | Na-gluconate | 10.0 |
| DL-Valine | 13.7 | Citric acid·$H_2O$ | 10.0 |
| DL-Leucine | 2.9 | $MgSO_4 \cdot 7H_2O$ | 2.5 |
| DL-Lysine·HCl | 2.2 | $FeSO_4 \cdot 7H_2O$ | 0.1 |
| L-Proline | 7.0 | $MnSO_4 \cdot H_2O$ | 0.01 |
| DL-Threonine | 2.7 | Thiamine·HCl | 0.003 |
| DL-Isoleucine | 7.3 | Ca-pantothenate | 0.002 |
| L-Cysteine·HCl | 4.0 | Biotin | 0.002 |
| | | D-Xylose | 66.7 |

inhibition produced per mole of D-serine. The results obtained with L-alanine and L-serine also indicated competitive effects against D-serine activity. The D-serine concentration tested in these experiments varied from 0.2 to 0.75 mM. It may be noted that the stimulatory effect of glycine on growth was also evident in media containing no D-serine (Smith and Higuchi, 1959) and that in the present studies L-serine, D- and L-alanine also were stimulatory in a glycine-deficient medium containing no D-serine.

*Reversal of inhibition by purines and derivatives.* A variety of purines and purine derivatives were tested for their ability to reverse the inhibitory action of D-serine. Inhibition by D-serine was partially antagonized by xanthine and adenosine (table 2). The degree of reversal was incomplete, even with high concentrations of these compounds. The addition of a combination of adenosine and xanthine resulted in less growth than that observed in the control culture containing neither compound. This effect was noted also in a medium containing no D-serine. Related compounds, adenine, guanine, guanosine, xanthosine, inosine, adenylic acid, and guanylic acid, were either ineffective or inhibitory.

*Reversal of inhibition by organic acids and derivatives.* The ability of glyoxylate, glycolate, and glyoxal to reverse the inhibition produced by D-serine is shown by data presented in table 3. Glyoxylate, in the concentration range of 1 to 2 mM, appeared to reverse completely the inhibition obtained with D-serine when autoclaved with the growth medium. Aseptic addition of glyoxylate sterilized either by autoclaving or by

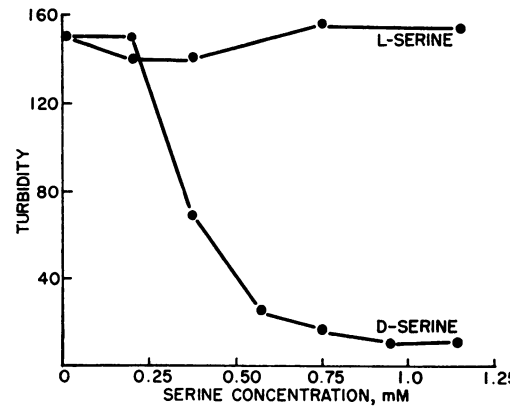


*Figure 1.* The inhibition of growth of *Pasteurella pestis* by D-serine.

filtration failed to reverse D-serine inhibition. When glyoxylate was autoclaved with L-glutamate at pH 7.5 for 15 min at 121 C, the resulting solution also reversed the inhibition of D-serine. Analysis of this solution by paper chromatography revealed the presence of glycine.

The activities of glycolate and glyoxal were not dependent on the autoclaving procedure. Glycolate was only one tenth as effective as glyoxylate on a molar basis. Furthermore, glycolate was not capable of reversing the inhibition of growth in the presence of high concentrations of D-serine (table 3). Glyoxal was toxic at concentrations above 2.1 mM, but at nontoxic levels it appeared more effective than comparable amounts of glycolate.

Another related compound, thioglycolate, showed only slight activity even at the high

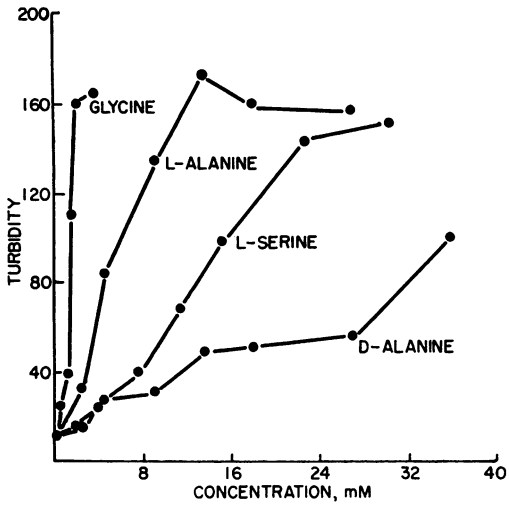


*Figure 2.* The effects of glycine, L-serine, and D- or L-alanine in reversing the inhibitory action of 0.76 mM D-serine on the growth of *Pasteurella pestis*.

