The Mechanism of Ethionine Toxicity to *Escherichia coli*[1] (35042)

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Ethionine, the ethyl analog of methionine, is toxic to a broad spectrum of biological systems. Its over-all effects range from microbial growth inhibition (1) to the genesis of a variety of tissue aberrations in mammals (2). Since ethionine is similar to methionine both in structure and chemical properties, and known effects of the analog are relieved by methionine (1), it is clear that the toxicity of ethionine is in some way related to an inhibition of the metabolism and/or synthesis of this key amino acid.

Aside from its role as a constituent of protein, methionine serves as a methyl donor (3) and as a precursor for spermidine biosynthesis (4) through an active form, *S*-adenosylmethionine. It also apparently functions in the initiation of protein synthesis as *N*-formylmethionine (5). In *Escherichia coli*, methionine represses enzymes involved in its *de novo* biosynthesis, and exerts feedback inhibition on homoserine *O*-transsuccinylase the first enzyme specific to the pathway (6). Interference at any one or more of these metabolic sites would be deleterious to living systems, and could be expressed in a number of ways depending on the particular biological system studied.

Despite the considerable literature describing the biological activity of ethionine, the precise site or sites of ethionine action remain to be defined. Data are presented in this paper which suggest that ethionine blocks the synthesis of methionine in *E. coli* by inhibiting the first enzyme unique for its biosynthesis.

Materials and Methods. The organisms used in this investigation were *Escherichia coli* ATCC 9637 and a methionine-requiring mutant derived from strain ATCC 9637 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (7) followed by penicillin selection and replica plating. The latter strain lacks the ability to convert *O*-succinylhomoserine to cystathionine, and when grown on minimal medium with limiting concentrations of methionine or cystathionine, it excretes *O*-succinylhomoserine into the growth medium. Cultures were grown and maintained on Anderson's (8) glucose–salts medium (minimal medium) solidified with 2% agar. The same medium supplemented with methionine or cystathionine was used for the growth and maintenance of the mutant strain. Inocula were prepared from cells grown in minimal media at 37° with shaking. Optical densities were determined at 660 mμ (1-cm light path) in a Bausch and Lomb "Spectronic 20" spectrophotometer. Standard curves relating viable counts, dry weight, and protein to OD were used to standardize all inocula.

Cells for enzyme extractions were grown to the late log phase (10^9 cells/ml), rapidly chilled, harvested by centrifugation, and washed three times with a buffer appropriate for subsequent experimental use. If not used immediately, washed cell pellets were rapidly frozen in a Dry Ice–alcohol bath and stored at −20°. Cell extracts were prepared by sonic disruption of buffered cell suspensions with a MSE sonic osillator (Instrumentation Associates, New York) for 1 mim at 0°. Cystathionine synthetase activity was determined with extracts purified through the first ammo-

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nium sulfate precipitation step according to the procedure of Kaplan (9). Extracts for the assay of methionyl-tRNA synthetase were prepared according to the procedure described by Zubay (10). Protein was determined by the method of Lowry *et al.* (11). All operations, unless otherwise indicated, were carried out at 4°.

Radioactivity was determined with a Packard Tri-Carb scintillation counter. Scintillants used were: (a) PPO, 5 g; dimethyl POPOP, 0.3 g; toluene to 1 liter, and (b) Bray's solution (12) for aqueous systems. For whole-cell incorporation studies, a rapid filtration method was used. Samples of 0.1 ml were withdrawn from experimental flasks and added to equal volumes of cold 10% trichloroacetic acid (TCA). Samples were allowed to stand for 30 min with occasional shaking before filtration. Millipore filters of pore size 0.22 μ gave consistent results with both whole cells and hot and cold TCA-precipitable fractions. Dried filters or measured samples of soluble fractions were placed in glass counting vials with 5 ml of the appropriate scintillant and assayed for radioactivity. Corrections for background and quenching were made for all determinations.

