Mechanism of the Growth Inhibitory Effect of Cysteine on *Escherichia coli*

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SUMMARY

Cysteine appeared to have two classes of growth inhibitory effect on *Escherichia coli*: (1) above 0.2 mM it inhibited growth on minimal medium by a mechanism which may involve interference with leucine, isoleucine, threonine and valine biosynthesis; (2) above 2 mM, in media with these amino acids, it had an effect which may involve interaction with membrane bound respiratory enzymes.

Cysteamine showed only effect (2).

INTRODUCTION

Roberts, Abelson, Cowie, Bolton & Britten (1957) reported that exogenous cysteine inhibits the growth of *Escherichia coli*, but the mechanism of this action is still unclarified. Nagy, Hernádi, Kovács, Vályi-Nagy (1968*a*) and Nagy, Kovács, Kari & Hernádi (1970) found that, in *E. coli* 15T⁻, the net synthesis of RNA and protein, but not that of DNA, was quickly inhibited by cysteine. An antagonism could be detected between cysteine and leucine (leu), isoleucine (ileu), threonine (threo) and valine (val) and it was suggested that the biosynthesis of these amino acids (AAs) is inhibited by cysteine (Kovács, Kari, Nagy & Hernádi, 1968). However, not all the effects of cysteine could be explained in this way (Nagy, Kari & Hernádi, 1969).

Since cysteine is an extremely reactive compound it is not surprising that it affects several enzymic reactions *in vitro* (Komlós & Erdős, 1959; Leavitt & Umbarger, 1961; Datta, 1967; Alföldi, Raskó & Kerekes, 1968) and a number of metabolic functions in *Escherichia coli*, as well as in other micro-organisms (Roberts *et al.* 1957; Pasternak, 1962; Bhuvaneshvaran & Sreenivasan, 1964; Bernheim, 1966; Koningsberger, 1967; Nagy, Hernádi *et al.* 1968*a*, 1968*b*, 1969, 1970; Nagy, Kovács, Balázs & Hernádi, 1968; Kari, Nagy & Hernádi, 1971). Each of these effects may be responsible for its cytotoxic action, but it is difficult to demonstrate which of these many reactions is involved in growth inhibition. Enzyme experiments *in vitro* and the various metabolic actions of cysteine need to be assessed in the light of detailed growth studies.

METHODS

Bacterial strains. *Escherichia coli* strain B (prototroph, from T. Alper of Hammersmith Hospital, London); Hfr CAVALLI *met⁻rel⁻* (a relaxed strain, auxotrophic for methionine); CP99 *his⁻arg⁻ser⁻B₁⁻rel⁺* (a stringent strain, auxotrophic for histidine, arginine, serine and B₁); and CP100 *his⁻arg⁻ser⁻B₁⁻rel⁻* (a relaxed mutant of CP99). The latter three strains were obtained from L. Alföldi (Medical University of Szeged, Hungary).

Growth. Organisms were cultivated on mineral salts medium C (Roberts *et al.* 1957)

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supplemented with 0.5 % (w/v) glucose; when required, amino acids (AAs) were added to 50 µg each/ml. and vitamin B₁ (as thiamine HCl) to 5 µg/ml. Overnight cultures were diluted 30-fold in prewarmed fresh medium and incubated aerobically at 37° in a water-bath shaker. The extinction (*E*) of cultures was measured with a Spectromom 360 spectrophotometer at 550 nm. using 0.5 cm. cuvettes. Exponentially growing cultures were used in all experiments. A formula for the calculation of growth inhibition was described earlier (Nagy *et al.* 1968*b*).

Incorporation of labelled compounds

[¹⁴C]-uracil. For the measurement of net RNA synthesis, cultures were exposed to labelled uracil (0.1 µCi/30 µg./ml.) and grown as described above. At intervals, duplicate 1 ml. samples were taken and added to 4 ml. ice-cold trichloroacetic acid (TCA, final concentration 5 %). After 60 min. the precipitates were collected on membrane filters (Sartorius MF 14), washed three times with 10 ml. cold 5 % TCA, glued to aluminium planchettes and counted in a windowless gas-flow counter (Gamma, Budapest).

[³⁵S]-sulphate. Bacteria were grown in mineral salts-glucose medium containing 0.2 µCi ³⁵S as 26.7 µg. [³⁵S]-sulphate per ml. The radiosulphate incorporation into the cold TCA-insoluble fraction was determined as described above.

