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Metabolism of D-Serine in *Escherichia coli* K-12: Mechanism of Growth Inhibition[1]

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Without significant killing, D-serine at concentrations greater than 50 μg/ml inhibits growth in minimal media of mutants of *Escherichia coli* K-12 unable to form D-serine deaminase. The mutants eventually recover at lower concentrations. There is no evidence of D-serine toxicity in rich media. Toxicity is partially reversed by L-serine. D-Serine does not interfere with L-serine activation, one-carbon metabolism, or (Cronan, personal communication) formation of phosphatidylserine. Pizer (personal communication) finds, however, that it is a powerful feedback inhibitor of the first enzyme of L-serine biosynthesis. In the presence of L-serine, the residual toxicity is largely and noncompetitively overcome by pantothenate, indicating that D-serine inhibits growth by affecting two targets: pantothenate biosynthesis and L-serine biosynthesis. L-Serine causes transient growth inhibition in *E. coli* K-12. Contaminating L-serine in D-serine preparations contributes to the D-serine inhibitory response.

D-Serine inhibits the growth of a number of microorganisms. Maas and Davis reported that in *Escherichia coli* W this inhibition was competitively reversed by β-alanine and noncompetitively reversed by pantothenic acid. It was concluded that D-serine prevented the coupling of β-alanine to pantoic acid, thus inhibiting the formation of pantothenic acid (13).

Durham and Milligan found that D-serine inhibited growth in a strain of *Flavobacterium* (7). β-Alanine reversed this inhibition, as did pantothenate (6), suggesting that in this system D-serine interfered with the biosynthesis of β-alanine. L-Aspartic acid, a precursor of β-alanine, also reversed the inhibitory effect of D-serine to a small extent in *Flavobacterium* (6), whereas it enhanced the inhibition in *E. coli* (13). D-Serine inhibits pantothenate synthesis in a species of *Erwinia* by blocking the synthesis of pantoic acid as well as β-alanine (9). D-Serine inhibits pantoic acid synthesis by blocking the hydroxymethylation of α-ketopantoic acid, the immediate precursor of pantoic acid (9). D-Serine also blocks the decarboxylation of aspartic acid to β-alanine (8). Thus, D-serine appears to have a different target in the sequence of pantothenate biosynthesis in each species.

In *Micrococcus lysodeikticus*, D-serine inhibited growth (20), and label from D-serine was found to be incorporated into cellular protein (21). L-Serine, which was most effective in overcoming incorporation of label from D-serine into the protein fraction (21), was also most effective in reversing growth inhibition (20).

D-Serine is toxic to *E. coli* K-12, but an inducible D-serine deaminase acts as a detoxifying agent by converting it to pyruvate and ammonia (14). Pantothenate alone does not affect D-serine toxicity in *E. coli* K-12. As we show below, D-serine has two targets in *E. coli* K-12: pantothenate synthesis and L-serine metabolism.

MATERIALS AND METHODS

Strains. *E. coli* strains EM 1101, EM 1301, and EM 1401, mutants unable to form D-serine deaminase (*dsdA*), were used in studies on the mechanism of D-serine toxicity. These strains have been described previously (15). EM 1305, a mutant defective in D-serine transport (*dagA*; 3) and auxotrophic for L-serine (*serA*), was used to assay a possible D-serine racemase, and ATCC 13762, originally B. D. Davis W 99-1, a pantothenate auxotroph, was used in the bioassay of pantothenate.

Growth. All media have been described previously (15).

Growth in liquid media was measured as optical density on a Klett-Summerson colorimeter, with the use of a 420-nm filter. Cultures were grown in 250-ml Erlenmeyer flasks fitted with side arms which served

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as nephelometer tubes. Cells were always grown with aeration at 37 C.

On solid media, cultures were streaked, and growth was recorded after 24 or 48 h on a relative basis, with +4 indicating maximal growth.

Sonic treatment. Cell suspensions were subjected to sonic oscillation at a frequency of 21 ± 0.5 kc in a Branson Sonic Power Sonifier for 1 min. The cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4 C.

ATP-pyrophosphate exchange. Before carrying out the serine-dependent adenosine triphosphate (ATP)-pyrophosphate exchange, it was necessary to partially purify seryl-transfer ribonucleic acid (tRNA) synthetase to remove inorganic phosphatase activity. The enzyme was therefore purified about 2.5-fold according to the methods of Muench and Berg (16).

The procedure followed for the L-serine-dependent ATP-pyrophosphate exchange was that of Calender and Berg (2), with modifications in buffer and pyrophosphate concentration as used by Katze and Konigsberg (11).

The ^{32}P-pyrophosphate used in the exchange was prepared from ^{32}P-orthophosphate (E. R. Squibb & Sons, carrier free) by the method of Bergman (1).

Racemase activity. The reaction mixture of Diven et al. (5) was used to determine whether EM 1101 contained any serine racemase activity.

The reaction mixtures were incubated for 1 h at 37 C, and the reaction was stopped by immersion in a boiling-water bath for 2 min. The reaction mixtures were sterilized by passage through a sterile membrane filter (Millipore HA, 0.45 μm pore size) fitted in a Swinnex filter holder attached to a 10-ml syringe.