β-Galactosidase was estimated in whole cells following the method described by Wallenfels (13). A unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 7 nmoles of *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in 15 min under the conditions employed. Assay of cystathionine synthetase was based on the method of Flavin (14). Partial purification of the enzyme was necessary to eliminate high endogenous NADH oxidase activity present in crude preparations. Homoserine-*O*-transsuccinylase activity was estimated by measuring the conversion of ^{14}C-succinyl-CoA to ^{14}C-*O*-succinylhomoserine. The reaction mixture consisted of 2 m*M* potassium phosphate buffer (pH 7.6) containing 1.0 m*M* dithiothreitol, 6 μmoles homoserine, 8 μmoles ^{14}C-succinyl-CoA, and 0.8 mg protein. The final volume was 2.7 ml. The reaction mixture was incubated for 30 min at 37° under helium, rapidly chilled, and the entire contents passed through a 1 × 10-cm Dowex-1-acetate column. Homoserine was eluted with 20 ml of water, and *O*-succinylhomoserine was eluted with 30 ml of 2 *M* acetic acid (15). The acid eluant was concentrated to 1 ml and analyzed chromatographically and for radioactivity. A unit of enzyme is defined as the amount of protein that forms 1.0 μmole of *O*-succinylhomoserine in 30 min under the conditions described above.

Methionyl-tRNA synthetase activity was estimated by measuring the appearance of ^{14}C-ethionine or ^{14}C-methionine in the hot TCA-soluble fraction. The assay system contained in 2.0 ml: 20 μmoles Tris buffer (pH 7.4), 30 μmoles $MgCl_2$, 20 μmoles ATP, 80 μmoles ammonium acetate, 3.5 mg prepared tRNA, 1 mg protein, and amino acids to concentrations indicated in text. The mixture was incubated for 10 min at 37°, and the reaction terminated by the addition of an equal volume of cold 10% TCA. After 30 min, the precipitate was collected, washed, and extracted with hot TCA. Hot soluble fractions were assayed for radioactivity after filtration.

Ethyl-l-^{14}C-ethionine, methyl-l-^{14}C-methionine, ^{14}C-L-valine (UL), and ^{14}C-succinic acid (UL) were purchased from Tracerlab. Homoserine, cystathionine, and methyl-β-D thiogalactopyranoside (TMG) were obtained from Mann Chemical Company. Homocysteine was formed just prior to use by treating its thiolactone with 2 equiv of NaOH at 45° for 10 min under helium. The solution was then neutralized with 1 equiv of HCl. ^{14}C-succinic anhydride was prepared by refluxing ^{14}C-succinic acid with unlabeled carrier in acetylchloride according to the method described by Shemin (16). Succinyl-CoA was prepared just before use by mixing 6 mg of succinic anhydride with 5 mg CoA, 0.5 ml water, and 0.1 ml of *M* $NaHCO_3$ under helium. Transfer RNA was isolated from the test organism following the method described by Zubay (10) with the modification of Sarin (17) to remove terminal amino acids. The concentration of tRNA was estimated by its extinction coefficient at 258 mμ (10).

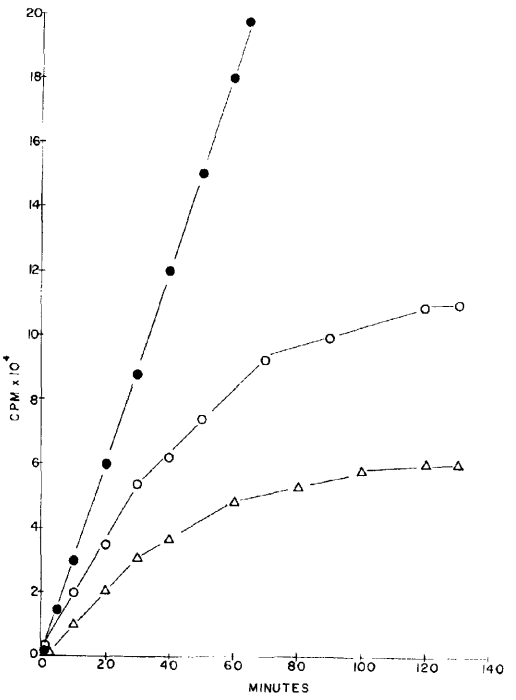

FIG. 1. Effect of ethionine on incorporation of ^{14}C-valine (0.3 mCi/mmole) into the hot TCA-insoluble fraction. ●—●, control-no addition; ○—○, plus L-ethionine ($3 \times 10^{-3} M$); △—△, plus L-ethionine ($6 \times 10^{-3} M$). Ethionine and/or ^{14}C-valine ($3 \times 10^{-4} M$) were added at 0 min.