Chemicals. Cysteamine-HCl (Fluka), [¹⁴C]2-uracil and [³⁵S]Na₂SO₄ (specific activity 25.35 mCi/mmol and 26 mCi/mmol respectively, NAEC Institute of Isotopes, Budapest) were used. All other chemicals used were reagent grade as obtained from Reanal (Budapest). Cysteamine (MEA) and cysteine (L-cysteine-HCl. 1 H₂O) were sterilized by filtration. Only the freshly prepared solutions of these compounds were used.

Stability of cysteine. Under our experimental conditions (liquid culture medium, aeration by shaking, pH 6.5 to 7.0) cysteine decomposes. Nagy *et al.* (1969) established that the rate of decomposition of cysteine was independent of initial cysteine concentration between 0.2 and 40 mM and that about 20 % of the cysteine decomposed during 60 min. Our growth tests reported here were made during this time. The compounds added did not affect the stability of cysteine or MEA; the stability of the latter compound was measured by the Ellman's method (Ellman, 1959).

RESULTS

Growth studies. Growth of *Escherichia coli* is inhibited by cysteine (Roberts *et al.* 1957; Nagy, Hernádi *et al.* 1968*b*), but resumes at the normal exponential rate after removal of the cysteine from the medium (Nagy *et al.* 1970). Fig. 1 shows the progressive inhibition of growth of *E. coli* B with increasing cysteine concentration. The first significant growth inhibition was observed with 0.2 mM-cysteine and almost complete inhibition was obtained with 10 mM-cysteine (Fig. 1, curve A). This growth inhibitory effect of cysteine was partially relieved by simultaneous addition of leu, ileu, threo or val (Kovács *et al.* 1968), leu and ileu being more effective than threo and val. The most powerful anti-cysteine action was obtained with a mixture of all four (Table 1). Only the L-forms of these AAs were effective, the D-isomers of the other three AAs behaving as the ileu shown in Table 1. The growth inhibitory effect of cysteine was not overcome by other AAs, *nor*-leu, *nor*-val, *N*-acetyl-val, benzoyl-val, or by uracil.

Growth of *Escherichia coli* B was inhibited by cysteine at concentrations higher than 2 mM, even in the presence of a mixture of leucine, isoleucine, threonine and valine (leu-ileu-threo-val), but to a considerably lesser extent than in their absence (Fig. 1, curve B).

The growth inhibitory effect of MEA was also analysed. Fig. 1 shows that MEA was about as effective in inhibiting growth as was cysteine in the presence of leu-ileu-threo-val.

Fig. 1. Progressive inhibition of growth of *Escherichia coli* B with increasing concentrations of cysteine and MEA. *Escherichia coli* B was grown aerobically on Roberts C mineral salts-glucose medium and the cultures in mid-log phase were treated as follows: ■, cysteine; ●, cysteine+ leu-ileu-threo-val (50 µg./ml. of each); △, MEA. The degree of growth inhibition was calculated as described by Nagy *et al.* (1968*b*).

Table 1. Effect of leu, ileu, threo and val on inhibition of growth of *Escherichia coli* B by 2 mM cysteine

Escherichia coli B was grown aerobically on Roberts C mineral salts-glucose medium at 37°. Logarithmically growing cultures were treated with the compounds indicated. Leu, ileu, threo and val were each added to 50 µg./ml. The degree of growth inhibition was calculated as described by Nagy, Hernádi *et al.* (1968*b*).

Treatments	Growth inhibition (%)
Cysteine	76
Cysteine + L-leu	40
Cysteine + L-ileu	42
Cysteine + L-threo	52
Cysteine + L-val	54
Cysteine + L-leu-ileu-threo-val	20
Cysteine + L-ileu	42
Cysteine + D-ileu	71
Cysteine + DL-ileu	57

No antagonism was detected between MEA and the above-mentioned compounds, including leu-ileu-threo-val.

Effect of cysteine on [¹⁴C]-uracil incorporation into rel⁻ and rel⁺ strains. We presented evidence that cysteine does not directly inhibit RNA synthesis in *Escherichia coli* B (Kari *et al.* 1971) and suggested that cessation of RNA synthesis in the presence of cysteine might be the consequence of inhibition of biosynthesis of leu, ileu, threo and val. This implies that cysteine may inhibit net synthesis of RNA via the action of *rel* gene. If this were true, one would expect to find that cysteine treatment induces a similar pattern of incorporation of labelled uracil as does AA deprivation in a *rel*⁻ mutant of *E. coli*. In this section we describe experiments to test this prediction.