The amount of D-serine converted to L-serine was determined by a bioassay. Small petri dishes (10 by 15 mm) were filled with 15 ml of minimal agar medium supplemented with 1 ml of the sterilized reaction mixture. About 40 cells of an overnight culture of EM 1304 (*serA*, *dagA*) were spread on top of each plate and incubated for 48 h at 37 C.

Pantothenate synthesis. The production of pantothenate in resting-cell suspensions was measured according to the method of Maas and Davis (13).

Incorporation of radioactive components. Uptake of ^{14}C-L-serine (New England Nuclear Corp., 10 Ci/mol) into whole cells was carried out as previously described (3).

Incorporation of ^{14}C-leucine (New England Nuclear Corp., 261 Ci/mol), ^{3}H-thymidine (New England Nuclear Corp., 5 Ci/mmol), and ^{3}H-uridine (New England Nuclear Corp., 1 mCi/0.0094 mg) was examined as described in the text. Samples were treated with trichloroacetic acid as described, filtered on Whatman GF/C glass filters, and washed with 75% ethanol. The filter pads were dried under an infrared lamp.

All samples were counted in a Mark I Nuclear-Chicago Scintillation Counter in a Liquifluor scintillation fluid.

RESULTS

Effects of D-serine on growth. Figure 1 illustrates the effect in minimal medium of various concentrations of D-serine on the growth of EM 1101, a mutant of *E. coli* K-12 unable to form D-serine deaminase. The mutant was grown to a density of about 10^8/ml. At time zero, the culture was subdivided. Different concentrations of D-serine were added to the various fractions, and turbidity increase during subsequent growth was recorded as indicated. At 1 μg/ml, D-serine did not affect growth; 5 μg/ml resulted in a lag of about 1 h, and 10 and 25 μg/ml resulted in lags of about 5 h each. In all cases, when growth resumed, the cells attained a doubling time similar to that of the control without D-serine. At D-serine concentrations of 50, and 150 and 500 μg/ml (not shown), growth was inhibited to 10% of the control. On minimal agar media, growth of EM 1101 was permanently inhibited by D-serine concentrations of $>$ 25 μg/ml. There was no obvious inhibition at 10 μg/ml. The wild-type parent strain grew readily on plates containing D-serine up to 500 μg/ml.

It should be noted that commercial D-serine (Nutritional Biochemicals Corp.) is contaminated with 3 to 5% L-serine (4). Concentrations of L-serine of 4 μg/ml or greater are sufficient to cause a transient toxicity in the strains of *E. coli* K-12 used in these studies (see below). This toxicity is largely reversed by a combination of L-threonine and L-isoleucine or L-threonine and L-leucine, and in growth studies at high D-serine concentrations these components were added to the media.

To determine whether resumption of growth in media containing 1, 10, and 25 μg/ml was due to removal of D-serine from the medium by the cells, we performed the following experiment. Sonic extracts of EM 1101 cultures which had been grown in minimal medium and in minimal medium supplemented with 25 μg of D-serine/ml were prepared after each culture attained a density of about 3×10^8/ml. After sterilization by filtration, they were used to resuspend cells of EM 1101 previously grown in minimal medium to a density of 10^8/ml, and growth of these cells was continued. Growth in each sonic extract was measured at intervals (Table 1). There was no inhibition of growth in the D-serine sonic extract. Addition of 25 μg of D-serine/ml to the sonic extract of the culture grown without D-serine did inhibit the growth of EM 1101.

It thus appears that D-serine had been removed from the medium by either breakdown or conversion to some nontoxic product, and this removal was what allowed the cells to recover from D-serine inhibition at 25 μg/ml and presumably also at 5 and 10 μg/ml.

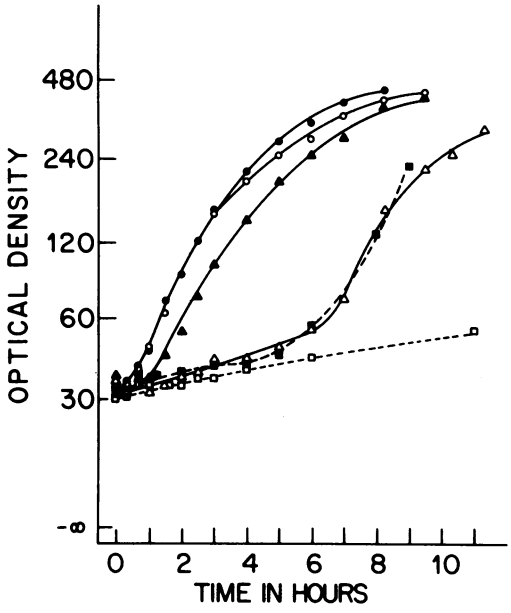

FIG. 1. *Effect of different concentrations of* D-*serine on the growth of EM 1101 in minimal medium. The growth is presented as optical density in Klett units.* D-*Serine concentrations:* ●, *no* D-*serine;* ○, *1 μg/ml;* ▲, *5 μg/ml;* △ *10 μg/ml;* ■ *25 μg/ml;* □ *50 μg/ml.*

TABLE 1. *Growth of EM 1101 in a sonic extract of cells which have recovered from* D-*serine growth inhibition*

Time of incubation (min)	Turbidity in Klett units[a]		
	a	b	c
0	30	31	29
30	43	41	33
75	67	67	36
150	110	112	40

[a] EM 1101 was grown to a density of 3×10^8/ml and diluted to a density of 10^8/ml in sonic extracts of cells from cultures: (a) grown in minimal medium; (b) grown in minimal medium supplemented with 25 μg of D-serine/ml until recovery from growth inhibition occurred, and (c) sonic extract (a) plus 25 μg of D-serine/ml. Growth was recorded as turbidity in Klett units.