Results. Effect of ethionine on growth and viability. The addition of ethionine to *E. coli* growing in minimal media resulted in growth inhibition and loss of viability. The effective inhibitory concentration of ethionine ranged from 1×10^{-4} *M*, the lowest concentration at which a significant change in growth rate occurred, to 6×10^{-3} *M*, a concentration which completely inhibited growth after addition.

Ethionine inhibition of protein synthesis. The effect of ethionine on protein synthesis in the test organism was studied by measuring ^{14}C-valine incorporation into protein, and β-galactosidase synthesis in the presence of the inhibitor. For ^{14}C-valine incorporation studies, the labeled amino acid was added to growing cultures in the early log phase. At the same time inhibitory levels of ethionine was added. Growth was followed turbidimetrically, samples withdrawn with time, and the amount of label in the hot TCA-insoluble cell fractions determined. The results show that valine incorporation was reduced within 5–10 min after addition of the analog and all valine incorporation ceased 2 hr after drug addition. At higher ethionine concentrations a more pronounced effect was observed (Fig. 1).

Comparable results were observed when β-galactosidase was used as an index of protein synthesis. Glycerol (0.2%) was used as a carbon source for these studies and TMG was employed as the inducer. When TMG and ethionine were added simultaneously to growing cultures enzyme synthesis was initiated within 2 or 3 min, but ceased abruptly some 30 min later. If the analog was added 15 min after the inducer, enzyme synthesis continued for the same time period and then stopped. No detectable enzyme was formed when TMG was added 15 min after ethionine (Fig. 2).

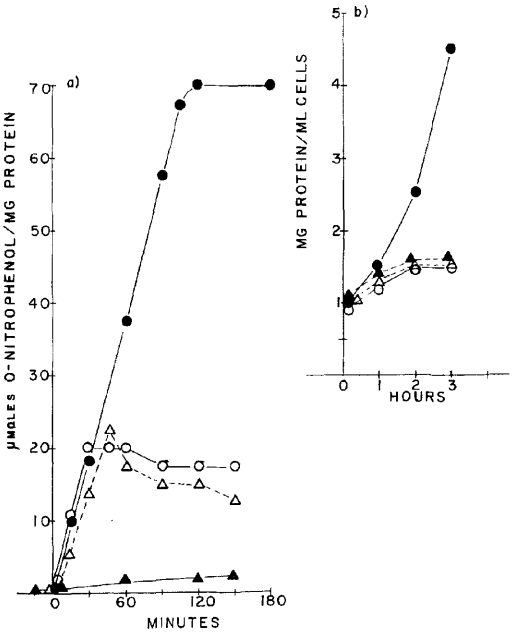

FIG. 2. Effect of ethionine on synthesis of β-galactosidase. Cells were grown in glycerol–salts medium until early log phase (a). At this time TMG ($5 \times 10^{-4} M$) and/or L-ethionine ($3 \times 10^{-3} M$) were added. ●—●, control; ○—○, TMG and ethionine simultaneously; △—△, Ethionine added 15 min after TMG; ▲—▲, ethionine added 15 min before TMG. (b) Insert at upper right indicates growth of the same culture measured in terms of cell protein.

Effect of ethionine or methionine–tRNA synthesis. A series of preliminary experiments conducted during this investigation indicated that the observed inhibition of growth and protein synthesis was not due to an earlier effect on RNA or DNA synthesis. Since all known effects of ethionine are reversed by methionine, two possible mechanisms of ethionine toxicity were suggested: inhibition of methionine–tRNA synthetase or interference with methionine synthesis *de novo*. The data showed, under the experimental conditions used, that ethionine did not inhibit the transfer of methionine to RNA. When ethionine alone was used as a substrate significantly lower amounts of the analog were found in the hot TCA-soluble fraction. At higher ethionine concentrations the amount of ethionine transferred to RNA was not significantly increased.

Reversal of ethionine effects by methionine precursors. If ethionine inhibits an enzyme(s) critical to methionine synthesis, a supply of the intermediate beyond the point affected would relieve the effects of the analog. By the same reasoning, intermediates occurring prior to the point of ethionine inhibition would not be expected to reverse the inhibition. For these studies, both the reversal of ethionine inhibition of β-galactosidase synthesis in whole cells and growth inhibition were used as an index of the efficacy of methionine precursors to serve as reversing compounds.

