# EFFECTS OF 9-HYDROXYELLIPTICINE ON GROWTH AND MACROMOLECULAR SYNTHESIS IN *ESCHERICHIA COLI*

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## 1. Introduction

Several drugs known to interact with nucleic acids through intercalation such as ethidium bromide, actinomycin D, daunomycin or mithramycin have a strong inhibitory effect on the growth of micro-organisms and eukaryotic cells [1–3]. These cytotoxic properties were probably result from the interaction of these drugs with nucleic acids or modifications of other metabolic process or synthesis.

The ellipticines (dimethyl-5-11[6 H]-pyrido [4-3-b]carbazoles) are heterocyclic compounds also able to intercalate into DNA [4,5]. In this series, 9-hydroxyellipticine (fig.1) which displays a high affinity for DNA has been thoroughly investigated [6]. This compound is very active against L1210 mice lymphoid leukemia [7].

As a preliminary attempt to comprehend the mechanism of actions of this drug, we report here the kinetics of several of its effects on the growth and macromolecular biosynthesis of *Escherichia coli* K 12. Pronounced and immediate effect on the growth and viability were found depending both on the drug and extracellular magnesium ion concentration. RNA and protein synthesis were found to be inhibited while DNA synthesis was only slightly reduced.

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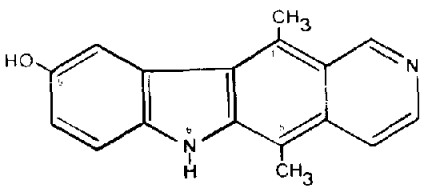


9-HYDROXYELLIPTICINE

Fig.1. Chemical structure of 9-hydroxyellipticine.

## 2. Materials and methods

### 2.1. *Bacterial strains and media*

*E. Coli* CR 341 ($F^-$, thy A, thi, leu, thr, met, $lacy^A$, str A) a K 12 strain was used in this study. The cells were grown in a modified Cohen and Richenberg medium [8] of the following composition (g/l): $(NH_4)_2SO_4$, 2.0; $FeSO_4 \cdot H_2O$, 0.0005; KCl, 0.075; $NaH_2PO_4 \cdot H_2O$, 0.138; and triethanolamine, 7.5. The pH was adjusted to 7.8 with HCl. This mineral medium was supplemented for growth with 0.5% sodium succinate and with the required amino acids (100 μg/ml of each), thymine (100 μg/ml or 10 μg/ml for incorporation studies with the labeled precursor) and thiamine (50 μg/ml). The concentration of $Mg^{++}$ critical in these experiments was ajusted to a final value of $10^{-4}$ M unless otherwise stated.

### 2.2. *Culture conditions*

Inocula were grown overnight at 37°C and then diluted 20-fold in the same medium. Cultures were

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grown at 37°C in erlenmeyer flasks on a rotary shaker and the growth was followed by measuring the turbidity at 578 nm with an Eppendorf photometer. In that case the drug was added in the mid exponential phase. Survival experiments were performed on samples diluted to $3 \cdot 10^{-5}$ in mineral medium and were plated on complete drug-free agar medium.

2.3. *Synthesis of cellular macromolecules*

In order to detect changes in the rate of macromolecular syntheses, radioactive precursors were added at the same time as 9-hydroxyellipticine. Protein synthesis was followed by measuring the incorporation of [$^{14}$C]leucine after precipitation of bacteria by 10% cold trichloroacetic acid and filtration on Millipore HAWP (0.45 μm) filters. The dry filters were counted in Toluene-PPO-POPOP scintillation liquid with a Packard Tric-Carb liquid scintillation spectrometer. RNA and DNA synthesis were followed by measuring the incorporation of [$^{3}$H]uracil (final concentration, 50 μg/ml) and [$^{3}$H]thymine (final concentration, 10 μg/ml) into cold trichloracetic acid-precipitable material.

2.4. *Determination of the nucleotide pool*

The incorporation of $^{32}PO_4^{3-}$ into ATP, GTP and ppGpp was measured in the medium described above but with a low phosphate concentration (0.1 mM). Nucleotides were determined according to Cashel et al. [9,10] by thin-layer chromatography on polyethyleneimine-cellulose of the material soluble in 1 M cold formic acid were performed. The solvent was either 0.8 M or 1.5 M $KH_2PO_4$.

