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Short Communications

The Role of H_2O_2 in the Reversible Inhibition of RNA Synthesis by Thiols in *E. coli*

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Cysteine has been shown by Roberts *et al.*[1] to inhibit the growth of *E. coli*. Vergroesen *et al.*[2] reported that thiols with $pK_a < 10$ were toxic at concentrations between 0.1 and 2.0 mM in human kidney tissue culture. Nagy *et al.*[3,4] demonstrated that cysteine had an inhibitory action on RNA and protein synthesis in *E. coli*. According to Kari *et al.*[5] 0.4 mM cysteine inhibits inducible synthesis of β-galactosidase, and at high concentrations cysteine as well as cysteamine give rise to energy depletion and inhibition of RNA synthesis by inhibiting membrane bound respiratory enzymes.

In the present communication we report some experiments carried out in order to clarify the mechanism of the RNA synthesis inhibiting action of thiols in cells of *E. coli*. Thiols (*e.g.* mercaptoethanol) are known not to affect RNA-polymerase *in vitro*. Therefore the inhibition *in vivo* appears to be caused by some reaction products of thiols. Possibly, cytotoxicity of thiols is due to the same products.

Materials and methods. Chemicals. (5-^{3}H)Uracil was purchased from Radiochemical Centre, Amersham, inorganic chemicals for analytical purpose were obtained from Merck, Darmstadt, and all other chemicals and biochemicals from Sigma Chemical Co.

Medium I. Phosphate buffer, pH 6.8, 60 mM; $MgSO_4$, 0.8 mM; 19 amino acids, 0.1 mM of each (except cysteine); cysteine, 0.05 mM; thymine, 0.015 mM; uracil, 0.02 mM; glycerol, 22.0 mM.

Medium II. Phosphate buffer, pH 6.8, 75 mM; $MgSO_4$, 1.0 mM; 19 amino acids, 0.125 mM of each, (except cysteine); thymine 0.01 mM; glycerol, 5 mM; (5-^{3}H)uracil, 2.5 μCi per ml, 500 mC per mol.

Washing medium. Phosphate buffer, pH 6.8, 60 mM; NaCl, 120 mM; $MgSO_4$, 0.5 mM.

RNA-SDS solution. 50 mg yeast RNA (Sigma, grade VI) and 5 g SDS dissolved in 100 ml distilled water and, to eliminate RNase contamination, incubated with 0.6 ml diethyl pyrocarbonate at 90 °C for 30 min.[6]

Cell suspension A. E. coli TAU⁻ CP 107 (rel+) cells (kindly provided by Dr. L. Alföldi, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary) from an overnight culture on nutrient agar were suspended in saline to yield a cell suspension with a density equal to OD = 0.2, measured at 520 nm, in 1 cm cuvettes, with a Unicam model SP 600 spectrophotometer.

Cell suspension B. 0.5 ml Cell Suspension A was diluted 10 times with Medium I and the culture was aerated for about 3 h by shaking at 37 °C until a cell density equal to OD = 0.4 – 0.5 was reached. The cell suspension was cooled to 0 °C, the bacteria were collected and washed with Washing medium at 0 °C on Sartorius membrane filter (pore size 0.45 μm) and then suspended in Washing medium to yield a cell suspension with a cell density equal to OD = 0.2.

Assay for RNA synthesis. 0.1 ml Cell Suspension B was added to 0.4 ml prewarmed Medium II and the suspension was incubated by shaking at 37 °C. At given times 0.1 ml RNA-SDS solution was added and after 5 min incubation at 37 °C the lyzed cells were chilled and mixed with 5 ml 5 % cold trichloroacetic acid. The precipitate was collected on Sartorius membrane filter (pore size 0.45 μm) and washed with cold 5 % TCA. The filters were placed into scintillation vials and nucleic acids were hydrolyzed with 0.2 ml 5 % TCA at 60 °C for 30 min. The filters were then dissolved in 5 ml methyl cellosolve, and the radioactivity was measured after the addition of 10 ml scintillation liquid (0.5 % PPO and 0.005 % POPOP in toluene) in a Liquid Scintillation Counter Intertechnique SL 30 with an efficiency of 29 %.