Cells were cultured in a glycerol–salts medium and allowed to grow until the early log phase at which time TMG was added. Ethionine was added 15 min later, and the cells were incubated an additional hour to ensure complete inhibition of protein synthesis. Intermediates were added to a final concentration of 6.7×10^{-3} *M* and incubation continued for 4 hr. Samples were withdrawn periodically during the experimental period for the estimation of β-galactosidase activity. Detectable enzyme synthesis stopped approximately 30 min after drug addition, and resumed within 2 hr after addition of methionine, homocysteine, or cystathionine; no reversal of drug inhibition was observed with homoserine or *O*-succinylhomoserine (Fig. 3). The same reversal pattern was obtained when growth was used as an index of reversing activity. However, in the latter instance reversing effects occurred 45–60 min after addition of the intermediates.

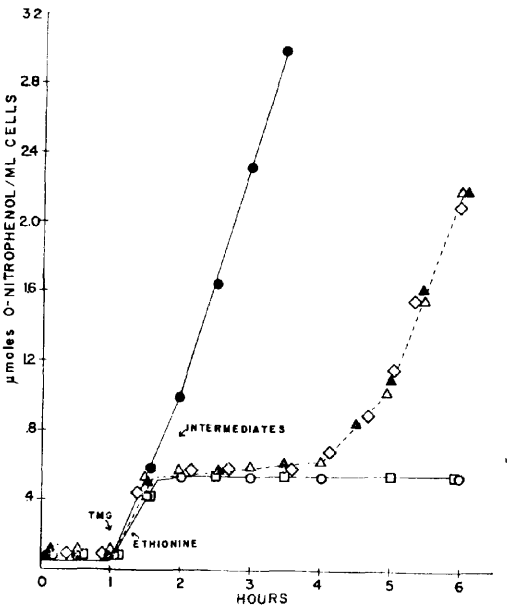

FIG. 3. Reversal of the effects of ethionine on β-galactosidase synthesis by methionine and intermediates of the methionine biosynthetic pathway. TMG (5×10^{-4} *M*) was added to all cultures at 1 hr. Ethionine was added to all cultures, except control at 1¼ hr; intermediates were added at 2 hr as follows: ●—● no additions; ○—○ homoserine; □—□, *O*-succinylhomoserine; △—△, homocysteine; ▲—▲, methionine; open diamond—open diamond, cystathionine.

Effect of methionine and ethionine on cystathionine synthetase and on homoserine O-transsuccinylase. The failure of *O*-succinylhomoserine and homoserine to reverse ethionine inhibition of β-galactosidase synthesis and growth, while other methionine precursors relieved the ethionine effect, suggested that ethionine inhibits methionine at a po'nt prior to cystathionine formation. The effect of ethionine on cystathionine synthetase and on homoserine *O*-transsuccinylase was further investigated.

Cystathionine synthetase catalyzes the conversion of *O*-succinylhomoserine to cystathionine in the presence of cysteine; in the

absence of cysteine, the enzyme converts its substrate to succinate and α-ketobutyrate (18). By coupling the reaction with lactic dehydrogenase and NADH, cystathionine synthetase may be conveniently assayed by following the oxidation of NADH at 340 mμ. Both ethionine and methionine were investigated as possible inhibitors of this enzyme. Neither compound inhibited the enzyme, even at concentrations 80-fold greater than that of the substrate *O*-succinylhomoserine.

Since these results clearly indicated that ethionine did not inhibit cystathionine synthetase *in vitro*, the effect of ethionine on homoserine *O*-transsuccinylase, the first enzyme unique for *de novo* methionine synthesis in *E. coli*, was investigated. A methionine-requiring mutant derived from *E. coli* ATCC 9637 was used as a source of the enzyme. When these cells are grown in a medium containing 3.5×10^{-5} *M* cystathionine, *O*-succinylhomoserine transsuccinylase is derepressed and *O*-succinylhomoserine is secreted into the growth medium. The cells were accordingly grown in a minimal medium supplemented with 3.5×10^{-5} *M* cystathionine until the late log phase (10^9 cells/ml), harvested by centrifugation, suspended in 0.5 *M* potassium phosphate buffer (pH 7.0) containing 1.0 m*M* dithiothreitol, disrupted sonically, and centrifuged to remove cell debris.

