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EFFECT OF TETRACYCLINE DERIVATIVES AND SOME CATIONS ON THE ACTIVITY OF ANHYDROTETRACYCLINE OXYGENASE

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SUMMARY. Anhydrotetracycline oxygenase was partially purified on a Sephadex G-25 column and DEAE-cellulose. The purified enzyme was inhibited by tetracycline derivatives added to the reaction mixture. Certain concentrations of calcium and magnesium ions stimulated the enzymic activity; further increase of the concentration resulted in a sharp decrease of the enzymic activity. The enzyme activity was also stimulated by Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+} and Mn^{2+} present in suitable concentrations in the reaction mixture. Michaelis constant of the enzyme was found to be $2.2.10^{-5}$.

INTRODUCTION. Tetracyclines are synthesized by a complex of enzymes called tetracycline synthetase. The last but one reaction step is catalyzed by anhydrotetracycline oxygenase (ATC-oxygenase). This enzyme was demonstrated in Streptomyces aureofaciens and in the oxytetracycline-producing strain of Streptomyces rimosus. Both anhydrotetracycline and anhydrooxytetracycline could serve as substrates for ATC-oxygenase isolated from both streptomycetes (Běhal, 1982). The production of tetracyclines is proportional to the level of ATC-oxygenase in the cells (Běhal et al. 1979). This level increases in the presence of benzylthiocyanate in the medium and depends on the amount of inorganic phosphate in the medium (Běhal et al. 1982). The synthesis of ATC-oxygenase begins after the inorganic phosphate has been exhausted from the medium.

In the present communication the effect of tetracycline (TC) derivatives and some metal ions on the activity of ATC-oxygenase was investigated. The effect of metal ions on the production of secondary metabolites was studied in a number of antibiotic producers, although mostly in vivo (Weinberg, 1982). The mechanism of their effect can be explained in many ways. They can influence both the synthesis of enzymes and their activity. The effect of

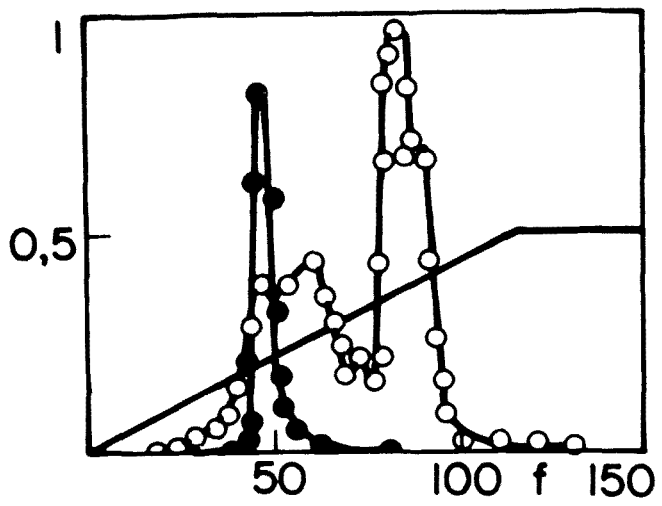

Figure 1. Purification of ATC-oxygenase on DEAE cellulose.
-o- A_{280} (protein) -●- Enzyme activity x 2(nkat/ml) —— KCl x 0.5(Mol)

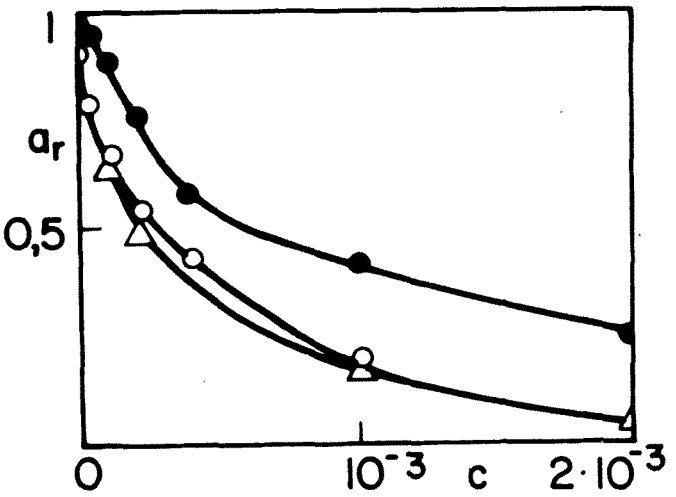

Figure 2. Effect of tetracyclines [c, concentration (M)] on ATC-oxygenase activity (a_r, relative activity).
-o- tetracycline -●- chlortetracycline -Δ- oxytetracycline