Low-level D-serine metabolizing activity. An attempt was made to detect D-serine racemase activity in extracts of EM 1101 in order to determine whether a racemase was responsible for removal of low concentrations of D-serine from the growth medium. Accordingly, cells of EM 1101 from a culture grown in minimal medium to a density of about 5×10^8/ml were washed and resuspended to one-fourth the original volume of the culture in 0.05 M $KHPO_4$ buffer (pH 8.1). An extract was then prepared by sonic treatment. The racemase assay consists of two steps: an incubation of extract with D-serine, followed by a bioassay for the presence of newly formed L-serine in the reaction mixture. Two concentrations of cell extract were assayed for racemase activity. Controls were as follows: reaction mixtures lacking D-serine at both extract concentrations and a reaction mixture containing D-serine as substrate but lacking extract.

The strain used for the bioassay was EM 1305 *serA dagA*, i.e., a D-serine-resistant, L-serine auxotroph. In addition to the control reaction mixtures, minimal agar plates were prepared with reaction mixtures containing extract at both concentrations, lacking D-serine but supplemented with 50 or 200 μg of L-serine/ml.

Table 2 presents the results of such an assay as average colony size (in millimeters) per 20 colonies measured. There appears to be no significant racemase activity. The average colony size on the two control media lacking D-serine substrate, but supplemented with 50 μg of L-serine/ml, was about 60% larger than that on corresponding experimental media.

TABLE 2. *Bioassay for* D-*serine racemase activity*[a]

Amt of extract (μg)	D-Serine, step 1	L-Serine (μg/ml), step 2	Avg colony size (mm)
57	+	—	0.99
285	+	—	1.14
57	–	—	0.91
57	–	50	1.53
57	–	200	1.53
285	–	—	0.94
285	–	50	1.88
285	–	200	2.02
–	+	—	0.95

[a] Cell extracts prepared from EM 1101 were incubated with D-serine and reaction mixture components (step 1, see text). The entire reaction mixtures were sterilized, and 1 ml was added to 14 ml of minimal agar medium. Results of growth of EM 1305 are presented as the average colony size per 20 colonies on each medium. Controls for the bioassay consist of media containing reaction mixture lacking D-serine (enzyme substrate), media as just described but supplemented with 50 or 200 μg of L-serine/ml, and media containing reaction mixtures lacking enzyme extract. Each 1 ml of complete reaction mixture contained: 0.04 M $KHPO_4$ buffer, pH 8.1, 0.9 μmol of pyridoxal phosphate, 6.7 μmol of reduced glutathione, 80 μmol of D-serine, and either 57 or 285 μg of cell extract.

A possible source of the low-level D-serine detoxifying activity may be an enzyme discovered by M. Heincz of this laboratory upon purification of D-serine deaminase. This activity appeared to have a high affinity for D-serine and L-serine, but a very low activity in the conversion of D-serine to pyruvate (unpublished data).

Bacteriostatic effect of D-serine. D-Serine is bacteriostatic, as was demonstrated in Fig. 1 and confirmed by the data of Table 3. EM 1101 was grown to a density of 2×10^8/ml, diluted 1:2 into fresh minimal medium supplemented with either 150 or 500 μg of D-serine/ml, and incubated for 18 h. Although the turbidity increased slightly, the viable counts remained similar, indicating that D-serine inhibits growth without appreciable killing. Microscopic examination showed no obvious effect of D-serine on the cells.

Reversal of D-serine inhibition. Preliminary experiments indicated that D-serine growth inhibition on solid medium was reversed to some extent by a variety of amino acids, the most effective by far being L-serine: EM 1301 showed significant growth after 1 day and excellent growth after 2 days on minimal agar plates containing 500 μg of D-serine/ml and 50 μg of L-serine/ml. The toxic effect was not overcome by pantothenate alone, as in *E. coli* W.

An experiment was performed to determine what effect a rich medium would have on D-serine toxicity. Strain EM 1101 was grown to a density of 10^8/ml in LB broth. The culture was divided into two equal parts, and 500 μg of D-serine/ml was added to one part. Growth was continued and turbidity was measured (Fig. 2). D-Serine was not toxic to EM 1101 in this medium. The doubling time for both cultures was about 21 min, and there was no lag in growth after the addition of D-serine. Since L-serine partially overcame D-serine growth inhibition and a rich medium completely prevented it, it appeared that D-serine has two targets in *E. coli* K-12, one in L-serine metabolism and the other in the metabolism of some component of the rich medium.