Escherichia coli Hfr CAVALLI *met*⁻ *rel*⁻ was grown to mid-log phase in glucose-mineral salts medium containing [¹⁴C]-uracil. The culture was then treated with cysteine (2 mM) and the methionine was simultaneously withdrawn. Unexpectedly, net RNA synthesis was inhibited by cysteine in this relaxed strain (Fig. 2). Leu-ileu-threo-val had no affect on this action of cysteine. Similar results were obtained when 20 mM MEA was used in place of cysteine. On the other hand, if methionine was also present at the time of cysteine treatment leu-ileu-threo-val completely abolished the inhibitory effect of cysteine (Fig. 3).

These experiments were repeated with another three strains with similar results. Thus, the inhibitory effect of cysteine on net RNA biosynthesis could not be attributed to the action of *rel* gene.

Fig. 2. Effect of cysteine on [¹⁴C]-uracil incorporation into *Escherichia coli* Hfr CAVALLI *met*⁻*rel*⁻ during methionine starvation. Bacteria were grown on Roberts C-glucose-methionine (50 µg./ml.) medium supplemented with [¹⁴C]-uracil (0.1 µCi/30 µg./ml.). Organisms in the mid-log phase of growth were centrifuged, washed three times and resuspended in the same, warmed, but methionine-free medium. The culture was divided into 3 portions, treated and incubated further at 37°. Treatments (at 0 min.): ○, control; ●, 2 mM-cysteine; △, 2 mM-cysteine + 50 µg./ml. of each of leu-ileu-threo-val. The incorporation of labelled uracil was measured as described in Methods.

Fig. 3. Effect of cysteine on [¹⁴C]-uracil incorporation into *Escherichia coli* Hfr CAVALLI *met*⁻*rel*⁻. Bacteria were grown as described in the legend to Fig. 2 and in the mid-log phase of growth were treated at the indicated time as follows: ○, ●, control; □, ■, 2 mM cysteine; ▽, ▼, 2 mM cysteine + 50 µg./ml. of each of leu-ileu-threo-val. The extinction (●, ■, ▼) and [¹⁴C]-uracil incorporation (○, □, ▽) were measured as described in Methods.

Breakdown of preformed 'relaxed' RNA during cysteine treatment. Dalgarno & Gros (1968) reported that net synthesis of 'relaxed' RNA ceases after incubating *Escherichia coli* EA₂ (*met*⁻*rel*⁻) in the absence methionine for 150 min.; subsequent incubation at 37° without an energy source, or with 2,4-dinitrophenol, induces a rapid breakdown of labelled 'relaxed' RNA. This was confirmed by glucose omission (Fig. 4). A similar breakdown was observed with glucose and 2 mM-cysteine. When glucose was omitted at the time of cysteine addition, no additional breakdown was obtained (not shown in Figure), indicating a common mechanism for these two treatments. This effect of cysteine was not influenced by leu-ileu-threo-val. Similar results were obtained when MEA (20 mM) was used in place of cysteine.

This suggests that the 'MEA-like' effect of cysteine induces an energy depletion of bacteria.

Fig. 4. Breakdown of 'relaxed' RNA during cysteine treatment in *Escherichia coli* Hfr CAVALLI *met*⁻*rel*⁻ cells. Bacteria were grown to mid-log phase on Roberts C-glucose-methionine (50 µg./ml.) medium. The organisms were washed free of methionine (see the legend to Fig. 2), resuspended in Roberts C-glucose-[¹⁴C]-uracil (0.2 µCi/30 µg./ml.) medium and incubated at 37°. After 150 min. the bacteria were treated as described below and reincubated at 37° for a further 120 min. Treatments: ○, control; ●, glucose omission (centrifugation, washing three times and resuspension in Roberts C-[¹⁴C]-uracil medium); △, 2 mM-cysteine. Acid-precipitable radioactivity was determined as described in Methods.

Effect of cysteine on [³⁵S]-sulphate incorporation. Incorporation of radiosulphate into various micro-organisms is prevented by cysteine (Roberts *et al.* 1957; Pasternak, 1962). In the case of *Escherichia coli*, however, cystine was used instead of cysteine, since the former compound fails to inhibit the growth of *E. coli*. Nevertheless, it was suggested that cysteine is the actual repressor of sulphate activating enzymes in *E. coli* (Wheldrake, 1967; Jones-Mortimer, 1968).