2.5. *Reagents*

9-hydroxyellipticine was synthesized in our laboratory according to Dalton et al. [11] and was a generous gift of M. Lecointe. Its structure was ascertained by nuclear magnetic resonance and mass spectroscopy, and elemental analysis. Its purity was checked routinely by thin layer chromatography and U.V. spectrophotometry. Labelled phosphoric acid; [$^{14}$C]leucine (240 mCi/mmole); [$^{3}$H]uracil (22 Ci/mmole) and [$^{3}$H]thymine (25 Ci/mmole) were products of the Commissariat à l'Energie Atomique, Saclay, France. The polyethyleneimine-cellulose sheets were from Macherey-Nagel (Düren, Germany). Reagent grade chemicals were used throughout.

## 3. Results and discussion

3.1. *Effect on growth and viability*

The overall effects of various concentrations of 9-hydroxyellipticine on the growth and viability of *E. coli* strain CR 341 are shown in fig.2. Cell growth (measured turbidimetrically) was slowed immediately after addition of the drug and remain exponential for generations. The inhibitory effect was linearly proportional to the concentration of drug added and was complete when this concentration exceeded 25 μM. 9-Hydroxyellipticine had a bacteriostatic effect on *E. coli* when its concentration was less than 15 μM. Under these conditions the number of viable cells remained constant after two hours of incubation in the presence of the drug. When 9-hydroxyellipticine-treated cells were filtered, washed and quickly immersed in fresh prewarmed medium, growth resumed quite immediately reaching balanced conditions within some minutes.

For concentrations higher than 15 μM there was a rapid decrease in viable counts depending on the time of incubation with the drug. For concentrations of 9-hydroxyellipticine of 25 μM, there is an almost complete loss of colony forming ability after two hours of treatment.

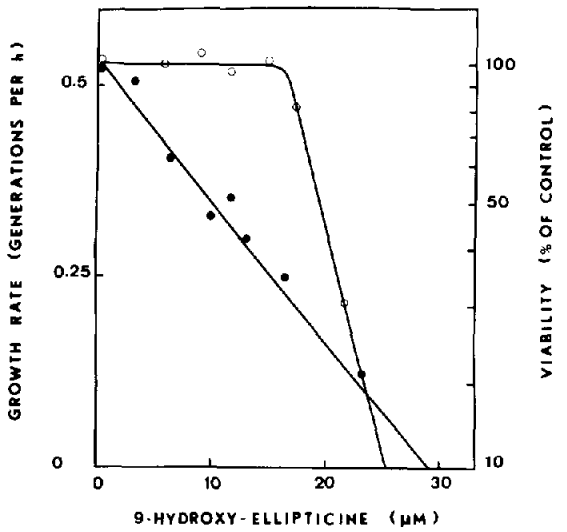


Fig.2. Effect of 9-hydroxyellipticine on the growth rate (•) and the viability (○) of *E. Coli* CR 341. The growth medium used is described under Materials and methods. The viability was determined by plating dilution on broth agar 2 h after addition of the drug.

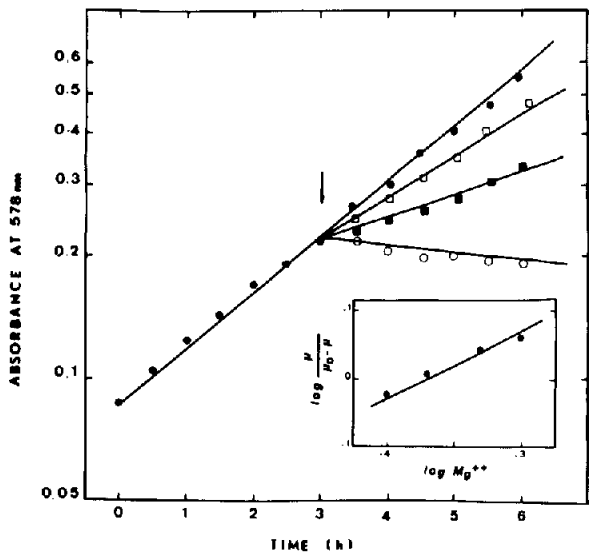


Fig.3. Reduction of growth rate of *E. Coli* CR 341 by 15 μM of 9-hydroxyellipticine as a function of $Mg^{++}$ concentration (●–●) 10 mM; (□–□) 1 mM; (■–■) 0.1 mM; (○–○) 0.01 mM $Mg^{++}$. The inset shows the determinations of $K_M$ of $Mg^{++}$ for growth process in the presence of 9-hydroxyellipticine. $\mu_0$ is the growth rate before addition of the drug (generations per hour); $\mu$ is the growth rate in the presence of the drug.