TABLE 2

*Partial reversal by xanthine and adenosine of the D-serine inhibition of growth of Pasteurella pestis*

| Antagonist, mM | Growth* Turbidity | | |
|---|---|---|---|
| | Without D-serine | With D-serine | |
| | | 0.38 mM | 0.76 mM |
| Xanthine | | | |
| 0.00 | 164 | 18 | 6 |
| 0.26 | 158 | 42 | 19 |
| 0.52 | 172 | 71 | 27 |
| 0.78 | 155 | 59 | 30 |
| 1.04 | 142 | 60 | 23 |
| 1.30 | 149 | 65 | 27 |
| Adenosine | | | |
| 0.00 | 170 | 48 | 13 |
| 0.15 | 152 | 96 | 26 |
| 0.30 | 152 | 89 | 31 |
| 0.45 | 161 | 88 | 35 |
| 0.60 | 175 | 80 | 38 |
| 0.75 | 184 | 71 | 37 |

* Growth of 1:10 dilution of culture measured nephelometrically.

concentrations used (28 mM). The additions of other amino acids, B vitamins, and Krebs cycle intermediates to the medium showed no significant activity in reversing the inhibition produced with D-serine. However, D-glucosamine at a high concentration (18 mM) produced a slight stimulation of growth in cultures inhibited by D-serine.

TABLE 3

*Reversal by glyoxylic acid, glyoxal, and glycolic acid of the D-serine inhibition of growth of Pasteurella pestis*

| Antagonist, mM | Growth* Turbidity | | |
|---|---|---|---|
| | Without D-serine | With D-serine | |
| | | 0.38 mM | 0.76 mM |
| Glyoxylic acid | | | |
| 0.00 | 170 | 33 | 8 |
| 0.43 | 148 | 112 | 23 |
| 0.86 | 144 | 158 | 54 |
| 1.30 | 143 | 164 | 92 |
| 1.70 | 132 | 152 | 175 |
| 2.20 | 149 | 142 | 178 |
| 3.20 | 169 | 174 | 156 |
| 4.30 | 176 | 162 | 158 |
| Glyoxal | | | |
| 0.00 | 157 | 43 | 10 |
| 0.69 | 163 | 59 | 13 |
| 1.40 | 173 | 96 | 18 |
| 2.10 | 189 | 127 | 24 |
| 2.80 | 122 | 95 | 19 |
| 3.50 | 47 | 50 | 18 |
| 5.20 | 4 | 6 | 5 |
| Glycolic acid | | | |
| 0.0 | 164 | 58 | 19 |
| 5.3 | 157 | 69 | 23 |
| 11.0 | 169 | 108 | 23 |
| 21.0 | 170 | 146 | 25 |
| 32.0 | 166 | 166 | 33 |
| 42.0 | 162 | 155 | 39 |

* Growth of 1:10 dilution of culture measured nephelometrically.

## DISCUSSION

The results presented in this paper indicate that the inhibition of growth of *P. pestis* by D-serine is probably related to an interference with glycine metabolism. This conclusion is supported by the fact that glycine was the most effective amino acid in reversing the D-serine inhibition. Moreover a competitive antagonism between the activities of glycine and D-serine was indicated by the constant ratio calculated from the concentrations of glycine required to counteract the various inhibitory effects of graded levels of D-serine employed in the medium.

The partial, noncompetitive reversals of inhibition obtained with adenosine and xanthine are compatible with the hypothesis that interference in glycine metabolism obstructed the normal entry of glycine into purine biosynthesis. Complete reversal of inhibition was not obtained by purine supplements alone because it can be presumed that glycine is involved in other essential physiological processes, e. g., synthesis of porphyrins and cellular proteins. The antagonism against the growth of *P. pestis* produced by the combination of xanthine and adenosine is difficult to understand in view of the growth promoting effects of each when tested singly. The activity of L-serine in the reversal of inhibition produced with D-serine can be related to its biochemical conversion to glycine. However, the possibility that L-serine may function as a D-serine antagonist on the basis of its structural similarity to the inhibitor has not been excluded.

The role of L-alanine in reversing the effect of D-serine is difficult to assess. It is not known to be a precursor to glycine. However, it may be significant that under conditions of glycine deficiency, D- or L-alanine was found to be stimulatory for the growth of *P. pestis* in a medium containing no D-serine.

The growth activity of glyoxylate in cultures containing D-serine can be explained on the basis of the conversion of glyoxylate to glycine during the autoclaving process. The mechanism of this reaction was studied by Metzler *et al.* (1954), who showed that nonenzymatic transamination between glutamate and glyoxylate produced nearly quantitative yields of glycine. The growth activity of glyoxal may involve mechanisms similar to that of glyoxylate, except that its more reactive dicarbonyl structure may not require high temperatures for the amination step. Subsequent oxidation of the aminated glyoxal may yield glycine.

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SUMMARY

The inhibition of the growth of *Pasteurella pestis* by D-serine and its reversal by various compounds have been studied.

Glycine was the most effective antagonist of D-serine. It was approximately 4 times more effective than L-alanine and 10 times more effective than L-serine on a molar basis. Glyoxylate was capable of reversal of D-serine inhibition only when it was autoclaved in the growth medium. A nonenzymatic conversion of glyoxylate to glycine was demonstrated.

Xanthine and adenosine were capable of only partial reversal of the effects of D-serine. Other purines and purine derivatives were not effective. Glycolate, thioglycolate, and D-glucosamine were only slightly active even at high concentrations.

Competitive reversal of D-serine activity was observed with glycine, L-alanine, L-serine, and glyoxylate. A noncompetitive type of reversal was observed with the nucleic acid derivatives.

It is postulated that D-serine exerts its inhibitory effects on the growth of *P. pestis* by blocking entry of glycine into purines and other compounds.

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