TABLE 3. *Bacteriostatic effect of D-serine*[a]

Incubation time (h)	Optical density		Viable counts	
	150 μg/ml	500 μg/ml	150 μg/ml	500 μg/ml
0	36	36	1×10^8	0.9×10^8
18	68	73	1.4×10^8	1×10^8

[a] EM 1101 was grown and diluted into minimal media containing 150 or 500 μg of D-serine/ml. Optical densities were recorded as Klett units. Viable counts were measured by diluting the cultures in minimal salts and plating on LB agar.

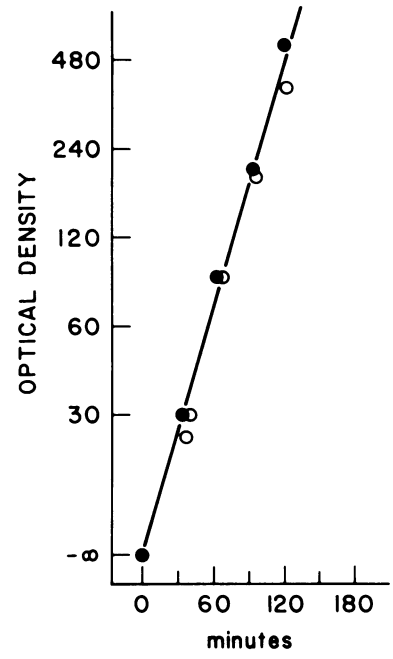

FIG. 2. *Effect of D-serine on growth of EM 1101 in LB broth. Growth is presented as optical density in Klett units in the absence (●) and presence (○) of 500 μg of D-serine/ml. D-Serine was added at an optical density of 30 Klett units.*

Effect of D-serine on L-serine metabolism. There are several possible mechanisms by which D-serine may affect L-serine metabolism, some of which could cause a harmful effect on the cell. D-Serine might compete with L-serine as substrate in some reaction(s): it might compete with L-serine for entry; it might compete for activation by the L-seryl tRNA synthetase; or it might interfere with formation of membrane phosphatidylserine or formation of one-carbon units. Also, D-serine could block the synthesis of L-serine, thereby creating an L-serine deficiency.

L-Serine reversal of the D-serine effect is not due to decreased uptake of D-serine, since the two amino acids have different transport systems (3), and we found no interference by D-serine with the uptake of ^{14}C-L-serine (Table 4). The slight decline in L-serine uptake at high D-serine concentration is accounted for by the small (3 to 5%) contamination of D-serine by L-serine (4).

To determine whether D-serine interfered with L-serine entry into protein, we examined the serine-dependent ATP-pyrophosphate exchange. Figure 3 presents Lineweaver-Burk (12)

plots of the pyrophosphate exchange reaction with L-serine and D-serine as substrates. The apparent K_m values derived as a mean of two determinations are $2.65 \pm 0.65 \times 10^{-5}$ and $3.80 \pm 0.5 \times 10^{-4}$ M for L- and D-serine, respectively. D-Serine did not act as a substrate in the reaction, and it seemed likely that the K_m of the D-serine reaction reflected that of the contaminating L-serine (4). This was confirmed by Katze and Konigsberg, who used a purified seryl synthetase (Correction, J. R. Katze, and W. Konigsberg, J. Biol. Chem. **245**:5874, 1970).

The data of Table 5 also confirm this supposition. Similar amounts of ^{32}P-pyrophosphate were incorporated into ATP when 2 mM D-serine or 0.1 mM L-serine (the amount of L-serine which contaminates 2 mM D-serine) was used as substrate, thus demonstrating that D-serine does not participate in the reaction. D-Serine does not inhibit the use of L-serine as substrate, as demonstrated by the similarity in results observed when 2 mM L-serine or 2 mM L-serine plus 20 mM D-serine were used as substrates.

TABLE 4. *Effect of D-serine on the uptake of L-serine*[a]

D-Serine (μg/ml)	L-Serine taken up (nmol/mg)
—	46.9
10	50.8
40	43.1
100	41.8
200	31.3
400	31.8

[a] Results are presented in nanomoles of ^{14}C-L-serine (10 μg/ml; specific activity, 0.1 Ci/mol) taken up per milligram (wet weight) of EM 1401 after 30 min of incubation at 37 C. The cells were treated with 200 μg of chloramphenicol/ml for 30 min at 37 C before addition of ^{14}C-L-serine and the various concentrations of D-serine.