### 3.2. *$Mg^{++}$ and growth inhibitory effect of 9-hydroxyellipticine*

9-Hydroxyellipticine (15 μM) completely inhibited the growth of *E. Coli* CR 341 in the presence of 10 μM $Mg^{++}$ but the growth rate was not reduced when $Mg^{++}$ was raised to 10 mM (fig.3). Moreover, raising the $Mg^{++}$ concentration subsequent to 9-hydroxyellipticine inhibition allowed the culture to resume growth provided no loss of viability had occurred. When 9-hydroxyellipticine was 15 μM the $Mg^{++}$ concentration required for half maximal growth was 0.2 mM (fig.3, insert). For untreated *E. Coli* cells this effect occurs when the $Mg^{++}$ concentration is around 1 μM [12].

The relationship between growth inhibition by a given concentration of 9-hydroxyellipticine and the external $Mg^{++}$ ions concentration suggests a sort of competitive inhibition between 9-hydroxyellipticine and one of the numerous processes required for growth and involving $Mg^{++}$. It does not rule out the simple explanation that the penetration of the drug across the outer membrane of *E. Coli* is prevented by $Mg^{++}$ ions closely bound to this structure.

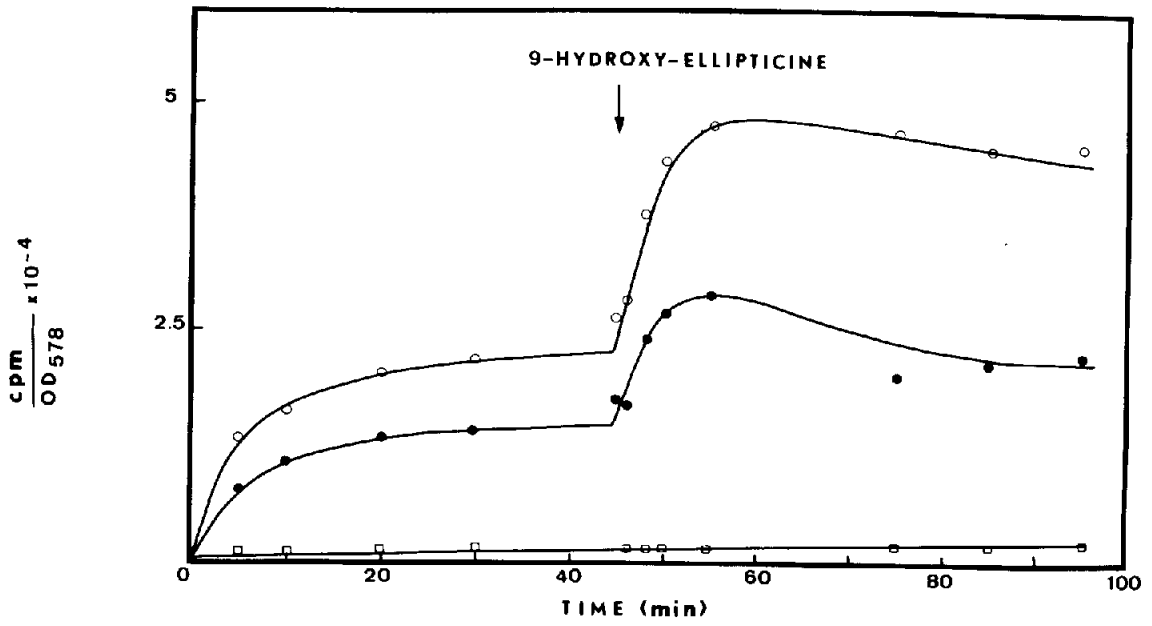


Fig.4. Effect of 9-hydroxyellipticine on the accumulation of ATP (○), GTP (●) and ppGpp (□) in *E. Coli* CR 341. An exponential growing culture (6 ml) in succinate medium was labeled with $^{32}PO_4$ (around 200 μCi) for 45 min before 9-hydroxycellipticine (20 μM) was added. Nucleotides were extracted and measured as described in Materials and methods.