Results and discussion. The action of cysteine, cysteamine, and 2-mercaptoethanol on the RNA synthesis is summarized in Fig. 1. The time curve of RNA synthesis in control and in cysteine-inhibited cells is shown in Fig. 2. Catalase or a peptide, called Fraction V (FV) isolated from Red Kidney beans,[7,8] completely counteracted the inhibitory effect of cysteine (heat inactivated catalase was inactive). Inhibition of RNA synthesis by cysteine in *E. coli* was reversible by catalase, as demonstrated by adding catalase after preincubation with cysteine for 20 min (Fig. 2). RNA synthesis started 10 min after the addition of catalase and proceeded with the same rate as in the control cells.

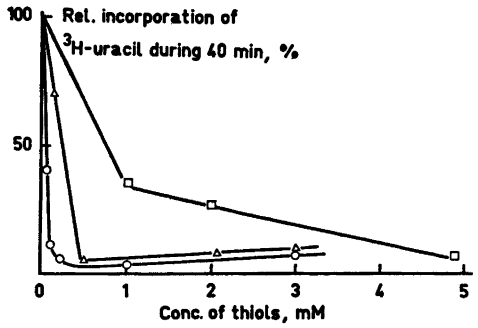

Fig. 1. The effect of cysteine (○), cysteamine (△) and 2-mercaptoethanol (□) on the RNA synthesis. (100 % corresponds to 3.3×10^5 cpm incorporated into *E. coli* TAU⁻ CP 107 (rel⁺) cells during 40 min incubation in the absence of thiols, *cf.* control in Figs. 2 and 3).

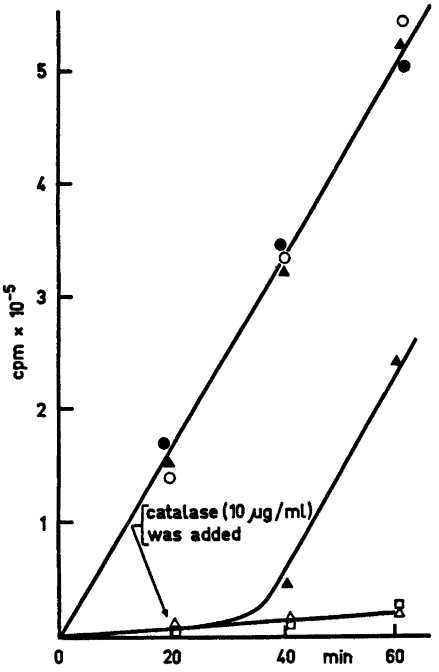

Fig. 2. The effect of catalase and FV on the inhibition of RNA synthesis caused by cysteine. Control (○); cysteine, 0.1 mM (△); cysteine, 0.1 mM plus catalase, 10 μg/ml (▲); cysteine, 0.1 mM plus heat inactivated catalase, 10 μg/ml (□); cysteine, 0.1 mM plus FV, 50 μg/ml (●), Catalase (Sigma C-100 40 000 units/mg) was added either at 0 or at 20 min.

Catalase did not reduce the concentration of cysteine.[9] From the fact that in the presence of catalase RNA synthesis was found to be unaffected by thiols, we conclude that peroxide formed during the oxidation of thiols rather than thiols as such was responsible for the inhibition of RNA synthesis. Indeed, we found that H_2O_2 inhibited RNA synthesis in *E. coli* cells and that this inhibition could be reversed by catalase in the same way as the inhibition caused by thiols (Fig. 3).

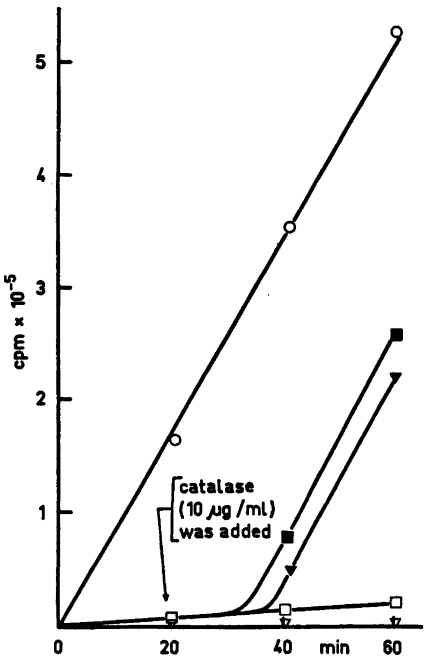

Fig. 3. The effect of H_2O_2 and catalase on the RNA synthesis. Control (○); H_2O_2 0.1 mM (□); H_2O_2, 1.0 mM (▽); H_2O_2, 0.1 mM plus catalase, 10 μg/ml (■); H_2O_2, 1.0 mM plus catalase, 10 μg/ml, (▼). Catalase (Sigma C-100 40 000 units/mg) was added at 20 min.