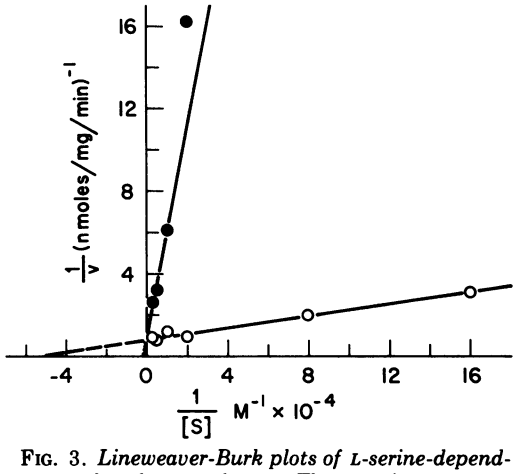

FIG. 3. *Lineweaver-Burk plots of L-serine-dependent pyrophosphate exchange. The reaction was carried out in the presence of 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.4), 2 mM ATP, 5 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, 10 mM mercaptoethanol, 4 μM ^{32}P-pyrophosphate (specific activity, 0.004 Ci/mol), and 19 μg of partially purified seryl-tRNA synthetase. The reactions were incubated at 37 C for 15 min, under which conditions the reaction was linear.* (○) *L-Serine as substrate;* (●) *D-serine as substrate.*

TABLE 5. *Effect of D-serine on the amino acid-dependent pyrophosphate exchange*[a]

Substrate	Pyrophosphate incorporated into ATP (nmol)
L-Serine (2 mM)	95
D-Serine (2 mM)	42
L-Serine (0.1 mM)	44
—	10
L-Serine (2 mM) + D-serine (20 mM)	100

[a] The reaction was performed in the presence of 100 mM sodium cacodylate (pH 7.0), 50 μM $MgCl_2$, 2 mM ATP, 0.1 mg of bovine serum albumin, 10 mM mercaptoethanol, 10 mM NaF, 2 mM ^{32}P-pyrophosphate (specific activity, 0.1 Ci/mol), and 95 μg of partially purified seryl-tRNA synthetase. The reactions were incubated at 37 C for 15 min.

Since D-serine did not appear to inhibit protein synthesis by interfering with the activation of L-serine, it was of interest to determine whether it had any direct effect on protein synthesis in an isolated system. In the following experiment, kindly performed by J. H. Schwartz, a cell-free system with phage f_2 as messenger was prepared (19). The effect of D-serine on this system as measured by incorporation of ^{14}C-lysine into trichloroacetic acid-precipitable material is shown in Table 6. In the first set of experiments, asparagine was the only amino acid besides ^{14}C-lysine added to the cell-free system. Samples were withdrawn from the reaction mixture as indicated to filter pads. The pads were immersed in 5% trichloroacetic acid, washed, and counted. At 0.1 M, D-serine inhibited ^{14}C-lysine incorporation, but it did not affect the system at 0.001 M and at 0.01 M.

In the next set of experiments, all of the amino acids except L-serine were added to the system. Samples were withdrawn as before. In this set, D-serine at low concentrations, 0.001 and 0.01 M, stimulated the incorporation of ^{14}C-lysine into trichloroacetic acid-precipitable material. Since the reaction mixture contained no L-serine, the stimulation was probably due to contaminating L-serine (4). At 0.1 M, the high-

TABLE 6. *Cell-free protein synthesis in the absence and presence of* D-*serine*[a]

Amino acids in system	D-Serine (M)	Counts/min in trichloroacetic acid-precipitable material		
		10 min	25 min	45 min
Asparagine only	—	2,263	3,004	3,891
	0.001	2,355	3,147	4,114
	0.01	2,177	3,016	4,131
	0.1	527	1,346	1,839
All except L-serine	—	4,188	7,930	8,510
	0.001	4,424	11,597	16,563
	0.01	3,747	9,545	11,331
	0.1	762	1,727	2,180

[a] The results are presented as counts per minute in cold trichloroacetic acid-precipitable material from samples taken at 10, 25, and 45 min of incubation. Protein synthesis was carried out under the following conditions: reaction mixture at 35 C containing 3 mM adenosine triphosphate, 0.2 mM guanosine triphosphate, 10 mM phosphoenolpyruvate, 30 μg of pyruvate kinase, 0.09 mM $MgCl_2$, 20 mM reduced glutathione, 30 mM KCl; 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.8), *E. coli* cell-free extract (N. Zinder strain 56) containing 3 mg total protein/ml, and 13 μM folinic acid. Phage f_2 RNA was added at about 0.8 mg/ml. Unlabeled amino acids, except asparagine and D-serine, were added at 100 μM. Asparagine was added at 20 μM and D-serine was added at 1, 10, and 100 mM. ^{14}C-lysine (specific activity, 144 Ci/mol) was added at 3 μM.

est concentration used, D-serine inhibited protein synthesis. This, however, is probably not significant physiologically.

L-Serine gives rise to glycine and one-carbon units. To determine whether this reaction is a target of D-serine, we examined the effect of compounds of one-carbon metabolism on D-serine inhibition. Table 7 presents results of two experiments. EM 1101 was streaked on minimal medium supplemented with sodium formate (500 μg/ml), glycine (50 μg/ml), and D-serine from 50 to 500 μg/ml. Glycine must be added to enable the cells to use sodium formate (E. Newman, personal communication). Glycine at a concentration of 50 μg/ml is not as effective as L-serine in overcoming the toxic effects of D-serine, but it does allow slow growth of EM 1301 on minimal agar plates containing 500 μg of D-serine/ml (McFall, unpublished data). Thus, although glycine is transported by the D-serine permease (3), the presence of D-serine does not exclude it from the cell. EM 1101 was also streaked on minimal medium containing glycine, methionine, thymine, guanine, and adenosine each at a concentration of 50 μg/ml, and various concentrations of D-serine. None of these conditions reversed the D-serine effect to any greater extent than addition of glycine alone.