3.3. *Effects on triphosphoribonucleotides and ppGpp*

The kinetics of $^{32}PO_4$ labeling of purine nucleotides were followed upon addition of the drug. The level of ATP and GTP (fig.4) and CTP (data not shown) increase rapidly as soon as the drug was added and reached values about twice higher than the control ones. The level of the unusual guanosine nucleotide which accumulates during the stringent responses [9,10,13,14] did not increase over the control levels after drugs addition.

3.4. *Effects on macromolecule synthesis*

The addition of 9-hydroxyellipticine (12 μM) to a culture of *E. coli* CR 341 growing exponentially in a succinate medium resulted in an immediate 40% inhibition of the rate of [$^{14}$C]leucine and [$^{3}$H]uracil incorporation respectively into protein and RNA (fig.5). DNA elongation was much less affected and continue at about 80% of its initial velocity. When the concentration of 9-hydroxyellipticine reached 20 μM the incorporation of each precursor was completely stopped. In the above experiments, synthesis was followed by measuring the incorporation of radioactive precursors into acid insoluble material in an auxotroph (except for uracil). Different mechanisms of action can account for the observed effects: (a) interference with transport of the added precursor in relation to membrane permeability, (b) inhibition of respiration, (c) inhibition of the synthesis of ribonucleotides triphosphates, (d) degradation of RNA or (e) direct inhibition of the polymerizing systems. It is unlikely that 9-hydroxyellipticine inhibits the synthesis of ribonucleotides triphosphates since the action of the drug was accompanied by an increase in the content of nucleic acids precursors. It is also unlikely that the formation of aminoacids is affected since the drug did not induce the stringent response. However it appeared that no specific inhibition for RNA and protein synthesis occurs since the rate of precursors incorporation in these macromolecules are identical. Interestingly, drugs in the ellipticine series have been shown to decrease the rate of oxygen consumption in yeast (Pinto and Paoletti, unpublished results). Such an inhibition of respiration could also explain the effects presently observed on bacteria. Work is in progress to delineate between some of the other possibilities.

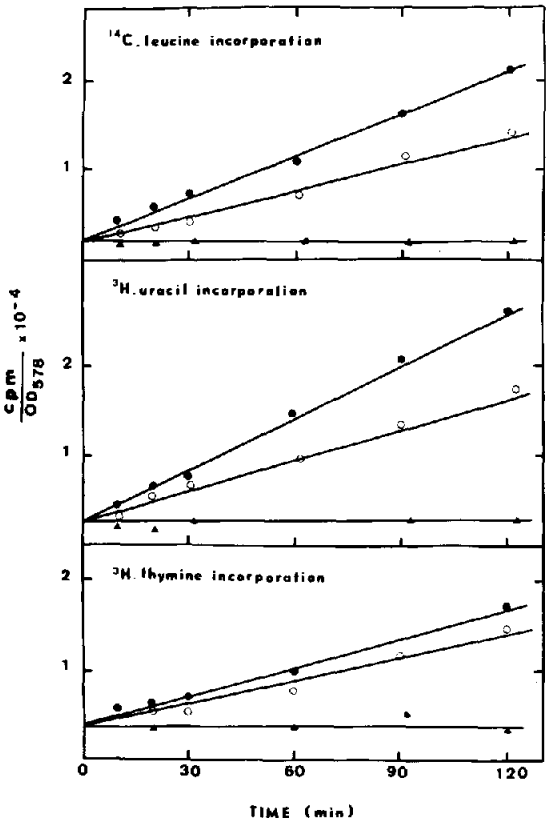


Fig.5. Incorporation of [$^{14}$C]leucine, [$^{3}$H]uracil and [$^{3}$H] thymine into the acid insoluble fraction of *E. Coli* strain CR 341. Drug and labeled precursors were added simultaneously to a logarithmically growing culture. Aliquots (0.1 ml) were removed for acid precipitation and counting as described in Materials and methods. (●–●) Growing control; (○- ○) 12 μM, (▲–▲) 20 μM, 9-hydroxyellipticine.

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