Homoserine *O*-transsuccinylase activity was assayed by measuring the conversion of ^{14}C-succinyl-CoA to ^{14}C-O-succinylhomoserine. The results, (Table I), show that methionine inhibits *O*-succinylhomoserine at all concentrations tested. Ethionine also inhibited the enzyme, although the concentration of the analog required to produce the same inhibition as methionine was approximately 5–10 times that of the natural amino acid.

Discussion. The data presented in this report suggest that ethionine is toxic to *E. coli* ATCC 9637 primarily by inhibiting homoserine *O*-transsuccinylase. Since this is the first enzyme in *E. coli* unique for *de novo* methionine biosynthesis, an inhibition at this point could create a state of intracellular methionine starvation and result in growth stasis. This interpretation is further supported by the complete reversal of ethionine inhibition by cystathionine, homocysteine, and methionine, and the lack of ethionine inhibition of methionyl-RNA synthetase. The failure of *O*-succinylhomoserine to reverse ethionine effects cannot be explained by the available data. Since cystathionine synthetase was not affected *in vitro* by high concentrations of ethionine, it may be that the cells were either unable to take up exogenous *O*-succinylhomoserine, or if they did, the compound was de-esterified prior to or during cellular incorporation.

The inhibition of methionine synthesis by ethionine and the failure of the analog to

TABLE I. Effect of L-Ethionine and Methionine on the Activity of Homoserine *O*-Transsuccinylase in Crude Extracts.[a]

Addition	Conen (m*M*)	Enzyme activity (units/mg)	Inhibition of *O*-succinylhomoserine formation (% control)
None (boiled enzyme)	—	0.00	—
None	—	2.00	—
Methionine	10.0	0.12	94
Methionine	2.0	0.40	80
Methionine	1.0	0.62	69
Methionine	0.4	0.93	53
Methionine	0.2	1.38	31
Ethionine	10.0	0.46	77
Ethionine	2.0	1.38	31
Ethionine	1.0	1.50	25
Ethionine	0.4	1.76	12
Ethionine	0.2	1.95	2.5

[a] The homoserine concentration was 2 m*M*. The amount of *O*-succinylhomoserine formed in each reaction flask was calculated from the radioactivity of the 0.2 *M* acetic acid fraction eluted from a Dowex 1-acetate column. Thin-layer chromatography (silica gel) of a sample of this fraction in *n*-butanol–propionic acid–water (74:22:31) revealed one ninhydrin-positive spot which contained all the radioactivity. The R_F value (0.25) matched that of an authentic sample of *O*-succinylhomoserine generously supplied by Dr. Martin Flavin of NIH.

substitute for methionine would not only result in an inhibition of protein synthesis but also affect other reactions in which this multifunctional amino acid is involved. Reports exist which indicate that ethionine-grown *E. coli* contains submethylated ribosomes (19), and submethylated DNA (20). The submethylated ribosomes differed physically from normal ribosomes. It was postulated that submethylated DNA resulted in impaired replication. The observed viability loss in the presence of inhibitory ethionine concentrations may be due to submethylated RNA and DNA or to the inhibition of other vital reactions requiring methionine.

The ability of an amino acid analog to mimic the naturally occurring amino acid as a feedback inhibitor has been reported for several other amino acids (21, 22). Schlesinger (23) demonstrated inhibition of homoserine *O*-transsuccinylase with α-methylmethionine, another methionine analog. Since methionine is involved in several areas of cellular metabolism, ethionine may prove to be a useful tool for further studies on the role methionine plays as a metabolite in cellular synthesis as well as its role in the regulation of macromolecular synthesis.

Summary. Toxic effects of ethionine on *Escherichia coli* in a glucose salts medium are manifested by growth inhibition and loss of viability. ^{14}C-valine incorporation studies and β-galactosidase studies indicate that ethionine rapidly inhibits protein synthesis. The methionine precursors, homocysteine and cystathionine, as well as methionine itself, reverse all ethionine effects while *O*-succinylhomoserine and homoserine do not. Homoserine *O*-transsuccinylase, the first enzyme unique for methionine biosynthesis, is inhibited by ethionine while the analog does not inhibit cystathionine synthetase or methionine tRNA synthetase. The available data indicate that the initial effect of ethionine on *E. coli* is to act as an inhibitor of homoserine *O*-transuccinylase resulting in a severe depletion of methionine available for protein and other cellular synthesis.

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