D-Serine does not inhibit the formation of phosphatidylserine in the *E. coli* strains used in this study (Cronan, personal communication), and D-serine does not act as a substrate in the same reaction in *E. coli* B (10). Thus, D-serine toxicity is not due to the formation of faulty membranes, at least insofar as phosphatidylserine is concerned.

A major target of D-serine could be interference with the biosynthesis of L-serine, by faulty feedback inhibition of phosphoglyceric acid dehydrogenase, the first enzyme in the L-serine biosynthetic pathway (17). Using a cell-free system, Lewis Pizer has shown that D-serine does, in fact, exert severe inhibition on phosphoglyceric acid dehydrogenase in *E. coli* K-12 (personal communication). No such inhibition occurs in *E. coli* B, which is not sensitive to D-serine (McFall, unpublished data).

Pantothenate synthesis. We next attempted to determine what the second target of D-serine toxicity might be. Strains EM 1101 and EM 1301, the latter a double mutant that is resistant to L-serine (see below), were streaked on minimal agar plates supplemented with D-serine and various combinations of amino acids

TABLE 7. *Effect of components of "one" carbon metabolism on* D-*serine growth inhibition*

Medium[a]	D-Serine (μg/ml)	Growth[b]	
		24 h	48 h
Na formate + glycine	—	+4	+4
	50	—	SC
	100	—	SC
	250	—	SC
	500	—	SC
Gly, met, thy, gua, and ade	—	+4	+4
	50	—	±
	150	—	—
	500	—	+1

[a] Cells were streaked on minimal medium, either supplemented with 500 μg of Na formate/ml and 50 μg of glycine/ml plus various concentrations of D-serine, or with glycine, methionine, thymine, guanine, and adenosine, each at 50 μg/ml, plus various concentrations of D-serine.

[b] Growth was recorded after 24 and 48 h of incubation at 37 C. All growth was recorded on a relative basis, with +4 indicating the amount of growth found on minimal medium after 24 h of incubation. SC indicates single colonies, apparently revertants.

and vitamins. Table 8 summarizes a number of such experiments. A combination of L-serine and pantothenate or β-alanine overcame D-serine toxicity completely in strain EM 1301 but not in strain EM 1101. Other amino acids, such as threonine and leucine, were helpful in strain EM 1101 because they overcome inhibitory effects of L-serine (4).

Data on quantitative reversal of the D-serine inhibition by pantothenate and β-alanine in strain EM 1301 are presented in Table 9. L-Serine was added to all media as a nutritional supplement, and threonine was used, as above, to overcome any inhibition by L-serine. Pantothenate at a concentration of 100 ng/ml, but not at 30 ng/ml, completely overcame D-serine inhibition at all concentrations tested. The concentration of β-alanine required to reverse D-serine toxicity, however, increased with increasing D-serine. These results suggested that D-serine interferes with the conversion of β-alanine to pantothenate.

The effect of D-serine on pantothenate synthesis was studied directly by measuring the extent to which resting-cell suspensions of *E. coli* synthesize pantothenate in the absence and presence of D-serine. It had been previously shown that, in the presence of β-alanine, resting cells excrete pantothenate into the medium (13). Accordingly, resting-cell suspensions were incubated in buffer containing β-alanine and D-serine at different concentrations. The amount of pantothenate excreted into the medium was measured by bioassay (Table 10). D-Serine inhibited the excretion of pantothenate, but as the concentration of β-alanine increased the inhibitory effect of D-serine decreased. This experiment confirmed that D-serine is a competitive inhibitor of β-alanine in the synthesis of pantothenate.

TABLE 8. *Effect of various compounds on D-serine growth inhibition*[a]

Additions[b]	EM 1101	EM 1301
D-Ser, L-ser	—	+
D-Ser, L-ser, thr, leu, β-ala	+1	+4
D-Ser, L-ser, thr, leu, pan	+1	+4
D-Ser, leu, pan	—	±
D-Ser, leu, β-ala	—	±
D-Ser, L-ser, pan	—	+3

[a] Growth was recorded after 24 h of incubation at 37 C. All growth was recorded on a relative basis, with +4 indicating the amount of growth found on minimal medium after 24 h of incubation.

[b] The final concentration of D-serine was 250 μg/ml; all other additions were at 50 μg/ml. D-Ser, D-serine; L-ser, L-serine; thr, L-threonine; leu, L-leucine; β-ala, β-alanine; pan, pantothenate.

TABLE 9. *Inhibition of growth by D-serine and its reversal by Ca pantothenate and β-alanine*[a]

Supplement	D-Serine			
	None	50 μg/ml	166 μg/ml	500 μg/ml
None	+4	±	±	±
Ca pantothenate				
0.001 μg/ml	+4	±	±	±
0.003 μg/ml	+4	+3	+1	±
0.01 μg/ml	+4	+4	+4	+2
0.03 μg/ml	+4	+4	+4	+3
0.1 μg/ml	+4	+4	+4	+4
0.3 μg/ml	+4	+4	+4	+4
β-Alanine				
0.001 μg/ml	+4	±	±	±
0.003 μg/ml	+4	±	±	±
0.01 μg/ml	+4	+1	±	±
0.03 μg/ml	+4	+4	±	±
0.1 μg/ml	+4	+4	+3	+½
0.3 μg/ml	+4	+4	+4	+3

[a] EM 1301 was streaked on minimal agar plates containing the above supplements and also L-serine and L-threonine, each at 50 μg/ml. Growth was recorded after 24 h at 37 C on a relative basis, with +4 indicating the amount of growth found on minimal agar with no additions after 24 h at 37 C.