The concentration of H_2O_2 in aerobic systems containing thiols seems to be low, because H_2O_2 generated according to eqn. (1) is continuously eliminated according to eqn. (2).[10]

$$2RSH + O_2 \rightarrow RSSR + H_2O_2 \quad (1)$$

$$H_2O_2 + 2RSH \rightarrow 2H_2O + RSSR \quad (2)$$

From rate constants determined for reactions (1) and (2) the steady concentration of H_2O_2 in a 1 mM aerated cysteamine solution was estimated to be 0.01 – 0.1 mM.[9] In this concentration range H_2O_2 clearly inhibits RNA

synthesis. Additional experiments show that the cytotoxic action of thiols is also due to peroxide.[9] Evidently, some cell constituent(s) are highly sensitive towards H_2O_2. Therefore, H_2O_2 should always be considered as a potential cytotoxic agent and/or as an efficient inhibitor of RNA synthesis in aerobic biochemical and biological systems containing thiols or other autoxidable materials. These effects can be prevented by catalase or by peptide FV. Catalase rapidly decomposes H_2O_2 formed in reaction (1). FV has no H_2O_2 decomposing activity [9] but prevents the formation of H_2O_2 by inhibiting reaction (1). Reaction (1) requires trace amounts of metal catalyst. FV was found to bind metal ions, especially copper, and by doing so to inhibit generation of H_2O_2 and aerobic oxidation of thiols.[9] This peptide has also been shown to counteract the inhibition of RNA synthesis caused by thiols in lymphocytes.[11]

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The Absolute Configuration of α-(Benzotriazolyl-1)propionic Acid. Synthesis of α-(4,5,6,7-Tetrahydrohydrobenzotriazolyl-1)propionic Acid

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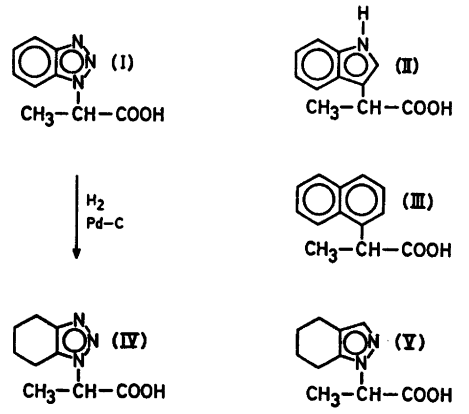
α-(Benzotriazolyl-1)propionic acid (I) has been resolved by Fredga and Lindgren.[1] Its absolute configuration has now been determined by X-ray powder photogram studies and CD measurements.

Attempts were first made to correlate I with α-(indolyl-3)propionic acid (II) of known configuration [2] by the quasi-racemate method.[3] However, neither melting-point diagrams nor X-ray powder photograms gave any indication of formation of quasi-racemates or solid solutions.

The acid I was then tested against α-(naphthyl-1)propionic acid (III).[4] Here the great difference in melting-point (~120 °C) is not favourable for thermal analysis. X-Ray powder photograms showed no quasi-racemate, but (+)-I and *S*-(+)-III gave solid solutions indicating the same absolute configuration. Thus (+)-I has *R*-configuration.

(−)-I was also hydrogenated yielding (−)-α-(4,5,6,7-tetrahydrobenzotriazolyl-1)propionic acid (−IV) (Scheme 1). The CD-spectrum of



Scheme 1.

(−)-IV was compared with that of *S*-(+)-α-(4,5,6,7-tetrahydroindazolyl-1)propionic acid (+V), whose absolute configuration is known.[5] (−)-IV and *S*-(+)-V give the same type of CD-curves (Fig. 1). This is also the case for (+)-I