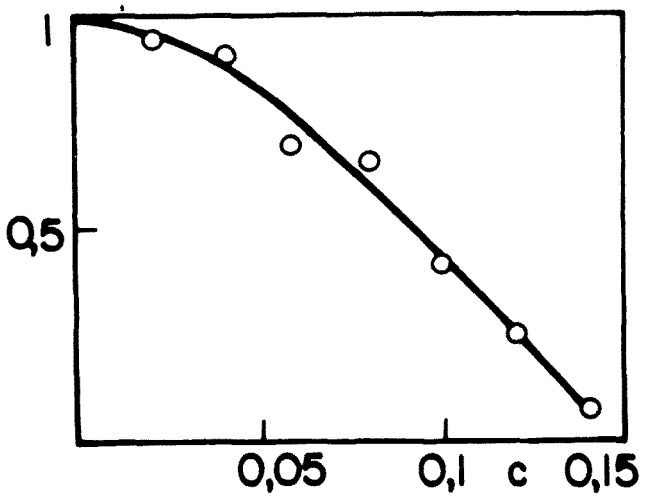

Figure 3. Effect of $(NH_4)_2SO_4$ [c, concentration (M)] on relative activity (a_r) of ATC oxygenase.

metal ions on the enzymic system synthesizing secondary metabolites was studied by Froyshov et al. (1980) in bacitracin synthetase. It follows from the data obtained that the studied ions differ in their mechanism of action.

MATERIAL AND METHODS. Microorganism (Streptomyces aureofaciens), cultivation conditions, preparation of the crude enzyme extract and the assay of ATC-oxygenase have already been described (Běhal et al. 1979). The enzyme was purified on a set of Sephadex G-25 columns (volume 5 ml) and on DEAE-cellulose (DE-52), on a column of 2.5 x 30 cm. The enzyme was eluted with a KCl gradient (0.02 - - 1 M).

RESULTS. The purification of the crude enzyme preparation on Sephadex G-25 and DEAE-cellulose made it possible to remove major portions of contaminating proteins and increase the specific activity of ATC-oxygenase by 10 - 13 times (Fig. 1). The purification of the enzyme by ammonium sulphate precipitation was not successful. The effect of tetracycline derivatives on the enzyme purified on DEAE-cellulose is shown in Fig. 2. All studied derivatives decreased the enzyme activity; chlortetracycline and oxytetracycline were more effective than tetracycline. Ammonium sulphate present in the reaction mixture also decreased the enzyme activity (Fig. 3). The enzyme activity increased in the presence of calcium and magnesium ions at concentrations of 10^{-4}-2.10^{-3}M and 10^{-3}-10^{-4}M, respectively (Fig. 4). At higher concentrations the enzyme activity rapidly decreased. The enzyme activity was stimulated by the above concentrations of calcium and magnesium ions even in the presence of tetracycline added to the reaction mixture at a concentration causing a decrease of the enzyme activity to 57 %. The resulting activity during a simultaneous effect equaled to the sum of partial effects of calcium and magnesium ions and tetracycline.

The effect of Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+} and Mn^{2+} was also investigated. The relationship between the activity of ATC-oxygenase and concentrations of individual ions is presented in Fig. 5. The concentration of ions required for the maximal

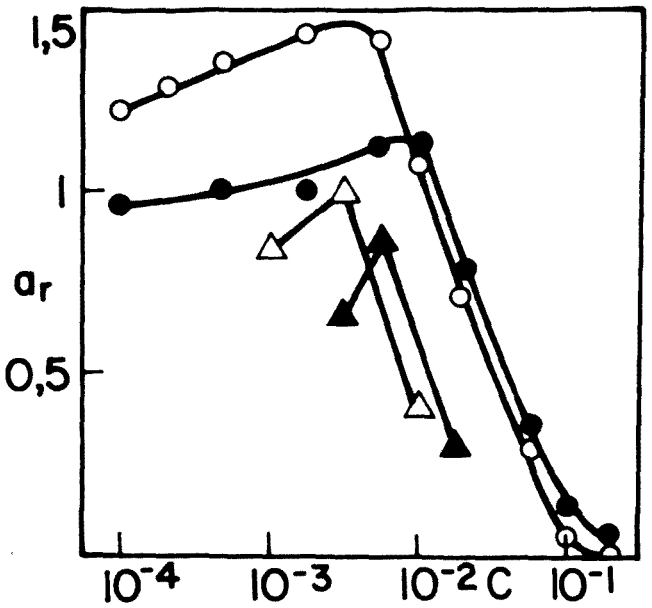

Figure 4. Effect of Ca and Mg ions + tetracycline, (c, molar concentration), on relative activity (a_r) of ATC oxygenase.