TABLE 10. *Inhibition by D-serine of pantothenate production in resting-cell suspensions*[a]

Supplement (μg/ml)		Pantothenate produced (μg/ml)
β-Alanine	D-Serine	
0.5	—	1.4
0.5	1,000	0.2
1.5	—	4.5
1.5	1,000	0.2
3	—	8.9
3	1,000	0.8
10	—	13.5
10	1,000	1.3
100	—	26.5
100	1,000	8.8

[a] All tubes contained 1.0 ml of a 15 times concentrated overnight culture of EM 1101 that had been grown in LB broth: 0.2 ml of 10% glucose, 0.2 ml of 1 M sodium phosphate buffer (pH 7.0), 0.3 ml of 0.1% pantoyl lactone. Total volume = 3.0 ml (adjusted with distilled water). Incubated for 4 h at 25 C.

Transient sensitivity to L-serine. Although L-serine and pantothenate reversed the toxic effect of D-serine in strain EM 1301, there was a transient residual toxicity at high (>150 μg/ml) D-serine concentrations in strains EM 1101 (Table 8), W 3828, and some other *dsdA* mu-

tants derived from strain W 3828. This residual toxicity was partially relieved by L-threonine, L-leucine, and L-isoleucine (data not shown). We previously described a class of mutants derived from strain W 3828 whose growth is permanently inhibited by low concentrations of L-serine. The L-serine inhibition is reversed by L-threonine, L-isoleucine, and L-leucine. These mutants appeared when mutagenized cultures were screened for variants unable to deaminate D-serine, because the D-serine used (Nutritional Biochemicals Corp.) contains 3 to 5% L-serine (4). The reversal by amino acids of the isoleucine-valine pathway suggested that the residual toxicity in strain EM 1101 might be due to an inherent transient sensitivity of W 3828 and its derivatives to L-serine, and that strain EM 1301 might, in fact, be a double mutant, both *dsdA* and L-serine-resistant. Accordingly, we measured the effect of 15 μg of L-serine/ml, or approximately the amount contained in 500 μg of D-serine/ml, on the growth of W 3828, EM 1101, and EM 1301 (Fig. 4). This amount of L-serine inhibited growth of all three strains almost immediately. EM 1301 began to recover quickly, after 30 min, but there was a much longer lag before the onset of recovery of W 3828 or EM 1101. Thus, EM 1301 is a double mutant, more resistant to L-serine than other W 3828 strains. If the EM 1101 culture was supplemented with L-threonine and L-isoleucine, however, the lag period was reduced to that seen with EM 1301. In further experiments with strain EM 1101, we found that as little as 4 μg of L-serine/ml caused a transient growth inhibition, but the duration of the growth lag decreased with the decreasing level of L-serine.

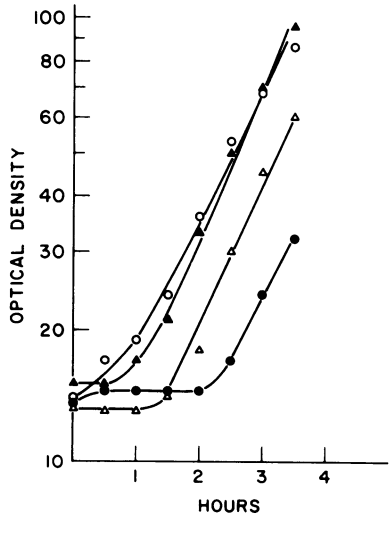

FIG. 4. *Effect of L-serine on the growth of various strains of E. coli K-12. L-Serine (15 μg/ml) was added to cultures at optical densities of about 14 Klett units, at which point growth ceased. (●) EM 1101; (▲) EM 1301; (△) W 3828; (○) EM 1101 in the presence of L-threonine and L-isoleucine, each at 50 μg/ml.*

It thus appears that the immediate cessation of growth observed in strains of *E. coli* K-12 that are exposed to high levels of D-serine (Fig. 1) is actually due to contaminating L-serine. The partial reversal by certain amino acids suggested an interference with protein synthesis. The effect of 150 μg of D-serine/ml (4.5 to 7.5 μg of L-serine/ml) on macromolecular synthesis in strain EM 1101 is consistent with this supposition (Fig. 5 and 6), resembling that of a severe shift down. RNA and protein synthesis are stopped immediately, with deoxyribonucleic