The effect of cysteine on [³⁵S]-sulphate incorporation could be measured without growth inhibition when the bacteria were cultured in glucose-mineral salts medium supplemented with leu-ileu-threo-val. Fig. 5 shows that the incorporation of radiosulphate ceased immediately after the addition of cysteine, but that growth continued at the same rate (cysteine was used at a concentration of 0.82 mM, the sulphur content of which equals that of the medium). When MEA was added to the culture at the above concentration no change was detected in the incorporation of [³⁵S]-sulphate.

These results agree with those cited above, as well as with Ginsberg's (1966) and with our previous data (Kovács *et al.* 1968; Nagy *et al.* 1969; Nagy & Quintiliani, 1970).

DISCUSSION

We have clearly distinguished between two kinds of growth inhibitory effect of cysteine on *Escherichia coli*. These will be called primary and secondary effects of cysteine.

When the organisms are cultured on glucose-mineral salts medium, growth is inhibited by the primary effect. The mechanism of this effect can be deduced from the following data: (i) the most powerful antagonism of cysteine was obtained using very low (50 $\mu\text{g./ml.}$) concentration of leu-ileu-threo-val (Fig. 1); (ii) cysteine inhibited the inducible synthesis of

Fig. 5. Effect of cysteine and MEA on [^{35}S]-sulphate utilization of *Escherichia coli* B. Bacteria were grown on Roberts C-glucose-leu-ileu-threo-val medium (50 $\mu\text{g./ml.}$ of each), containing [^{35}S]Na $_2$ SO $_4$ (0.2 $\mu\text{Ci/26.7 } \mu\text{g./ml.}$). At the indicated time the organisms were treated as follows: $\circ$, $\bullet$, control; $\square$, $\blacksquare$, 0.84 mM-cysteine; $\triangle$, $\blacktriangle$, 0.84 mM-MEA. The extinction ($\bullet$, $\blacksquare$, $\blacktriangle$) and radio-sulphate incorporation ($\circ$, $\square$, $\triangle$) were measured as described in Methods.

β -galactosidase in a manner reminiscent of the way in which synthesis of this enzyme is prevented by withdrawal of a required AA from an auxotrophic mutant. This action was also overcome by addition of leu-ileu-threo-val (Kari *et al.* 1971); (iii) there are two enzymes, concerned in the biosynthesis of these four AAs in *E. coli*, which are inhibited *in vitro* by cysteine: homoserine dehydrogenase (HSDH, Datta, 1967, 1969) and acetohydroxy acid synthetase (AHAS, Leavitt & Umbarger, 1961). A good quantitative correlation exists between the degree of growth inhibitory and enzyme inhibitory effects of cysteine (Fig. 1, curve A; Fig. 1 of Datta, 1967; Table 4 of Leavitt & Umbarger, 1961, respectively). If the activities of HSDH and AHAS are simultaneously inhibited by cysteine the bacteria will be starved for threo, val, ileu and leu (Umbarger & Davis, 1962). Thus, one may explain in this way the necessity to add all four AAs simultaneously for optimal anti-cysteine action (Table 1).

When the primary action of cysteine is overcome by leu-ileu-threo-val, growth of *Escheri-*

chia coli is inhibited by the secondary effect of cysteine, which requires higher concentrations of this compound (Fig. 1, curve B). Though the nature of this secondary effect is less clear than that of the primary one, we can deduce a possible mechanism from the following data: (i) the effect of cysteine was analogous to that produced by omission of glucose (Fig. 4) and by 2,4-dinitrophenol treatment (Dalgarno & Gros, 1968). This is evidence that cysteine, as its secondary effect, causes energy depletion; (ii) at 10 mM, cysteine inhibits the oxygen uptake of yeasts (Bhuvaneshvaran & Sreenivasan, 1964); (iii) cysteine inhibits the activity of cytochrome oxidase (Komlós & Erdős, 1959); (iv) though the secondary effect of cysteine is also produced by MEA (Fig. 1, 4), there is a great deal of evidence which suggest that MEA is not absorbed by the bacteria (this problem is discussed in detail by Ginsberg, 1966). Our results are also compatible with this view (Fig. 5 and Nagy *et al.* 1969). It is therefore reasonable to think that the sites of the secondary action of cysteine are at the cell surface. This is in good agreement with the findings cited in (iii), since cytochromes are known to be attached to the cell membrane (Reynolds, 1968).

In conclusion, we suggest that cysteine as its secondary action may inhibit the activities of membrane bound respiratory enzymes and via this action causes an energy depletion of the organisms. The inhibition of net synthesis of RNA in *rel*⁻ mutants and the breakdown of 'relaxed' RNA are the consequences of this effect.

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