-o- Ca^{++} alone; -Δ- + TC

-●- Mg^{++} alone; -▲- + TC

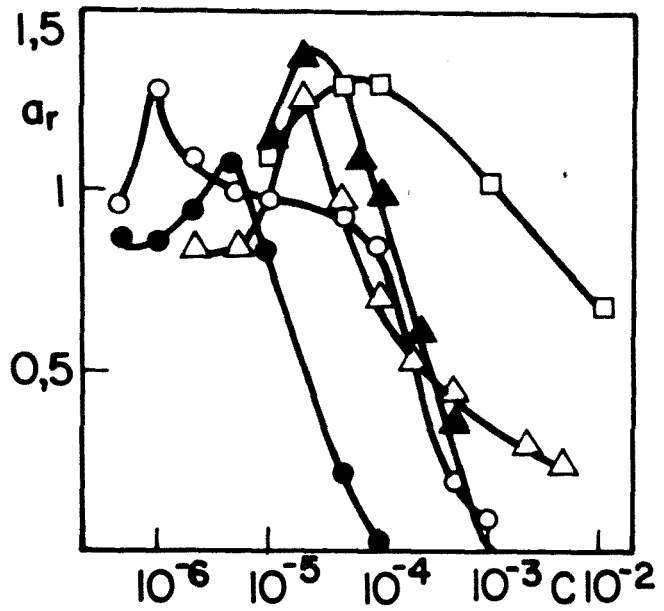

Figure 5. Effect of metal ions (molar concentration c) on relative activity (a_r) of ATC oxygenase.

-o- Cu^{++} -●- Ni^{++} -Δ- Co^{++}

-□- Mn^{++} -▲- Fe^{3+}

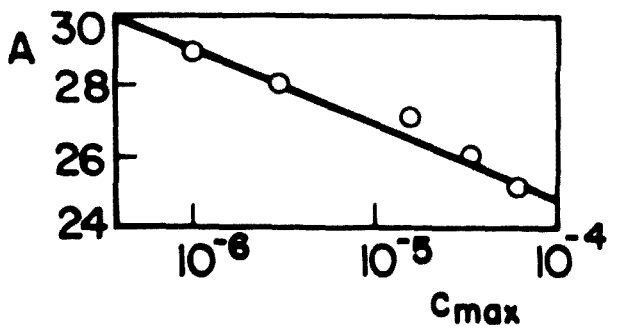

Figure 6. Variation of the metal ion concentration at maximum stimulating effect (c, molar) with atomic no. A

stimulatory effect decreases with increasing atomic number of the element. It follows from Fig. 6 that this relationship is linear. In the simultaneous presence of Fe^{3+} and Ca^{2+} the inhibitory effect of the ion added at the inhibitory concentration compensated for the stimulatory effect of the ion added at the concentration increasing the enzyme activity (Table 1). Michaelis constant with anhydrotetracycline as substrate was determined to be $K_M = 2.2 \times 10^{-5}$ mol x L^{-1}.

DISCUSSION. It follows from the results obtained that metal ions play an important role in the function of the tetracycline-synthesizing system. The curves illustrating the relationship between the enzyme activity and ion concentration are of rather similar course. After reaching the maximal stimulatory effect the enzyme activity sharply decreases with a relatively small increase of the ion concentration. This fact indicates that the production microorganism is sensitive to the concentration of metal ions. However, the mechanism of their action is not known. The mode of action of calcium and magnesium apparently differs from that of other studied ions as demonstrated by the results of the simultaneous effect of calcium and iron ions. On the basis of Fig. 6 it may be assumed that copper, nickel, cobalt, iron and manganese ions have a similar mode of action on ATC-oxygenase.

The interaction of metal ions with tetracyclines consists primarily in chelation properties of tetracyclines (Williamson and Everett 1975). Changes in the activity of ATC-oxygenase caused by added cations, however, cannot be explained by a differe degree of chelation of the substrate and product which would shift the equilibrium of the enzymic reaction. Concentrations of Ca^{2+} and Mg^{2+} stimulating and then inhibiting the enzyme activity are by two order of magnitudes higher than that of the substrate. Also other metal ions, except for Cu^{2+} and Ni^{2+}, vary at concentrations higher by an order of magnitude.

Table I
Influence of Fe^{3+} and Ca^{2+} at the activity of ATC-oxygenase

c_{Fe} (mol/L)	c_{Ca} (mol/L)	activity (1=100 %)
2.10^{-5}		1,35
3.10^{-4}		0,38
	5.10^{-3}	1,42
	3.10^{-2}	0,46
2.10^{-5}	5.10^{-3}	1,47
2.10^{-5}	3.10^{-2}	0,26
3.10^{-4}	5.10^{-3}	0,33
3.10^{-4}	3.10^{-2}	0,25

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