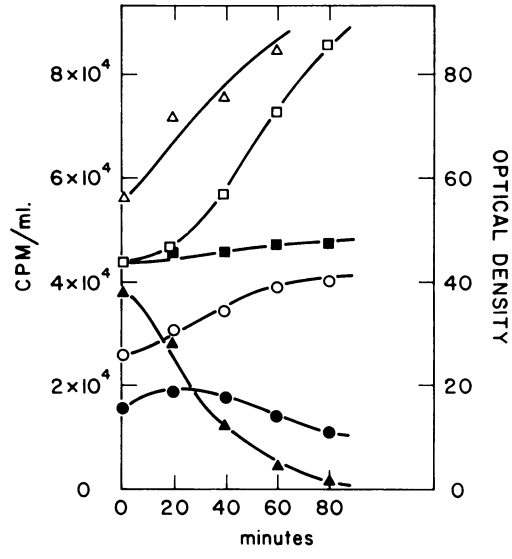

FIG. 5. *Effect of D-serine on synthesis of RNA and DNA. Strain EM 1101 was grown to a density of about 10^8/ml in minimal medium. The culture was divided into two parts, D-serine was added to one part, and the cells were reincubated. At 20-min intervals, 0.5-ml samples of cells growing either in the absence (open symbols) or presence (closed symbols) of 150 μg of D-serine/ml were withdrawn and added to tubes containing either ^{3}H-uridine at a final concentration of 0.04 μM (specific activity, 2 Ci/mol) plus 10 μM unlabeled thymidine or ^{3}H-thymidine at a final concentration of 0.2 μM (specific activity, 5 mCi/μmol). The tubes were incubated at 37 C for 5 min; such a pulse period was used to avoid complications due to the formation of the inducible thymidine phosphorylase (18). The results are presented as counts per minute of trichloroacetic acid-precipitable material per milliliter of sample for each pulse period. (△, ▲), ^{3}H-uridine; (○, ●), ^{3}H-thymidine; (□, ■), optical density of cultures measured in Klett units.*

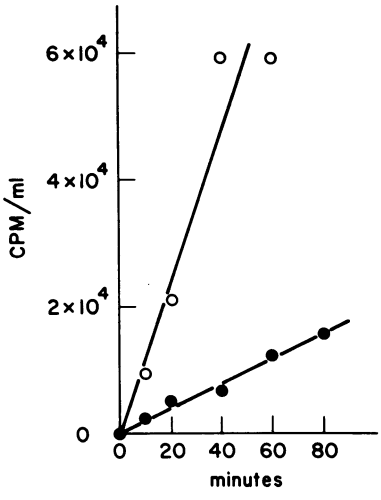

FIG. 6. *Effect of* D-*serine on protein synthesis. Strain EM 1101 was grown to a density of* 10^8*/ml in minimal medium. The culture was divided into two parts;* ^{14}*C-*L*-leucine (5 μM final concentration; specific activity, 10 Ci/mol) was added to both, and* D*-serine at 150 μg/ml final concentration was added to one part. The cultures were reincubated, and samples were withdrawn from the control* (○) *and the culture growing in the presence of* D*-serine* (●) *to tubes containing trichloroacetic acid at a final concentration of 10%. The tubes were heated for 15 min and then chilled. Results are presented as counts per minute of* ^{14}*C-leucine incorporated into trichloroacetic acid-precipitable material per milliliter of sample.*

acid (DNA) synthesis allowed to continue for a time, apparently to finish the round of replication.

To determine whether the inhibitory effect of L-serine on growth is an aberration, specific to strain W 3828 and its derivatives, or a general characteristic of *E. coli* K-12, we tested the growth response in minimal medium of several other *E. coli* K-12 strains presently in use in this laboratory: 1012B, K 140, Hfr 1, KL 16-99, and AB 444. L-Serine at a final concentration of 15 μg/ml immediately inhibited the growth of all of these strains in minimal medium. Growth resumed after periods of 2 to 5 h. Transient sensitivity to L-serine is thus a general property of *E. coli* K-12 strains.

DISCUSSION

D-Serine causes cessation of growth of *E. coli* K-12, which is completely reversed when D-serine is removed from the medium. Moreover, when the D-serine in the medium is depleted, the cells soon resume a nearly normal growth rate (Fig. 1). Thus, although D-serine is extremely toxic, it does no permanent damage and is a bacteriostatic agent.

The inhibitory effect of D-serine is reversed by L-serine to some extent, and it does not occur in rich medium. This suggested that D-serine may block a reaction critical to growth, which involves L-serine, and another reaction of unknown nature, whose function can be replaced by a rich medium.

As shown by Pizer, D-serine is an inhibitor of phosphoglyceric acid dehydrogenase. Thus, its major target in L-serine metabolism is probably a block in the biosynthesis of L-serine.

The second target of D-serine appears to be in the coupling of β-alanine to pantoic acid to form pantothenate. In the presence of L-serine, β-alanine, and pantothenate, reversal of the D-serine effect is similar to that found by Maas and Davis in *E. coli* W (13).

This is not quite the whole story. There is also a transient but severe inhibition of growth and protein synthesis at high levels of D-serine that is attributable to contaminating L-serine. Since this inhibition is largely reversed by amino acids of the isoleucine-valine pathway, it appears that L-serine interferes either with their synthesis or utilization in protein synthesis. It is ironic that the compound which is most important in overcoming the D-serine inhibition should itself be inhibitory.

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